

**ANDROGEN RECEPTOR EXPRESSION  
IN THE HUMAN AND RAT  
UROGENITAL TRACT**

**Androgeenreceptor expressie in de tractus urogenitalis  
van de rat en de mens**

**PROEFSCHRIFT**

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ME VELLE CIVEM ESSE TOTIUS MUNDI NON UNIUS OPPIDI

(I would rather be a citizen of the world, than of one city)

Erasmus Desiderius Roterodamus (ca. 1469-1536)

Voor Myriam

Robbert, Barend

en Falco

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## ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
ADF	age dependent factor
AIS	androgen insensitivity syndrome
AMH	anti müllerian hormone
AR	androgen receptor
BL	bladder
bp	base pairs
bw	body weight
cDNA	complementary deoxyribonucleic acid
DES	diethylstilbestrol
DU	density units
DHT	5 $\alpha$ -dihydrotestosterone
ED	efferent ductules
EGF	epidermal growth factor
EH	epididymal head
EJ	ejaculatory ducts
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ET	epididymal tail
FGF	fibroblast growth factor
GC	gubernacular cone
GF	genital fold
GL	gubernacular ligament
GS	genital swelling
GT	genital tubercle
HGF	hepatocyte growth factor
hCG	human chorionic gonadotropin
im	intramuscular
KGF	keratinocyte growth factor
kb	kilo base
kDa	kilo Dalton
la/bbc	levator ani/bulbocavernosus
MD	müllerian duct
ML	midline
Mr	molecular mass
mRNA	messenger ribonucleic acid

## Abbreviations

NP	nipple anlagen
OV	ovary
PA	prostate anlagen
PAGE	polyacrylamide gel electrophoresis
PE	perineum
PGE	prostaglandin E
pn	postnatal
PSA	prostate specific antigen
RAR $\beta$	retinoic acid receptor $\beta$
rGH	rat growth hormone
rPRL	rat prolactine
sc	subcutaneously
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SRY	sex-determining region of Y chromosome
SV	seminal vesicle
T	testosterone
TE	testis
Tfm	testicular feminization
US	urogenital sinus
VD	vas deferens
VE	verumontanum
WD	wolffian duct
UR	urethra





## **Introduction**

## **Chapter 1**

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### **Introduction and Scope of the Thesis**

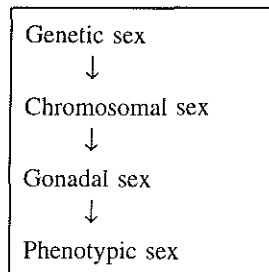
## 1.1. Introduction

### 1.1.1. Sexual differentiation

Sexual differentiation is a sequential process beginning with the establishment of the genetic sex at fertilization of the oocyte by a sperm. In the fertilized oocyte the testis determining gene drives the process of sexual differentiation into the male direction. In normal development the SRY gene that codes for the testis determining factor is located on the short arm of the Y chromosome. In males the indifferent gonad will turn into a testis, in females the gonad becomes an ovary. In the mammal, the heterogametic sex (XY) is male and the homogametic sex (XX) is female. The male phenotype is formed as a consequence of endocrine secretions of the testis. Female characteristics will develop in the presence of an ovary or if no gonads are present.

The presence or absence of the testis determining gene and the presence of XY or XX chromosomes are known as genetic sex and chromosomal sex, respectively (Table 1). Subsequently, the type of gonad depicts the gonadal sex, the male or female anatomic characteristics form the phenotypic sex. Ambiguous differentiation of sexual characteristics occurs if one of the steps is disturbed or lacking.

**Table 1.** Sequence of events in sexual differentiation.



### 1.1.2. Development of the sexual phenotype

In our society the distinction between male and female phenotype is considered absolute, and these terms are often used to emphasize opposites. Usually the components of an individual determining the sexual phenotype are indeed dominantly either male or female, conform to the sex established at the time of fertilization of the oocyte. During sexual differentiation almost all sexual phenotypic differences emerge from sexual indifferent bipotential precursors in the embryo (Table 2).

male	indifferent stage	female
testis spermatogonia Sertoli cells Leydig cells rete testis	indifferent gonad	ovary oögonia granulosa cells theca cells rete ovary
efferent ductules  paradidymis  appendix epididymis ductus epididymis  vas deferens seminal vesicle ejaculatory duct  trigone ureter renal pelvis collecting ducts	mesonephric tubules   mesonephric duct (wolffian duct)   (ureteral) bud of mesonephric duct	epoöphoron (transversal ductules) paroöphoron  appendix vesiculosa epoöphoron (longitudinal duct) gärtner's duct  trigone ureter renal pelvis collecting ducts
appendix testis	müllerian duct (paramesonephric duct)	hydatid fallopian tubes uterus
seminal colliculus posterior urethral valves	müllerian tubercle (sino- vaginal bulb)	hymen
prostatic utricle (masculine vagina)	urogenital sinus	vagina
bladder posterior urethra	urogenital sinus	bladder whole urethra vaginal vestibule
prostate	urogenital sinus	paraurethral gland (of Skene)
bulbourethral gland (of Cowper)	urogenital sinus	major vestibular gland (of Bartholin)
penis corpus cavernosum glans penis	urogenital tubercle	clitoris corpus cavernosum glans clitoridis
anterior urethra corpus spongiosum preputium	urogenital fold	labium minus  preputium clitoridis
scrotum	urogenital swelling	labium major
gubernaculum testis (cremaster muscle)	gubernaculum	ligamentum teres uteri

Table 2. Homologies between male and female urogenital structures.

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During organogenesis, the external genitalia of both sexes are derived from common primordia, the genital tubercle, folds, and swellings. In the female fetus these genitalia elongate but change very little: the genital tubercle becomes the clitoris, the genital folds become the labia minora, and the genital swellings become the labia majora. In the male fetus, phallic development begins with the appearance of the genital tubercle. Fusion and growth of the genital folds cause formation of the urethra and shaft of the penis and ultimately bring the urethral orifice to the end of the genital tubercle (glans penis) (Hunter, 1933). In man, the urethral groove starts fusing in the pelvic region at eight weeks of gestation, progressively bringing the ostium to the top of the urogenital tubercle in week 14 of gestation. If the urethral groove fails to fuse anywhere along its extent, hypospadias will be the result.

The embryologic origin of the prostatic duct epithelium is the urogenital sinus. In the 10th week of gestation, prostatic ducts originate as solid epithelial outgrowths from the urogenital sinus, which later canalize to form a ductal lumen. The mesenchyme of the urogenital sinus, which surrounds and supports the epithelial outgrowths, is the origin of the stromal component of the prostate.

Dimorphism in the development of the breast in human males and females has not been documented before puberty. The transient milk secretion in male and female newborn suggests no functional difference of the potency of differentiation into the male or female direction until puberty (Pfaltz, 1949; Wilson, 1991). This supports the idea that different time windows exist for different tissues considering sexual differentiation.

In contrast to the external features and the urogenital sinus, some structures of the internal genital tracts arise from different anlagen in male and female. The internal genitalia (as well as the renal collecting ducts) are derived from cells of the mesonephric kidney and ducts system. The mesonephric kidney develops from intermediate mesoderm in the thoracolumbar region. In both sexes, the wolffian ducts are the excretory ducts of the mesonephric kidney system and are connected anatomically with the indifferent gonad. In the human embryo, at approximately 6 weeks of gestation the development of the paramesonephric (müllerian) ducts begin as invaginations in the coelomic epithelium just laterally to the mesonephric ducts (Gruenwald, 1941). The müllerian ducts, which are not adjacent to the gonads, result in later life in fallopian tubes, uterus, and upper part of the vagina. The wolffian as well as the müllerian ducts are initially present in early embryos of both sexes. In the male fetus, the wolffian ducts give rise to the epididymides, vasa deferentia, and seminal vesicles; the müllerian ducts disappear. In the female fetus, the wolffian ducts disappear (or persist in some cases as gärtner's ducts).

### 1.1.3. Testosterone and Anti-Müllerian Hormone

Once the testes are present in the embryo, sexual differentiation becomes an endocrine process, as deduced by Alfred Jost (1947, 1953). He concluded that the development of the fetal testis is critical for male differentiation. Jost castrated male rabbit embryos before the onset of phenotypic differentiation. He established that castrated rabbit embryos of both sexes develop as females. When the extirpated gonad was replaced by a crystal of testosterone, the wolffian ducts virilized bilaterally, but the müllerian ducts did not regress. In contrast, when a fetal testis was implanted adjacent to a fetal ovary, the wolffian duct persisted, and in addition müllerian ducts regressed on the ipsilateral side. Masculinization of the fetus is the positive result of action by testicular hormones, whereas development of the female phenotype does not require hormones from the fetal ovary. Therefore, two types of testicular hormones are essential for establishing the male phenotype: anti-müllerian hormone (AMH), which causes regression of the müllerian ducts in male, and testosterone, which virilizes the embryo. AMH is a glycoprotein formed by the Sertoli cells, and prevents development of the uterus and fallopian tubes in male. The second hormone, testosterone can be converted to a more potent metabolite  $5\alpha$ -dihydrotestosterone.

The main androgen secreted by the Leydig cells of the fetal testis is testosterone. In adults the testicular androgen production is under control of the hypothalamic-pituitary-gonadal axis. Testosterone concentrations in the spermatic vein are approximately 75 times higher than the levels detected in serum of peripheral venous blood (see Coffey, 1992 for a review). Peripheral levels range from 10-40 nmol/l. Testosterone circulates mainly bound to two proteins: sex hormone binding globulin and albumin. The unbound or free hormone comprises 1 to 3 percent of the total serum level. In fetal life Leydig cells appear in the gonad at week eight of gestation (Baillie et al., 1966). It is not known whether testosterone synthesis at the onset of hormonal production in the human fetal testis is either regulated by the fetal pituitary (Huhtaniemi et al., 1977, Molsberry et al., 1982) or is initiated by chorionic gonadotropin produced by the placenta (Frowein and Engel, 1974), or is autonomous (Catt et al., 1975; George et al., 1978; George and Wilson 1980). There is a peak in testosterone production at week sixteen of gestation (Kaplan et al., 1978), followed by a decline because of Leydig cell involution. From week twenty onwards androgen production reaches levels equal to that seen in female fetuses. After the first one-third of gestation, Leydig cell function is definitively controlled by fetal pituitary gonadotropin secretion (Kaplan et al., 1976).

#### 1.1.4. 5 $\alpha$ -Dihydrotestosterone

Differentiation of the male phenotype from the stage, when the genitalia of both sexes are indifferent, except for the gonads, is caused by two androgens (or "man-makers"): testosterone and 5 $\alpha$ -dihydrotestosterone. In most target cells testosterone is reduced to 5 $\alpha$ -dihydrotestosterone. Both androgens bind to the same receptor. The finding that testosterone and 5 $\alpha$ -dihydrotestosterone act via the same androgen receptor protein in cell nuclei is substantiated by studies of the androgen insensitivity syndrome (see chapter 1.1.5). 5 $\alpha$ -Dihydrotestosterone has a higher affinity for the receptor than testosterone (Grino et al., 1990). The 5 $\alpha$ -dihydrotestosterone complex enhances activity of a reporter gene system approximately 10 times more efficiently than testosterone does (Deslypere et al., 1992). Consequently, 5 $\alpha$ -dihydrotestosterone amplifies a rather weak testosterone signal.

However, testosterone and 5 $\alpha$ -dihydrotestosterone play separate roles in male differentiation. In the regulation of some genes testosterone and 5 $\alpha$ -dihydrotestosterone have a specific function (van der Schoot, 1990; George et al., 1991). Testosterone is responsible for the regulation of the secretion of luteinizing hormone by the hypothalamic-pituitary system (Griffin and Wilson, 1989), penile erection (Heaton and Varrin, 1994), sexual behavior (Vreeburg et al., 1972), muscle development, voice deepening, scrotal rugation and pigmentation, axillary and pubic hair development (Randall, 1994a). Normal spermatogenesis relies on locally higher testosterone levels than in serum (Roberts and Zirkin, 1991). In male sexual development testosterone stabilizes the wolffian ducts in the male fetus, in contrast to the female fetus in which the wolffian duct system regresses.

5 $\alpha$ -Dihydrotestosterone is responsible for differentiation of the male external genitalia, male urethra and prostate during organogenesis. 5 $\alpha$ -Dihydrotestosterone initiates most of the androgen mediated events of male sexual maturation at puberty (growth of facial and body hair, temporal regression of scalp hair, maturation of external genitalia). The enzyme 5 $\alpha$ -reductase is present in these tissues before initiation of male differentiation takes place (Siiteri and Wilson, 1974). 5 $\alpha$ -Reductase which catalyzes the conversion of testosterone to 5 $\alpha$ -dihydrotestosterone, exists in more than one form (Andersson et al., 1991). 5 $\alpha$ -Reductase type 2 shows an acidic pH optimum of enzyme activity of 5.5, the enzyme with a more alkaline pH optimum of 7.5, is termed 5 $\alpha$ -steroid-reductase type 1. The type 1 enzyme gene was characterized first, and is located on chromosome 5. The gene encoding type 2 is located on chromosome 2. The type 2 isoenzyme is expressed in normal fetal and adult prostate and external genitalia (Silver et al., 1994). Type 1 is expressed in skin and a few other tissues. Type 2 is more sensitive to the 5 $\alpha$ -reductase inhibitor finasteride. Deficiency of the 5 $\alpha$ -reductase enzyme type 2 is one of the causes of impaired androgen action in the fetus (Thigpen et al., 1992). It results in male pseudohermaphroditism (Walsh et al., 1974; Imperato-McGinley et al., 1974). Affected individuals have a syndrome called pseudovaginal

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perineoscrotal hypospadias: the external genitalia fail to virilize, while the wolffian duct derivatives (epididymis, vas deferens and seminal vesicle) develop normally. At puberty, a partial masculinization of external genitalia can occur by testosterone either by a rise of hormone production by the testes or by exogenous supply of androgens. The molecular cause of  $5\alpha$ -reductase enzyme type 2 deficiency are mutations in the gene. However, the frequency of type 2 gene mutations and consequently a lower or no production of  $5\alpha$ -dihydrotestosterone, is low (28 different mutations in 37 families reported, see Wilson et al., 1993 for a review).

It is intriguing in which way the hormonal signal transduced by one androgen receptor is translated into different physiological effects, depending on the ligand. Testosterone is needed for virilization of the wolffian duct structures, reduction to  $5\alpha$ -dihydrotestosterone is a prerequisite for virilization of the remaining tissues. Although testosterone and  $5\alpha$ -dihydrotestosterone perform different physiological roles, the two hormones both bind to the same androgen receptor (Griffin and Wilson, 1989). One explanation for the differential action is that the androgen receptor in the wolffian duct is processed differently in comparison to the receptor in the external genitalia of the fetus and adult man. Another possibility could be that the differential action in testosterone-dependent tissues requires as yet unknown cell-specific factors (Randall, 1994b). A third hypothesis is that the androgen receptor in wolffian duct is the same, but it recognizes testosterone due to its locally high concentration. Veyssi re et al. (1980) proposed that testosterone virilizes the wolffian duct supplied to this structure in an exocrine fashion at a much higher concentration than to the other androgen target organs, presumably either by direct transport from the testis down the lumen of the wolffian or mesonephric duct, or by diffusion. This hypothesis is consistent with the observation that male differentiation starts by stabilization of the wolffian duct. Before testosterone serum concentration begins to increase, testosterone appears in the testis, one to two weeks prior to external virilization (Price et Ortiz, 1965). It has been demonstrated that high concentrations of testosterone reach the wolffian duct by transport from the testis down the lumen (Veyssi re et al., 1982). Furthermore in the wolffian duct of some mammalian species  $5\alpha$ -reductase activity is absent until the completion of sexual differentiation (Wilson and Lasnitzki, 1971; Siiteri and Wilson, 1974; Tsuji et al., 1994): consequently,  $5\alpha$ -dihydrotestosterone is not synthesized in cells of the wolffian duct during sexual differentiation.

Compelling evidence of testosterone transport from the testis down the lumen of the wolffian duct was found in human hermaphrodites, who develop a male phenotype, with an unilaterally developed vas deferens at the side of the testis (Grumbach et al., 1955). Consistent with this theory are cases of women with virilizing congenital adrenal hyperplasia, a syndrome characterized by increased levels of circulating androgen in the absence of a testis. In these females the external genitalia are masculinized, while wolffian ducts are not stabilized (Griffin and Wilson, 1992).

**Table 3.** Summary of hormonal control of male phenotypic sex differentiation.

<i>Phase of phenotypic differentiation</i>				
<i>Gonadal hormone</i>	<i>Active hormone at the target cell</i>	<i>Müllerian duct regression</i>	<i>Wolffian duct differentiation</i>	<i>Virilization of urogenital sinus and external genitalia</i>
<i>Anti-Müllerian hormone</i>	<i>Anti-Müllerian hormone</i>	+		
<i>Testosterone</i>	<i>Testosterone</i>		+	
<i>Testosterone</i>	<i>5<math>\alpha</math>-Dihydro-testosterone</i>			+

### 1.1.5. Androgen Insensitivity Syndrome

In 1817 the first female was described with intra-abdominal testes, where ovaria were expected (Steglehner, 1875). Testosterone-dependent tissues of wolffian duct origin and 5 $\alpha$ -dihydrotestosterone-dependent tissues of the urogenital sinus and urogenital tubercle fail to masculinize in humans and animals with impaired androgen receptor function. The syndrome of testicular feminization has been described in many different species: man, chimpanzee, mouse, rat, and domestic cat. The syndrome is associated with variable defects of virilization of the genetic male. Androgen insensitivity syndrome (AIS) is subdivided in the complete form and incomplete forms (Patterson et al., 1994). Testicular feminization is also referred as the complete form of AIS. In the complete AIS, the phenotype is female with underdevelopment of the labia and a blind ending vagina, paucity of axillary and pubic hair. Incomplete AIS is associated with partial virilization of the external genitalia. The labioscrotal folds are partially fused and clitoromegaly is common. Wolffian duct structures are present in contrast to the syndrome of complete feminization. The ejaculatory ducts empty in the vagina. A milder form of incomplete AIS is the Reifenstein syndrome, predominantly a male phenotype with penoscrotal hypospadias, cryptorchidism and gynecomastia at the time of expected puberty. The infertile male syndrome is a forme fruste, which implies a male with azoospermia or extreme oligospermia (with or without a positive family history for the Reifenstein syndrome) and low binding capacity of cultured genital skin fibroblasts (Aiman et al., 1979; Yong et al., 1994; Puscheck et al., 1994). Complete or gross deletions of the



androgen receptor gene are rare in patients with the complete AIS. Point mutations at several different sites in exons 2-8 encoding the DNA- and androgen-binding domains respectively, have been reported for partial and complete forms of androgen insensitivity (Brinkmann et al., 1994). The frequency of the syndrome is estimated on 1 in 20,000 to 1 in 64,000 male births, and in 1 to 2 percent of phenotypical women with inguinal hernia (Griffin and Wilson, 1989). The relative high frequency of the syndrome is due to the X-linked recessive inheritance, which becomes clinically manifest in all affected males (XY). The syndrome has no impact on life expectancy. The trait is transmitted from carrier females to half of their genetic sons and daughters according to a pattern of single gene (mendelian) inheritance.

### 1.1.6. Androgen receptor expression pattern during fetal development

During early development of primordia of male genitalia the expression pattern of androgen receptors differs from that of older stages, in that androgen receptors are initially observed only in the mesenchymal cells. Androgens induce epithelial differentiation of reproductive organs via androgen action in the mesenchymal cells (Cunha, 1991). For example, the epithelium of the fetal urogenital sinus of the rat remains androgen receptor negative during prenatal morphological differentiation of the prostate when epithelial prostatic buds appear and when prostatic mesenchyme is androgen receptor positive yet. Only postnatally androgen receptor is detected in prostatic epithelium (Chapter 4 of this thesis; Cooke et al., 1991; Takeda et al., 1985, Husmann et al., 1990).

Tissue recombination studies in mice yielded a role for the mesenchyme in androgen-mediated differentiation of the urogenital tract (Cunha et al., 1983). For example, when tissue recombinants of embryonic urinary bladder epithelium and urogenital sinus mesenchyme are exposed to androgens, the epithelium develops prostatic buds. However, recombinants of epithelium from the urogenital sinus and mesenchyme from other embryonic sites are incapable of prostatic development under similar circumstances. Furthermore, mesenchyme from the urogenital sinus of the androgen-insensitive testicular feminization (Tfm) mouse fails to mediate prostatic growth when recombined with normal urogenital sinus epithelium, whereas epithelium from the urogenital sinus of the Tfm mouse undergoes prostate formation in the presence of androgen when recombined with normal mesenchyme (Cunha and Lung, 1978; Lasnitzki and Mizuno, 1980). Similarly, androgen-mediated regression of the nipple primordia of the mammary gland in the male mouse requires an androgen-responsive mesenchyme (Wasner et al., 1983). When normal, wild-type mouse mammary epithelium, containing a nipple rudiment, is recombined with mammary mesenchyme from an androgen-resistant Tfm mouse, normal androgen-dependent regression of the bud does not occur, whereas mammary bud regression does occur in recombinants of wild-type mesenchyme and Tfm epithelium (Kratochwil and Schwartz, 1976). These well-designed studies indicate that

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tissue responses to androgens in male fetuses are either a stimulation or an inhibition of epithelial cell proliferation mediated via the mesenchyme. In the mesenchyme-epithelial interactions, the growth factor keratinocyte growth factor (KGF), a member of the FGF family, has been suggested to act as a paracrine mediator between mesenchyme and epithelium (Finch et al., 1989; Cunha et al., 1994). KGF expression is androgen dependent and production has been demonstrated in mesenchymal prostate cells (Yan et al., 1992). The KGF receptor (Johnson and Williams, 1993) is exclusively expressed in the epithelial cells. Another candidate is Hepatocyte growth factor (HGF) produced by mesenchymal cells, with the Met proto-oncogene as its receptor (Birchmeier and Birchmeier, 1993; Tsarfaty et al., 1994). Other candidates for induction of epithelial differentiation are the retinoic acid receptor  $\beta$  (RAR  $\beta$ , Dolle et al., 1990), the prostaglandins PGE<sub>2</sub> and PGE<sub>2a</sub> (Gupta and Bentlejewski, 1992) and epidermal growth factor (EGF, Gupta and Jaumotte, 1993).

### 1.1.7. Regulation of androgen receptor expression

Modulation of androgen receptor levels by androgens has been studied in several human model systems, but in particular in the prostate (Shan et al. 1990; Ruizeveld de Winter et al. 1992; van der Kwast et al. 1991, 1994). Androgens are essential for prostate development, and important for maintenance of the structure and the function of the adult prostate. Androgen ablation or blockage of androgen receptor function by anti-androgens results in a rapid reduction in prostate size via programmed cell death (apoptosis) (Kerr and Searle, 1973; Kyprianou and Isaacs, 1988). This feature is used in the treatment of benign prostatic hyperplasia and tumors of the prostate (Wilson, 1980; Schröder 1993, 1994, Brinkmann and Trapman 1995). The beneficial effect of castration on growth of prostate cancer was first scrutinized by Huggins and Hodges (1941), (a detailed discussion on the role of the androgen withdrawal in prostatic diseases is beyond the scope of this thesis).

In rats, androgen withdrawal causes a decrease in androgen receptor levels in the prostate (Husmann et al., 1990; van Doorn et al., 1976). The size of the remaining rat prostate is less than 15% of that in the intact animal. Involution of the prostate is a reversible process. Administration of testosterone results in complete prostate regeneration, including androgen receptor expression (Prins and Birch, 1993). The stability of the androgen receptor is enhanced by its ligand. Androgen regulated genes detected in the regenerating prostate might be important in normal prostate development. The knowledge about the genes which are induced in the prostate after testosterone administration to castrated animals is a field of important research these days (Furuya and Isaacs, 1993).

The regulation of the androgen receptor expression is organ-specific and age-dependent. In the rat phallus, androgen receptor mRNA levels decrease in response to elevated levels of androgen at sexual maturation. Androgen receptor expression shows a permanent decrease

after puberty, which can be induced by administration of androgens. In the adult male rat, the limitation of penile growth is related to the decrease in the number of androgen receptor molecules in the corpora cavernosa, the os penis and other parts of the body of the penis, but in the nuclei of the skin epithelium and in the cells of the urethra androgen receptor is still expressed (Takane et al., 1990, 1991; Gonzalez-Cadavid et al., 1991).

Another pattern of expression is found in the rat testis where androgen receptor expression is stage dependent. The receptor in Sertoli cells is only detectable in specific spermatogenic stages. The mechanism of the stage dependent expression is unknown. Androgen receptor expression in peritubular myoid cells, arterioles, and Leydig cells is unrelated to the stage of the adjacent seminiferous tubules (Bremner et al., 1994). Testosterone or its metabolites control the overall expression of the androgen receptor in the rat testis. No androgen receptor was detected after withdrawal of endogenous testosterone.

In defined cell models, the level of androgen receptor mRNA was decreased by androgen (Quarmby et al., 1990; Krongrad et al., 1991), while transiently the cellular level of immunoreactive androgen receptor increased (Krongrad et al., 1991). Kemppainen et al. (1992) showed in vitro an increase of half-life of androgen receptor in the presence of androgen. The improved stability of the receptor protein induced via its ligand may yield more androgen receptor protein despite a decreased transcription rate of the genes.

### 1.1.8. Molecular structure of the androgen receptor

The androgen receptor belongs to the superfamily of ligand-dependent transcription factors (Evans, 1988). This family includes the receptors for androgens, estradiol, progesterone, glucocorticoids, mineralocorticoids, retinoids, thyroid hormone, and vitamin D. These receptors have a characteristic three domain structure in common: (1) an amino-terminal domain thought to be involved in gene regulation; (2) a DNA binding domain that contains two zinc fingers for binding to DNA; and (3) a C-terminal domain that binds the ligand. The preservation of the nucleotide sequence is highest in the DNA binding domains and the ligand binding domains (O'Malley, 1990). The amino-terminal domains of these receptors show little homology. The ligand binding domain and the DNA binding domain are connected via a hinge region. The function of the hinge region is unclear, part of it plays a role in the nuclear transport of the receptor (Jenster et al., 1993).

The human androgen receptor protein is encoded by one gene located on the long arm of the X chromosome (Lubahn et al., 1988; Chang et al., 1988; Trapman et al. 1988; Faber et al., 1989; Tilley et al., 1989). The androgen receptor cDNA has also been cloned for the mouse, rat, dog, guinea pig and clawed frog (He et al., 1990). Sequence comparison of the rat androgen receptor with the human androgen receptor revealed 83% nucleotide homology, and 85% homology at the amino acid level (Tan et al., 1988). The human androgen receptor

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cDNA has a length of 10.6 kb. The 5'- and 3'-untranslated regions are remarkable long, 1.1 and 6.8 kb, respectively. The open reading frame is 2.7 kb. The reported cDNA sequences represent a protein of approximately 910 amino acid residues, depending on the length of two long, hypervariable amino-acid stretches in the amino-terminal domain, the polyglutamine and polyglycine stretches, respectively (Faber et al., 1989; Tilley et al., 1989; Chang et al., 1988; Lubahn et al., 1988). The protein is encoded by eight exons: the amino-terminal domain is encoded by one large exon (Faber et al., 1989), the DNA binding domain by exons 2 and 3, the hinge region and the ligand binding C-terminal domain by the remaining five exons (Lubahn et al., 1989; Kuiper et al., 1989; Marcelli et al., 1990).

The androgen receptor protein is complexed with heat shock proteins in the absence of ligand (Veldscholte et al., 1992). Ligand binding results in dissociation of the steroid-receptor heat shock protein-complex, and in activation of the receptor. Upon ligand-binding, phosphorylation of the androgen receptor increases (Van Laar et al., 1991; Kuiper et al., 1993). Phosphorylation of steroid receptors provides a mechanism for cell specific and differential regulation of gene expression by cell or tissue specific kinases (Ortí et al., 1992; Kuiper and Brinkmann, 1994). The activated androgen receptor-ligand complex binds as a dimer to specific enhancer sequences called androgen responsive elements present in the 5'-flanking region of genes that are androgen regulated (Riegman et al., 1991).

The transcription start site of the androgen receptor gene is located approximately 1 kb upstream of the translation initiation codon. The promoter of the rat (Baarends WM et al., 1990), the mouse (Faber et al., 1991b) and the human androgen receptor (Tilley et al., 1990; Faber et al., 1991a) show two major sites of transcription initiation. In the mouse androgen receptor a second promoter, which is regulated by 5 $\alpha$ -dihydrotestosterone (Grossmann et al., 1994) and a suppressor element in the 5'-flanking region have been found (Kumar et al., 1994). No TATA or CCAAT box were found. The promoter contains one Sp1 binding site upstream of one of the two major closely linked transcription initiation sites, which are located in a 13 bp region. Expression of Sp1 is elevated during sexual differentiation of the mouse. Sp1 may have a regulatory function in the transcription of the androgen receptor gene (Saffer et al., 1991). Sequences both upstream and downstream of the two start sites are involved in androgen receptor expression, as shown by deletion analysis (Faber et al., 1993). An age-dependent factor (ADF) has been reported as a regulatory element in the rat androgen receptor promoter associated with age dependent expression of the androgen receptor (Supakar et al., 1993).

### 1.1.9. The undescendent testis

Testicular descent is a complex process that involves transabdominal movement of the developing testis, formation of the processus vaginalis, and movement of the testis through the inguinal canal into the scrotum (Backhouse, 1982). In the persistent müllerian duct syndrome the testes are located at the same position as the ovaries in females. These observations suggest, that transabdominal movement of the testis is regulated by anti-müllerian hormone (Josso et al., 1983).

Inguinoscrotal descent of the testis is thought to be directly or indirectly mediated by androgens. Compelling evidence is provided by the findings that cryptorchidism is a common clinical feature associated with genetic disorders. These involve abnormalities in gonadotropin production and androgen secretion or action (Bardin et al., 1969; Santen and Paulsen, 1973; Cattanach et al., 1973; Steiner and Boggs, 1965). Furthermore, in the rat and the rabbit, estradiol treatment completely inhibits testicular descent. Presumably, primarily gonadotropin secretion by the pituitary is inhibited and secondarily testicular androgen production (Rajfer and Walsh, 1977; Rajfer, 1982). Degeneration of the cranial suspensory ligament of the gonad is androgen dependent (van der Schoot and Elger, 1992). Fentener van Vlissingen et al. (1988) suggested an additional unidentified testicular substance, named "descendin", responsible for gubernacular outgrowth. Van der Schoot and Elger showed failure of androgens to affect perinatal growth of the gubernaculum (1993). However, consistent with the concept that the gubernaculum is a site of androgen action in testicular descent, George and Peterson first identified a low amount of specific, high affinity androgen receptor in the newborn rat gubernaculum (1988). The androgen receptor in the gubernaculum has been located predominantly in the mesenchymal cells in the core of the tissue (Husmann and McPhaul, 1991).

Since 1762, when John A. Hunter described for the first time the fetal gubernaculum, various mechanisms have been proposed for the final placement of the testes into the scrotum. These include the following events. (1) Traction of the testis by the gubernaculum and/or cremaster muscle. Support for this concept comes from the observation that severance of the genitofemoral nerve, which innervates the gubernaculum, prevents testicular descent in rodents. Recent studies have shed some light on the genitofemoral nerve with respect to the release of neurotransmitters such as calcitonin gene-related peptide. The release is controlled by androgens (Terada et al., 1994; Cain et al., 1994). (2) The immobile gubernaculum anchors the testis during growth of the body wall (Shono et al., 1994) and leads the testis to the scrotum. (3) Intra-abdominal pressure pushes the testis through the inguinal canal that is increased in diameter by a swollen gubernaculum. (4) Development and maturation of the epididymis may be responsible for testicular migration. In boys with undescended testes 80% revealed anatomical anomalies (an underdeveloped or abnormal ending processus vaginalis, a

mechanical obstruction in the inguinal canal), that would explain the failure of testicular descent (Hazebroek et al., 1987). In conclusion, it has become clear, that multiple events are involved in testicular descent (Takeshi and Hutson, 1994). In spite of these data, the mechanism by which androgens act to promote testicular descent is still not clear.

### 1.1.10. Molecular causes of hypospadias

Hypospadias is a congenital defect of the penis. The abnormal urethral opening (meatus) is located at the ventral side of the penis, and may be anywhere along the shaft, or along the scrotal or perineal raphe. The anatomical features consist of an ectopic urethral meatus, a lack of a frenulum with an abnormal distribution of preputial skin causing a cleft preputial skin at the ventral side and a surplus of skin on the dorsal side, and a chordee of fibrous tissue, causing a bending of the penis with a concave ventral curvature. The most common classification of hypospadias relates to the location of the ectopic meatus (glandular, on the distal shaft or the proximal shaft of the penis, scrotal, or perineal). Hypospadias is the most common congenital anomaly of the penis. The incidence is about 1 out of 300 live male births (Duckett, 1992). An increase in registrations of hypospadias has been noted (Kallen and Winberg, 1982; Matlai and Beral, 1985). The increase may be related to higher estrogen exposure in utero (Sharpe and Skakkebaek, 1993). Early in pregnancy, when the placenta produces low levels of progesterone and estrogens, exposure to external estrogens and progestins (Aarskog, 1979) causes disorders in outgrowth of male reproductive organs. In sons of mothers treated with the synthetic estrogen diethylstilbestrol (DES) the incidence of hypospadias increased to 1 out of 25 boys (Henderson et al., 1976).

In man, elevated levels of estrogens may cause congenital abnormalities in the first trimester (Mittwoch et al., 1993). In rats (Vannier and Raynaud, 1980) and in mice (Beckman et al. 1994) fetal exposure to estrogen induces male pseudohermaphroditism and persistence of müllerian ducts (Newbold et al., 1984). In which way estrogens are effective in the etiology of hypospadias is a major unsolved problem, hence two possible explanations can be considered:

- (1) decreased testicular androgen production by suppression of the hypothalamus-pituitary-testis axis via a feed-back mechanism. Estrogens may decrease the release of gonadotropins from the pituitary gland and subsequently retard the gonadal development and function.
- (2) direct action of estrogens via the estrogen receptor during male sexual differentiation in the first trimester of gestation. Male external genitalia lack the estrogen receptor in the second and third trimester of gestation (Kalloo et al., 1993).

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The balance between androgens and estrogens at the level of the developing urogenital tubercle may determine the regulation of the expression of the androgen receptor and estrogen receptor. In the monkey prostate, estrogen receptor expression is increased after castration and decreased by androgens in the circulation (West et al., 1988). The role of estrogens in normal male phenotypic development is unclear, however, in absence of the estradiol-mediated responsiveness by disruption of the estrogen receptor both in male and female mice the reproductive tracts are severely affected (Lubahn et al., 1993).

Disorders of male sexual differentiation can result from defective or delayed androgen action. Hypospadias can occur in individuals with impaired androgen action due to either abnormalities of androgen production or expression of abnormal androgen receptors caused by mutations in the receptor gene (Kaspar et al., 1993), or by lack of conversion of testosterone to 5 $\alpha$ -dihydrotestosterone due to mutations in the 5 $\alpha$ -reductase enzyme type 2 gene (Griffin and Wilson, 1989).

Only rarely, hypospadias is due to male pseudohermaphroditism. The majority of hypospadias patients have no mutations in the androgen receptor gene (Evans et al., 1991), nor in the 5 $\alpha$ -reductase enzyme type 2 gene, and have normal expression levels of both proteins (Chapter 6 of this thesis; Silver et al., 1994). In the majority of hypospadias cases there is no history of estrogen intake during pregnancy or familial occurrence (de Vries, 1986). In a few patients with isolated hypospadias and cryptorchidism who did not have any other congenital abnormalities sex chromosomal anomalies have been reported (Yamaguchi et al., 1991).

Allen and Griffin (1984) presented evidence in support of the concept that hypospadias is rather an endocrinopathy of the child. Abnormal penile development would be more a local manifestation of a general delay in androgen action than a local dimorphic problem. Often hypospadias is presented in combination with cryptorchidism or an utriculus (Devine et al., 1980). The associated abnormalities suggest a general cause for hypospadias. Certainly, these data are consistent with an insensitivity to androgens in the target cells during differentiation as a possible causative factor.

The possibility that an abnormal response to 5 $\alpha$ -dihydrotestosterone is due to abnormal binding capacities of the androgen receptor has been studied extensively (Svensson and Snochowski 1979; Terakawa et al., 1990; Gearhart et al., 1988). In the past, biopsies of foreskin at the time of reconstructive surgery have been studied. Genital skin fibroblasts were cultured for radioligand binding studies. Using this technique the maximum binding capacity and the affinity of 5 $\alpha$ -dihydrotestosterone for the androgen receptor can be determined (Brown and Migeon, 1981). Results, concerning the content of androgen receptors in penile skin is decreased in hypospadias, have been either controversial or inconclusive. The etiology of hypospadias is unknown in most cases.

### 1.1.11. The rat as a model for androgen action in differentiation of the genital tract

The rat has been used for studies on normal penile development (Kluth et al., 1988), on microphallus development (Husmann and Cain, 1994), on hypospadias (Clark et al., 1993; Imperato-McGinley et al., 1992), on testicular descent (Spencer et al. 1991; van der Schoot 1992; Husmann and McPhaul, 1991) and on prostate development (Timms et al., 1994, Prins and Birch, 1995). It is known already for more than 30 years that progesterone interferes with urethral groove fusion in male rat fetuses (Junkmann and Neumann, 1964). Severe hypospadias in most of male rat offspring occurred after administration of progesterone to pregnant rats. On the other hand, in female fetuses progestins had a virilizing effect. The apparently paradoxical effect of progestins on genital organogenesis of the male and female fetuses is analogous to that observed in children born with a severe form of congenital virilizing adrenal hyperplasia due to  $3\beta$  hydroxysteroid dehydrogenase deficiency (Bongiovanni, 1964). Genetic males are born with hypospadias, whereas genetic females have clitoral hypertrophy after prenatal exposure to elevated levels of dehydroepiandrosterone.

Antiandrogens, like cyproterone acetate, given to pregnant rats can induce feminization of male fetuses (Neumann et al., 1970). Abnormal penile development in the rat has been studied after prenatal exposure of  $5\alpha$ -reductase inhibitors (Clark et al., 1993) and also after androgen receptor blockade (Imperato-McGinley et al., 1992).

Table 1. Effect of Flutamide on sexual differentiation

Pregnant rats were treated with 25 mg/kg x day of Flutamide  
from day 12 to day 21 of gestation.

Analysis of the male offspring was done at the age of 95 days pn.

Group	Hypospadias Cleft Prepuce Chordee	Penile Length mm $\pm$ SD	Unilateral Undescend. Testis	Anogenit. Distance mm $\pm$ SD
Control	0/8	10.4 $\pm$ 1.3	0/8	48.5 $\pm$ 4.9
Flutamide	11/15*	6.7 $\pm$ 1.2	11/15* (9 left)	25.3 $\pm$ 2.5

\*significantly different from control,  $p < 0.001$

The period of phenotypic sex differentiation in the rat is between day 14 and day 18. On day 14 testicular differentiation is visible. On day 15 the testicular secretion of androgens starts, while at the same time initiation of regression of the müllerian duct in the male fetus



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occurs. On day 16 regression of the wolffian duct starts in female fetuses. On day 17 of pregnancy seminal vesicles appear, and on day 18 the first prostatic buds are starting to develop. Term is day 22. Hypospadias was investigated in this thesis by exposing fetal male rats to flutamide during the period of sexual differentiation. For this experiment pregnant Wistar rats have been treated with 25 mg/kg of the anti-androgen flutamide from day 12 to day 21 of gestation. The male offspring was examined for hypospadias.

Figure 1 shows a photograph of a rat 95 days old with a normal penis and a male rat of the same age with hypospadias after treatment with 25 mg/kg flutamide during fetal sexual differentiation. In the male rats exposed to flutamide 11 out of 15 developed hypospadias. Hypospadias was characterized by an abnormal location of the urethral orifice, a cleft prepuce at the ventral side, and a bending of the penis causing a concave curvature at the ventral side of the penis. These studies show the development of a model for hypospadias in rat when androgen binding to the androgen receptor is inhibited. The treatment resulted in undescendent testis, while penile length and anogenital distance were decreased (Table 1).

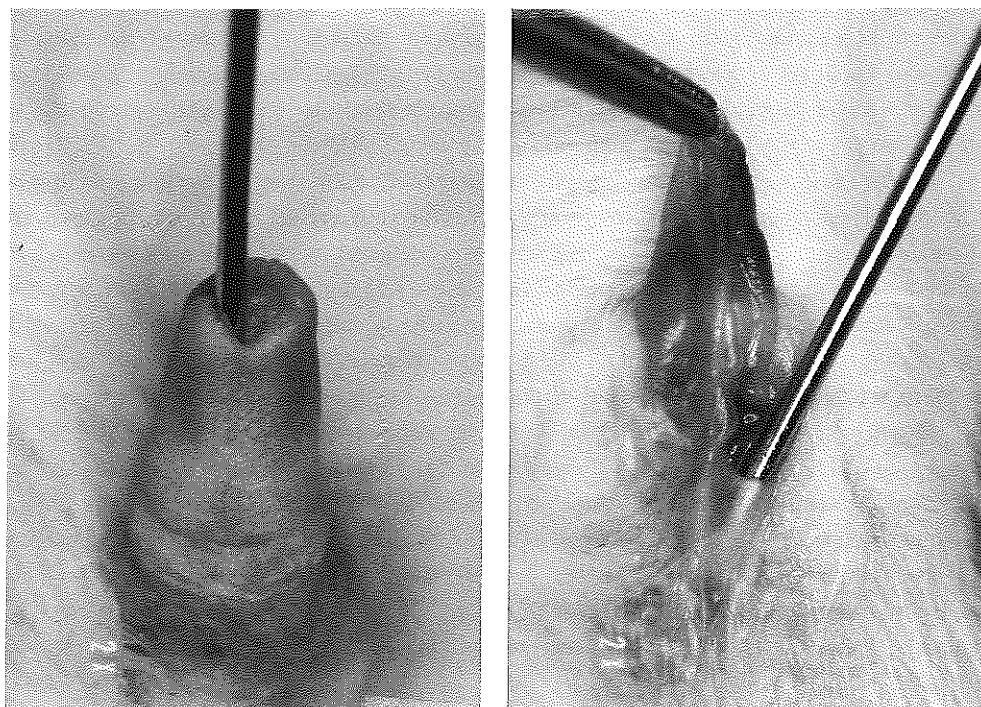


Fig. 1. Effect of 25 mg/kg flutamide, given to pregnant rats, on hypospadias development in the male offspring a) a normal rat penis, b) a hypospadias penis in rat (rats are 95 days pn).

### 1.2 Scope of the thesis

The aim of this study was to determine the level and the pattern of expression of the androgen receptor in target tissues of the fetal and adult rat, and to understand the mechanism behind the hormonal influence on androgen receptor protein expression during fetal sexual differentiation. In addition, androgen receptor expression in the adult rat was studied. To perform this work, immunological assays have been developed to overcome the limitation of radio-ligand binding assays. Quantitative determination of the androgen receptor levels directly in tissue homogenates with a radio-ligand assay is complicated by endogenous testosterone levels either bound to plasma proteins or to the androgen receptor itself. Time-consuming exchange assays affect considerably the integrity of the androgen receptor protein, because of proteolytic breakdown. Another drawback from which this technique suffers, is the amount of tissue needed to perform the assay. In this thesis a sensitive method has been developed and applied in which relatively small amounts of fetal tissue were sufficient for immunoreactive androgen receptor protein content determination by an immunoblotting technique. The effect of manipulation of the expression in adult male rats is presented and discussed in Chapter 2. In Chapter 3 the androgen receptor in tissues of the fetus is discussed. The level of expression in male and female urogenital tissues has been established in the developing urogenital tract and androgen receptor levels are investigated after androgen administration and after androgen inhibition during sexual differentiation.

In the past steroid autoradiography studies have been used to examine androgen receptors in fetal and neonatal histological tissue sections. However, long exposure times are needed for this type of studies. Only radioactivity is detected, which includes metabolites of androgens with different binding characteristics for the receptor. In the cells that are negative in the autoradiographs, occupancy by endogenously bound ligand cannot be excluded, resulting in a false negative picture. Mono- and polyclonal specific antibodies against the androgen receptor have been used in an immunohistochemical assay for rapid detection of cellular receptor distribution. In Chapter 4, cellular and tissue androgen receptor distribution during sexual differentiation in the fetal rat was studied as assessed by immunohistochemistry, showing mesenchymal localization of the androgen receptor in the urogenital tissues before epithelial expression. The chronological order of androgen receptor expression in fetal and neonatal tissues is presented. Additionally, the question, whether androgens enhance expression of the androgen receptor in developing rat genital tract by increasing the frequency of androgen receptor positive cells or by the density per cell, was addressed.

During fetal and neonatal development impaired androgen action has been described as a cause of the undescendent testis and hypospadias. For a better understanding of androgen action in normal testicular descent and penile formation and in certain diseased states, a

## **Introduction**

detailed study of androgen receptor expression in the normal and pathological situation is described in Chapters 5 and 6. Androgen receptor expression was investigated in a rat model, and in boys with and without hypospadias respectively. Finally, in Chapter 7 the results from the former chapters are discussed in a broader context. The pathological disturbance of androgen receptor expression and the consequence for impaired virilization of the wolffian duct and the urogenital tubercle are considered in the general discussion. Proposals for future research on androgen receptor expression are made with respect to unsolved physiological processes and important clinical questions.



## Chapter 2

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### **Tissue distribution and regulation of the immunoreactive androgen receptor in the adult rat**

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Submitted

**Abstract**

Androgen receptor (AR) distribution in male and female rats, measured by a Western (immuno-)blot assay, revealed a broad distribution of the receptor in both male and female tissues. Highest amounts of AR (10-100% compared to prostate) were detected in tissues of the male and female urogenital tract and in the adrenal. In another set of tissues, including the androgen-dependent levator ani/bulbocavernosus muscles, the preputial glands, scrotal skin, vagina, uterus, and ureter, AR levels were 1-6% of those seen in prostate. In other tissues including skeletal and cardiac muscles, kidney, lung, liver, gut, spleen, abdominal skin, cerebrum, cerebellum, hypothalamus, and pituitary, the receptor is expressed at low, barely detectable levels (<1% of the prostate control). Castration caused an 80% decrease in AR level in ventral prostate and a 67% decrease in the levels in seminal vesicle but had no effect on adrenal AR. In contrast, hypophysectomy caused a profound decrease in AR levels in the adrenal, ventral prostate and seminal vesicle. Growth hormone and, to a lesser extent, prolactin treatment caused an increase in detectable AR levels in prostate, and seminal vesicle, but not in adrenal of hypophysectomized rats. High doses of dexamethasone (10 µg/100 g bw/d, sc) caused a reduction in adrenal wet weight, but had no effect on adrenal AR levels. These studies confirm that in the urogenital tract, AR is modulated by androgen and indicate that AR in the adrenal is under the control of as yet unidentified hormone(s).

**Introduction**

Androgen receptor (AR), the intracellular protein that mediates androgen action, is a member of the steroid-thyroid hormone-vitamin D-retinoid class of transcription regulatory factors (Evans 1988). This protein (Lubahn et al. 1988; Chang et al. 1988; Trapman et al. 1988; Tilley et al. 1989) is encoded on the X chromosome and mediates the virilization of the male fetus and androgen action during postembryonic male sexual maturation (Griffin & Wilson 1989; Goldstein JL & Wilson JD 1975).

Two techniques have been widely utilized to examine AR distribution. Ligand binding assays have provided insight into the tissue distribution of the receptor, and immuno-histochemical studies have provided insight into the cellular distribution of the receptor within tissue (Takeda et al. 1990; Ruizeveld de Winter et al. 1991). However, each technique has its limitations: neither method can demonstrate whether the AR protein is processed similarly in all tissues and quantitation using either method can be imprecise. We have previously used SDS polyacrylamide gel electrophoresis and an immunoblot assay to determine the size and amount of immunoreactive AR in cultured human skin fibroblasts (Wilson et al. 1992). In the present study, we examine the characteristics and distribution of the AR in various tissues of adult rats of both sexes and the effects of castration, ACTH suppression, hypophysectomy, and hypophysectomy with replacement of growth hormone or prolactin.

## MATERIALS AND METHODS

**Animals and tissue preparation.** To examine AR tissue distribution, young, adult male and female Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN), weighing 175 to 275 g, were killed with an overdose of ether. Organs were dissected and stored in liquid nitrogen or processed fresh. In preliminary experiments, we found that storage of the tissue for as long as 3 weeks in liquid nitrogen did not influence the amount of immunoreactive AR detected. Animals were maintained under controlled lighting conditions (12 h of light, 12 h of darkness) and temperature (24°C) and given free access to standard laboratory chow and water. The drinking water of the hypophysectomized animals contained 10% sucrose and 0.45% saline.

**Tissue extraction.** Tissue samples were weighed and minced. Fragments were suspended (0.1 g /0.9 ml ) in sample buffer containing 110 mM sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 80 mM Tris [pH 6.9], 0.002% bromphenol blue, and 10% glycerol and homogenized on ice in an all-glass Dounce homogenizer (10 strokes of a loose pestle, followed by 20 strokes of a tight pestle). The homogenate was sonicated (Branson Model W 350, setting 7) for 10 sec, boiled for 5 min, and centrifuged at 200,000 g for 30 min. Aliquots of the supernatant, termed tissue extract, were used immediately for Western blotting or stored at -80°C and thawed only once. The loss of protein during processing of the tissues ranged from 6% to 61% with an average loss of 30%. Two percent or less of the immunoreactivity in the extract was recovered when the 200,000 g pellet was reextracted in the same volume of sample buffer.

**Immunoblot assay.** Extracts were boiled for 3 min immediately before loading on the gel. Aliquots (50 µl) of each sample were applied to 1.5 mm x 10 cm x 10 cm 7.5% polyacrylamide gels containing 3.5 mM SDS and 0.2% N,N'-methylene-bis-acrylamide (Laemmli 1970). Electrophoresis was performed at room temperature in a SE 600 Hoefer unit at 50 mA until the bromphenol blue dye front reached the bottom of the running gel. Proteins were then transferred to nitrocellulose membrane filters using a Hoefer Transphor at 25 V in buffer containing 192 mM glycine, 25 mM Tris, and 20% (vol/vol) methanol at room temperature for approximately 12 hours. Immunoreactive AR was visualized as described (Husmann et al. 1990) by incubating the nitrocellulose filters with affinity purified antibodies directed at the N-terminal 21 amino acid sequence of the human AR (rabbit U402), followed by the addition of [<sup>125</sup>I] goat, antirabbit immunoglobulin G, F(ab')<sub>2</sub>. Immunoblots were exposed to Kodak XAR2 film at -80°C for different periods ranging from overnight to 4 days. The specificity of the detection method was established by competition studies in which affinity purified U402 antibodies were preabsorbed with an excess of synthetic N-terminal 21 amino acid peptide (Husmann et al. 1990). The sizes of the visualized proteins were inferred from the position of radiolabelled molecular weight standards (Rainbow markers, Amersham Corp., Arlington Heights, IL) included in each gel. The apparent Mr of the major band AR

(110K Mr) is consistent with previous reports (Wilson et al. 1992, Husmann et al. 1990, van Laar et al. 1989).

**Densitometry.** A computing densitometer (Model 300A, Molecular Dynamics, Sunnyvale, CA) was used to measure the density of the 110K Mr AR bands visualized by autoradiography. Several dilutions of a standard rat prostate preparation were included in each gel. This preparation was arbitrarily assigned a value of 1 immunoreactive AR unit per mg protein or per mg wet tissue weight. The standard prostate extract was prepared at the start of this study and stored in aliquots at  $-80^{\circ}\text{C}$ . The relative amount of immunoreactive AR in each tissue was estimated by comparing the density of the 110K Mr AR band to values obtained with the prostate standard in the same gel.

**Protein measurement.** Protein levels in tissue extracts were measured by the method of Lowry et al. (1951) after precipitation with 10% (wt/vol) trichloroacetic acid in the presence of 0.8 mM sodium deoxycholate.

**Hormonal manipulations.** During these studies rats were treated according to three protocols:

**Protocol 1.** Levels of AR in tissues from intact male rats were compared to levels in males that had been castrated seven days previously or treated with dexamethasone ( $10\text{ }\mu\text{g}/100\text{ g BW}\cdot\text{day}$ ) dissolved in 0.2 ml 10% ethanol in triolein given sc for seven days. Dexamethasone was purchased from Sigma Chemical Co. (St. Louis, MO). Control and castrated animals received triolein only. Tissues from 3 animals in each group were pooled.

**Protocol 2.** Hypophysectomized and sham-operated males were obtained from Harlan Laboratories, Indianapolis, IN and examined eight days after surgery. The completeness of hypophysectomy was documented by demonstrating a marked reduction in prostate weight in the hypophysectomized ( $53 \pm 2\text{ mg}$ ) compared to the sham-operated group ( $234 \pm 20\text{ mg}$ ). Tissues from 2-6 animals per group were pooled for extraction.

**Protocol 3.** In animals treated with growth hormone or prolactin the completeness of hypophysectomy was verified by the absence of change in body weight over a period of 11 days, by visual inspection of the sella turcica at autopsy, and by demonstrating a difference in prostate weight ( $238 \pm 11\text{ mg}$  for control and  $<25\text{ mg}$  for the hypophysectomized groups). The hormones were dissolved in physiological saline and administered as daily sc injections ( $80\text{ }\mu\text{g}/100\text{ g BW}$ ) for seven days. Rat Growth Hormone (rGH) and rat Prolactin (rPRL) were supplied by the National Hormone and Pituitary Program. Tissues from 3 animals per group were pooled.



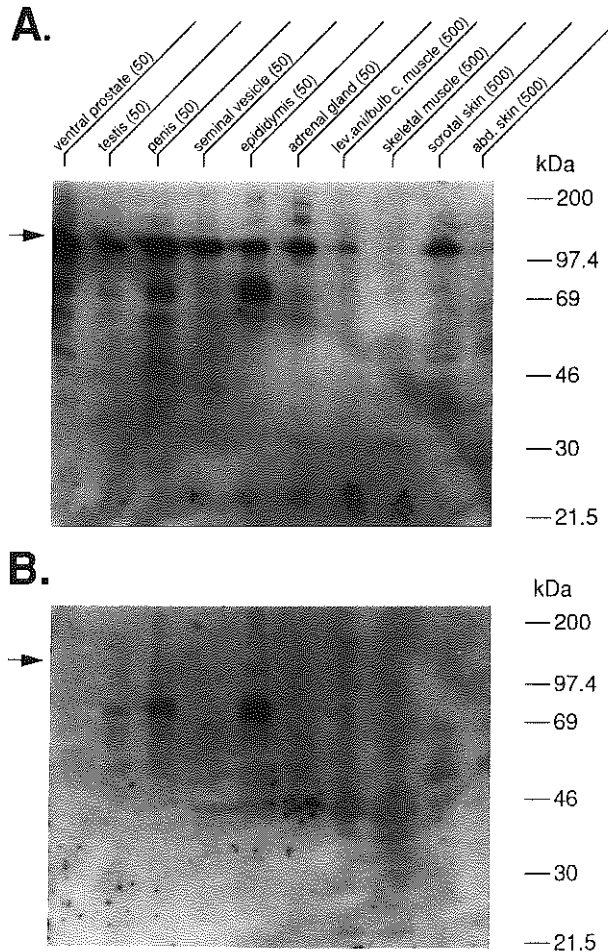


Figure 1. Immunoblots of androgen receptor protein in rat tissue extracts. (A) Aliquots of tissue extracts containing 50 or 500  $\mu$ g protein were applied to 7.5% SDS-polyacrylamide gels. Electrophoresis, transfer to nitrocellulose membrane, and immunoblotting were performed as described in the text. Locations and sizes ( $M_r \times 10^{-3}$ ) radiolabeled molecular weight markers run in the same gel are indicated. The arrow on the left side indicates specific protein bands at approximately 110K. Lev.ani, levator ani muscle; bulb. c., bulbocavernosus muscle; abd., abdominal. (B) The effect of pre-incubation of the anti-androgen receptor antibodies with a peptide corresponding to the N-terminal 21 amino acids of the androgen receptor. The protocol was identical to that in A, except that the affinity purified anti-N-terminal antibodies from rabbit U402 were preincubated overnight with excess peptide antigen.

## RESULTS

Relatively high levels of a 110K Mr AR band were recognized by U402 antibodies in extracts of ventral prostate, testis, penis, seminal vesicle, epididymis, and adrenal (Fig 1A). Lower levels of the same band were evident in extracts of levator ani/bulbus cavernosus muscle and scrotum, and very low to undetectable levels were present in extracts of non-genital skin and skeletal muscle. When these extracts were pre-incubated with an excess of the N-terminal 21 amino acid AR peptide used as immunogen no 110K Mr band was seen, indicating that the 110K Mr band recognized by these antibodies is specific (Fig 1B). In subsequent experiments, tissue levels of the 110K Mr AR bands were expressed relative to samples of a standard rat prostate extract equivalent to 5 mg wet weight of prostate included in each gel. Two representative curves obtained with the rat ventral prostate used as an internal standard are illustrated in Figure 2.

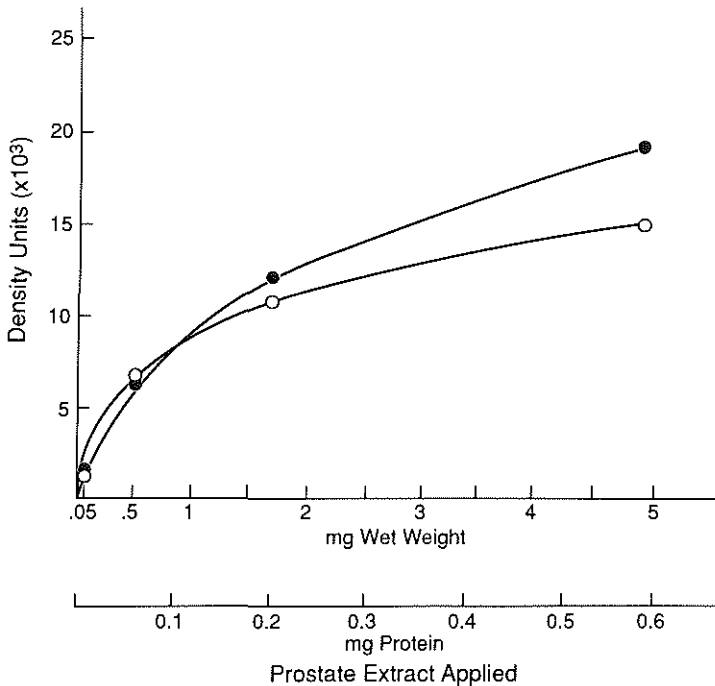


Figure 2. Relationship between the amount of rat prostate extract assayed and the density of radiolabeled 110K bands obtained in two immunoblots with several dilutions of the standard rat prostate extract. Protein bands with apparent Mr of 110K were recognized by antibodies to the N-terminal region of AR and detected using [ $^{125}$ ] antirabbit antibody. The specific bands were visualized by autoradiography and quantified by densitometry as described in the text.

In Table 1, the results of eight experiments have been summarized. The tissues have been grouped into three categories based on the amount of 110K Mr AR band detected. Category 1 includes ventral prostate, penis, epididymis, seminal vesicle, testis, ovary and adrenal gland in which AR levels are relatively high, ranging from a tenth that in prostate (testis) to levels similar to prostate (penis). In category 2, the level of immunoreactive AR was lower (1-5% compared to the prostate) but clearly demonstrable. Tissues in this group include levator ani/bulbus cavernosus muscle, preputial gland, scrotal skin, ureter, urinary bladder, uterus, vagina, lung and heart. Tissues in category 3 had undetectable or barely detectable AR levels that were less than 1% compared to prostate.

We next investigated the endocrine regulation of the AR levels in ventral prostate, seminal vesicle, testis and adrenal gland. The results of these experiments are summarized in Table 2. Castration caused a profound decrease in the amount of AR in prostate (80%) and seminal vesicle (67%) but had no effect on levels in the adrenal gland implying that receptor levels in the adrenal are regulated by non-testicular hormones.

These results suggested that the regulation of AR expression in the adrenal gland is different from that in the prostate. To examine this possibility, we studied the regulation of AR expression in hypophysectomized and sham-operated rats. Results are shown in Table 3. Hypophysectomy caused an 86% decrease in immunoreactive AR in the adrenal gland and a 62% decrease in the testis. To further explore the mechanism of AR expression in adrenal glands of intact rats the adrenal axis was suppressed by high doses of dexamethasone. The weight of the adrenal glands in these animals (Table 2) decreased to values similar to those were found in the hypophysectomized animals (Table 3), but AR levels did not change. Administration of growth hormone (rGH) caused a 22% increase of body weight of hypophysectomized rats (Table 4), documenting bioactivity of the hormone, but no change in AR levels in adrenal glands was observed. Administration of rat prolactin (rPRL) also did not influence immunoreactive AR levels in the adrenals. However, both rGH and rPRL caused an increase in the level of immunoreactive AR in ventral prostate, and rGH caused a similar increase in immunoreactive AR in seminal vesicles.

Table 1. Relative levels of androgen receptor in tissues of the rat as assessed by immunoblot assay

Relative amounts of immunoreactive androgen receptor			
		per mg wet weight	per mg protein
<b>Males</b>			
Category	Ventral prostate	1.0	1.0
1	Penis	0.7	0.9
	Adrenal	0.5	0.5
	Epididymis (head)	0.4	0.3
	Epididymis (tail)	0.4	0.5
	Seminal Vesicle	0.3	0.5
	Testis	0.1	0.2
2	La/bbc muscle	0.04	0.04
	Preputional gland	0.03	0.02
	Scrotal skin	0.02	0.05
	Bladder	0.01	0.02
	Ureter	0.01	0.04
	Lung	0.01	0.01
	Heart	0.01	0.01
3	Skeletal muscle	<0.01	<0.01
	Abdominal skin	<0.01	<0.01
	Kidney	<0.01	<0.01
	Cerebrum	<0.01	<0.01
	Pituitary	<0.01	<0.01
	Hypothalamus	<0.01	<0.01
	Liver	<0.01	<0.01
	Spleen	<0.01	<0.01
	Stomach	<0.01	<0.01
	Small intestine	<0.01	<0.01
	Colon	<0.01	<0.01
<b>Females</b>			
Category	Ovary	0.2	0.2
1	Adrenal	0.1	0.2
2	Uterus	0.06	0.05
	Vagina	0.05	0.06
	Lung	0.02	0.02
	Heart	0.01	0.01
3	Skeletal muscle	<0.01	<0.01

The results of 8 experiments have been combined. Electrophoresis, transfer, immunoblotting and densitometry were performed as described in the text. In each gel aliquots of the same standard prostate extract were used to obtain standard curves and AR immunoreactivity per mg wet tissue weight or per mg protein is arbitrarily set at 1. The amount of protein applied for the category 1 tissues varied from 290-740 µg protein; for category 2 tissues from 190-840 µg protein; and for category 3 tissues from 340-950 µg protein. Levator ani/bulbocavernosus muscles are abbreviated la/bbc.

## DISCUSSION

This study provides additional information about the distribution and regulation of the AR in tissues of the rat. AR is detectable (albeit in relatively low abundance) in many tissues not previously demonstrated to be androgen target tissues. These include the uterus, vagina, heart, lung, ovary and adrenal. In all tissues examined the molecular weight of the predominant form of AR identified with anti N-terminal antibodies is approximately 110K, suggesting that the size of the AR is similar in all rat tissues. Additional minor, specific bands observed in several tissues were assumed to be degradation products and were not investigated further. Relative levels of the AR were assessed by comparing the intensity of the radiolabeled 110K bands from different tissues to a standard rat prostate preparation. Utilizing this method, we have grouped the tissues into three categories: category 1, those tissues with a high level of expression of the AR (the gonads, other tissues of the urogenital tract and the adrenal); category 2, those tissues with low but detectable levels of AR (preputional gland, levator ani/bulbuscavernosus muscle, scrotal skin, bladder, lung and heart); category 3, tissues in which the AR is too low to be reliably detected by these methods.

In general, these findings confirm previous results in that high levels of AR are present in tissues of the urogenital tract. Low levels of expression in the urinary bladder and ureter might be explained as a consequence of the embryogenesis of these tissues. The ureter arises from a bud of the wolffian duct and contributes to the embryogenesis of the bladder. The uterus is derived from the müllerian duct, which in turn is derived from the wolffian duct (Greunwald 1941). Although AR has previously been described in rat uterus, using immunologic assays (Young et al. 1988), the physiological significance of the AR in urinary bladder, ureter, uterus, lung, and heart is not clear.

The presence of high levels of AR in the rat ovary is also of interest. Androgen receptor in ovary is localized predominantly in granulosa cells in rat (Bentvelsen & McPhaul, unpublished) and in human (Horie et al. 1992), suggesting that androgens may play a role in follicular growth and maturation.

High affinity androgen receptors have been detected in rat and mouse adrenal previously (Takeda et al. 1990, Calandra et al. 1978, Rifka et al. 1978). Our findings confirm these observations and suggest that levels of AR in the adrenal of the male rat are approximately half those found in the ventral prostate. The role of adrenal AR in adrenal physiology is unclear, although it has been postulated that adrenal AR mediates androgen-induced hypertension in the rat (Colby 1970, Gallant et al. 1991). The hormonal regulation of the adrenal AR is different from the regulation of AR in the ventral prostate. For example, castration caused the level of the AR in the prostate to decline dramatically, but had no effect on AR levels in the adrenal. Failure of supraphysiological doses of dexamethasone to suppress expression of the adrenal AR, coupled to the observation that hypophysectomy

caused a dramatic decrease in adrenal AR levels, suggest that a hormone(s) other than testicular androgens or ACTH is involved in regulation of the adrenal AR. Furthermore, although it has been reported that AR expression in rat prostate and seminal vesicles responds to growth hormone and prolactin treatment (Colby 1970, Gallant et al. 1991, Prins 1987, Prins et al. 1992, Reiter et al. 1992, this study), neither hormone caused an increase in immunoreactive AR in the adrenal when administered to hypophysectomized rats. The precise hormonal control of AR expression in the adrenal is yet to be identified.

In summary, we have employed an immunoblot assay to examine the quantity and distribution of immunoreactive AR in tissues of the adult rat. This study demonstrates that the receptor is detectable not only in tissues of the urogenital tract but also in many tissues not thought to be androgen target tissues. Highest levels of the receptor were detected in the urogenital tract and the adrenal, however among these tissues, the mechanism controlling AR expression must be diverse. AR in ventral prostate and seminal vesicle is modulated by androgen and, to a lesser degree by growth hormone and prolactin, whereas AR expression in the adrenal is dependent on intact hypophyseal function. The mechanisms of this diversity remain to be determined.

Table 2. Effect of castration or dexamethasone on androgen receptor levels in male rats

Treatment Protocol	Relative amounts of immunoreactive androgen receptor		Average tissue weight (mg $\pm$ SEM)	
	per mg wet weight	per mg protein		
I Intact (+ triolein)				
Ventral prostate	0.5 $\pm$ 0.2	0.5 $\pm$ 0.2	229 $\pm$ 20	
Adrenal	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1	25 $\pm$ 0.5	
Testis	0.02, 0.1	0.01, 0.08	1459 $\pm$ 55	
Seminal vesicle	0.05, 0.07	0.05, 0.03	180 $\pm$ 14	
II Castrate (+ triolein)				
Ventral prostate	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	37 $\pm$ 5	
Adrenal	0.5 $\pm$ 0.1	0.5 $\pm$ 0.1	26 $\pm$ 1	
Seminal vesicle	0.02, 0.02	0.02, 0.02	43 $\pm$ 4	
III Intact (+ dexamethasone)				
Ventral prostate	1.0, 0.5	0.7, 0.3	200 $\pm$ 17	
Adrenal	0.4, 1.0	0.5, 0.5	14 $\pm$ 2	
Testis	0.04, 0.1	0.04, 0.08	1465 $\pm$ 36	
Seminal vesicle	0.1, 0.1	0.1, 0.08	184 $\pm$ 31	

Intact male rats were given dexamethasone (10  $\mu$ g/100 g body weight  $\cdot$  day) dissolved in triolein or triolein alone for seven days. Castrate rats were given triolein alone. Data from two (individual values) or three (average  $\pm$  SEM) experiments are given. Mean body weights  $\pm$  SEM for groups I, II and III prior to treatment were 232  $\pm$  3 g, 230  $\pm$  5 g and 229  $\pm$  6 g, respectively; final body weights were 273  $\pm$  8 g, 251  $\pm$  6 g and 242  $\pm$  5 g, respectively.

Table 3. Effect of hypophysectomy on androgen receptor levels in male rats

Relative amounts of immunoreactive androgen receptor			Average tissue weight (mg $\pm$ SEM)	
Treatment Protocol	per mg wet weight	per mg protein		
Sham-operated				
Ventral prostate	0.7 $\pm$ 0.1	0.7 $\pm$ 0.1	234 $\pm$	20
Adrenal	0.7 $\pm$ 0.1	0.7 $\pm$ 0.2	25 $\pm$	2
Testis	0.07, 0.06	0.1, 0.06	1307 $\pm$	41
Seminal Vesicle	0.04, 0.03	0.04, 0.03	157 $\pm$	19
Hypophysectomized				
Ventral prostate	<0.01	<0.03	53 $\pm$	2
Adrenal	0.1 $\pm$ 0.1	0.1 $\pm$ 0.1	11 $\pm$	1
Testis	0.03, 0.02	0.04, 0.03	1054 $\pm$	48
Seminal Vesicle	0.01, <0.01	0.02, <0.02	43 $\pm$	8

Hypophysectomized male rats were compared to sham-operated male rats eight days after surgery. Each experiment included 2 - 6 animals per group. Data from one, two (individual values) or three (average values  $\pm$  SEM) experiments are combined in the table. Mean body weights ( $\pm$  SEM) prior to surgery were  $220 \pm 4$  g for the hypophysectomized animals and  $219 \pm 3$  g for the sham-operated animals; final body weights were  $193 \pm 3$  g and  $259 \pm 5$  g for the hypophysectomized and sham-operated animals, respectively.

Table 4 Effect of hypophysectomy, hypophysectomy and rat growth hormone, or hypophysectomy and rat prolactin on androgen receptor levels in male rats

Treatment Protocol	Relative amounts of immunoreactive androgen receptor		Average tissue weight (mg $\pm$ SEM)	
	per mg wet weight	per mg protein		
Sham-operated				
Ventral prostate	0.5	0.9	238 $\pm$	11
Adrenal	0.4	0.3	36 $\pm$	2
Seminal vesicle	0.07	0.05	199 $\pm$	13
Hypophysectomized				
Ventral prostate	<0.01	<0.01	24 $\pm$	3
Adrenal	<0.01	<0.02	7 $\pm$	1
Seminal vesicle	<0.01	<0.01	12 $\pm$	1
Hypophysectomized and rGH				
Ventral prostate	0.03	0.04	21 $\pm$	2
Adrenal	<0.01	<0.02	7 $\pm$	1
Seminal vesicle	0.02	0.04	11 $\pm$	1
Hypophysectomized and rPRL				
Ventral prostate	0.02	0.05	18 $\pm$	1
Adrenal	<0.01	<0.01	7 $\pm$	1
Seminal vesicle	<0.01	<0.03	11 $\pm$	1

Male rats were sham-operated or hypophysectomized. Hypophysectomized animals received no further treatment or were treated for seven days with rat growth hormone (rGH) or with rat prolactin (rPRL). rGH (80  $\mu$ g/100 g body weight) or rPRL (80  $\mu$ g/100 g body weight) were administered daily as sc injections starting 11 days after hypophysectomy. Data from one experiment are given. Mean body weights  $\pm$  SEM for the sham-operated, hypophysectomized, hypophysectomized and rGH, and hypophysectomized and rPRL were 239  $\pm$  2 g, 152  $\pm$  3 g, 147  $\pm$  4 g and 147  $\pm$  2 g prior to treatment, respectively; final body weights were 305  $\pm$  2 g, 143  $\pm$  5 g, 179  $\pm$  5 g and 150  $\pm$  3 g.



## Chapter 3

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**The androgen receptor of the urogenital tract of the fetal rat is regulated by androgen**

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**Abstract**

To provide insight into androgen-mediated virilization, we measured the androgen receptor in tissues of male and female rat fetuses prior to and during the period of phenotypic sex differentiation. Western immunoblotting was performed utilizing an antibody directed against the 21 amino-terminal segment of the androgen receptor. In immunoblots prepared from urogenital tract tissues of day 17 male and female fetal rats, this antibody specifically recognizes a 110K protein band characteristic of the androgen receptor. Androgen receptor levels were low to undetectable in a variety of non-urogenital tract tissues. After day 18 of fetal development, the amount of androgen receptor decreased in female urogenital tissues, and by day 22 the amount of immunoreactive androgen receptor was higher in the male urogenital sinus and tubercle than in the corresponding tissues of the female. Administration of 5 $\alpha$ -dihydrotestosterone to pregnant rats at a dose of 50 mg/kg body weight per day from day 12 to day 22 caused an increase in immunoreactive androgen receptor in the female urogenital sinus and tubercle to levels approaching those in male tissues. Administration of the androgen antagonist flutamide (100 mg/kg body weight per day) during the same interval caused a reduction in androgen receptor level in the urogenital sinus and tubercle of the male. These findings suggest that androgens modulate the amount of androgen receptor in the embryonic urogenital tract either by inducing the proliferation of androgen-responsive cells or by increasing androgen receptor levels in individual cells.

**1. Introduction**

During embryogenesis, two hormones from the fetal testis are responsible for formation of the male phenotype. Müllerian-inhibiting hormone, secreted by the Sertoli cells, causes regression of the müllerian ducts in the male, and testosterone, from the Leydig cells, causes virilization of the wolffian ducts, urogenital sinus, and the urogenital tubercle (George and Wilson, 1994). Physiological evidence indicates that embryos of both sexes have similar androgen receptor mechanisms. The virilization of the male urogenital tract is mediated by the formation of testosterone by the fetal testis at the appropriate time, and the administration of androgens to female embryos causes full masculinization of the urogenital tract (Schultz and Wilson, 1974; George and Peterson, 1988). The genetic evidence is also clearcut that the same androgen receptor protein that mediates androgen action in post-embryonic life is also responsible for androgen action during embryogenesis; mutations that impair function of the androgen receptor in the human (Griffin and Wilson, 1989) or the mouse (Lyon and Hawkes, 1970; Goldstein and Wilson, 1972) prevent androgen action both during embryogenesis and in mature animals.

Although we have demonstrated that the ligand binding characteristics of androgen receptors isolated from the embryonic urogenital tract are similar to those in the reproductive tract of mature animals (George and Noble, 1984; George et al., 1988), it has not been

feasible to do more detailed studies of the distribution or regulation of androgen receptor levels in the embryo.

In the current study, we used polyclonal antibodies directed at the amino terminus of the androgen receptor (Husmann et al., 1990) to examine the receptor in immunoblots of embryonic tissues. Our findings indicate that as in the adult, the androgen receptor is expressed predominantly in tissues of the urogenital tract, that the level of expression is initially similar in male and female urogenital tissues, that the levels of the androgen receptor fall with time in the female urogenital tract, and that the decrease in androgen receptor levels in the female can be prevented with androgen treatment. We conclude that in the developing urogenital tract, androgen receptor levels are initially hormone independent but that continued androgen receptor expression in the embryo is androgen dependent.

## **2. Materials and methods**

### **2.1. Animals**

Timed pregnant, Holtzman-Sprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN), maintained under controlled conditions of lighting (12 h light/12 h dark) and temperature (23°C), and allowed free access to standard laboratory chow and water. The day on which sperm were detected in the vagina was considered day 1 of pregnancy. Dams were killed on days 15, 17, 18, 20 and 22 of pregnancy. Sex was confirmed by gonadal inspection. Urogenital tubercles were obtained from day 15 fetuses, but microdissection of other tissues was not done prior to day 17. After dissection, tissues were stored frozen in liquid nitrogen for not more than 3 weeks (preliminary experiments showed that storage for this period did not decrease the level of immunoreactive androgen receptor detected in the samples).

In some experiments, pregnant rats were given either flutamide (Schering Corp., Bloomfield, NJ) (100 mg/kg body weight per day) or DHT (50 mg/kg body weight per day). Both drugs were dissolved in 10% ethanol/ 90% triolein and were administered daily by subcutaneous injection from day 12 to day 22 of gestation. Fetuses were delivered surgically on day 22; the urogenital tubercles and urogenital sinuses of male and female fetuses were pooled separately and analyzed for androgen receptor by Western blotting (see below). The drug treatments were without obvious effects with the exceptions that DHT treatment resulted in wolffian duct virilization and enlargement of the urogenital sinus and tubercle of female fetuses, whereas flutamide treatment impaired epididymal development and caused a reduction in the size of the urogenital tubercle of males (results not shown).

### **2.2. Homogenization**

Frozen pools of tissue (50-250 mg wet weight from 15-40 fetuses for urogenital tissues) were thawed and homogenized in nine volumes (100 mg tissue/0.9 ml buffer) of sample

buffer (110 mM sodium dodecyl sulfate (SDS), 100 mM dithiothreitol, 80mM Tris (pH 6.9), 0.002% bromophenol blue and 10% glycerol) in an all-glass, Dounce homogenizer (10 strokes of a loose pestle; 20 strokes of a tight pestle). The homogenate was then sonicated (Branson Model W350, setting 7) for 10s, boiled for 5 min, and centrifuged at 200 000 x g for 30 min. Aliquots of the supernatant (termed tissue extract) were either directly loaded on gels or stored at -80°C. When frozen samples were analyzed, the samples were thawed only once before electrophoresis.

### 2.3. Western blotting

Before loading on SDS-polyacrylamide gels, extracts were again boiled for 3 min, sonicated, and centrifuged in a microfuge for 5 min. Aliquots (50 µl) were applied to 10 cm x 10 cm x 1.5 mm 7.5% polyacrylamide gels containing 3.5 mM SDS and 0.2% N,N'-methylene-bis-acrylamide (Husmann et al., 1990). Electrophoresis was performed at room temperature in an SE 600 Hoefer unit at 50 mA until the bromophenol blue dye front reached the bottom of the running gel. Proteins were then transferred to nitrocellulose membranes using a Hoefer Transphor at 25 V in buffer containing 192 mM glycine, 25 mM Tris, and 20% (v/v) methanol at room temperature for 12h. Immunoreactive androgen receptor was visualized as previously described (Husmann et al., 1990) by incubating the nitrocellulose membranes with affinity purified antibodies directed against the N-terminal 21 amino acid sequence of the human androgen receptor (antibody from rabbit U402) After washing, specifically bound antibody was visualized using [<sup>125</sup>I]\_labeled goat, antirabbit immunoglobulin G, F(ab')<sub>2</sub>. Immunoblots were exposed to Kodak XAR2 film at -80°C for periods of time ranging from overnight to 4 days. The specificity of the method was established by competition studies in which the affinity purified U402 antibody was preabsorbed with excess synthetic N-terminal peptide (Husmann et al., 1990). The size of the visualized androgen receptor protein was inferred from the position of radiolabelled molecular weight standards (Rainbow Markers, Amersham Corp., Arlington Heights, IL) included in each gel. The apparent Mr of the androgen receptor (approx. 110K) is consistent with previous reports (Husmann et al., 1990; Van Laar et al., 1989).

### 2.4. Densitometry

A computing densitometer (Model 300A, Molecular Dynamics, Sunnyvale, CA) was used to measure the density of the 110K androgen receptor bands. For purposes of comparison, dilutions of tissue extract (prepared as above) from rat prostate were included in each gel as an internal standard. An estimate of the amount of androgen receptor in each tissue was obtained by comparing the density of the 110K androgen receptor band measured in each sample to the density obtained for the internal prostate standard. These comparisons were always made to a linear portion of the prostate standard curve (i.e. between 5 and 50 µg

of protein loaded). In Table 1, the relative levels of immunoreactive androgen receptor detected in the tissues are expressed as arbitrary densitometry units per microgram of protein.

### 2.5. Protein measurement

Proteins were measured in the original tissue extracts by the method of Lowry et al. (1951) after precipitation with 10% (w/v) trichloroacetic acid in the presence of 0.8 mM sodium deoxycholate. In experiments where equal amounts of protein were loaded in each lane of a gel, a volume adjustment was made using loading buffer.

## 3. Results

Immunoreactive androgen receptor that migrates with an apparent molecular mass of 110K was readily discernible in tissue extracts of both male and female urogenital sinus and urogenital tubercle from day 17 fetal rats (Fig. 1). The mobility of the immunoreactive androgen receptor was similar in these tissues, in adult prostate, and in fetal epididymis and testis. These findings indicate that no major size difference exists between the androgen receptor from the wolffian-derived epididymis and the androgen receptor from tissues derived from the external genitalia (urogenital tubercle and urogenital sinus).

Table 1

Immunoreactive androgen receptor levels in fetal (day 20) rat tissues

Relative amount of androgen receptor			
Male tissues		Female tissues	
Ug tubercle	1.5	Ug tubercle	0.6
Ug sinus	1.1	Ug sinus	0.4
Adrenal	0.9	Adrenal	1.0
Testis	0.7	Ovary	0.2
Epididymis	0.8	Uterus	0.1

Relative amounts of androgen receptor in tissues of the day 20 fetal rat as measured by the Western immunoblotting technique described in the text. The amounts of androgen receptor detected in individual experiments (density units/  $\mu$ g protein) were expressed relative to the amount measured in an adult rat prostate standard which was arbitrarily given a value of 1. In other experiments (results not shown), relative amounts of androgen receptor were  $<0.1$  in lung, heart, urinary bladder, intestine, skeletal muscle, kidney, brain, and liver. Ug, urogenital.

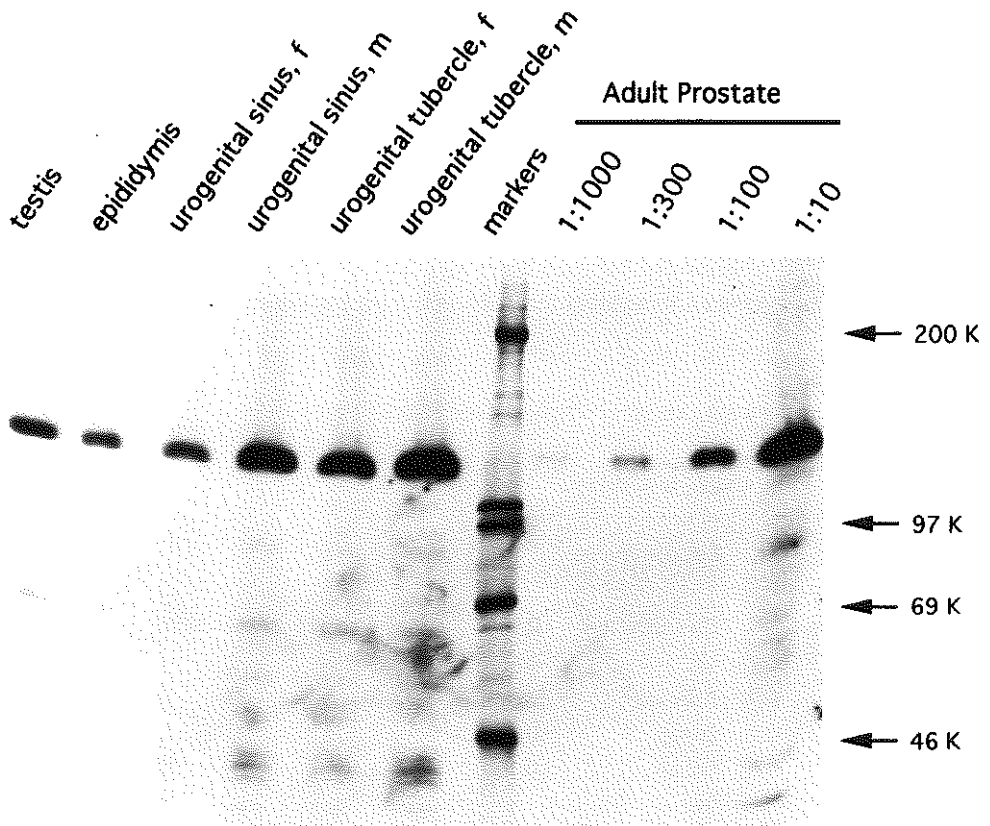


Fig. 1. Visualization of the androgen receptor by immunoblotting in tissues of day 17 fetal (male and female) rats and adult prostate. Tissues were homogenized in loading buffer (100 mg/0.9 ml loading buffer), and aliquots of tissue extracts (approx. 500  $\mu$ g protein) were electrophoresed as described in the text. Serial dilutions of adult prostate homogenate (5-500  $\mu$ g protein) provides a reference for the relative levels of immunoreactive protein in the other tissues. The position of the radiolabeled protein standard are shown on the right.

## AR in fetal rat

Microdissection of the undifferentiated wolffian duct before day 17 was not technically feasible. Specificity of the immunoblotting was established by demonstrating that pre-absorbing the anti-androgen receptor antibodies with excess amino terminal peptide that was used as immunogen blocked visualization of the 110K protein (Fig. 2).

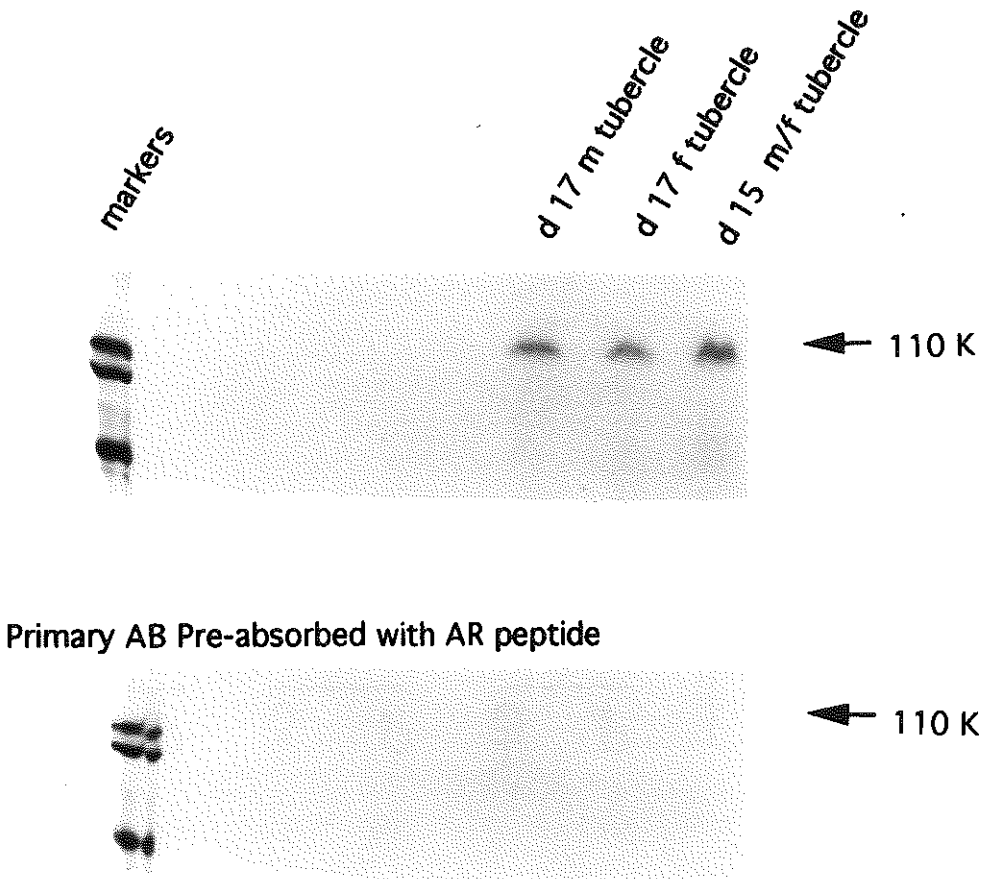


Fig. 2. The androgen receptor antibody (U402) specifically recognizes a 110K protein in day 15-17 urogenital tubercle. Tissues were homogenized in loading buffer (100 mg tissue/0.9 ml buffer). Upper panel-primary antibody, U402; lower panel-primary antibody, U402 pre-incubated with an excess of N-terminal peptide used as immunogen. Radiolabeled protein standards are shown on the left.

Having established the specificity of the immunoblot procedure in fetal rat tissues, we examined the tissue distribution and relative level of immunoreactive androgen receptor in the day 20 rat fetus. These results are summarized in Table 1. High levels of immunoreactive androgen receptor were detected in testis, epididymis, ovary, and in both male and female urogenital sinus, urogenital tubercle, and adrenal. Low levels of androgen receptor were present in a variety of other tissues examined including bladder, heart and lung.

The amount of androgen receptor in tissues of the day 20 male fetus was consistently higher than in corresponding female tissues. Since androgen receptor levels were similar in most preparations of day 17 male and female urogenital tubercle (Fig. 2), this finding suggested that androgens produced by the fetal testes might influence the level of androgen receptor in male urogenital tissues. This concept was supported by a developmental study of the androgen receptor level in male and female urogenital tubercle (Fig. 3). After day 18 of gestational age, the amounts of androgen receptor in male urogenital tubercle were consistently higher than in the female urogenital tubercle.

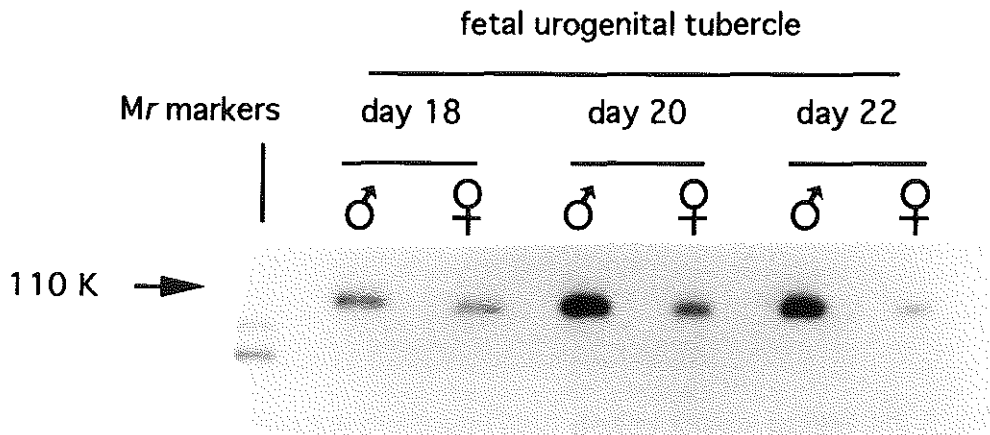
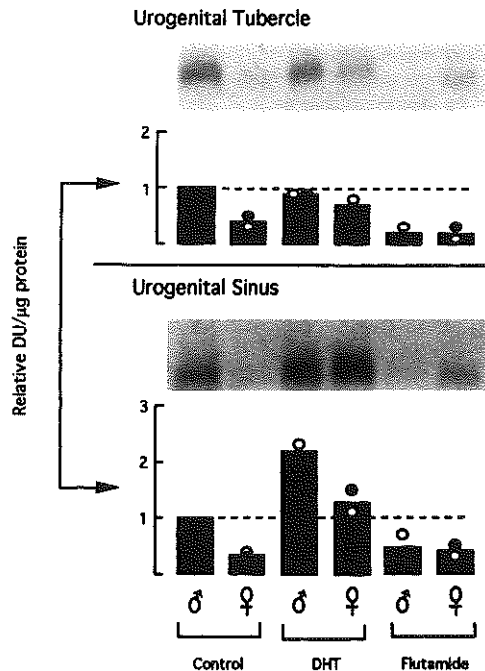


Fig. 3. Developmental profile of immunoreactive androgen receptor in male and female urogenital tubercle. Extracts of male and female urogenital tubercles were made as described in the text. Equal amounts (100  $\mu$ g protein) of each tissue sample were applied.



To establish whether this difference was due to fetal androgens, we next measured the level of immunoreactive androgen receptor in the urogenital sinus and tubercles of male and female fetuses that had been treated in utero with either the anti-androgen flutamide or with DHT. As shown in Fig. 4, in female embryos from pregnant rats treated with DHT, androgen receptor levels in the urogenital sinus and tubercle were similar to those in control males. DHT administration caused a further increase in the amount of the androgen receptor in the urogenital sinus, but not the urogenital tubercle, of the male. The administration of flutamide (100 mg/kg body weight per day) caused a consistent reduction in the amount of androgen receptor detected in the urogenital sinus and tubercle of male offspring to levels similar to those in untreated females.

Fig. 4. Effect of *in vivo* DHT or flutamide treatment on androgen receptor levels in urogenital sinus and tubercle of male and female rat embryos. Timed pregnant rats were treated with either DHT (50 mg/kg body weight per day), flutamide (100 mg/kg body weight per day), or vehicle (trioline oil) from gestational day 12-22. On day 22, the pups were delivered surgically, and the urogenital tubercles and urogenital sinuses were dissected, homogenized and analyzed by the Western immunoblotting procedure described in the text. The results of two experiments are shown in the bar graphs. The values corresponding to the autoradiogram shown are represented by the solid symbols. Approximately 30  $\mu$ g protein was loaded in each lane of the gel. DU, densitometric units.



#### 4. Discussion

In the eutherian mammal, virilization of the male urogenital tract commences immediately after the onset of testosterone synthesis by the fetal testis, suggesting that the mechanisms that mediate the action of androgen in these tissues are fully expressed at this

time (George and Wilson, 1994). In keeping with this concept, It is not surprising that androgen receptor levels were demonstrable by day 17 of fetal rat development and were higher in urogenital tract tissue than in non-urogenital tract tissues.

A developmental study revealed that the levels of androgen receptor increased moderately in male urogenital tract tissues between days 17 and 22 at the time testosterone is synthesized by the fetal testis (Habert and Picon, 1984) but decreased in tissues of the female urogenital tract during the same time interval. This decrease in the levels in females was prevented by administration of DHT to the mothers. Furthermore, a decrease in androgen receptor levels in urogenital tract tissues of male fetuses was seen following the administration of the anti-androgen flutamide to pregnant animals. These findings suggest that the androgen receptor levels in the fetal urogenital tract are initially hormone independent but that continued expression of the androgen receptor is androgen-dependent. Modulation of the levels of androgen receptor by androgen has been studied in a number of model systems. Androgen withdrawal causes a decrease in androgen receptor levels in the rat prostate (Prins and Birch, 1993; van Doorn et al., 1976). The rat phallus, in contrast, shows a permanent, developmentally associated decrease in androgen receptor content and androgen receptor mRNA levels in response to androgen (Takane et al., 1990, 1991; Gonzalez-Cadavid et al., 1991). In defined cell models, androgen decreases the level of androgen receptor mRNA (Quarmby et al., 1990; Krongrad et al., 1991) and in some instances transiently increases the cellular level of immunoreactive androgen receptor (Krongrad et al., 1991). Thus, the regulation of the androgen receptor is complex and tissue or cell specific, and the current studies do not identify the mechanism responsible for the androgen-dependent changes in androgen receptor content in the fetal urogenital sinus and tubercle. Histochemical studies of androgen receptor expression in the fetal urogenital tract will be necessary to discern whether these changes are due to alterations in the cellular content of androgen receptor or whether they are due to changes in the number of cells that express androgen receptor.

The findings in the present study are interesting in another regard. Testosterone itself mediates the virilization of the wolffian ducts, whereas DHT formation is necessary for virilization of the urogenital sinus and external genitalia (George and Wilson, 1994). Genetic evidence suggests that a single androgen receptor mediates the action of both hormones (George and Wilson, 1994), and ligand binding studies of the androgen receptor in the rabbit fetus indicate that all tissues of the urogenital tract bind DHT preferentially (George and Noble, 1984; George et al., 1988). We did not observe any distinguishing characteristic of androgen receptors isolated from the wolffian-derived fetal epididymis compared to receptors isolated from the fetal urogenital tubercle and sinus and the adult prostate, but we still are not certain whether testosterone-mediated virilization of the wolffian duct is due to post-translational modification of the receptor that we did not detect or whether the receptor is identical in all androgen-target tissues.

## Chapter 4

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### **Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat**

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### Abstract

Distribution and regulation of androgen receptor expression in genitalia of the rat during fetal and neonatal development was assessed by immunohistochemistry. In mesonephric duct derivatives the androgen receptor expression became evident first in the efferent ductules and epididymis, subsequently in the vas deferens and finally in the seminal vesicle. Only the efferent ductules showed some epithelial cells positive for the androgen receptor in addition to positive mesenchymal cells at fetal day 14 (the first day of investigation). In all other mesonephric duct derived tissues, we found androgen receptor expression in the mesenchyme before the epithelium became positive.

In the mesenchyme of the urogenital tubercle, genital folds and swellings, cells were positive for androgen receptors already from day 14 onwards. In the mesenchymal part of the prostate anlagen in the urogenital sinus, androgen receptor positive cells were found first on fetal day 16. The epithelium of the tubercle and the epithelial part of the prostate became androgen receptor positive on postnatal day 5.

Administration of 5 $\alpha$ -dihydrotestosterone from day 11 to day 20 of gestation to pregnant rats caused a stabilization of the wolffian duct in female fetuses with an androgen receptor expression pattern similar as found in male fetuses. In addition, an increase in the density and frequency of androgen receptor positive cells was found in the female urogenital sinus and tubercle. Administration of the androgen antagonist flutamide during the same period caused a reduction in androgen receptor positive cells in male fetuses, and an underdevelopment of the wolffian duct, urogenital sinus and tubercle, respectively.

These findings indicate that androgens enhance the expression of androgen receptors in the developing rat genital tract by induction and by increasing the frequency of androgen receptor positive cells. The developmental pattern of androgen receptor expression during fetal and neonatal sexual differentiation in the rat mesonephric duct system reflects the androgen-responsiveness of the ducts, and is consistent with induction of the androgen receptor along the ducts by testosterone.

### Introduction

Normal development and functioning of male genitalia are primarily controlled by androgens (Wilson et al., 1981). In most androgen target cells testosterone is converted into its metabolite 5 $\alpha$ -dihydrotestosterone. Testosterone and 5 $\alpha$ -dihydrotestosterone play separate roles in fetal male differentiation: testosterone mediates wolffian duct virilization, while differentiation of the urogenital sinus and the external genitalia depends upon 5 $\alpha$ -dihydrotestosterone (Griffin and Wilson, 1989). The different roles of the two hormones is clearly reflected in the 5 $\alpha$ -reductase type 2 deficiency syndrome. In individuals with this syndrome masculinization of the urogenital sinus and external genitalia does not occur during fetal life, but wolffian duct development proceeds into epididymis, vas deferens and seminal

## Immunohistochemical localization of the AR

vesicle. Compelling evidence exists that both testosterone and 5 $\alpha$ -dihydrotestosterone act via the same receptor protein, because in individuals with the complete form of androgen insensitivity the wolffian duct stabilization, as well as virilization of the urogenital sinus and tubercle, are affected (Griffin and Wilson, 1989).

Differentiation of the wolffian ducts into male internal genitalia begins at week eight of gestation in man, and at fetal day 14 in the rat (Price and Ortiz, 1965). During early development of male genitalia the distribution of AR differs from that of later stages in that AR expression initially is present only in mesenchymal cells and later also appears in the epithelium (Cooke et al., 1991). Androgens induce epithelial differentiation of the genitalia via action in mesenchymal cells (Shannon and Cunha, 1983; Takeda et al., 1985).

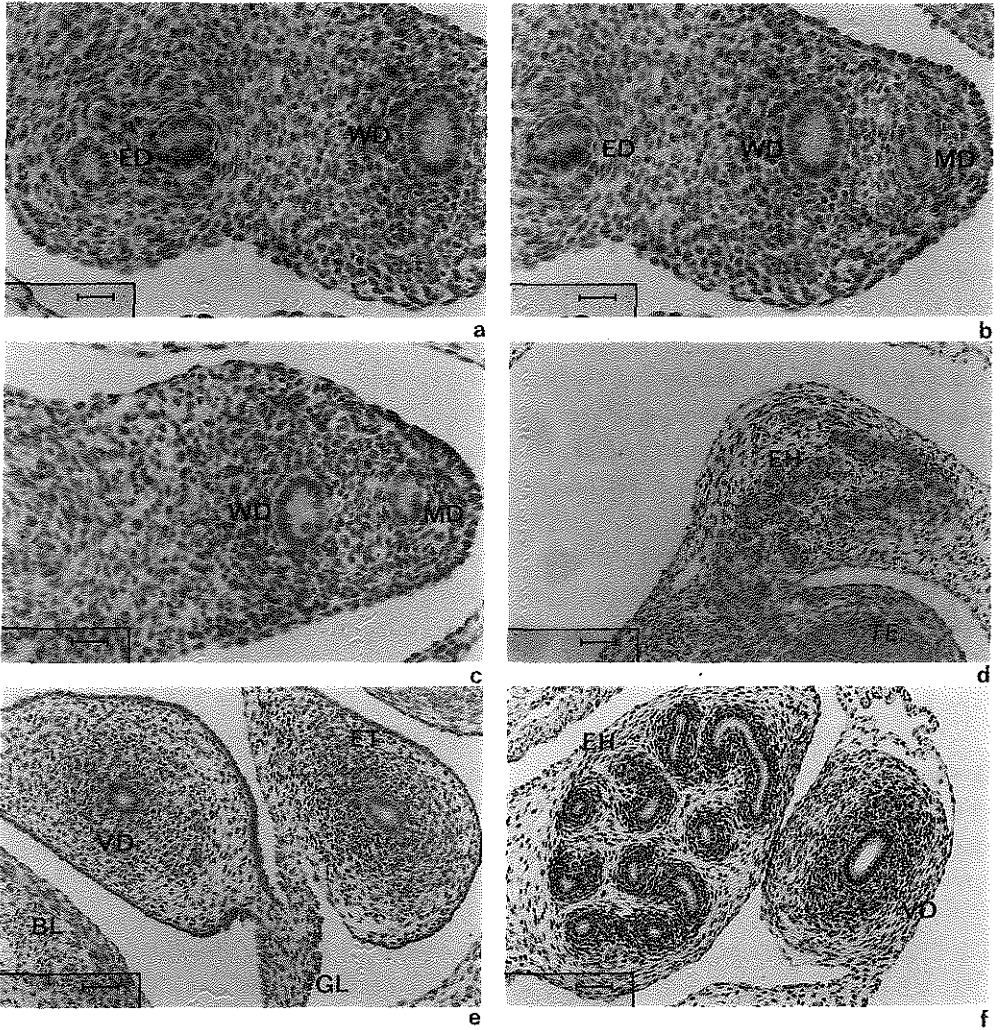
In previous work we have demonstrated that during early sexual differentiation in the male and female rat fetus ARs are present at equally high levels in the urogenital tubercle and sinus of both sexes (Bentvelsen et al., 1994). In the tubercle of male fetuses AR expression increases with time, while in female fetuses AR protein expression of the tubercle diminishes. Those immunoblotting studies, however, were by no means conclusive with respect to the cellular distribution of the AR, either these changes are caused by alterations in the cellular density of AR expression or in the frequency of AR positive cells. Studies on the cellular distribution of AR expression in the developing urogenital tract are based on autoradiography (Cooke et al., 1991; Shannon and Cunha, 1983; Takeda et al., 1985). In autoradiographic studies however, radioactivity is detected not only of the primary label, but it also includes the metabolites of androgens that might have binding characteristics different from the primary label. In the cells which are negative in the autoradiograms, occupancy of the AR by endogenous unlabelled ligand cannot be excluded. This consequently results in a false negative signal.

Since a sensitive specific immunohistochemical assay for paraffin-embedded tissues was developed recently (Janssen et al., 1994), we have used this assay to re-examine the developmental pattern of the differential expression of the AR at the protein level. Our observations indicate that androgens enhance the expression of AR in developing rat genital tract by increasing the density and frequency of AR positive cells.

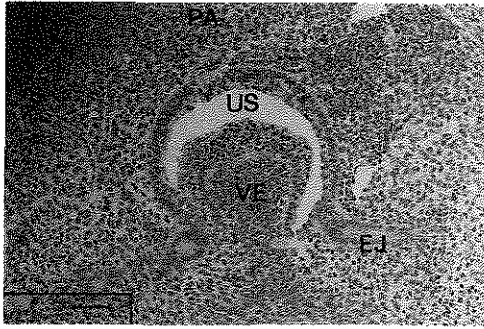
Fig.1. Immunohistochemical staining of the androgen receptor in the wolffian duct

a,b,c: Wolffian duct (WD), efferent ductules (ED) and müllerian duct (MD) are shown at fetal day 14. c: The specificity of the immunoreactivity was indicated by the lack of nuclear staining after preincubation of the antibodies with the peptide SP197. d: The epididymal head (EH) at fetal day 18, TE is testis. e The epididymis tail (ET) and the gubernacular ligament (GL), the vas deferens (VD) and the bladder (BL) at day 18. f (neonatal day 5): The vas deferens and epididymal tail. Bar = 25  $\mu$ m in a,b,c; bar = 50  $\mu$ m in d,e,f.

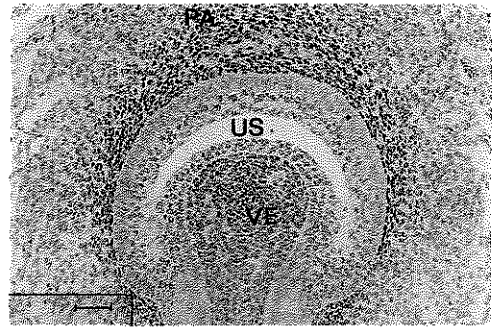
## Immunohistochemical localization of the AR



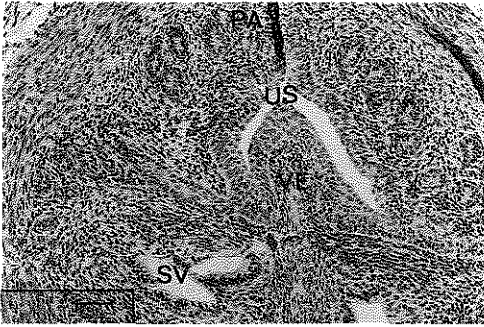
## Immunohistochemical localization of the AR



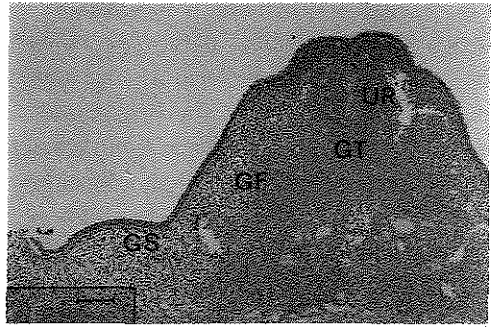
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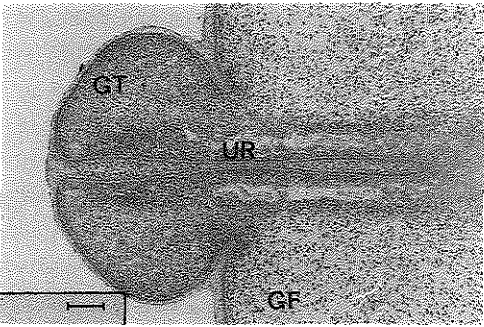
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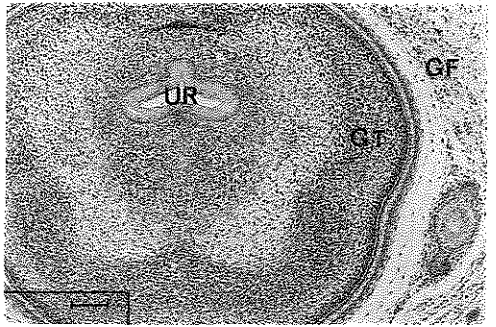
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## Immunohistochemical localization of the AR

Fig.2. Immunohistochemical staining of the androgen receptor in the genital tubercle, sinus and surrounding tissues in the male rat.

a: The urogenital sinus (US), the prostate anlagen (PA) and the verumontanum (VE) on fetal day 14 at the location, where the ejaculatory ducts (EJ) enter the sinus. b: fetal day 18. c: Budding of the prostate at postnatal day 5; SV are seminal vesicles. d,e,f: The urogenital tubercle (GT) with urethra (UR), genital fold (GF) and swelling (GS). d (day 14); e (day 18); f (2 days after birth). Bar = 50  $\mu$ m in a,b,c; bar = 100  $\mu$ m in d,e,f.

## MATERIALS AND METHODS

### *Animals*

Wistar rats (obtained from TNO, Rijswijk, The Netherlands) were maintained under controlled conditions of day and night (14 hours and 10 hours, respectively) and temperature ( $20 \pm 1^\circ\text{C}$ ) and allowed free access to standard laboratory chow and tap water. Timed pregnant dams (vaginal plug= day 0; term= day 22) were killed at the start of sexual differentiation of the mesonephric ducts on day 14, and further on days 16, 18, 20 and 21 of pregnancy. Fetuses were removed immediately. Newborn rats were sacrificed on days 2, 5 and 10. Flutamide (a kind gift from Schering Corp., Bloomfield, NJ) was administered 100 mg/kg body weight per day, and  $5\alpha$ -dihydrotestosterone (DHT) 50 mg/kg body weight per day. Both drugs were dissolved in 10% ethanol/ 90% olive oil and were administered daily by subcutaneous injection from day 11 to day 20 of gestation. The treated fetuses were killed on day 21.

Fetuses and neonates were fixed in 10% neutral phosphate-buffered formalin. After 24 h the bodies were cut into two halves. Sex was confirmed by gonadal inspection. From each age 2-4 animals were analyzed. The lower body halves (without intestines, extremities and tail) were embedded in paraffin.

### *Antiserum against AR*

The antiserum used was raised in rabbits against a synthetic peptide identical to the first 20 amino acid residues of the N-terminal domain of the human and rat AR. The polyclonal antibodies were developed as previously described (Zegers et al., 1991). The oligopeptides were conjugated to keyhole limpet hemocyanin (KLH, Calbiochem, San Diego, CA) for immunization of rabbits (Flemish Giant random bred, MBL-TNO, Rijswijk, The Netherlands). The sera were tested in a direct ELISA for anti-peptide response, and in an immunoprecipitation assay for AR specificity. The antibodies will be referred to as SP197 (Janssen et al., 1995).

### *Immunohistochemistry*

Details for immunohistochemical procedures have been reported previously (Janssen et al., 1994). All sections (5  $\mu$ m) were cut transversely and mounted on slides coated with 3-



## Immunohistochemical localization of the AR

aminopropyl triethoxy-silane (Sigma Chemical Co., S. Louis, MO). Slides were dried overnight at 60°C, dewaxed and rehydrated, after endogenous peroxidase activity was blocked with 3% H<sub>2</sub>O<sub>2</sub> in methanol. Sections were then microwaved three times for 5 min at 700 W under 0.01 M citric acid monohydrate buffer (pH 6.0), after which they were cooled to room temperature and rinsed with PBS (pH 7.4). The sections were pre-incubated with 10% normal goat serum (Dako, Glostrup, Denmark) in PBS for 15 min. Then sections were incubated overnight at 4°C with the primary antibodies SP197. The immune serum SP197 was diluted 1: 8,000 in PBS (vol/vol). Control sections were incubated either with nonimmune rabbit serum, diluted to the same extent as the primary antibodies, or with the primary antibodies, after incubation of the antibodies overnight with an excess of the free peptide (1 mg/ml), which was used to raise the antibodies. The antibodies and peptide were diluted to 1: 8,000 in PBS (vol/vol) for blocking experiments. Staining was carried out by the avidin-biotin peroxidase method. Cells were considered AR positive, when a nuclear staining was observed. Nuclear counterstaining was performed with Mayer's hematoxylin.

## RESULTS

### *Immunohistochemistry*

#### *Efferent ductules*

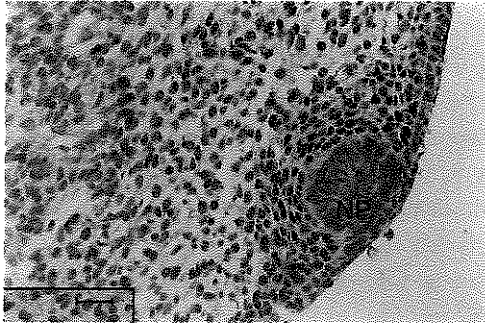
Day 14 is the onset of sexual differentiation of the efferent ductules and wolffian duct in the rat fetus. The efferent ductules contained some AR+ mesenchymal cells on day 14 (Fig. 1a). At this age the efferent ductules were the first sites of the mesonephric duct system in which AR expression also occurred in epithelial cells. Preincubation of the antibodies with the corresponding peptide blocked the nuclear staining pattern (Fig. 1c). During the whole further period of gestation from day 14 to day 10 after birth both epithelium and mesenchyme of the efferent ductules were AR+.

#### *Wolffian duct*

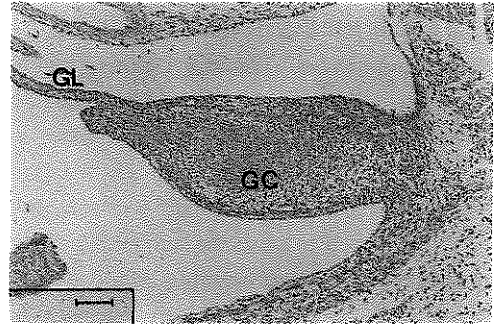
Fig.3. Immunohistochemical staining of the androgen receptor in the nipple anlagen, in the gubernaculum and in the perineum of the male fetus, and in the müllerian and wolffian ducts, in the genital sinus and in the tubercle of the female fetus.

a (day 14): The nipple anlagen (NP). b (day 18): The gubernacular cone (GC) and part of the ligament (GL). c Perineum (PE) at fetal day 18. The midline (ML) between the urethra and the skin is indicated. d (day 18): The müllerian duct (MD), the wolffian duct (WD) regresses. e (day 18): The female urogenital sinus. f (day 18): The female urogenital tubercle (GT), genital fold (GF) and swelling (GS). Bar = 25 µm in a,d,e; bar = 50 µm in c; bar = 100 µm in b,f.

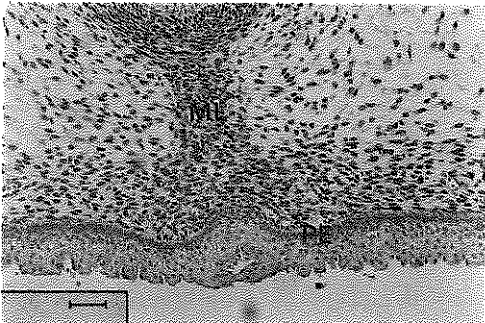
## Immunohistochemical localization of the AR



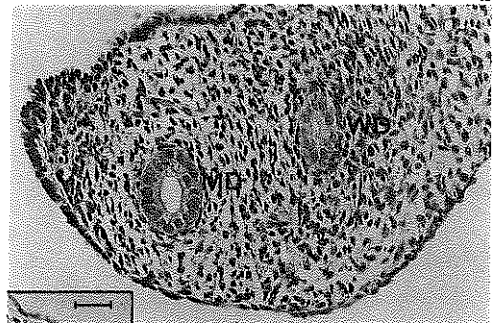
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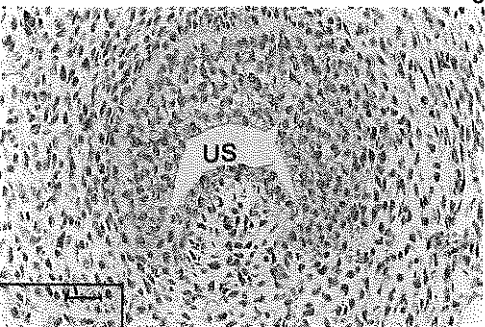
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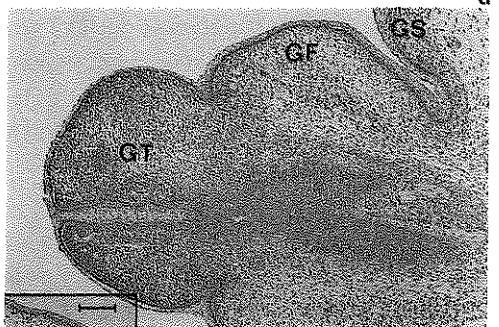
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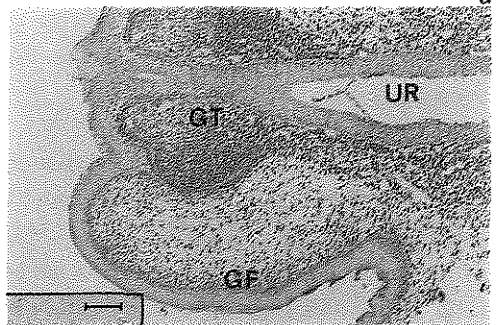
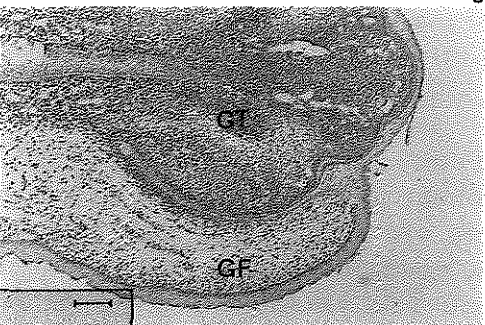
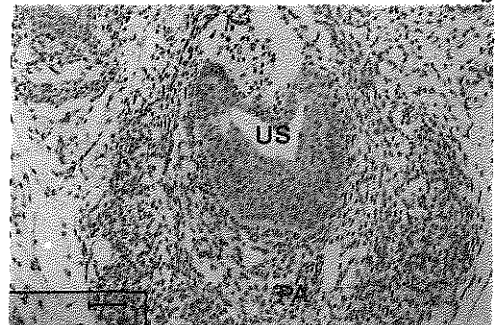
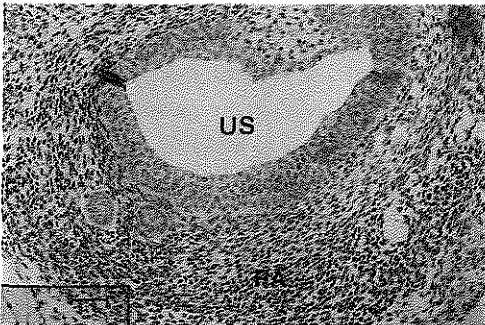
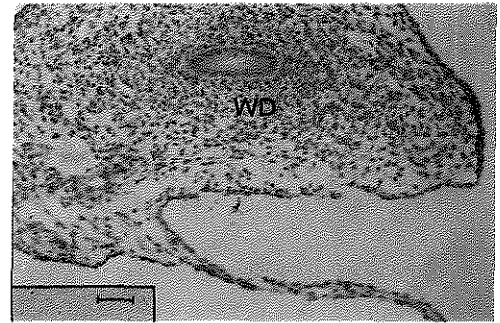
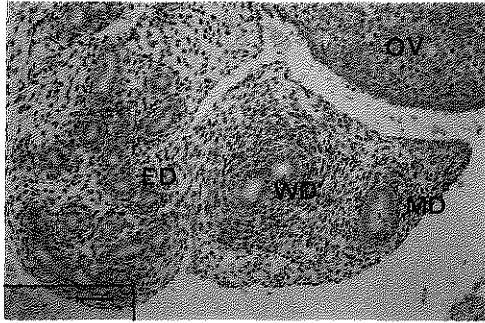


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## Immunohistochemical localization of the AR



## Immunohistochemical localization of the AR

Fig.4. Effect of 5 $\alpha$ -dihydrotestosterone on the urogenital tract in the female rat on fetal day 21, and the effect of the anti-androgen flutamide on the genitalia in the male fetus of the same age.

a: Wolffian duct (WD) and the efferent ductules (ED) were present in the female (MD: müllerian duct, OV: ovary). b: Wolffian duct after exposure to flutamide in the male. c: Prostate anlagen (PA) ventral of the urogenital sinus (US) in the female fetuses treated with DHT. d: Differentiation of the prostate anlagen (PA) was retarded in the flutamide treated male fetuses. e: The virilized female genital tubercle (GT) and genital fold (GF). f: Underdevelopment of the male genital tubercle with a cleft urethra (UR). Bar = 50  $\mu$ m in a,b,c,d; bar = 100  $\mu$ m in e,f.

The mesenchyme around the wolffian duct exhibited nuclear staining on day 14 (Fig. 1a,b). At the medial side of the wolffian duct more mesenchymal cells showed AR+ than at the lateral side, close to the müllerian duct (n = 4). The epithelia of the wolffian duct and the müllerian duct were negative at day 14 and 16. At day 18 of gestation epithelial cells of the epididymal head stained for AR (Fig. 1d). The epididymal tail showed mesenchymal cells expressing the AR from day 18 of gestation (Fig. 1e), but epithelium remained negative. Epithelial cells of the epididymal tail were weakly AR+ at day 2 after birth. At day 5 and day 10 the proportion of epithelial AR+ cells was increased (Fig. 1f) as compared to day 2.

In the vas deferens (distal wolffian duct) the AR was not detectable on day 14, 16 and 18, but became weakly detectable in the mesenchyme on fetal day 20 and in the epithelium from day 2 postnatally. On day 5, the epithelium of the vas deferens contained exclusively positive cells surrounded by a ring of AR negative tissue: the basal cell layer (Fig. 1f). This basal cell layer was surrounded by a ring of AR+ mesenchymal cells. The seminal vesicles exhibited AR+ cells in the mesenchyme from fetal day 21 onwards, and in the epithelium from day 2 after birth (Fig. 2c).

### *Urogenital sinus*

On fetal day 14 the prostate anlagen did not show the AR, but some staining of cells in the urothelium on top of the verumontanum was found, at the site where the ejaculatory ducts enter the sinus (Fig. 2a). On fetal day 16 AR+ cells were found only in the mesenchymal part of the prostate anlagen. The mesenchyme of the prostate stained intensively at fetal day 18 (Fig. 2b). At this stage a higher frequency of cells of the urothelium expressing the AR was observed. On day 2 a few epithelial cells of the prostate showed weak AR staining. The majority of epithelial cells of the prostate was AR+ on day 5 after birth (Fig. 2c).

### *Urogenital tubercle, folds and swellings*

The mesenchymal cells of the urogenital tubercle, of the genital folds and of the genital swellings, respectively were positive for AR from fetal day 14 onwards (Fig. 2d). During development of the external genitalia the amount of mesenchymal cells expressing the AR

## Immunohistochemical localization of the AR

increased progressively. (Fig. 2e,f). The epithelial cells in the skin of the tubercle showed AR expression from day 5 after birth.

*Differential AR expression pattern in the urogenital tract was compared with some other androgen dependent developmental structures: mammary gland, gubernaculum and perineum.*

### *Nipple primordia*

In the nipple anlagen of the mammary gland were many AR+ mesenchymal cells found on fetal day 14 (Fig. 3a).

### *Gubernaculum*

At fetal day 18 the mesenchyme of the gubernacular cone showed AR+ cells, in particular in the core (Fig. 3b). Some positive cells were identified in the gubernacular ligament or cord (Fig. 3b and 1e).

### *Pubic region and the perineum*

An abundant amount of mesenchymal AR+ cells was noticed in the perineum from day 18 onwards (3c) and also in the prepubic region (not shown).

### *Female urogenital tissues*

Müllerian duct structures remained negative for AR at all examined ages in male and female fetuses. In female fetuses at fetal day 18 and 21, AR expression in the regressing wolffian duct (Fig. 3d), urogenital sinus (Fig. 3e) and tubercle (Fig. 3f), respectively, was low as compared to frequencies of AR+ cells observed in male fetuses.

*Effect of DHT or flutamide on AR expression in genital tracts of female and male rat fetuses, respectively.*

### *Wolffian duct*

Administration of 5 $\alpha$ -dihydrotestosterone from day 11 to day 20 of gestation to pregnant rats caused a stabilization of the wolffian duct in female fetuses with an androgen receptor expression pattern similar as found in male fetuses (Fig. 4a).

No AR was detectable in the mesenchyme around the wolffian duct from the male fetus on the fetal day 21, after exposure to flutamide given daily starting from fetal day 11 (Fig 4b).

### *Urogenital sinus*

High AR levels in the mesenchyme of the prostate anlagen in female fetuses after DHT were consistent with a masculine pattern of differentiation of the prostate anlagen (Fig. 4c).

In the flutamide treated male fetus AR expression was diminished and prostate development was retarded (Fig. 4d).

### *Urogenital tubercle*

## Immunohistochemical localization of the AR

In the female fetus DHT administration caused an outgrowth of the tubercle which differentiated into the male direction, and an increase in cells positive for AR (Fig. 4e).

Administration of the androgen antagonist flutamide caused in male fetuses a reduction in AR+ cells and an underdevelopment of the urogenital tubercle with a wide open urethra (Fig. 4f).

TABLE I.

AR expression in mesenchymal and epithelial cells during development of fetal and neonatal male genitalia.

### Tissue:

Efferent ductules	<div></div>																		
Epididymis (head)	<div></div>																		
Epididymis (tail)	<div></div>																		
Vas deferens	<div></div>																		
Seminal vesicle	<div></div>																		
Prostate anlagen	<div></div>																		
Tubercle	<div></div>																		
Gubernaculum	<div></div>																		

14	15	16	17	18	19	20	21	0	1	2	3	4	5	6	7	8	9	10	
								↑											
Fetal								Birth	Neonatal										
Age (days)																			

mesenchyme AR+ ( ——— ); both mesenchyme and epithelium AR+ ( ===== )

## DISCUSSION

This study provides in vivo evidence that expression of the androgen receptor (AR) is regulated by androgens during morphogenesis of the genitalia. The AR is first induced in the mesenchymal cells and subsequently in the epithelial cells. The sequential appearance of the AR in mesenchymal and in epithelial cells in fetal and neonatal male reproductive tissues is given in a timetable (Table I). At fetal day 14 (the first day of investigation) only the efferent ductules showed some AR positive epithelial cells as well as AR positive mesenchymal cells. In other tissues, we found AR expression in the mesenchyme before the epithelium became positive.

From studies of the 5 $\alpha$ -reductase type 2 deficiency syndrome (Wilson et al., 1981) it is

## Immunohistochemical localization of the AR

clear that the efferent ductules and the wolffian duct are stabilized by testosterone. Probably, testosterone interacts with these tissues in an "exocrine" fashion: high concentrations of testosterone are diffusing from the testis via the efferent ductules down the lumen of the wolffian duct, creating a testosterone gradient along the wolffian duct (Veyssi re 1982). This concept is supported by a recent study in which transport of fluorescence labelled testosterone down the wolffian duct was reported during sexual differentiation of the mouse (Tong and Hutson, 1994).

Since testosterone (and not 5 $\alpha$ -dihydrotestosterone) virilizes efferent ductules and wolffian duct, it is conceivable that testosterone stabilizes the efferent ductules and the upper part of the epididymis, followed by the remaining part of the epididymis, the vas deferens and finally the seminal vesicle. In some species, however, the distal part and to a smaller extent the middle part of the wolffian duct may be less dependent on virilization by testosterone (Ts ju et al., 1994). Relatively high levels of testosterone reach these tissues by diffusion through the lumen of the mesonephric duct system, increasing AR expression in the efferent ductules and the medial side of the wolffian duct close to the testis respectively, followed by the remaining part of the wolffian duct: the epididymis and later in fetal life the vas deferens and seminal vesicle. The higher content of androgen receptor observed at the medial part of the wolffian duct in comparison to the lateral side may reflect a higher testosterone gradient caused by the hormonal diffusion from the testis. Prevention of transport of testosterone through the mesonephric duct derivatives may affect AR expression in the target cells negatively. Consequently androgen dependent development may be inhibited, which results in degeneration of the vas deferens. According to this hypothesis, mechanical obstruction of hormonal transport in the epididymis could explain the congenital absence of the vas deferens as found in patients with cystic fibrosis (Tizzano et al., 1994).

Various modulating physiological effects of androgens during embryogenesis are mediated by AR expressed in the mesenchymal cells. For example, male-type differential growth of the urogenital tubercle stimulated by androgen action starts with an abundant AR positive mesenchyme (Shannon and Cunha, 1983; Takeda et al., 1985; Husmann et al., 1990), while the effect of androgen action in the nipple primordia of the mammary gland in the male rat implies the ultimate regression of the nipple primordia (Wasner et al., 1983). The androgen-induced elimination is induced by androgen-activated mesenchymal cells, since the mesenchyme contained many AR positive cells in contrast to the negative epithelium.

During the investigated period the genital tubercle showed an increasing proportion of cells expressing the AR in the mesenchymal cells. Five days postnatally AR expressing epithelial cells were noticed in the prepuce. The AR expression of the rat penis during maturation has been studied extensively before (Takane et al., 1991a,b; Gonzales-Cadavid et al., 1991). After sexual maturation AR staining is only present in epithelial nuclei of preputial skin and urethra.

## Immunohistochemical localization of the AR

The presence of large amounts of AR in the perineum suggests a role for androgens in perineal outgrowth. During sexual differentiation the rat penis migrates to a more ventral position than the female tubercle. George (1989) showed, that exposure of male rat fetuses to a 5 $\alpha$ -reductase inhibitor during sexual differentiation reduced the distance between the anus and tubercle, indicating that this process is highly 5 $\alpha$ -dihydrotestosterone dependent. Furthermore, in this caudal region in the rat, androgen action is reflected in the sexual dimorphism in the levator ani/bulbocavernosus muscle group (Jung and Baulieu, 1972).

The role of androgen action in testicular descent is still under debate (Van der Schoot, 1992). Our study confirms the presence of AR in the rat gubernaculum (Husmann and McPhaul, 1991; Bentvelsen and George, 1993). AR positive cells are present in the gubernacular cord (or ligament) and in the core of the gubernacular cone, before inversion of the cone into a cremaster sac.

In the female genital tract AR does not seem to play a significant role. The protein disappears when the indifferent sexual stage has changed into a female direction. These data coincide with the observation that the critical period of AR action on the developing genitalia ends soon after birth (Schultz and Wilson, 1974). The decrease in the amount of AR positive cells in female fetuses could be prevented by administration of DHT to the pregnant dams. Exposure to androgen caused a stabilization of the wolffian duct in the females. Furthermore, a decrease in the number of AR containing cells in the genital tract of male fetuses was seen following the administration of the anti-androgen flutamide to pregnant rats.

In conclusion, these findings demonstrate the AR distribution in various tissues of the developing male genital tract of the male rat. Our data provide support for a mechanism by which androgens modulate the expression of AR in the genital tract by induction and subsequently proliferation of AR containing cells. It is consistent with the idea that virilization of the efferent ducts and the wolffian duct, is accomplished by large amounts of testosterone reaching these structures in an exocrine fashion. In these tissues testosterone induces the AR, in the mesenchyme first, and subsequently in the epithelium in a temporal fashion. The earliest expression is found in tissues with the smallest distance to the testis. In tissues virilized in an endocrine way by 5 $\alpha$ -dihydrotestosterone ARs appear later in time, but also in the mesenchymal cells first, and hereafter in the epithelial cells. The factors in the mesenchyme mediating the epithelial induction of ARs remain to be determined.



## Chapter 5

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**The fetal gubernaculum contains higher levels of androgen receptor than does the postnatal gubernaculum**

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### Abstract

The androgen receptor of the rat gubernaculum was measured by a sensitive immunoblotting technique from day 19 of fetal development to day 20 of postnatal development. In relative terms (densitometric units/ $\mu\text{g}$ . protein), it was found that the amount of the gubernacular androgen receptor decreased dramatically from fetal to postnatal development, coincident with the transition of the gubernaculum from a tissue primarily composed of undifferentiated mesenchymal cells in the fetus to a tissue that is primarily made up of muscle during postnatal development. We conclude that the undifferentiated mesenchyme of the fetal gubernaculum is a primary target of androgen action.

### 1. Introduction

Testicular descent is a complex process that involves transabdominal movement of the developing testis, formation of the processus vaginalis, and movement of the testis through the inguinal canal into the scrotum (Backhouse, 1982).

The first stage, transabdominal movement of the testis, probably involves, at a minimum, degeneration of the peritoneal fold that anchors the cranial part of the testis to the abdominal wall and expansion (outgrowth) of the caudal gonadal ligament (gubernaculum). Degeneration of the cranial gonadal ligament and transabdominal movement of the testis may be regulated by müllerian-inhibiting hormone (Josso et al., 1983; Hutson, 1985). Other aspects of testicular descent are thought to be directly or indirectly mediated by androgens. Compelling evidence that this is so is provided by the findings that cryptorchidism is a common clinical feature associated with genetic disorders involving abnormalities in gonadotropin production and androgen secretion or action (Bardin et al., 1969; Santen and Paulsen, 1973; Cattanach et al., 1973; Steiner and Boggs, 1965). Furthermore, in the rat and the rabbit, estradiol treatment completely inhibits testicular descent, presumably by primarily inhibiting gonadotropin secretion by the pituitary and secondarily inhibiting testicular androgen production (Rajfer and Walsh, 1977; Rajfer, 1982).

The formation of the gubernaculum testis, an anatomical structure found in all animals in which the testes descend, is critical for the development of the processus vaginalis and descent of the testis into the scrotum (Wells, 1944; Bergh et al., 1987; Frey and Rajfer, 1984). Consistent with the theory that the gubernaculum is the site of androgen action in testicular descent, we previously identified a relatively low amount of specific, high affinity androgen receptor in the newborn rat gubernaculum (George and Peterson, 1988). The androgen receptor in the gubernaculum seems to be located predominantly in the mesenchymal cells lining the core of the tissue (Husmann and McPhaul, 1991). If this is so, then it would be predicted that the amount of androgen receptor would be greater in the fetal gubernaculum, which is almost entirely composed of undifferentiated mesenchyme, than in the postnatal gubernaculum, which is made up of muscular tissue. Our analysis of the

androgen receptor in the developing gubernaculum of the rat by Western immunoblotting indicates that this prediction is true and substantiates the theory that the gubernaculum is a primary site of androgen action in testicular descent.

## **2. MATERIALS AND METHODS**

### *2.1. Animals and tissue preparation.*

Timed pregnant HoltzmanSprague-Dawley rats were obtained from Harlan Laboratories (Indianapolis, Indiana), maintained under controlled conditions of lighting (12 hours of light/12 hours of dark) and temperature (23°C), and allowed free access to standard laboratory chow and water. The day sperm were detected in the vagina was considered day 1 of pregnancy. Gubernacular tissue was dissected from fetuses on gestational days 19 and 22 and from newborn rats on days 2, 5, 10 and 20. The pooled tissues were weighed and stored in liquid N<sub>2</sub>. Preliminary experiments demonstrated that this method of tissue storage did not affect the immunoreactivity of the androgen receptor (results not shown). Tissue pools were thawed and homogenized (1 gm./9 ml.) in sample buffer (110 mM. sodium dodecyl sulphate, 100 mM. dithiothreitol, 80 mM. Tris [pH 6.9], 0.002% bromphenol blue and 10% glycerol) in an all glass Dounce homogenizer (10 strokes of a loose pestle, followed by 20 strokes of a tight pestle). The samples were then sonicated for 10 seconds (Model W185, Ultrasonics, Inc., Plainview, New York), boiled (5 minutes), and centrifuged for 30 minutes at 200,000 g. Aliquots of the supernatant (tissue extract) were electrophoresed on polyacrylamide gels which were then subjected to immunoblotting (see below). In preliminary experiments, it was shown that the 200,000 g pellet did not contain immunoreactive androgen receptor (results not shown).

### *2.2. Immunoblotting and densitometry.*

Extracts (50 µl.) were loaded onto 7.5% polyacrylamide gels containing 3.5 mM. sodium dodecyl sulfate and 0.2% N,N'-methylene-bis-acrylamide. The gels were run at 30 mA until the dye front reached the bottom of the running gel. The proteins were then transferred to nitrocellulose membrane filters using a Hoefer transphorblotting apparatus at 25 V in buffer containing 190 mM. glycine, 25 mM. Tris, and 20% (v/v) methanol at room temperature for 12 hours. The nitrocellulose filter was then incubated with affinity-purified anti-androgen receptor antibody U402, which is a rabbit polyclonal antibody directed against the aminoterminal 21 amino acids of the human androgen receptor (Husmann et al., 1990). <sup>125</sup>I-labelled goat anti-rabbit immunoglobulin G F(ab')<sub>2</sub> fragments were used to detect antibody binding. Immunoblots were exposed to Kodak XAR film at -80°C for 5 days. The apparent molecular weights of the visualized protein bands were inferred from the position of radiolabeled Rainbow Molecular Weight standards (Amersham Corp., Arlington Heights, Illinois).

The specificity of the method was assessed by preincubating the primary antiserum with

## AR in rat gubernaculum

excess peptide that was used as immunogen. For these experiments, the affinity-purified antibody (U402) was mixed with an excess (12 hours, 4°C) of the peptide used as immunogen (amino-terminal residues 1-21 of the human androgen receptor) prior to incubation with the nitrocellulose filter (Husmann et al., 1990). The density of the specific 110 kd androgen receptor band was measured on a computing densitometer (Model 300A, Molecular Dynamics, Sunnyvale, California).

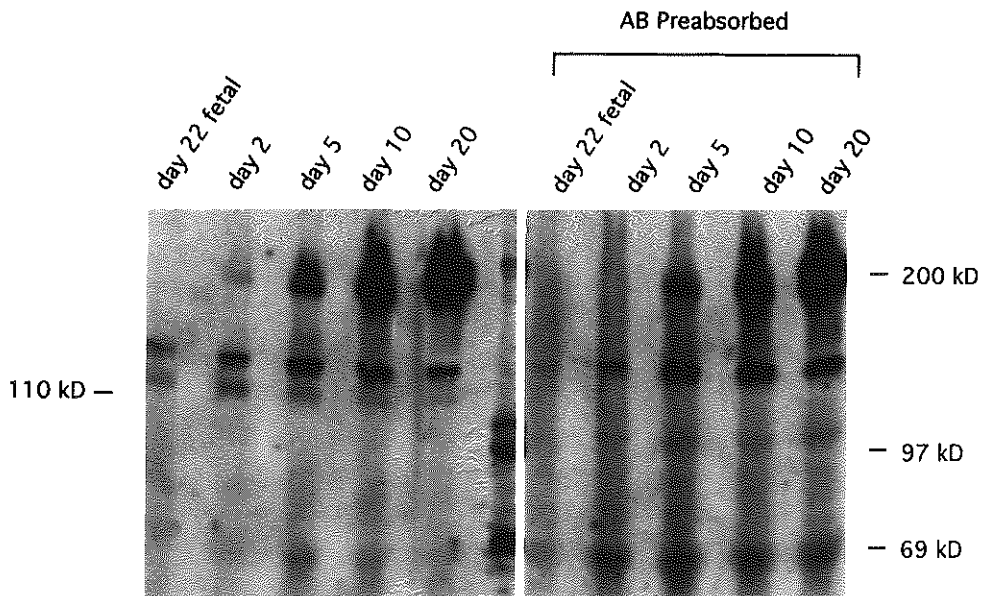


Fig. 1. Immunoreactive androgen receptor in male rat gubernaculum as function of age. Gubernacula were dissected from rats aged fetal day 22 to postnatal day 20, homogenized in loading buffer, and aliquots containing 0.2 to 0.78 mg. protein were loaded onto polyacrylamide gels as described in text. Immunoblotting was as described, and specificity was assessed by immunoabsorption of primary antibody with excess immunogen as described (Husmann et al., 1990).

### 3. RESULTS

When extracts of gubernacular tissue from male rats aged fetal day 22 to postnatal day 20 were electrophoresed on poly-acrylamide gels which were subsequently probed with an anti-androgen receptor antibody, the 110 kd protein characteristic of the androgen receptor of other androgen dependent tissues, such as prostate, is clearly recognizable (fig. 1). The specificity of the 110 kd band was verified by preabsorbing the primary androgen receptor antibody with an excess of the amino-terminal amino acid peptide used as immunogen (Fig. 1, right). At least two nonspecific proteins of higher molecular weight were detected in the gubernacular tissue by the androgen receptor antibody (Fig. 1). The identity of these proteins is not known and was not investigated. However, we suspect that these proteins are associated with the outer muscular portion of the developing gubernaculum since they appear to increase with age of the gubernacular tissue.

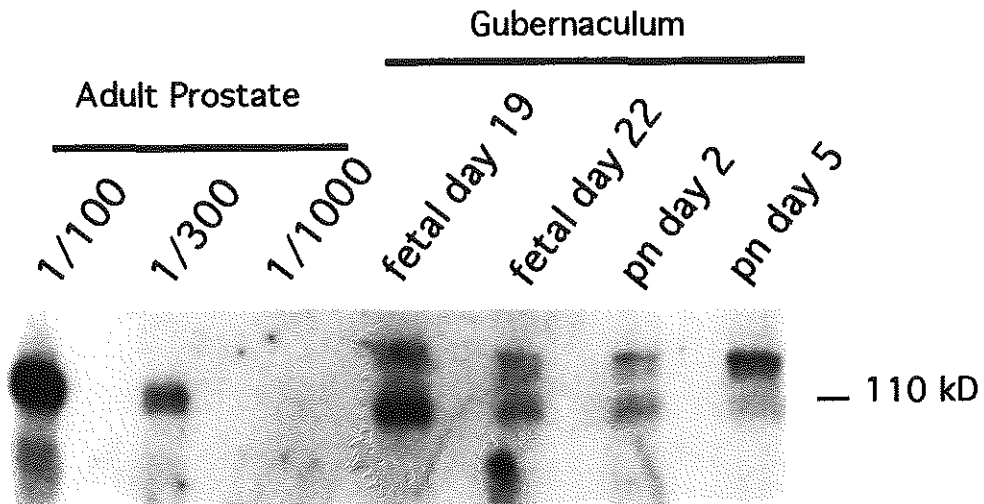


Fig. 2. Comparison of immunoreactive androgen receptor in adult rat prostate and fetal and newborn rat gubernacula. Gubernacula and prostate were dissected from rats at ages indicated and homogenized in loading buffer at ratio of 1 gm. tissue to 9 ml. buffer. Adult prostate samples were further diluted such that amount loaded represented 1/100 to 1/1000 (gm. tissue per ml. buffer). Immunoblotting was as described. Amount of protein loaded in wells corresponding to fetal day 19 was 0.20 mg.; fetal day 22 was 0.27 mg.; postnatal (pn) day 2 was 0.35 mg.; pn day 5 was 0.52 mg.

### AR in rat gubernaculum

Figure 2 demonstrates that when nearly identical amounts of gubernacular protein are loaded on the polyacrylamide gels, the decline in the 110 kd androgen receptor with age can be clearly seen. Furthermore, the results depicted in this figure confirm that the androgen receptor antibody recognizes a protein of similar size (110 kd) in both adult prostate and gubernaculum, and that the androgen receptor is much more abundant in the prostate than in the gubernaculum as has been previously reported (George and Peterson, 1988).

In figure 3 we have plotted gubernacular wet weight and the amount of gubernacular 110 kd androgen receptor as a function of age from fetal day 19 to postnatal day 20. The gubernaculum increased in size approximately 40-fold (from 0.5 mg. per gubernaculum to 21 mg. per gubernaculum). Over this same developmental period, the androgen receptor rapidly declined in concentration from a level of 110 densitometric units (DU)/ $\mu$ g. protein on day 19 of fetal life to a level of 13 DU/ $\mu$ g. protein by day 2 of postnatal development. By the time that testicular descent occurs in the rat, about day 20 of postnatal development, the amount of androgen receptor was 5 DU/ $\mu$ g. protein.

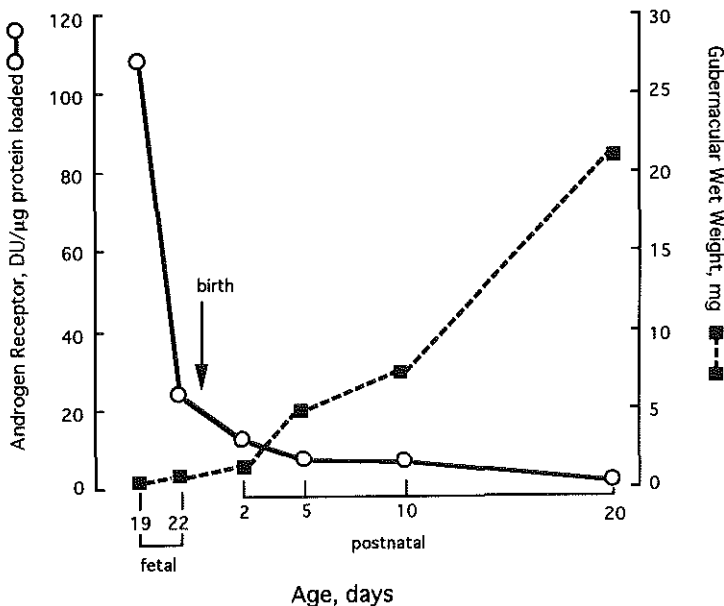


Fig. 3. Developmental pattern of immunoreactive androgen receptor in rat gubernaculum. Immunoblotting of gubernacular protein with anti-androgen receptor antibody was as described. Relative amount of androgen receptor is expressed as densitometric units (DU)/ $\mu$ g. protein loaded on the gel. Individual gubernacular wet weights were determined on pools of 16 to 52 gubernacula.

#### **4. DISCUSSION**

Previous work has indicated that the gubernaculum of the male rat contains a relatively modest amount of specific androgen receptor and is, therefore, likely to be a target tissue for androgen (George and Peterson, 1988). Furthermore, the site of androgen action in the gubernaculum appears to be the undifferentiated mesenchymal cells in the core of the tissue (Husmann and McPhaul, 1991). If this is true, it would be predicted that the amount of androgen receptor in the gubernaculum should decline with age as the mesenchymal cell core diminishes. In an attempt to clarify the role of androgen in testicular descent, we have measured the amount of androgen receptor in the developing gubernaculum by a sensitive Western immunoblotting technique. The 110 kd androgen receptor was clearly detectable by Western immunoblot analysis of gubernacular tissue from male rats at all ages, examined from fetal day 19 to postnatal day 20, and was found to decline dramatically during the prenatal period. These findings are consistent with the theory that the site of androgen action during the process of testicular descent is focused on the prenatal development of the gubernaculum. Prenatally, the gubernaculum is characterized as a mass of poorly organized, undifferentiated mesenchymal cells (George, 1989). Immunohistochemical evidence is also consistent with the theory that the site of androgen action is localized in the mesenchymal cells of the developing gubernaculum (Husmann and McPhaul, 1991). We assume that the amount of androgen receptor in the mesenchyme does not change with age, and that the amount of androgen receptor measured simply reflects the amount of mesenchyme.

Androgens do not influence the postnatal inversion of the gubernaculum that leads to descent, of the testis into the scrotum during the final stage of testicular descent (van der Schoot, 1992). Furthermore, Spenser et al. (1991) and Husmann and McPhaul (1991) have reported that the only period in which anti-androgens are effective in blocking testicular descent in the rat is from days 15.5 to 17 of fetal life, a finding which is consistent with the theory that the mesenchymal cells of the caudal gonadal ligament (the gubernaculum) are a site of androgen action.





## Chapter 6

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### **Immunoreactive androgen receptor levels in preputional skin of boys with isolated hypospadias**

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## Abstract

**Objective** To elucidate whether diminished levels of androgen receptor (AR) are the underlying cause for the development of hypospadias by determining AR levels in the foreskins of boys with hypospadias.

**Patients and methods** The content of AR was determined by a sensitive immunoblotting technique in extracts from preputial tissue obtained from 15 patients (mean age 2.5, SD  $\pm$  1.5 years) with hypospadias and from seven controls (mean age 2.5, SD  $\pm$  1.5 years). Immunoreactivity of the protein was measured by densitometry.

**Results** No significant difference in mean AR content was found between those boys with hypospadias ( $2.1 \pm 0.9$  fmol/mg protein,  $\pm$  SD) and the age-matched control group ( $2.2 \pm 0.3$  fmol/mg protein). Stratification by the severity of hypospadias (distal and proximal on the shaft, and penoscrotal hypospadias) showed that the severity was not related to tissue AR levels.

**Conclusion** It is unlikely that hypospadias in general is caused by a decreased expression of AR.

## Introduction

Hypospadias is a congenital anomaly occurring in 3 out of every 1,000 live male births. Rarely, hypospadias is related to mutations in the androgen receptor (AR) (McPhaul et al. 1993, Hiort et al. 1994, Batch et al. 1993, Kaspar et al. 1993) or even less frequently to the steroid 5 $\alpha$ -reductase type 2 deficiency syndrome (Wilson et al. 1993). It is unknown whether the development of hypospadias in general is caused by a decrease of androgen action due to diminished testicular androgen production or to a decrease in androgen receptor levels. Attempts to define an underlying endocrinopathy in androgen metabolism as a common cause of the disorder have been unsuccessful: Studies to determine whether the content of ARs in penile skin is decreased in hypospadias have been inconclusive (Svensson & Snochowski 1979, Terakawa et al. 1990, Gearhart et al. 1988, Keenan et al. 1984, Allen & Griffin 1984, Schweikert et al. 1989). The significance of the results of these studies was limited because AR was determined in cultures of genital skin fibroblasts by ligand binding assays which measure the maximum binding capacity of androgen to the receptors (Brown & Migeon 1981) in cultures, but do not reflect the number of receptors in the whole penile skin tissue. If only cultured fibroblasts are investigated, the contribution by other tissues containing target cells for androgens, such as the epithelium, is not included. Determination of the androgen receptor levels in foreskin tissue homogenates with a radio-ligand assay is complicated by endogenous testosterone levels either bound to plasma proteins or to the AR (Roehrborn et al. 1987). Time-consuming exchange assays affect considerably the integrity of the AR protein, because of the proteolytic breakdown. Fibroblast cultures are not a feasible system in which

to determine the amount of AR in foreskin: in immunohistochemical studies of human genital skin the basal cells of the squamous cell epithelium express many more ARs than do the fibroblasts (Ruizeveld de Winter et al. 1991).

The aim of the present investigation was to determine whether AR levels in prepuces of boys with isolated hypospadias are lower than those in normal foreskin obtained from circumcisions. Receptor levels were determined in homogenates of prepuces by measuring immunoreactivity of the AR protein in an immunoblot assay.

### MATERIALS AND METHODS

The study was comprised 15 patients (mean age  $\pm$  SD,  $2.5 \pm 1.5$  years) with isolated hypospadias (no associated anomaly) and seven control patients (mean age  $2.5 \pm 1.5$  years) undergoing circumcision; their ages at surgery ranged from 8 months to 5 years. Samples of preputional tissue were obtained during reconstructive surgery for those with hypospadias and during circumcision for the controls. The meatus was located on the glans in two patients, distal on the shaft in three, on the proximal shaft in seven and three had penoscrotal hypospadias.

*Tissue extraction.* Tissue samples were weighed and pulverized under liquid nitrogen, the powder suspended (0.1 g/0.9 ml) in sample buffer (100 mM Tris, pH 6.8, 1 mM EDTA, 20% glycerol, 20 mM dithiothreitol, 4% sodium dodecyl sulphate) containing protease inhibitors (0.6 mM phenylmethylsulfonylfluoride, 1 mM leupeptin and 0.5 mM bacitracin) and homogenized on ice in a glass/Teflon Dounce homogenizer (10 strokes of a loose pestle, followed by 20 strokes of a tight pestle). After centrifugation at 100,000 g for 10 min at 4°C the supernatant was used immediately for SDS-PAGE and Western immunoblotting or stored at -80°C and thawed only once.

*Immunoblot assay.* Aliquots (25  $\mu$ l) were boiled for 3 min before loading on 7% polyacrylamide gels. Nitrocellulose filters were incubated with monoclonal antibodies directed against amino acids 301-320 in the N-terminal domain of the human AR (F39.4) and immunoreactive AR was visualized by chemiluminescence as described previously (Veldscholte et al. 1992). Molecular mass values were estimated by comparison with protein molecular mass markers (Sigma, St. Louis, MO) run in the same gel. The films were scanned using a video densitometer (BIO-RAD, model 620) and the amount of AR in each sample was determined by comparison with the density of an internal standard included on each gel (Bentvelsen et al. 1994) which, in the present study, comprised several dilutions of a cytosol fraction of a 110 kDa AR preparation, obtained after expression in murine erythroleukemia cells of an AR expression vector (Needham et al. 1992). The standard curve is shown in Fig. 1 and only the linear section of the curve was used to calculate AR values.

In a hormone binding assay, performed as described previously (Veldscholte et al. 1992), the AR content of the cytosol preparations was 85 fmol/mg protein and the relative

levels of immunoreactive AR detected are expressed as fmol (equivalent) per mg protein. Protein levels were determined by the BCA Protein Assay (Pierce, Rockford, IL).

*Immunohistochemistry.* Immunohistochemistry was performed on 5µm-thick formalin-fixed, paraffin-embedded foreskins using a microwave antigen retrieval method (Janssen et al. 1994).

## RESULTS

The clinical features and AR level of the 15 patients and seven controls are shown in Table 1. No significant difference in AR content was found in the hypospadias group ( $2.1 \pm 0.9$  fmol/mg protein) when compared with the age-matched control group ( $2.2 \pm 0.3$  fmol/mg protein, Mean  $\pm$  SD). Stratification of the severity of hypospadias into glandular and distal shaft hypospadias, proximal shaft and penoscrotal hypospadias ( $1.9 \pm 0.8$ ,  $2.3 \pm 1.2$  and  $2.3 \pm 0.4$  fmol/mg protein, respectively) showed that the severity of hypospadias was not related to tissue AR levels. Two patients showed a relatively low level of AR expression ( $<0.15$  fmol/mg protein); one had glandular hypospadias and the other a proximal shaft hypospadias. Immunohistochemical staining showed no significant differences in tissue distribution of the AR in prepuces from patients with hypospadias and a control patient (who underwent circumcision for phimosis); AR was localized in the stratified squamous epithelium basal cells. The squamous cell epithelium expressed more AR than the stromal fibroblasts (Fig. 2).

## DISCUSSION

In human fetal development the penis is formed between week 8 and 14 of gestation. When the formation of the urethra is almost complete, the prepuce starts to develop, and by 15 weeks of gestation it completely covers the glans penis. Disorders in penile formation can result from abnormal hormonal activity during virilization of the external genitalia (Devine et al. 1980). Hypospadias has been related to estrogen exposure of the fetus in the first trimester. Early in pregnancy, exposure to external estrogens causes abnormal outgrowth of male reproductive organs. The incidence of hypospadias in sons of mothers treated with the synthetic estrogen, diethylstilbestrol (DES) was increased to one in 25 (Henderson et al. 1976). Also synthetic progesterone might affect urethral outgrowth, because a correlation was found between the time of maternal hormonal intake and the abnormal location of the urethral meatus (Aarskog 1979). These observations suggest aberrations in the hormonal environment can influence penile outgrowth.

The relationship between hypospadias and mutations in the AR gene has been established. Hypospadias is part of the disorders in individuals with the Reifenstein syndrome (Eberle et al. 1993). In the steroid 5 $\alpha$ -reductase type 2 deficiency syndrome testosterone is insufficiently metabolized to 5 $\alpha$ -dihydrotestosterone (Wilson et al. 1993). This impaired 5 $\alpha$ -dihydrotestosterone synthesis can result in hypospadias due to insufficient concentrations of

## AR in hypospadias

the active androgen in the target cells during fetal development of the penis. However, isolated hypospadias is rarely detected in families with 5 $\alpha$ -reductase type 2 deficiency syndrome.

An abnormal response to 5 $\alpha$ -dihydrotestosterone caused by abnormal binding capacity of the androgen receptor has been investigated extensively as a cause for hypospadias (Svensson & Snochowski 1979, Terakawa et al. 1990, Gearhart et al. 1988, Keenan et al. 1984, Allen & Griffin 1984, Schweikert et al. 1989). Biopsies of foreskin have been used to produce genital skin fibroblast cultures and assessed by radioligand binding. The results of these studies have been controversial and the method is not suitable to determine the amount of receptor protein in tissues.

Previously, a decreased level of AR was found in male rat fetuses after exposure to flutamide during sexual differentiation (Bentvelsen et al. 1994) and most of the rats exposed to flutamide developed hypospadias [unpublished observations]. Thus, by analogy, the present study investigated a correlation between decreased levels of AR and hypospadias. No differences in immunoreactive AR levels were found, nor were subgroups of distal and proximal hypospadias different from the control group. In general, no differences in AR distribution between AR containing cells in epithelium and stroma was observed in hypospadias and controls.

In conclusion, it is unlikely that decreased expression of AR causes hypospadias in general. However, our study has limitations, including that prepuce biopsies were only taken at a mean patient age of 2.5 years, when any differences in AR content between normal subjects and patients with hypospadias may no longer be present.

**Acknowledgements:** We are grateful to Mr. P.J. Janssen and Dr. Th. H. van der Kwast for the help with the immunohistochemistry.

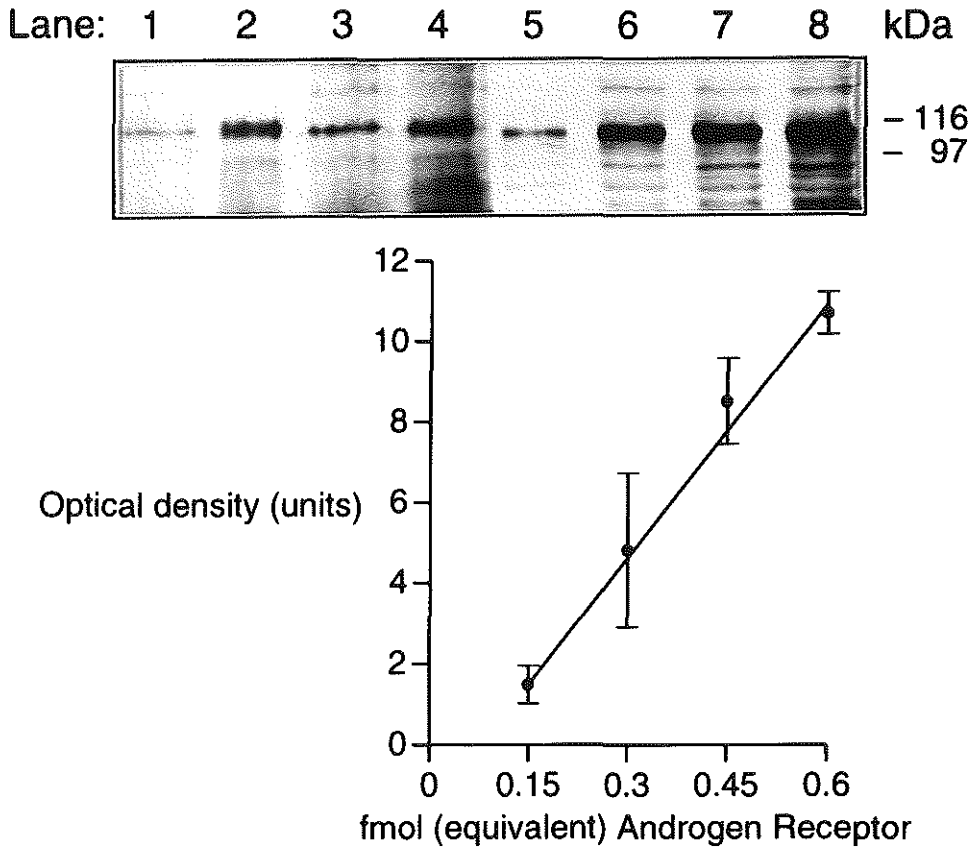


Fig. 1. Immunoblots of AR protein: 0.1 mg protein was loaded per lane. Lane 1. 0.16 fmol (proximal hypospadias). Lane 2. 0.18 fmol (glandular hypospadias). Lane 3. 0.19 (control). Lane 4. 0.24 (control). Lane 5, 6, 7 and 8, standard preparation of 0.15, 0.30, 0.45 and 0.60 fmol, respectively. The relationship between several dilutions of the AR standard preparation and the density of the 110 kDa bands obtained by immunoblotting is shown as an internal standard curve (combined from five experiments).

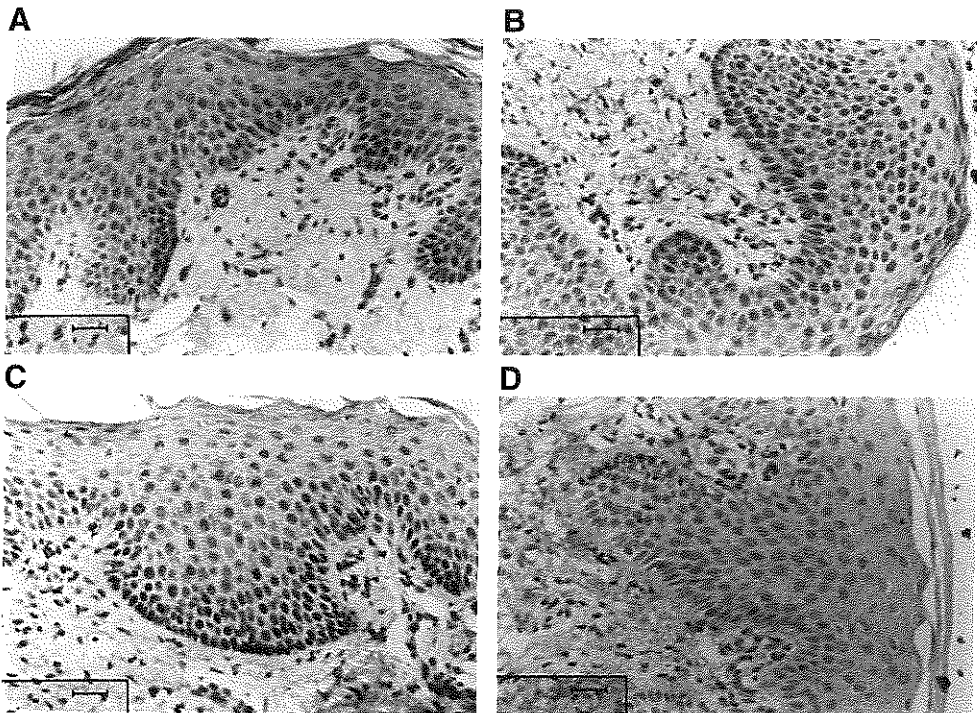


Fig. 2. Immunostaining of AR with the monoclonal antibody F39.4.1 in foreskins (A) from hypospadias patients with normal (2.6 fmol/mg protein) and (B) low expression of AR (<1.5 fmol/mg protein) and (C and D) a control patient (2.6 fmol/mg protein; D is a negative control). Nuclear counterstaining with Mayer's hematoxylin. Original magnification x 400. Bar= 7.5  $\mu$ m.

## AR in hypospadias

Table 1. Androgen Receptor (AR) Levels in Preputional Skin (a) from Boys with Hypospadias ( $2.1 \pm 0.9$  fmol/mg protein, Mean  $\pm$  SD) and (b) from a age-matched control group ( $2.2 \pm 0.3$  fmol/mg protein).

Table 1a

Hypospadias: location of the meatus	age (yr) at operation	AR (fmol/mg protein)
glandular	4 5/12	1.8
glandular	2 1/2	<1.5
distal shaft	2 3/4	2.6
distal shaft	1 5/6	2.2
distal shaft	1 2/3	2.2
proximal shaft	11/12	<1.5
proximal shaft	1 4/12	3.6
proximal shaft	11/12	1.8
proximal shaft	1 1/12	4.0
proximal shaft	2 2/3	2.5
proximal shaft	5 1/6	1.6
proximal shaft	1 1/3	1.8
penoscrotal	1 1/12	2.7
penoscrotal	4 5/12	2.3
penoscrotal	5 1/4	1.9



Table 1b

Controls: indication for operation	age (yr) at operation	AR (fmol/mg protein)
phimosis	2 5/6	1.8
phimosis	5 1/12	2.3
phimosis	1 1/12	2.3
phimosis	2 7/12	1.9
phimosis	2	2.4
phimosis	2/3	2.7
phimosis	3 3/4	2.6





### 7.1 Introduction

During specific periods of fetal and early neonatal life the genital tract is orchestrated in part by the sex hormones, acting through steroid hormone receptors. Hormones that control development of the genitalia appear to have a function in preparing tissues for a reproductive role in adult life, while survival *a priori* is not compromised by ineffective reproductive functions. The male sex hormones are essential for the development of the male phenotype during embryogenesis as well as male virilization during postnatal life. Impaired androgen action in the male fetus due to mutations in the androgen receptor gene causes impaired sex-specific development, which results in an intersex syndrome in man known as the androgen insensitivity syndrome.

This thesis deals with the role of the androgen receptor protein in the development and maintenance of the male phenotype. The physiological response to androgens is mediated by the androgen receptor, an intra-cellular protein, which functions as a ligand-dependent transcription factor. The role of the androgen receptor is associated with male phenotypical development. Administration of androgens to female rat embryos from day 12 until day 22 of gestation causes full masculinization (Schultz and Wilson, 1974). Androgen action depends on time-specific expression of the androgen receptor, and on tissue-specific conditions. Much detailed insight into human sexual development has generated from two main sources. The first is the analysis of the androgen insensitivity syndrome. The second is the close similarity between the syndrome and experimentally induced aberrancies in sexual development in animals. In this thesis the relationship of androgen receptor expression and hypospadias was explored in boys with hypospadias. An animal model, developed in the rat, was used for studying the pattern and role of androgen receptor expression.

### 7.2 Quantitative Western immunoblotting assay

Expression of the androgen receptor could be investigated by valid immunological methods, recently developed in laboratories in Dallas and Rotterdam (Wilson et al., 1992; Husmann et al., 1990, Janssen et al., 1993, Chapter 5). In the studies performed in Dallas, an affinity-purified polyclonal antibody (U402) raised against the first 21 amino acid residues of the androgen receptor N-terminal domain, has been used for western immunoblotting experiments. After some modification the method could be used as a quantitative androgen receptor assay. The androgen receptor levels were expressed as immunoreactive androgen receptor equivalent and determined with a standard curve obtained with adult rat prostate tissue (Bentvelsen et al., 1994). Prostate extract either used immediately after preparation, or stored at -80°C and thawed only once, showed no difference in immunoreactivity after storage. In Rotterdam, a monoclonal antibody (F39.4.1) (Zegers et al., 1991) was used in the same assay to analyze the level of human androgen receptor expression in preputional tissue by immunoblotting. The amount of immunoreactive androgen receptor in each tissue was

## General discussion

determined by comparing the density of the 110 kDa AR band with that of an internal standard with a known amount of androgen receptor, determined in binding studies.

### 7.3 Immunohistochemistry

Paraffin-embedded fetal and neonatal rat specimens were investigated with an immunohistochemical assay. Tissue sections expressing high levels of androgen receptor, were investigated with a polyclonal antibody (SP197) raised against the first 20 amino acid residues of the N-terminal domain of the human and rat androgen receptor (similar as U402). Preabsorption of the antibody with an excess of the oligopeptide utilized for raising the antibodies, completely blocked the interaction with the androgen receptor protein. It confirmed that androgen receptor was detected by the immunological staining. The results obtained with respect to androgen receptor up-regulation by androgens with Western immunoblotting were confirmed in an immunohistochemical study, showing an increase of frequency and density of androgen receptor containing cells after exposure to androgen. The differential stabilization by androgens (T versus DHT) of wolffian duct structures versus urogenital sinus and urogenital tubercle is still a subject under debate. In this thesis this issue has been addressed to some extent, in which the expression of androgen receptors in different tissues was compared by Western immunoblotting. So far no indications were found that different types of androgen receptors are expressed in tissues regulated extensively and exclusively by either T or DHT. The development pattern found by immunohistochemistry in the rat fetus (Bentvelsen et al., 1995b) was not substantially different from the pattern reported in the mouse, based on autoradiographic studies (Cooke et al., 1991).

### 7.4 Androgen receptor expression pattern and regulation

In the fetal rat, androgen receptor expression was stimulated by androgens and inhibited by androgen receptor blockade. In the adult rat, in accessory sex organs the same regulatory pattern was observed, however, adrenal androgen receptor expression was under the control of another yet unidentified non-testicular hormone, because androgen receptor levels in the adrenal gland did not change after castration. The regulatory effect of ACTH suppression was investigated by treatment of male rats with high doses of dexamethasone. When the animal received supraphysiological doses of dexamethasone, androgen receptor levels did not decrease that dramatically as was the case in hypophysectomized animals. It implies that androgen receptor levels in rat adrenal may not decrease after suppression of ACTH release. However, dexamethasone itself may have prevented a decrease in androgen receptor levels, and thereby counteracting a possible decrease caused by ACTH suppression. Therefore, at this moment ACTH cannot be ruled out as a candidate for androgen receptor regulation in the adrenal. Other pituitary gland hormones that may influence the androgen receptor expression in the rat adrenal are growth hormone and prolactin. The role of the adrenal androgen receptor is not

## General discussion

understood. For tying down androgen receptor regulation in the adrenal, additional experiments in hypophysectomized rats receiving ACTH should be performed (see Chapter 2). In the human adrenal androgen receptor expression could not be demonstrated (Ruizeveld de Winter et al., 1991; unpublished observations).

Evidence was obtained that the efferent ductules and the wolffian duct are virilized by testosterone which reaches the target tissues in an exocrine fashion. The concept that large amounts of testosterone activate the androgen receptor in the cranial part of the wolffian duct, close to the testis and earlier, than the distal part of the wolffian duct, is consistent with the temporal sequence of androgen receptor expression in these tissues. Androgen receptor expression in the wolffian duct proceeds during sexual development in a cranial-caudal sequence. The earliest expression was found in wolffian duct derivatives with the shortest distance to the testis. The expression in the mesenchymal cells precedes the expression in the epithelial cells.

In the rat fetus it is shown that reproductive tissues, in particular the urogenital tubercle, express androgen receptors at a higher level than other tissues in both male and female fetuses at the onset of sexual differentiation (Bentvelsen et al., 1994). The same is true during early stages for the estrogen receptor in male and female fetal mice (Greco et al., 1992, 1993). Presumably, for both androgen receptor and for estrogen receptor expression there exists a sexual indifferent stage of bipotential receptor availability. At what time androgen receptor expression starts and which factors are involved in early androgen receptor expression, is currently not known.

Exposure to flutamide during sexual differentiation led to down-regulation of the androgen receptor protein. Since the flutamide treated animals develop hypospadias a relation between the development of hypospadias and decreased androgen receptor levels is highly suggested. In contrast, in boys with hypospadias, no significant changes in androgen receptor levels were found (Chapter 6). However, the clinical study had its limitations, because the time point the prepuce biopsies were taken, was far beyond the period of differentiation of the urogenital tubercle. At the average age of 2½ years a possible difference of androgen receptor content between normals and hypospadias patients may not be present anymore.

### 7.5 Proposals

Several aspects of the study described in this thesis raise questions related to male sexual differentiation. The factors that initiate androgen receptor transcription have not been studied. Whether androgen receptor was expressed before sexual differentiation is still not unravelled. How the preference of expression for the reproductive tissues is established, prior to androgen production by the gonad, is unknown. Which mechanisms regulate androgen receptor expression?

Androgen receptor measurements and subcellular localization studies after administra-

## General discussion

tion of finasteride treatment of fetal rats in utero may give more insight in the role of testosterone and 5 $\alpha$ -dihydrotestosterone during sexual differentiation. Finasteride should affect the levels of 5 $\alpha$ -dihydrotestosterone in the urogenital sinus and urogenital tubercle, but should not alter the high androgen levels in the wolffian duct derivatives. If this occurs, the androgen receptor should be expressed in a normal way in wolffian duct tissues, but the expression should be inhibited in urogenital tubercle and sinus. It should reveal which part of the distal wolffian duct is stabilized by testosterone. Substitution by 5 $\alpha$ -dihydrotestosterone should overcome the blockade.

In humans at eight weeks of fetal age, testicular production of testosterone in the fetus may be initiated autonomously and subsequently regulated by chorionic gonadotropin (hCG) produced by the placenta and/or by LH. Aberrant penile development may be caused by delayed placental or pituitary gonadotropin secretion or incomplete transmission of hCG to the fetus. Our data showed an increase of androgen receptor protein expression in the rat after androgen treatment at the fetal age. Whether boys with micropenis should be treated with high doses of androgens to increase penile length is an unsolved problem (Husmann and Cain, 1994). From earlier animal studies it was concluded that down-regulation of androgen receptor expression can be expected when androgens are given before the pubertal age (Takane et al., 1991a,b). Earlier cessation of penile growth caused by a decreased number of androgen receptors would give a smaller penis at the adult age. For this reason, Husmann (1994) abandoned yet the use of testosterone cream or testosterone i.m. to increase penile size before hypospadias repair. However, androgen receptors are still present at high levels in the epithelium of preputional tissue of adults (unpublished observations). To answer the question whether androgen administration to prepubertal boys should be limited, androgen receptor levels should be measured in hypospadias patients before and after hormonal treatment. The final penile length after androgen treatment should be compared to an age-matched untreated control group. Clearly, more clinical data are necessary. A study on penile length in precocious puberty can possibly clarify this matter. Hopefully, the proposed investigations will lead to a better understanding of the role of androgens and the androgen receptor in normal and pathological phenotypical development of the urogenital tract.

## REFERENCES

- Aarskog D **1979** Maternal progestin as a possible cause of hypospadias. *N Engl J Med* 300: 75-78.
- Aiman J, Griffin JM, Gazak JM, Wilson JD, MacDonald PC **1979** Androgen insensitivity as a cause of infertility in otherwise normal men. *N Engl J Med* 300: 223-227
- Allen TD, Griffin JE **1984** Endocrine studies in patients with advanced hypospadias. *J Urol* 131: 310-314.
- Andersson S, Berman DM, Jenkins EP, Russell DW **1991** Deletion of steroid 5 $\alpha$ -reductase 2 gene in male pseudohermaphroditism. *Nature* 354: 159-161
- Baarends WM, Themmen APN, Blok LJ, Mackenbach P, Brinkmann AO, Meyer D, Faber PW, Trapman J, Grootegoed JA **1990** The androgen receptor promotor. *Mol Cell Endocrinol* 74: 75-84
- Backhouse KM **1982** Embryology of testicular descent and maldescent. *Urol Clin North Am*, 9: 315-325
- Bardin CW, Ross GT, Rifkind AB, Cargille CM, Lipsett MB **1969** Studies of the pituitary-Leydig cell axis in young men with hypogonadotropic hypogonadism and hyposmia: comparison with normal men, prepubertal boys and nonpituitary patients. *J Clin Invest* 48: 2046-2056
- Batch JA, Evans BAJ, Hughes IA, Patterson MN **1993** Mutations of the androgen receptor gene identified in perineal hypospadias. *J Med Genet* 30: 198-201
- Baillie AH, Ferguson MM, Hart DMcK **1966** Histochemical evidence of steroid metabolism in the human genital ridge. *J Clin Endocrinol Metab* 26: 738
- Beckman WC jr, Newbold RR, Teng CT, McLachlan JA **1994** Molecular feminization of mouse vesicle by prenatal exposure to diethylstilbestrol: Altered expression of messenger RNA. *J Urol* 151: 1370-1378.
- Bentvelsen FM, George FW **1993** The fetal gubernaculum contains higher levels of androgen receptor than does the postnatal gubernaculum. *J Urol* 150: 1564-1566.
- Bentvelsen FM, George FW, McPhaul MJ, Wilson JD **1994** The androgen receptor of the urogenital tract of the fetal rat is regulated by androgen. *Mol Cell Endocrinol* 105: 21-26.
- Bentvelsen FM, van der Linden JETM, Brinkmann AO, Schröder FH, Nijman JM **1995a** Immunoreactive androgen receptor levels in preputional skin of boys with isolated hypospadias. *Brit J Urol*, in press.
- Bentvelsen FM, Brinkmann AO, van der Schoot P, van der Linden JETM, van der Kwast ThH, Boersma WJA, Schröder FH, Nijman JM **1995b** Development pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. *Mol Cell Endocrinol*, in press
- Bergh A, Hendler HR, Wahlquist L **1978** Studies on factors governing testicular descent in the rat -particularly the role of the gubernaculum testis. *Int J Androl* 1: 342-356
- Birchmeier C, Birchmeier W **1994** Molecular aspects of mesenchymal epithelial interactions. *Ann Rev Cell Biol* 9: 511-540
- Bongiovanni AM **1962** The adrenogenital syndrome with deficiency of 3 $\beta$  hydroxysteroid dehydrogenase. *J Clin Invest* 41: 2086-2092
- Bremner WJ, Millar MR, Sharpe RM, Saunders PTK **1994** Immunohistochemical localization of androgen receptors in the rat testis: Evidence for stagedependent expression and regulation by androgens. *Endocrinology* 135: 1227-1234



## References

- Brinkmann AO, Jenster G, Ris-Stalpers C, van der Korput JAGM, Brüggewirth HT, Boehmer ALM, Trapman J **1994a** Androgen receptor abnormalities. In: Motta M, Serio M eds. Sex hormones and antihormones in endocrine dependent pathology: Basic and clinical aspects 1th edn. Amsterdam: Elsevier Science B.V., 33-44
- Brinkmann AO **1994b** Steroid hormone receptors: activators of gene transcription. *J Ped Endocrinol* 7: 275-282
- Brinkmann AO and Trapman J **1995** Mutant androgen receptors in endocrine-related cancer. *J Endocrinology* 2(3): 1-12
- Brown TR, Migeon CJ **1981** Cultured human skin fibroblasts: a model for the study of androgen action. *Mol Cell Biochem* 36: 3-21
- Cain MP, Kramer SA, Tindall DJ, Husmann DA **1994** Expression of androgen receptor protein within the lumbar spinal cord during ontologic development and following antiandrogen induced cryptorchism. *J Urol* 152: 766-769
- Calandra RS, Purvis K, Naess O, Attramadal A, Dloseland O, Hansson V **1978** Androgen receptors in the rat adrenal gland. *J Steroid Biochem* 9: 1009-15
- Catt K, Dufau ML, Neaves WB, Walsh PC, Wilson JD **1975** LH-hCG receptors and testosterone content during differentiation of the testis in the rabbit embryo. *Endocrinology* 97: 1157
- Cattanach BM, Iddon CA, Charlton H M, Chiappa SA, Fink G **1977** Gonadotropin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature* 269: 338-340
- Chang C, Kokontis J, Liao S **1988** Structural analysis of complementary DNA and amino acid sequences of human and rat androgen receptors. *Proc Natl Acad Sci USA* 85: 7211-7215
- Clark RL, Anderson CA, Prahallada S, Robertson RT, Lochry EA, Leonard YM, Stevens JL, Hoberman AM **1993** Critical developmental periods for effects on the male rat genitalia induced by finasteride, a 5 $\alpha$ -reductase inhibitor. *Toxicol Appl Pharmacol* 119: 34-40
- Coffey DS **1992** The molecular biology, endocrinology, and physiology of the prostate and seminal vesicles. In: Walsh PC, Gittes RF, Perlmutter AD, Stamey TA, eds. *Campbell's Urology* 6th edn; vol 2, Chapt 6. Philadelphia: WB Saunders & Co, pp 221-266
- Colby HD, Skelton FR, Brownie AC **1970** Testosterone-induced hypertension in the rat. *Endocrinology* 86: 1093-1101
- Cooke PS, Young P, Cunha GR **1991** Androgen receptor expression in developing male reproductive organs. *Endocrinology* 128: 2867-2873
- Cunha GR, Lung B **1978** The possible influence of temporal factors in androgenic responsiveness of urogenital tissue recombinants from wild-type and androgen-insensitive (Tfm) mice. *J Exp Zool* 205: 181-194
- Cunha GR, Chung, LWK, Shannon JM, Taguchi O, Fujii H **1983** Hormone induced morphogenesis and growth: Role of mesenchymal-epithelial interactions. *Recent Prog Horm Res* 39: 559-598
- Cunha GR, Cooke PS, Bigsby R, Brody JR **1991** Ontogeny of sex steroid receptors in mammals. In: Parker MG ed. *Nuclear Hormone Receptors* 1th edn; Chapt 11. London: Academic Press Ltd., 235-268
- Cunha GR, Foster BA, Donjacour AA, Rubin JS, Sugimura Y, Finch PW, Brody JR, Aaronson SA **1994** Keratinocyte growth factor: a mediator of mesenchymal-epithelial interactions in the development of androgen target organs. In: Motta M, Serio M eds. Sex hormones and antihormones in endocrine dependent pathology: Basic and clinical aspects 1th edn. Amsterdam: Elsevier Science B.V., 45-57
- Deslypere JP, Young M, Wilson JD, McPhaul MJ **1992** Testosterone and 5 $\alpha$ -dihydrotestosterone

## References

- interact differently with the androgen receptor to enhance transcription of the MMTV-CAT reporter gene. *Mol Cell Endocrinol* 88: 15-22
- Devine CJ, Gonzales-Serva L, Stecker JF, Devine PC, Horton CE **1980** Utricular configuration in hypospadias and intersex. *J Urol* 123: 407-411
- de Vries JDM **1986** Hypospadias repair with the transverse island flap repair technique. Thesis, Katholieke Universiteit Nijmegen
- Dolle P, Ruperte E, Leroy P, Moriss-Kay G, Chambon P **1990** A systematic study of their differential pattern of transcription during mouse organogenesis *Development* 110: 1133-1151
- Duckett JW **1992** Hypospadias. In: Walsh PC, Gittes RF, Perlmutter AD, Stamey TA, eds. *Campbell's Urology* 6th edn; vol 2, Chapt 50. Philadelphia: WB Saunders & Co, pp 1893-1919
- Eberle J, Überreiter S, Radmayr C, Janetschek G, Marberger H, Bartsch G **1993** Posterior hypospadias: Long term follow-up after reconstructive surgery in the male direction. *J Urol* 150: 1474-1477
- Evans RM **1988** The steroid and thyroid hormone receptor superfamily. *Science* 240: 889-895
- Evans BAJ, Williams DM, Hughes IA **1991** Normal postnatal androgen production and action in isolated micropenis and isolated hypospadias. *Archives of Disease in Childhood* 66: 1033-1036
- Faber PW, Kuiper GGJM, van Rooij HCJ, van der Korput JAGM, Brinkmann AO, Trapman J **1989** The N-terminal domain of the human androgen receptor is encoded by one, large exon. *Mol Cell Endocrinol* 61: 257-262
- Faber PW, van Rooij HCJ, van der Korput JAGM, Schipper HJ, Brinkmann AO, Trapman J **1991a** Characterization of the human androgen receptor transcription unit. *J Biol Chem* 266: 10743-10749
- Faber PW, King A, van Rooij HCJ, Brinkmann AO, de Both NJ, Trapman J **1991b** The mouse androgen receptor: functional analysis of the protein and characterization of the gene. *Biochem J* 278: 269-278
- Faber PW, van Rooij HCJ, Schipper HJ, Brinkmann AO, Trapman J **1993** Two different overlapping pathways of transcription activation on a TATA-less human androgen receptor promotor: the role of Sp1. *J Biol Chem* 268: 9296-9301
- Fentener van Vlissingen JM, van Zoelen EJ, Ursum PJ, Wensing CJ **1988** In vitro model of the first phase of testicular descent: identification of a low molecular weight factor from the fetal testis involved in proliferation of gubernaculum testis cells and distinct from specified polypeptide growth factors and fetal gonadal hormones. *Endocrinology* 123: 2868-2877
- Finch PW, Rubin JS, Miki T, Ron D, Aaronson SA **1989** Human KGF is FGF-related with properties of a paracrine effector of epithelial cell growth. *Science* 245: 752-755
- Frey HL, Rajfer J **1984** Role of the gubernaculum and intra-abdominal pressure in the process of testicular descent. *J Urol* 131: 574-579
- Frowein J, W Engel **1974** Constitutivity of the HCG-receptor protein in the testis of rat and man. *Nature* 249: 377-379
- Gallant S, Alfano J, Charpin M, Brownie AC **1991** Expression of adrenal cytochrome P-450 in testosterone-induced hypertension. *Hypertension* 18: 523-528
- Gearhart JP, Linhard HR, Berkovitz GD, Jeffs RD, Brown TR **1988** Androgen receptor levels and 5 $\alpha$ -reductase levels in preputial skin and chordee tissue of boys with isolated hypospadias. *J Urol* 140: 1243-1246
- George FW, Catt KJ, Neaves WB, Wilson JD **1978** Studies on the regulation of testosterone synthesis in the rabbit fetal testis. *Endocrinology* 102: 106-107

## References

- George FW, Wilson JD **1980** Endocrine differentiation of the fetal rabbit ovary in culture. *Nature* 283: 861-863
- George FW, Noble JF **1984** Androgen receptors are similar in fetal and adult rabbits. *Endocrinology* 115: 1451-1458
- George FW, Peterson KG **1988** 5 $\alpha$ -Dihydrotestosterone formation is necessary for embryogenesis of the rat prostate. *Endocrinology* 122: 1159-1164
- George FW, Peterson KG, Frenkel PA, Wilson JD **1988** The androgen receptor in the fetal epididymis is similar to that in the mature rabbit *Proc Soc Exp Biol Med* 188: 500-503
- George FW, Peterson KG **1988** Partial characterization of the androgen receptor of the newborn rat gubernaculum. *Biol Rep* 39: 536-539
- George FW **1989** Developmental pattern of 5 $\alpha$ -reductase activity in the rat gubernaculum. *Endocrinology* 124: 727-732
- George FW, Russell DW, Wilson JD **1991** Feed-forward control of prostate growth: Dihydrotestosterone induces expression of its own biosynthetic enzyme, steroid 5 $\alpha$ -reductase. *Proc Natl Acad Sci USA* 88: 8044-8047
- George FW, Wilson JD **1994** In: Knobil E, Neill JD, eds. *The Physiology of Reproduction* 2nd edn. New York: Raven Press, pp 3-28
- Goldstein JL, Wilson JD **1972** Studies on the pathogenesis of the pseudohermaphroditism in the mouse with testicular feminization. *J Clin Invest* 51: 1647-1658
- Goldstein JL, Wilson JD **1975** Genetic and hormonal control of male sexual differentiation. *J Cell Physiol* 85: 365-378
- Gonzales-Cadavid NF, RS Swerdloff, CAE Lemmi, J Rajfer **1991** Expression of the androgen receptor gene in the penile tissue and cells during sexual maturation. *Endocrinology* 129: 1671-1678
- Greco TL, Furlow JD, Duello TD, Gorski J **1992** Immunodetection of estrogen receptors in fetal and neonatal mouse reproductive tracts. *Endocrinology* 130: 421-429
- Greco TL, TM Duello, J Gorski **1993** Estrogen Receptors, Estradiol, and Diethylstilbestrol in early development: The mouse as a model for the study of estrogen receptors and estrogen sensitivity in embryonic development of male and female reproductive tracts. *Endocr Rev* 14: 59-71
- Greunwald P **1941** The relation of the growing müllerian duct to the wolffian duct and its importance for the genesis of malformations. *Anat Rec* 81: 1-19
- Griffin JE, Wilson JD **1989** The androgen resistance syndromes: 5 $\alpha$ -reductase deficiency, testicular feminization and related disorders. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic Basis of Inherited Disease*. New York: Mc Graw-Hill, pp 1919-1944
- Griffin JE and Wilson JD **1992** In: PC Walsh, AB Retik, TA Stamey and ED Vaughan, Jr, eds. *Campbell's Urology* 6th edn; vol 2, Chapt 37. Philadelphia: WB Saunders, pp 1509-1542
- Grino PB, Griffin JE, Wilson JD **1990** Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone. *Endocrinology* 126: 1165-1172
- Grossmann ME, Lindzey J, Blok L, Perry JE, Kumar MV, Tindall DJ **1994** The mouse androgen receptor gene contains a second functional promoter which is regulated by dihydrotestosterone. *Biochemistry* 33: 14594-14600
- Grumbach MM, van Wijk JJ and Wilkins **1955** Chromosomal sex in gonadal dysgenesis (ovarian agenesis) : relationship to male pseudo hermaphroditism and theories of human sex differentiation. *J Clin Endocr Metab* 15: 1161-1193

## References

- Gupta C, Bentlejewski CA **1992** Role of prostaglandins in the testosterone dependent wolffian duct differentiation of the mouse. *Biol Reprod* 47: 1151-1160
- Gupta C, Jaumotte J **1993** Epidermal growth factor binding in the developing male reproductive duct and its regulation by testosterone. *Endocrinology* 133: 1778-1782
- Habert R, Picon R **1984** Testosterone, dihydrotestosterone and estradiol-17 $\beta$  level in maternal and fetal plasma and in fetal testis of the rat. *J Steroid Biochem* 21: 193-198
- Hazebroek FWJ, de Muinck Keizer-Schrama SMPF, van Maarschalkerweerd M, Visser HKA, Molenaar JC **1987** Why luteinizing hormone-releasing-hormone nasal spray will not replace orchiopexy in the treatment of boys with undescended testes. *J Pediatr Surg* 22: 1177-1182
- He WW, Fischer LM, Sun S, Bilhartz DL, Zhu X, Young C Y-F, Kelly DB, Tindall DJ **1990** Molecular cloning of androgen receptors from divergent species with a polymerase chain reaction technique: complete cDNA sequence of the mouse androgen receptor and isolation of androgen receptor cDNA probes from dog, guinea pig and clawed frog. *Biochem Biophys Res Commun* 171: 697-704
- Heaton JPW, SJ Varrin **1994** Effects of castration and exogenous testosterone supplementation in an animal model of penile erection. *J Urol* 151: 797-800
- Henderson BE, Benton B, Cosgrove M et al. **1976** Urogenital tract abnormalities in sons of women treated with diethylstilbestrol. *Pediatrics* 58: 505
- Hiort O, Klauber G, Cedron M, Sinnecker GHG, Keim L, Schwinger E, Wolfe H, Yandell DW **1994** Molecular characterization of the androgen receptor gene in boys with hypospadias. *Eur J Pediatr* 153: 317-321
- Horie K, Takakura K, Fujiwara H, Suginami H, Liao S, Mori T **1992** Immunohistochemical localization of androgen receptor in the human ovary throughout the menstrual cycle in relation to oestrogen progesterone receptor expression. *Hum Repr* 7: 184-190
- Huggins C, Hodges CV **1941** Studies on prostate cancer I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res* 1: 293-297
- Huhtaniemi IT, Korenbrot CC, Jaffe RB **1977** hCG binding and stimulation of testosterone biosynthesis in the human fetal testis. *J Clin Endocrinol Metab* 44: 963-967
- Hunter JA **1786** A description of the situation of the testis in the foetus, with its descent into the scrotum . In: *Observations on certain parts of the animal oeconomy*. London, pp 1-26
- Hunter RH **1933** Notes on the development of the prepuce. *J Anat* 70: 68
- Husmann DA, Wilson CM, McPhaul MJ, Tilley WD, Wilson JD **1990** Antipeptide antibodies to two distinct regions of the androgen receptor localize the receptor protein to the nuclei of target cells in the rat and human prostate. *Endocrinology* 126: 2359-2368
- Husmann DA, MJ McPhaul **1991** Localization of the androgen receptor in the developing rat gubernaculum. *Endocrinology* 128: 383-387
- Husmann DA, McPhaul MJ **1991** Time-specific androgen blockade with flutamide inhibits testicular descent in the rat. *Endocrinology*, 129: 1409-1416
- Husmann DA, Cain MP **1994** Microphallus: Eventual phallic size is dependent on the timing of androgen administration. *J Urol* 152: 734-739
- Hutson JM **1985** A biphasic model for the hormonal control of testicular descent. *Lancet* 2: 419-421
- Imperato-McGinley J, Guerrero L, Gautier T, Peterson RE **1974** Steroid 5 $\alpha$ -reductase deficiency in man: an inherited form of male pseudohermaphroditism. *Science* 186: 1213-1215

## References

- Imperato-Mc Ginley J, Sanches RS, Spencer JR, Yee B, Vaughan ED **1992** Comparison of the effects of the 5 $\alpha$ -reductase inhibitor finasteride and the antiandrogen flutamide on prostate and genital differentiation: Dose-response studies. *Endocrinology* 131: 1149-1156
- Janssen PJ, Brinkmann AO, Boersma WJ, Kwast Th H van der **1994** Immunohistochemical detection of the androgen receptor with monoclonal antibody F39.4 in routinely processed, paraffin-embedded human tissues after microwave pretreatment. *J Histochem Cytochem* 42: 1169-1175
- Janssen P.J., Zegers N.D., Boersma W.J., Verleun-Mooyman M.C.T., Brinkmann A.O., Kwast Th. H. van der **1995** Androgen receptor distribution in mouse, rat and human tissues; a comparative study using a novel polyclonal antibody homologous to the N-terminal receptor domain part of the mouse, rat and human androgen receptor. In preparation.
- Jenster G, Trapman J, Brinkmann AO **1993** Nuclear import of the human androgen receptor. *Biochem J* 293: 761-768
- Johnson DE, Williams LT **1993** Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res* 60: 1-35
- Josso N, Fekete C, Cachin O, Nezelof C, Rappaport R **1983** Persistent müllerian ducts in male pseudohermaphroditism, and its relationship to cryptorchidism. *Clin Endocrinol* 29: 247-258
- Jost A **1947** Recherches sur la différenciation sexuelle de l'embryon de lapin. III. Rôle des gonades foetales dans la différenciation sexuelle somatique. *Arch Anat microsc Morphol expér* 40: 271-316
- Jost A **1953** Problems of fetal endocrinology: the gonadal and hypophyseal hormones. *Recent Prog Horm Res* 8: 379-413
- Junkmann K, Neumann F **1964** Zum Wirkungsmechanismus von an Feten antimaskulin wirksamer Gestagen. *Acta Endocrinol (Kbh)* 45 Suppl 90: 139-153
- Jung I, Baulieu EE **1972** Testosterone cytosol "receptor" in the rat levator ani muscle. *Nature [New Biology]* 237: 24-26
- Kallen B, Winberg J **1982** An epidemiological study of hypospadias in Sweden. *Acta Paediatr Scand (suppl 293)*: 1-21
- Kalloo NB, Gearhart JP, Barrack ER **1993** Sexually dimorphic expression of estrogen receptors, but not of androgen receptors in human fetal external genitalia. *J Clin Endocrinol Metab* 77: 692-698
- Kaplan SL, Grumbach MM, Aubert ML **1976** The ontogenesis of pituitary hormones and hypothalamic factors in the human fetus: maturation of central nervous system regulation of anterior pituitary function. *Recent Prog Horm Res* 32: 161-243
- Kaplan SL, Grumbach MM **1978** Pituitary and placental gonadotropins and sex steroids in the human and sub-human primate fetus. *J Clin Endocrinol Metab* 7: 487-511
- Kaspar F, Cato ACB, Denninger A, Eberle J, Radmayr C, Glatzl J, Bartsch G, Klocker H **1993** Characterization of two point mutations in the androgen receptor gene of patients with perineoscrotal hypospadias. *J Steroid Biochem Mol Biol* 47: 127-135
- Keenan BS, McNeel RL, Gonzales ET **1984** Abnormality of intracellular 5 $\alpha$ -dihydrotestosterone binding in simple hypospadias: Studies on equilibrium steroid binding in sonicates of genital skin fibroblasts. *Pediatr Res* 18: 216-220
- Kemppainen JA, Lane MV, Sar M, Wilson EM **1992** Androgen receptor phosphorylation, turnover, nuclear transport, and transcription activation. *J Biol Chem* 267: 968-974
- Kerr JFR, Searle J **1973** Deletion of cells by apoptosis during castration induced involution of the rat prostate. *Virchows Arch* 13: 87-102
- Kluth D, Lambrecht W, Reich P **1988** Pathogenesis of hypospadias - More questions than answers. *J*

## References

Ped Surg 23: 1095-1101

Kratochwil K, Schwartz P **1976** Tissue interaction in androgen response of embryonic mammary rudiment of the mouse: Identification of the target tissue for testosterone. *Proc Natl Acad Sci USA* 73: 4041-4044

Krongrad A, Wilson CM, Wilson JD, Allman DR, McPhaul MJ **1991** Androgen increases androgen receptor protein while decreasing receptor mRNA in LNCaP cells. *Mol Cell Endocrinol* 76: 79-88

Kuiper GGJM, Faber PW, van Rooij HCJ, van der Korput HCJ, Ris-Stalpers JAGM, Klaassen C, Trapman J, Brinkmann AO **1989** Structural organization of the human androgen receptor gene. *J Mol Endocrin* 2: R1-R4

Kuiper GGJM, de Ruiter PE, Trapman J, Boersma WJA, Grootegoed JA, Brinkmann AO **1993** Localization and hormonal stimulation of phosphorylation sites in the LNCaP-cell androgen receptor. *Biochem J* 291: 95-101

Kuiper GGJM, Brinkmann AO **1994** Steroid hormone receptor phosphorylation: is there a physiological role? *Mol Cell Endocrinol* 100: 103-107

Kumar MV, Jones EA, Grossmann ME, Blexrud MD, Tindall DJ **1994** Identification and characterization of a suppressor element in the 5'-flanking region of the mouse androgen receptor gene. *Nucleic Acids Res* 22: 3693-3698

Kyprianou N, Isaacs JT **1988** Activation of programmed cell death in the rat ventral prostate after castration. *Endocrinology* 122: 552-562

Laemmli UK **1970** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685

Lasnitzki I, Mizuno T **1980** Prostate induction: Interaction of epithelium and mesenchyme from normal wild-type and androgen insensitive mice with testicular feminization. *J Endocrinol* 85: 423-428

Lyon MF, Hawkes SG **1970** X-linked gene for testicular feminization in the mouse. *Nature* 227: 1217-1219

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ **1951** Protein measurement with The Folin phenol reagent. *J Biol Chem* 193: 265-275

Lubahn DB, Joseph DR, Sullivan PM, Willard HF, French FS, Wilson EM **1988** Cloning of human androgen receptor complementary DNA and localization on the X chromosome. *Science* 240: 327-330

Lubahn DB, Joseph DR, Sar M, Tan J, Higgs HN, Larson RE, French FS, Wilson EM **1988** The human androgen receptor: complementary deoxyribonucleic acid cloning, sequence analysis, and gene expression in the prostate. *Mol Endocrinol* 2: 1265-1275

Lubahn DB, Brown TR, Simental JA, Higgs HN, Migeon CJ, Wilson EM, French FS **1989** Sequence of the intron/ exon junctions of the coding regions of the human androgen receptor gene and identification of a point mutation in a family with complete androgen insensitivity. *Proc Natl Acad Sci USA* 86: 9534-9538

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O **1993** Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci USA* 90: 11162-11166

Marcelli M, Tilley WD, Wilson CM, Griffin JE, Wilson JD, McPhaul MJ **1990** Definition of the human androgen receptor gene structure permits the identification of mutations that cause androgen resistance: premature termination of the receptor protein at aminoacid residue 588 causes complete androgen resistance. *Mol Endocrinology* 4: 1105-1116

Matlaj P, Beral V **1985** Trends in congenital malformations of external genitalia. *Lancet* 1: 108

## References

- McPhaul MJ, Marcelli M, Zoppi S, Griffin JE, Wilson JD **1993** The spectrum of mutations in the androgen receptor gene that causes androgen resistance. *J Clin Endocrinol Metab* 76: 17-23
- Mittwoch U, Burgess AMC, Baker PJ **1993** Male sexual development in "a sea of oestrogen". *Lancet* 342: 123-124
- Molsberry RL, Cau BR, Mendelson CR, et al. **1982** Human chorionic gonadotropins binding to human fetal testis. *J Clin Endocrinol Metab* 55: 791-794
- Needham M, Gooding C, Hudson K, Antoniou M, Grosveld F, Hollis M **1992** LCR/MEL: A versatile system for high-level expression of heterologous proteins in erythroid cells. *Nucleic Acids Research* 20: 997-1003
- Newbold RR, Suzuki Y, McLachlan JA **1984** Müllerian duct maintenance in heterotypic organ culture after in vivo exposure to diethylstilbestrol. *Endocrinology* 115: 1863-1868
- Neumann F, von Berswordt-Wallrabe R, Elger W et al. **1970** Aspects of androgen-dependent events as studied by antiandrogens. *Recent Prog Horm Res* 26: 337-410
- O'Malley B **1990** The steroid receptor superfamily: more excitement predicted in the future. *Mol Endocrinol* 4: 363-369
- Orti E, Bodwell JE, Munck A **1992** Phosphorylation of steroid hormone receptors. *Endocr Rev* 13: 105-128
- Patterson MN, McPhaul MJ, Hughes IA **1994** Androgen Insensitivity Syndrome. In: Sheppard M and Stewart P, eds. *Baillières Clinical Endocrinology and Metabolism*; vol 8, Chapt 8. Cambridge: University Press, pp 379-404
- Pfaltz CR **1949** Das embryonale und postnatale Verhalten den Männlichen Brustdrüse beim Menschen. II. Das Mammaorgan in Kinder-, Junglings-, Mannes- und Greisalten. *Acta Anat* 8: 293
- Price D, Ortiz E **1965** The role of fetal androgens in sex differentiation in mammals. In: RL Ursprung, H de Haan, eds. *Organogenesis*, Holt Rinehart and Wiston, New York 629-652
- Prins G **1987** Prolactin influence on cytosol and nuclear androgen receptors in the ventral, dorsal, and lateral lobes of the rat prostate. *Endocrinology* 120:1457-1464
- Prins GS, Cecim M, Birch L, Wagner TE, Bartke A **1992** Growth response and androgen receptor expression in seminal vesicles from aging transgenic mice expressing human or bovine growth hormone genes. *Endocrinology* 131: 2016-2023
- Prins GS, Birch L **1993** Immunocytochemical analysis of androgen receptor along the ducts of the separate rat prostate lobes after androgen withdrawal and replacement. *Endocrinology* 132: 169-178
- Prins GS, Birch L **1995** The development pattern of androgen receptor expression in rat prostate lobes is altered after neonatal exposure to estrogen. *Endocrinology* 136: 1303-1314
- Puscheck EE, Behzadian MA, McDonough PG **1994** The first analysis of exon 1 (the transactivation domain) of the androgen receptor gene in infertile men with oligospermia or azoospermia. *Fertil Steril* 62: 1035-1038
- Quarmby VE, Yarbrough WG, Lubahn DB, French FS, Wilson EM **1990** Autologous down-regulation of androgen receptor messenger ribonucleic acid. *Mol Endocrinol* 4: 22-28
- Rajfer J, Walsh PC **1977** Hormonal regulation of testicular descent: Experimental and clinical observations. *J Urol* 118: 985-990
- Rajfer J **1982** Endocrinological study of testicular descent in the rabbit. *J Surg Res* 33: 158-163
- Randall, VA **1994a** Androgens and human hair growth. *Clinical Endocrinology* 40: 439-457

## References

- Randall, VA **1994b** Role of 5 $\alpha$ -reductase in health and disease. In: Sheppard M and Stewart P, eds. Baillières Clinical Endocrinology and Metabolism; vol 8, Chapt 9. Cambridge: University Press, pp 405-431
- Reiter E, Bonnet P, Sente B, Dombrowicz D, de Leval J, Closset J, Hennen G **1992** Growth hormone and prolactin stimulate androgen receptor, insulin-like growth factor-I (IGF-I) and IGF-I receptor levels in the prostate of immature rats. *Mol Cell Endocrinol* 88: 77-87
- Riegman PHJ, Vlietstra RJ, van der Korput JAGM, Brinkmann AO, Trapman J **1991** The promoter of the prostate-specific antigen gene contains a functional responsive element. *Mol Endocrinol* 5: 1921-1930
- Rifka SM, Cutler GB Jr, Sauer MA, Loriaux DL **1978** Rat adrenal androgen receptor: A possible mediator of androgen induced decrease in rat adrenal weight. *Endocrinology* 103: 1103-1110
- Roberts KP, Zirkin BR **1991** Androgen regulation of spermatogenesis. *Ann N Y Acad Sci* 637: 90-106
- Roehrborn CG, Lange JL, George FW, Wilson JD **1987** Changes in amount and intracellular distribution of androgen receptor in human foreskin as a function of age. *J Clin Invest* 79: 44-47
- Ruizeveld de Winter JA, Trapman J, Vermey M, Mulder E, Zegers ND, van der Kwast Th H **1991** Androgen Receptor expression in human tissues: an immunohistochemical study. *J Histochem Cytochem* 39: 927-936
- Ruizeveld de Winter JA, van Weerden W, Faber PW, van Steenbrugge GJ, Trapman J, Brinkmann AO, van der Kwast ThH **1992** Regulation of androgen receptor expression in the human heterotransplantable prostate carcinoma PC-82. *Endocrinology* 131: 3045-3050
- Saffer JD, Jackson SP, Annarella MB **1991** Developmental expression of Sp1 in the mouse. *Mol Cell Biol* 11: 2189-2199
- Sar M, Lubahn DB, French FS, Wilson EM **1990** Immunohistochemical localization of the androgen receptor in rat and human tissues. *Endocrinology* 127: 3180-3186
- Santen RJ, Paulsen CA **1973** Hypogonadotropic eunichoidism. II. Gonadal responsiveness to exogenous gonadotropins. *J Clin Endocrinol Metab* 36: 55-63
- Schröder FH **1993** Endocrine therapy for prostate cancer: Recent developments and current status. *Br J Urol* 71: 633-640
- Schröder FH **1994** 5 $\alpha$ -Reductase inhibitors and prostatic disease. *Clin Endocrinol* 41: 139-147
- Schultz FM, JD Wilson **1974** Virilization of the Wolffian duct in the rat fetus by various androgens. *Endocrinology* 94: 979-986
- Schweikert HU, Schlüter M, Romalo G **1989** Intracellular and nuclear binding of [ $^3$ H] dihydrotestosterone in cultured genital skin fibroblasts of patients with severe hypospadias. *J Clin Invest* 83: 662-668
- Shan LX, Rodriguez MC, Jänne OA **1990** Regulation of androgen receptor protein and mRNA concentrations by androgens in rat ventral prostate and seminal vesicle in human hepatoma cells. *Mol Endocrinol* 4: 1636-1646
- Shannon JM, Cunha GR **1983** Autoradiographic localization of androgen binding in the developing mouse prostate. *Prostate* 4: 367-373
- Sharpe RM, Skakkebaek NE **1993** Are estrogens involved in falling sperm counts and disorders of the male reproductive tract? *Lancet* 341: 1392-1395
- Schultz F.M., Wilson J.D. **1974** Virilization of the wolffian duct in the rat fetus by various androgens. *Endocrinology* 94: 979-986



## References

- Shono T, Ramm-Anderson S, Hutson JM **1994** Transabdominal testicular descent is really ovarian ascent. *J Urol* 152: 781-784
- Siiteri PK, Wilson JD **1974** Testosterone formation and metabolism during male sexual differentiation in the human embryo. *J Clin Endocrinol Metab* 38: 113-125
- Silver RI, EL Wiley, AE Thigpen, JM Guileyardo, JD McConnel, DW Russel **1994** Cell type specific expression of steroid 5 $\alpha$ -reductase 2. *J Urol* 152: 438-442
- Spencer JR, Torrado T, Sanchez RS, Vaughan ED Jr, Imperato-McGinley J **1991** Effects of flutamide and finasteride on rat testicular descent. *Endocrinology*, 129: 741-748
- Steglehner G **1875** De hermaphroditorum natura. Bamberg and Leipzig, 1817 (cited by Leopold). *Archiv Gynakologie* 8: 487-490
- Steiner MM, Boggs JD **1965** Absence of the pituitary gland, hypothyroidism, hypoadrenalism and hypogonadism in a 17-year old dwarf. *J Clin Endocrinol Metab* 25: 1591-1598
- Supakar PC, Song CS, Jung MH, Slomczynska MA, Kim JM, Vellanoweth RL, Chatterjee B, Roy AK **1993** A novel regulatory element associated with age-dependent expression of the androgen receptor gene. *J Biol Chem* 268: 26400-26408
- Svensson J, Snochowski M **1979** Androgen receptor levels in preputial skin from boys with hypospadias. *J Clin Endocrinol Metab* 49: 340-345
- Takane KK, George FW, Wilson JD **1990** Androgen receptor of the rat penis is down-regulated by androgen. *Am J Physiol* 258: E46-E50
- Takane KK, DA Husmann, MJ McPhaul, JD Wilson **1991a** Androgen receptor levels in the rat penis are controlled differently in distinctive cell types. *Endocrinology* 128: 2234-2238
- Takane KK, Wilson JD, McPhaul MJ **1991b** Decreased levels of androgen receptor in the mature rat phallus are associated with decreased levels of androgen receptor messenger ribonucleic acid *Endocrinology* 129: 1093-1100
- Takeda H, Mizmo T, Lasnitzki I **1985** Autoradiographic studies of androgen binding sides in the rat urogenital sinus and postnatal prostate. *J Endocrinol* 104: 87-92
- Takeda H, Chodak G, Mutchnik S, Nakamoto T, Chang C **1990** Immunohistochemical localization of androgen receptors with mono- and polyclonal antibodies to androgen receptor. *J Endocrinol* 126: 17-25
- Takeshi S, Hutson JM **1994** Capsaicin increases the frequency of cryptorchism on flutamide-treated rats. *J Urol* 152: 763-765
- Tan JA, Joseph DR, Quarmby VE, Lubahn DB, Sar M, French FS, Wilson E **1988** The rat androgen receptor: primary structure, autoregulation of its messenger ribonucleic acid, and immunocytochemical localization of the receptor protein. *Mol Endocrinol* 2: 1276-1285
- Terakawa T, Shima H, Yabumoto H, Koyama K, Ikoma F **1990** Androgen receptor levels in patients with isolated hypospadias. *Acta Endocrinologica (Copenh)* 123: 24-29
- Terada M, Goh DW, Farmer PJ, Hutson JM **1994** Calcitonin gene-related peptide receptors in the gubernaculum of normal rat and 2 models of cryptorchism. *J Urol* 152: 759-762
- Timms BG, Mohs TJ, Dido LJA **1994** Ductal budding and branching patterns in the developing prostate. *J Urol* 151: 1427-1432
- Thigpen AE, Davis DL, Milatovich A, Mendonca BB, Imperato-McGinley J, Griffin JE, Francke U, Wilson JD, Russell DW **1992** Molecular genetics of steroid 5 $\alpha$ -reductase 2 deficiency. *J Clin Invest* 90: 799-809

## References

- Tilley WD, Marcelli M, Wilson JD, McPhaul MJ **1989** Characterization and expression of a cDNA encoding the human androgen receptor. *Proc Natl Acad Sci USA* 86: 327-331
- Tilley WD, Marcelli M, Wilson JD, McPhaul MJ **1990** Expression of the human androgen receptor gene utilizes a common promoter in diverse human tissues and cell lines. *J Biol Chem* 265: 13776-13781
- Tizzano EF, Silver MM, Chitayat D, Benichou JC, Buchwald M **1994** Differential cellular expression of cystic fibrosis transmembrane regulator in human reproductive tissues. *Am J Pathol* 144: 906-914
- Tong SYC, Hutson JM **1994** Exocrine hormone secretion at the onset of sexual differentiation. Program of the Annual Meeting of the Section on Urology, American Academy of Pediatrics, Dallas TX, p 83-84 (Abstract 34)
- Trapman J, Klaasen P, Kuiper GGJM, van der Korput JAGM, Faber PW, van Rooij HCJ, Geurts van Kessel A, Voorhorst MM, Mulder E, Brinkmann AO **1988** Cloning and expression of a cDNA encoding the human androgen receptor. *Biochem Biophys Res Comm* 153: 241-248
- Tsarfaty I, Rong S, Resau JH, Rulong S, Pinto da Silva P, Vande Woude GF **1994** The Met proto-oncogene mesenchymal to epithelial conversion. *Science* 263: 98-101
- Tsuji M, H Shima, N Terada, GR Cunha **1994** 5 $\alpha$ -Reductase activity in developing urogenital tracts of the fetal and neonatal male mice. *Endocrinology* 134: 2198-2205
- Veldscholte J, Berrevoets CA, Zegers ND, van der Kwast ThH, Grootegoed JA, Mulder E **1992** Hormone-induced dissociation of the androgen receptor- Heat shock protein complex: Use of a new monoclonal antibody to distinguish transformed from nontransformed receptors. *Biochemistry* 31: 7422-7430
- van der Kwast ThH, Schalken J, Ruizeveld de Winter JA, van Vroonhoven CCJ, Mulder E, Boersma W, Trapman J **1991** Androgen receptors in endocrine-therapy-resistant human prostate cancer. *Int J Cancer* 48: 189-193
- van der Kwast ThH, Dommerholt HBR, van Vroonhoven CCJ, Chadha S **1994** Androgen receptor expression in the cervix of androgen-treated female-to-male transsexuals: association with morphology and chain-specific keratin expression. *Int J Gynecol Pathol* 13: 133-138
- van der Schoot P **1990** Sexual differentiation of body, brain and behavior: Animal studies intended to gain insight in human sexuality. In: Slob AK, Baum MJ, eds. *Psychoneuroendocrinology of growth and development*. EUR Medicom 77-82
- van der Schoot P **1992** Androgens in relation to prenatal development and postnatal inversion of the gubernacula in rats. *J Reprod Fert* 1992; 95: 145-158
- van der Schoot P, Elger W **1992** Androgen-induced prevention of outgrowth of cranial gonadal suspensory ligaments in fetal rats. *J Androl* 13: 534-542
- van der Schoot P **1993** Foetal testis control the prenatal growth and differentiation of the gubernacular cones in rabbits - a tribute to the late Professor Alfred Jost. *Development* 118: 1327-1334
- van der Schoot P, Elger W **1993** Perinatal development of gubernacular cones in rats and rabbits: Effect of exposure to anti-androgens. *Anat Rec* 236: 399-407
- van Doorn E, Craven S, Bruchovsky N **1976** The relationship between androgen receptor and the hormonally controlled response of rat ventral prostate. *Biochem J* 160: 11-21
- van Laar JA, Bolt-de Vries J, Voorhorst-Ogink MM, Brinkmann AO **1989** The human androgen receptor is a 110 Kd protein. *Mol Cell Endocrinol* 63: 39-44
- van Laar, Berrevoets CA, Trapman J, Zegers ND, Brinkmann AO **1991** Hormone-dependent androgen receptor transformation in human lymph node carcinoma of the prostate cells. *J Biol Chem* 266: 3734-3738

## References

- Vannier B, Raynaud JP **1980** Long-term effects of prenatal oestrogen treatment on genital morphology and reproductive function in the rat. *J Reprod Fert* 59: 43-49
- Veyssi re G, Corre M, Berger M, Jean-Faucher Ch, de Turckheim M, Jean C **1980** Androg nes Circulants et organog nese sexuelle male chez le f etus de lapin. *Arch Anat Microsc Morphol Exp* 69: 17-20
- Veyssi re G, Berger M, Jean-Faucher Ch, de Turckheim M, Jean C **1982** Testosterone and dihydrotestosterone in sexual ducts and genital tubercle of rabbit fetuses during sexual organogenesis: effect of fetal decapitation. *J Steroid Biochem* 17: 149-154
- Vreeburg JTM, van der Vaart PDM, van der Schoot P **1972** Prevention of central defeminization but not masculinization in male rats by inhibition neonatally of oestrogen biosynthesis. *J Endocrinol* 74: 375-382
- Walsh PC, Madden JD, Harrod MJ, Goldstien JL, MacDonald PC, Wilson JD **1974** Familial incomplete male pseudohermaphroditism, type 2. Decreased dihydrotestosterone formation in pseudovaginal perineoscrotal hypospadias. *N Engl J Med* 291: 944-949
- Wasner G, Hennerman I, Kratochwil K **1983** Ontogeny of mesenchymal androgen receptors in the embryonic mouse mammary gland. *Endocrinology* 113: 1771-1780
- Wells LJ **1944** Descensus testicularum: descent after severance of the gubernaculum. *Anat Rec* 88: 465-466
- West NB, Roselli CE, Resko JA, Greene GL, Brenner RM **1988** Estrogen and progestin receptors and aromatase activity in rhesus monkey prostate. *Endocrinology* 123: 2312-2322
- Wilson CM, Griffin JE, Wilson JD, Marcelli M, Zoppi S, McPhaul MJ **1992** Immunoreactive Androgen Receptor in subjects with Androgen Resistance. *J Clin Endocrinol Metab* 75: 1474-1478
- Wilson JD, Lanitzki I **1971** Dihydrotestosterone formation in fetal tissues of the rabbit and rat. *Endocrinology* 89: 659-668
- Wilson JD **1980** The pathogenesis of benign prostatic hyperplasia. *Am J Med* 68: 745-756
- Wilson JD, George FW, Griffin JE **1981** Hormonal control of sexual development. *Science* 211: 1278-1284
- Wilson JD **1991** Gynecomastia, a continuing diagnostic dilemma. *N Engl J Med* 324: 334-335
- Wilson JD, Griffin JE, Russell DW **1993** Steroid 5 $\alpha$ -reductase 2 deficiency. *Endocr Rev* 14: 577-593
- Yamaguchi T, Kitada S, Osada Y **1991** Chromosomal anomalies in cryptorchidism and hypospadias. *Urol Int* 47: 60-63
- Yan G, Fukabori Y, Nikolaropoulos S, Wang F, McKeenan WL **1992** Heparin binding keratinocyte growth factor (FGF-7) is a candidate stromal to epithelial cell prostate andromedin. *Mol Endocrinol* 6: 2123-2128
- Yong EL, Ng SC, Roy AC, Yun G, Ratnam SS **1994** Pregnancy after hormonal correction of severe spermatogenic defect due to mutation in androgen receptor gene. *Lancet* 344: 826-827
- Young CYF, Murthy LR, Prescott JL, Johnson MP, Rowley DR, Cunningham GR, Killian CS, Scardino PT, VonEschenbach, Tindall DJ **1988** Monoclonal Antibodies against the androgen receptor: Recognition of human and other mammalian androgen receptors. *Endocrinology* 123: 601-610
- Zegers ND, Claassen E, Neelen C, Mulder E, Laar JH van, Voorhorst MM, Berrevoets CA, Brinkmann AO, Kwast Th H van der, Winter JAR de, Trapman J, Boersma WJA **1991** Epitope prediction and confirmation for the human androgen receptor: generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy. *Biochim Biophys Acta* 1073: 23-32

## SUMMARY

The steroid hormones testosterone (T) and 5 $\alpha$ -dihydrotestosterone (DHT) have separate roles in development of the male phenotype and maintenance through binding to the same intracellular high-affinity androgen receptor (AR). In the process of sexual differentiation almost all phenotypic differences between the sexes emerge from bipotential precursors and are virilized, when DHT is present. Some structures of the internal genital tracts arise from different anlagen in male and female fetus. In man, epididymis, vas deferens and seminal vesicle develop after stabilization of the wolffian duct under the influence of T and the müllerian duct regresses under the influence of anti-müllerian hormone. In the female fetus, the wolffian ducts disappear. The müllerian ducts give rise to the fallopian tubes, uterus, and vagina. From studies in individuals with the androgen insensitivity syndrome the target tissues for androgens are well-known. As a pre-requisite for androgen action the androgen receptor should be expressed in the target tissues. The regulation of the androgen receptor is organ-specific, time-specific and depends on hormonal conditions (Chapter 1). The aim of this study was to determine the level and expression pattern of the androgen receptor in target tissues of the fetal and adult rat, and to understand the factors that control the androgen receptor protein expression during fetal sexual differentiation and in the adult.

In Chapter 2 androgen receptor (AR) expression in male and female rats, measured by a Western (immuno-) blot assay is described. It revealed a broad distribution of the receptor in both male and female tissues. Highest amounts of AR were detected in tissues of the male and female urogenital tract and in the adrenal. Castration caused an 80% decrease in AR level in ventral prostate and a 67% decrease in the levels in seminal vesicle but had no effect on adrenal AR. In contrast, hypophysectomy caused a profound decrease in AR levels in the adrenal, ventral prostate and seminal vesicle. These studies confirm that in the urogenital tract, AR expression is enhanced by androgen and indicate that AR in the adrenal is under the control of some other pituitary hormone.

During the period of phenotypic sex differentiation in urogenital tract tissues from day 14 onwards in male and female fetal rats high levels of androgen receptor were found (Chapters 3 and 4). Physiological evidence, that embryos of both sexes have androgen receptor expression at initiation of sexual differentiation was confirmed. During morphogenesis of the genitalia, the AR is first induced in the mesenchyme, then the epithelium becomes a target for androgen action. Androgen receptor levels were low to undetectable in a variety of non-urogenital tract tissues. In a relatively short period during fetal sexual development, the amount of androgen receptor decreases in the female urogenital tissues to undetectable levels. Administration of 5 $\alpha$ -dihydrotestosterone to pregnant rats during the period of sexual differentiation of the fetuses induced immunoreactive androgen receptor in the female urogenital sinus and tubercle to levels approaching those in male

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tissues. Administration of the androgen antagonist flutamide during the same interval caused a reduction in androgen receptor level in the same tissues in male fetuses. The immunohistochemical findings indicated that androgens stimulate androgen receptor expression in the fetal urogenital tract by inducing the proliferation of androgen-responsive cells.

Interestingly, we did not observe a different type of androgen receptor in the testosterone-induced fetal epididymis as compared to androgen receptors isolated from the fetal urogenital tubercle and sinus and the adult prostate, that growth under  $5\alpha$ -dihydrotestosterone influences. Since in normal fetal life the efferent ductules and the wolffian duct are virilized by testosterone supplied to these structures in an exocrine fashion (and not by  $5\alpha$ -dihydrotestosterone), we suggest that relatively high amounts of testosterone reaching these tissues by direct transport through the lumen of the mesonephric duct system, increase AR expression, again in the mesenchyme first, and subsequently in the epithelium.

To clarify the role of androgen in testicular descent (Chapter 5), androgen receptor levels of the rat gubernaculum were determined from 3 days prenatally to day 20 of postnatal development. In relative terms, it was found that the amount of the gubernacular androgen receptor decreased dramatically from fetal to postnatal development, coinciding with the transition of the gubernaculum from a tissue primarily composed of mesenchymal cells in the fetus to a tissue that is primarily made up of muscle cell during postnatal development. Consequently, the mesenchyme of the fetal gubernaculum can be considered as a primary target of androgen action.

To elucidate whether diminished levels of AR are the underlying cause for the development of hypospadias, androgen receptor levels in foreskins of boys with hypospadias were studied (Chapter 6). The result was that it is unlikely that hypospadias in general is caused by a decreased expression of AR.

In summary, androgen receptor expression is bipotential in the sexual indifferent stage. In later life diverse patterns of androgen receptor distribution and the regulation discern differences between the sexes. Although not essential for individual life, androgen receptor plays undoubtedly an important role in reproduction.

## SAMENVATTING

De steroïdhormonen testosteron (T) en 5 $\alpha$ -dihydrotestosteron (DHT) spelen een verschillende rol in de ontwikkeling van de mannelijke geslachtsorganen en in het functioneren hiervan. T en DHT binden aan dezelfde intracellulaire receptor, die vervolgens aan het DNA bindt, hetgeen resulteert in transcriptie regulatie van androgeen responsieve genen. De mannelijke geslachtshormonen (androgenen) vormen een stabiel complex met deze androgeenreceptor. Tijdens de seksuele differentiatie ontstaan bijna alle fenotypische verschillen tussen man en vrouw uit voorloper organen die in potentie zowel in de mannelijke als de vrouwelijke richting kunnen differentiëren. Enkele inwendige geslachtsorganen ontstaan uit verschillende embryonale structuren. Als testosteron aanwezig is, zal de foetus het mannelijke geslacht aannemen. In de man ontwikkelen de epididymis, het vas deferens, en het zaadblaasje zich uit de gang van Wolff. Testosteron voorkomt regressie van de gang van Wolff in tegenstelling tot de situatie bij de vrouw. Bij de man verdwijnt de gang van Müller, terwijl bij de vrouw hieruit de tuba, de uterus en een deel van de vagina ontstaat. Uit onderzoek van personen met het androgeen ongevoeligheids syndroom weten we welke organen onder de invloed van de mannelijke geslachtshormonen uitgroeien. Een voorwaarde voor de werking van de androgenen is de aanwezigheid van een goed functionerende androgeenreceptor. De aanmaak van dit eiwit verschilt per orgaan en is afhankelijk van het ontwikkelingsstadium van het individu en de hoeveelheden geslachtshormonen die circuleren in de foetus (hoofdstuk 1).

Dit onderzoek had als doel, de bepaling van de hoeveelheid androgeenreceptor in verschillende organen; in welke weefsels de androgeenreceptor als eerste voorkomt; in welke celtypen de androgeen receptor te vinden is. Tevens werden factoren bestudeerd, die de aanmaak van het androgeenreceptor eiwit regelen tijdens de geslachtsdifferentiatie. Als proefdier werd de volwassen rat en de rattefoetus gebruikt. Hypospadie werd in de mens bestudeerd.

In hoofdstuk 2 wordt de expressie van de androgeenreceptor in verschillende organen in mannetjes- en vrouwtjes ratten beschreven. De androgeenreceptor concentraties werden door middel van een immunologische "blotting"-techniek gemeten. Per orgaan zijn de onderlinge verschillen groot. De hoogste waarden werden in de urogenitale organen en de bijnier gevonden, zowel bij de mannelijke- als bij de vrouwelijke rat. Castratie van de mannetjes veroorzaakte een daling van 80% in de prostaat en van 67% in de zaadblaasjes, maar had geen effect op de AR expressie van de androgeenreceptor in de bijnier. Verwijdering van de hypofyse veroorzaakte daarentegen wel een enorme afname van androgeenreceptor in de bijnier, evenals in de ventrale prostaat en in de zaadblaasjes. Deze studie toont aan, dat in de urogenitale tractus de androgeenreceptor in expressie toeneemt in de aanwezigheid van mannelijke geslachtshormonen, maar dat de aanmaak van de androgeenreceptor van de

rattebijnier door een mogelijk hypofysair hormoon wordt geregeld.

In de rattefoetus werden de meeste androgeenreceptoren ook in de urogenitale tractus gevonden, en wel vanaf 14 dagen draagtijd, als de geslachtsdifferentiatie van de foetus begint. Gedurende de aanleg van de geslachtsorganen verschijnt de androgeenreceptor eerst in de mesenchymale cellen, daarna in de epitheliale cellen. Buiten de urogenitale tractus komt de androgeenreceptor nauwelijks tot expressie. In de 8 dagen die de geslachtsontwikkeling duurt bij de rat vermindert de expressie van de androgeenreceptor in de vrouwelijke foetus snel. Toediening van  $5\alpha$ -dihydrotestosteron aan dragende ratten gedurende de geslachtsdifferentiatie van de foetussen induceerde het androgeenreceptor eiwit in de vrouwelijke urogenitale sinus en tuberkel tot een niveau dat vergelijkbaar is met dat van de mannelijke foetus. Blootstelling aan flutamide, een androgeenreceptor blokker, gedurende dezelfde foetale periode veroorzaakte een afname van het androgeenreceptor niveau in dezelfde organen, in de penis in aanleg en in de voorloper van de prostaat. Met behulp van de immunohistochemische technieken werd aangetoond, dat de totale toename van de hoeveelheid androgeenreceptor in het weefsel wordt veroorzaakt door een vermeerdering van het aantal cellen dat de androgeenreceptor aanmaakt en door meer androgeenreceptoren per cel.

Verschillen tussen de androgeenreceptor in de foetale epididymis en die in de urogenitale tuberkel en sinus en die in de prostaat van het volwassen dier werden niet gevonden. De gang van Wolff wordt op een exocrine manier van testosteron voorzien, dat wil zeggen, dat het hormoon de doelwitcellen niet bereikt door verspreiding via de bloedbaan, maar dat het testosteron wordt meegevoerd door de afvoergang van de mesonefros (gang van Wolff). De androgeenreceptor in de gang van Wolff komt eerst vlakbij de testis tot expressie en later in het deel, dat verder van de testis ligt, hetgeen overeenkomt met het concept, dat testosteron de androgeenreceptor langs de gang van Wolff tot expressie brengt, eerst in het mesenchym en daarna in het epitheel.

Om de rol van mannelijke geslachtshormonen bij de indaling van de testikel te verhelderen (hoofdstuk 5), werd de hoeveelheid androgeenreceptor in het gubernaculum bepaald vanaf 3 dagen voor de geboorte tot 20 dagen erna. Relatief nam de hoeveelheid androgeenreceptor na de geboorte dramatisch af, hetgeen overeenkomt met de samenstelling van het gubernaculum, dat aanvankelijk tijdens het foetale stadium vooral uit androgeen bevattende mesenchymale cellen bestaat, maar na de geboorte vooral androgeenreceptor negatieve gladde spiercellen bevat.

Om inzicht te krijgen in de oorzaak van hypospadie werd de hoeveelheid androgeenreceptor in de voorhuid van jongens geboren met een hypospadie bepaald (hoofdstuk 6). Er werd geen verschil gevonden met een groep normale jongens. Het is onwaarschijnlijk, dat een verlaagde hoeveelheid androgeenreceptor de oorzaak van deze aandoening is.

## **Samenvatting**

Concluderend kan worden gesteld dat, de expressie van de androgeenreceptor in potentie in beide geslachten gelijk is. Later ontstaan de verschillen tussen beide geslachten in de verdeling van de androgeenreceptor. Hoewel de androgeen receptor een belangrijke rol speelt in de fertiliteit van het individu, is een gestoorde werking van de androgeenreceptor niet bepalend voor de levensduur.



**LIST OF PUBLICATIONS**

Schröder Fritz H, Paul J Carpentier, Petar A Maksimovic, **Frans M Bentvelsen 1989** Transrectal ultrasonography volumetric applications to prostatic carcinoma. In: Diagnostic ultrasound of the prostate. Martin Resnick, Hiroke Watanabe, James P Karr (red.). Elsevier Science Publishing Co., Inc., New York, pp. 124-128

**Bentvelsen FM**, JFAT Bogdanowicz, R Oosterom, FH Schröder **1990** Een vergelijking tussen prostaatzure fosfatase en prostaat-specifiek antigeen bij de diagnostiek van het prostaatcarcinoom. Ned Tijdschr Geneesk 134: 1596-1600

Bogdanowicz JFAT, **FM Bentvelsen**, R Oosterom, FH Schroeder **1991** Evaluation of prostate-specific antigen and prostatic acid phosphatase in untreated prostatic carcinoma and benign prostatic hyperplasia. Scand J Urol Nephrol Suppl 138: 97-103

**Bentvelsen FM**, FH Schröder **1992** Prostate specific antigen as an aid in screening for prostate cancer. Tijdschr NVKC 17: 35-39

Oosterom R, JFAT Bogdanowicz, **FM Bentvelsen**, FH Schröder **1992** Comparison of prostate acid phosphatase and prostate specific antigen. Tijdschrift NVKC 17: 19-21

**Bentvelsen FM**, D van den Ouden, FH Schröder **1993** Prostate-specific antigen in monitoring for recurrence after radical prostatectomy for localized prostatic cancer. Br J Urol 72: 88-91

van den Ouden D, **FM Bentvelsen**, ER Boevé, FH Schröder **1993** Positive margins after radical prostatectomy: correlation with local recurrence and distant progression. Br J Urol 72: 489-494

**Bentvelsen FM**, FH Schröder **1993** Modalities available for screening for prostate cancer. Eur J Cancer 29A: 804-811

**Bentvelsen FM**, FH Schröder **1993** Eignet sich Prostata spezifisches Antigen (PSA) für ein Screening auf Prostata-Carcinom? Labor-Medizin 16.Jahrgang: 2-7

**Bentvelsen Frans M**, Fredrick W George **1993** The fetal gubernaculum contains higher levels of androgen receptor than does the postnatal gubernaculum. J Urol 150: 1564-1566

### **List of publications**

**Bentvelsen Frans M**, Fredrick W George, Michael J McPhaul, Jean D Wilson **1994** The androgen receptor of the urogenital tract of the fetal rat is regulated by androgen. *Mol Cell Endocrinol* 105: 21-26

**drs. F.M. Bentvelsen**, prof.dr. F.H. Schröder **1995** Hoofdstuk 4: Obstructie van de urinewegen. In: *Leerboek urologie*. Prof.dr. H.J. de Voogt, dr T.A. Boon (red.). Wetenschappelijke Uitgeverij Bunge B.V., Utrecht, in press

**Bentvelsen Frans M**, Annelies JETM van der Linden, Albert O Brinkmann, Fritz H Schröder, Rien JM Nijman **1995** Immunoreactive androgen receptor levels in preputional skin of boys with isolated hypospadias. *Brit J Urol*, in press

**Bentvelsen FM**, AO Brinkmann, P van der Schoot, JETM van der Linden, ThH van der Kwast, WJA Boersma, FH Schröder, JM Nijman **1995** Development pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. *Mol Cell Endocrinol*, in press

**CURRICULUM VITAE**

Frans Bentvelsen werd op 10 december 1958 in Den Hoorn (gemeente Schipluiden) geboren. In 1977 werd aan het St. Stanislascollege te Delft het Gymnasium  $\beta$  diploma behaald. Aansluitend studeerde hij Geneeskunde aan de Erasmus Universiteit Rotterdam, waar hij op 20 januari 1984 het artseneexamen aflegde. Tijdens zijn militaire dienst was hij werkzaam op de afdeling Interne Geneeskunde van Militair Hospitaal "Dr. A. Matthijsen" te Utrecht. Hierna werkte hij op de afdeling heelkunde van het St. Franciscus Gasthuis te Rotterdam en startte daar in 1986 met de chirurgische vooropleiding (opleider Dr. C.H.J. Stockmann) voor de opleiding tot uroloog. Van 1988 tot 1990 vond de opleiding plaats op de afdeling Urologie van het Academisch Ziekenhuis Rotterdam (opleider Prof. dr. F.H. Schröder) en op de afdeling Kinderurologie van het Sophia Kinderziekenhuis te Rotterdam (opleider Prof. dr. R.J. Scholtmeijer). In 1991 volgde het perifere gedeelte van de urologische opleiding in het St. Franciscus Gasthuis te Rotterdam (opleider Dr. S.I. Miranda). Op 1 januari 1992 werd hij ingeschreven in het specialistenregister. Vervolgens werkte hij gedurende anderhalf jaar als "research-fellow" op de afdeling "Endocrinology and Reproduction" van "the University of Texas, Southwestern Medical Center at Dallas" (hoofd Prof. J.D. Wilson, M.D.), alwaar een aanvang werd gemaakt met het in dit proefschrift beschreven onderzoek. Na terugkomst in Nederland in juli 1993 was hij als staflid werkzaam op de afdeling kinderurologie (subhoofd Dr. J.M. Nijman) en sinds 1995 op de afdeling urologie van het Academisch Ziekenhuis Rotterdam (hoofd Prof. dr. F.H. Schröder). Dit klinisch werk werd gecombineerd met experimenteel onderzoek op de afdeling Endocrinologie & Voortplanting van de Erasmus Universiteit (hoofd Prof dr. J.A.. Grootegoed) onder begeleiding van Dr. A.O. Brinkmann. Vanaf 1 juli 1995 werkt hij als uroloog in de Oosterscheldeziekenhuizen te Goes en Zierikzee in associatie met S. Geluk, A.J.J. van Stekelenburg en J.H. Vriesde. De schrijver is gehuwd met Mw. Myriam H.J.M. Koop, zij hebben drie kinderen.

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