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THE RELATIVE POTENCY OF TESTOSTERONE AND DIHYDROTESTOSTERONE IN THE RAT VENTRAL PROSTATE: ROLE OF 5α-REDUCTASE

by

A. Stuart Wright

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
November 1999

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by A. Stuart Wright

in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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DEDICATION

This thesis is dedicated to my father, Dr. Alex Wright (1937 – 1997) - for giving me the
courage and the tools.

"Scots, wha hae wi' Wallace bled,
Scots, wham Bruce has aften led,
Welcome to your gory bed, -
Or to victorie. -

Now 's the day, and now 's the hour:
See the front o' battle lour,
See approach proud Edward's power,
Chains and Slaverie. -

Wha will be a traitor knave?
Wha can fill a coward's grave?
Wha sae base as be a Slave?
-Let him turn, and flee: -

Wha for Scotland's King and law
Freedom's sword will strongly draw,
Free-man stand, or Free-man fa',
Let him follow me. -

By Oppression's woes and pains!
By your Sons in servile chains!
We will drain your dearest veins,
But they shall be free!

Lay the proud Usurpers low!
Tyrants fall in every foe!
Liberty's in every blow!
Let us Do - or die!!!"

Robert Burns (1759-1796)
"Robert the Bruce's March to Bannockburn"
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DHT vs. T in serum

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ABSTRACT

Testosterone (T), the major circulating androgen, must be converted to dihydrotestosterone (DHT) by the enzyme 5α-reductase (5α-R) for maximal activity in the prostate. Based on its higher affinity for the androgen receptor, its high intraprostatic concentration and the observation that men with congenital 5α-R deficiency have small prostates, DHT was considered to be the only active androgen in the prostate. In both rats and men, administration of 5α-R inhibitors such as finasteride results in prostate shrinkage but not to the same extent as castration. The most probable reason is the reciprocal rise in intraprostatic T that accompanies the decrease in DHT on 5α-R inhibition. The objective of the studies presented here was to investigate the relative potencies of T and DHT in prevention of rat ventral prostate regression and in regrowth.

To determine the relative potencies of intraprostatic T and DHT in preventing regression, castrated rats were implanted for 4 days with varying sizes of T pellets in the presence or absence of finasteride treatment. In the absence of finasteride, virtually all intraprostatic T was converted to DHT, creating a dose-response for DHT effects in the prostate. In the presence of finasteride, DHT was suppressed to the levels found in a castrated rat and a dose-response for T effects was achieved. Prostate regression consists of atrophy through reduced secretory activity and cell loss through apoptosis. DHT was 2.4 times more potent than T in maintaining normal prostate weight and lumen mass, a measure of epithelial cell function. However, intraprostatic T and DHT were able to inhibit apoptosis with equal potency, indicating that 5α-R inhibition in an intact animal results in atrophy and minimal apoptosis because of the potency of intraprostatic T.

In order to examine the relative potencies of T and DHT on prostate growth, rats were castrated for 2 weeks to allow the prostate to fully regress and were given T implants in the presence or absence of finasteride to create dose-responses for the effects of intraprostatic T and DHT, respectively. 1.6-1.9 times more T than DHT was needed to achieve half-maximal responses in markers of hypertrophy and hyperplasia and a 2 - 3 fold higher threshold was observed for T compared to DHT before significant growth occurred. A more pronounced potency difference was found when the relationship between serum T levels and prostate growth examined. 11-16 times more serum T was required for half-maximal prostate growth when DHT formation was blocked by finasteride, suggesting that 5α-R plays a role in prostatic androgen accumulation.

Whether T or DHT pellets were used to provide the circulating androgen, the concentration of DHT in the ventral prostate in the absence of finasteride was identical, indicating that in terms of prostate physiology, DHT could serve as the major circulating androgen. However, to achieve similar levels of T or DHT in serum, much larger DHT pellets were needed, consistent with the known rapid metabolism of DHT in tissues other than the prostate.

Collectively, these results indicate that the role of 5α-R in the prostate is two-fold: it converts T to a moderately more potent androgen and permits the accumulation of androgen at low serum T concentrations. T has probably arisen as the major circulating androgen instead of DHT because it can be aromatized to estradiol, which has important roles in male physiology.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>4-MA</td>
<td>17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one</td>
</tr>
<tr>
<td>5α-R</td>
<td>5α-reductase</td>
</tr>
<tr>
<td>AEC</td>
<td>3-amino-9-ethylcarbazole</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>apoptosis activating factor-1</td>
</tr>
<tr>
<td>AR</td>
<td>androgen receptor</td>
</tr>
<tr>
<td>ARA&lt;sub&gt;70&lt;/sub&gt;</td>
<td>androgen receptor associated protein-70</td>
</tr>
<tr>
<td>ARE</td>
<td>androgen response element</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BPH</td>
<td>benign prostatic hyperplasia</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CAT</td>
<td>chloramphenical acetyltransferase</td>
</tr>
<tr>
<td>CBP</td>
<td>cAMP response element binding protein-binding protein</td>
</tr>
<tr>
<td>CDK-2, -4, -5</td>
<td>cyclin dependent kinase-2, -4, -5</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl-pyrocarbonate</td>
</tr>
<tr>
<td>DES</td>
<td>diethylstilbestrol</td>
</tr>
<tr>
<td>DFF</td>
<td>DNA fragmentation factor</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotosterone</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>estrogen receptor</td>
</tr>
<tr>
<td>G3PDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GITC</td>
<td>guanidinium isothiocyanate</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>GR</td>
<td>glucocorticoid receptor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IGFBP</td>
<td>IGF binding protein</td>
</tr>
<tr>
<td>IGF-I or -II</td>
<td>insulin-like growth factor -I or -II</td>
</tr>
<tr>
<td>KGF</td>
<td>keratinocyte growth factor</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>LH</td>
<td>leutinizing hormone</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAPKKK1</td>
<td>mitogen activated protein kinase kinase kinase 1</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumor virus</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-[N-morpholino]-propanesulfonic acid</td>
</tr>
<tr>
<td>NRC</td>
<td>nuclear receptor co-activator</td>
</tr>
<tr>
<td>P/CAF</td>
<td>p300/CBP-associated factor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>PSBP</td>
<td>prostate specific binding protein</td>
</tr>
<tr>
<td>R1881</td>
<td>methyltrienolone</td>
</tr>
<tr>
<td>Rb</td>
<td>retinoblastoma</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SGP-2</td>
<td>sulfated glycoprotein-2</td>
</tr>
<tr>
<td>SHBG</td>
<td>steroid hormone binding globulin</td>
</tr>
<tr>
<td>T</td>
<td>testosterone</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyltransferase</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TIF2</td>
<td>transcriptional mediator/intermediary factor-2</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>TRPM-2</td>
<td>testosterone repressed prostate message-2</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT mediated dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
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</table>
ACKNOWLEDGEMENTS

I am indebted to Dr. Catherine Lazier for her insight, endurance and guidance. The direction of Dr. Rittmaster has been an inspiration as has the support of colleagues Robert Douglas and Lynn Thomas without whom research would never have been as enjoyable.

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Finally, I would like to thank my loving family, especially my wife Valerie and daughter Ceilidh, for everything so far and everything to come.
INTRODUCTION

In the aging male population, benign prostatic hyperplasia (BPH) and prostate cancer are major causes of morbidity and mortality (Geller 1991; Geller 1993). Autopsy studies indicate that by age 80, BPH will be present in 90% of men and prostate cancer in 50 – 80% (Geller 1991; Nomura and Kolonel 1991). Prostate cancer has recently taken over as the leading form of non-cutaneous cancer in Western men (Parker et al. 1997). From these statistics, it is obvious that understanding control of prostate growth is key to developing strategies to treat prostate diseases. The enzyme 5α-reductase (5α-R), found mainly in the prostate and skin, has become the subject of significant research and drug discovery because of its presumed role in the pathophysiology of the adult male. This NADPH-dependent enzyme catalyzes the reduction of testosterone (T) to dihydrotestosterone (DHT). Two types of 5α-R have been cloned and characterized, which differ in their pH optima, sensitivity to inhibitors, and tissue distribution (Andersson and Russell 1990; Jenkins et al. 1992; Russell and Wilson 1994). The androgens, T and DHT, are essential hormones for male phenotypic differentiation and maturation through their interaction with the androgen receptor (AR) (Bruchovsky et al. 1975; Wilson et al. 1983).

Aging and the presence of testes are two prerequisites for the development of BPH (Geller 1991). The dependence of the prostate and its hyperplastic disorders on testicular androgens has been known for some time, as the absence of functioning testes prior to age 40 prevents BPH and prostate cancer (Moore 1944). T from the testes acts as a
prohormone in the prostate where it is converted to DHT. DHT has been deemed
necessary for BPH from observations of men with 5α-R reductase type 2 deficiency, the
rare disorder of male pseudohermaphroditism, which is characterized by underdeveloped
external genitalia, normal male musculature and a small prostate composed primarily of
stroma (Imperato-McGinley et al. 1979; Maes et al. 1979; Imperato-McGinley et al.
1992). In addition, the unique physiology of androgen metabolism in the prostate is an
explanation for the lack of benign or malignant disease in the adjacent sex glands such as
the seminal vesicles (Griffiths et al. 1991; Krieg et al. 1993). With the emergence of
DHT as the primary androgen involved in benign and malignant hyperplasia, 5α-R
inhibition is a target for therapy in the prevention of prostate cancer (Brawley et al. 1994;
Gormley et al. 1995).

Inhibition of 5α-R using pharmacological agents has been shown to reduce the prostatic
content of the potent androgen DHT to levels associated with castration (Rittmaster
1997). The prostate, in turn, reduces in size despite the normal circulating levels of serum
T (Rittmaster 1994). However, as DHT levels in the prostate decrease, T levels rise, and
despite the low levels of DHT, the prostate does not shrink to the same extent as
castration (Rittmaster et al. 1995; Rittmaster et al. 1996). The androgenicity of T alone
on the rat prostate has not been fully elucidated because 5α-R normally converts virtually
all T into DHT. Thus, separating the effects of T from DHT awaited the development of
5α-R inhibitors. The following is an examination of the effect of T compared with DHT
on regression and regrowth of the rat ventral prostate. Dose-response studies have been
used to detect differences in potency, maximal activity and minimum thresholds for
androgen action. Lastly, the effect on the prostate of circulating DHT is compared with
circulating T and possible reasons for evolution of T as the principal circulating androgen instead of DHT are considered.

1. PROSTATE PHYSIOLOGY

During fetal development, the Wolffian ducts develop into the epididymis, seminal vesicles, and vas deferens as a result of stimulation by T. The urogenital sinus differentiates into the prostate, penis, and scrotum as a result of reduction of fetal T to DHT (Cunha et al. 1987).

The prostate is a male sex accessory tissue that plays a role in reproductive function. The prostate in both humans and rats lies immediately below the bladder surrounding the proximal portion of the urethra. The prostate in the adult rat weighs approximately 450 ± 50 mg (Cunha et al. 1987; Rittmaster et al. 1995) and is characterized by distinct lobes composed primarily of epithelium and some stroma. The ventral, dorsal and lateral lobes each have separate functions and androgen responsiveness (Matusik et al. 1986; Prins 1989; Banerjee et al. 1993; Banerjee et al. 1995). The ventral lobe has the highest androgen concentration, lowest zinc levels and is the most responsive to androgen withdrawal or replacement. This is followed by the dorsal lobe, which is less responsive, has lower androgen levels, higher zinc concentrations and more rapid cell turnover. The lateral lobes are the least sensitive to androgen and have the highest zinc concentration (Matusik et al. 1986; Yamashita et al. 1996).

The human prostate resembles a horse chestnut, weighs approximately 20 ± 6 g and does not have distinct lobes but rather zones that lack distinct boundaries (Berry et al. 1984).
The prostate has been described as having four zones: the peripheral zone contains most of the ducts and is the initial site of carcinomas, the central zone contains the second highest concentration of ducts and surrounds the ejaculatory ducts, the preprostatic zone is the smallest zone and surrounds the urethra, and the transition zone which has some glandular components and is the initial site of BPH (McNeal 1980a; McNeal 1980b). In addition there is the anterior fibromuscular stroma which has no glandular components and is primarily smooth muscle.

The prostates from both rats and humans are similar in the formation of ducts that branch into the urethra. Epithelial cells supported by fibroblastic stromal cells line these ducts. The ductal branching has been likened to a tree, with growth occurring primarily in the distal tips (Sugimura et al. 1986). The prostate ductal system of the rat can be divided into three regions based on proximity to the urethra: proximal, intermediate, and distal (Verhagen et al. 1988; Prins et al. 1992; Prins and Birch 1993; Nemeth and Lee 1996; Banerjee et al. 1998). In the distal tips the epithelial cells are tall, columnar and active in cell proliferation. The cells in the intermediate region are also columnar but cell turnover is not apparent. This region is where the majority of cell secretion takes place. Cell proliferation does not appear in the proximal region. The epithelial cells in this region are low and cuboidal in shape and stain heavily for cathepsin D, a marker associated with cell death (Lee et al. 1990). The secretions of the prostate provide the bulk of the volume of the ejaculate with high concentrations of prostaglandins, spermine, fructose, citric acid, zinc, immunoglobulins, and various enzymes such as proteases, esterases and phosphatases. The necessity of the prostate is in question since sperm in the epididymis
are capable of fertilizing the ovum, and removal of the ventral lobe of the prostate does not abolish male fertility (Queen et al. 1981).

a. Structure: epithelium and stroma

Epithelial cells line the duct of the prostate and are composed of three major cell types: the secretory epithelial cells, the basal cells, and the neuoroendocrine cells (reviewed in (Luke and Coffey 1994)). As with most glands with cell-renewing populations, the epithelial cells turn over slowly and are replaced to maintain homeostasis of the organ. The rate of epithelial cell turnover is in the range of 1-2% per day (Isaacs 1984). The tall, columnar secretory cells are terminally differentiated and do not appear to enter a proliferative cycle. These cells rest next to each other connected by cell adhesion molecules attached to the basement membrane through integrin receptors. The nucleus is located near the base of the cell below an abundant Golgi apparatus and upper cytoplasm rich with secretory granules and enzymes. Microvilli line the apical (lumenal) plasma membrane. Basal cells are small, undifferentiated keratin-rich cells, that number less than 10% of the total epithelial cell population. These cells have large irregular shaped nuclei, with little cytoplasm and lack secretory products. The cells are located along the basement membrane and appear to be wedged between the bases of the secretory epithelial cells. These cells are thought to be involved in active transport but the major role is their proliferative capacity (Evans and Chandler 1987; Bonkhoff et al. 1994). These cells are believed to function as stem cells, giving rise to secretory epithelial cells. Neuroendocrine cells are found among the secretory epithelium and carry out their regulatory activity by the secretion of hormonal polypeptides and biogenic amines such
as serotonin. The most probable role of these cells is in the regulation of secretory activity and cell growth (Guthrie et al. 1990).

The basement membrane on which the epithelial cells rest acts as an interface to the stromal compartment. It is a complex structure of collagen types IV and V, glycosaminoglycans, polysaccharides and glycolipids. The stroma is comprised of fibroblasts, smooth muscle cells, axons, capillary and endothelial cells. Stromal cells play a central role in determining the fate of epithelial cells in the prostate. Although androgens are mitogenic to the prostate in animals, they have no proliferative effect on epithelial cells in culture. Addition of growth factors from serum or co-culture with fibroblasts (stroma) is necessary for a mitogenic response in isolated epithelial cells (Sutkowski et al. 1992; Yan et al. 1992; Bayne et al. 1998).

b. Endocrine overview

The prostate maintains its size and secretory function through the continued presence of serum T that acts as a prohormone and is converted in the prostate to DHT for hormonal action. T is synthesized from progesterone through reversible reactions, however, the formation of DHT or estradiol from T is irreversible. All of these steroids have a significant impact on tissues in the body, ranging from neonatal imprinting to differentiation. The dependence of the prostate on androgens is well documented as is the ability of T and DHT to promote differentiation and proliferation (Bruchovsky et al. 1975). An example of negative neonatal imprinting is the observation that brief estrogen exposure in the neonatal male rat has been shown to result in permanent suppression of prostate growth and reduced prostatic responsiveness to testosterone in adulthood (Prins
Thus, maintenance of metabolic pathways and androgen production is essential to normal development of the reproductive tract.

Fig. 1 illustrates the pathways of hormonal control that affect the prostate. The decapeptide, gonadotropin-releasing hormone (GnRH) is released from the hypothalamus and acts on the anterior pituitary to release luteinizing hormone (LH). LH signals the Leydig cells of the testes to produce T which is transported through the blood associated with albumin and in humans, steroid hormone binding globulin (SHBG). Androgens from adrenal origins, which include androstenedione (a reversible T metabolite), are not a major pathway for androgen action since adrenalectomy in rats has no effect on castration-induced prostate regression (Kyprianou and Isaacs 1987a). T and certain metabolites negatively feed back on the hypothalamus and pituitary to reduce steroidogenesis. Estrogens in the male are derived from peripheral conversion of T through a P450 aromatase enzyme. Estrogens have also been used therapeutically to inhibit androgen action (i.e. diethylstilbestrol) (Makela et al. 1990; Yamashita et al. 1996). Their action is not to act directly to block androgen action but rather to act indirectly by inhibiting pituitary release of LH thereby reducing steroidogenesis. The same principle can be seen with GnRH analogues which disrupt the pulsatile pattern of GnRH release necessary before LH will be secreted (Santen and Warner 1985). In essence, these factors act as a medical castration by suppressing steroidogenesis.

Since the testes produce the major serum androgen for growth and maintenance of reproductive tissues it is important to briefly review this function (Luke and Coffey
Fig. 1 Pathways of hormonal control that affect the prostate. Broken lines indicate negative feedback. GnRH, gonadotropin releasing hormone; ACTH, adrenocorticotropic hormone; LH, leutinizing hormone; FSH, follicle stimulating hormone; GH, growth hormone; CRH, corticotropin releasing hormone; DHEA, dehydroepiandrosterone.
1994). T is the major circulating androgen in males and is almost exclusively from testicular origins. In both the rat and the human, more than 95% of circulating T is from the testes. Fig. 2 illustrates an overview of T biosynthesis, plasma concentrations and metabolism from the human (Luke and Coffey 1994). Circulating levels of T in humans are approximately 600 ng/dl, with production rates around 6 mg/day. Serum T levels gradually decrease with age to approximately 500 ng/dl after the age of 70. Only 2% of serum T is not protein-bound and free to diffuse into cells for androgen action or metabolism (approximately 15 ng/dl or 1 nM). T uptake by the liver leads to 17-ketosteroids that are secreted into urine as water-soluble conjugates with either sulfuric or glucuronic acid. The testes produce a small amount of estrogen but the majority, approximately 75 – 90%, of serum estrogens are derived from peripheral conversion of androstenedione and T to estrone and estradiol (Siiteri 1979). Removal of the 19-methyl group and aromatization of the steroid convert the androgenic C19 steroids to the estrogenic C18 steroids. The daily production rate of estradiol in the male is about 40 – 50 µg, of which only 5 – 10 µg can be accounted for by direct testicular secretion (Luke and Coffey 1994). Adipose tissue is the supposed site of peripheral aromatization but the exact location has not been quantitated. Of the 6 mg T produced in a man each day, only 0.40% is converted to estrogens. Estrogen production, unlike T, does not fall off with age but actually increases, however, the level of free estrogen remains constant. The ratio of free estradiol to free T increases 40% with age because free T decreases.

In the normal male, androgens act to prevent cell death in the prostate and to sustain proliferation rates such that overgrowth or involution does not occur. Sufficient levels of serum T are necessary to maintain the balance of cell turnover, but if the levels fall, the
Fig. 2 Overview of serum T production and metabolism. The numbers given are for the human. Similar pathways prevail in the rat except that rat does not express SHBG (Luke and Coffey 1994).
prostate will rapidly involute. The androgen-dependence of epithelial cells is evident from the 90% decrease in number, 80% decrease in volume and 60% reduction in cell height after androgen withdrawal (English et al. 1987). The regression of the prostate on castration is not passive whereby the depressed rate of proliferation fails to compensate for the normal rate of cell death. This mechanism would be far too slow and result in only 8% cell loss after three days (Bruchovsky et al. 1975). The process must be active since four days after castration in the rat, the cell loss is about 50% (Rittmaster et al. 1995). Thus, androgens act specifically to initiate prostate growth, antagonize cell death and maintain cell survival.

c. Apoptosis

Apoptosis, or programmed cell death, is a mechanism by which target organs such as the prostate eliminate cells. Apoptosis can be differentiated from necrosis, another form of cell death, because it is an orderly, ATP-dependent process, and is morphologically distinct (Kerr et al. 1972). Expression of a complex balance of genes whose products inhibit or enhance cell death regulates the pathway. Deregulation of this process through mechanisms such as mutation can contribute to the clonal expansion of neoplastic cells (Reed 1995). While apoptosis is a complex deliberate mechanism that is used for such functions as maintaining the status of an organ, necrosis is more the result of accidental death. Necrotic death is a response to harmful conditions outside of the cell such as hyperthermia, hypoxia, ischemia, metabolic poisons, and direct cell trauma (reviewed in (Trump et al. 1997)). Membrane damage or energy depletion causes the disappearance of membrane ion-pumping activities, resulting in plasma membrane permeability changes. In turn, cytoplasmic and organelle swelling occurs, especially within the mitochondria.
The cell eventually ruptures and cellular contents leak out into the extracellular space, invoking an inflammatory response in adjacent viable tissue. In necrotic death, the cell has only a passive role in the cell death process.

Programmed cell death can be initiated by endogenous factors (such as hormones or lack thereof) and exogenous factors (such as radiation, chemicals and viruses). The apoptotic process has been observed to occur in two discrete stages (reviewed in Schwartzman and Cidlowski 1993). First, the cell undergoes nuclear and cytoplasmic condensation, where genomic DNA is irreversibly degraded into nucleosomal fragments (multiples of approximately 150-200 bp). DNA is also degraded during necrotic death, but that is a late event and results in a continuous spectrum of fragment sizes. In apoptosis, the cell eventually breaks up into membrane-bound fragments that contain structurally intact organelles. In the second stage, these membrane-bound fragments, termed apoptotic bodies, are then phagocytosed by macrophages or neighboring cells and rapidly degraded.

Epithelial cell turnover occurs on a daily basis in the prostate, but the balance of cell death with cell proliferation is able to maintain the prostate at a constant size. In the non-castrated normal adult male rat, the rate of apoptosis and thus proliferation is a low 2.1% per day (Isaacs 1984). An imbalance in the factors that maintain the prostate will alter the prostate cell number and size. One of the most obvious methods of imbalancing the factors that maintain the prostate is androgen withdrawal through castration. In an androgen-replete state, the epithelia are tall, columnar secretory cells, but after androgen withdrawal, they shrink in size and rapidly become low and cuboidal in shape before the
majority of them undergo apoptosis. As mentioned earlier, epithelial cells make up 80-90% of the total number of cells in the ventral prostate of the adult male rat (English et al. 1987). By seven days after castration, 66% of these glandular cells have been eliminated by apoptosis (English et al. 1987). There is some evidence of stromal loss but the degree of cell loss is less than that with the epithelial cells (Banerjee et al. 1995). It is interesting to note that within the prostate, apoptosis seems to coincide with AR expression. The lumenal epithelial cells undergo apoptosis and express AR, but the basal epithelial cells do not express AR and do not undergo apoptosis after androgen withdrawal (Prins et al. 1991; Hayward et al. 1996).

Within 6 hours after castration, serum T levels drop to 1% of their original value (Kyprianou and Isaacs 1988). By 24 hours after castration, DHT levels in the prostate are 5% of the levels in non-castrated controls, which in turn leads to a loss of detectable AR within 24 - 48 hours (Kyprianou and Isaacs 1988; Prins 1989). The loss of AR relieves the transcriptional repression of genes encoding proteins such as transforming growth factor-β (TGF-β) and Bax which have demonstrated the ability to reduce proliferation and initiate apoptosis in lumenal epithelial cells (Martikainen et al. 1990; Sutkowski et al. 1992; Lee et al. 1999; Perlman et al. 1999). However, loss of AR after castration also relieves transcriptional repression of genes encoding proteins that can prevent apoptosis such as TRPM-2 (Buttyan et al. 1989; Tenniswood et al. 1992) and Bcl-2 (McDonnell et al. 1992; Raffo et al. 1995; Perlman et al. 1999). The mRNA for various growth factors in the rat prostate also demonstrate distinctive regulation after castration. The mRNA of the mitogens epidermal growth factor (EGF), TGF-α and their receptor EGF-R are all down-regulated after castration (Nishi et al. 1996). TGF-β type II receptor mRNA is
increased after castration, as is basic fibroblast growth factor (bFGF). The regulation of keratinocyte growth factor (KGF) and the FGF-receptor is complex in that they both increase in mRNA expression after castration but also increase during T-induced regrowth (Nishi et al. 1996). The effects of the mitogens insulin-like growth factor (IGF-I and II) are mediated in part by the level of their serum binding proteins (Cohen et al. 1991), particularly IGFBP-5 which is increased after castration (Thomas et al. 1998). An increase in the binding protein presumably would make less of the mitogen available for stimulation of proliferation.

The complexity of apoptosis in the prostate is made obvious when the time course for the peak in apoptosis is compared with the time course for the drop in androgen levels after castration. Intraprostatic androgen is ablated by 24 h yet the peak in cell loss, where the number of cells dying per day reaches 20%, does not occur until three days after castration (Isaacs 1984; Tenniswood et al. 1992; Furuya and Isaacs 1993). In addition, cells do not all die at once, but rather in an asynchronous manner. It appears that programmed cell death of glandular cells may be initiated when some survival factors, possibly regulated by DHT, fall below a critical level (Denmeade et al. 1996). Other factors, such as the balance of the anti-apoptotic and pro-apoptotic proteins, Bcl-2 and Bax, will determine a cell’s fate (Perlman et al. 1999).

Denmeade et al. (1996) described an orderly series of biochemical and molecular changes for apoptosis in comparison to steps in progression through the proliferative cell cycle (Fig. 3). The proliferative cell cycle is divided into the traditional $G_0$, $G_1$, synthesis (S), $G_2$, and mitotic (M) phases. The cell leaves the quiescent $G_0$ state,
Fig. 3 The cell cycle and programmed cell death pathway as applied to a $G_0$ prostatic glandular cell. D1 denotes the period during which new gene and protein expression required for induction of the DNA fragmentation period (F phase) occurs as part of the programmed cell death pathway. D2 denotes the period during which the cell itself fragments into apoptotic bodies as part of its programmed death (Denmeade and Isaacs, 1996)
re-enters G₁ and is pushed through the stage by the association of cyclin D with cyclin dependent kinase (CDK) 2, 4 or 5. The retinoblastoma (Rb) protein, which acts as a guardian making sure the cell is ready to enter the S phase, must be inactivated by phosphorylation. Cyclin E and CDK2 combine to phosphorylate Rb, allowing the cell to enter the S phase, while cyclin A and CDK2 drive the cell through the S phase of the cell cycle. Cyclin B/A and CDK2 leads the cell through G₂ and into M phase where the cell finishes dividing. Reactivating Rb through its dephosphorylation halts the cell cycle. In many cases, including prostate cancer cells, accumulation of hypophosphorylated Rb results in apoptosis.

As illustrated in Fig. 3, the first phase of the apoptotic process is the signal to the cell that it is to undergo programmed cell death (phase D₁). This stage of the apoptotic process in the prostate can be reversed by the addition of exogenous T. The cell does not enter into a defective proliferative cell cycle but rather undergoes epigenetic reprogramming such as changes in DNA methylation, histone acetylation and deacetylation, and alterations in growth factor responsiveness (Denmeade et al. 1996; Rennie and Nelson 1998). This results in an increase in gene expression and proteins associated with cell death, particularly Ca²⁺ and Ca²⁺-dependent endonucleases (Kyprianou et al. 1988; Arends et al. 1990; Gutierrez et al. 1999). At the end of the D phase, early F phase, polyamines and histone H1 are decreased. Both are involved in DNA compaction, and their loss allows access for the DNA fragmentation by the endonucleases. The most significant and irreversible step in apoptosis is DNA fragmentation (the F phase). This can be detected in the cells by the TUNEL technique where the cleaved strands of DNA are labeled by nick-end labeling using terminal deoxynucleotidyltransferase (TdT). Another technique used
to detect apoptosis is by staining for the protein, tissue transglutaminase, which has been shown to form a protective scaffolding around the apoptotic cell (Tenniswood et al. 1992; Wunsch et al. 1993; Rittmaster et al. 1995; Cummings 1996). During the last phase (D₂), the cell breaks up into apoptotic fragments finalizing the cell death process.

Activators and inhibitors of the protein kinase C family appear to be important in regulation of apoptosis in prostate epithelial cells (Day et al. 1994; Powell et al. 1996). Inducible overexpression of the serine/threonine kinase, PKCα, or the addition of 12-O-tetradecanoylphorbol-13-acetate (TPA) in the prostate cancer cell line, LNCaP, results in apoptosis (Zhao et al. 1997). The mechanism appears to involve the accumulation of hypophosphorylated Rb and increased levels of the CDK inhibitor, p21. DU-145 cells, which lack functional Rb, fail to undergo TPA-induced programmed cell death. Inhibitors of Rb (E1a, the viral oncoprotein) and of PKC (staurosporine) both suppress PKC-mediated apoptosis. However, PKC-mediated apoptosis is not just a simple matter of increased p21 and Rb hypophosphorylation. Apoptosis is not induced in PKCα-transfected LNCaP cells grown in serum-depleted medium demonstrating the need for some serum factor(s). Rb may signal the cell to undergo apoptosis but the execution machinery appears to be the caspase family of cysteine proteases. Addition of the caspase inhibitor, Z-Val-Ala-Asp-fluoromethylketone, protected against PKC-induced apoptosis in prostate cells (Zhao et al. 1997). Recently, caspase-7 was shown to be involved in most modes of apoptosis in LNCaP cells (Marcelli et al. 1999). In fact, overexpression of caspase-7 was able to induce apoptosis in LNCaP cells and in LNCaP cells that overexpress Bcl-2 (Marcelli et al. 1999).
After androgen ablation, the prostate epithelial cells do not enter into the S phase of the cell cycle to activate a futile attempt at DNA repair. The presence of p53 is not required for androgen-ablation induced apoptosis (Berges et al. 1993). Castrated p53-deficient mice showed no difference in prostate cell loss compared to wild-type mice, indicating that the p53 tumour suppressor is not required for androgen-induced cell death (Berges et al. 1993), unlike in many other systems.

Genes that are expressed in the first phase (D1) of the apoptotic process induced by androgen ablation are listed in Table I, as are genes induced during prostate cell proliferation. Expression of TRPM-2, also known as sulfated glycoprotein-2 (SGP-2) or the clusterin gene, is one of the most widely used means to assess apoptosis in the prostate (Buttyan et al. 1989). TRPM-2, calmodulin (Dowd et al. 1991) and TGF-β, (Furuya and Isaacs 1993) are only up-regulated in cells undergoing apoptosis. However, several genes such as c-myc, H-ras and tissue transglutaminase are also involved in the proliferative cell cycle (Furuya and Isaacs 1993).

The cloning and characterization of TRPM-2 was first reported by Leger et al. (1987). Its pattern of expression was further characterized by Buttyan et al. (1989). The androgen-repressed state of TRPM-2 is inferred because of its absence from the prostates of non-castrated rats. Androgen withdrawal results in up-regulation of the gene in the prostate and correlates with the level of apoptosis (Buttyan et al. 1989; Tenniswood et al. 1992; Russo et al. 1994). The peak in cell death occurs 3-4 days after castration, as does the peak in TRPM-2 message levels (Connor et al. 1988; Furuya and Isaacs 1993). Furthermore, calcium channel antagonists that will slow the onset of cell
Table I. Changes in mRNA expression during proliferation and apoptosis induced by androgen manipulation (from (Denmeade et al. 1996). Recent work on alterations in Bax and Bcl-2 expression is discussed in the text (Perlman et al. 1999).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Proliferation</th>
<th>Apoptosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ornithine decarboxylase</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>Thymidine kinase</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>H(_2)-histone</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>c-fos</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>Glucose-reg. Protein 78 kDa</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>Cyclin C</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>Cyclin D(_1)</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>Cyclin E</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>DNA polymerase (\alpha)</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>C(_5)-prostatein</td>
<td>Induced</td>
<td>Decreased</td>
</tr>
<tr>
<td>TRPM-2</td>
<td>Decreased</td>
<td>Induced</td>
</tr>
<tr>
<td>TGF-(B(_1)</td>
<td>Decreased</td>
<td>Induced</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>Decreased</td>
<td>Induced</td>
</tr>
<tr>
<td>(\alpha)-prothymosin</td>
<td>Decreased</td>
<td>Induced</td>
</tr>
<tr>
<td>c-myc</td>
<td>Induced</td>
<td>Induced</td>
</tr>
<tr>
<td>H-ras</td>
<td>Induced</td>
<td>Induced</td>
</tr>
<tr>
<td>Tissue transglutaminase</td>
<td>Induced</td>
<td>Induced</td>
</tr>
</tbody>
</table>
death in the prostate will coordinately reduce the expression of TRPM-2 (Connor et al. 1988).

Although TRPM-2 expression is associated with programmed cell death, its exact role remains unclear. It may play a role in the prevention of cell death, since not all cells that express TRPM-2 are destined for apoptosis (Lee et al. 1990; Sensibar et al. 1990). The androgen-responsive prostate cancer cell line, LNCaP, will undergo apoptosis as a result of TNFα administration (Sherwood et al. 1990). TRPM-2 expression increases and peaks two hours following TNFα exposure, and declines afterwards. At the onset of DNA fragmentation, TRPM-2 protein falls to undetectable levels, indicating that TRPM-2 depletion may be necessary for cell death to proceed. To confirm these observations, TRPM-2 was overexpressed in LNCaP cells that were then treated with TNFα. The transfected cells became resistant to the cytotoxic effects of TNFα, suggesting a role for TRPM-2 protein in the protection from TNFα-induced cell death. Further support for an anti-apoptotic role for TRPM-2 is found in Noble rats that have cancerous lesions in the dorsolateral lobes of their prostates induced by estrogen and T treatment (Ho et al. 1998). Apoptotic activity is not increased, yet TRPM-2 expression is up-regulated. Expression in the ventral lobe is not detected. It appears that expression of TRPM-2 is no longer androgen-repressed and is associated with neoplastic transformation of prostate cells. The fact that up-regulation leads to inappropriate cell survival, is reminiscent of the oncoprotein, Bcl-2.

Bcl-2 is one of the most extensively studied oncogenes involved in the negative regulation of apoptosis (Reed 1994; Boise et al. 1995). The ability of Bcl-2 to block
programmed cell death from a wide variety of apoptotic stimuli provides the strongest evidence for a shared or common pathway of cell death in mammals.

The mechanism for the anti-apoptotic ability of Bcl-2 is believed to be through its capability to heterodimerize with a series of death proteins, Bax (Bcl-2-associated x-protein) being the main one (Oltvai et al. 1993; Yin et al. 1994; Reed 1998). In the prostate, normal secretory epithelia do not express the Bcl-2 protein but a subset of primary adenocarcinomas obtained from untreated prostate cancer patients as well as tissue from hormone-refractory prostatic adenocarcinomas, immunostained positively for the Bcl-2 oncoprotein (Raffo et al. 1995). Overexpression of bcl-2 in LNCaP cells protects against apoptotic stimuli, such as serum withdrawal and treatment with TPA (Raffo et al. 1995). In vivo, these transfected LNCaP cells formed tumours rapidly, even in the castrated nude mouse. Fig. 4 illustrates how Bax/Bcl-2 ratios may act as the "rheostat" of programmed cell death, whereby higher Bcl-2 levels may be used to sequester excess Bax, generating a cell that is relatively insensitive to apoptotic stimuli. In support of this model, Buttyan's group has recently shown that castration of rats results in a transient increase in bax mRNA peaking three days after castration, and a more sustained elevation in bcl-2 mRNA. The ratio of bax/bcl-2 mRNA was increased during periods of most intense apoptosis (Perlman et al. 1999).

Fig. 4 also shows the integration of signals from Bax and Bcl-2 to the execution machinery (Liu et al. 1997; Wilson 1998). Apoptotic stimuli trigger the release of cytochrome c from the outer mitochondrial membrane, which forms a tetrameric complex with apoptosis activating factor (Apaf-1), pro-caspase-9, and dATP. The complex results
Fig. 4 Hypothetical model for the control of apoptosis by negative and positive signals affecting the cell death machinery. Positive regulation of Bcl-2 or negative regulation of the Apaf-1 - cytochrome c - pro-caspase-9 – dATP complex or caspases would result in inhibition of apoptosis. Pro-apoptotic signals could act by negatively regulating Bcl-2 (elevated levels of Bax), or by positively regulating the Apaf complex or caspases.
in the activation of caspase-9 which in turn activates caspase-3 (Li et al. 1997).

Activation of caspase-3 cleaves a subunit from DNA fragmentation factor (DFF) generating an active DNAase, which results in apoptotic DNA cleavage.

Several studies have shown that the bcl-2 gene appears to be androgen-repressed, directly or indirectly. In rats that have been castrated, bcl-2 mRNA was elevated for 10 days before falling to pre-castrate levels (McDonnell et al. 1992). Addition of exogenous T reduced the elevated bcl-2 mRNA. In ZR-75-1 estrogen-responsive breast cancer cells, androgen, acting via the AR, down-regulates bcl-2 expression (Lapointe et al. 1999).

There is evidence linking Bcl-2 expression to progression of prostate cancer (McDonnell et al. 1992; Moul et al. 1996; Tang and Porter 1997) but other studies disagree, because Bcl-2 alone is not a good prognostic indicator of tumour progression (Cohen et al. 1995; Moul et al. 1996)

d. Proliferation

The general process of proliferation is illustrated in Fig. 3 and Table I (as discussed earlier), and involves receptor-mediated signals and activation of various cyclin-dependent kinases in recruiting cells from the quiescent state into the cell cycle. Sites of androgen action via AR in the cell cycle in the normal prostate are not well understood. Recently, there have been numerous reports exploring de-regulation of particular cell cycle components in prostate cancer. For example, in a prostate cancer cell line, DHT stimulates proliferation, enhances CDK-2 activity and down-regulates the CDK inhibitor p27Kip1 (Ye et al. 1999). There is some suggestion that decreased p27Kip1 is associated with progression of prostate cancer (Guo et al. 1997; Fernandez et al. 1999; Tsihlias et al.
1999) but not all studies agree (Erdamar et al. 1999). Another CDK inhibitor, p21, has been shown to be up-regulated by DHT via an androgen response element (ARE) in its promoter region (Lu et al. 1999). This action may relate more to anti-apoptotic activity of androgen, than to its effects on proliferation directly.

The concept has emerged in the last decade that androgens do not directly mediate growth of prostate epithelial cells but rather induce stromal cells to secrete growth factors that act through cell receptors to enhance their proliferation (Cunha 1994). In both the rat and human prostates, AR is found in lumenal epithelial cells and to some extent in the stroma but not in basal epithelial cells (Prins et al. 1991; Leav et al. 1996). Basal cells, however, are the only epithelial cells with proliferative potential. Thus, AR may have a survival, but not a proliferative potential in lumenal epithelial cells. However, this does not detract from the function of androgens to promote expression in the lumenal epithelial cells of differentiated products such as PSA, whose gene promoter is regulated by androgen (Henttu and Vihko 1994; Luke and Coffey 1994).

In the stroma, several growth factors have been identified which have autocrine growth regulatory activity (reviewed in (Peehl 1996)). Basic FGF is a mitogen produced by stromal cells to promote stromal proliferation, and is found in increased levels in BPH (Story et al. 1989; Story et al. 1996). However, epithelial cells do not respond to bFGF. Stroma also appears to be the major source of IGF (Byrne et al. 1996). In culture, epithelial cells do not produce IGF-I or II while stromal cells produced IGF-II. In vivo, both epithelial and stromal cells have IGF type I receptors (which will bind IGF-II) so IGF-II could have either, or both, autocrine and paracrine interactions (Peehl et al. 1995).
Stromal cells secrete KGF, a member of the FGF family, but the receptor is only found in epithelial cells (Peehl et al. 1995). KGF is mitogenic on epithelial cells and this was supposedly an example of the stromal-epithelial interaction that may regulate cell survival (Denmeade et al. 1996). KGF mRNA has been shown to decrease after castration and increase after androgen replacement, indicating some degree of androgen-dependence (Fasciana et al. 1996). However, another group reported that KGF, as detected immunohistochemically, is not related to functional differences in prostate epithelial cells and its expression is not greatly influenced by androgen (Nemeth et al. 1998).

EGF is the only mitogenic growth factor whose mRNA is up-regulated by androgens and not by castration as well (Culig et al. 1996; Nishi et al. 1996). As mentioned previously, the mRNA expression of TGF-α, EGF-R, and KGF is up-regulated after 3-5 days of T replacement in castrated rats but is also up-regulated after castration (Nishi et al. 1996).

Prostate cancer often alters the expression of growth factors involved in proliferation and inhibition of apoptosis (Foster et al. 1998; Russell et al. 1998). The most significant change is the move from paracrine stimulation requiring an epithelial-stromal interaction to autocrine stimulation where the prostate cancer cell produces both the growth factor and the receptor (Rennie and Nelson 1998). The epithelium eventually becomes independent of the stroma and often, the androgen signaling axis itself. Prime examples of these growth factors systems are: IGF-I and IGF type I receptor (Kaplan et al. 1999);
KGF and KGF receptor (McGarvey and Stearns 1995); EGF, TGF-α and EGF-R (Seth et al. 1999).

e. Pathology of the prostate

The first and most common pathology of the prostate is BPH (also termed nodular prostatic hyperplasia). By age 50 one quarter of all men have some degree of hyperplasia and by age 80, over 90% of males will have BPH (Geller 1991). The mechanism for hyperplasia may be related to the accumulation of DHT in the prostate, since men with 5α-R deficiency have small prostates and do not appear to get BPH (Imperato-McGinley et al. 1992). The normal prostate, which weighs 20-30 g, can weigh up to 50-100 g with BPH. Hyperplasia begins in the verumonatum in the transition zone and the enlargement can impinge on the urethra leading to urinary difficulty and hesitation. In BPH, both glandular and stromal compartments are increased with larger glands having more infolding as seen in Fig. 5. Although BPH is a clinical problem, it is not a precursor to neoplasia.

Prostate cancer is the most common non-cutaneous form of cancer and the leading cause of cancer related death in Western men (Parker et al. 1997). Digital rectal exam or blood screening for PSA detects prostate cancer. A rising PSA score over time indicates prostatic cell proliferation, which can be from BPH or prostate cancer. The carcinomas usually arise from the peripheral zone and are often multifocal. Fig. 6 shows the characteristics and stages of prostate cancer as it progresses. The adenocarcinomas start as increased glandular growth composed of small glands back to back with little or no intervening stroma. Histologically, the cancerous cells are large, round and have a
Fig. 5 Slides of a normal prostate (A), BPH (B), and a comparison of BPH and prostate cancer (C). Panel C shows prostate cancer on the left with characteristic tight packing and little stroma or lumen. On the right is BPH, with larger glands and still visible stroma. Panels A and B are from (http://155.37.5.42/eAtlas/Nav/msProsta.htm) and Panel C is from (http://www-medlib.med.utah.edu/WebPath). Magnification: panels A and C - 100x; panel C - 40x.
Fig. 6 Prostate cancer sections with increasing Gleason score (from http://155.37.5.42/eAtlas/Nav/msProsta.htm). Panel A, the cancer is still has a definitive boundary and a low Gleason score. Panel B shows infiltration of the tumor glands into the stroma and a much higher Gleason score. Panel C shows a loss of differentiation and glandular appearance; the tumor now freely infiltrates the stroma. Panel D illustrates the loss of organization in the ducts and the large nucleolus of the tumor cells. Magnification: panels A,B and C - 100x; panel D - 400x.
prominent nucleolus. As the carcinomas become less differentiated the glands fuse and form solid nests or solid sheets. As the carcinomas progress they become less differentiated and ductal arrangement is all but lost. With the decrease in differentiation comes the risk of the tumours becoming androgen-independent.

Prostate tumours are initially androgen dependent requiring DHT for survival. If androgens are withdrawn, the tumour and normal prostate cells will die through apoptosis. Androgen ablation by medical or surgical castration continues to be the predominant treatment for metastatic prostate cancer (Geller 1993). The side effects associated with this therapy, such as impotence, gynecomastia and depression, can be quite devastating. The therapy is highly effective initially, achieving regression of the tumour and suppression of PSA. However, after an average time of 2 years the tumour will recur, characterized by increasing PSA levels and androgen-independent growth.

There are two postulated mechanisms cells can take to survive in an androgen-depleted environment: bypass reliance on the AR or sensitize the AR (reviewed in (Jenster 1999)). The cells can continue to survive by 1) mutating the AR to be more promiscuous in ligand-binding, 2) amplify the AR, 3) activate the AR in a ligand-independent manner or 4) amplify co-activators.

Loss of AR expression leads to the assumption that the cells have bypassed the AR pathway. Expression of bcl-2 and ras oncogenes and mitogen activated protein kinase (MAPK) are possible mechanisms by which the cells survive. Prostate cancer cell lines (i.e. LNCaP) transfected with these genes become androgen-independent (Voeller et al. 1991; McDonnell et al. 1992; Raffo et al. 1995; Gioeli et al. 1999). In many cases,
growth factors and their receptors are expressed in the tumour cell allowing for autocrine stimulation (reviewed in (Rennie and Nelson 1998; Russell et al. 1998)). The pathways that might be involved in hormone-independent cancer are numerous, underscoring the heterogeneous nature of prostate cancer.

2. ANDROGEN ACTION

The concepts of androgen action were formulated as a result of studies on physiology and androgen metabolism in normal animals (reviewed in (Wilson 1975)). Testosterone diffuses into the cell by passive diffusion along an activity gradient. Once inside the cell, T can be reduced to DHT or aromatized to estradiol. DHT and T bind to the same high-affinity receptor protein in the nucleus. The androgen receptor – androgen complex can then act as a transcription factor and alter cell function and signaling in various ways. Fig. 7 shows the classical roles for T and DHT in androgen-target cells. As will be discussed, T shows the ability to mimic DHT in androgen action in many respects.

a. Androgen receptor structure

Although androgens can diffuse into target and non-target cells, androgen action will only occur in cells that contain the androgen receptor (AR). The AR is a member of the steroid hormone receptor family and like all its members, once bound by an appropriate ligand the receptor will act as a transcription factor to regulate target gene transcription (Tsai and O'Malley 1994). The members of the steroid receptor superfamily have a common structural arrangement of their functional domains. The main functional domains are an N-terminal transactivation region, a DNA binding domain, a hinge region and a hormone binding domain in the C-terminus. The androgen-bound AR will dimerize
Fig. 7 Androgen action in a target cell (Wilson et al. 1993). Dashed line represents the classical roles for DHT in androgen action.
and bind specific DNA sequences termed androgen response elements (AREs) and alter gene transcription. Androgen-dependent growth, differentiation and development all require functional AR.

The AR gene consists of 8 exons and encodes a 110 kD protein of 918 amino acid residues (reviewed in (Zhou et al. 1994; Keller et al. 1996; Wiener et al. 1997)). The AR gene was mapped to the X chromosome at Xq11-12 and because it is X-linked, men only have one copy of the gene. Fig. 8 illustrates the domains and important features of the AR protein. The N-terminal region contains the transactivation domain responsible for the regulation of gene transcription. There are polyglutamine and polyproline residues that may be important for transcriptional regulation. There are two phosphorylation sites at Ser31 and Ser34, but these are not necessary for transactivation (Zhou et al. 1995). Exons 2 and 3 encode the DNA binding domain located between amino acid residues 556 and 623. At two sites, four cysteine residues non-covalently bind a zinc ion, contorting the protein to form zinc fingers capable of interacting with specific DNA sequences. Mutations at this site can result in a non-functional receptor. For example, a mutation at position 616 converting a leucine residue to arginine, greatly reduced the ability of the receptor to bind to DNA, and resulted in partial androgen insensitivity syndrome (De Bellis et al. 1994). Point mutations in the AR gene were found in 92% of the patients with androgen insensitivity syndrome and the mutations were most commonly found in the hormone binding and DNA binding domain (Quigley et al. 1995).

The remaining 295 amino acid from exons 4-8 form the C-terminus and include the hinge region, nuclear localization and ligand (hormone) binding domains. As can be inferred
Fig. 8 Schematic of the functional domains of the human androgen receptor (adapted from Keller et al. 1996, Zhou et al. 1994). The exons are labeled A-H. The numbers above the box indicate the amino acid number at the junction between exon and intron. Phosphorylation sites are indicated by lines and the amino acid numbered below. The functional domains and are indicated by lines bracketed by the corresponding amino acid number in the receptor.
from the name of the region, mutations in the hormone-binding domain will alter the affinity or specificity of the receptor. The result can be a non-functional receptor or one that functions only at high steroid levels or has altered specificity such as the AR found in LNCaP prostate cancer cells (Montgomery et al. 1992).

b. Androgen receptor distribution

As expected, both AR mRNA and protein levels are high in male reproductive tissues (Takeda et al. 1990; Berman and Russell 1993). The adrenal gland has extremely high levels of AR mRNA and medium to high levels of protein. Medium levels of AR expression are also found in the testes and ovary. Low levels of expression are found in the levator ani and bulbocavernosus muscles, scrotal skin, vagina, and preputial gland. Muscle, pituitary and hypothalamus show a medium level of AR by immunohistochemistry (Sar et al. 1990).

The AR is present in lumenal epithelial cells, smooth muscle cells and a variable percentage of stromal cells in the ventral, lateral and dorsal lobes of the adult rat prostate (Prins et al. 1991). Lumenal epithelial cells from the ventral lobe stained more intensely than did epithelial cells from the lateral or dorsal lobes. For all three lobes, basal epithelial cells and endothelial cells were negative for AR immunostaining, while periductal smooth muscle was strongly positive for AR as was perivascular smooth muscle. Although some stromal cells in the ventral and dorsal lobes were AR positive, the majority were AR negative. For comparison, AR staining in human BPH samples was prominent in the nuclei of epithelial cells and was also present in the stroma of
fibromuscular BPH regions (Sar et al. 1990). In prostate cancer, heterogeneous staining in stromal and epithelial cells was observed (Sar et al. 1990; Culig et al. 1998).

Within the rat prostatic lobes, the ducts exhibit distinct regions of morphology and androgen-responsiveness (Lee et al. 1990). AR expression along the proximal-distal axis of the rat ventral lobe was examined as a possible mechanism for regional differences in androgen responsiveness (Prins et al. 1992). Immunohistochemistry and autoradiography were used to examine the level of AR staining and relative ability to bind ligand, respectively. Quantitation of AR staining intensity in epithelial cells revealed no significant difference between proximal, intermediate and distal regions. Approximately 30% of fibroblastic stromal cells stained positive for AR, although with a lower intensity than that seen in the epithelial cells and there was also no variation between regions. Autoradiography, used to determine whether the staining AR was functional, confirmed the findings of the immunohistochemistry. Thus, the regional heterogeneity in prostatic growth and function is not a result of differences in levels of AR. Other region-specific structural, intracellular, or paracrine factors may be responsible for the differences in androgen responsiveness along the prostatic duct.

c. Androgen receptor regulation in the prostate

In most AR positive cells, the level of AR gene transcription is regulated by androgens themselves (Quarmby et al. 1990). In development, or in a background of low androgens, AR expression can be up-regulated by androgens. Excess androgen in the adult will cause down-regulation of the AR mRNA. The human AR gene contains an androgen-responsive region, to which AR protein binds, and is involved in up-regulation of AR
transcription (Dai and Burnstein 1996). This 350-bp cDNA fragment of the AR was confirmed to have two androgen response elements that function synergistically and are separated by 182 bp. The sequence termed ARE-1 (5'-TGTCCCT-3') resembles a site recognized by several steroid receptors including the AR. ARE-2 (5'-AGTACTCC-3') is identical to a portion of the ARE found in the rat probasin gene promoter (Rennie et al. 1993; Claessens et al. 1996).

Other steroids acting via their receptors, have been shown to inhibit AR mRNA expression. AR mRNA was down-regulated when high T levels were aromatized to estradiol in smooth muscle cells (Lin et al. 1993). In COS-1 cells co-transfected with glucocorticoid receptor (GR) and AR cDNAs, dexamethasone mimicked androgen-mediated down-regulation of AR mRNA (Kumar et al. 1998). TPA can control AR expression and it has recently been found to have a response element of its own within the 5'-flanking region of the mouse AR (Kumar et al. 1998).

In the majority of the studies in rats, most agree that prostatic AR mRNA increases after androgen withdrawal, but there are conflicting results as to what happens after several days (Quarmby et al. 1990; Prins and Woodham 1995). AR mRNA increased 3-fold after castration and remained elevated for four days (Quarmby et al. 1990). Administration of T to normal rats decreased AR mRNA. Testosterone given to testicular-feminized rats that lack a functional AR did not decrease AR mRNA. Down-regulation of AR mRNA by androgen therefore appears to be a receptor-mediated process. In a separate study, AR mRNA expression in the absence of androgen was shown to increase transiently and return to normal levels after three days (Prins and Woodham 1995). This occurs in all the
lobes of the prostate except in the region that makes up the second half of the lateral lobe known as LP2. Regulation of AR mRNA appears to occur at the transcriptional and post-transcriptional levels (Mora et al. 1996). AR mRNA in rats castrated 24 h prior to an injection of a high concentration of T remains unchanged after 1 h. However, in the presence of the protein synthesis inhibitor cyclohexamide, AR mRNA levels decreased, indicating that a protein synthesis-dependent mechanism may be involved in the regulation of the stability and/or the translation of AR mRNA in the prostate. The AR protein levels in these rats, as detected by immunohistochemistry, show strong nuclear AR staining after 1 h. In the presence of cyclohexamide, there was no increase in nuclear AR staining, which indicates that new AR protein is being produced during T treatment.

Differential regulation of AR protein has been shown in the different lobes of the prostate (Prins 1989; Prins and Birch 1993). Both ligand-binding and immunohistochemical assays showed that AR levels fall in the ventral and dorsal lobes to undetectable levels within ten days of castration. No change occurred in the lateral lobe. Testosterone replacement returned AR levels to normal after five days in the ventral and dorsal lobes. In the lateral lobe, despite the absence of a response to androgen withdrawal, androgen replacement resulted in a transient increase in AR.

Another study on AR regulation at the protein level showed that AR immunostaining fell significantly after castration, but that administration of T or DHT resulted in increased nuclear AR staining within 15 min but return to normal staining intensity took 72 hours (Sar et al. 1990). The increase in AR staining after 15 min appears to be too rapid for new protein synthesis, but the region of the prostate examined was probably the distal tips of
the ventral prostate which expresses AR even in the castrated animal (Prins and Birch 1993).

Overall, many studies show that AR is regulated by androgen, this becomes important as the larger issue of androgenic manipulation of prostate growth and regression is addressed.

d. Transactivation by AR

Once in the nucleus, the AR complex will dimerize and bind to AREs in the promoter region of target genes. Binding to DNA will allow interaction of AR with the basal transcription machinery either directly or through various co-activators. Fig. 9 illustrates the classical transactivation and transrepression functions of the AR. Transcriptional activation by AR may occur through binding of the N-terminal region of the AR (amino acids 142-485) to the transcription factor TFIIF and the TATA-box-binding protein (McEwan and Gustafsson 1997). Recruitment of receptor to the promoter regions results in increased levels of target gene transcription. Recently, there have been a number of new proteins revealed that interact with AR to assist in activation of transcription. These co-activators include TIF2 (transcriptional mediator/intermediary factor) (Voegel et al. 1996), ARA₇₀ (androgen receptor associated protein) (Yeh and Chang 1996), ARA₁₆₀ (Hsiao 1999), ARA₅₄ (Kang 1999), and a compound NRC (nuclear receptor co-activator) (Tan et al. 1999).

The co-activators bind to various regions of the AR and interact to increase transactivation activity. In human prostate cancer DU145 cells transfected with a
Fig. 9 Transactivation and transrepression functions of the AR. The androgen diffuses through the plasma membrane to bind to the AR. The receptor undergoes a conformational change and translocates to the nucleus. The complex forms a homodimer and binds to the DNA at specific ARE and enhances gene transcription through direct interaction with the basal transcription apparatus or through various co-activators. The AR can negatively regulate gene expression through interaction with transcription factors already bound to DNA or through competitive binding to sites occupied by other transcription factors.
functional AR, ARA\textsubscript{70} functions as an activator to enhance AR transcriptional activity 10-fold in the presence of 10\textsuperscript{-10} M DHT or 10\textsuperscript{-9} M T, but not 10\textsuperscript{-6} M hydroxyflutamide (Yeh and Chang 1996). ARA\textsubscript{70} interacts with the C-terminal domain, which contains the ligand-binding domain. This probably explains why flutamide does not increase the transcription rate. Presumably, flutamide does not allow the receptor to be in an active conformation after ligand binding and may mask co-activator binding sites. Within the AR transfected DU-145 cells, the specific ligand-induced AR conformation and transactivation activity is also illustrated by the fact that there is a potency difference between T and DHT. Ten times more T is required for the same activation achieved with DHT (Yeh and Chang 1996). ARA\textsubscript{160}, a newly characterized co-activator, enhanced AR-mediated transactivation in transiently transfected PC-3 cells (Hsiao 1999). However, ARA\textsubscript{160} acts as an N-terminal co-activator, and ligand specificity perhaps will not play a role in transactivation potency, but this has yet to be determined. ARA\textsubscript{54} is another co-activator, similar in function to ARA\textsubscript{70} which increases AR transactivation activity (Kang 1999). It is possible that these co-activators act simultaneously and synergistically, but these complex interactions have yet to be investigated. Fig. 10 from the McGill University website illustrates the most up-to-date series of co-activators and co-repressors.

The co-activator CBP ((cAMP response element binding protein)-binding protein) will bind the AR complex and also recruits a histone acetyltransferase, P/CAF (p300/CBP-associated factor) to target gene promoters (Yang et al. 1996). The AR, CBP and P/CAF multi-protein complex does not seem to act on the basal transcription apparatus but will
Fig. 10 Androgen receptor-interacting proteins (from http://www.mcgill.ca/androgendb/interact.gif). Co-activator abbreviations: ARA_{55, 77} (AR co-activator 55 or 77), GRIP1 (GR interacting protein 1), TIF2 (transcriptional intermediary factor 2), RAF (receptor activating factor), SNURF (small nucleus RING finger), F-SRC-1 (steroid receptor co-activator), RIP_{140} (receptor interacting protein 140), and CBP/300 (CREB protein-binding protein). Co-repressors abbreviations: Ets-TF (Ets transcription factor), MCM7 (mini-chromosome maintenance 7), ARIP3 (AR activating protein 3), and SRC-1 (steroid receptor co-repressor 1). Other protein abbreviations: NJ (NSC-34 clone J), and hsp5 (heat shock proteins).
enhance acetylation of chromatin, which can overcome inhibitory effects of chromatin on gene expression. Simultaneous binding of CBP and any of the ARAs is not known.

e. Transrepression by AR

As discussed earlier, apoptosis in the prostate after castration is characterized by a proportional increase in expression of the gene for TRPM-2 (Buttyan et al. 1989; Rittmaster et al. 1991; Furuya and Isaacs 1993; Woolveridge et al. 1998). The implication is that the AR in the presence of androgen negatively regulates the TRPM-2 gene. Androgen ablation relieves AR transrepression and the gene’s expression is up-regulated. The main mechanism for AR negative regulation of gene expression appears to be through protein-protein interaction. For example, in a ligand-dependent manner, AR has been shown to inhibit c-Jun binding to the AP-1 site in DNA, thus down-regulating c-Jun activity (Kallio et al. 1995). However, AP-1 is not always the target for down-regulation of expression. In the case of human collagenase I gene expression, AR negatively regulates expression through a physical interaction with Ets-related transcription factors that are also required for positive regulation (Schneikert et al. 1996).

This negative regulation is specific for the AR. In fact, most of the genes negatively regulated by AR carry Ets-binding sites in their promoter regions (Metsis et al. 1992; Schneikert et al. 1996; Heckert et al. 1997). The mapsin gene has a negative ARE (5’-GTACTCTGATCTCC-3’) which down-regulates gene expression upon binding the liganded AR-complex (Zhang et al. 1997). The human ornithine decarboxylase promoter also carries a negative ARE (5’-GxxCxCxxxGTGCT-3’, where x is in variation) (Bai et al. 1998). The mechanism for DNA binding to lead to transcriptional repression is unclear. Co-repressors that have histone deacetylase activity, could bind to the AR and
deacetylate chromatin to bring it into a repressed state. The negative ARE of the mapsin
gene is also close to an Ets-binding site. Therefore, it would be possible to have AR
binding to the negative ARE and the Ets-related proteins bound to the Ets site.

f. Crosstalk with AR and growth factors

The third method of AR-regulated gene expression involves growth factor-mediated
enhancement of transactivation. Essentially this involves crosstalk with membrane-
initiated signal systems. Several growth factors have demonstrated the ability to enhance
androgen-dependent AR transactivation or to initiate ligand-independent transactivation
by AR. For example, CV-1 and HeLa cells were transfected with AR expression vectors
and a CAT reporter linked to an MMTV promoter. In the presence of androgen, EGF,
IGF-I and the phosphotyrosine phosphatase inhibitor, vanadate, all enhanced AR-
dependent transactivation by 1.5- to 2.5-fold (Reinikainen et al. 1996). Genistein, a
tyrosine kinase inhibitor, diminished AR transactivation by two thirds. Also, Culig et al.
(1994) have demonstrated ligand-independent AR transactivation by EGF and IGF-I
through the tyrosine phosphorylation pathway. It can be speculated that this growth
factor-mediated increase in tyrosine phosphorylation signals AR to enhance its
transactivation activity. The increased activity may occur through phosphorylation of the
receptor itself or the co-activators that enhance receptor activity. Recently, a possible role
of mitogen-activated protein kinase kinase kinase 1 (MAPKKK1 or MEKK1) in AR
transactivation in the presence or absence of ligand has been demonstrated (Abreu-Martin
et al. 1999). Activation of the ligand-independent AR activity has important implications
in hormone-independent prostate cancer.
g. T and DHT effects on the AR

Textbooks generally state that DHT has a higher affinity for AR than T and that this explains the higher potency of DHT. Early studies on the $K_d$ for DHT and T binding to AR report values that show a 4-10 fold difference with DHT having the higher affinity (Wilbert et al. 1983; Carlson and Katzenellenbogen 1990). Apparently, the absolute values vary considerably depending on assay conditions.

The conversion of T to DHT results in a more stable steroid-AR complex which enhances prostate function and growth (Grino et al. 1990; Deslypere et al. 1992; Zhou et al. 1995). Using recombinant AR expressed in COS cells, the increased stability of the DHT-AR complex compared to the T-AR complex was demonstrated by the fact that T dissociates from AR 3 times faster than DHT or methyltrienolone (R1881) (Zhou et al. 1995). DHT stabilizes the receptor by slowing the rates of ligand dissociation and AR degradation. Previous in vitro studies have indicated that DHT is ten-fold more active than T in enhancing transcription of the MMTV-CAT reporter gene in transfected cells (Deslypere et al. 1992). In a yeast two-hybrid system examining the effect of ARA20 on AR-mediated gene transcription, Yeh et al. (1996) demonstrated that ten times more T than DHT was need to get the same amount of activity. In both cases, sufficiently high concentrations of T can mimic the action of DHT.

Although all of these studies have focused on the increased potency of DHT, there is at least one example of a gene that demonstrates preferential regulation with the T-AR complex over the DHT-AR complex (Lin and Chang 1997). Using differential display PCR, the gene TDD5 was identified from T cell hybridomas (which lack 5α-R activity)
stimulated with T and DHT. TDD5 was repressed by 10 nM T in 2 hours while 8 hours was needed for the same effect with 10 nM DHT. TDD5 is expressed almost exclusively in the mouse kidney. To date the promoter specificity has yet to be determined.

The relative potency of T and DHT thus can vary, depending on the endpoint measured. This is no doubt concerned with the nature and conformation of the ligand-AR complex, its interaction with particular response elements and the cell and promoter context specificity of the response elements.

3. 5α-REDUCTASE

a. 5α-Reductase in normal physiology

5α-R catalyzes the reduction of T across the double bond at the 4, 5 position using NADPH as the H⁺ donor, as seen in Fig. 11. This reaction is predominant in the prostate and skin of the adult male. The importance of T reduction to DHT was not identified until the late 1960's when work by Bruchovsky et al. (1968) and Anderson and Liao (1968) identified that 5α-R might be a regulatory step in androgen action and not just involved in steroid catabolism. It was observed that administration of ³H-T to rats resulted in the accumulation of ³H-DHT in the nuclei of the ventral prostate cells. The AR was also found to bind DHT preferentially, indicating that DHT was indeed important in androgen action in the prostate (Fang et al. 1969; Mainwaring 1969; Carlson and Katzenellenbogen 1990).

The finding of an inborn error with a 5α-R enzyme confirmed the importance of this protein in the development of the male reproductive tract (Imperato-McGinley et al. 
Fig. 11 5α-R - catalyzed conversion of T to DHT using NADPH as a cofactor, from (Frye et al. 1998). The transition state for reduction is also depicted.
1979; Maes et al. 1979; Imperato-McGinley et al. 1991; Imperato-McGinley et al. 1992; Wilson et al. 1993). In men with pseudohermaphroditism from 5α-R type 2 deficiency, the Wolffian duct structures (epididymis, vas deferens, and seminal vesicles), that respond to T, develop normally. However, the urogenital sinus structures (prostate, penis and scrotum) arise from DHT stimulation, and are underdeveloped. The condition known as 5α-R deficiency, results in genotypic males who display a female phenotype until puberty when they undergo varying degrees of virilization including penile enlargement, testicular descent, and development of male musculature. Body and facial hair is reduced in these individuals, and the prostate remains small, at approximately 1/6th the normal size and is composed mostly of stroma (Imperato-McGinley et al. 1992). Examining cultured fibroblasts from genital skin was initially the most effective way to determine 5α-R activity in an individual. The activity from cultured genital skin fibroblasts of a normal subject has a peak at pH 5.5 with a broad shoulder of activity over an alkaline range. The results in a subject with 5α-R type 2 deficiency display low rates of activity at pH 5.5 but are otherwise normal. The importance of this curve would not be realized until after the enzyme was cloned, as will be discussed later.

5α-R activity in cultured genital fibroblasts from the families from Dallas and the Dominican Republic from which 5α-R deficiency was originally diagnosed, had the same profile as outlined in Fig. 12. The afflicted individuals in a family from Los Angeles showed measurable 5α-R activity but it was kinetically abnormal. Contrary to the first form of 5α-R type 2 deficiency in which the enzyme did not bind T, this mutated enzyme had a normal $K_m$ for T but a decreased affinity for NADPH, the cofactor needed for the formation of DHT. This enzyme was unstable and exhibited a rapid turnover. Fig. 13
illustrates 28 different mutations in the 5α-R type 2 gene. Although there are many mutations, the outcome is either altered affinity for T or altered affinity for NADPH.

5α-R plays a role in other tissues such as the liver and skin. In the liver, 5α-R has been suggested to be a catabolic step for the clearance of androgens (Russell and Wilson 1994). 5α-R in skin has been suggested to regulate activity and may be a contributor to male pattern baldness (Schroder 1994). In the brain, calcium signaling of gonadotrophs in the pituitary appears to be regulated by DHT (Tobin and Canny 1998). However, other functions of 5α-R in the brain are poorly understood (Celotti et al. 1997).

b. 5α-Reductase structure

Because of the hydrophobicity and insolubility of 5α-R, detergents had to be used to solubilize the enzyme from cell membranes. Unfortunately, after chromatography to purify the enzyme, all activity was lost. The protein was finally isolated using the technique of expression cloning in Xenopus oocytes (Andersson et al. 1989). The cDNA was used to isolate a human homologue of the rat cDNA by cross-hybridization screening of a prostate cDNA library (Jenkins et al. 1992). The 5α-R picked up by the screen, however, did not possess the same activity as the 5α-R in the prostate. This 5α-R was only weakly inhibited by finasteride (a potent 5α-R inhibitor in the prostate, capable of lowering DHT concentrations to near that of a castrated animal). The pH for optimal 5α-R activity for this new 5α-R was alkaline. This was dissimilar from the acidic pH optimum for 5α-R activity in the prostate. Lastly, it appeared that the chromosomal locus for this gene was not the same as that for the families with 5α-R deficiency (Jenkins et al. 1992). The existence of a second 5α-R gene was the conclusion of this strong evidence.
Fig. 12 Mutations in the 5α-R type 2 gene and protein. A schematic diagram of the 5α-R gene is shown above. The locations of 28 different mutations are indicated on the left and the sites of recurring mutations in various populations are on the right. Delta NG represents the deletion in the cohort found in New Guinea; 359 delta TC indicates a deletion of the TC nucleotide at position 359 of the cDNA. R227* indicates a premature termination codon; 725+1, G to T indicates a splice junction mutation. The other mutations represent single amino acid substitutions. The initiating methionine is designated “Met” in exon 1 and the normal termination codon is designated by an asterisk in exon 5. From (Wilson et al. 1993).
The isolation of the second 5α-R cDNA was accomplished by expression cloning in cultured human cells (Andersson et al. 1991). This 5α-R had the more classical activity similar to that seen in the prostate. It had an acidic pH optimum, was sensitive to finasteride, and the gene from which the cDNA was derived was found to be mutated in patients with 5α-R deficiency (Normington and Russell 1992).

The two 5α-R isozymes have been identified as 5α-R type 1 and type 2; named for the chronological order in which they were detected (Andersson et al. 1989; Andersson and Russell 1990; Jenkins et al. 1992). Because of the aforementioned difficulty in isolating the protein from cells, the primary structures of both rat and human 5α-R isozymes were determined from their respective cDNAs (reviewed in (Russell and Wilson 1994)). They have predicted molecular weights of 28 – 29,000, based on proteins composed of 254-260 amino acids. The amino acid sequences of the type 1 and 2 isozymes are 50% identical. For rat and human 5α-R type 1 and 2, the amino acid sequence identities were 60% and 77% similar, respectively. Within the protein, 37% of the residues have side chains found in the hydrophobic interior of globular proteins (Cys, Ile, Leu, Met, Phe, and Val), but the arrangement of these hydrophobic amino acids does not suggest clear-cut transmembrane domains. The inference from this distribution pattern is that 5α-R isozymes are intrinsic membrane proteins embedded in the lipid bilayer. This explains the difficulty in attempts to isolate and purify the protein (Andersson et al. 1989; Andersson and Russell 1990).

The genes for both 5α-R isozymes contain five exons and four introns (Labrie et al. 1992). Although the position of the introns in the two genes is basically the same, the
genes are located on different chromosomes. Human type 1 isozyme, also termed
SRD5A1, is located on the distal short arm of chromosome 5 (band p15). The type 2
isozyme (SRD5A2) is located in band p23 of chromosome 2 (Thigpen et al. 1992).

c. Characterization of type 1 and type 2 5α-reductase

The characterization of the optimum pH for 5α-R enzyme activity was carried out in
transfected cell lysates (Andersson and Russell 1990; Normington and Russell 1992). In
this system, the 5α-R type 1 isozyme has a broad alkaline pH optimum (pH 6 – 8.5) for
which its activity remains high. The pH optimum range for the type 2 isozyme was
narrow, acidic and centered around pH 5.0. The pH optimum, being characteristic of each
isozyme, was used to describe the presence of 5α-R isozymes in particular tissues and
cell types. For example, the most activity in cultured genital skin fibroblasts is in the
acidic range while in non-genital skin the majority of 5α-R activity is in the alkaline
range (reviewed in (Wilson et al. 1993)).

The kinetics of 5α-R type 1 and type 2 show that the type 2 isozyme has a higher affinity
for T (K_m = 0.5-1.0 µM for type 2 (Andersson and Russell 1990) and K_m = 1-5 µM for type
1 5α-R (Andersson et al. 1991; Thigpen et al. 1993)). The K_m for the NADPH cofactor is
in the low micromolar range for both isozymes (Thigpen et al. 1993). When the K_m is
examined in intact or permeabilized cells at pH 7.0, the value for the type 2 isozyme was
in the low nanomolar range (4-50 nM) (Thigpen et al. 1993). This suggests that although
5α-R type 2 has an acidic pH optimum in the cell lysate assay system, in the cell it is
most effective at neutral pH.
Pulse-chase experiments with transfected CHO cells indicate that both 5α-R isozymes have long half-lives of around 20-30 hours (reviewed in (Russell and Wilson 1994)). Micromolar concentrations of finasteride did not alter the half-life of the enzymes, which suggests that the regulation of the enzyme, at least in CHO cells, is not at the level of protein degradation. Post-translational modification (i.e. phosphorylation) of the enzymes is also not indicated.

d. 5α-Reductase distribution

The subcellular localization of 5α-R in the rat prostate has been considered nuclear for both immunoreactivity and enzymatic activity but the issue is controversial. In crude subcellular fractions of the rat prostate, type 1 5α-R was found preferentially in the nuclear fraction, while type 2 5α-R had a predominantly microsomal localization (Span et al. 1996). Using antibodies raised against human 5α-R isozymes, the type 1 5α-R was localized immunohistochemically in the human prostate to the nucleus, and the type 2 isozyme to the cytoplasm (Aumuller et al. 1996). Based on the acidic pH optimum of 5α-R type 2, the subcellular localization is liable to be within an acidic subcellular compartment such as the endosome or lysosome. However, in CHO cells transfected with 5α-R type 2, compounds that neutralize the acidic compartments do not affect 5α-R activity (Thigpen et al. 1993). Immunohistochemical studies in these transfected CHO cells reveals that 5α-R type 1 and 2 appear to reside in the endoplasmic reticulum (a neutral pH compartment). In a study with 5α-R type 1 or 2 cDNA transfected COS cells, subfractionation localized both isozymes to both the crude nuclear and crude cytoplasmic fractions (Savory et al. 1995). However, cytoimmunofluorescence localized both isozymes to just the nuclear periphery (Savory et al. 1995). This study concluded that the
5α-R isozymes were part of the outer nuclear membrane/endoplasmic reticulum. It has been suggested that in its native state within the endoplasmic reticulum, the isozyme functions at a neutral pH (Thigpen et al. 1993). The shift in pH when the cells are physically lysed for analysis of 5α-R activity may reflect a conformational change.

The tissue distribution of the 5α-R type 1 and type 2 isozymes in the rat is different from the human (Normington and Russell 1992). Table II illustrates some of the differences. In the skin, 5α-R type 1 is dominant in the sebaceous glands (Luu-The et al. 1994), while 5α-R type 2 predominates in the dermal papilla of beard hair follicles (Itami et al. 1991) and is highly expressed in the inner epithelial sheath of hair follicles over much of the body (Eicheler et al. 1995). In the adult rat prostate, the basal epithelial cells express type 1 5α-R, while type 2 5α-R is confined to the stroma (Berman and Russell 1993). Basal epithelial cells retain proliferative capacity and are suspected to differentiate into luminal epithelial cells, which do not express 5α-R (Verhagen et al. 1988). It has been suggested that DHT is produced in the basal cells for autocrine stimulation in order to differentiate into luminal epithelial cells. Conversely, DHT produced from basal cells may act in a paracrine fashion to stabilize or stimulate the division of adjacent androgen-dependent luminal epithelial cells (Berman and Russell 1993). All three lobes of the rat prostate have 5α-R activity, express both isozymes and only vary subtly in pH profile for 5α-R activity (Prahala et al. 1998).

In Table II, the human prostate shows only type 2 5α-R. Since those data were tabulated several studies have shown 5α-R type 1 activity in the human prostate, although the distribution is still controversial (Aumuller et al. 1996; Bonhoff et al. 1996; Bruchovsky
Table II. Tissue distribution of 5α-R isozymes (Russell and Wilson 1994).

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>5α-R type 1</th>
<th>5α-R type 2</th>
<th>Method of detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Ventral prostate</td>
<td>+</td>
<td>+</td>
<td>mRNA, protein</td>
</tr>
<tr>
<td></td>
<td>Epididymis</td>
<td>+</td>
<td>+++</td>
<td>mRNA, protein</td>
</tr>
<tr>
<td></td>
<td>Seminal vesicles, vas deferens, testes</td>
<td>+</td>
<td>+</td>
<td>mRNA</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>+++</td>
<td>-</td>
<td>mRNA, protein</td>
</tr>
<tr>
<td></td>
<td>Adrenal, brain, colon, intestine, kidney</td>
<td>++</td>
<td>+</td>
<td>mRNA</td>
</tr>
<tr>
<td></td>
<td>Heart</td>
<td>-</td>
<td>-</td>
<td>mRNA</td>
</tr>
<tr>
<td></td>
<td>Lung</td>
<td>++</td>
<td>-</td>
<td>mRNA</td>
</tr>
<tr>
<td></td>
<td>Muscle, spleen, stomach, ovary</td>
<td>+</td>
<td>-</td>
<td>mRNA</td>
</tr>
<tr>
<td>Human</td>
<td>Prostate, epididymis, seminal vesicle, genital skin</td>
<td>-</td>
<td>++</td>
<td>mRNA, protein</td>
</tr>
<tr>
<td></td>
<td>Testis, ovary, adrenal, brain, kidney</td>
<td>-</td>
<td>-</td>
<td>mRNA, protein</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>+</td>
<td>+</td>
<td>mRNA, protein</td>
</tr>
<tr>
<td></td>
<td>Non-genital skin</td>
<td>++</td>
<td>-</td>
<td>mRNA, protein</td>
</tr>
</tbody>
</table>
et al. 1996; Pelletier et al. 1998). Analysis of the cell types with Northern blots and RT-PCR indicate that the prostate epithelium expresses 5α-R type 1 mRNA exclusively, while mRNA for both isozymes was found in the stroma (Bruchovsky et al. 1996). In situ-hybridization for 5α-R type 1 and 2 mRNA showed that mRNA for both isozymes was present in the epithelium and stroma, and that the labeling for 5α-R type 1 mRNA was stronger than for 5α-R type 2 mRNA (Pelletier et al. 1998). It was also shown that the ratio of silver grains in the epithelial cells was 2–3 times higher than that in the stroma. Immunohistochemical analysis of human prostates indicate that luminal epithelial cells express 5α-R type 1 primarily, while basal epithelial cells express both isozymes (Bonkhoff et al. 1996). The type 2 5α-R has been shown immunohistochemically to reside in basal epithelial and stromal cells, but not the luminal epithelial cells (Silver et al. 1994). However, Aumuller et al. (1996) demonstrated weak, diffuse staining for 5α-R type 2 in luminal epithelial cells as well as basal epithelial and stromal cells. Thus, it appears that both 5α-R isozymes may reside in all prostate cells, and that co-expression is likely. 5α-R type 1 is localized to nuclei, which challenges its perceived role as a catabolic enzyme, and is found in higher concentrations in the epithelium than in the stroma. 5α-R type 2 is localized to the cytoplasmic region, most likely the endoplasmic reticulum, and is also found in higher concentrations in the epithelium than stroma but to a lesser extent.

5α-R expression is altered in prostate cancer. Prostate cancer is initially androgen-responsive but after androgen withdrawal therapy, will lapse into an androgen-insensitive state (Denmeade et al. 1996). Most carcinomas, even those that are androgen-independent, have an intact AR (Ruizeveld de Winter et al. 1994). 5α-R was detected in
prostate cancer, particularly in high-grade and androgen-independent tumours, but
eexpression within the cancerous cell is shifted from that of a normal cell (Bonkhoff et al.
1996). Type 1 5α-R was not only found in the nucleus but also in the cytoplasm. The type
2 isozyme was increased both the cytoplasm and nucleus. 5α-R was even expressed in
tumour cells that had lost AR expression.

A mutation of the 5α-R type 2 gene has been proposed as a risk factor for prostate cancer.
Somatic mutations in SRD5A2 have been reported in 57% of a small population of
tumour samples (Akalu et al. 1999). Some mutations in populations that have a higher
incidence of prostate cancer have been linked to increased 5α-R activity and DHT
formation (Reichardt et al. 1995; Makridakis et al. 1999). In addition, other mutations
have been found predominantly in Asian men that actually reduce DHT levels and, at
least in an epidemiologic sense, reduce the risk of prostate cancer (Makridakis et al.
1997; Lunn et al. 1999).

e. Developmental role of 5α-reductase

The critical period for 5α-R in the development of the rat was determined using maternal
dosing with the 5α-R inhibitor, finasteride, beginning on gestational day 15 and ending
on postpartum day 21 (Clark et al. 1993). It was determined that the critical time in which
DHT is required for complete prostate and external genitalia formation is days 16 and 17
of gestation. The human equivalent of this time would be 8-12 weeks gestation.

Immunoblot examination of 5α-R expression revealed unique human tissue-specific and
developmental expression patterns of the 5α-R isozymes (Thigpen et al. 1993). The type
1 isozyme is expressed in the liver at birth, and is transiently expressed in newborn skin and scalp. At puberty, the expression of 5α-R type 1 reappears in skin and scalp and becomes permanent throughout adulthood. Balding scalp showed no differences in type 1 expression from non-balding scalp. 5α-R was detected in the prostate at all ages that were examined, even in malignant neoplastic tissue. Type 2 5α-R is transiently expressed in the skin and scalp of newborns and is the sole 5α-R in the seminal vesicle, epididymis and fetal genital skin. The liver contains both isozymes but only after birth.

With 5α-R type 2 deficiency, there are no other apparent symptoms besides abnormal development of genitalia and prostate, which indicates that the chief developmental role of 5α-R type 2 is virilization of the external genitalia and urogenital sinus (Wilson et al. 1993). However, at puberty 5α-R type 2 deficient males will begin to virilize to different extents (Imperato-McGinley et al. 1991) and some researchers (Thigpen et al. 1993) attribute this to the role of 5α-R type 1.

The role of 5α-R from birth to puberty was examined in rats using the pharmacological agent, finasteride (George et al. 1989). Serum DHT values were suppressed to 25-50% of control values while serum T levels were almost double that of controls. The prostate, penis, seminal vesicles, and epididymis were all reduced in size to 30-50% of controls. Several tissues that are known to be androgen-dependent, such as the perineal muscles (i.e. levator ani) and preputial gland, were unaffected by the withdrawal of DHT. Testicular histology and sperm production were also unaffected by finasteride treatment. The fact that even after birth, 5α-R inhibition was able to retard prostate, epididymis and seminal vesicle growth further suggests that DHT is the trophic hormone in these tissues.
The lack of effect on perineal muscles indicates that these tissues respond to the presence of T and not DHT for growth. As will be discussed later, the profile of the enzymes involved in steroid metabolism for muscle also indicates that T retention and action are favored over DHT (Carlson and Katzenellenbogen 1990; Pasupuleti and Horton 1990).

"Knockout mice," in which a particular gene is ablated by embryonic stem cell technology, are an excellent tool for examining the role of specific factors in normal function. A type 1 5α-R isozyme knockout mouse was developed and overall, the male mice appear normal (Mahendroo et al. 1996). However, knockout female mice experience a delay in parturition that is maternal in origin and can be reversed by the addition of the 5α-reduced androgen, 3α-androstanediol. This effect suggests a role for 5α-R type 1 and 3α-androstanediol in parturition in mice. Although 3α-androstanediol was used as a supplement, it is most likely oxidized to DHT by 3α-hydroxysteroid oxidoreductase, which was unfortunately not measured in this study. The litter size of these female mice is also approximately 1/3rd that of normal litter sizes (2.7 vs. 8.0 pups. respectively) (Mahendroo et al. 1997). It was determined that the time of fetal death corresponds with an upsurge in T production. Aromatase inhibitors were able to reverse the induction of fetal death indicating that an excess of estrogen, due to aromatization from an excess of T, is responsible for the fetal death midgestation. Thus, 5α-R type 1 does not have any obvious effects in male mice but is necessary for correct fetal androgen metabolism and clearance. The absence of 5α-R type 1 blocks 3α-androstanediol formation and results in an excess of T, leading to excessive estrogen levels.
The question may arise as to why we do not see 5α-R type 1 mutations in humans. It is possible that there are 5α-R type 1 mutations, but in the absence of any symptoms in men, they go undetected. In women, the facts that fetal death due to estrogen excess would appear early in gestation, that delayed parturition would be treated medically and that these mutations are likely to be rare, contribute to the reasons type 1 isozyme mutations have not been found.

Although a 5α-R type 2 knockout mouse has not been reported, treatment with finasteride, as described above, can result in 5α-R type 2 loss. However, finasteride is not rigorously selective for type 2 5α-R, but merely is preferential, so there is still some question as to specific roles for 5α-R type 2 in the rodent model.

f. Regulation of 5α-reductase by androgen

5α-R expression appears to be unique in its regulation because it is under feed-forward control by its own steroid product, DHT (George et al. 1991). In the experiment described by George et al. (1991), finasteride administered to rats for 7 days reduced prostate weight by 55% and 5α-R activity by 87%, compared to normal controls. The level of 5α-R activity in the treated rats was close to that seen in castrated rats. To determine if the reduction in 5α-R was a direct consequence of DHT deficiency, androgen effects on regrowth of the prostate were examined. Rats were castrated for 7 days, which allowed the prostate to regress and 5α-R activity levels to drop to 5% of levels found in non-castrated animals. Rats were then treated with T or DHT propionate in the presence and absence of finasteride. T-P and DHT-P in the absence of finasteride, increased prostate weight and 5α-R activity. The T-P group treated with finasteride did
not show an increase in prostate weight or 5α-R activity. Finasteride had no inhibitory effect in the animals given DHT-P. 5α-R mRNA levels from these groups mimic the results with 5α-R activity. In the T-P treated animals, finasteride prevented an increase in 5α-R mRNA, maintaining it at levels near those of a castrated animal. In the DHT-P treated group, finasteride did not suppress 5α-R mRNA levels, which rose to control levels. Therefore, the evidence suggests that DHT controls prostate 5α-R levels and that the mechanism for increased 5α-R mRNA relies on increased levels of DHT, supporting a feed-forward mechanism of control, probably at the transcriptional level.

Horton et al. (1993) examined a mechanism in which androgen induction of 5α-R may be mediated by IGF-1. IGF-1 is a potent mitogen and its expression has been shown to be increased by androgens (Hobbs et al. 1993). Using rat and human cultured genital skin fibroblasts, an increase in 5α-R activity was observed with $10^{-7}$ M DHT, while T at the same concentration had a negligible effect. However, the growth factor, IGF-1, was able to promote the same increase in 5α-R activity found with DHT at 1/100th the concentration ($6.4 \times 10^{-9}$ M). When the cultured fibroblasts were incubated with DHT and an IGF-1 or an IGF-1 receptor antibody, the effect of DHT on 5α-R activity was reduced. Thus, it appears that IGF-1 can enhance androgen action by increasing 5α-R expression and thus intraprostatic DHT concentrations. The mechanism could be described as feed-forward and supports the hypothesis by George et al. (1991).

g. 5α-Reductase inhibitors

Numerous pharmacological 5α-R inhibitors have been developed that compete for the androgen-binding site on the 5α-R enzyme. Characteristics of a good inhibitor are: 1)
potency, reflected in a long half life and low $K_i$, ensuring that only low levels of the drug are required, 2) selectivity, so that the inhibitor should not bind hormone receptors and that no other enzymes should be inhibited, thus reducing side effects, and 3) activity, such that the inhibitor should not be metabolized to a hormonally active compound.

One of the earliest examples of a pharmacological $5\alpha$-R inhibitor was 4-MA (17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one), seen in Fig. 13.

![4-MA](image)

**Fig. 13 4-MA (17β-N,N-diethylcarbamoyl-4-methyl-4-aza-5α-androstan-3-one) structure**

This compound is a potent inhibitor of $5\alpha$-R but is not strictly selective because it is also weakly binds the AR (Brooks et al. 1981; Liang and Heiss 1981), and is also inhibits aromatase (Stone et al. 1986).
The $5\alpha$-R inhibitor, finasteride, illustrated in Fig. 14, is widely known and has been available to the public under the tradenames, Proscar or Propecia.

![Finasteride molecule]

**Finasteride**

Fig. 14 structure of finasteride (also called MK-906 or Proscar)

Finasteride preferentially inhibits $5\alpha$-R type 2 but can also weakly inhibit $5\alpha$-R type 1. In human fibroblasts, the $K_i$ for finasteride on $5\alpha$-R type 2 is 3–5 nM, but for the type 1 enzyme the $K_i$ is over 300 nM (Russell and Wilson 1994). Using recombinantly expressed isozymes, the $K_i$ for type 2 is approximately ten-fold lower than that for type 1 $5\alpha$-R, but there is some dispute as to what the values are (Iehle et al. 1995; Azzolina et al. 1997). In one study, the $K_i$ was 7.8 nM for type 2 $5\alpha$-R and 108 nM for type 1 $5\alpha$-R (Iehle et al. 1995), which contrasts with the result found by other researchers ($K_i = 1.19$ and 10.2 nM for $5\alpha$-R type 2 and 1, respectively).
Finasteride has been an invaluable tool for study of the role of 5α-R in prostate development, growth and regression. Finasteride was used to elucidate the relative potency of T and DHT in the rat prostate for the results presented in this thesis. However, the background for examining T and DHT effects on the adult ventral prostate was derived from several inhibitors besides finasteride, such as FK143 (Homma et al. 1997), turbosteride (Iehle et al. 1995), CGP 53153 (Hausler et al. 1996), FCE 27837 (di Salle et al. 1995), SK&F 105657 (Lamb et al. 1992), PNU 157706 (Zaccheo et al. 1998) and LY300502 (Sutkowski et al. 1996).

Inhibitors of 5α-R type 1 have been developed, however their efficiency for prostate regression appears to be in doubt. The inhibitor LY191704, is active against cultured stromal cells, but is inactive against freshly isolated prostate cells (Hirsch et al. 1993). It was proposed that long-term culture conditions in the cultured stromal cells may alter the expression for the 5α-R enzymes rendering them vulnerable to type 1 inhibition. The prostate cancer cell line, LNCaP, is derived from a lymph node metastases and is the standard for prostate cancer in vitro studies because it is well differentiated and androgen responsive (Lee et al. 1995). LNCaP cells only express 5α-R type 1, and are more sensitive to selective 5α-R type 1 inhibitors (Sutkowski et al. 1996) but will respond to finasteride (Klus et al. 1996). However, the AR in LNCaP cells has a substitution of A for G at nucleotide 876 of the steroid binding domain, which results in more promiscuous ligand binding (Montgomery et al. 1992). An illustration of this point is the fact that finasteride (Bologna et al. 1995; Klus et al. 1996) can inhibit DHT action directly, acting as an anti-androgen in these cells.
As previously mentioned, 5α-R inhibition in rats will suppress intraprostatic DHT concentration to levels near that of a castrated animal, but will also increase the intraprostatic T concentration up to 20-fold (di Salle et al. 1995; Rittmaster et al. 1995; George 1997). Finasteride administered daily will result in a decrease in prostate weight but not to the same extent as castration (Rittmaster et al. 1991; Shao et al. 1993; Rittmaster et al. 1995). Castration significantly reduces intraprostatic T and DHT levels and is associated with waves of apoptosis. By 21 days after castration, the prostate is reduced in weight by 93%, whereas, normal rats treated with finasteride for 21 days show a decrease in prostate weight of 65% (Rittmaster et al. 1995). Castration decreased prostate DNA content by 88% after 14 days, while finasteride treated animals lost 52% of prostate DNA content compared with age matched controls. If these results are compared with rats from the beginning of treatment and not with age-matched controls, the DNA content drop with finasteride treatment was only 41% while castration reduced content 82%. This interpretation reduces the perceived effectiveness of each treatment, especially finasteride, but it may be more accurate in terms of assessing actual cell loss. Shao et al. (1993), observed that RNA/DNA and protein/DNA ratios fell 30-36% with 28 days of finasteride treatment while these ratios fell 70% after 28 days of castration.

Expression of TRPM-2, which correlates with the level of apoptosis, was only found in the prostates of castrated animals, and not in those from rats treated with finasteride (Rittmaster et al. 1991; Shao et al. 1993). Finasteride had a definitive effect on prostate lumenal atrophy and atrophy of the luminal epithelial cells but again it was not to the same extent as castration (Rittmaster et al. 1995). Finasteride reduced duct mass 47% and epithelial cell mass by 60% by 4 and 9 days of treatment, respectively. By 9 days,
castration caused a 95% reduction in duct mass and 93% reduction in epithelial cell mass. Similar results were obtained by Lamb et al. (1992), using the 5α-R inhibitor SK&F 105657. After 14 days of inhibitor treatment, ventral prostate weight was 35% of control animals from day 0, and castration reduced the prostate weight to 10% of controls. 5α-R inhibition only resulted in a drop of 30% in the prostate DNA content compared to day 0 controls, again demonstrating that cell loss is not the major mechanism of regression of the prostate after 5α-R treatment. Therefore, 5α-R reduces prostate size and weight but not to the same extent as castration, despite the fact that the intraprostatic DHT concentration was reduced to near castrate levels. However, finasteride treatment results in a near 20-fold increase in intraprostatic T levels. Thus, the attenuation of the response to DHT withdrawal must be due to the increase in intraprostatic T. However, because these studies only examine the effects in non-castrated animals, the true nature of the T effects and DHT effects are difficult to assess. The possibility that the low level of DHT may be preventing full inhibition of the prostate cannot be excluded. It would be possible to designate the effects of either T or DHT if thresholds for DHT and T action in the prostate were known.

Despite the fact that 5α-R inhibition does not induce high levels of apoptosis, the prostate significantly decreases in size, especially compared with age matched controls (Rittmaster et al. 1995). 5α-R inhibition may have more of an effect on suppressing cell proliferation than on promoting cell death. In an early study with the 5α-R inhibitor MDL 18,341, Blohm et al. (1986) castrated rats for 7 days before T replacement in the presence and absence of 5α-R inhibitor. The increase in prostate weight was repressed by MDL 18,341, but the levator ani muscle, which does not contain high levels of 5α-R, was
unaffected. Finasteride has also been shown to inhibit prostate regrowth in 7 day castrated rats treated for 3 days with T-P (Shao et al. 1993). In this case, $^3$H-thymidine incorporation into DNA was also measured and finasteride reduced the level of incorporation compared to rats not treated with finasteride. Finasteride reduced the prostate weight increase in castrated rats treated with 100 µg T-P by 53%, whereas, it had no effect in castrated rats given 100 µg DHT-P (Brooks et al. 1991). A limitation of these studies is that just one dose of T was examined. Since it is known that high concentrations of T can mimic the effects of DHT in vitro (Grino et al. 1990; Deslypere et al. 1992; Yeh and Chang 1996), it is essential to carry-out dose-response experiments in comparing the two androgens.

In clinical trials with men with BPH, finasteride decreased intraprostatic DHT concentration by about 90%, while prostatic T concentrations rose severalfold (Norman et al. 1993). Finasteride decreased prostate size by about 20%, increased urinary-flow rate by 23% and improved BPH symptom scores (Gormley et al. 1992).

Morphometrically, epithelial cell and ductal width decreased with increasing duration of treatment, but the stromal compartment showed little change (Montironi et al. 1996; Rittmaster et al. 1996). In another one-year double-placebo comparative trial using finasteride, terazosin (an α-1 adrenergic blocker which relaxes smooth muscle) and a combination of the two, finasteride was shown to have no effect. This study was criticized for its selection of patients without an enlarged prostate, and a later meta-analysis of the data showed a correlation with initial prostate size and the degree of symptom improvement (Boyle et al. 1996). Clearly finasteride is not as effective as
originally predicted (Geller and Sionit 1992) and the most probable reason is the potency of intraprostatic T. However, the extent of that potency was still unknown.

**h. 5α-Reductase inhibitors and prostate cancer**

5α-R inhibitors have been tested in models of prostate cancer, to varying degrees of success. The Dunning rat prostatic carcinoma, R3327H, shares some characteristics of human prostate cancer such as slow growth rate, responsiveness to hormonal therapy and histology similar to that of a well differentiated carcinoma (Isaacs and Coffey 1981).

When Dunning tumour-bearing rats were treated with 5α-R inhibitors, they did not show sensitivity to either finasteride (Brooks et al. 1991) or SK&F 105657 (Lamb et al. 1992). However, the prostates of these animals were decreased compared to controls, indicating that the tumour may not contain 5α-R type 2 or that it responds equally well to T as DHT, perhaps due to a defective AR. Finasteride may just be a poor choice for 5α-R inhibition in this particular tumour. With regard to that issue, rats bearing the Dunning R3327 prostate carcinoma that were treated with a dual 5α-R isozyme inhibitor, PNU 157706, did show inhibition of tumour growth (Zaccheo et al. 1998). After the tumours had reached a palpable, 1-cm in diameter, the rats were treated for 9 weeks with inhibitor.

Tumour growth was inhibited 53% and 82% by 5α-R inhibitor treatment and castration, respectively. The 5α-R type 2 inhibitor turosteride, studied by the same researchers, had no effect at reducing tumour incidence rate when given to animals immediately after implantation while castration reduced tumour incidence rates by 38% (Zaccheo et al. 1998). Nevertheless, dual 5α-R inhibition did result in decreased tumour growth compared to controls.
In F344 rats treated with the drug, 3, 2'-dimethyl-4-aminobiphenyl (DMAB), which will induce prostatic lesions, finasteride reduced the incidence of macroscopic carcinoma by 33% (Tsukamoto et al. 1998). Finasteride treatment, 15 mg/kg twice per week (which is a relatively low dose), was compared with Casodex (an antiandrogen) treatment. Microscopic lesions were only reduced at the highest dose of Casodex and not with finasteride. However, over half the rats treated with the high level of Casodex died due to toxicity of the drug. It would be interesting to evaluate the effect of higher levels of finasteride on microscopic lesions.

Another 5α-R inhibitor, FK143, was evaluated in rats that spontaneously develop prostate cancer (Homma et al. 1997). Two doses of inhibitor were evaluated, both reduced prostatic DHT levels, but the higher dose resulted in elevated serum T levels. The incidence of prostate cancer was slightly lower in the low FK143 group, but there was zero occurrence of macroscopic lesions compared to control groups. The high dose of FK143, which resulted in elevated serum T, showed no difference from controls in terms of prostate cancer incidence and development of macroscopic lesions. Thus, it seems that for rodent models of prostate cancer, administration of 5α-R inhibitors suppresses tumour growth and delays tumour progression but has little effectiveness for reducing tumour incidence (Andriole et al. 1987; Lamb et al. 1992; Umekita et al. 1996). Two caveats to 5α-R inhibitor effects in prostate cancer are that in order for androgen action to be attenuated, the inhibitor must block the isozyme expressed by the cancer, and that serum T must not be significantly elevated above normal values because T may be able to mimic the effects of DHT by sheer mass.
It is hoped that the results of finasteride on tumour xenografts in rodents would be similar in humans but the work is limited (Brawley et al. 1994; Gormley et al. 1995; Brawley and Thompson 1996). However, it has been observed that in men who have undergone radical prostatectomy for prostate cancer, treatment with finasteride resulted in a delay in the rise of PSA for up to 14 months (Andriole et al. 1995). Finasteride in combination with other AR antagonists and GnRH analogues can give maximal androgen ablation but the usefulness of maximal androgen ablation has yet to be proven (Kirby et al. 1999). In some cases the combination of finasteride and flutamide (an AR antagonist) allowed some preservation of libido and potency by preserving serum T levels (Brufsky et al. 1997), but the study was small, and no mechanism for the action of T in the presence of flutamide was given.

4. ANDROGEN METABOLISM AND SERUM ANDROGENS

An issue that arose in the course of the work is the question of why T, and not DHT, is the major circulating androgen. It has been speculated that uptake of DHT by the prostate may not be as efficient as uptake of T (Lasnitzki et al. 1974). Indeed, Pollard et al. demonstrated antiandrogenic effects on the prostates of rats supplemented with DHT (Pollard et al. 1987; Pollard 1998). Non-castrated L-W rats predisposed to developing prostate cancer were supplemented with equal concentrations of either T or DHT for 14 months (Pollard et al. 1987). Prostate adenocarcinomas developed in 24% of the T-supplemented rats but not in the DHT-supplemented rats. In a clinical study of hypogonadal men supplemented with DHT, prostate size was observed to decrease after a mean time of 1.8 years of treatment (de Lignieres 1993). It has been suggested that these
observations are the result of an impaired capacity of exogenous DHT to accumulate in the prostate. However, they may reflect the overall lower levels of circulating androgen because tissues such as muscle metabolize DHT more rapidly than T.

Androgen metabolism is an important aspect of hormone accumulation in target tissues and therefore has a direct bearing on androgen action. As mentioned, the major source of T in rats and humans is the Leydig cells of the testes that produce T in response to LH stimulation (Fig. 1). Adrenal sources are present, but as seen in the rat, are too low to have an effect on the prostate (Kyprianou and Isaacs 1987a; Kyprianou and Isaacs 1987b). T circulates bound to large proteins such as albumin and steroid hormone binding globulin (only found in humans). At any given time, up to 98% of T in circulation is bound with 2% free to diffuse into cells.

Fig. 15 outlines the various metabolic pathways accessible to the androgens. Depending on enzyme and co-factor availability, T can be irreversibly aromatized to estradiol, 5α-reduced to DHT or reversibly converted to the inactive metabolite androstenedione. DHT has the option to be irreversibly glucuronidated by UDP-glucuronosyltransferase, reversibly oxidized to 5α-androstanedione, or reduced to 3α/β-androstanediol. 3α/β-androstanediol can be irreversibly glucuronidated at the 3- or 17-position or reversibly oxidized at the 17-position to 3α/β-androsterone which can also be glucuronidated at the 3α or β positions.

In the prostate, DHT accumulation for androgen action is the typical result. In terms of androgen metabolism, this is achieved through a low rate of reduction of DHT to the
Fig. 15 Metabolic pathways for T.
inactive metabolite, 3α-androstanediol, and a high rate of oxidation of circulating 3α-androstanediol back to DHT (Pasupuleti and Horton 1990). The pathway for DHT metabolism in the prostate is illustrated in Fig. 16. Pasupuleti et al. (1990) determined that the conversion rate in the prostate for 3H-DHT to 3H-3α-androstanediol was 6% after 3 hours, while the rate for the reverse reaction was 31.5%. DHT inactivation through glucuronidation was a low 0.1-0.4%. The pathway for DHT metabolism in skeletal muscle is illustrated in Fig. 17. Muscle, which contains no 5α-R, favors the accumulation of T. In fact, DHT reduction rates are 37% over 3 hours, whereas the oxidation rate of 3α-androstanediol rate is only 2.7%. Glucuronidation rates are as low as they are in prostate. The skin and liver are two other sites that are responsible for androgen metabolism. There is no net gain in DHT or 3α-androstanediol in these tissues, however, they are responsible for the majority of androgen glucuronidation (Pasupuleti and Horton 1990).

The conversion of DHT to 3α-androstanediol effectively reduces androgen action because of the reduced affinity of 3α-androstanediol for androgen binding proteins (Bartsch 1980). However, in order to completely reduce androgen potential, the body must rid itself of the androgen. Glucuronidation is the non-reversible reaction to make androgens soluble in water so that the body can excrete steroids. Rittmaster et al. (1988), determined that the predominant form of 3α-androstanediol-glucuronide is 3α-androstanediol, 17-glucuronide.

The metabolic pathway for T in the prostates of rats treated with finasteride is no longer 5α-reduction to DHT, but rather oxidation to the inactive metabolite
Fig. 16 DHT metabolic pathways in the prostate.

Fig. 17 DHT metabolic pathways in the muscle.
androstenedione (George 1997). The formation of androstenedione is not rapid because T accumulates in the prostate at significantly high levels. However, androstenedione is not retained in the cells with the same affinity as T or DHT, and the overall androgen level in the prostates of rats treated with finasteride is slightly lower than the overall androgen level in prostates of normal rats. The same trend can be observed in the prostates of men treated with finasteride (Norman et al. 1993).

As would be predicted, the predominant pathway for T metabolism in BPH tissue slices and cell suspensions is 5α-reduction to DHT (Smith et al. 1994). The metabolism in prostate cancer cell lines is not as clear as it is in BPH (Smith et al. 1994). In the poorly differentiated and androgen unresponsive DU-145 cells, T was converted to DHT similar to BPH, although the rate of metabolism was over 100-fold slower. In contrast, the similarly poorly differentiated, androgen unresponsive PC-3 cells contained high 17 β-HSD activity forming large amounts of androstenedione, and converting T at a rate twice that in BPH cell suspensions. LNCaP, the differentiated and androgen responsive prostate cancer cell line rapidly converted T exclusively to a glucuronide conjugate at about the same rate as BPH cells form DHT (Smith et al. 1994). Interestingly, LNCaP was the only cell line whose growth and colony-forming ability was stimulated by testosterone and DHT.

Understanding tissue-specific and cell-line specific variations in androgen metabolism is fundamental to understanding the overall regulation of androgen action in normal and pathological physiology.
5. AIMS OF THE PRESENT WORK

When this work was initiated, Dr. Rittmaster’s laboratory was focused on the effect of finasteride in rats and men. The observation that 5α-R inhibition was not as effective as anticipated based on early AR affinity and gene activation work led to the study of the relative potency of T and DHT in vivo.

In the first study, the relative potency of testosterone and DHT on prostate regression was examined. Rats were castrated and then immediately implanted with T pellets that formed a dose response from castrate to physiologic replacement. This was done in the absence and presence of finasteride to create intraprostatic dose responses for T and DHT. The rats were sacrificed 4 days later. Atrophy was assessed using morphometrics to determine the size of prostate ducts as a measure of epithelial cell activity. Apoptosis was evaluated using two markers of programmed cell death: intraprostatic expression of the gene for TRPM-2 and staining for DNA fragmentation by in situ end-labeling of damaged DNA strands, an early event in the apoptotic process. The results were plotted as a function of intraprostatic T or DHT. As can be seen from the complex reactions, pathways and mechanisms whereby we get androgen induced alteration in function of the prostate, it is important which markers of prostate growth and regression are examined. For this reason, markers of prostate regression that reflect overall changes in the prostate with castration and immediate replacement with a dose range of T in the presence and absence of the 5α-R inhibitor, finasteride, were chosen. The TRPM-2 and prostate specific binding protein (PSBP) genes were chosen to reflect the level of apoptosis and cell secretory function in the prostate, respectively. AR immunostaining was examined to determine the level of AR induced by the various concentrations of intraprostatic T and DHT.
The second study examines the relative potency of T and DHT on prostate regrowth after castration, in order to elucidate the role of 5α-R in stimulation of prostate regrowth. Rats were first castrated and their prostates allowed to regress for 2 weeks. The animals were then implanted with varying sizes of T pellets, in the presence or absence of finasteride. In the absence of finasteride a dose-response for the potency of DHT on prostate regrowth was generated, while in the presence of finasteride, DHT is suppressed to near castrate levels at all concentrations of T used and a dose-response for T resulted. Of particular importance was the ability of intraprostatic T or DHT to stimulate proliferation in the prostate. In the assessment of the relative potency of these two intraprostatic androgens, we compared not only the levels of T and DHT, but also the relationship between serum and prostatic androgen levels. Hypertrophy was measured by duct mass and hyperplasia was assessed through measurement of the mass of DNA per prostate. Overall changes in the prostate were evaluated by measuring prostate weight.

The ability of exogenous DHT to accumulate in the prostate was compared with the ability of DHT to accumulate in the prostate from conversion of serum T. Rats were castrated and left for 2 weeks to allow their prostates to fully regress. They were then implanted with DHT or T pellets that covered a dose range from castration androgen levels to supraphysiologic. The rats were sacrificed 7 days later and prostate weight, intraprostatic and serum androgen levels were measured.

The work presented in this thesis has resulted in two publications and five abstract presented at various conferences:
Publications:

"Regrowth of the involuted prostate in the castrated rat: role of 5α-reductase"  
Wright, AS., Douglas, RC., Thomas, LN., Lazier, CB., and Rittmaster, RS.,  

"Relative potency of testosterone and dihydrotestosterone in preventing atrophy  
and apoptosis in the prostate of the castrated rat." Wright, AS., Thomas, LN.,  

Abstracts:

Canadian Fertility and Andrology Society, 44th Annual Meeting. Mont  
Tremblant, Quebec, Sept. 9-12, 1998 "Why isn't dihydrotestosterone the major  
circulating androgen in rats ... Or men? The role of 5α-reductase." A.S. Wright,  
R.C. Douglas, L.N. Thomas, C.B. Lazier, R.S. Rittmaster. Deps. of Physiology  
and Biophysics, Biochemistry and Medicine, Dalhousie University, Halifax, N.S.

ENDO '98, The Endocrine Society 80th Annual Meeting, New Orleans, June 24-  
7, 1998 "Relative potency of testosterone and DHT on regrowth of the involuted  
rat prostate." A.S. Wright, R.C. Douglas, L.N. Thomas, C.B. Lazier, R.S.  
Rittmaster, Deps. Of Physiology and Biophysics, Biochemistry, and Medicine  
Dalhousie University, Department of Medicine, Research Day. Halifax, N.S. May  
7, 1998 "The role of the 5α-reductase enzyme in stimulating prostate growth."  
Division of Endocrinology, Departments of Physiology and Biophysics, and  
Biochemistry

Canadian Fertility and Andrology Society, 42nd Annual Meeting. Lake Louise,  
Alta, Nov. 20-23, 1996 "Relative potency of testosterone and dihydrotestosterone  
in preventing atrophy and apoptosis in the prostate of the castrated rat." A.S.  
Wright, R.C. Douglas, L.N. Thomas, C.B. Lazier and R.S. Rittmaster. Division of  
Endocrinology, Departments of Physiology and Biophysics, and Biochemistry

Oral Presentation - at ICE '96 (10th International Congress of Endocrinology) San  
Francisco, CA June. 11-15, 1996 "Relative potency of testosterone and  
dihydrotestosterone in preventing atrophy and apoptosis in the prostate of the  
castrated rat." A.S. Wright, R.C. Douglas, L.N. Thomas, C.B. Lazier and R.S.  
Rittmaster. Division of Endocrinology, Departments of Physiology and  
Biophysics and Biochemistry
METHODS

ANIMAL PROTOCOLS

Validation of the animal model

For this experiment and all others that followed the animals used were male Sprague
Dawley rats (55 days old, post-pubertal) purchased from Charles River Laboratories
(Montreal, PQ), housed in the Carelton Animal Care Facility, and fed water and rat chow
ad libidum.

The implants of T were validated for constant delivery in rats (n=30) that were castrated
and immediately implanted with 5 mg T pellets (Innovative Research of America,
Sarasota, FL). The rats were killed via CO₂ asphyxiation on after 0, 2, 4, 8, 9, 10, and 11
days and blood and ventral prostates taken. Blood was collected via cardiac puncture and
the prostates were weighed prior to be storage in liquid nitrogen. The blood was allowed
to clot at room temperature for 2 h and spun at 1500 x g for 20 min. The serum was
drawn off using a pipette, transferred to 5 ml glass vials and stored at -70°C until needed
for extraction.

Prevention of rat ventral prostate regression after castration using T pellet implants
with and without finasteride treatment ("The Regression Study")

Rats were castrated and immediately implanted with 21-day release T pellets from a
range of sizes. The first group of rats were not treated with finasteride and was divided
into six subgroups: intact, castrated, and castrated plus 0.10, 0.30, 0.50, or 0.70 mg T
pellets. The number of rats per subgroup was 19, 14, 35, 18, 16, and 17, respectively.
Rats in the second group were treated with the 5α-R inhibitor finasteride (Proscar; Merck, Sharp & Dohme Research Laboratories, Rahway, NJ) to inhibit intraprostatic conversion of T to DHT. The rats were divided into eight subgroups: intact, castrated, intact plus finasteride, and castrated and 0.10, 0.25, 2.5, 5.0, or 15.0 mg T implants plus finasteride. The number of rats per subgroup was 14, 16, 15, 29, 15, 15, 14, and 15, respectively. Finasteride-treated rats were given 40 mg/kg daily subcutaneous (sc.) injections in 1 ml sesame oil, 10% ethanol vehicle (Rittmaster et al. 1991). Normal (non-castrated) and castrated rats were used as controls for these experiments. Castration was performed via the scrotal incision under ketamine/xylazine anesthesia. Ligatures were tied above the testes using 3-0 softgut, the testes removed and the incision closed with 1 or 2 stitches of 3-0 silk. At the time of castration, a T pellet was implanted into a pocket made under the skin in the midscapular region. The incision was closed using 3-0 silk and 1 or 2 stitches. The rats were killed after 4 days of treatment. The ventral prostates were immediately removed, weighed, and prepared for histological examination or frozen in liquid nitrogen for determination of androgen concentration and mRNA levels. Blood was collected via cardiac puncture, clotted at room temperature and the serum was drawn off and stored at −70°C until assayed for androgen content.

**Regrowth of the castrated rat ventral prostate using T implants in the presence or absence of finasteride ("The Regrowth Study")**

In this study, 21-day release T or DHT pellets were implanted in rats which had been castrated 2 weeks previously. A preliminary experiment was carried out to compare 7 and 14 days of regrowth in rats castrated 2 weeks previously and given T pellet implants. In rats not treated or treated with finasteride, the T pellet sizes used were the same as below
but there were only 3 rats per subgroup for 7 and 14 days. In the main study, the first
group of 2-week castrated rats did not receive finasteride treatment and had implants of T
pellets in the following sizes: 0.1, 0.3, 0.5, 1.0, 5.0, and 15.0 mg. The numbers of rats per
subgroup were 11, 11, 19, 7, 6, and 6, respectively. In the second group of rats, the
selective 5α-R inhibitor finasteride was used to inhibit intraprostatic conversion of T to
DHT. These 2-week castrated rats were implanted with T pellets of the following doses:
0.1, 0.3, 0.5, 1.0, 2.5, 5.0, and 15.0 mg, and were given 40 mg/kg/day sc. injections of
finasteride in 1 ml sesame oil with 10% ethanol vehicle. The numbers of rats per
subgroup for the rats treated with finasteride were 11, 11, 11, 11, 9, 8, and 6, respectively.
In a separate experiment, DHT pellets were implanted in 2-week castrated rats. There
were 5 rats per group and the sizes of the DHT pellets used were 0.1, 0.3, 0.5, 1.0, 5.0,
15, 30, and 60 mg.

Rats were killed 7 days after implantation while the prostates were actively growing. All
pellets were implanted sc. into the subscapular region while the rats were under
ketamine/xylazine anesthesia. Rats were killed via CO₂ asphyxiation, exsanguinated, and
the ventral prostates were removed, weighed and prepared for histological examination or
immediately frozen in liquid nitrogen.

HISTOLOGICAL EXPERIMENTS

Formalin Fixing and Paraffin Embedding

Excised prostates were immediately weighed and fixed in 10% PBS-buffered formalin
for paraffin embedding. The histology technician Shirley Dean handled the paraffin
embedding, block cutting and hematoxylin and eosin staining. However, the following is
a synopsis of the procedure. Fixed tissues were embedded using an autotechnicon
(Autotechnicon Co. Chauncey, NY). The process of embedding the prostates follows 16
h of agitation in solutions of 70% ethanol, 95% ethanol and 100% ethanol, 50%
ethanol/50% xylene, 100% xylene and paraffin. The prostates were placed in molds,
covered with paraffin, and allowed to set. Prior to cutting the tissue sections using a
microtome (Jung, Heidelberg, Germany), the paraffin embedded tissue blocks were
further hardened by placing them at −20°C for 30 min. Sections were cut (5 μm thick) and
transferred to a 45°C water bath. In the water bath, individual sections were placed onto
silinated microscope slides and dried overnight at 37°C to fix the tissue to the slide.

**Hematoxylin and Eosin (H&E) Staining**

The slides were deparaffinized by incubating in 4 successive baths of xylene for 2 min
each. Incubating for 30 sec each in 100% ethanol, 100% ethanol, 95% ethanol, 70%
ethanol and tap water rehydrated the slides. The slides were covered for 1-3 min with
Harris’ Hematoxylin (0.9% hematoxylin, 12% ammonium aluminum sulfate, 0.3%
mercuric oxide, and 0.05% sodium iodate 4% glacial acetic acid), immersed 4 times in
0.2% nitric acid, rinsed in tap water and incubated in Scott’s tap water (10.53% MgSO₄,
1.84% NaHCO₃) until blue (approximately 5 min). They were then rinsed in tap water
again and stained with eosin Y (2% eosin Y in 100-200 ml water acidified with 5-20
drops of glacial acetic acid) for 10-40 sec. To remove excess eosin the slides were rinsed
in tap water and dehydrated by incubating in 70%, 95% and 100% ethanol baths for 30
sec each. Finally, slides were incubated in 4 successive xylene baths and #1 thickness
coverslips were mounted with DPX or Permount.
**Lumen / epithelial cell mass determination**

The procedures for determining lumen and epithelial cell mass were essentially the same, differing only in the structure isolated, therefore only the lumen mass determination will be described. A Nikon microscope (Melville, NY), JVC RGB video camera (Model No. TK-1070U, Elmwood Park, NJ) and a Truevision NuVista+ frame grabber card (Indianapolis, IN) were used to capture microscopic images (100x magnification) from H&E stained slides to a Macintosh computer. For determination of lumen mass on H&E stained slides, 10 random captures of each slide were made. Fig. 18 shows the steps involved in analyzing digitized images of the ventral prostate for lumen mass. Using Adobe Photoshop 3.0 (Adobe Systems Inc., Mountain View, CA) the lumen area was isolated from other morphometric structures and re-coloured to red. This does not alter the image but simply allows the image analysis program to visualize that structure more easily. The lumen area was quantitated by using the public domain program, NIH Image 1.61 (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Briefly, the image was converted to grayscale and using the “Measurement Macros” add-in, a threshold was chosen that selects only the lumen area and nothing else in the image; the area selected is highlighted in red. The program was then asked to compute the percent area selected. The result was reported as a percentage of the entire field. All of the images are the same total area to make comparisons accurate. To determine lumen mass, the mean percentage of the lumen area for each tissue was multiplied by the corresponding prostate weight. (Note: The lumen area determination for the regression study was accomplished using an older set of equipment before an image analysis system was acquired by Dr. Rittmaster’s laboratory.)
Fig. 18 Lumen mass determination:

a) The slide below is one of ten random captures from an intact normal control prostate (100x mag.). The lumen area is the area of the duct minus the epithelial cell area.

b) The lumen area is isolated by selecting the area using the application Photoshop®, and recoloring it for image analysis.
c) The image is converted to grayscale (256 grays) by the program NIH Image 1.61

d) A threshold that highlights the lumen was employed to determine the percent area of the slide that was comprised by lumen.
The two systems were validated and the results were identical. In the regression study, lumen area was determined using a Zeiss Videoplan morphometric computer and a Zeiss microscope fitted with a JVC video camera.)

**TUNEL Staining**

Apoptotic cells were quantified by staining for double stranded DNA fragmentation using a modification of the TUNEL technique (Rittmaster et al. 1995). Slides were deparaffinized and rehydrated as described previously. The slides were washed 3 times, transferred to a humidity chamber and covered with 0.5% pepsin (in HCl, pH 2.0) for 45 min at 37°C. After washing 4 times with distilled water, the slides were incubated for 5 min in 2% H₂O₂ at room temperature to quench endogenous peroxidase activity. The slides were washed again 4 times with distilled water and equilibrated in TdT buffer (30 mM Tris pH 7.2, 140mM sodium cacodylate, 1mM cobalt chloride) for 10 min. The buffer was drained and the tissues covered with TdT (0.4 μg/μl) and biotin-14-dATP (12.5 pmol/μl) in TdT buffer and incubated for 60 min at 37°C in the humidity chamber. The negative control was a slide that was known to be positive for TUNEL staining, and was incubated with biotin-14-dATP, but no TdT. The reaction was terminated by transferring the slides to TB buffer (30 mM sodium citrate, 0.3 M sodium chloride) in coplin jars. The slides were washed 4 times with distilled water and the slides returned to the humidity chamber where the tissue sections were covered with 2% bovine serum albumin (BSA) for 10 min to inhibit non-specific binding of streptavidin. The slides were washed 3 times in PBS and the tissues were covered with streptavidin-horseradish peroxidase (1:1000) for 60 min at room temperature. The slides were washed 3 times in PBS and 2 times in distilled water prior to incubation in AEC chromagen
buffer (0.2 mg/ml: v 3-amino-9-ethylcarbazole (AEC), 0.1M sodium acetate, 0.02M acetic acid, plus 200 µl H₂O₂ just before use) for 15 - 30 min at 37°C. The slides were rinsed in distilled water and counterstained with Mayer’s hematoxylin for 30 sec. and Scott’s tap water for 2 min. Coverslips were mounted with glycerol gelatin (Sigma Diagnostics, St. Louis, MO). The degree of staining was quantitated by counting of the number of positive epithelial cells in a minimum of 1200 epithelial cell per slide and in 3 to 5 prostates per subgroup. Results were expressed as the percent of epithelial cells staining positively for DNA fragmentation.

**Androgen Receptor Staining**

The slides were deparaffinized and rehydrated as described above. The slides were transferred to a Coplin jar and rinsed 3 times in PBS (0.9%). Because a nuclear protein was being examined, the tissues were subjected to a heat antigen retrieval procedure to expose the antigen. Slides were autoclaved at 15 atm for 2 min in 0.01 M sodium citrate (pH 6.0) using a pressure cooker. The slides were cooled under tap water and then transferred to a coplin jar and rinsed 3 times in PBS. Endogenous peroxidase activity was quenched by incubating in 2% H₂O₂ for 5 min. The slides were rinsed 3 times in PBS and placed in a humidity chamber. A kit containing blocking reagent (5% normal serum in 0.01M PBS containing 0.1% sodium azide), linking reagent (biotinylated anti-immunoglobulin serum in 0.01M PBS containing 0.1% sodium azide, carrier protein, and human serum), and labeling reagent (peroxidase labeled ultra streptavidin in 0.01M PBS containing 1% carrier protein and 0.04% preservative and stabilizer) was used for the staining procedure (Signet Laboratories, Dedham, MA). The procedure was carried out at room temperature unless otherwise stated. The slides were incubated for 20 min with 1-3
drops of the blocking agent. Excess blocking agent was tapped off and the area surrounding the tissue was wiped carefully. Polyclonal AR antibody (Novocastra, New Castle, England) was pipetted onto the slides (50 μl, 1:80 dilution) and the slides were incubated in the humidity chamber for 20 min. The control slide was a ventral prostate slide from a normal animal that did not receive the polyclonal antibody but was otherwise treated the same as the others. The slides were rinsed 3 timed in PBS, 1-3 drops of the secondary antibody was added and the slides were returned to the humidity chamber for 20 min. The slides were rinsed 3 times in PBS, 1-3 drops of the streptavidin-horseradish peroxidase was added and the slides were again returned to the humidity chamber again for 20 min. The slides were rinsed a final time in PBS followed by 2 washes in distilled water then incubated for 20 min at 37°C in AEC chromagen buffer. The slides were rinsed in tap water and coverslips were mounted with glycerol gelatin.

The AR staining was found predominantly in the nucleus of epithelial cells. AR results were expressed as a percentage of epithelial cell area. Individual staining cells cannot be counted because the receptor was found in all epithelial cells. The greater the amount of receptor, the larger the area of staining. Images (100x magnification) from the slides stained for AR were captured as described earlier for lumen mass. Fig. 19 shows the steps involved in quantitating the AR staining and the epithelial cell area. Essentially, the epithelial cells were isolated from the image to eliminate non-specific staining that may interfere with the analysis using the program Adobe Photoshop 3.0. The corrected image was opened in NIH Image 1.61, which converts it to a 256 shade grayscale. A threshold was chosen (100) that encompassed the staining in the grayscale image in a similar pattern to that seen in the colour image. This threshold was used for all calculations
Figure 19: AR nuclear staining in epithelial cells. Steps involved in image analysis:

a) Image capture (200x magnification) of intact normal control

b) Stroma and lumen areas are cut from the image to eliminate non-specific interference
c) The image is converted to grayscale (256 grays) by the program NIH Image 1.61

d) A threshold of 100 grays is chosen to represent the AR staining seen in the prostate epithelial cells. Thereafter, this threshold was used, allowing all slides to be compared as a percentage of normal.
e) The AR staining was expressed as a function of epithelial cell area, which was determined by measuring at full threshold.
reported in the Results. The images were also analyzed using thresholds of 85, 125 and 150 to bracket the selection and ensure that there were no anomalies at differing thresholds. In essence, the bracketing demonstrated that the trends were the same for all the images at a given threshold. If the data are expressed as a percentage of staining in an intact rat, the results were the same regardless of threshold level. The staining was expressed as a percentage of epithelial cell area. Total epithelial cell area was determined by analyzing the digitized image at full threshold to measure the isolated area. The results for each group represent 5 random captures per slide and 3 slides per group.

RNA EXPRESSION

Plasmid Preparations

Bacteria (E. Coli DH5αF') previously transformed with plasmid DNA containing the PSBP, TRPM-2 or G3PDH gene insert (Rittmaster et al. 1991) were suspended in 1 ml of sterile Luria-Bertani (LB) medium (1% bactotryptone (w/v), 0.5% yeast extract (w/v), 1% NaCl (w/v), final pH 7) supplemented with ampicillin (50 μg/ml). The cells were grown overnight in 25 ml of LB media supplemented with ampicillin at 37°C with constant shaking. A 5 ml aliquot of the cell suspension was removed, sterile glycerol added to a final concentration of 30%, and frozen at -70°C for storage. The remaining 20 ml of cell suspension was added to 1 L of sterile M9 medium (22 mM sodium phosphate, 22 mM potassium phosphate, 8.5 mM sodium chloride, 18.7 mM ammonium chloride, 11.1 mM glucose, 10 g/L casein-hydrolyzed amino acids, 1 mM magnesium sulfate, 0.1 mM calcium chloride, 50 g/ml ampicillin, pH 7.4) in a 4 L Erlenmeyer flask and incubated, with vigorous shaking at 37°C. When the optical density of the solution
reached 0.8 (at 600 nm; an indication that the culture was still in log phase growth; approximately 5 h at 37°C), 5 ml of chloramphenicol (34 mg/ml in ethanol) was added and the incubation continued, with shaking, overnight. The cells were then pelleted by centrifugation at 4000 x g for 5 min at 4°C and the supernatant discarded. The bacteria were re-suspended in 10 ml of TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5) and transferred into two polycarbonate ultracentrifuge tubes. The cells were re-pelleted by centrifugation at 4000 x g for 5 min at 4°C and the supernatant discarded. Each pellet was re-suspended in 4 ml of cold sucrose (10% in 50 mM Tris, pH 8.0) and 0.5 ml of lysozyme (30 mg/ml) was then added to each tube to digest the bacterial cell walls. The solution was incubated on ice for 10 min and then 1 ml of EDTA (0.5 M, pH 8.0) was added to each tube, mixed, and incubated a further 5 min on ice. Triton X-100 (2.5 ml of 1% in water) was added to each tube, mixed and placed in a 37°C water bath for 2 - 5 min. The mixture was centrifuged at 192,000 x g for 30 min at 4°C, the supernatant collected and the total volume brought to 20 ml with sterile distilled water. 19.0 g of cesium chloride was added, dissolved by gentle mixing, and 400 µl of ethidium bromide (10 mg/ml) added. The solution was transferred to two heat sealable (crimp-top) centrifuge tubes, topped up with liquid paraffin, sealed and centrifuged at 225,000 x g overnight. The centrifuge was stopped without a brake. The presence of two distinct DNA bands was checked using a UV light and the lower one removed by syringe and needle inserted through the side of the tube. To each milliliter of solution removed, 3 ml of TE buffer was added. N-Butanol (4 ml) was added, gently mixed and centrifuged at 1500 x g for 5 min to separate the phases. The top (n-Butanol) phase was removed and discarded and the aqueous phase re-washed with 4 ml of n-Butanol until all of the orange colour was removed. The aqueous phase was transferred to a 50 ml polypropylene
centrifuge tube and 8 ml of cold ethanol was added for each ml of solution removed from the seal-top centrifuge tube. This was placed on ice for 20 min and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the pellet was re-suspended in 400 μl of TE buffer and transferred to a 1.5 ml centrifuge tube. Tris-saturated phenol (0.5 ml) and 0.5 ml of chloroform were added, mixed and centrifuged for 2 min. The aqueous phase was transferred to another 1.5 ml centrifuge tube and 50 μl of 3.0 M sodium acetate was added and mixed, 1 ml of ethanol was added and mixed and the tube placed on ice for 10 min. The tube was centrifuged for 5 min and the supernatant discarded. The pellet was washed twice in 80% ice cold ethanol followed by centrifugation for 5 min. The pellet was air-dried and re-suspended in 250 – 500 μl of TE buffer. The concentration of the plasmid was determined by spectrophotometry and the remaining sample was stored at -20°C until use.

Isolation of cDNA probes from plasmids

The TRPM-2, PSBP and G3PDH cDNA inserts were isolated from plasmid DNA using specific restriction endonucleases. Double digests were required for TRPM-2 and G3PDH. Approximately 6 μg of plasmid DNA was brought up to a total volume of 16 μl with distilled water and 2 μl each of restriction buffer, and both restriction enzymes were added and incubated for 2 h at 37°C. For the G3PDH insert, restriction enzyme buffer #2 (reACT #2; Gibco BRL reaction mix) was used with XbaI and PstI to isolate a 750 bp fragment. For the TRPM-2 insert, restriction enzyme buffer #3, BamHI, and EcoRI were used to isolate a 1.4 kb fragment. For the PSBP insert restriction enzyme buffer #2 and PstI were used to isolate a 0.5 kb fragment. The reactions were run on a low melting point 0.8% agarose gel using TAE as a running buffer. Using a scalpel, the appropriate
bands were cut from the gel and placed in eppendorf tubes. The purification of the fragment was accomplished using a GeneClean kit (BioCan Scientific, Mississauga, ON) according to the manufacturer’s instructions. To each tube, 1 ml of NaI solution was added and the tubes heated at 55°C until the agar melted (about 5 min). Glassmilk (10 µl) was added to the tubes and incubated at room temperature for 10 min. The tubes were spun in a picofuge (Stratagene, La Jolla, CA) to pellet the glassmilk. The supernatant was removed and the pellet washed 3 times with 200 µl of New Wash (GeneClean kit). After the third wash was discarded, the pellet was dried at 55°C for 5 min. Incubating at 55°C for 5 min in 10 µl TE eluted the DNA. The tubes were centrifuged for 4 min at 15,000 rpm in a microfuge and the supernatant removed. For the G3PDH insert, TE extraction was repeated. The concentration of the isolated insert was estimated using 1 and 5 µl of the sample, 1 µg of 1 kb ladder standard and another 0.8% agarose gel and. The band intensities were compared to estimate the concentration of the insert.

**Probe labeling and purification**

The cDNA probes were radioactively labeled using a random priming kit (Pharmacia Biotech, Montreal, PQ). The insert DNA (50 ng) was denatured for 2 min at 100°C and then quick-cooled on ice. Reaction mix (10 µl) consisting of dATP, dTTP, and dGTP was added along with 1 µl of Klenow fragment polymerase and 50 µCi of α[32P] dCTP and the mixture was diluted with distilled water to 50 µl. The mixture was incubated at 37°C for 30 min. The reaction was stopped by cooling on ice and adding 5 µl EDTA (0.5 M, pH 7.5).
The labeled probe was assessed for the percent radioactivity incorporation, radioactive counts (cpm) per µl and percent purity after being purified over a Sephadex G50 (Pharmacia Biotech, Montreal, PQ) spin-column. An aliquot of the labeled mixture (2 µl) was diluted 1:50 with distilled water. To determine the total radioactivity, 5 µl of the diluted probe was spotted onto a Whatman GF/C glass microfibre filter disc (Whatman International, Maidstone, England) and air-dried. The filter disc was placed in a scintillation vial and scintillation cocktail was added then the tube counted in a Beta-counter. The DNA was isolated from the mixture by pipetting 5 µl of the diluted mixture into a tube containing 25 µl of carrier DNA. One ml of 10% TCA was added and tube was placed on ice for 5-10 min. The precipitated DNA was transferred to filter paper using a vacuum apparatus and washed twice with 5 ml of 10% TCA. The filter papers were transferred to scintillation vials, scintillation cocktail was added and the tubes counted in a beta-counter. The percent incorporation was calculated by dividing the cpm for the precipitated DNA by the total counts (usually 60-70% incorporation). To purify the probe, the remaining labeled mixture (approximately 18 µl) was added to a Sephadex G50 column along with 180 µl of water and spun in a centrifuge for 5 min. The spin-column was made from a 1-ml syringe plugged with glass wool and filled with Sephadex G50. The column was centrifuged at 2500 x g for 10 min, topped up with more Sephadex G50 and centrifuged again as above. From the purified probe, 2 µl was spotted onto filter paper for cpm per µl determination. To ascertain the percent purity of the probe mixture, 2 µl was added to 98 µl of distilled water. From this tube, 5 µl was spotted onto filter paper and 5 µl was added to 25 µl of carrier DNA and 1 ml of 10% TCA. The last tube was placed on ice for 5-10 min. After the precipitated DNA was transferred to filter paper using a vacuum apparatus, the filter papers were transferred to scintillation vials.
scintillation cocktail was added and the tubes counted in a beta-counter. The percent purity was calculated by dividing the total counts from the diluted mixture by the counts from the precipitated DNA (results were typically in the 75-85% range).

**RNA Isolation**

In order to reduce the possibility of contamination from Rnases, gloves were worn at all times and all glassware, mortars, pestles, and spatulas were baked at 200°C for at least 2 h. Millipore de-ionized water was used for all reagents and where necessary, water was treated with diethyl-pyrocarbonate (DEPC, 1:1000 dilution) overnight to chemically cleave any protein matter left in the water. DEPC was removed by autoclaving at 15 lb. per sq. inch for 20 min at 120°C.

RNA isolation for preliminary experiments in the Regression Study was accomplished using a modification of the acid guanidinium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi 1987). RNA was isolated from 200 - 400 mg of frozen prostate. Prostates were pulverized under liquid nitrogen with mortar and pestle and suspended in 1 ml/100 mg tissue of solution D (4M G1TC, 25 mM sodium citrate (pH 7.0), 0.5% n-lauryl sarcosyl and 0.1 M 2-mercaptoethanol). Samples were homogenized with a Polytron homogenizer (Brinkman, Rexdale ON) at 8000 rpm for 30 sec. Sodium acetate (0.1 ml/100mg tissue, 0.2 M, pH 4.0) and 1 ml/100 mg tissue of DEPC water-saturated phenol (pH 6.5) were added and the sample was vortexed. Chloroform:isoamyl alcohol, 49:1 (0.2 ml/100 mg tissue) was added and the mixture was vortexed and left on ice for 2 h. The samples were centrifuged at 1700 x g for 10 min and the aqueous phase was removed and transferred to a new tube, after the volume was
measured. An equal volume of ice cold isopropanol was added to the aqueous phase and the sample gently mixed. The mixture was stored at -20°C overnight. The sample was then centrifuged at 11200 x g for 30 min at 4°C to pellet the RNA and the supernatant removed and discarded. The pellet was air dried briefly and washed twice with 70% ethanol. The pellet was re-suspended in 0.75 ml of solution D, vortexed, 0.75 ml of isopropanol added, and incubated overnight at -20°C. The RNA was pelleted by centrifugation at 3000 x g for 30 min. The supernatant was discarded and the pellet washed twice with 70% ethanol and air-dried. The RNA was re-dissolved in 500 µl of TE (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, pH 8.0) at 4°C overnight. The solution was extracted with 500 µl phenol (pH 6.5) and 100 µl chloroform/iso-amyl alcohol. Again, the samples were centrifuged, the aqueous phase removed, 50 µl 2M sodium acetate (pH 4.5) and 1000 µl 95% ethanol added and the sample stored at -20 °C overnight. Samples were pelleted by centrifugation at 3000 x g for 30 min, washed twice with 70% ethanol and air-dried. The RNA pellet was dissolved in 50-100 µl of TE and the concentration determined by spectrophotometry (Milton Roy Spectronic 1001 plus UV/VIS Spectrophotometer, Rochester, New York). Briefly, an aliquot of nucleic acid solution was diluted 1:50 with distilled water and the absorbance was measured at 260 nm and 280 nm. The nucleic acid concentration was calculated by the equation:

\[ \text{Abs (260nm) x (1 cm/cuvette path length cm) x dilution factor x } 0.04 \text{ g/l} \]

\[ = \text{g/l nucleic acid} \]

The ratio of absorbance at 260 nm to that at 280 nm was also calculated and the sample either discarded or re-extracted if this ratio was less than 1.6. For prostate tissues in the
preliminary experiments, the extra purification steps were necessary to remove enough protein to get a relatively pure sample (OD 260:280 ratio above 1.6). Because the excess of steps was time consuming and the yield was low, an alternate method of RNA isolation (described below) was employed for the prostates in the main part of the Regression Study.

RNA was isolated from prostatic tissue using a modification of a cesium chloride ultracentrifuge method (Davis et al. 1986). Prostates were pulverized under liquid nitrogen with mortar and pestle and suspended in 1 ml/100 mg tissue solution D (4M GITC, 25 mM sodium citrate (pH 7.0), 0.5% n-lauryl sarcosyl and 0.1 M 2-mercaptoethanol). Samples were homogenized with a Polytron homogenizer (Brinkman, Rexdale ON) at 8000 rpm for 30 sec. Samples were made up to 9 ml with Solution D and layered over 4 ml of 5.7M CsCl with 0.1M EDTA in Beckman ultracentrifuge tubes (Fullerton, CA) and spun overnight at 35000 rpm in a Ti-70 rotor. The solution D was siphoned off by syringe and 20 gauge needle by piercing the sides of the tube. The tube was cut and the remaining CsCl was poured off, and the pellet was air dried. The RNA pellet was then reconstituted in TE buffer and transferred to eppendorf tubes. 500 µl of phenol: chloroform (4:1) was added and the tubes vortexed and centrifuged at 10,000 rpm for 4 min. The aqueous layer was removed, 500 ml chloroform was added and the tubes were vortexed and centrifuged for 4 min. The aqueous layer was transferred to a new tube and the RNA precipitated overnight at -20°C with 50 ml 2M sodium acetate (pH 4.5) and 1000 ml 95% ethanol. Samples were pelleted by centrifugation at 4°C for 15 min, then washed twice with 70% ethanol and air dried. The RNA was suspended in 200-400 µl TE
buffer because of the increased yield rather than 50-150 \( \mu \)l, as used in the previous method. The samples were stored at \(-70^\circ C \) if not used immediately.

**Northern Blots**

Northern blots were used to confirm that the probes used in the slot blots identified single bands. Total RNA was separated by electrophoresis through a 5-6 mm thick denaturing agarose gel (1% agarose, 5.4% formaldehyde, 10% 10 x MOPS). RNA samples (10 \( \mu \)g, in <6 \( \mu \)l TE) were diluted with 18 \( \mu \)l of loading buffer (10 \( \mu \)l formamide, 3.5 \( \mu \)l 30% formaldehyde, 2 \( \mu \)l of 10 x MOPS buffer, 2 \( \mu \)l 10 x Loading dye (5% glycerol, 0.1M EDTA, pH 8.0, 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol) and 0.25 \( \mu \)l 10 mg/ml ethidium bromide), heated for 5 min at 65\( ^\circ \)C then cooled on ice until loaded onto the gel. Electrophoresis was carried out at 80 volts in 1 x MOPS (40 mM MOPS, 100 mM sodium acetate, 20 mM EDTA, pH 8.0, balanced to a final pH of 7.0) for 30 min and then at 5 volts overnight (14-18 h). The gel was carefully removed and photographed using Polaroid 52 film (Kodak, Mississauga, ON) on a UV light box. The gel was rinsed (with shaking) in alkaline hydrolysis buffer (50 mM NaOH, 10 mM NaCl) for 45 min, neutralizing buffer (0.1M Tris-HCl, pH 7.5) for 45 min and 10x SSC (3M NaCl, 0.3M trisodium citrate, pH 7.0) for 1 h. The gel was placed on a wick apparatus, well-side down and the RNA transferred to GeneScreen (Dupont, Mississauga, ON) using 25 mM Na\(_2\)HPO\(_4\) (pH 6.5) buffer. The membrane was patted dry with filter paper and crosslinked in a UV Stratalinker (Stratagene, La Jolla, CA).
Slot Blots

RNA samples were serially diluted eight times from 5 μg to 0.04 μg and made up to 20 μl with distilled H₂O. Loading buffer was added, and the samples were heated for 15 min at 65°C. The samples were placed on ice and 15 μl of TE buffer was added. GeneScreen Plus was equilibrated in HPLC-grade H₂O along with precut Whatman 3M filter paper. The slot blot apparatus was soaked in 10% SDS and then successive washings with HPLC-grade H₂O. GeneScreen Plus and filter paper were assembled with the slot blot apparatus and the wells run through with 100 μl of TE buffer. The samples were loaded into their respective wells and allowed to incubate for 30 min. Gentle suction was applied and then the wells washed again with 100 μl of TE. The membrane was UV crosslinked using a UV Stratalinker 2400.

Hybridization

Labeled cDNA probes were made for TRPM-2, PSBP and G3PDH mRNA as described earlier in this section. The membranes from either Northern or slot blots were prehybridized in 20 ml hybridizing mixture (50% deionized formamide, 0.05M NaH₂PO₄ (pH 6.5), 5X SSC, 0.5 mg/ml yeast tRNA, 1X Denhardt’s solution, 1% SDS) for two h. The probes (approximately 20 million cpm) were placed in boiling water for 5 min, quick cooled on ice, and added to 20 ml fresh hybridizing mixture. The prehybridizing mixture was poured off and the probe containing mixture poured onto the membrane and incubated for 36 h in a 42°C shaking water bath. The blots were removed and washed as follows: twice with 2X SSC for 15 min at room temperature, once with 2X SSC, 1% SDS for 15 min at room temperature, and once with 0.1X SSC, 1% SDS for 30 min at 42°C. Membranes were given a final rinse in 2X SSC and patted dry with Whatman 3M
filter paper. The blots were wrapped in plastic wrap and exposed to x-ray film (Kodak, X-OMAT AR, Mississauga, ON) for varying lengths of time while stored at -70°C. Films were developed using a Kodak automatic film processor.

In order to re-probe the membrane, the original probe was removed by 4 successive 5 min washes in a boiling stripping solution (0.1X SSC, 1% SDS) with shaking, followed by a 1 h incubation in the stripping solution at 70-80°C with shaking. The blot was washed a final time in boiling stripping solution and allowed to cool to room temperature. The membrane was given a final rinse with 0.1X SSC. The membrane could be re-probed using the hybridization procedure described above.

Densitometry was performed on the films by scanning at 300 dots per inch (dpi) with a Macintosh Colour One Scanner and a Macintosh computer. The bands were analyzed using NIH Image 1.58 and relative expression determined by calculating the ratio of the signals for the band of interest (TRPM-2 or PSBP mRNA) and the band used for standardization (G3PDH mRNA). For the slot blots, the resulting pixel area from the analysis of a dilution series was plotted against the dilution concentration. The area for 1 µg of RNA was used for comparison purposes between labeled probes.

**DNA MASS PER PROSTATE ASSAY**

The mass of DNA per prostate measurement is a modification of the diphenylamine assay by Burton et al (Burton 1968). Each frozen prostate was ground under liquid nitrogen and suspended in 10 vol/g of tissue of homogenization buffer (10 mM Tris-HCl, pH 7.0, 100
mM NaCl, 10 mM EDTA, pH 8.0). The samples were homogenized on ice using a Polytron homogenizer at 8000 rpm for 30 sec. Aliquots of 800 μl (72.7 mg prostate) were taken from the homogenate and stored at −20°C until ready for assay. For samples that did not have an 800 μl aliquot available, the sample volume of the homogenate was determined and the remaining assay volumes adjusted accordingly. Procedural losses were accounted for using a $^{32}$P – labeled calf thymus DNA trace. Briefly, using a nick translation kit (Boehringer Mannheim, Montreal, PQ), 50 ng calf thymus DNA was labeled with 20 μCi of α($^{32}$P) dCTP, DNA polymerase I, and for 1 h at 15°C. Incorporation and purification were carried out as described in the probe labeling methods section.

Prostate homogenate samples were thawed for assay on ice and 10 μl (3000 cpm) of calf thymus tracer was added to each sample and to 3 total count control samples containing 3 ml of 5% TCA. The following calculations are for an 800 μl sample. The samples were homogenized at 6000 rpm using a Polytron homogenizer for 60 sec then precipitated on ice for 30 min with 800 μl of 10% TCA. Samples were centrifuged at 1700 x g for 5 min. The supernatant was discarded and the pellet re-suspended in 4 ml of 5% TCA. The sample was centrifuged (5 min) and the pellet re-suspended two more times.

The DNA content of the sample was determined from the pellet using the diphenylamine assay. The pellet was re-suspended in 1 ml of 5% TCA and incubated at 85°C for 20 min to hydrolyze the DNA. The samples were cooled on ice and an additional 2 ml of 5% TCA added. The samples were centrifuged at 1700 x g for 5 min and the supernatant saved. Two samples of 700 μl of supernatant were added to 1.4 ml of diphenylamine dye.
(1.5% diphenylamine (w/v), 1.5% H₂SO₄, and 0.5% acetaldehyde in glacial acetic acid) and incubated for 16 h at 30°C. Calf thymus DNA standards were prepared by dissolving calf thymus DNA in 5nM NaOH to a final concentration of 0.3 mg/ml. The samples were read at 600 nm in a spectrophotometer, and the DNA concentration per sample determined from the standard curve.

Procedural loss of DNA was calculated by counting duplicate 500 µl aliquots from the samples in vials with scintillation cocktail in a beta-counter. Recovered counts were compared with those from the total count control tubes. Overall recovery was calculated as the ratio of recovered counts over total counts followed by a correction for tracer purity.

**DETERMINATION OF INTRAPROSTATIC AND SERUM ANDROGENS**

Intraprostatic T and DHT were measured by radioimmunoassay (RIA) after extraction and column chromatography. It was determined in the Rittmaster laboratory that in order to extract sufficient levels of androgens it was necessary to treat the glassware. All glassware for the extraction of androgens from the prostate must be siliconized. Prostates were ground under liquid nitrogen, transferred to labeled siliconized tubes and 5 ml of Delsal’s solution (80:20 dimethoxymethane: methanol) added. To track the experimental loss of T and DHT, trace amounts of these radioactive androgens were employed. ³H-T and ³H-DHT (NEN, Boston, MA) each around 2000 cpm were added to the samples and total count tubes. The samples were homogenized (Polytron, Brinkman Instruments, Rexdale, ON) on ice for 15 sec intervals with 30 sec cooling off periods 6 times with
washing of the homogenizer probe and sides of the tube with more Delsal's after the fourth interval. The samples were spun for 20 min at 3000 g (Beckman 6R centrifuge, Fullerton, CA) and the supernatant transferred to siliconized tubes and dried under nitrogen gas in a 37°C water bath. The samples were reconstituted in 500 µl benzene and applied to a silica Sep-pak (Waters, Milford, MA) which had been pre-equilibrated with hexane. The column was washed with 10 ml of hexane and the steroid fraction eluted with 5 ml of ethyl acetate. The samples were dried under nitrogen gas at 37°C. T and DHT were separated by Celite column chromatography. To make the columns, dried Celite (Fisher Scientific, Fair Lawn, NJ) was mixed 2:1 (w/v) with ethylene glycol and packed into 5 ml pipettes. The columns were equilibrated with a solution of 95% iso-octane and 5% benzene. The samples were reconstituted with 200 µl iso-octane and added to the chromatography columns. The elution pattern for T and DHT from the chromatography columns was determined in validation experiments. To elute the DHT fraction, 20 ml of 95% iso-octane: 5% benzene was run through the column with the first 8 ml were discarded, the next 9 ml collected, and the last 3 ml discarded. To elute the T fraction, after the 20 ml of 95% iso-octane: 5% benzene, the solution was changed to 70% iso-octane and 30% benzene and the first 3 ml were discarded and the next 9 ml were collected. The T and DHT fractions were dried down in a Speed-Vac and stored at -30°C until assayed.

T and DHT fractions for serum were separated, as described above for prostates; however, the extraction of the steroids from serum was different. Trace ³H-T and ³H-DHT (2000 cpm per sample) were added to 500 µl of serum. The sample was extracted twice with 5 ml of ether (Fisher Scientific, Fair Lawn, NJ) and the ether fractions
evaporated to dryness using nitrogen. The samples were reconstituted with 200 µl iso-octane and added to the Celite chromatography columns to separate the T and DHT fractions.

Standards of T and DHT (2-500 pg/tube) for the RIA were serially diluted in RIA buffer. For the samples that were expected to have low T and DHT concentrations, the volume of buffer added was 300 µl. For intermediate levels of T or DHT the sample were reconstituted in 500-1000 µl of RIA buffer and for high levels, the sample 2300 µl of buffer were added. The procedure is described for the T RIA but the RIA for DHT is analogous. RIA buffer (400 µl) was added to scintillation vials followed by 100 µl of the diluted sample, 100 µl of RIA buffer containing 10-15,000 cpm ³H-T, and 100 µl of diluted T antibody (1:4000, Endocrine Sciences, Calabasas Hills, CA). The tubes were shaken, and incubated overnight at 4°C. Dextran-coated charcoal was used to separate antibody-bound ³H-T from unbound steroid and the supernatant radioactivity determined in the beta-counter after addition of 5 ml scintillation cocktail. All samples and standards were assayed in duplicate. A computer program that used weighted regression of the logit-log-transformed data analyzed the RIA data. The results were corrected for extraction recovery. Samples with a recovery below 50% were re-assayed. Procedural recoveries were 70 ± 7% (mean ± SD) for T and 65 ± 11% for DHT. Mean assay sensitivities (assuming a mean recovery) were 0.18 ng/g for T and 0.21 ng/g for DHT. All reported T and DHT measurements were within the working range of the assay. Interassay coefficients of variation were 11.1% for T and 9.9% for DHT.
STATISTICS

Values were expressed as the mean + SEM. Statistical analyses of significance were performed using StatView (Abacus Concepts, Berkeley, CA) on a Macintosh computer. For each intraprostatic androgen and its effect on various prostatic measurements, an analysis of variance (ANOVA) was performed to determine significance. If significance was detected, post-hoc testing was done to determine which means were different from each other. Pairwise comparisons were performed using Fisher's protected least squares significant difference (Fisher’s PLSD).

Multiple regression analysis was used to determine whether linear regression lines were significantly different from each other (p<0.05) in the Regression Study. A dummy variable was assigned for T and DHT dose responses and used in the equation (Weisberg 1985):

\[ Y = b_0 + b_1 x + b_2 Z + b_3 Zx \]

where \( y \) = prostate weight or lumen mass, \( b_0 \) = the intercept, \( x \) = the intraprostatic androgen concentration, and \( Z \) = the dummy variable (0=T and 1=DHT).

When \( Z=0 \) the equation gives the equation of the line for the T dose response and when \( Z=1 \) the equation gives the equation of the line for the DHT dose response. p<0.05 determined statistical significance of the dependent variable from the three independent variables. Analysis of non-linear graphs for statistical significance between T and DHT dose responses was performed using two-way ANOVA. TRPM-2 mRNA and TUNEL
staining were assigned as dependent variables, while intraprostatic androgen concentration was grouped according to T and DHT ranges and a dummy variable was assigned for T or DHT dose response. p<0.05 was considered statistically significant.
RESULTS

1. VALIDATION OF THE ANIMAL MODEL

Accurate T delivery is the cornerstone of the animal model used for the experiments presented herein. Inconsistent absorption and diffusion into circulation are problems associated with T administration via depot injections or silastic implants (personal communication, B. Robaire). The method of T delivery used for these experiments employed a constant-release pellet whose matrix can contain any substance (Innovative Research of America, Sarasota, FL). By virtue of this relationship, larger pellets release larger amounts of steroid into the circulation. The validity of the pellet’s constant delivery was assessed using 21-day release T pellets in castrated rats. Rats (n=30) were castrated and immediately given 5 mg T pellets. The implants were placed under the skin in the mid-scapular region 1-2 cm from the site of incision. Care was taken not to damage the pellets, which might affect the surface area and release rate, and the wound was closed with a sterile stitch, to reduce the chance of infection or inflammation. The animals were killed on days 0, 2, 4, 8, 9, 10, and 11 and blood and ventral prostates were collected. Fig. 20a and 20b show the measurements of serum T and prostate weight as a function of time in days. These parameters remained stable for the duration of the experiment with no significant fluctuations over time (ANOVA, f=1.26, p>0.05). Serum T and prostate weight were maintained in the range of 349 ± 18 ng/dl and 447 ± 15 mg, respectively. With the constant delivery using T pellets confirmed, the larger studies could be instituted with confidence.
Fig. 20 Validation study results for serum T (A) and prostate weight (B) in castrated rats implanted with a 5 mg, 21-day release T pellet for 11 days. Animals (n=3 per day) were sacrificed on days 0, 2, 4, 8, 9, 10, and 11. There was no significant variation in prostate weight or serum T over the time period (p>0.05).
2. PREVENTION OF RAT VENTRAL PROSTATE REGRESSION AFTER CASTRATION USING T PELLET IMPLANTS WITH AND WITHOUT 5α-R INHIBITION ("The Regression Study")

As discussed in the introduction, inhibition of 5α-R has been shown to reduce prostate size in both rats and humans, but not to the same extent as castration. With 5α-R inhibition, DHT in the prostate is reduced near to the level associated with castrated animals, however, T levels rise in a reciprocal manner (George et al. 1989; Rittmaster et al. 1989; Geller 1990; Brooks et al. 1991; Gormley et al. 1992; McConnell et al. 1992; Shao et al. 1993). Thus, it is important to understand the relative potencies of T and DHT in vivo on several different responses, including inhibition of apoptosis and stimulation of growth and cell function. As mentioned earlier, the ability to separate the effects of intraprostatic T and DHT in vivo in the rat awaited development of a compound like finasteride that was a specific competitive inhibitor of 5α-R and did not have any antiandrogenic activity. The ability to distinguish between T and DHT responses in the prostate was the driving factor for determining the relative potency of T and DHT in preventing regression of the rat ventral prostate in the castrated rat.

In the model used to investigate the ability of T and DHT to prevent regression, two groups of rats were castrated and immediately implanted with T pellets of various sizes. The first group was given finasteride injections while the second group was not, and four days later, blood and prostates were taken. As mentioned previously, in the prostates of rats not treated with finasteride, virtually all T from serum is converted to DHT by 5α-R. Theoretically, varying the size of the T pellet implanted would result in a dose-response
of serum T, which in turn would result in a dose-response for DHT in the prostate. Rats castrated and given T implants in the presence of finasteride treatment would exhibit a dose-response for serum T concentrations, and a dose-response for T in the prostate. The intraprostatic T and DHT would then be related to a number of measures for prostatic atrophy and apoptosis.

a. Serum and intraprostatic DHT and T levels

A number of preliminary experiments using the model described above were carried out. Initially it was determined that T pellet implants in the absence of finasteride gave rise to intraprostatic DHT levels that increased with increasing pellet size. Prostate weight varied linearly with intraprostatic DHT concentration up to a DHT level of about 15 ng/g, similar to that found in a normal animal. Above the ventral prostate weight found in a normal rat (approximately 450 mg) increasing intraprostatic DHT had only a small effect on weight. This preliminary experiment was repeated in the presence of finasteride treatment and similar results observed, with the major intraprostatic androgen being T, instead of DHT.

Further preliminary experiments were carried out to determine the sizes of T pellets that could be used to establish dose-response curves that would have an adequate number of points in the linear range of the prostate response to androgens.

For the principal Regression Study, 119 rats were used in the group not treated with finasteride (DHT dose-response) and 133 rats in the group treated with finasteride (T dose-response). Table III shows the data for both groups for serum and intraprostatic
androgen levels resulting from T pellets of varying sizes. Serum T levels increased with increasing pellet size and ranged from 4.3 – 110 ng/dl in the rats not treated with finasteride. Serum DHT levels for rats not treated with finasteride ranged from 6.5 – 9.0 ng/dl, with no discernable difference among the groups (ANOVA, f=0.79, p>0.05). Rats that were given finasteride had serum T levels that also increased with increasing pellet size, although a greater range of pellet sizes was used, hence the range of serum T was also greater, 12.5 – 495 ng/dl. The serum DHT concentrations were near the limit of detection and ranged from 0.50 – 2.69 ng/dl.

Fig. 21 graphs the pellet size as a function of T pellet size in (A) the presence of finasteride treatment and (B) the absence of finasteride treatment. Serum T increased abruptly with low T pellet size in the presence of finasteride and then showed a linear relationship between T pellets 0.25 – 15.0 mg. Relatively small T pellets were used for the rats not treated with finasteride and a large increase in serum T occurred with the 0.7 mg pellets. The use of these pellet sizes was justified, however, as seen in Fig. 23 (prostate weight vs. intraprostatic T or DHT), these small pellets resulted in range of prostate weights from the level just above that for a castrated rat to above the weight seen in a normal rat.

Serum T levels for the normal rat, castrated rat and non-castrated rat treated with finasteride were 255 ± 21, 5.70 ± 1.38, and 268 ± 25 ng/dl, respectively. These results indicate that finasteride has does not substantially elevate the serum T levels of the normal rat (f=0.143, p>0.05).
Table III. The Regression Study: T pellet size and the resulting serum and intraprostatic androgen levels

<table>
<thead>
<tr>
<th>T pellet size</th>
<th>Finasteride Treatment</th>
<th>Serum T (ng/dl)</th>
<th>Serum DHT (ng/dl)</th>
<th>n</th>
<th>Prostate T (ng/g)</th>
<th>Prostate DHT (ng/g)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mg</td>
<td>No</td>
<td>4.27 ± 0.52</td>
<td>7.15 ± 1.45</td>
<td>3</td>
<td>0.27 ± 0.05</td>
<td>2.29 ± 0.42</td>
<td>6</td>
</tr>
<tr>
<td>0.3 mg</td>
<td>No</td>
<td>19.5 ± 2.4</td>
<td>9.03 ± 2.89</td>
<td>3</td>
<td>0.34 ± 0.09</td>
<td>6.93 ± 0.54</td>
<td>3</td>
</tr>
<tr>
<td>0.5 mg</td>
<td>No</td>
<td>24.1 ± 2.1</td>
<td>6.50 ± 2.48</td>
<td>3</td>
<td>0.29 ± 0.02</td>
<td>9.97 ± 1.23</td>
<td>3</td>
</tr>
<tr>
<td>0.7 mg</td>
<td>No</td>
<td>110 ± 11</td>
<td>6.53 ± 1.73</td>
<td>3</td>
<td>0.42 ± 0.13</td>
<td>12.9 ± 1.11</td>
<td>3</td>
</tr>
<tr>
<td>0.1 mg</td>
<td>Yes</td>
<td>12.5 ± 2.0</td>
<td>1.64 ± 0.28</td>
<td>3</td>
<td>1.83 ± 0.14*</td>
<td>0.52 ± 0.09</td>
<td>6</td>
</tr>
<tr>
<td>0.25 mg</td>
<td>Yes</td>
<td>44.7 ± 5.0</td>
<td>1.61 ± 1.18</td>
<td>3</td>
<td>7.27 ± 0.83</td>
<td>0.72 ± 0.29</td>
<td>3</td>
</tr>
<tr>
<td>2.5 mg</td>
<td>Yes</td>
<td>115 ± 6.0</td>
<td>2.69 ± 1.56</td>
<td>3</td>
<td>9.91 ± 1.55</td>
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<td>211 ± 34</td>
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<td>13.9 ± 0.98</td>
<td>0.88 ± 0.06</td>
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<tr>
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<td>495 ± 50</td>
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<td>3</td>
<td>25.9 ± 3.5</td>
<td>0.74 ± 0.10</td>
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<tr>
<td>Intact</td>
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<td>255 ± 21</td>
<td>11.8 ± 3.3</td>
<td>3</td>
<td>0.73 ± 0.10</td>
<td>17.6 ± 1.6</td>
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<tr>
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<td>267 ± 25</td>
<td>3.59 ± 2.20</td>
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<td>19.0 ± 0.6</td>
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<td>Castrate</td>
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<td>0.52 ± 0.19</td>
<td>3</td>
<td>0.61 ± 0.21</td>
<td>0.60 ± 0.12</td>
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</tbody>
</table>
Fig. 21 Serum T concentrations resulting from the implantation of various sizes of T pellets into castrated rats for 4 days in the Regression Study. A) Rats given daily finasteride injections. B) Rats not treated with finasteride.
Within the prostates, the T pellets resulted in a dose-range of 2.29 – 12.29 ng/g for intraprostatic DHT in rats not treated with finasteride and 1.83 – 25.9 ng/g for intraprostatic T in rats treated with finasteride (Table III). The mean intraprostatic T and DHT concentrations were 0.73 ± 0.10 and 17.6 ± 1.6 ng/g (n=10) in normal animals, 0.61 ± 0.21 and 0.60 ± 0.12 ng/g in castrated animals (n=6), and 19 ± 0.61 and 0.98 ± 0.17 ng/g in non-castrated animals given finasteride (n=3).

The relationship between the intraprostatic androgen concentration and serum T is illustrated in Fig. 22. As expected, the increasing concentration of serum T in both finasteride-treated and untreated rats resulted in increasing intraprostatic T or DHT levels, respectively. In rats not given finasteride, virtually all of the T was converted to DHT, and the intraprostatic T concentration varied from 0.27 - 0.42 ng/g, with no significant difference in the T concentration among the dose-range (ANOVA, f=4.44, p>0.05). This indicates that in the absence of finasteride, of the two androgens measured, DHT was the only intraprostatic androgen that responded to differing levels of T in serum. In the rats treated with finasteride, intraprostatic T ranged from 0.60 – 25.9 ng/g while the maximum intraprostatic DHT concentration was 0.88 ng/g. It is worth noting that finasteride, a competitive inhibitor of 5α-R, was able to suppress DHT to close to levels found in castrated rats even at the highest concentrations of T. Also, the contribution of the level of intraprostatic DHT found in finasteride-treated rats to androgenic effects in the prostate would probably be very slight. It has been shown by Kyprianou et al. (1987) that a threshold concentration of DHT approximately 2.5 times
Fig. 22 Prostate T and DHT concentration as a function of serum T in the Regression Study. Rats that did not receive finasteride and therefore have DHT as the major intraprostatic androgen are represented by open squares □, while rats that were given finasteride resulting in T being the major intraprostatic androgen are represented by closed circles ●. As seen in Table 1, intraprostatic T from rats not treated with finasteride and DHT from finasteride-treated rats was suppressed below 1.0 ng/g and also does not significantly vary with serum T. Therefore, these values were not reported in the graphs.
higher than the DHT concentration seen here with finasteride treatment must be reached before there is a significant effect on the prostate in castrated rats.

b. Prostate weight

An appropriate global measure of changes as a result of androgen manipulation, is the mass of the excised ventral prostate. Therefore, prostate weight was measured as part of the dose-response experiment described above. Fig. 23 shows prostate weight as a function of intraprostatic T or DHT. The results from this experiment are best represented by linear equations. As expected, prostate weight decreases as the intraprostatic T or DHT concentration decreases. The slope of the curve for DHT is 2.4 times higher than the slope for intraprostatic T, indicating that DHT is 2.4 times more potent than T. Another way to examine the difference in potency between intraprostatic T and DHT is the concentration of each androgen that is necessary to maintain normal prostate weight. In Fig. 23 the dashed line represents normal prostate weight (449 ± 16 mg). The concentration of DHT required to maintain normal prostate weight was only 9.5 ng/g, while 22.9 ng/g of T was needed to match the effects of DHT. Thus, in the prostate, 2.4 times more T than DHT was needed to maintain normal prostate weight.

c. Measures of atrophy

(i) Lumen mass

Decreases in prostate weight consist of two main components: atrophy and apoptosis. Atrophy is the loss of secretory activity, which was assessed by two morphometric methods and one molecular method. As mentioned earlier, the secretory component of the prostate is the epithelial cells, which secrete into the lumen area of the duct. Thus, the
Fig. 23 Prostate weight as a function of intraprostatic T or DHT in the Regression Study. The rats were castrated and immediately implanted with a T pellet from a dose range in the presence or absence of finasteride treatment. Prostate weights and intraprostatic androgens were measured after 4 days. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The dashed line represents the prostate weight from a normal control rat (449 ± 16 mg). For purposes of comparison, the ventral prostate wet weights for non-castrated rats treated with finasteride or for rats castrated for 4 days were 311 ± 13 mg and 206 ± 18 mg, respectively, and are significantly different from normal (f=27.41, p<0.0001).
first experimental measure for atrophy in the Regression Study was that for duct lumen mass. Briefly, a slide from a paraffin-embedded prostate was stained with hemotoxylin and eosin to visualize the architecture. A digital image of a random section of the slide was captured and the percent area occupied by the ducts were multiplied by the weight of the ventral prostate. This was done ten times for each prostate with three prostates per point.

Fig. 24 shows the important structures in the morphometry of the ventral prostate. Epithelial cells line the ducts and secrete prostatic fluid into the lumen. If secretions into the lumen decrease, then the lumen will shrink. The stroma supports the ducts and contains blood vessels. The second image from a castrated 4d previously rat illustrates the atrophied ducts but also shows apoptotic bodies interspersed with the epithelial cells.

Fig. 25 is a comparison of prostate morphometry using images captured at 100x magnification. An image from the prostate of a normal control rat is compared with an image from a rat castrated 4 days previously, an non-castrated rat treated with finasteride, and rats castrated, given T implants and not treated or treated with finasteride. Of the rats castrated, given T implants, but not treated with finasteride, two images are shown; one from a rat given a 0.3 mg T implant or approximately half the maximal intraprostatic DHT concentration, and one from a rat given a 0.7 mg T implant or the maximum intraprostatic DHT concentration used in this study. Two images are shown for rats castrated, given T implants and treated with finasteride; one from a rat given a 2.5 mg T implant which results in an intraprostatic T concentration slightly higher than the intraprostatic DHT concentration with the 0.3 mg T pellet (no finasteride), and one from
Fig. 24 Results for prostate images from an intact normal rat (A) (100x mag.) and from a rat castrated for 4 days (B) (200x mag.). The lumen area is the area of the duct minus the epithelial cell area.
Fig. 25  Morphometry from slides of ventral prostates from the Regression Study (100x magnification). Images are from: A) a normal control rat, B) a rat castrated 4 days, C) an non-castrated rat treated with finasteride for 4 days, D) 0.3 mg T pellet in a castrated rat, E) 2.5 mg T pellet plus finasteride injections in a castrated rat, F) 0.7 mg T pellet in a castrated rat, G) 15.0 mg T pellet plus finasteride injections in a castrated rat.
a rat given a 15.0 mg T implant or the highest concentration of intraprostatic T from the study. In the normal animal (fig. 25-A), lumen volume and mass are high, the ducts are large, displaying very little infolding, and the epithelial cells are for the most part, tall and columnar with no apoptotic bodies. Very little stroma is evident because the ducts are swollen and occupy most of the prostate. One thing to note is that not many whole ducts are visible within the images for the normal rat. Rats castrated, or given small T implants have more ducts and cells in the field of view of the image. Animals castrated 4 days previously (fig. 25-B) had small ducts with low cuboidal, epithelial cells and apoptotic bodies scattered throughout the epithelium. Secretory function is diminished, thus, the lumen is collapsed and the duct displays increased infolding. The stroma is now a prominent feature of the image from the castrated rat. The image from an non-castrated rat treated for 4 days with finasteride (fig. 25-C) shows smaller ducts than those from the normal rat. There is also more infolding and stroma, which are indications of atrophy. In rats given a 0.3 mg T implant in the absence of finasteride (fig. 25-D), the prostate has regressed as seen by the infolding and the less well-rounded ducts. In comparison, rats given a 2.5 mg T implant in the presence of finasteride (fig. 25-E) had smaller ducts with more infolding and the stroma was becoming a prominent feature. The prostate images from rats given a 0.7 mg T implant (fig. 25-F) or 15.0 mg T implant plus finasteride (fig. 25-G) were similar to the image from the normal rat. The ducts were large with little infolding and the stroma is not prominent. In addition, very few apoptotic bodies were evident. Thus, the highest level of intraprostatic T (fig. 25-G) is able to mimic the prevention of prostate regression seen with the highest level of intraprostatic DHT used in this study (fig. 25-F). At lower levels of intraprostatic T (fig. 25-E), there is more atrophy evident than for an equivalent concentration of intraprostatic DHT (fig. 25-D).
Fig. 26 Lumen mass as a function of intraprostatic T or DHT in the Regression Study.

Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The lumen mass was 177 ± 18, 121 ± 6, 85 ± 9 mg for normal controls, rats treated with finasteride and castrated rats, respectively, with finasteride and castrated rat values significantly different from normal (f=5.66, p<0.016). The dashed line represents normal duct lumen mass.
Fig. 26 illustrates the lumen mass, as determined from morphometry, as a function of intraprostatic T or DHT levels. The relationship between each androgen and the lumen mass was linear. As indicated earlier, lumen mass decreased as either intraprostatic T or DHT decreased. The slope for the data points from the DHT dose-response was 2.4 times higher than the slope for the T dose-response. This indicates a higher potency for DHT but it is not as high as was predicted from *in vitro* experiments (Grino *et al.* 1990; Deslypere *et al.* 1992; Yeh and Chang 1996). In Fig. 26, the dashed line represents the lumen mass from the ventral prostate of a control rat (177 ± 18 mg). The concentration of intraprostatic T that was needed to maintain normal secretory activity was 2.5 times greater than the concentration of DHT (8.4 ng/g DHT versus 21.2 ng/g T), very similar to the prostate weight response.

**(ii) Epithelial cell mass**

Epithelial cell mass was calculated on the same principle as that for lumen mass. Fig 27 shows the digital image of the prostate from a normal rat. The epithelial cells were isolated and the image analyzed to determine the epithelial cell area. The percent area from 10 random sections was multiplied by the prostate weight to give the epithelial cell mass. This measure has been used to describe differences in secretory function in similar circumstances (Rittmaster *et al.* 1995). Fig. 28 shows that a dose-response was evident in that epithelial mass decreased with decreasing androgen. Epithelial cell mass values for normal rats, finasteride-treated non-castrated rats and 4 day castrated rats were significantly different from each other (f=9.86, p<0.02). The result for rats treated with finasteride for 4 days shows that 5α-R inhibition reduces epithelial cell mass by 26%. This is expected because finasteride treatment also reduces prostate weight after 4 days of
Fig. 27 Determination of epithelial cell area. Depicted is the epithelial cell area (outlined in red) from the prostate of an intact normal rat (100x magnification). Epithelial cell mass was determined in a similar manner to lumen mass.
Fig. 28 Epithelial cell mass as a function of intraprostatic T and DHT in the Regression Study. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The epithelial cell mass for normal rats, finasteride-treated rats and rats castrated 4 days previously was 159 ± 17, 118 ± 4, and 58 ± 7 mg, respectively, with finasteride-treated and castrated rat values significantly different from normal (f=9.86, p<0.02).
treatment by 31%. The data show that there is little if any difference in the ability of intraprostatic T or DHT to prevent regression of epithelial cell mass. However, with this particular physical measure, it could be argued that four days of treatment after castration may be too early to resolve differences in the potency between T and DHT in preventing regression.

(iii) Prostate specific binding protein (PSBP) mRNA

PSBP has been associated with epithelial cell function (Ritmaster et al. 1991). RNA was isolated from some of the prostates from each group of the intraprostatic T and DHT dose-response experiments. Using a cDNA probe for PSBP mRNA, Northern blots were carried out to examine PSBP mRNA variation with differing levels of androgen in the prostate (Fig. 29). The blot shows that PSBP mRNA is high in normal rats and in those with high DHT levels, and decreases as intraprostatic DHT decreases. In order to quantitate the level of staining, slot blots were used. The results for PSBP mRNA were normalized against expression of the gene encoding G3PDH. As expected, Fig. 30 shows that PSBP expression decreases with decreasing T or DHT concentration. Although there was a lot of variability in the blots at high concentrations of T and DHT, it appears that T is approximately equipotent with DHT for maintaining PSBP expression in the Regression Model. The variability within points in this experiment does not preclude a small difference in potency, but it clearly does not show the 10 times potency difference between T and DHT seen with in vitro studies on the expression of individual genes (Deslypere et al. 1992; Yeh and Chang 1996).
Lane 1 2 3 4 5 6 7

Fig. 29 Northern blot analysis of ventral prostate mRNA using the PSBP cDNA probe and showing the 0.67 kb PSBP mRNA. Lane (1) mRNA from normal rats, (2) castrated, and (3)–(7) increasing sizes of T pellets implanted into castrated animals for 4 days.

Fig. 30 Normalized PSBP mRNA expression from slot blots as a function of intraprostatic T and DHT in the Regression Study. Rats that did not receive finasteride are represented by open squares □, while rats that did receive finasteride are represented by closed circles ⊗. Each point represents mRNA from 3-4 animals. The value for finasteride treated intact rats was 74 ± 28% and rats castrated for 4 days was 10 ± 3%.
In summary, all three features of secretory function measured above exhibit androgen responsiveness by decreasing with decreasing intraprostatic androgen concentration. PSBP mRNA and epithelial cell mass did not show a discernable potency difference between intraprostatic T and DHT, however, lumen mass, which is a direct measure of epithelial cell output, did show a 2.5 times difference in androgen potency. The involution of the prostate is a complex cascade of physiologic changes where some factors will show a differential response to T and DHT and some may not. The most conspicuous measure (lumen mass) demonstrates a potency difference between T and DHT in the ability to maintain secretory function. Nevertheless, the difference is far less than was predicted from in vitro studies, which indicated that T is ten-fold less potent than DHT (Deslypere et al. 1992; Yeh and Chang 1996).

d. Measures of apoptosis

(i) Testosterone repressed prostate message – 2 (TRPM-2) mRNA

The second measure of prostate involution is cell loss through apoptosis. Long-term castration results in a 90% loss in total prostate mass, with more than 80% of epithelial cells eliminated. Two markers of cell death, TRPM-2 mRNA levels and TUNEL staining of epithelial cells, peak at 4 days post-castration (Leger et al. 1987; Rittmaster et al. 1991; Furuya and Isaacs 1993; Russo et al. 1994; Rittmaster et al. 1995). TRPM-2 mRNA which is highest in the prostates of castrated rats, correlates with the level of programmed cell death during prostate regression and is virtually absent in normal controls (Leger et al. 1987; Buttyan et al. 1989). Northern blots were used in the Regression Study to verify that TRPM-2 in the prostate is revealed as a single band and that it increases in expression as androgen concentration decreases. Fig. 31 shows a
Fig. 31 Northern blot analysis of ventral prostate mRNA using the TRPM-2 cDNA probe and showing the 2.0 kb TRPM-2 band. Lane (1) RNA from a normal intact rat, (2) an intact rat treated with finasteride for 4 days, (3) a rat castrated for 4 days, (4) – (9) rats castrated and given 0.1 – 15.0 mg T and finasteride treated. The decrease in lane 4 is due to low loading of RNA. The blot is overexposed and was not used for quantitative purposes.
Northern blot from the dose-response group treated with finasteride. The blot shows very low levels of TRPM-2 in the normal rat, non-castrated rat treated with finasteride, as well as at the higher concentrations of intraprostatic T. The mRNA levels for both intraprostatic dose-response experiments were quantitated using slot blots and radiolabeled probes for TRPM-2 and the gene, encoding G3PDH. To compare levels of the mRNA from the different treatment groups, RNA from the prostates of the same castrated and normal rats was used on each slot blot as standards. Every sample was normalized against G3PDH mRNA and expressed as a percentage of the castrate control. Fig 32 shows that TRPM-2 mRNA levels were reduced to the range found in normal animals by 10 ng/g of intraprostatic T or DHT. In addition, the two curves were not statistically different from each other when analyzed by two-way ANOVA (f=1.75, p>0.05). Thus, T and DHT are equipotent in repressing expression of TRPM-2. It is also worth noting that the concentration of androgen at which TRPM-2 staining returns to normal values is nearly half the DHT concentration of a normal rat and half the intraprostatic T concentration of an non-castrated rat treated with finasteride.

(ii) TUNEL staining

Using the enzyme TdT, fragmentation of DNA strands (which is an early event in apoptosis) was detected by nick end labeling with biotinylated dATP. This physical measure of the concentration of fragmented DNA in cells also correlates with the apoptotic activity in a tissue (Rittmaster et al. 1995). Fig. 33 illustrates that the percentage of cells that stain positively using the TUNEL method is highest in castrated rats and decreases with increasing androgen concentration. Normal rats and non-castrated rats treated with finasteride show very little evidence of TUNEL staining in the epithelial
Fig. 32 TRPM-2 mRNA levels from slot blots expressed as a percentage of those found in the 4 day castrated-rat prostate, plotted versus intraprostatic T and DHT concentrations in the Regression Study. TRPM-2 is not expressed in the ventral prostates of normal controls nor in intact animals treated with finasteride. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T).
Fig. 33 The percent of epithelial cell that stain positively for DNA fragmentation as a function of intraprostatic T and DHT in the Regression Study. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The dashed line represents the levels of staining in the ventral prostate from a normal rat. The level of staining for a rat treated with finasteride was slightly higher.
cells of the ventral prostate. The percentage of cells staining for DNA breaks was reduced to the normal range (0.13 ± 0.09, n=8) by an intraprostatic concentration of 10 ng/g for either T or DHT. Using two-way ANOVA, the two curves for DHT and T dose-response experiments were not significantly different from one another (f=0.02, p>0.05). Thus, T and DHT demonstrate equal potency for inhibition of DNA fragmentation; a hallmark of programmed cell death.

(iii) Mass of DNA per prostate
Measuring the mass of DNA per prostate is an assessment of cell number. The concentration of DNA per prostate is highest in normal controls and decreases upon castration. However, the four day time period is early to detect the small differences in the mass of DNA per prostate for the dose-response experiments. The values for normal rats, rats treated with finasteride for 4 days and rats castrated 4 days previously were 990 ± 45, 802 ±41, 718 ± 69 µg DNA/prostate, respectively. As expected, Fig. 34 shows that the mass of DNA per prostate decreased with decreasing androgen level, however, at low T and DHT levels there was little difference in their relative potencies. At the highest level detected, DHT appears to prevent cell loss and even to promote cell proliferation more effectively than T but more experimental points are necessary to confirm the trend.

In summary, these results indicate that for inhibition of apoptosis there does not appear to be a relative potency difference between intraprostatic T and DHT. One supposition could be that the AR need only be occupied by an androgen, and that specific coactivators may stabilize the AR complex long enough to elicit the action of inhibition of apoptosis. Other investigations may reveal a potency difference between these
Fig. 34 Mass of DNA per prostate as a function of intraprostatic T and DHT in the Regression Study. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The point at 9.9 ng/g of T was dropped because only one prostate was available for the DNA assay. Each of the rest of the points represents 3 animals. The values for normal rats, rats treated with finasteride and castrated rats were 990 ± 45, 802 ± 41 and 718 ± 69 µg DNA/prostate, respectively. Values from the castrated rats but not the non-castrated, finasteride-treated rats were significantly different from normal (p<0.05).
andro gens for particular endpoints but in the larger scheme, it appears that 5α-R inhibition has little effect in promoting apoptosis in the ventral prostate. As long as the intraprostatic androgen level remains above 10 ng/g, there will not be a great deal of apoptotic activity. This concentration, it should be noted, is approximately half the normal intraprostatic DHT concentration of a normal animal and half the intraprostatic T associated with finasteride treatment in an non-castrated animal. This explains why finasteride treatment only results in a 40% decrease in ventral prostate mass (Rittmaster et al. 1995). Most of the weight loss appears to be through loss of secretory function rather than through programmed cell death. As has been mentioned, the difference in potency between T and DHT is much less than would be predicted by AR affinity and in vitro studies. Despite the small difference in potency between intraprostatic T and DHT in rats, it has been observed that men with 5α-R deficiency have extremely small prostates. This cannot be explained only by 5α-R inhibition effects on prostate regression. Thus, the relative potency of T and DHT to promote growth of the ventral prostate was examined in the next phase of the studies.

3. REGROWTH OF THE CASTRATED RAT VENTRAL PROSTATE USING T IMPLANTS IN THE PRESENCE OR ABSENCE OF 5α-R INHIBITION ("The Regrowth Study")

The role of 5α-R may not lie with the inhibition of prostate regression, but rather with the stimulation of prostate growth. As mentioned previously, studies have been done using a single dose of T (in the presence or absence of 5α-R inhibitor) given to rats castrated 7 or 14 days previously, but no dose-response has been performed in vivo that could separate
T from DHT effects on prostate growth. Often the T was injected, which results in spikes in circulating T levels introducing more variables into the response of the prostate to high levels of T. Therefore, in the Regrowth Study, rats were castrated and left until their prostates were fully regressed then implanted with a constant release T pellet (Innovative Research of America, Sarasota, FL) in the presence or absence of finasteride treatment. The time course for full prostate regression was determined to be 14 days based on previous experiments (Rittmaster et al. 1995).

Preliminary studies were done to determine the sizes of T pellets necessary to give a broad range of intraprostatic androgen levels and prostate growth (data not shown). Once the dose range was determined, a comparison was made between 7 and 14 days of regrowth. In order to examine the prostate while it was actively growing, 7 days post-implantation was chosen as the time point. Fig 35 shows that in the absence of finasteride, prostate weight increased the most with the larger T pellets, and that with the smaller size T pellets, the dose-response did not continue to increase prostate weight beyond 7 days. In the presence of finasteride, prostate weight increased with increasing pellet size, and again prostate weight did not progress beyond 7 days in the smaller sizes of T pellets.

a. Serum and intraprostatic DHT and T levels

Fig. 36 illustrates that serum T levels rose with increasing T pellet size and that the relationship was not linear. A large jump in serum T levels occurs between the 0.5 mg T and 1.0 mg T pellets regardless of treatment with finasteride. Table IV indicates that the range for androgen measurements runs from near castrate levels to one and a half times
Fig. 35 Pilot experiment for Regrowth Study. A range of T pellets was implanted into castrated rats in the presence or absence of finasteride treatment. Prostate weight for each T pellet implant size was plotted as a function of days. A) T pellets in the absence of finasteride, B) T pellets in the presence of finasteride.
Fig. 36  Serum T levels resulting from T pellet implants in the Regrowth Study. Rats were castrated 14 days prior to receiving T pellet implants. Blood and prostates were taken after 7 days with the implant. The open squares □ represent the animals not treated with finasteride (resulting in DHT being the major intraprostatic androgen), while the closed circles ⨿ represent the animals that received daily finasteride injections (resulting in T being the major intraprostatic androgen).
Table IV. The Regrowth Study: T pellet size and the resulting serum and intraprostatic androgen levels.

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<th>Finasteride Treatment</th>
<th>Serum T (ng/dl)</th>
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<th>Prostate T (ng/g)</th>
<th>Prostate DHT (ng/g)</th>
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<td>1.0 mg</td>
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<td>17.8 ± 2.21</td>
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<td>500 ± 10</td>
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<td>0.53 ± 0.12</td>
<td>25.8 ± 3.46</td>
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<td>0.1 mg</td>
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<td>8.5 ± 2.4</td>
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<td>1.41 ± 0.54</td>
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<td>26.9 ± 1.7</td>
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<td>1.0 mg</td>
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<td>155 ± 5.8</td>
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<td>2.5 mg</td>
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<td>200 ± 4.8</td>
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<td>14.2 ± 1.05</td>
<td>0.80 ± 0.12</td>
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<tr>
<td>5.0 mg</td>
<td>Yes</td>
<td>419 ± 14</td>
<td>3</td>
<td>20.6 ± 1.78</td>
<td>0.83 ± 0.21</td>
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<td>15.0 mg</td>
<td>Yes</td>
<td>516 ± 11</td>
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<td>24.6 ± 1.83</td>
<td>1.16 ± 0.17</td>
<td>5</td>
</tr>
<tr>
<td>Intact</td>
<td>No</td>
<td>286 ± 23</td>
<td>5</td>
<td>0.85 ± 0.13</td>
<td>19.1 ± 1.15</td>
<td>5</td>
</tr>
<tr>
<td>Intact</td>
<td>Yes</td>
<td>307 ± 83</td>
<td>3</td>
<td>16.0 ± 2.05</td>
<td>0.30 ± 0.26</td>
<td>2</td>
</tr>
<tr>
<td>Castrate</td>
<td>No</td>
<td>5.0 ± 1.5</td>
<td>3</td>
<td>0.42 ± 0.18</td>
<td>0.51 ± 0.18</td>
<td>3</td>
</tr>
</tbody>
</table>
normal androgen replacement values. In the absence of finasteride, the resulting serum T from T pellets 0.1-15 mg (21-day release) was 6.4 – 500 ng/dl. In the presence of finasteride, the range of serum T that results from the similar T pellet range, 0.1 – 15 mg, was 8.5 – 516 ng/dl. The serum T levels for the experimental controls were 286 ± 23, 307 ± 83, 5.0 ± 1.5 for untreated, finasteride-treated and castrated rats, respectively. This study shows that as in the Regression Study, intraprostatic DHT was suppressed to the levels found in a castrated rat in the presence of finasteride. In the absence of finasteride, virtually all T in the prostate is converted to DHT. Normal rat prostate DHT and T levels were 19.1 ± 1.5 and 0.85 ± 0.13 ng/g, while castrated rats had intraprostatic DHT and T levels of 0.51 ± 0.18 and 0.42 ± 0.18, respectively. For rats castrated and given T pellets in the absence of finasteride treatment, intraprostatic DHT levels ranged from 3.34 – 25.8 ng/g, while the T levels remained at 0.4 –0.8 ng/g. In the presence of finasteride, rat intraprostatic T levels ranged from 1.41 – 24.6 ng/g while DHT was suppressed between 0.4 – 1.1 ng/g. These data show that dose-responses were established for intraprostatic T and DHT from T pellet implants in the presence and absence of finasteride, respectively. For the T and DHT dose-responses, the corresponding DHT and T levels were low. The intraprostatic DHT level associated with finasteride treatment was found to be below the threshold necessary before DHT can induce prostate growth (Kyprianou and Isaacs 1987a; Kyprianou and Isaacs 1987b). Consequently, only the major intraprostatic androgen that increases with increasing serum T concentration was reported in the figures. It is worth restating that, in the face of increasing T pellet size and thus serum T concentration; finasteride was able to maintain suppression of intraprostatic DHT levels.
b. Prostate weight

The response of the involuted prostate to androgen stimulation is complex; therefore global markers of prostate growth were used. Prostate weight encompasses both increases in hypertrophy and cell number. Fig. 37 shows the relationship between increasing prostate weight and increasing intraprostatic T or DHT. As fig. 37 illustrates, there was a threshold for intraprostatic T (5-10 ng/g) below which there was no discernable difference in prostate weight from that seen in the castrated rat. The threshold for intraprostatic DHT was approximately 3 ng/g. Fig. 37 also clearly indicates that the slopes of the curves for intraprostatic T and DHT, after the respective thresholds, were comparable. In order to compare the relative potency of intraprostatic T and DHT on prostate growth, the concentration of each androgen needed to achieve a half-maximal increase in prostate weight was examined. On the figure, a horizontal dashed line denotes the half-maximal prostate weight. The concentration of T required to stimulate a half-maximal increase in prostate weight was 1.8 times the concentration of intraprostatic DHT. The results for half-maximum comparisons can also be found in Table V. The highest concentrations of both T and DHT resulted in similar increases in prostate weight underlining the observation that high levels of T can mimic the effect of DHT on prostate regrowth in this and other studies (Grino et al. 1990; Zhou et al. 1995).

c. Hypertrophy

The morphometry of the ventral prostates from the Regrowth Study is shown in Fig. 38. The prostate images are from rats that were left intact, castrated, given a 0.5 mg T pellet without finasteride treatment (approximately half-maximal intraprostatic DHT concentrations), a 1.0 mg T pellet with finasteride treatment (approximately the half-
Fig. 37 Prostate weight as a function of intraprostatic T and DHT in the Regrowth Study. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The dashed line is the half-maximal increase in prostate weight (141 mg). The regression line for rats treated with finasteride was derived using all points significantly higher than castrate (p<0.05) plus the last point that was not significantly different (p>0.05) using Fisher’s PLSD for post-hoc analysis.
Table V. Intraprostatic T and DHT concentrations associated with the half-maximal responses in markers of prostate regrowth.

<table>
<thead>
<tr>
<th></th>
<th>Half-maximal level</th>
<th>T Dose-Response (ng/g)</th>
<th>DHT Dose-Response (ng/g)</th>
<th>Ratio T:DHT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prostate weight</strong></td>
<td>141 mg</td>
<td>19.2</td>
<td>10.9</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>Lumen mass</strong></td>
<td>49 mg</td>
<td>16.6</td>
<td>10.6</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>DNA content</strong></td>
<td>317 μg</td>
<td>17.9</td>
<td>9.2</td>
<td>1.9</td>
</tr>
</tbody>
</table>
maximal intraprostatic T concentrations), or 15.0 mg T pellets with or without
finasteride-treatment (maximal intraprostatic DHT and T concentrations). As expected,
the ducts from the castrated animals were severely atrophied. The stroma makes up a
large part of the field of view and the epithelial cells can be described as low and
cuboidal. The ducts in rats that had approximately 10 ng/g of intraprostatic DHT were
much larger, the epithelial cells were more columnar and there was less stroma between
the ducts. In comparison, the ducts from animals that received approximately 10 ng/g
intraprostatic T were not as large, displayed more infolding and the stroma was more
prominent. At the highest concentrations of T and DHT, the difference in lumen size and
prostate morphometry is no longer apparent.

Hypertrophy was determined by the morphometric measure of lumen mass. This physical
measure derived from image analysis of digital images exhibits androgen responsiveness.
Fig. 39 demonstrates that there was a threshold for intraprostatic T (5-10 ng/g) below
which the lumen mass was not significantly different (ANOVA p>0.05) from the lumen
mass of a castrated rat. The threshold for intraprostatic DHT was 3 ng/g. Above these
thresholds, the slopes for the linear regression lines for the lumen mass versus
intraprostatic T or DHT concentrations were similar. The dashed line denotes the half-
maximal response in lumen mass. The concentration of T in the prostate needed to
achieve the half-maximal lumen mass was 1.6 times more than the concentration of DHT
required for the same effect (Table V). Fig. 39 shows that the highest concentrations of T
are able to mimic the response of the prostate to DHT for increasing secretory activity.
Fig. 38  Morphometry of ventral prostates from animals in the Regrowth Study. Images from A) a normal intact rat, B) a rat castrated 14 days, a rat castrated for 14 days and given a C) 0.5 mg T pellet, D) a 1.0 mg T pellet plus finasteride, E) a 15.0 mg T pellet, or F) a 15.0 mg T pellet plus finasteride for 7 days. (100x magnification)
Fig. 39  Lumen mass as a function of intraprostatic T and DHT in the Regrowth Study. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The dashed line represents the half-maximal increase in lumen mass (49 mg).
d. Hyperplasia

The second factor in ventral prostate growth is an increase in cell number. Hyperplasia in the Regrowth Study for the ventral prostate was determined by measuring the DNA content of each sample. Fig. 40 demonstrates that the amount of DNA per prostate increases as a function of intraprostatic T and DHT, and that there are thresholds for both androgens before increases from castrate levels of DNA per prostate are observed. The threshold for intraprostatic T occurs near 10 ng/g, while the threshold for intraprostatic DHT is 3 ng/g. Once again the slopes of the curves after the thresholds were reached were similar. The half-maximal increase in mass of DNA per prostate was denoted by the dashed line and the concentration of T at this point was 1.9 times higher than the concentration of DHT (Table V). At the highest intraprostatic concentrations, T resulted in the same amount of DNA/prostate as DHT, indicating again the ability of high concentrations of T to mimic the maximal androgenic effect of DHT.

The degree of both hypertrophy and hyperplasia differed in response to intraprostatic T compared to intraprostatic DHT. However, the difference was only in the range of 1.6-1.9 times. A functional advantage of 5α-R for prostate growth is not particularly reflected in these results. However, focusing on intraprostatic androgen levels ignores the relative ability of the prostate to concentrate the two androgens intracellularly. Therefore, the results were reinterpreted as a function of serum T. As Fig. 41 shows, when the intraprostatic androgen level is plotted as a function of serum T, DHT accumulates in the ventral prostate preferentially at low serum T concentrations. As serum T levels rise, the advantage of 5α-R for accumulation of DHT over T in the prostate diminishes. Half-maximal prostatic accumulation of DHT occurs at a serum T concentration of
Fig. 40 Mass of DNA per prostate as a function of intraprostatic T and DHT in the Regrowth Study. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The dashed line represents the half-maximal increase in DNA per prostate (317 µg).
Fig. 41 Prostate androgen concentration as a function of serum T in the Regrowth Study.

Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). The x-error bars represent the error associated with the serum T measurements, while the y-error bars are the error from the intraprostatic androgen results. The intraprostatic DHT dose-response data best fit a logarithmic curve, while the relationship within the intraprostatic T dose-response is best described as linear.
approximately 49 ng/dl, while half-maximal intraprostatic accumulation of T in the
presence of finasteride requires 4.6 times more serum T (227 ng/dl). Therefore, a major
role of 5α-R appears to relate to the accumulation of DHT at low serum T levels.

When prostate weight, lumen mass and μg DNA per prostate are expressed as a function
of serum T rather than intraprostatic androgen concentration, the results are considerably
different, as illustrated by fig. 42. In the absence of finasteride, all three parameters rose
quickly with increasing serum T and then approached a plateau. These data are best
represented by a logarithmic curve. In the presence of finasteride, considerably more
serum T was needed before a response was seen in prostate weight, lumen mass and μg
DNA per prostate. An exponential curve best describes this portion of the data. However,
if more data points were added after the highest dose, the curve would probably look
sigmoidal where a plateau similar to that seen in the rats not treated with finasteride, is
reached. Thus, in the absence of finasteride, the majority of prostate regrowth occurs at
low serum T concentrations. In the presence of finasteride, high levels of serum T were
required for prostate regrowth. Table VI displays the half-maximal levels of prostate
weight, lumen mass and μg DNA per prostate and the serum T concentrations, in the
presence and absence of finasteride, needed to achieve these levels. In the presence of
finasteride, 11-15 times more serum T was necessary to obtain the half-maximal
responses, than in the absence of finasteride. This result reflects the 1.6 - 1.9 times
difference in potency between the two intraprostatic androgens and the concentration of
serum T needed in the presence of finasteride to accumulate T in the prostate. Thus, 5α-R
appears to provide a modestly more potent androgen (DHT), but also leads to the
accumulation of androgen in the prostate at much lower levels of T in serum.
Fig. 42 Measures of prostate regrowth [lumen mass (A), DNA/prostate (B) and prostate weight (C)] as a function of the serum T concentration. Rats that received treatment with finasteride are represented by open squares (□), while rats that did not receive finasteride are represented by closed triangles (▲). The horizontal line indicates the half-maximal effect on each parameter of prostate growth. The data for rats not treated with finasteride correlate best with a logarithmic curve, while the results from rats treated with finasteride best fit an exponential curve.
Table VI. Serum T concentrations associated with the half-maximal responses in markers of prostate regrowth

<table>
<thead>
<tr>
<th></th>
<th>Half-maximal level (mg)</th>
<th>Serum T with Finasteride (ng/dl)</th>
<th>Serum T without Finasteride (ng/dl)</th>
<th>Ratio T:DHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate weight</td>
<td>141</td>
<td>406</td>
<td>36</td>
<td>11.2</td>
</tr>
<tr>
<td>Lumen mass</td>
<td>49</td>
<td>360</td>
<td>33</td>
<td>10.9</td>
</tr>
<tr>
<td>DNA content</td>
<td>317 (µg)</td>
<td>379</td>
<td>24</td>
<td>15.8</td>
</tr>
</tbody>
</table>
At high levels of serum T, there is little advantage to 5α-R conversion of T to DHT, because the intraprostatic androgen levels are the same and at these supraphysiologic levels, intraprostatic T is nearly as effective as intraprostatic DHT.

e. Androgen receptor staining

A possible mechanism to explain why intraprostatic DHT is more potent than T may be through regulation of AR levels. Fig. 43 shows examples of AR immunohistochemical staining in the ventral prostates from the Regrowth Study. The prostates are from a normal rat, a rat castrated 14 days previously, a castrated rat with a 0.5 mg T implant (approximately the half-maximal intraprostatic DHT concentration), a castrated rat with a 1.0 mg T implant and treated with finasteride (approximately the half-maximal intraprostatic T concentration), a castrated rat with a 15.0 mg T implant (maximal intraprostatic DHT for the dose-response) and a castrated rat with a 15.0 mg T implant and treated with finasteride (maximal intraprostatic T for the dose-response). These images represent the low (castrate), middle (half-maximal androgen replacement) and high (largest T implant) levels of AR staining. It is readily apparent that AR staining is found primarily in the nucleus of the lumenal epithelial cells. With normal intraprostatic DHT levels, the staining intensity in the ventral prostate of the normal rat is high and the color is bright red. In the castrated rat, there was no evidence of nuclear cellular staining. The red color in the lumen is non-specific staining of proteins in the lumen and blood vessels. In the rats that have small T implants, nuclear staining is evident in most of the epithelial cells but the intensity is very weak. As the T or DHT concentration in the prostate increases so does the intensity of the AR staining. In the prostates from rats with the half-maximal DHT concentration, the staining is definitely higher than in castrated rat
Fig. 43 Androgen receptor staining in ventral prostates from rats in the Regrowth Study. Images from A) a normal intact rat, B) a rat castrated 14 days, a rat castrated for 14 days and given a C) 0.5 mg T pellet, D) a 1.0 mg T pellet plus finasteride, E) a 15.0 mg T pellet, or F) a 15.0 mg T pellet plus finasteride for 7 days. (100x magnification)
but less than that seen in the normal rat. In the rats treated with finasteride that have a half-maximal T concentration, AR staining is evident but not to the same extent as with the same concentration of DHT. At the high concentrations of intraprostatic DHT and T the AR staining appears to match or almost match that seen with the normal controls.

T and DHT effects on AR levels were assessed by image analysis. Because the epithelial cells were the only cells that stain positively for the AR, the epithelial cell area was isolated from the images. By selecting only these cells, a more accurate measurement of the staining intensity for AR in the prostate can be made. Fig. 44 reports the percentage of epithelial cell area staining positively for AR plotted against intraprostatic T and DHT concentrations. Low concentrations of intraprostatic T or DHT display low AR staining levels and there is little evidence of a potency difference between T and DHT. As the intraprostatic androgen levels increase toward DHT levels in normal rats (~19 ng/g) and T levels in rats treated with finasteride (~16 ng/g), a difference in the AR staining intensity is apparent. Although there was a lot of variability at high concentrations of intraprostatic T and DHT (above androgen levels in normal animals), the level of staining is similar to that seen in the prostates of normal rats and a potency difference is not evident. Thus, these results can be interpreted to reflect a small potency difference between intraprostatic T and DHT similar to that seen in the other parameters measured in the Regrowth Study. The difference, however, is slight, once again underscoring the fact that the potency of T is higher than was predicted by in vitro studies (Deslypere et al. 1992; Yeh and Chang 1996) and in vivo studies done without a dose-response (George et al. 1991; Rhodes et al. 1993; Shao et al. 1994; Hausler et al. 1996).

Fig. 44
Fig. 44 AR staining as a function of intraprostatic T or DHT in the Regrowth Study. Rats that did not receive finasteride are represented by open squares □ (intraprostatic DHT), while rats that were given finasteride are represented by closed circles ● (intraprostatic T). For comparison, values for the normal and 14 day castrated rat are 0.202 ± 0.052 and 0.027 ± 0.011, respectively, and significantly different from each other (p<0.05).
f. DHT pellets and androgen accumulation in the prostate

It has been shown that T, and not DHT, is the major circulating androgen in the body and at high levels it can mimic the effects of DHT in the prostate. It has been suggested that T is necessary as the circulating androgen because DHT uptake in the prostate is less efficient (Lasnitzki et al. 1974), and that circulating DHT is less androgenic in the prostate, to the point of preventing cancerous lesions in the gland (Pollard et al. 1987; Pollard 1998). To investigate whether DHT can replace T as the major circulating androgen and the efficiency of DHT accumulation in the prostate, another dose-response study was performed. Rats were castrated and their prostates allowed to involute for fourteen days. DHT pellets were implanted under the scapular skin and the rats left for seven days before blood and ventral prostates were taken. The serum DHT dose-response was compared with the serum T dose-response in the absence of finasteride treatment (Fig. 45). The figure and the results in Table VII indicate that large DHT pellets were needed for rather modest increases in serum DHT concentration. The intraprostatic DHT concentrations achieved by varying T or DHT pellet size gave similar results (Fig. 46). Intraprostatic DHT increased with increasing T or DHT pellet sizes, but significantly larger DHT pellets were needed to sustain the DHT level. Based on these results it might appear that DHT does not enter the prostate with the same efficiency as T. However, expressing intraprostatic DHT as a function of serum T or DHT is very informative (Fig 47). The dose-response curves are virtually indistinguishable. Whether T or DHT is the androgen in serum, just as much DHT accumulates in the prostate. There is no efficiency difference in the ability of serum androgens to accumulate DHT in the prostate. Under these conditions, 5α-R does not appear to be a rate-limiting step for intraprostatic DHT formation from serum T. However, much larger DHT pellets than T pellets were needed
to achieve equal prostate and serum androgen concentrations. This result most likely reflects the rapid metabolism of DHT in tissues other than the prostate, particularly in muscle (Pasupuleti and Horton 1990).
Fig. 45  Serum T and DHT resulting from T and DHT pellets. The black bars represent serum T from T pellets, while the hatched bars are serum DHT from DHT pellets.

Fig. 46  Prostate DHT concentration resulting from varying sizes of T and DHT pellets. The T (black bars) and DHT (hatched bars) pellets both resulted in DHT accumulation in the ventral prostate.
Table VII. Comparison of serum androgen levels with size of pellet implant used in rats (values reported as mean ± SEM).

<table>
<thead>
<tr>
<th>Pellet Size</th>
<th>Serum T from T Pellets (ng/dl)</th>
<th>Serum DHT from DHT Pellets (ng/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 mg</td>
<td>6.4 ± 1.1</td>
<td>2.4 ± 0.4</td>
</tr>
<tr>
<td>0.3 mg</td>
<td>22.8 ± 2.3</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>0.5 mg</td>
<td>20.4 ± 2.3</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>1.0 mg</td>
<td>212 ± 18</td>
<td>6.9 ± 1.6</td>
</tr>
<tr>
<td>5.0 mg</td>
<td>447 ± 17</td>
<td>12.7 ± 5.3</td>
</tr>
<tr>
<td>15.0 mg</td>
<td>500 ± 10</td>
<td>27.8 ± 9.3</td>
</tr>
<tr>
<td>30.0 mg *</td>
<td></td>
<td>55.2 ± 2.9</td>
</tr>
<tr>
<td>60.0 mg *</td>
<td></td>
<td>71.5 ± 11.7</td>
</tr>
</tbody>
</table>

* Size was used only for DHT pellet
Fig. 47 Intraprostatic DHT concentration as a function of serum T or DHT. The closed triangles ▲ represent the results from using T pellet implants while the open circles ○ are the results from DHT pellet implants.
DISCUSSION

The relative potency of T and DHT in prevention of ventral prostate regression

As discussed in the introduction, androgens in the prostate play a dual role of inhibiting cell death and promoting cell growth. DHT has been determined to be the active androgen in the prostate based on its higher intraprostatic concentration, selective retention in the nuclei of prostate cells (Anderson and Liao 1968; Bruchovsky and Wilson 1968) and the observation that men who cannot form DHT have small prostates (Imperato-McGinley et al. 1979). Androgen action is carried out by the AR, which acts as a transcription factor to enhance or repress gene transcription. The AR can bind both T and DHT but has higher affinity for DHT and is more stable when bound by DHT (Carlson and Katzenellenbogen 1990; Grino et al. 1990; Zhou et al. 1995). Based on this and other studies in vitro showing that DHT has 10-fold greater transcription activation activity (Deslypere et al. 1992), the effect of high concentrations of T from 5α-R inhibition was initially thought to be negligible (Geller and Sionit 1992).

In the rat, inhibitors such as finasteride reduce DHT concentrations in the prostate to the level seen in a castrated animal; a concentration that has been shown not to be androgenic (Kyprianou and Isaacs 1987a; Kyprianou and Isaacs 1987b). As a result of such inhibition, the prostate is reduced in size, but not to the same extent as with castration (Brooks et al. 1981; Wenderoth et al. 1983; Lamb et al. 1992; Shao et al. 1993; Rittmaster et al. 1995). Along with the reduction in intraprostatic DHT levels, 5α-R inhibition results in a near reciprocal rise in intraprostatic T. The serum T level in a
finasteride-treated intact rat is essentially the same as that seen in an intact rat, but the intraprostatic T level has risen nearly 20-fold. The androgenic ability of T is the most likely reason 5α-R is not as effective as castration. Prior to this work, the relative potency of T and DHT had not been fully assessed *in vivo*.

In order to assess the relative potency of T and DHT on any parameter of androgen action in the ventral prostate, accurate delivery of T had to be assured. At the time these studies were initiated, silastic tubing filled with T was the preferred method of long-term steroid delivery. The technique for producing these implants was learned in Dr. B. Robaire's lab at McGill University in Montreal, PQ. The length of the silastic tube implant was supposed to correlate with the serum T concentration. Factors that affect the serum T level associated with a particular length of tubing include the packing tension and level of handling. Dr. Robaire also cautioned that the tubing membrane becomes overloaded with T and that after surgically implanting the steroid-filled tubing under an animal's skin, there was a massive release of T which leveled after a few days. Thus, a large number of these implants would be put into a host rat to allow the membranes to equilibrate and then they were transferred to new rats for study. Owing to the variability and complexity of this technique, it was decided to use commercially available constant release T pellets instead (Innovative Research of America, Sarasota, FL). The constant release for a given pellet size was confirmed in castrated rats and the results indicated that the pellets were a superior alternative to silastic tubes for delivery of constant levels of T supplementation. Various sizes of T pellets were then used in the castrated rats to achieve a range of serum T levels. Concurrent finasteride treatment was used to block DHT formation in the
prostate and a dose-response for intraprostatic T was accomplished. In the absence of
finasteride treatment, a dose-response for intraprostatic DHT was attained from the
various levels of serum T.

In the Regression Study, the ability of a range of intraprostatic T levels to inhibit atrophy
and apoptosis in the ventral prostate was compared with that for a range of intraprostatic
DHT concentrations. The rats were castrated and immediately given a T pellet and the
rats being examined for intraprostatic T were given an injection of finasteride. The
treatment was continued for 4 days, which is the peak time period for cell death
(Rittmaster et al. 1991; Furuya and Isaacs 1993; Guenette et al. 1994; Rittmaster et al.
1995). The results from the Regression Study indicate that the relative potency of these
two androgens depends on the endpoint being measured.

One of the concerns with the model for the Regression Study was that the small amount
of DHT present in the prostate after treatment with finasteride might be enough to
potentiate the high concentration of T. Results indicated, however, that low levels of T or
DHT are unable to significantly inhibit cell atrophy or apoptosis in the model. Low
concentrations of T and DHT in both the dose-response studies had no significant effect
on maintenance of prostate weight, lumen mass, or the markers of apoptosis. Therefore,
the low DHT concentration within the prostates of the T dose-response experiment is
unlikely to enhance T effectiveness. In previous in vivo studies it was demonstrated that
the level of intraprostatic DHT that results from castration is not high enough to have an
effect on inhibition of cell atrophy, inhibition of apoptosis or cell proliferation
(Kyprianou and Isaacs 1987a). Kyprianou et al. (1987a) also showed that complete elimination of androgens in male Sprague Dawley rats by castration and adrenalectomy did not induce any further reduction in the prostates than castration alone. DHT appears to have a critical threshold above which the prostatic concentration must rise in order to stimulate prostatic cell number.

Prostate weight is a global measure of androgen action in the prostate. Two weeks after castration, the prostate shrinks to 10% of its original weight (Rittmaster et al. 1995). In the Regression Study, after four days of castration the prostate had regressed by 54%, while in intact rats treated with finasteride for four days, the mean weight reduction was 31%. Prostate weight in rats four days after castration and implantation of various sizes of T pellets in the absence of finasteride ranged from 48% to 120% of the weight in a normal intact rat. After four days, the prostate weight in the rats castrated, given T implants and treated with finasteride, ranged from 46% to 104% of the weight in an intact rat. The prostate weight reduction in the rats showed a linear relationship with the corresponding intraprostatic DHT or T levels. However, the concentration of intraprostatic T required to maintain normal prostate weight was 2.4 times the concentration of DHT, indicating that in the prostate, DHT is 2.4 times more potent than T for prevention of regression.

Prostate weight gives an overall estimate of androgen action, but it encompasses both atrophy and apoptosis in the regressing prostate. To get a clearer depiction of the relative
potency of intraprostatic T and DHT in the regressing prostate, separate measures of atrophy and apoptosis were made.

a. Atrophy in the ventral prostate

Lumen mass is a morphometric measure of the secretory activity of the luminal epithelial cells. In the castrated rat, the lumen atrophies significantly, the ducts shrink in size and display infolding where the epithelial cells push into the lumen. After four days, finasteride-treated intact rats show a reduction of lumen mass of 32% compared to intact controls. Castration, by comparison, reduced lumen mass by 52%. For the intraprostatic T and DHT dose-responses, the decrease in lumen mass with decreasing androgen concentration was linear, and the slope for intraprostatic DHT was 2.4 times higher than that for intraprostatic T. Similar to the response in prostate weight, the concentration of intraprostatic T required to maintain normal lumen mass was 2.5 times higher than the concentration of intraprostatic DHT, indicating a potency difference of 2.5.

Epithelial cell mass is a morphometric measure of atrophy within the androgen-dependent luminal epithelial cells. The epithelial cell mass decreased by 26% after four days of finasteride treatment and 63% after four days of castration. For the intraprostatic T and DHT dose-responses, epithelial cell mass decreased with decreasing androgen concentration but there was no clear difference in potency for the two androgens. In determining the mean epithelial cell area, the random sampling for each prostate does not differentiate between proximal and distal ducts. The proximal ducts contain epithelial cells that are largely androgen-independent and do not undergo apoptosis after castration.
(Lee et al. 1990). These epithelial cells in the proximal ducts are squamous even in the intact rat but still have normal lumen sizes. Thus, analysis of the separate regions of the prostate in the Regression Study may have shown a difference in epithelial cell mass in the intraprostatic T and DHT dose-response experiments.

PSBP mRNA has been used as a marker of androgen action and secretory activity in lumenal epithelial cells (Rittmaster et al. 1991). Castration and finasteride treatment reduce the levels of PSBP mRNA by 90 and 26%, respectively. As expected, the mRNA level decreases with decreasing androgen concentration in the Regression Study, but unexpectedly, no difference was seen in the ability of T and DHT to maintain PSBP mRNA expression. The variability in the slot blot results could mask a small difference in potency but not the ten-fold difference seen with in vitro studies of AR transfected into cells (Deslypere et al. 1992; Yeh and Chang 1996). However, if indeed T and DHT as AR ligands are equipotent in their action on PSBP transcription, it would be interesting to compare features of the PSBP promoter with the MMTV promoter, where T and DHT have a 10-fold difference in activity. Regulatory regions of the PSBP-C3 gene contain several progesterone and glucocorticoid response element-like sequences that can act as AREs. These are present both in the 5'-flanking region and in the first intron. One of the intronic AREs is very similar to a potent response element in the MMTV gene (Tan et al. 1992). Differences in liganded AR behavior with PSBP and MMTV promoters might depend on the complexity and context of the promoters and possibly also on the ability of co-activators to interact with the ligand-receptor complex and the basal transcription machinery (Katzenellenbogen et al. 1996).
By comparing epithelial cell size and PSBP mRNA expression, there appeared to be little
difference between T and DHT in terms of their potency. Four days of treatment, it could
be argued, are not enough to see a difference in potency between the effect of the two
androgens in epithelial cell mass or PSBP mRNA expression. However, a measure of
overall secretory activity such as lumen mass, that is physical and not dependent on
particular gene expression levels, gives a more representative picture of altered cell
physiology. The secretions involve numerous components, not just PSBP. Thus, as
measured by lumen mass, DHT is approximately 2.5 times more potent than T for
maintaining secretory activity.

b. Apoptosis in the ventral prostate
An unexpected result was the lack of a potency difference between T and DHT in
preventing programmed cell death in the prostate. Measurement of two different
apoptotic characteristics, along with an evaluation of the loss in DNA per prostate was
used to quantify the dose-response effects for intraprostatic T and DHT. TRPM-2 has
long been associated with the degree of apoptotic activity in the prostate and peak
expression occurs approximately four days after castration (Leger et al. 1987; Rittmaster
et al. 1991; Tenniswood et al. 1992). In the intact rat and intact rat treated with
finasteride, TRPM-2 mRNA expression was low to undetectable (Rittmaster et al. 1991;
Shao et al. 1993; Russo et al. 1994; Shao et al. 1994). The dose-responses for
intraprostatic T and DHT showed that TRPM-2 mRNA expression levels increased as
androgen levels fall but both T and DHT were able to maintain normal levels of TRPM-2
expression at the same concentration. More data points in the steep part of the curve for TRPM-2 mRNA expression may have indicated a small difference in potency, but this was not possible based on the T pellet implants available. However, the androgen concentration by which TRPM-2 mRNA expression is returned to normal levels is only a little over half the intraprostatic DHT concentration in an intact rat and also just over half the intraprostatic T concentration seen in intact rats treated with finasteride. This result provides one explanation for the fact that finasteride treatment does not reduce prostate weight as much as castration; T is equipotent with DHT in its ability to prevent apoptosis. Finasteride treatment does not reduce T in the prostate to the point at which high levels of TRPM-2 mRNA are expressed.

Fragmentation of DNA, a hallmark of apoptosis, was detected by TUNEL immunostaining. The level of TUNEL staining in intact rats and intact rats treated with finasteride was low but more epithelial cells stain positively with finasteride treatment than without. As expected, the castrated rats had the highest level of epithelial cells staining for DNA fragmentation and this dropped as the androgen level increased in the T-implanted animals. Similar to the results with TRPM-2 mRNA, T showed no difference from DHT in the concentration that reduces TUNEL staining. The concentration of intraprostatic T or DHT at which TUNEL staining returned to near baseline levels was 10 ng/g for both androgens.

The results from the DNA per prostate measurement were consistent with the markers of atrophy discussed above. Castration resulted in the largest loss in the mass of DNA per
prostate. In the T and DHT dose-responses, DNA content was reduced in a non-linear manner as androgen levels fell and there was little difference between results for intraprostatic T or DHT. Although four days after castration is the peak for apoptosis markers, the prostate regress for two weeks before it reaches its nadir. Thus, the time point may be early to detect subtle differences in potency, and large differences may still be evident.

Results from the markers of apoptosis and DNA per prostate indicate that intraprostatic T can inhibit apoptosis and cell loss at the same concentration as intraprostatic DHT. This concentration is approximately half the intraprostatic DHT level in an intact control rat and just over half the intraprostatic T concentration associated with finasteride treatment in an intact rat. Unless the concentration of either intraprostatic DHT or T is reduced below this level, apoptosis will not occur in large amounts. Consequently, the decrease in prostate weight after finasteride treatment is mostly due to atrophy, although there is a slightly higher than normal level of apoptosis. Antiandrogens, such as flutamide, also appear to result in prostate shrinkage without significant TRPM-2 induction or apoptosis. Treatments that lower serum T levels, such as DES, did result in prostatic apoptosis and TRPM-2 expression (Russo et al. 1994).

The results from the Regression Study show that AR regulation of TRPM-2 does not show ligand specificity. The actual mechanisms by which T and DHT suppress TRPM-2 expression and apoptosis are unknown. It is not at all certain that the increase in TRPM-2 expression after castration has a direct relationship to the apoptotic machinery. Indeed,
TRPM-2 appears to be more analogous to bcl-2. In the rat prostate, the proximal ducts are lined with relatively androgen-independent epithelial cells that normally express high levels of TRPM-2 protein (Sensibar et al. 1991). Overexpression of TRPM-2 protein in LNCaP cells was able to prevent TNF-α induced cell death (Sensibar et al. 1995). Unlike LNCaP cells, PC-3 cells are not responsive to TNF-α. When examined for TRPM-2 protein levels, it was observed that PC-3 cells constitutively express high levels and that incubation with TRPM-2 antisera renders these cells sensitive to the pro-apoptotic signal of TNF-α (Sintich et al. 1999). Thus, castration results in up-regulation of survival factors in an attempt to subjugate the signal to undergo apoptosis. Presumably, the cell survives if TRPM-2 protein expression is high enough, but the mechanism of action has yet to be elucidated.

It is possible that androgens directly suppress expression of “death” genes, or that they regulate anti-apoptotic genes. The anti-apoptotic gene bcl-2 demonstrates androgen-responsiveness either directly or indirectly (McDonnell et al. 1992). Castration in rats results in an increase of bcl-2 mRNA which can be reduced by the addition of exogenous T (McDonnell et al. 1992). Bax, a Bcl-2-related protein, has been shown to inhibit the anti-apoptotic property of Bcl-2 (Boise et al. 1995). Recently, in the prostate the ratio of bax to bcl-2 expression has been shown to determine a cell’s fate (Perlman et al. 1999). After castration the level of bax rose significantly and declined after three days, while bcl-2 mRNA remained elevated for up to seven days. The same results were found using the Bax and Bcl-2 protein levels (Perlman et al. 1999). Once the direct target genes for
androgen action in apoptosis suppression are identified, it will be of interest to investigate the action of T or DHT-bound AR on transcriptional regulation of these genes.

In the Regression Study, antibodies for Bcl-2 and Bax were used to stain the paraffin embedded prostates sections. It was expected that the epithelial cells in prostates from normal intact controls would not exhibit Bcl-2 staining, while the prostates from castrated animals would have high levels of Bcl-2. Despite manufacturer’s claims that these antibodies would work in paraffin-embedded tissues, extensive testing with numerous methods of staining and several dilution series could not resolve adequate and reliable results. It was finally determined that the antibodies were not specific in the rat paraffin-embedded prostates.

The discrepancy between the rapidity of the drop in androgen levels after castration and the delayed peak activity of apoptosis belies direct action of androgen in preventing cell death, although TGF-β provides a possible model for direct androgenic action on apoptosis. TGF-β has been shown to result in prostatic cell death in culture (Martikainen et al. 1990; Sutkowski et al. 1992; Byrne et al. 1996) and androgen regulation of the TGF-β gene is implied through its increase in expression after castration (Nishi et al. 1996). An indirect mechanism for the involution of the prostate after androgen withdrawal is through hypoxia induced by reduced blood flow (Shabsigh et al. 1998). After castration, changes in prostate vasculature are rapid and involve apoptosis of endothelial cells (Shabsigh et al. 1999). The trophic factor for vascularization in the prostate, vascular endothelial growth factor (VEGF), is regulated by androgens,
presumably through the AR (Sordello et al. 1998). Androgen withdrawal after castration results in decreased expression of VEGF from epithelial cells in the prostate, which in turn results in endothelial cell apoptosis and reduced blood flow. The hypoxic environment is then a trigger for large waves of apoptosis. The reduction in blood flow is gradual, which explains why apoptotic death occurs rather than necrotic death and ischemia. The prostate regresses to the level that can be sustained by the extent of the vasculature.

The Regression Study shows that DHT is 2.4-times more potent than T in maintaining prostate mass. This may be related to effects in the vasculature. However, it is not related to differential effects on apoptosis inhibition.

The relative potency of T and DHT on regrowth of the rat ventral prostate

The results from the Regression Study suggested that while T and DHT were equipotent in inhibition of apoptosis, they might not be equipotent in promoting growth of the prostate. The Regrowth Study addressed the relative potency of intraprostatic T and DHT on regrowth of the fully involuted prostate.

The results from the Regrowth Study indicate a small potency difference between T and DHT. The conclusion must be qualified, however, because at very high concentrations T can mimic the action of DHT on prostate growth. Before a significant increase in prostate weight occurred, a threshold for intraprostatic T and DHT had to be reached. The threshold for intraprostatic DHT was less than half that for intraprostatic T. After the
threshold, though, the slopes for the increase in weight as a function of intraprostatic androgen concentration were nearly identical. Using the half-maximal increase in prostate weight to compare potency, DHT was 1.8-fold more potent than T.

**a. Hypertrophy**

Just as there are two components to prostate regression, prostate regrowth consists of hypertrophy and hyperplasia. The morphometric measure lumen mass was used to assess the relationship between secretory activity and intraprostatic T or DHT concentration. The threshold required before intraprostatic T significantly increased lumen mass was twice the threshold for intraprostatic DHT. However, after the threshold was achieved, the linear regression curves for intraprostatic T or DHT *vs.* lumen mass had similar slopes. The same phenomenon was evident for prostate weight. The potency difference between intraprostatic T and DHT as measured by the half-maximal increase in lumen mass, which reflects secretory activity, showed that DHT was 1.6 times more potent than intraprostatic T. Lumen mass is a global marker for secretory activity. It would have been of interest to investigate the dose-responsiveness of T and DHT in multiple individual components of rat prostatic secretions, such as PSBP, phosphatases and other enzymes. A preliminary experiment in prostate regrowth suggested that DHT was more potent than T in induction of PSBP mRNA, but no dose-response was carried out (Manning 1995).

**b. Hyperplasia**

Hyperplasia was measured by the mass of DNA per prostate. Similar to the results seen for prostate weight and lumen mass, there is a threshold concentration required for T and
DHT before a significant increase in DNA per prostate was seen. However, the threshold for intraprostatic T is close to three times that for intraprostatic DHT. The difference in potency for stimulation of a half-maximal increase in the mass of DNA per prostate shows that DHT is 1.9-fold more potent than intraprostatic T.

The threshold required for DHT to stimulate prostate growth was much higher than the concentration of intraprostatic DHT found after 5α-R inhibition. Simply put, the androgen effects in the prostates of the rats treated with finasteride are due to the intraprostatic T concentration since the DHT concentration is too low to be effective.

As was seen in the Regression Study, intraprostatic DHT was somewhat more potent than intraprostatic T in promoting secretory activity during regrowth of the prostate. In contrast, although the Regression Study showed no difference in the ability of intraprostatic T and DHT to inhibit apoptosis, there was a difference in the ability of these androgens to promote cell growth. DHT in the prostate is nearly twice as potent as intraprostatic T, although T at very high concentrations is able to mimic the action of DHT. The overall role of 5α-R in prostate physiology is not readily apparent from the potency difference between intraprostatic T and DHT. However, consideration of serum T levels revealed that another consequence of 5α-R activity is the increased accumulation of DHT in the prostate at low serum T concentrations. DHT accumulation from serum T is best described as logarithmic, while the relationship between serum T and intraprostatic T is linear. If 5α-R in the prostate is inhibited, 4.6 times more serum T is needed to accumulate the same half-maximal concentration of T in the prostate as DHT.
When the markers of prostate regrowth are expressed in terms of serum T levels, the serum T concentration required for the half-maximal responses of prostate regrowth is 10.9 – 15.8 times higher in the presence of finasteride than in its absence. In contrast, the potency difference based on the intraprostatic levels of the two androgens is only 1.6 – 1.9 fold. Expressing the results in terms of the serum T levels encompasses both the intraprostatic potency and the enhanced ability of the prostate to accumulate and retain DHT. Thus, the role of 5α-R is two-fold: to convert T into a modestly more potent hormone and to promote the accumulation of DHT at low serum T concentrations.

The most likely explanation for the increased retention of DHT in the prostate is that DHT has a higher affinity for the AR and is more capable of stabilizing the AR complex (Carlson and Katzenellenbogen 1990; Zhou et al. 1995). Stabilizing the receptor complex against degradation means that DHT is able to increase the time it is bound within the nucleus, and with a lower turnover rate, less serum T is needed to maintain the supply of androgen within the prostate. The results for AR staining in the Regrowth Study indicate that AR levels were lower in the rats treated with finasteride and mid-size T pellets. At higher androgen concentrations, intraprostatic T appears to be able to promote AR expression at approximately the same potency as DHT, however, there was considerable variability in the measurements. Investigation with other AR antibodies and antigen retrieval systems would be informative.

Proliferation in the prostate is a consequence of the presence of androgens. However, as has been discussed, the relationship may not be direct and probably involves paracrine
stromal-epithelial interactions and growth factors. KGF has been implicated as a stromal-
epithelial mediator of androgen action (Sugimura et al. 1996). The evidence for growth
factor, specifically KGF, involvement in mediating androgen induced growth is: (a)
epithelial cells require the stroma for growth, differentiation and function (Cunha et al.
1987); (b) KGF is produced in the stroma but the receptor (FGF-R2) is only expressed on
epithelial cells (Yan et al. 1992); (c) in organ culture, KGF stimulates ductal
morphogenesis with about 70% of the efficiency of T (Sugimura et al. 1996); (d)
androgen-induced morphogenesis is blocked by a neutralizing antibody to KGF
(Sugimura et al. 1996); and (e) activity of the KGF promoter transiently transfected into
LNCaP cells can be stimulated up to eleven-fold by the synthetic androgen R1881
(Fasciana et al. 1996). KGF is not present in epithelial cells in the normal human
prostate, but is found in epithelial cells in BPH and prostate cancer. AR is co-localized in
the BPH and cancer cells that express KGF. This supports the notion that KGF action
may change from paracrine to autocrine as cells become malignant (Rennie and Nelson
1998; Planz et al. 1999).

KGF regulation has been shown to be mediated by androgens, and antiandrogens can
inhibit KGF-mediated growth (Thomson et al. 1997). The regulation of KGF-mediated
growth in the prostate is more complicated than a direct relationship between AR levels
and KGF expression. Recently, a study has shown that KGF mRNA and protein levels do
not decrease significantly after castration (Nemeth et al. 1998). However, the regulation
of KGF signaling for proliferation may be at the KGF-receptor level in the androgen-
dependent epithelial cells. This concept needs experimental confirmation because to date,
no study has examined KGF-receptor levels in castrated and androgen replaced animals. If the KGF axis is indeed regulated by androgens, the reduced proliferative response seen in the T dose-response of the Regrowth Study may be due to decreased KGF responsiveness (i.e. reduced KGF-receptor levels). AR interactions with the KGF system may prove to be ligand-dependent but further study is needed to assess this.

The IGF axis is also a candidate for a role in proliferation in the ventral prostate. Although this system has not been extensively studied in the normal rat prostate, it has been shown that IGF-I and II stimulate epithelial cell proliferation in vitro (Cohen et al. 1991). The regulation of IGF action in the prostate is complex because of its interaction with several IGFBPs (Peehl et al. 1995). Castration and 5α-R inhibition increase the concentration of IGFBPs and suppress mRNA levels of IGF-I and its receptor (Huynh et al. 1998; Thomas et al. 1998). Thomas et al. (1998) were able to show, however, that the peak in IGFBP-5 immunostaining is later than the peak in apoptotic activity, suggesting that IGFBP-5 functions as an inhibitor of cell proliferation rather than as a signal for apoptosis. The high levels of IGFBP staining after finasteride treatment offer an additional mechanism for the reduced prostatic cell proliferation observed in the rats from the T dose-response in the Regrowth Study. However, it is not unanimously agreed that IGFs serve a proliferative function in the rat prostate: Foster et al. (1998) suggest that IGFs maintain terminal differentiation and inhibit apoptosis, but do not serve a proliferative role.
Mechanisms for differential effects of T and DHT on the prostate

The *in vivo* effects that are reported here differ from previous *in vitro* studies. Deslypere *et al.* (1992) used CHO cells co-transfected with a plasmid encoding AR cDNA and a reporter plasmid containing the androgen-responsive MMTV promoter to determine whether binding parameter differences could explain the physiologic differences between T and DHT action. CHO cells show very little conversion of steroids to other metabolites and finasteride was added to prevent even trace amounts of DHT forming from T administration. Both hormones achieved the same maximal reporter gene activity but DHT was approximately ten times more potent than T for half-maximal activity. In an examination of the relative binding affinities of various androgens and androgen analogues to the rat AR from cell preparations, T was found to have a ten-fold less affinity than DHT (Carlson and Katzenellenbogen 1990). Grino *et al.* (1990) used cultured fibroblasts from the genital skin of a 5α-R deficient male to measure T interaction with the human AR. The fibroblast monolayer has abundant AR but very little 5α-R and what residual activity remained was further inhibited by the addition of finasteride. It was found that T and DHT have the same maximal binding to receptor but the affinity of T binding is about half that of DHT. Kinetic experiments indicated that T exhibits a five-fold faster dissociation rate from AR than DHT. Thermolability experiments, which measure receptor complex stability, demonstrated that ten times the concentration of T was required to mimic the stability of receptor seen with DHT. Zhou *et al.* (1995) used recombinant AR in COS cells to investigate the relative effectiveness of T and DHT in receptor stabilization. It was demonstrated that the half-maximal dissociation rate was three times faster for T than DHT and that T was less effective at
stabilizing the receptor against degradation. These results along with those of Grino et al. (1990) lead to the conclusion that the DHT-AR complex was more potent than the T-AR complex and that the decreased potency of T could be overcome by mass action. The results for measurement of prostate weight and atrophy in the Regression Study support this conclusion, but the fact that T and DHT are equipotent in the prevention of cell death was not predicted by any of these in vitro models. It appears that the relative potency of T and DHT for regulation of the genes involved in apoptosis suppression in the prostate is different from their relative potency for the genes involved in prostate secretory activity.

The influence of the ligand on nuclear receptor structure and action is beginning to be understood. For example, the crystal structure of the ligand-binding domain of the ER has been shown to undergo distinct conformational alterations in the transactivation domain, depending upon whether the ligand bound was an agonist or an antagonist (Brzozowski et al. 1997). The specific effects of different androgen agonists and antagonists are likely to vary depending on the particular AR conformation achieved with a given ligand, as well as with the promoter context and complexity, and the cell type specificity of sensitive genes. Thus, T may be more or less potent than DHT in regulation of different genes. Recently, Avila et al. (1998) have identified genes in the prostate that are in fact modulated differently by finasteride treatment. Using differential display PCR, they have classified genes that are upregulated only in intact rats and genes that are upregulated only with finasteride treatment, thereby comparing DHT vs. T actions in the whole animal. Individual genes identified as being differentially regulated have not yet been identified. However, it must be kept in mind when studying individual genes that in
complex responses such as promotion of growth, or inhibition of apoptosis, many
different androgen-responsive genes are probably involved, and it is always important to
show whether an alteration in expression of a given gene is causal or peripheral to the
physiological process being observed.

**DHT accumulation in the prostate as a function of circulating**

**T or DHT**

Since DHT is more potent than T for most endpoints, the question arises as to why DHT
is not the major circulating androgen in males. The present study shows that serum T and
DHT can serve equally well as precursors for intraprostatic DHT in castrated rats.
However, an important disadvantage for the role of DHT as the major peripheral
androgen is that it is not aromatizable to estrogen. Estrogen has been demonstrated to be
essential for normal bone maturation in male rodents and men and for normal fertility in
male rodents (Smith et al. 1994; Morishima et al. 1995; Carani et al. 1997; Lindzey and
Korach 1997). Another disadvantage to having DHT as the major circulating androgen is
its rapid metabolism in tissues other than the prostate. For example, in muscle, T has been
considered to be the active androgen because of the low 5α-R activity (Russell and
Wilson 1994) and low DHT levels in this tissue (Saartok et al. 1984). Conversion of
DHT to androstanediol, catalyzed by 3α-hydroxysteroid oxidoreductase, predominates in
muscle, while the reverse reaction predominates in prostate (Pasupuleti and Horton
1990). Therefore, given the large mass of muscle tissue in relationship to prostate tissue,
it is not surprising that the resulting serum DHT concentrations are much lower than the
those of T.
**Contribution of estrogen to prostate physiology**

Because T can be metabolized to estradiol, it is possible that the intraprostatic T dose-responses seen in both studies reported here may not be due solely to the effects of T but also to the effects of estrogens. The effect of estrogen on the prostate is unclear and appears to depend of the animal used. Excess estrogen in the canine model will act synergistically with androgens to produce glandular hyperplasia (Walsh and Wilson 1976). In the Noble rat, dysplasia and adenocarcinomas of the prostate can be induced by the combination of estrogen and T administration (Lau et al. 1998). One caveat to the estrogen synergism with T for hyperplastic formations is that it is unlikely that these two steroids would co-exist at such high levels because negative feedback of estrogen would reduce T production. Nevertheless, estrogen action on the prostate is presumably is mediated through its receptors. In the rat, ERα has been localized immunohisto-chemically to the periductal stromal cells and not to epithelial cells (Chang et al. 1999).

In contrast, ERβ is found exclusively in the epithelial cells (Prins et al. 1998). The distribution of ERβ also varies along the proximal distal axis of the prostate with the highest levels in the androgen-dependent distal region and the lowest in the less androgen-sensitive proximal region. The levels of ERβ are controlled by androgen and not estrogen as shown by the observation that castration reduces ERβ mRNA levels by 24 h and androgen replacement rapidly restores these levels (Prins et al. 1998; Shughrue 1998). Chang and Prins speculate that ERβ is a marker of epithelial cell differentiation (Chang and Prins 1999).
The affinities of each form of estrogen receptor for estrogens in the prostate are similar (Kuiper et al. 1996). Because the serum T concentration is similar in normal rats and those treated with finasteride, any increase in estrogen concentration would have to arise from local aromatization of androgens to estrogens. Aromatase was found in both the stroma and epithelium of human prostates by immunocytochemistry by Matzkin et al. (1992). However, a subsequent study using more sensitive techniques, did not detect aromatase in the epithelium (Hiramatsu et al. 1997). In addition, this study only detected the presence of aromatase and aromatase activity (which do not necessarily coincide) in the stroma of hyperplastic and carcinomatous glands. Using direct measurement of estrogen production, Brodie et al. (1989) could find no evidence of aromatase activity in human prostate, even when T metabolism was inhibited with 5α-R inhibitors. In addition, aromatase inhibitors have no effect on canine BPH (Oesterling et al. 1988) and have little effect on human BPH (Schweikert et al. 1993; Radlmaier et al. 1996). Although it would be unlikely that estrogens could mimic the ability of androgens to prevent apoptosis in the rat prostate, a role for local estrogen production in the T dose-response experiments reported here cannot be ruled out.
CONCLUSIONS

The Regression Study

Inhibition of 5α-R results in a decrease in the prostatic DHT concentration and an increase in intraprostatic T. The Regression Study examined the relative potencies of T and DHT in prevention of prostate regression after castration. The results showed that DHT is 2.5 times more potent than prostatic T in prevention of prostate regression from atrophy and in maintenance of epithelial cell secretory function. However, both intraprostatic T and DHT were able to inhibit apoptosis with equal potency. The intraprostatic androgen concentration at which apoptosis is reduced to near normal levels is half that of an intact rat treated or not with finasteride. This study provides an explanation for the inability of 5α-R inhibition in an intact animal to reduce prostate weight to the same extent as castration, despite the reduction of DHT to levels seen with castration.

The Regrowth Study

The Regression study suggested a role for 5α-R in prostate proliferation. The Regrowth Study clarified this role by demonstrating that: (1) the threshold concentration for intraprostatic T before significant growth occurs is over twice that for intraprostatic DHT, (2) DHT in the prostate is approximately twice as potent as T in the prostate in stimulating a half-maximal increase in both prostate secretory activity and proliferation, (3) very high concentrations of T can mimic the effects of intraprostatic DHT, (4) 5α-R allows accumulation of DHT in the prostate at low serum T concentrations while in the
presence of 5α-R inhibition, significantly higher serum T is needed to accumulate T in the prostate, and (5) when prostate growth is expressed as a function of serum T levels, in the presence of 5α-R inhibition, 10-15 times more serum T is needed for half-maximal stimulation of prostate growth. Thus, the role of 5α-R is two-fold: to convert T to a more potent androgen and to allow accumulation of DHT in the prostate at low serum T concentrations.

**DHT vs. T in the serum**

Because DHT is more potent than T for most endpoints, the question of whether DHT could replace T as the major circulating androgen was examined. Significantly larger DHT implants were needed to achieve modest increases in serum DHT levels in castrated rats, however, DHT from serum accumulates in the prostate with the same efficiency as DHT from serum T in the absence of finasteride. Therefore, while DHT could replace T as the major circulating androgen, the production rates would have to be immense because of the rapid peripheral metabolism of DHT. The other major disadvantage to having high levels of DHT in circulation is the fact that it cannot be aromatized to estrogen. Estrogen has been demonstrated to be essential for normal fertility and bone maturation in males. Thus, 5α-R allows normal virilization of the external genitalia and prostate with high circulating levels of T for use in tissues other than the prostate where T acts as a prohormone for estrogen for fertility and bone maturation.
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