

# Androgen receptor mutations and androgen insensitivity

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## Abstract

The androgen receptor (AR) is a high affinity receptor protein encoded on the human X-chromosome that mediates the actions of androgens during development and in the adult. Defects in this receptor protein result in a wide range of abnormalities of male sexual development. Studies in a number of different laboratories have identified mutations of the AR gene in subjects with androgen resistance syndromes. Defects that interrupt the AR open-reading frame have been traced to a number of distinct types of genetic alterations, have been identified in widely separated segments of the AR gene, and are invariably associated with the phenotype of complete androgen insensitivity. By contrast, mutations that cause single amino acid substitutions within the AR are localized to the DNA- or ligand-binding domains of the receptor protein and have been associated with the full range of androgen resistant phenotypes. The diversity of mutations that have been identified has prompted a consideration of the relationship between AR mutation and phenotype. Analyses of AR abundance and function suggest that the phenotypic abnormalities that result from mutation of the AR reflect the extent to which AR activity is impaired in target tissues. Such decreases in AR function may be the result of the diminished receptor function, decreases in receptor concentration, or a combination of these two effects.

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## 1. Introduction

In vertebrates, androgens regulate a large number of developmental and homeostatic processes, ranging from the regulation of events critical to the development of the normal male phenotype during embryogenesis, to those required for normal function in adults, such as spermatogenesis. In mammals, two steroid hormones, testosterone (T) and 5 $\alpha$ -dihydrotestosterone (DHT), serve as the major circulating androgens. While these steroids differ only in the presence of a single double bond, in selected circumstances the actions of each hormone has been associated with the control of specific processes and the actions of both hormones are required to account for the entire spectrum of processes regulated by androgen.

The effects of androgen are exerted via the androgen receptor (AR) protein, which is encoded on the human X chromosome. Analysis of the sequences of cDNAs encoding this receptor revealed that it is a member of the

nuclear receptor family of transcription factors (Mangelsdorf et al., 1995). Consistent with this, the AR contains discrete domains responsible for the binding of ligand (LBD) and for the recognition of target DNA sequences (DBD).

## 2. Clinical syndromes of androgen resistance

Mutations of the AR gene cause a range of phenotypic abnormalities of male sexual development (Quigley et al., 1995; Griffin et al., 2001). At one end of the spectrum are individuals with complete androgen insensitivity (complete testicular feminization) who exhibit normal breast development and female external genitalia (Morris, 1953; Morris and Mahesh, 1968). At the other extreme are individuals with male phenotypes that are characterized by either subtle undervirilization or infertility. Between these two poles are intermediate phenotypes characterized by differing degrees of virilization. Patients with predominantly female phenotypic development, but with clitoromegaly and posterior labial fusion have been characterized as incomplete

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testicular feminization. Patients with severe forms of perineal hypospadias and gynecomastia have been termed the Reifenstein phenotype. More detailed criteria have been proposed to permit a more careful description of the degree to which virilization is affected (Quigley et al., 1995).

### *2.1. Perspectives from which to characterize patients with clinical defects of androgen action*

The initial studies to characterize the defects of the AR in patients with clinical forms of androgen resistance employed the use of tritiated androgen in binding assays to measure the levels and nature of AR expression in fibroblasts from patients and normal controls (Pinsky et al., 1981; Griffin and Durrant, 1982; Brown et al., 1982). The application of these methods in a number of different laboratories permitted the identification of subjects that displayed different alterations of ligand binding: absent binding, decreased binding, qualitative abnormalities of androgen binding, and individuals in whom no defect of ligand binding could be measured. In specimens with qualitatively abnormal AR, the levels of AR binding are normal, but difference in the stability or affinity of androgen binding by the AR could be detected. In specimens from patients with normal androgen binding, no qualitative or quantitative defect of androgen binding is detected, despite clear-cut hormonal and genetic data suggestive of an AR defect.

The cloning of cDNAs encoding the human AR permitted the development of techniques to identify genetic alterations of the AR that cause androgen resistance. A large number of different mutations have been identified in different laboratories and many can be found compiled within the AR database (<http://ww2.mcgill.ca/androgendb/>). Despite the large number of different mutations that have been identified within the AR gene, a number of generalizations can be made. In the following discussion, these are grouped according to the type of alteration of AR structure that has been identified.

One of the first types of mutations of the AR gene that were identified was a group in which single nucleotide substitutions resulted in the insertion of premature termination codon within the open reading frame of the AR (Fig. 1A). In different pedigrees, such mutations have been localized to each of the eight coding exons of the AR gene. Owing to the location of the critical hormone and DNA binding domains of the AR at the carboxyl terminus of the AR protein, the resulting truncated receptor proteins are predicted to lack segments of the critical LBD, at the least. Such mutant receptors are incapable of binding androgen with high affinity and are inactive in assays of AR function. Such alterations of the AR open reading frame are uniformly associated with the phenotype of complete

androgen insensitivity (complete testicular feminization). Although caused by distinct types of genetic mutation, other alterations that interrupt the integrity of the AR (alterations of splicing, nucleotide insertions or deletions that result in frameshifts of the open reading frame) are also uniformly associated with complete androgen resistance and absent ligand binding.

Defects of the AR associated with normal androgen binding represent another relatively homogenous class of defects. When studied in monolayer binding assays, patients in this category were found to have no discernible abnormalities of ligand binding using the variety of qualitative and quantitative tests. When the nucleotide sequence analysis of the AR gene was determined in patients with this type of androgen resistance, the defects within this category were localized exclusively to the region that mediates the binding of the receptor protein to target DNA sequences within the genome (Fig. 1B). When such mutations were recreated in cDNAs and studied in heterologous cells, ligand binding by the receptor proteins was found to be normal. Despite normal kinetics of ligand binding, when assayed functionally this class of receptors was found to exhibit defects in the activation of model androgen-responsive reporter genes. Mutant receptors in which amino acid substitutions have been identified within the DNA-binding domain of the receptor protein have been associated with a broad range of androgen-resistant phenotypes, including complete and partial androgen insensitivity.

Amino acid substitutions within the ligand-binding domain of the AR have been grouped into two major types (Fig. 1C). In the first, studies of this category of patient sample had demonstrated an absence of ligand binding. When the AR genes in this category of “ligand binding absent” fibroblasts were studied, the mutations were uniformly localized to the ligand-binding domain of the receptor protein. When carefully studied, these mutations could be shown to have two discernibly different effects on ligand binding by the receptor protein. Infrequently, such substitution mutations completely abolished the capacity of the receptor to bind ligand with high affinity (mutant N69 in panel C). Much more commonly, ligand binding could be identified in heterologous cells transfected with cDNA encoding the receptor proteins (mutant N105 in panel C). In this latter circumstance, however, ligand binding was found to display considerable instability at physiologic temperatures. Regardless of the pattern of ligand binding detected when such cDNAs are assayed following expression in heterologous cells, these receptor proteins are found to exhibit markedly impaired capacity to stimulate model androgen-responsive reporter genes.

The final category of mutations is that associated with qualitative abnormalities of ligand binding (Fig. 1D). Investigations in a number of laboratories have identi-

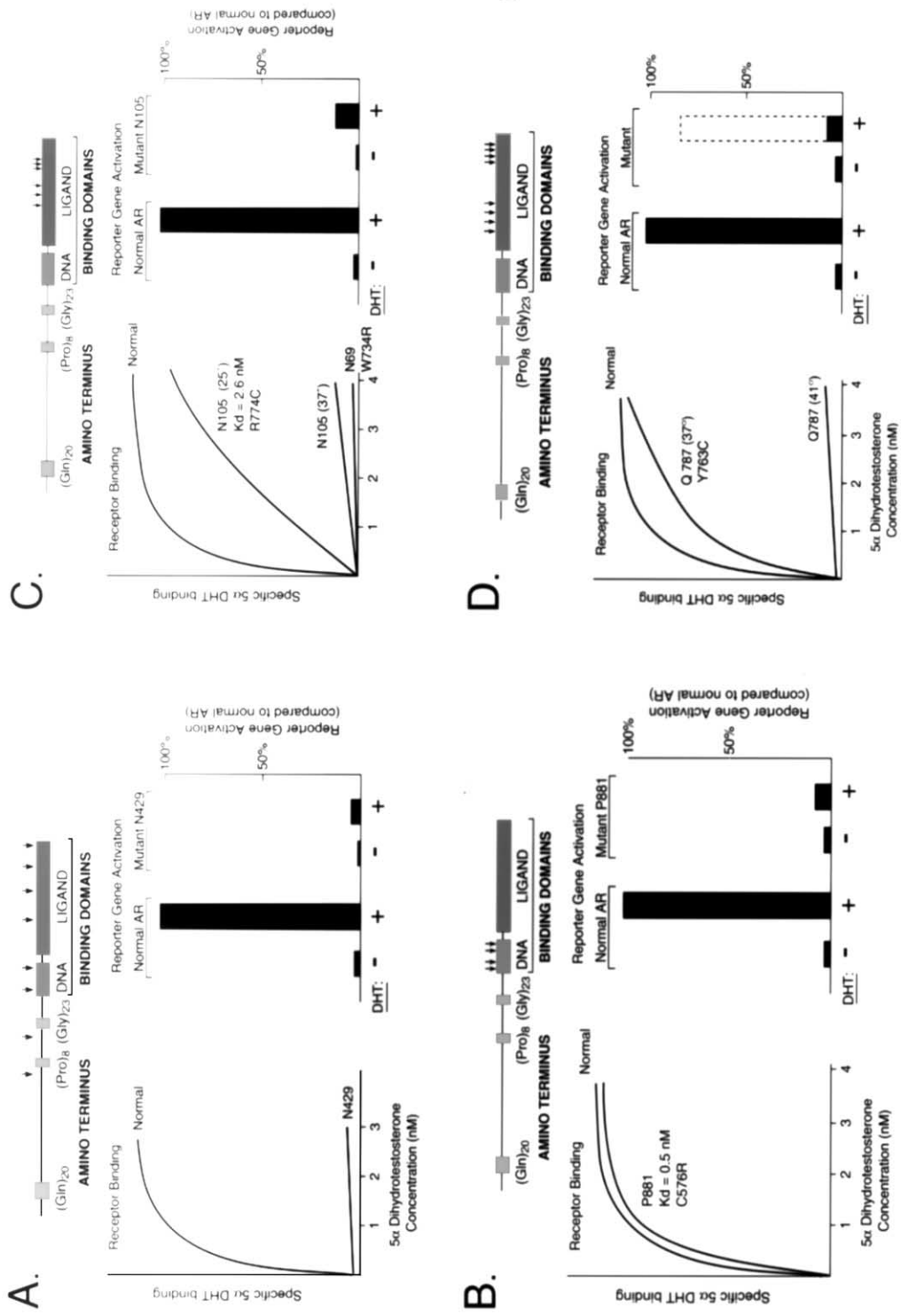


Fig. 1

fied mutations of the AR gene associated with a variety of the qualitative defects of androgen binding, including alterations of ligand affinity, thermal instability of ligand binding, and rapid dissociation of ligand from the receptor protein. Uniformly, these defects have been traced to amino acid substitution mutations within the ligand-binding domain of the receptor protein. Introduction of cDNAs encoding the predicted mutant ARs in heterologous cells have demonstrated that such changes are necessary and sufficient for the alterations of binding that have been observed. In addition, such mutant receptors have been shown to display a range of functional defects when introduced into cells and assayed using functional assays. Mutations of the ligand-binding domain associated with qualitatively abnormal binding by the receptor protein have been associated with the entire range of androgen-resistant phenotypes.

Substitution mutations of the ligand-binding domain of the AR protein account for over half of the mutations identified in syndromes of androgen insensitivity. As noted above, these mutations are associated with a wide range of effects on ligand binding by the receptor. Furthermore, the mutations associated with absent and qualitatively abnormal ligand binding are localized to similar regions within the ligand-binding domain of the receptor (McPhaul et al., 1992). Such observations have raised the question of how tightly associated alterations of ligand binding are with the function of the receptor protein. To investigate this, Marcelli et al. examined the functional capacities of a wide range of mutant ARs containing substitution mutations within the ligand-binding domain of the receptor that were associated with different alterations in ligand-binding by the mutant receptor (Marcelli et al., 1994). Differing responses were observed for each of the mutant ARs when compared with the function of the normal AR assayed in parallel. These experiments demonstrated that the defective function of the mutant ARs could be most

easily demonstrated using saturating concentrations of testosterone. Several of the mutant ARs showed substantially higher degrees of activation in saturating concentrations of  $5\alpha$ -DHT were used in place of testosterone. All the mutant receptors showed highest activity when saturating concentrations of a substituted testosterone derivative were employed (mibolerone; 7,17-dimethyl-19-nortestosterone). These results suggested either that the mibolerone-receptor complex possessed unique properties or implied an important influence of androgen metabolism in the cells in which the mutant AR function was being assayed. Subsequent experiment using pulses of metabolizable and nonmetabolizable androgen analogs demonstrated the effect of these mutations on the stability of the hormone-receptor complex and the influence androgen levels during the incubation period. These results underscore the importance of the stability of the hormone receptor complex and demonstrated that in this collection of mutant ARs, it was not possible to dissociate alterations of ligand binding and measures of AR function. These findings also demonstrated that the activity of many mutant ARs that retained the capacity to bind ligand could be modulated pharmacologically to near normal levels, depending on the ligand and incubation conditions employed. Clinical observations supporting such conclusions have been made in a limited number of individuals that have been treated with supraphysiologic concentrations of androgen.

## 2.2. Direct measurements of AR function

In addition to the implications mentioned above, these latter studies also demonstrated the impact that assay methods can have on measures of AR function. In the preceding examples, alterations in the method of incubation or the type of androgen employed had major effects on the extent to which defective AR function could be observed. Such observations have caused our

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Fig. 1. Mutations of the AR that cause androgen resistance. (A) Termination codons interrupt the synthesis of the AR protein and remove carboxyl terminal portions of the receptor. Owing to these alterations of AR structure, portions of the ligand-binding domain are missing. As a result, the receptor protein is unable to bind ligand with high affinity in binding assays (left) and is inactive in functional assays (right). The same considerations apply to mutations that cause premature termination of the receptor as a result of alterations in AR mRNA splicing, frameshift mutations, deletions or insertions. (B) The analysis of AR gene structure and patients in whom fibroblasts contained normal levels of qualitatively normal AR revealed mutations localized to the DNA-binding domain of the receptor protein. When expressed in eukaryotic cells, such receptor proteins displayed normal ligand binding affinity, but were impaired in functional assays. (C) In some patients, absent ligand binding in monolayer binding assays was traced to single amino acid substitutions within the ligand-binding domain of the receptor. Careful analysis in heterologous cells revealed that in some instances, the expressed receptor protein was incapable of binding ligand (mutant N69). In other instances, the ligand binding can be detected, but the binding activity was unstable under physiologic conditions (mutant N105). In both instances, the receptor is markedly impaired in functional assays. (D) Qualitative abnormalities of ligand binding are the result of amino acid substitutions within the ligand-binding domain of the receptor. Such abnormalities include alterations of ligand affinity and thermal instability (depicted). While the degree of functional impairment varies between individual mutant ARs of this class, alterations in ligand concentrations may result in substantial recovery of receptor function (depicted as the dotted lines). Panels A–D. In this schematic summary, more severe disturbances of AR function are depicted. Although mutations that cause premature termination (A) are uniformly associated with inactive receptors and more severe clinical defects of androgen action, alterations of receptor function that result from single amino acid substitutions within the DNA- and ligand-binding domains of the receptor (B–D) are associated with the entire spectrum of the androgen insensitivity.

group to explore methods by which AR function could be directly assayed in samples obtained from patients with different forms of androgen insensitivity. While one could theoretically approach such a problem by transfection of model reporter genes into recipient fibroblast strains, we have approached this problem by employing recombinant adenoviruses to deliver model androgen-responsive reporter genes directly into patient fibroblast cultures. These methodologies have permitted us to demonstrate the ability to discriminate normal fibroblast from patients with complete forms of androgen insensitivity using this functional assay (McPhaul et al., 1993). Subsequent application of this same method to strains established from patients with a broader range of defects showed that this method could be used to identify AR defects associated with the Reifenstein phenotype (McPhaul et al., 1997). Interestingly, in addition to the potential applications in the diagnosis of androgen insensitivity, these experiments have also identified a small number of patients in whom discernible alternations of AR function are present, but in whom no defect of AR structure or expression can be detected (Alléra et al., 1995).

### 2.3. Immunoblot assays

A number of different methodologies have been employed to characterize the mutations of the AR associated defects of the androgen action. Following the cloning of cDNAs encoding the human AR, high affinity antibodies capable of recognizing the AR were produced and used in immunoblot assays. A large number of studies have been reported in which mutations have been identified in the AR gene and the effects of these mutations on receptor function have been analyzed using transfection and in vitro measures of AR function. The effect of these mutations on the level of AR that is expressed in tissues has been less well studied.

Avila et al. recently applied such a method to the study of 27 patients with the androgen insensitivity in which ligand binding could not be detected in monolayer binding assays (Avila et al., 2002). The results of these immunoassays were correlated the genetic defects that were identified in each subject.

The results of these analyses are summarized in Fig. 2 and permit several generalizations to be made. First, the

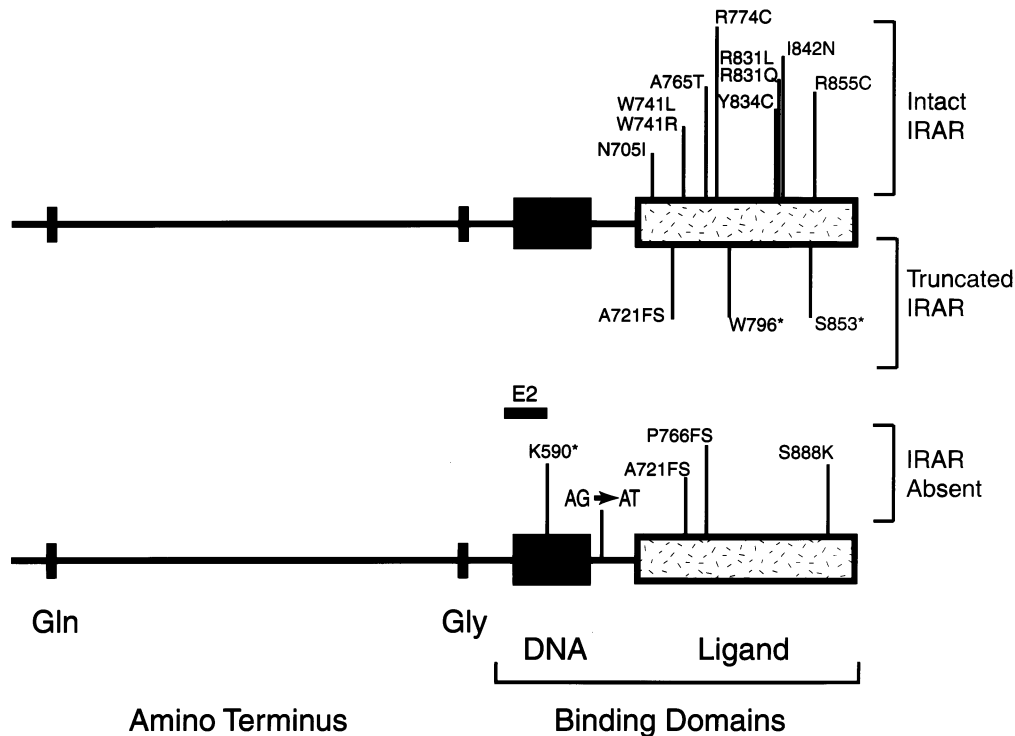


Fig. 2. Mutations of the human AR identified in a group of 27 patients with complete androgen insensitivity and absent ligand binding in monolayer binding assays. *Upper panel:* A summary of the mutations identified in fibroblasts in which immunoreactive AR was measurable. In most instances, single amino acid substitutions were identified in those strains in which normal size AR was detected in immunoblots. In a small proportion of samples, a truncated receptor protein was identified. In this latter group of samples, point mutations leading to an interruption of the AR open reading frame were identified. *Lower panel:* A summary of the mutations identified in fibroblasts in which AR could not be detected using sensitive immunoblot assays. In four instances, mutations were identified which predict an interruption of the AR open reading frame. It is presumed that the AR proteins synthesized in these strains are unstable and do not accumulate to levels that could be detected in the immunoblot assays. In two related strains, exon 2 could not be amplified and these patients are presumed to harbor a deletion of all or part of exon 2 (indicated by the bold bar).

detection of immunoreactive AR in samples in which ligand binding is absent is uniformly associated with mutations that disrupt the structure of the ligand-binding domain. This is most frequently the result of single amino acid substitutions with the ligand-binding domain of receptor protein. And some instances, small amounts of truncated immunoreactive AR are identified. In these instances, premature termination codons were localized to distinct portions of receptor protein.

In a smaller group of patient samples, the AR could not be detected using sensitive the immunoblot assay. In the vast majority of instances, this was found to be the result of mutations that interrupt the integrity of the AR open reading frame. Such mutations include stops codons, alterations of AR mRNA splicing, and frame-shift mutations. It is presumed that the truncated receptor proteins that would be predicted to accumulate (or the mRNAs encoding them) are unstable compared with those instances in which truncated AR proteins can be identified (see above).

Although these generalities can be applied to most of the individual samples within this group of 27 patients, two intriguing exceptions were evident. First, was the identification of a single amino acid substitution (serine to lysine at amino acid residue 888) in a patient in whom immunoreactive receptor was undetectable. Such an observation has not been made previously, and suggests that this amino acid substitution may induce marked instability of the receptor protein. Second, was the demonstration that no mutations were present within the AR open reading frame of one patient in which IRAR was absent. This finding suggests that in this individual, mutations may be present within non-coding segments of the receptor gene, such as the promoter, 5' or 3' untranslated segments, or deep within intronic regions of the AR gene that were not analyzed.

#### 2.4. AR mutations and phenotype

At the outset, it was possible that specific mutations might be correlated with distinctive androgen resistant phenotypes. The breadth of mutations that have now been identified have instead permitted an appreciation that the effects of an AR mutation can exerted through alterations of the level of AR expression, the degree to AR function is disrupted, or contributions at both levels.

In considering the large number of mutations that have been identified, there is in general good agreement in terms of the phenotype that is observed when the same mutation is identified in different pedigrees. While this agreement is best in patients with complete forms of androgen insensitivity, a greater degree of variation is observed in patients with partial forms of androgen insensitivity. This is likely because at the time of sexual development, relatively small variations in AR activity

can have discernible effects on the degree of virilization that is observed. To this point, alterations in the level of 5 $\alpha$  reductase activity (Boehmer et al., 2001) and somatic mosaicism (Holterhus et al., 1999; Gottlieb et al., 2001) have been implicated as contributors to the degree of variation that has been observed.

### 3. Unanswered questions

Despite the wealth of information that has accumulated regarding the nature of AR mutations associated with the different androgen resistant phenotypes, a number of questions remain. First, while the frequency of detecting an AR mutation is high in subjects in whom endocrine studies or family history suggest a defect of the X-linked AR gene, AR mutations are identified much less frequently in patients with defects of virilization in whom such information is not available, particularly in patients with partial forms of androgen insensitivity (Batch et al., 1992; Alléra et al., 1995). In patients in which the AR is not detected using sensitive assays of AR abundance, this may represent mutation of segments of the AR gene outside of the AR ORF, including the promoter, 5' or 3' untranslated segments, or sequences within the introns. Even more intriguing are those individuals in whom AR can be shown to be present. In some studies, assays of AR function have suggested that the AR does not function normally in fibroblasts obtained from such individuals (Alléra et al., 1995). Such findings would be consistent with a defect in gene(s) other than the AR (such as a coactivator) that are required for normal AR function, as has been proposed by Adachi et al. (2000). Equally plausible would be the effects of mutations in genes other than the AR that have a selective effect on androgen action during embryogenesis. Such mutations might exert an effect on the timing or level of AR expression during embryogenesis or exert a direct effect on androgen synthesis or metabolism during this critical period.

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