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# Minireview: Sex Differentiation

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**Mammalian sex differentiation is a hormone-dependent process in the male following the determination of a testis from the indifferent gonad through a cascade of genetic events. Female sex differentiation is not dependent on ovarian hormones, yet there is evidence that members of the Wnt family of developmental signaling molecules play a role in Müllerian duct development and in suppressing Leydig cell differentiation in the ovary. The testis induces male sex differentiation (including testis descent) through a time-dependent production of optimal concentrations of anti-Müllerian hormone, insulin-like factor(s) and androgens. Observations in several human syndromes of disordered fetal sex development corroborate findings in murine embryo studies, although there are exceptions in some gene knockout models. The ubiquitously expressed AR interacts in a ligand-dependent manner with coregulators to control the expression of androgen-responsive genes. Preliminary studies suggest the possibility of hormone resistance syndromes associated with coregulator dysfunction. Polymorphic variants in genes controlling androgen synthesis and action may modulate androgenic effects on sex differentiation. (*Endocrinology* 142: 3281–3287, 2001)**



SEX DIFFERENTIATION is defined as the phenotypic development of structures consequent upon the action of hormones produced following gonadal determination. In reality in mammals, sex differentiation is gonad-dependent only in males because in XX females, phenotypic development is female whether an ovary develops or not. Hence, male development can only occur when the fetal testis secretes two key hormones at a critical period in early gestation, an embryological phenomenon clarified 50 yr ago by the studies of Jost (1, 2). Sex determination is defined as the commitment of the indifferent gonad to a testis or an ovary, a development that is genetically programmed in a critically timed and gene dosage-dependent manner. Much about what is known of this process has been obtained from studies in early mouse embryos and observations from gene knockout experiments. Similar information in humans has mainly been obtained through studies of patients with sex reversal syndromes, particularly in 46,*XY* complete and partial females (3, 4). The focus of this review is on the mechanisms that control androgen production and action in mediating differentiation of the internal and external genitalia in the male. Nevertheless, it is necessary to briefly review the embryology of fetal sex development and the genetics of sex determination.

## Embryology

The temporal events in fetal sex development are shown schematically in Fig. 1. Primordial germ cells migrate from the yolk sac to the urogenital ridge, which develops as a thickening on the ventral surface of the primitive mesonephros. This tortuous cell migration is impaired in mice with the Steel mutation, rendering them sterile but appropriately sex differentiated (5, 6). Thus, germ cells play no part in sex determination. The first sign of testis determination is aggregation of pre-Sertoli cells (probably derived from the adjacent mesonephros) around the germ cells to form primary sex cords at about 6–7 wk of gestation. By the end of wk 9, the mesenchyme that separates the seminiferous cords gives rise to the interstitial cells, which differentiate as steroid-secreting Leydig cells. Figure 1 denotes the increase in testosterone levels that approach concentrations in the fetal serum to within the lower end of the adult male range (7, 8). Concomitantly, there is Leydig cell proliferation, increased expression of steroidogenic enzymes (particularly 3$\beta$-hydroxysteroid dehydrogenase and P450 17$\alpha$-hydroxylase/C17–20-lyase) and expression of the AR in the peritubular myoid cells (9).

Primordia for both the male and female internal genital ducts are present initially and are derived, respectively, from mesonephric ducts and a coelomic epithelial cleft between the genital ridge and the mesonephros. Müllerian duct regression starts at 8 wk of gestation in the male through the action of anti-Müllerian hormone (AMH) secreted by Sertoli cells, which binds to the type II AMH receptor expressed in the surrounding mesenchyme of the Müllerian ducts (10, 11). Stabilization of Wolffian ducts to differentiate as the vas deferens, epididymis, and seminal vesicle is dependent on testosterone primarily but is also responsive to weaker androgens such as androstenedione (12). Wolffian ducts regress in the female in the absence of androgens. Differentiation of the male external genitalia is androgen regulated; this appears to be dihydrotestosterone (DHT)-specific based on the expression profile of 5$\alpha$-reductase type II enzyme, and observations in human sex reversal syndromes characterized by a deficiency of this enzyme (13, 14). Estrogens do not appear necessary for normal sex differentiation of either sex as shown by murine estrogen receptor knockout models and

Abbreviations: AF-2, Activation function region; AIS, androgen insensitivity syndrome; AMH, anti-Müllerian hormone; CAIS, complete AIS; CSL, cranial suspensory ligament; DM, DNA-binding motif; DHT, dihydrotestosterone; Insl3, insulin-like factor 3; PAIS, partial AIS; P450scc, P450 side chain cleavage enzyme; StAR, steroidogenic acute regulatory protein.

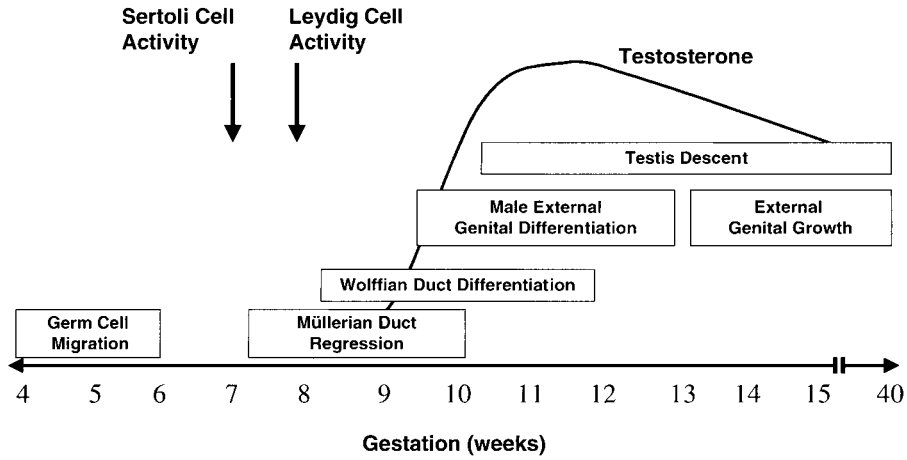


FIG. 1. Embryologic events in male sex differentiation depicted in temporal fashion. The line depicts the increase in fetal serum testosterone concentrations. The word activity refers indirectly to the action of AMH in causing Müllerian duct regression and androgens to induce male sex differentiation.

normal genital development in males with a mutant *ER* gene or aromatase deficiency (15–17).

## Genetic Control of Sex Determination

Numerous genes are involved in controlling determination of gonad type. The process is fundamental to programming sex differentiation and has been reviewed in detail recently (18–22). Because this minireview is focused on sex differentiation, only a few key features of sex determination are highlighted in this section. *SRY* is the principal initiator of the cascade of gene interactions that determine the development of a testis from the indifferent gonad. *SOX9* plays a crucial role in this pathway where it is up-regulated by *SRY* and *SF1* to initiate differentiation of pre-Sertoli cells to Sertoli cells. That *SOX9* lies downstream of *SRY* in a cascade of testis development is illustrated by a mouse transgene insertion that deletes a regulatory element repressing *SOX9* in XX fetal gonads and leads to XX sex reversal (23). Thus, in XY male development, this repressor function upstream of *SOX9* is normally repressed or inhibited by *SRY*, thereby allowing *SOX9* to induce testis formation. In normal female development, *SOX9* is repressed and no testis forms.

Only a minority (15–20%) of XY patients with gonadal dysgenesis and sex reversal have a mutation in *SRY* to account for the phenotype (24). Furthermore, the *SRY* gene is not detected in 20% of XX males. Other genes required for testis determination in humans remain to be identified, despite several characterized in mouse gonadal development. Even though the human syndrome of campomelic dysplasia and XY sex reversal is caused by mutations in *SOX9* (25), no mutations of this gene have been found in XY gonadal dysgenesis alone (26). Similarly, *SOX3* from which *SRY* is believed to have evolved (27), was normal in mutation analysis of a group of patients with unexplained XX sex reversal and XY gonadal dysgenesis (28). A novel gene, *tescalcin*, was recently identified as specifically expressed in early fetal mouse testis cords using the technique of representational difference analysis (29). When the human homolog is cloned, it is possible that this gene may be implicated in some forms of XY gonadal dysgenesis. Some progress has been made in characterizing a locus on terminal 9p, which leads to XY sex reversal when deleted (30). Two candidate genes at 9p24.3 have been identified, which are evolutionarily conserved and homologous with *doublesex* (*dsx*) and *mab3* genes involved in sex development in Drosophila and Caenorhabditis, respectively (31). They encode proteins with a DNA-binding motif (DM domain). The human genes are termed *DMRT1* and *DMRT2*, *doublesex* and *mab*-3 *r*elated *t*ranscription factors. Extensive studies in a large number of XY sex-reversed patients have yet to identify mutations in these genes (32, 33).

The dogma of mammalian sex development attributes no specific active gene regulatory events to ovarian determination and internal genital development. However, it now appears that female development in the mouse at least, is regulated by members of the Wnt family of developmental signaling molecules (34, 35). *Wnt-4* is expressed in the developing mesonephros and hence involved in gonad development (36). It is down-regulated in the testis (perhaps by *Sry*) but remains in the ovary; it is also expressed in the Müllerian ducts but is absent from Wolffian ducts. Disruption of *Wnt-4* in females results in masculinized ovaries, which produce androgens from Leydig-like cells, stabilization of Wolffian ducts and absence of Müllerian ducts (37). Consequently, *wnt-4* is normally required for initial Müllerian duct development in both sexes and subsequent suppression of Leydig cell differentiation in the developing ovary. Another signaling molecule, *wnt-7a*, is needed to complete the further development of Müllerian ducts into the internal female genital tract (38, 39). Although human homologues for *wnt-4* and *wnt-7a* are identified, their precise role in human female sex development remains to be established. There are many other factors implicated in sex determination not covered in this brief section but Fig. 2 summarizes their hierarchy in the formation of a testis or an ovary. It is emphasized that much of the information is gleaned from expression studies in mouse genital ridges and from the result of gene knockout models. Thus *WT1*, *SF1*, *Liml*, and *Emx2* depicted in Fig. 2 are genes involved in formation of the genital ridge as well as other primordia such as the adrenals and kidneys. The precise role for some of these factors for human sex determination is not established.

## Hormonal Control of Sex Differentiation

The post gonad determination phase of sex differentiation is almost exclusively hormone-dependent and is an active sexually unimorphic process for the male. AMH and testos-

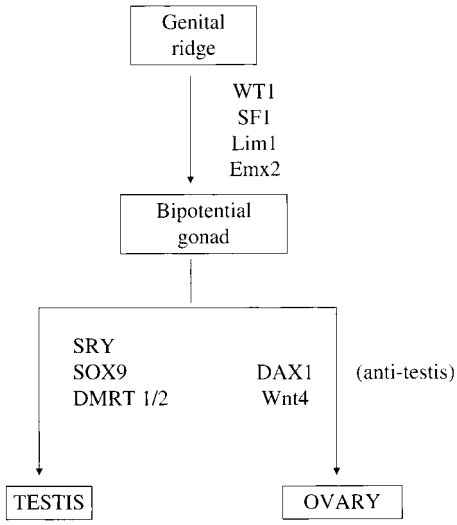


FIG. 2. Factors controlling gonad determination. DAX-1 may have an indirect role in ovarian development by acting as an anti-testis factor.

terone are the two key hormones produced by the testis in optimal concentrations during a critical time frame in early gestation to ensure male development. Also a key component in the process is the developmental expression of cognate receptors for these hormones in target tissues. In later gestation, the testis migrates transabdominally from its origin adjacent to the developing kidney before final inguino-scrotal descent. This can be regarded as part of the completion of sex differentiation in the male and recent studies describe the role of *insulin-like* 3 gene (*Insl3*) and its product in this process.

### *AMH*

AMH is a glycoprotein produced in fetal Sertoli cells and belongs to the TGF-$\beta$ superfamily, which includes inhibin and activin (40). The primary role for AMH in sex development is to cause a gradient of cranial to caudal regression of Müllerian ducts during a short period from 8–10 wk of gestation in the human. This is achieved by the protein binding to a similarly expressed gradient of AMH type II receptor in mesenchymal cells which, presumably by a paracrine mechanism, induce apoptosis of the epithelial cells of the Müllerian ducts. AMH signaling via the membrane-bound serine/threonine kinase type II receptor requires recruitment and phosphorylation also of a type I receptor. This mode of action for the TGF-$\beta$ family involves signal transduction via the Smad pathway (41). The AMH type I receptor has yet to be firmly identified, but a candidate is the bone morphogenic protein type IB receptor, which forms a complex with the AMH type II in a ligand-dependent manner (42).

The role of AMH in male sex differentiation is illustrated by the persistence of Müllerian duct derivatives in males with inactivating mutations of either the *AMH* or *AMH type II* receptor gene, but who otherwise develop normally (43, 44). Maldescent of the testes in the human syndrome is probably the result of anatomical connection of the gonads to the persistent Müllerian ducts rather than indicating a specific role for *AMH* in testis descent. Furthermore, targeted disruption of *AMH* and *AMH type II* receptor genes in mice does not prevent testis descent (45, 46).

### *Control of testis descent*

Migration of the testis from the lower pole of the kidney on the abdominal wall or ovarian position into the extra-abdominal scrotal sac is a two-stage process of transabdominal migration and inguino-scrotal descent (47). Cryptorchidism affects up to 3% of male newborns and the prevalence may be increasing (48). Abdominal wall connections to the testis are through the cranial suspensory ligament (CSL) and caudally, via the gubernaculum. This latter mesenchymal tissue in the male contracts, thickens and develops a bulbar outgrowth which, with regression of the CSL, results in the testis located in the lower abdomen by the internal inguinal ring. CSL regression appears to be an androgen-dependent process (49). The gubernaculum remains a thin cord in the female and preservation of the CSL anchors a stationary position for the ovary.

Insulin-like factor 3 (Insl3) or relaxin-like factor is a member of the insulin-like hormone superfamily and is expressed early in fetal mouse Leydig cells. *Insl3*−/− male mice are bilaterally cryptorchid; the gubernacular bulbs fail to develop and resemble normal female gubernacular structures (50, 51). The majority of *Insl3*+/− male mice also have some degree of testis maldescent (unilateral or bilateral) at birth but which rectifies itself by adult life. Leydig cell function and male urogenital development is otherwise normal. A role for INSL3 in transabdominal testis migration in humans is less persuasive as recent studies in boys with bilateral cryptorchidism suggest *INSL3* gene mutations are rare (52–54). However, unique mutations which were reported in two boys with crytorchidism were heterozygous, suggesting that *INSL3* haploin sufficiency may cause some sporadic cryptorchidism apparent only at birth (53). If there is a specific receptor for INSL3, that may be a cause of dysfunctional signaling. Inguino-scrotal descent is androgen dependent as illustrated by observations in patients with hypogonadotropic hypogonadism and the siting of testes in the androgen insensitivity syndromes (56, 57). Exposure to estrogens has been implicated as a causal factor in boys with cryptorchidism (48, 57). Leydig cell expression of *insl3* in mice is inhibited during prenatal exposure to diethyl stilbestrol (58, 59).

## Androgen Control of Sex Differentiation

### *Role of gonadotropins*

Next to testis determination, the production and action of androgens is the essential requirement for male sex differentiation. Gonadotropic control of fetal testicular steroidogenesis, mediated initially by human CG and later by LH, operates through the well characterized seven transmembrane G protein-coupled LH/CG receptor (60). Regulation of testosterone biosynthesis in early fetal rabbit gonads appears to be gonadotropin independent (61), and recent studies of targeted disruption of the *LH/hCG* receptor gene in mice showed normally differentiated, but hypoplastic genitalia (62, 63). Inactivating mutations of the LH receptor in humans result in varying phenotypes in males, including complete

sex reversal, ambiguous genitalia, or only isolated micropenis (64).

*Testosterone biosynthesis and metabolism*

The enzymatic steps and their genetic control in the testicular biosynthesis of testosterone from cholesterol and further metabolism to the potent androgen, DHT, are well documented (65–69). All steps are necessary for androgen production but key points include the rate limiting step controlled by the steroidogenic acute regulatory protein (StAR) in conjunction with the P450 side chain cleavage enzyme (P450scc) and the enzyme P450c17 which, by virtue of having two enzyme activities (17$\alpha$-hydroxylase and 17,20lyase), as a qualitative regulator of steroidogenesis. The enzymes 17$\beta$-hydroxysteroid dehydrogenase and 5$\alpha$-reductase function to amplify the androgenic signal through the synthesis of the more potent androgens, testosterone, and DHT.

Much can be learned about the role of androgens in male sex differentiation by studying patients with male undermasculinization secondary to deficiencies of androgen biosynthetic enzymes (70, 71). What is intriguing is the extent of Wolffian duct stabilization and development prenatally in disorders such as 17$\beta$-hydroxysteroid dehydrogenase and 5$\alpha$-reductase enzyme deficiencies, in contrast to the almost complete lack of male development of the external genitalia at birth. In both enzyme deficiencies, the external genitalia virilize at puberty if the testes are left *in situ*; this androgenic effect has been attributed to substrate conversion by other nonmutant isoenzymes. This does not necessarily explain the difference in fetal internal and external male phenotypes, but in the case of 17$\beta$-hydroxysteroid dehydrogenase deficiency, it is possible that a weaker-acting androgen such as androstenedione is sufficient to stabilize Wolffian ducts.

## Mechanism of Androgen Action

The cellular and molecular actions of androgen in developmental regulation are key to understanding male sex differentiation. Central to this process is the AR, a nuclear transcription factor that controls androgen-dependent gene expression.

A single AR is ubiquitously expressed and binds all androgens intracellularly in target cells (Fig. 3). Unliganded AR is an inactive oligomer complexed to heat shock proteins (*e.g.* Hsp90, Hsp70) and located in the cytoplasm. The oligomeric complex dissociates on ligand binding, undergoes a conformational change while transporting into the nucleus to bind as a homodimer to DNA hormone response elements (72). In common with other nuclear receptors, the AR comprises three functional domains involved in transcriptional regulation, DNA and ligand binding. The least conserved, large N-terminal domain contains an activation function (AF-1) region which is autonomously involved in gene transactivation. The AR has a unique N-terminal polymorphic glutamine region as a result of a variable number of CAG repeats. Variations in CAG repeat length affect AR transcriptional efficiency (73). The central DNA-binding domain is the most conserved region; the C-terminus contains a second activation function region (AF-2) and mediates heat shock protein interactions, dimerization, nuclear localization signaling as well as ligand binding.

The AF regions interact with an intermediary group of proteins termed co-regulators to form protein: protein interactions in a ligand-dependent manner to either increase (co-activator) or decrease (co-repressor) gene transcription (74, 75). Figure 4 illustrates the interaction of ligand-bound AR homodimers in a multiprotein complex with SRC-1 and CBP, representative members of the nuclear receptor coregulator family (76). AF-2 is ligand-dependent and is located within one of the $\alpha$-helices (helix 12) which binds to receptor-interacting motifs (LXXLL; L is leucine, X is any amino acid) of co-regulators (77). The AR is unique in displaying constitutive activity *in vitro* based on deletion experiments of the ligand-binding domain (78). This suggests a critical role for AF-1 in gene transactivation; interaction with SRC-1 is not apparently via LXXLL motifs but with a conserved, glutamine-rich region in the C-terminal region (79). Also depicted in Fig. 4 as part of the multiprotein complex is SRA (steroid receptor RNA activator) which is AF-1 selective and functions uniquely as an endogenous RNA transcript (80). A co-regulator having relatively specific interaction with the AR ligand-binding domain is ARA70 (81).

The X-linked disorder of androgen resistance characterized by the androgen insensitivity syndromes has provided useful information on androgen action and on what may be the phenotypic outcome with a defect in this complex, multistep process.

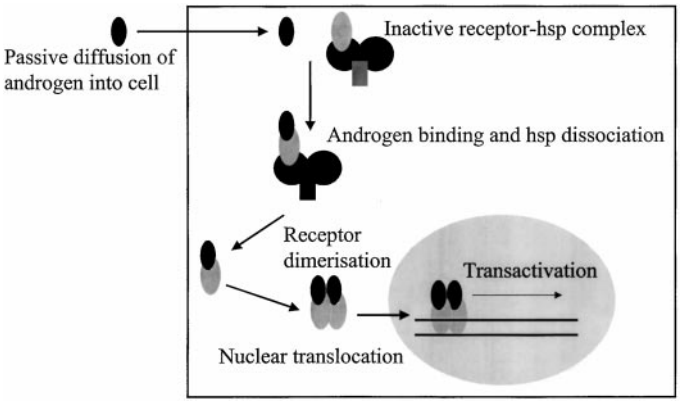


FIG. 3. Schematic of androgen action.

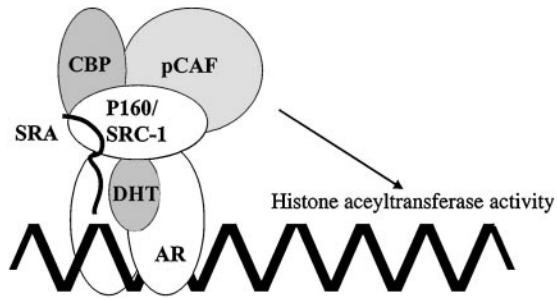


FIG. 4. Schematic of ligand-bound AR interacting with co-regulator proteins. p160/SRC-1 (steroid receptor coactivator 1), CBP (CREB-binding protein), pCAF (CBP-associated factor), SRA (steroid receptor RNA activator).

*Syndromes of androgen insensitivity*

The androgen insensitivity syndrome (AIS) is defined by the complete AIS (CAIS) or partial AIS (PAIS) absence of signs of androgen responsiveness in XY males with normal testis determination and androgen biosynthesis (82, 83). It is the clinical paradigm of hormone resistance that relates to numerous examples of both nuclear receptor and cell membrane receptor-related cell signaling systems (84). A form of PAIS is also recognized where infertility is the sole manifestation in normally sex differentiated males (85).

Numerous *AR* gene mutations are reported in AIS and they are detailed on an international database (86; http//www.mcgill.ca/androgendb/). A preponderance of mutations affect the AR ligand binding domain. Functional analysis provides indirect evidence about critical regions in support of the recently reported crystal structure of the AR ligand binding domain (87). Homology modeling based on the known crystal structure of the related progesterone receptor can also be used. For example, arginine 779 is critical to ligand binding and subsequent transactivation whereas a histidine 874 alanine substitution has only a minimal effect on androgen binding (88).

*Coregulator dysfunction*

Compelling evidence for the role of coregulators in hormone action comes from studies in *SRC-1* mutant mice (89). Sex hormone-dependent organs showed reduced growth response *in vivo* to sex steroids compared with intact *SRC-1* mice. Only a few studies of coregulators in human hormone resistance syndromes are reported to date. Two sisters with clinical and biochemical evidence of resistance to glucocorticoids, mineralocorticoids, and androgens but not thyroid hormones were postulated to have a coactivator defect, but no molecular studies were performed (90). A patient with CAIS in whom the *AR* gene was normal was recently reported to lack a 90-kDa band protein, which interacted with the AF-1 region of the AR in control genital skin fibroblasts, thus raising the possibility of a novel explanation for some forms of androgen resistance (91, 92).

In another recent study, the *ARA70* cDNA was screened in a group of XY patients with varying degrees of undermasculinization in whom defects in the AR had been excluded; no mutations were identified (93). The large family of nuclear co-regulators influence transcriptional regulation in a combinatorial and ligand-dependent manner. Whether the action of any one member when disturbed is so specific as to cause a hormone resistance state has yet to be determined in humans.

## Modulating Factors in Androgen Action

Variations in the number of AR CAG repeats within the normal range (11–31) are associated with male reproductive disorders such as decreased spermatogenesis in otherwise normal males (94, 95). Longer repeats within the normal range are also associated with varying degrees of undermasculinization of unknown cause (96). In a subsequent study of a larger number of males with abnormal genital development, there was evidence that a longer repeat may contribute to the cause of genital maldevelopment, particularly when less severe (97). On the basis of these findings, a model for how the AR polymorphism may modulate androgen action in sex differentiation is proposed (Fig. 5). Several of the numerous genes involved in androgen biosynthesis and action are polymorphic; the coordinated functional consequences of such variants may be relevant for optimal androgen synthesis and action during the critical developmental phase of sex differentiation.

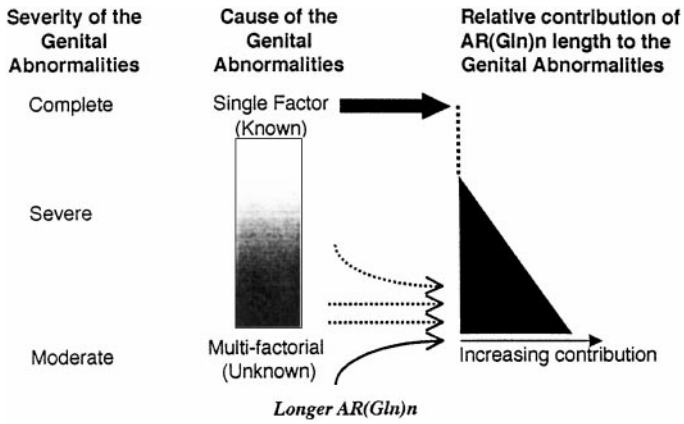


FIG. 5. A model incorporating the effect of an AR polymorphism on the etiology of genital abnormalities. The influence of a longer glutamine repeat is greater when multifactorial causes lead to moderate genital abnormalities. Reproduced with permission from *The Journal of Clinical Endocrinology & Metabolism*.

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