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CHARACTERIZATION OF TRPM-2, A GENE INVOLVED IN THE REGRESSION OF THE RAT VENTRAL PROSTATE AFTER CASTRATION.

by

JOCELYNE G. LÉGER

submitted in partial fulfilment of the requirements for the

degree of

Doctorate of philosophy

in Biochemistry

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UNIVERSITÉ D'OTTAWA
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ABSTRACT

The normal growth and function of the rat ventral prostate (RVP) are under the control of androgens. After castration, the prostate undergoes regression via the process of apoptosis. This cell loss involves the luminal epithelial cells at the distal region of the prostatic ducts. The androgen depletion also results in a decrease of the expression and mRNA levels of prostate specific genes, such as prostate steroid-binding protein (PSBP). Prostate regression has been shown to be an active process requiring RNA and protein synthesis. A number of castration specific mRNA species have been identified in the RVP. The most abundant of these Testosterone Repressed Prostate Messages is 2000 nucleotides in size, and is named TRPM-2. In this study, a cDNA clone specific for TRPM-2 has been isolated and sequenced, and used to study the expression of TRPM-2 after castration and anti-androgen treatment by Northern and in situ hybridization analysis. The expression of TRPM-2 is maximal when the rate of cell death is greatest, and is localized in the luminal epithelial cells of the distal region of the prostatic ducts. These results imply that TRPM-2 plays an active part in the apoptotic process. Sequence analysis suggests that TRPM-2 is a membrane associated protein which likely plays a role in maintaining membrane integrity during the remodeling stages of apoptosis.

DEDICATION

A mes parents, qui m'ont donné " l'éducation du coeur", et qui ont fait de moi ce que je suis aujourd'hui

et

A Pierre, qui a su se montrer encourageant et très patient, et qui, avec sa constante bonne humeur, a toujours pu me faire rire, même pendant les moments les plus difficiles.

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Schering AG for the gift of cyproterone acetate.

Schering Corporation for the gift of flutamide.

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Felix Bursch for the stimulating discussions, and for giving me a more complete vision of cell death and apoptosis.

My supervisor, Dr. Martin Tenniswood, for all his help and guidance, for being supportive and encouraging when the going was tough and results scarce, for being enthusiastic when good results finely rolled in, but mostly I thank him for making these past 5 years fun.

TABLE OF CONTENTS

1. INTRODUCTION

1.1. ANDROGENIC EFFECTS ON THE PROSTATE

Current interest in the prostate and the mechanism of action of androgens on the gland was initiated by the observation that androgen ablation was effective in controlling the spread of prostatic cancer (PC) (Huggins & Hodges, 1941; Huggins, Stevens & Hodges, 1941). This disease and benign prostatic hyperplasia (BPH) are very prevalent in men of 50 years and older. There is evidence of BPH in approximately 80% of males over the age of 50, and this disease is the second leading cause of all surgery in males. Prostatic cancer is the second leading cancer site in males, and the third cause of cancer deaths (McNeal, 1983; Coffey & Pienta, 1987).

The normal growth and function of the prostate is under the control of androgens. The classical mechanism of action of androgens is shown in figure 1. The main circulating testicular androgen in the rat is testosterone, which circulates bound to serum albumin (Terniswood, Bird & Clark, 1982). The mechanism by which testosterone enters the cells of the prostate has not been firmly established. It is usually assumed that steroid hormone enters the target cell by passive diffusion, although the possibility of active transport has not been completely eliminated (Giorgi, Shirley, Grant & Stewart, 1973). However, several recent lines of evidence, primarily from studies on estrogens, progestins, and glucocorticoids, suggest that the uptake of steroids across the plasma membrane of target cells

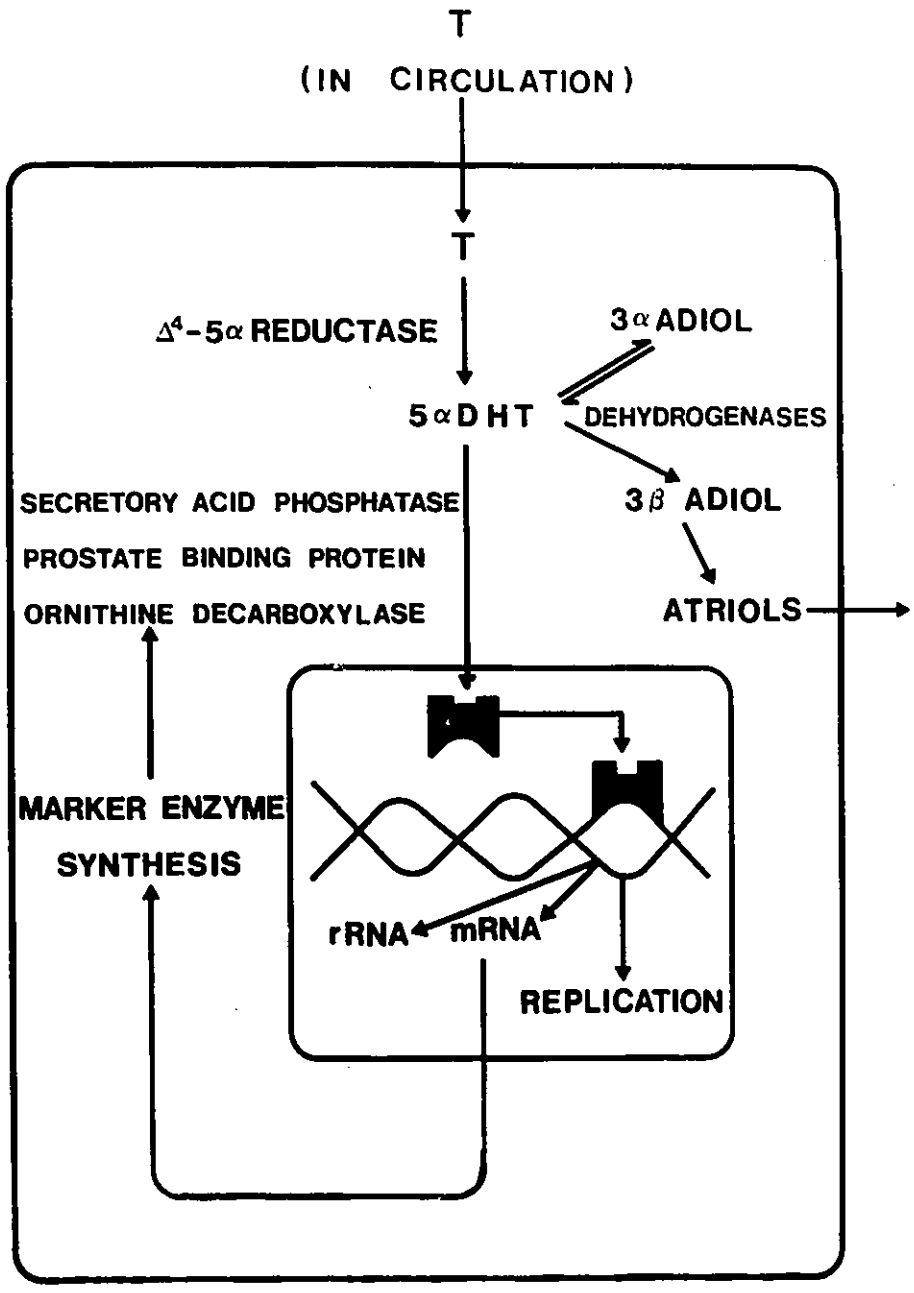
FIGURE 1: Mechanism of action of androgens.

T : Testosterone

5α-DHT : 5α-dihydrotestosterone

 : androgen receptor

 : chromatin


T
(IN CIRCULATION)
T
Δ4-5α REDUCTASE
3α ADIOL
5α DHT
DEHYDROGENASES
3β ADIOL
SECRETORY ACID PHOSPHATASE
PROSTATE BINDING PROTEIN
ORNITHINE DECARBOXYLASE
ATRIOLS
MARKER ENZYME
SYNTHESIS
rRNA
mRNA
REPLICATION

is carrier-mediated (Milgrom, Atger & Baulieu, 1973; Suyemitsu & Hiroshi, 1975; Pietras & Szego, 1977; Fant, Yearkley & Harrison, 1983; Blondeau & Baulieu, 1984; Bression, Michard, Le Dafniet, Pagesy & Peillon, 1986), although there are still a number of objections to this hypothesis (Muller & Wotiz, 1979; Muller, Johnston & Wotiz, 1979; Giorgi & Stein, 1981; Duval, Durant & Homo-Delarche, 1983).

Testosterone itself is probably inactive in the prostate, and must be metabolized to 5α-dihydrotestosterone (5α-DHT) before it is active. The metabolism of testosterone to 5α-DHT is catalyzed by the enzyme NADH, Δ^4-5α-reductase, which reduces the Δ^4 double bond. 5α-DHT is further metabolized in the prostate to 3α- and 3β-androstanediol by the enzymes 3α- and 3β-hydroxysteroid dehydrogenases. The 3β-androstanediol is further metabolized to the 6β- and 7α-androstanetriols by the enzymes 6β- and 7α-hydroxylases (Van Doorn, Bird & Clark, 1975; Isaacs, McDermott & Coffey, 1979). These metabolites appear to be involved in the clearance of the steroid from the cell, and 5α-DHT is most probably the only active androgen in the prostate (Tenniswood, Bird & Clark, 1982).

It has been shown that 5α-DHT is the androgen which preferentially binds to the androgen receptor (Fang, Anderson & Liao, 1969; Mainwaring, 1969), and which accumulates in the nucleus of the target cell (Anderson & Liao, 1968; Bruchovsky & Wilson, 1968; Fang & Liao, 1971). The binding of 5α-DHT to the androgen receptor initiates the androgenic effects of the steroid. The androgen

receptor was originally thought to be localized in the cytosol, similarly to the estrogen receptor (Jensen, Suzuki, Kawashima, Stumpf, Jungblunt & De Sombre, 1968; King & Mainwaring, 1974). After binding of the active androgen, the steroid-receptor complex was thought to be translocated to the nucleus. However, recent autoradiographic methods suggest that the androgen receptor is localized exclusively in the nucleus of both the rat and human prostate (Peters & Barrack, 1987a; 1987b). Similar experiments using monoclonal antibodies raised against the estrogen receptor also suggested that the unoccupied estrogen receptor may be localized in the nucleus and not the cytoplasm (King & Green, 1984; Welshons, Lieberman & Gorski, 1984; Welshons, Krummel & Gorski, 1985). There are several groups that have presented evidence recently demonstrating the presence of the estrogen receptor in the cytoplasm and the nucleus (Antakly & Eisen, 1985; Szego & Pietras, 1985). Similar discord has arisen regarding the subcellular localization of the glucocorticoid receptor (Govindan, 1980; Antakly & Eisen, 1985; Fuxe, Wikstrom, Okret, Agnati, Harfstrand, Yu, Granhelm, Zoli, Vale & Gustafsson, 1985; Hollenberg, Weinberger, Ong, Cerelli, Orom Lebo, Thompson, Rosenfield & Evans, 1985; Wilkstrom, Bakke, Okret, Bronmegand & Gustafsson, 1987; Picard & Yamamoto, 1987).

Regardless of the initial subcellular localization of the receptor, the nuclear 5α-DHT-receptor complex exerts its effects on specific gene transcription by binding to acceptor sites on the prostatic chromatin that appear to be associated

with the nuclear matrix (Mainwaring, Symes & Higgins, 1976; Nyberg, Hu, Loor &Wang, 1976; Colvard & Wilson, 1984; Rennie & Foekens, 1984; Wang, Luo & Xu, 1984; Buttyan & Olsson, 1986).

It is still unclear how the receptor protein enters the nucleus. Small proteins (15 kDa) appear to diffuse freely across the nuclear envelope, whereas larger proteins enter the nucleus more slowly (67 kDa) or are excluded (450 kDa) (Paine & Feldherr, 1972; Paine, Moore & Horowitz, 1975). There must therefore be some recognition signal(s) and a mechanism for the entry of steroid hormone receptors into the target cell nucleus. Androgen binding sites have been identified on the nuclear envelopes of a number of androgen-responsive tissues, such as the rat ventral prostate, the rat liver and the Shionogi mouse mammary carcinoma (Lefebvre, Howell & Golsteyn, 1985). Similar hormone binding sites have been identified for thyroid hormone (Lefebvre & Venkatraman, 1984; Venkatraman & Lefebvre, 1985; 1987). The glucocorticoid receptor has been found to contain a signal sequence which permits nuclear entry of the protein (Picard & Yamamoto, 1987; Lefebvre, personal communication). It has also been determined that transport across the nuclear envelope requires ATP (Richardson, Mills, Dilworth, Laskey & Dingwall, 1988; Newmeyer & Forbes, 1988), suggesting that the phosphorylation status of the hormone receptor may be an important factor in the translocation process. It has been demonstrated that the phosphorylation state affects binding of androgen to the androgen receptor (Golsteyn, Graham, Goren

& Lefebvre, 1989). It remains to be seen whether this also affects the interaction of the androgen receptor with the nuclear envelope.

The androgen receptor itself remains the least well-characterized steroid receptor. It has only recently been established that the human androgen receptor is a 110 kDa protein with distinct DNA binding and steroid-binding domains (Van Laar, Bolt-de Vries, Voorhorst-Ogink & Brinkmann, 1989; Brinkmann, Klaasen, Kuiper, van der Korput, Bolt, de Boer, Smit, Faber, van Rooij, van Kessel, Voorhorst, Mulder & Trapman, 1989). cDNA clones for both the rat and human androgen receptor have recently been characterized (Chang, Kokontis & Liao, 1988; Lubahn, Joseph, Sullivan, Willard, French & Wilson, 1988; Trapman, Klaassen, Kuiper, van der Korput, Faber, van Rooij, Geurts van Kessel, Voorhorst, Mulder & Brinkmann, 1988), and these will facilitate future studies on the localization, translocation across plasma and nuclear membranes, and synthesis of the receptor.

1.2. PROSTATIC PROTEINS

The effects of androgens on the prostate can be divided into two categories: those related to growth and replication, and those related to function and secretion. The androgen-regulated proteins involved in growth of the gland include DNA polymerase (Rennie, Symes & Mainwaring, 1976) and unwinding

protein (Mainwaring, Rennie & Keen, 1976; Mainwaring, Keen & Stewart, 1976), both of which are involved in DNA replication and synthesis.

One group of androgen-regulated proteins associated with function and/or secretion are those involved in RNA synthesis, in particular RNA polymerases I and III (Mainwaring, Mangan & Peterken, 1971; Davies, & Griffiths, 1974; Thomas, Davies & Griffiths, 1977 & 1978). After castration, the levels of these enzymes are greatly decreased, as synthesis of RNA coding for prostatic proteins decreases. There are a number of prostate-specific secretory and non-secretory proteins which are androgen-dependent. These include spermine binding protein (Liang, Mezzetti, Chen, & Liao, 1978; Hiipakka, Chen, Schilling, Oberhauser, Saltzman & Liao, 1984; Chang, Saltzman, Hiipakka, Huang & Liao, 1987), ornithine decarboxylase (Piik, Rajamaki, Guha & Janne, 1977; Danzin, Jung, Claverie, Grove, Sjoerdsma & Koch-Weser, 1979), aldolase (Mainwaring, Mangan, Irving & Jones, 1974), nuclear protein kinases (Wilson, Davies, & Ahmed, 1980; Goueli & Ahmed, 1984), β_2-adrenergic receptor (Collins, Quarmby, French, Lefkowitz & Caron, 1988), prostatic acid phosphatase (Woodward, 1959; Vanha-Perttula, Niemi, & Helminen, 1972; Helminen, Ericsson, Rytoluoto, Vanha-Pertulla, 1975; Tenniswood, Bird & Clark, 1976; Tenniswood, Abrahams, Bird & Clark, 1978), kallikrein-related protease (Chapdelaine, Potvin, Ho-Kim, Larouche, Bellemare, Tremblay & Dubé, 1988; Winderickx, Swinnen, Van Dijck, Verhoeven & Heyns, 1989), and prostate

-steroid binding protein (Heyns & DeMoor, 1977; Heyns, van Damme & DeMoor, 1978; Parker & Scrace, 1979).

Prostate steroid-binding protein (PSBP) is the most abundant secretory protein in the rat ventral prostate (Heyns & De Moor, 1977; Lea, Petrusz & French, 1979). The protein is composed of three distinct polypeptide chains, C1, C2 and C3, that are arranged as a tetramer consisting of 2 subunits, one containing the C1 and C3 polypeptides, the other the C2 and C3 polypeptides (Heyns, Peeters, Mous, Rombouts & De Moor, 1978; Heyns, Van Damme & De Moor, 1978). Each of these polypeptides is coded for by a unique gene (Parker, Needham, White, Hurst & Page, 1982; Hurst & Parker, 1983; Viskochil, Perry, Lea, Stafford, Wilson & French, 1983). The expression of the C1, C2 and C3 genes is dependent on androgens and the level of each mRNA decreases dramatically following castration. This decrease can be reversed by readministering testosterone (Parker & Scrace, 1979; Bossyns, Delaey, Rombauts & Heyns, 1986). Although testosterone affects the transcription rate of PSBP genes (Peeters, Mous, Rombauts & Heyns, 1980; Page & Parker, 1982; Perry, Viskochil, Ho, Fong, Stafford, Wilson & French, 1985), the major influence on the steady state level of the PSBP genes appears to be at the post-transcriptional level since the hormone was shown to stabilize the newly transcribed hnRNA at the nuclear level (Zhang & Parker, 1985). Due to its high abundance and strong dependence on androgens, PSBP is commonly used as a marker of the hormonal status in the rat ventral prostate.

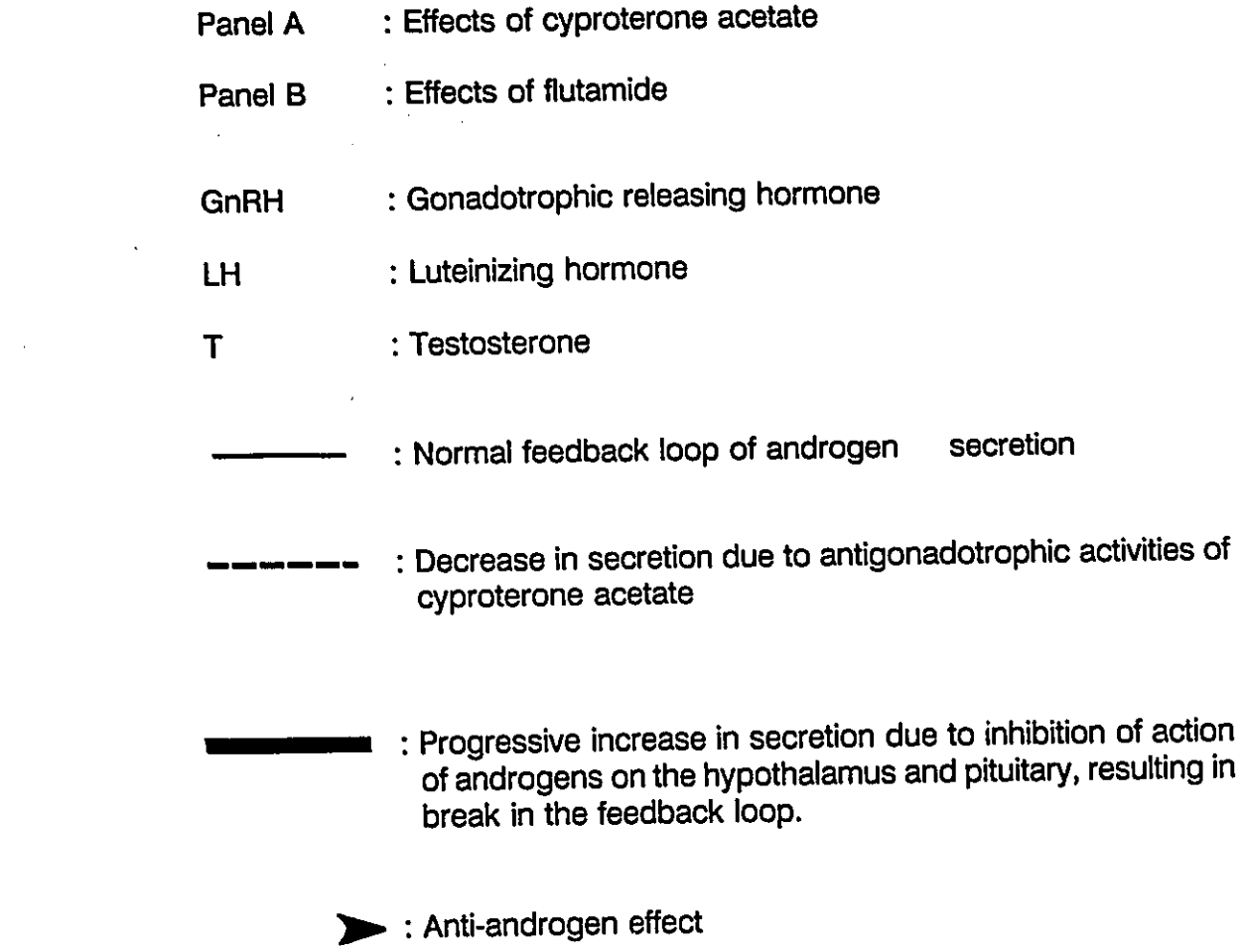
1.3. EFFECTS OF ANTI-ANDROGENS ON THE PROSTATE

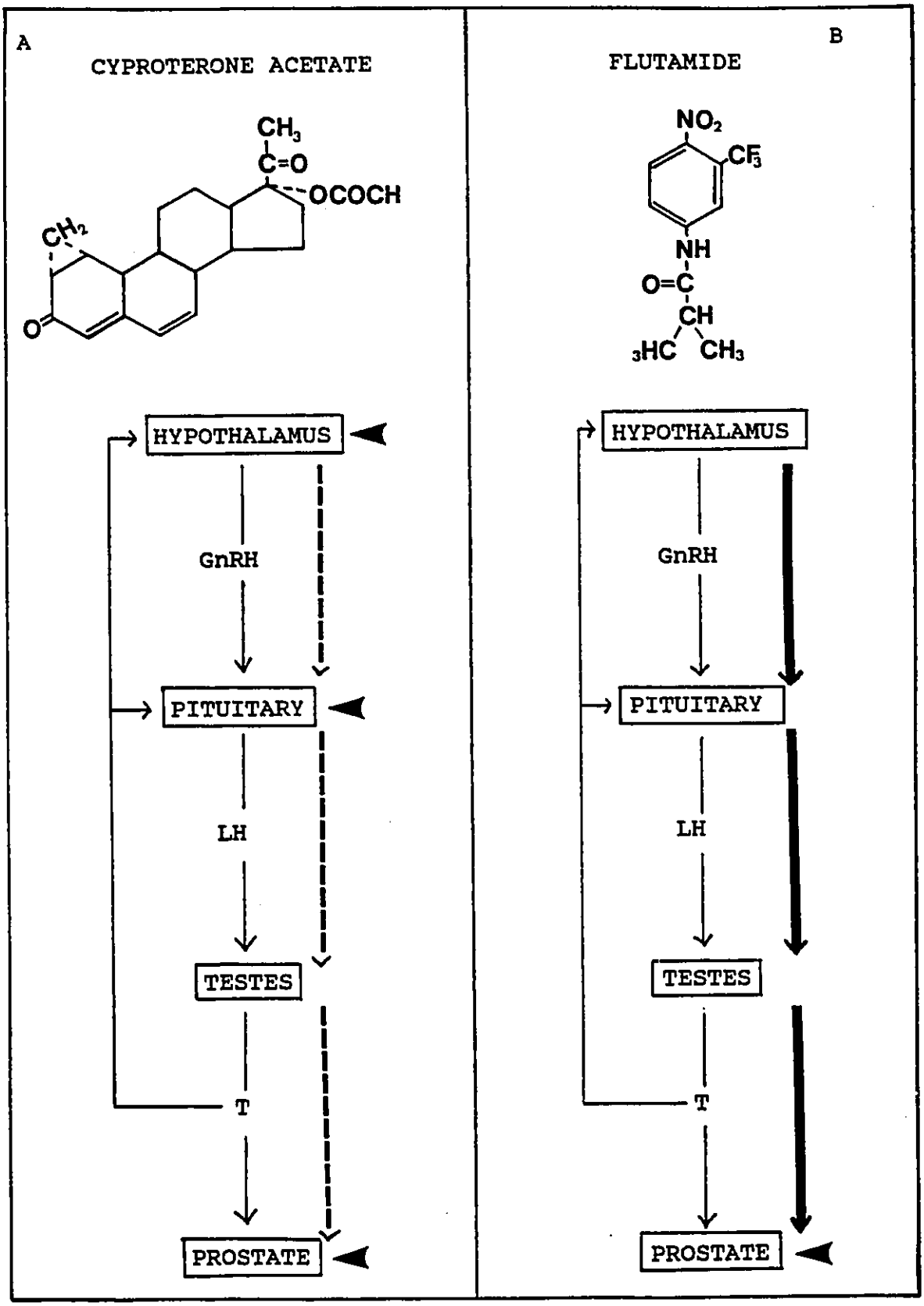
1.3.1. Anti-androgenic therapies

Since the observation that prostate tumor regression can be induced by castration (Huggins et al., 1941), most of the therapies for prostatic cancer are designed to mimic the effect of castration by suppressing the androgenic stimulation of prostatic growth and function. These therapies include treatment with anti-androgens, such as cyproterone acetate and flutamide, which interfere with the steroid receptor mechanism (Neri, Florance, Koziol & van Cleave, 1972; Neumann, 1977; Neri & Peets, 1975; Morales & Nickel, 1985) and with compounds which interfere with steroid metabolism, such as ketoconazole (Trachtenberg, 1984; Heyns, Drochmans, van der Schueren & Verhoeven, 1985; English, Santner, Levine & Santen, 1986; Habenicht & El Etreby, 1987; Aabo, Kjaer & Hansen, 1988).

Two of the most commonly used anti-androgens are flutamide and cyproterone acetate. Cyproterone acetate is a steroidal anti-androgen, whereas flutamide is nonsteroidal (Fig. 2). The effect of flutamide is limited to the level of the receptor, inhibiting formation and/or translocation of the androgen-receptor complex. The effects of cyproterone acetate are more complex since this anti-androgen interferes with the formation of the hormone-receptor complex and also

FIGURE 2: Effects of cyproterone acetate and flutamide on the hypothalamus-pituitary-testes-prostate axis.




A
CYPROTERONE ACETATE
CH3
C=O
OCOCH
CH2
O
HYPOTHALAMUS
GnRH
PITUITARY
LH
TESTES
T
PROSTATE
B
FLUTAMIDE
NO2
CF3
NH
O=C
CH
3HC
CH3
HYPOTHALAMUS
GnRH
PITUITARY
LH
TESTES
T
PROSTATE

acts at the level of the pituitary via its progestogenic and antigonadotrophic properties (Fig. 2) (Neumann, Habenicht & Schacher, 1984; Knuth, Hano & Nieschlag, 1984; Neumann & Topert, 1986). Although both these anti-androgens operate via different mechanisms, treatment with these compounds results in atrophy of the prostate, which is the main aim of the therapy.

In order to eliminate both testicular and adrenal androgens and to provide complete androgen blockade a variety of combination therapies have also been developed which include using anti-androgens in combination with GnRH analogues, anti-prolactin agents or inhibitors of adrenal steroid biosynthesis (Drago, Santen, Lipton, Worgul, Harvey, Boucher, Manni & Rohner, 1984; Labrie, Dupont, Bélanger, Giguere, Lacoursière, Emond, Monfette & Bergeron, 1985; Mathé, Schally, Comaru-Schally, Mauvernay, Vovan, Machover, Misset, Court, Bouchard, Duchier, Morin, Keiling, Scharzenberg, Kerbrat, Achille, Tronc, Fendler, Pappo, Metz & Prevot, 1986; Ahmann, Crawford, Kreis, Levasseur & the aminoglutethimide study group, 1987; Schroeder, Lock, Chadcha, Debruyne, Karthaus, de Jong, Klijn, Matroos & de Voogt, 1987; Labrie, Dupont, Giguere, Borsanyi, Lacourcière, Bélanger, Lachance, Emond & Monfette, 1987; Labrie, Dupont, Bélanger & Lachance, 1987; Dupont, Labrie, Giguere, Borsanyi, Lacourcière, Bergeron, Cusan, Bélanger & Emond, 1988; Dowsett, Shearer, Ponder, Malone & Jeffcoate, 1988).

1.3.2. Ineffectiveness of treatments

While the short term response to ablative therapy is encouraging, the long term effectiveness of the treatments described above is limited. There has been no significant improvement in the disease-free interval or the overall 5 year survival of patients with prostatic carcinoma over the last forty years (Scott, Menon & Walsh, 1980; Slack, Lane, Priore & Murphy, 1986; Pont, 1987; Vanyustel, Ang, Vantongelen, Drochmans, Baert & van der Schueren, 1987; Schulze, Isaacs & Senge, 1987). Very often the recurrent tumor growth is resistant to anti-androgen therapy, probably due to the emergence of hormonally independent cells. These cells are presumed to arise either through an alteration in the gene expression of the normal androgen dependent cells which releases them from the controlling influence of androgens, or from androgen independent cells already in the prostate. There is now increasing evidence for the existence of such androgen independent cells in the prostate of rats (McKeehan, Adams & Rosser, 1984; Montpetit, Abrahams, Clark & Tenniswood, 1988; Montpetit & Tenniswood, 1989), dogs (Chevalier, Bleau, Roberts & Chapdelaine, 1980; McKercher, Chevalier, Roberts & Chapdelaine, 1984) and humans (Merchant, Clarke, Ives & Harris, 1983). Thus, the failure of treatment for prostatic diseases appears to be due to the fact that they are aimed at the androgen-dependent cells, while ignoring the androgen-independent cell population.

1.4 MORPHOLOGY OF THE PROSTATE

1.4.1. Two major cell types of the prostate

The prostate is composed of two major cell types: epithelial and stromal. They are arranged in ductal networks, with the epithelial cells facing the lumen, and resting on a layer of stromal cells (Figure 3). These two cell types have very different morphologies and also differ biochemically.

The epithelial cells synthesize and secrete the prostatic specific, androgen dependent proteins (listed in section 1.3) and also synthesize polyamines (Williams-Ashman, Corti & Sheth, 1975) and citrate (Slaunwhite & Sharma, 1977). It has been shown that the majority of the Δ^4-5α-reductase activity (which converts testosterone to 5α-DHT) resides in the stromal fraction of the human BPH tissue (Cowan, Cowan, Grant & Elder, 1977; Krieg, Klotzl, Kaufmann & Voigt, 1981). In the rat ventral prostate both 5α-reductase and 3α-hydroxysteroid dehydrogenase activities are present in the stromal and epithelial cells, although the majority of the activity is localized in the epithelial cells (Djoseland, Bruchovsky, Rennie, Otal & Hoglo, 1983; Orlowski, Bird & Clark, 1983). The levels of androgen receptor appear to be higher in the epithelial cells of the human prostate (Krieg et al., 1981; Peters & Barrack, 1987b), whereas the stroma contains the majority of the estrogen (Jung-Testas, Groyer, Bruner-Lorand, Hechter, Baulieu & Robel, 1981;

FIGURE 3: Morphology of rat ventral prostate.

Panel A: Random section of rat ventral prostate.

Scale bar: 45 μm
S: Stroma; L: Lumen

Panel B: Schematic representation of a cross-section

Panel C: Schematic representation of a longitudinal section.


: fibroblasts


:columnar secretory epithelial cells

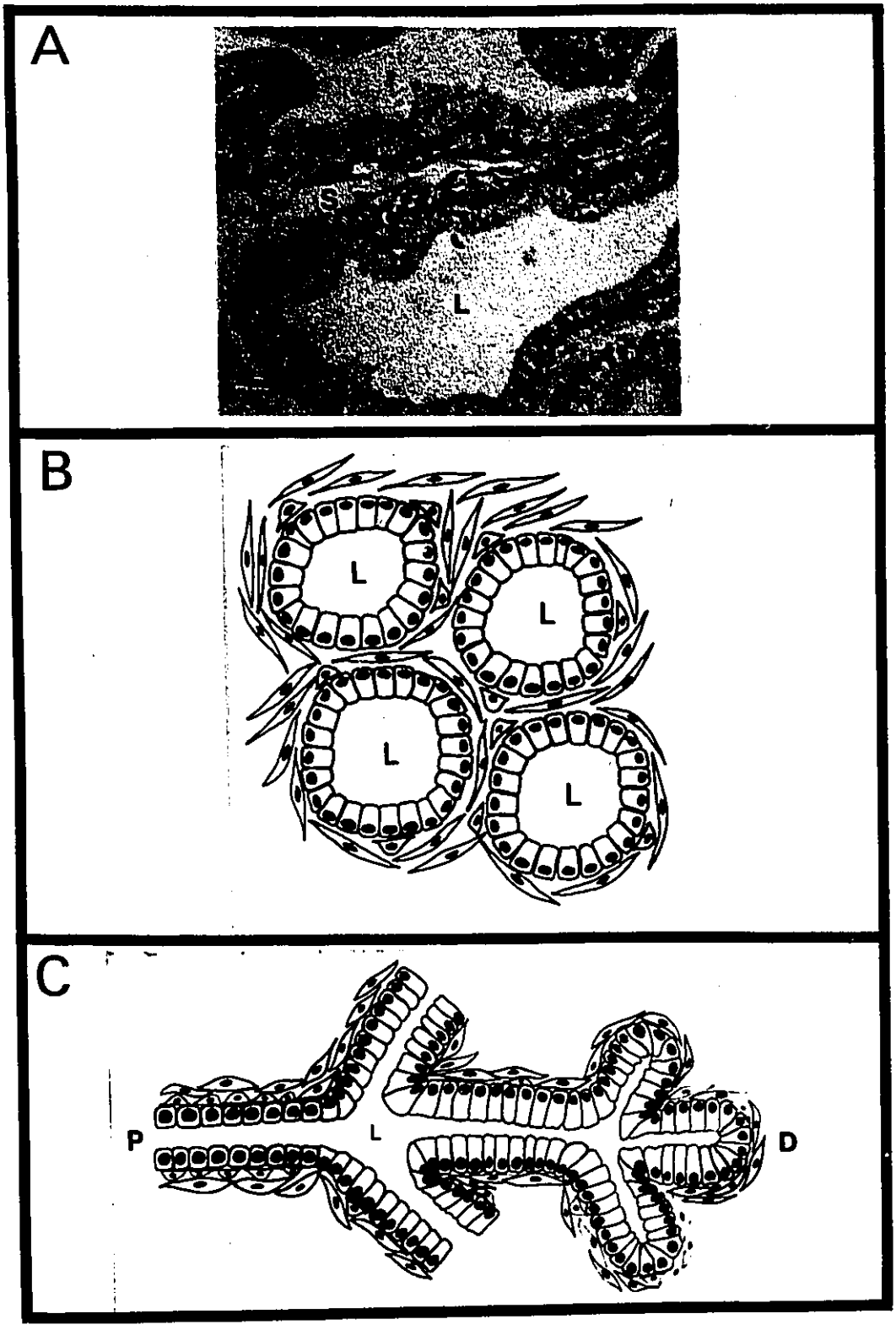

:cuboidal non-secretory epithelial cells


: basal cells

P : proximal region

D : distal region

L : Lumen


A
S
L
B
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L
L
L
C
P
L
D

Krieg, et al., 1981; Lamarre, Chevalier, McKercher, Bleau, Roberts & Chapdelaine, 1985) and the progesterone receptors (Cowan, Cowan & Grant, 1977) in several species including rat, dog and human. The steroid levels also differ between the two cell types, as demonstrated in human BPH tissues. The levels of 5α-DHT, androsterone, 5α-androstane-3α, 17β-diol are higher in the stroma than in the epithelial cells, while testosterone levels are similar in the two cell types (Bartsch, Krieg, Becker, Morhmann & Voigt, 1982; Lahtonen, Bolton, Kukkarinen & Vihko, 1983).

These characterizations imply that both epithelial cells and stromal cells are actively involved in androgen metabolism and presumably in mechanisms of hormonal action.

1.4.2. Epithelial-stromal interactions

Until recently it was thought that the majority of androgen-mediated mechanisms occurred in the epithelial cells of the prostate, and that the role of the stromal cells was restricted to providing a structural support for the secretory cells. It is now clear that interaction between the two cell types is essential for normal growth and function of the prostate. It has been observed that human prostate epithelial cells do not grow when separated from the stroma (Franks, Riddle,

Carbonell & Grey, 1970), and that the stroma is necessary for growth of epithelial cells in organ cultures (Lasnitzki & Mizuno, 1977; 1979).

The importance of the stroma for the development, growth and function of the epithelial cells has been clearly demonstrated by recombination studies using isolated cell types from the urogenital sinus (UGS), the embryonic precursor of the prostate. In the presence of androgens, the normal UGS differentiates into prostatic tissue (Lasnitzki & Mizuno, 1977). The stromal component of the urogenital sinus, the mesenchyme (UGM), can be conveniently separated from the epithelial component (UGE), and recombined to investigate interactions between the two. It has been shown that secretory cyto-differentiation of isolated epithelial cells (UGE) only occurs when the mesenchymal cells (UGM) are present (Cunha, 1976; Cunha, Chung, Shannon & Reese, 1980; Cunha, Chung, Shannon, Taguchi & Fujii, 1983). Moreover, the UGM elicits prostatic secretory cyto-differentiation in epithelium of the urinary bladder or vagina (Cunha, 1975; Cunha & Lung, 1978; Cunha, Lung & Reese, 1980; Cunha, Fujii, Neubauer, Shannon, Sawyer & Reese, 1983). Recombination experiments using the UGE and UGM from testicular feminized (Tfm) mice has provided very important insights into the role of the androgen receptor in the cyto-differentiation of the prostate. The androgen receptor in these mice is inactive. As a result the urogenital sinus develops into a vagina even in male rats. However, when UGE cells from Tfm mice are recombined with UGM from normal mice, they grow and cyto-differentiate to form a prostate

(Cunha, 1972; Cunha & Lung, 1978; Lasnitzki & Mizuno, 1980; Cunha & Chung, 1981; Cunha, 1984; Shannon & Cunha, 1984). Autoradiographic studies have demonstrated that in the recombinant prostatic tissue, the epithelial cells do not contain the androgen receptor, even though they have cyto-differentiated (Cunha, Chung, Shannon, Taguchi & Fujii, 1983; Shannon & Cunha, 1984). The hormonal effects must therefore be mediated via the mesenchymal androgen receptors. Indeed, when normal UGE cells are recombined with UGM from Tfm mice, they cyto-differentiate to form a vagina, demonstrating that the stromal androgen receptor is absolutely required for normal epithelial development (Cunha, Sugimura & Bigsby, 1985).

The requirement for the stromal cells for prostatic growth and differentiation is further supported by studies on the neural components of the gland. It has been demonstrated that the expression of PSBP in the epithelial cells of the prostate is partially controlled by norepinephrine (Chung, Thompson, Chao, Bell & Ruth, 1986; Thompson & Chung, 1986; Thompson, Zhau & Chung, 1987). Since the neural innervation is mostly found in the stroma (Vaalasti & Hervonen, 1979), it appears that the neural influences on the epithelial synthesis of PSBP may also be mediated through the stroma.

1.4.3. Mediators of stromal-epithelial interactions

1.4.3a. Growth and inhibitory factors

The mechanisms involved in the interaction between the epithelium and stroma have not yet been identified or characterized. An elaborate hypothesis has been developed which involves several growth and inhibitory factors (Tenniswood, 1986). It has been suggested that these factors are involved in the early stages of embryonic development and in the normal growth and function of the adult prostate (Fig.4). Briefly, in this model, the stromal cells, under androgenic stimulation, secrete a growth factor, SDGF (stromally derived growth factor), which stimulates growth of the epithelial cells. Simultaneously, epithelial cells produce EDGF (epithelially derived growth factor) which stimulates stromal growth. At a certain stage of development (probably prior to puberty), synthesis of the androgen receptor in the epithelial population occurs. Once the prostate attains maturity, the epithelial cells produce EDIF (epithelially derived inhibitory factor), which inhibits formation of SDGF, therefore inhibiting further growth. Once the synthesis of SDGF and EDGF ceases the epithelial cells synthesize and secrete the prostate specific proteins, such as PSBP and SAP (secretory acid phosphatase). The nature of these growth factors is not clear, and they may be either secreted soluble proteins or components of the basement membrane.

FIGURE 4: Growth and inhibitory factors involved in stromal-epithelial interactions in the developing and normal adult prostate.

T : Testosterone

5α-DHT : 5α-dihydrotestosterone

SDGF : Stromally derived growth factor

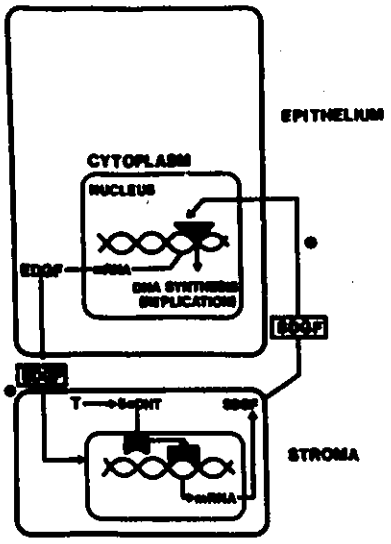
EDGF : Epithelially derived growth factor

EDIF : Epithelially derived inhibitory factor

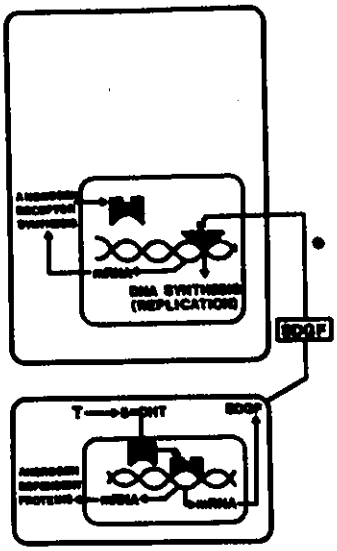
 : Androgen receptor

 : Chromatin

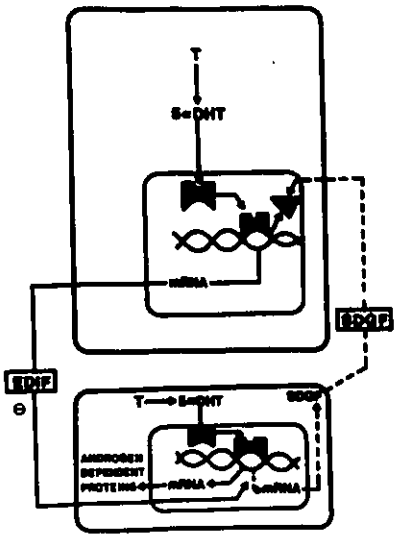
(Reproduced, with permission from Tenniswood, 1986)



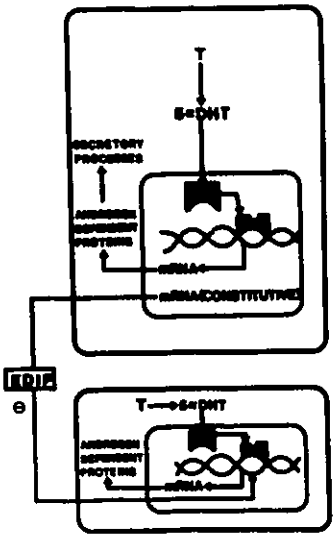
A. INITIAL STAGES OF DEVELOPMENT



B. INDUCTION OF THE ANDROGEN RECEPTOR



C. SYNTHESIS OF EDIF AND REPRESSION OF SDQF



D. NORMAL STATUS OF THE ADULT PROSTATE

While some soluble growth factors have been isolated from human, canine and rat prostate (Parrish, Heston, Pletscher, Tackett & Fair, 1984; Story, Jacobs & Lawson, 1984; Chevalier, Bleau, Roberts & Chapdelaine, 1984; Chapdelaine & Chevalier, 1985; Matuo, Nishi, Matsui, Sandberg, Isaacs & Wada, 1987), these growth factors do not appear to be putative SDGF and EDGF. They have been isolated from tissue homogenates or epithelial cells, and these factors have only been tested on epithelial cells or non-prostatic cells. An inhibitory factor has been identified in human prostatic tissue, but its nature and mechanism of action have not been characterized (Parrish et al., 1984).

1.4.3b. Extracellular and cellular structures

The growth and inhibitory effects may also be mediated through the basement membrane, via the cytoskeleton and nuclear matrix of both cell types, since all these cellular matrices are interrelated. Such effects have been thoroughly investigated in the mammary gland, where the matrix on which the epithelial cells are grown affects the shape of the cells, and the expression of a differentiated phenotype (Emerman, Enami, Pitelka & Nandi, 1977; Emerman & Pitelka, 1977; Shannon & Pitelka, 1981; Wicha, Lowrie, Kohn, Bagavandoss & Mahn, 1982; Haeuptle, Suard, Bogenmann, Reggio, Racine & Kraehenbuhl, 1983; Lee, Parry & Bissell, 1984; Lee, Lee, Kaetzel, Parry & Bissell, 1985; Blum, Zeigler & Wicha, 1987; Medina, Li, Oborn & Bissell, 1987; Blum & Wicha, 1988). The matrix also

determines the nature and distribution of glycosaminoglycans produced by the epithelial cells. These products are major components of the basement membrane, and play a role in maintaining the differentiated state (Parry, Lee, Farson, Koval & Bissell, 1985; Rapraeger, Jalkanen & Bernfield, 1986). Changes in cell shape are modulated by the basement membrane via the cytoskeleton, which is connected to the basement membrane by several proteins including laminin, integrin and talin (Terranova, Rao, Kalebic, Margulies & Liotta, 1983; Tamkun, DeSimone, Fonda, Patel, Buck, Horwitz & Hynes, 1986; Horwitz, Duggan, Buck, Beckerle & Burridge, 1986). The changes in cell shape are thought to be transduced to the level of gene expression via the nuclear matrix. It has recently been demonstrated that the intermediate filaments (containing the cytokeratins) and the nuclear matrix are contiguous (Fey & Penman, 1984; Katsuma, Swierenga, Marceau & French, 1987; Carmo-Fonseca, Cidadao & David-Ferreira, 1987). Since it is well established that actively transcribed genes, such as ovalbumin in the chicken oviduct (Robinson, Nelkin & Vogelstein, 1983), vitellogenin in chicken liver (Jost & Seldran, 1984) and PSBP in the rat prostate (Buttyan & Olsson, 1986) are associated with the nuclear matrix, this provides a physically continuous connection between the basement membrane and expressible DNA that can be readily altered by changing the tension exerted on the cytoskeletal network. Thus, changes in the composition of the basement membrane (or transmembrane receptors) of one cell type can affect the transcription of genes in the other cell type.

Thus, it is quite possible that such effects occur in the prostate via basement membrane components synthesized by both the stromal and epithelial cells. Indeed, it has been demonstrated that in the adult prostate, androgens stimulate the fibroblasts to produce collagen, which is a major component of the basement membrane (Muntzing, 1981; Mariotti & Mawhinney, 1981), while the epithelial cells produce and secrete collagenase (Muntzing, 1981). The balance between the synthesis of collagen by the stromal cells and its destruction by the collagenase from the epithelial cells has been proposed to be a major component for the maintenance of homeostasis in the prostate (Muntzing, 1981). Collagen synthesis and collagenase secretion may represent SDGF and EDGF respectively.

1.4.4. Ductal morphology

In addition to the stromal and epithelial components of the prostate, there is a heterogeneity within the luminal epithelial cell population along the prostatic ducts. The cells at the distal tips of the prostatic ducts are involved in growth and replication (Sugimura, Cunha, Donjacour, Bigsby & Brody, 1986; Sugimura, Cunha & Donjacour, 1986a). After replication some of these cells differentiate, and become secretory cells, which are the tall columnar epithelial cells characteristic of the prostate. These cells are dependent on androgens for the maintenance of their normal morphology and function (Cunha et al., 1980; Shannon & Cunha, 1983). The prostate also contains some cells that are cuboidal in shape and

appear to be androgen-independent. These cells are localized in the proximal region (closer to the ureter) of the prostatic ducts (Cunha, Sugimura & Bigsby, 1985; Cunha, Donjacour, Cooke, Mee, Bigsby, Higgins & Sugimura, 1987) (Fig. 3C).

Recently, by immunostaining for intermediate filament proteins of the cytoskeleton, the epithelial cells have been further sub-divided into cuboidal non-secretory luminal cells in the proximal region, tall columnar secretory luminal cells in the distal region and basal epithelial cells which are localized predominantly in the proximal region (Rouleau, Léger & Tenniswood, submitted). These results have shown that the basal cells are interposed between the stromal and luminal epithelial cells of the proximal region, where they likely attenuate stromal-epithelial interactions (Fig. 3C). The heterogeneity in morphology and function of the epithelial cells along the ducts may thus be due to differences in interactions with the stromal cells. The growth/inhibitory factors, basement membrane composition and/or innervation state may also vary in the different regions of the prostatic ducts, and contribute to the epithelial heterogeneity.

1.5. INVOLUTION OF THE PROSTATE

1.5.1. Changes after castration

During androgen depletion, such as after castration or during anti-androgen treatment, many changes occur in the morphology and function of the prostate. The nuclear androgen receptor decreases to undetectable levels within the first twelve hours after castration (Bruchovsky, et al., 1975; Kyprianou & Isaacs, 1988). There is a marked reduction in prostatic size, and a dramatic decrease in androgen dependent protein synthesis (see section 1.3). The prostatic protein, total RNA and DNA contents decrease 80-85% following castration as a result of autophagia (Bruchovsky, et al., 1975; Lee, 1981; Isaacs, 1984). The loss of epithelial cells is considerably greater than that of stromal cells (Fig. 5). The loss of DNA content corresponds to the loss of approximately 80% of the epithelial cells of the gland (DeKlerk & Coffey, 1978; English, Drago & Santen, 1984), via the process referred to as "programmed cell death" or "apoptosis" (Kerr & Searle, 1973; Sandford, Searle & Kerr, 1984).

FIGURE 5: Random sections of rat ventral prostate before and 6 days after castration.

Panel A: Before castration. Scale bar: 110 μm

Panel B: After castration. Scale bar: 110 μm

Panel C: Before castration. Scale bar: 45 μm

Panel D: After castration. Scale bar: 45 μm

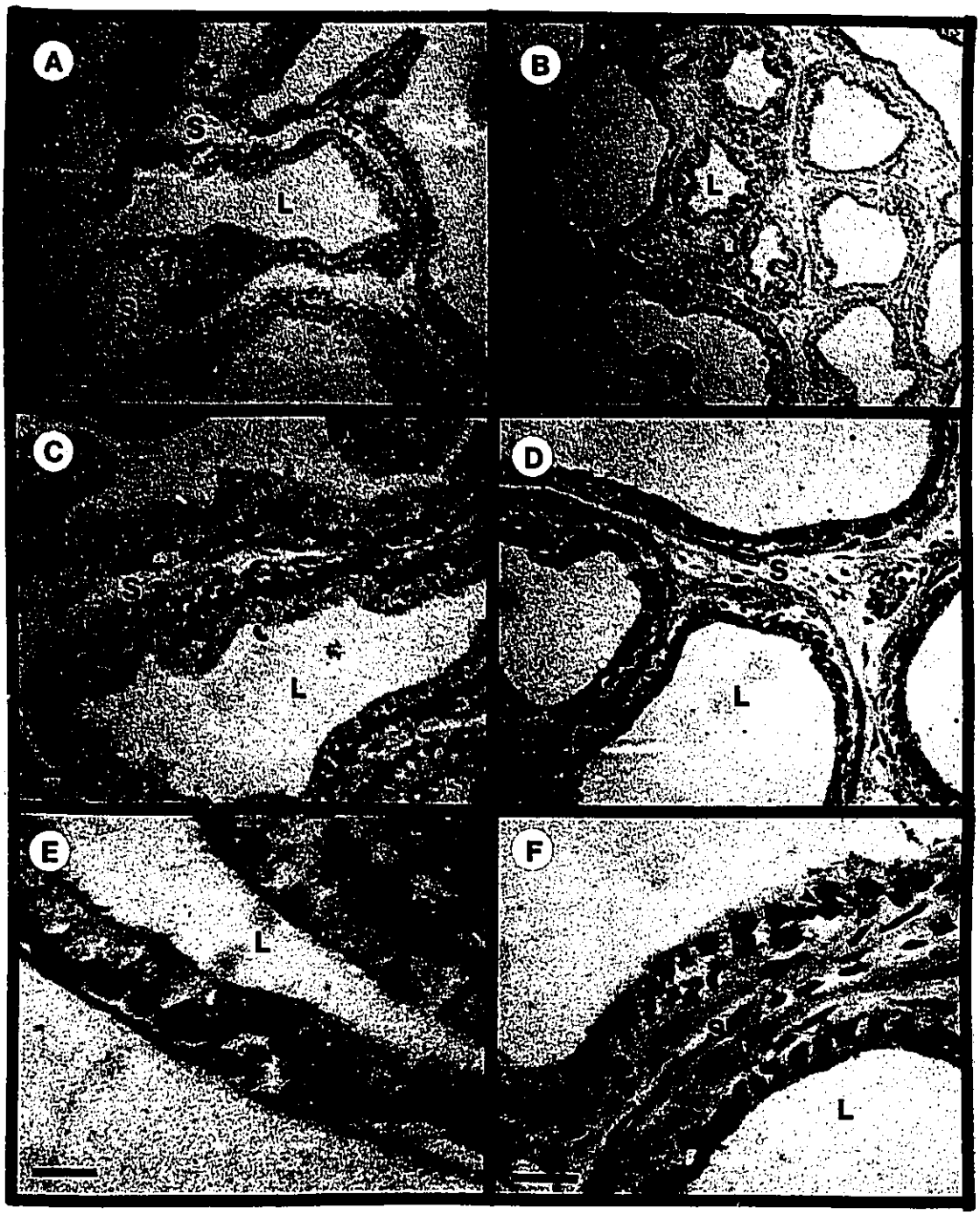
Panel E: Before castration. Scale bar: 18 μm

Panel F: After castration. Scale bar: 18 μm

S: Stroma; L: Lumen

Arrowhead: Apoptotic body

NOTE: Panel C same as Fig. 3A, page 16.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1.5.2. Forms of cell death

1.5.2a. Necrosis

There are two major modes of cell death which have been characterized: necrosis and apoptosis. Necrosis follows injury to the tissue, usually by toxic agents or ischemia. It affects cells in groups, and results in swelling of the cells, followed by rupture of plasma and organelle membranes, and dissolution of organized structure. A number of marker enzymes, such as glutamate oxaloacetate transferase (GOT), are released into the intercellular spaces and into the circulati[illegible], (Wyllie, Kerr & Currie, 1980).

1.5.2b. Apoptosis

There is another mode of cell death which occurs under physiological conditions. It has been termed "apoptosis" , a term derived from the Greek which describes the falling of leaves from trees in autumn (Kerr, Wyllie & Currie, 1972). It has been well characterized morphologically, as schematized in Fig. 6. This diagram has been adapted from the stages described in the liver (Bursch, Kleine & Tenniswood, 1990), and redrawn to adapt the stages to prostate morphology. Apoptosis consists of four major histologicaly detectable steps: contraction of cell volume, nuclear chromatin condensation, cellular fragmentation and swift

FIGURE 6: Morphological stages of apoptosis in the prostate.


:Stromal cell


:Epithelial cell


: Macrophage


:Apoptotic body

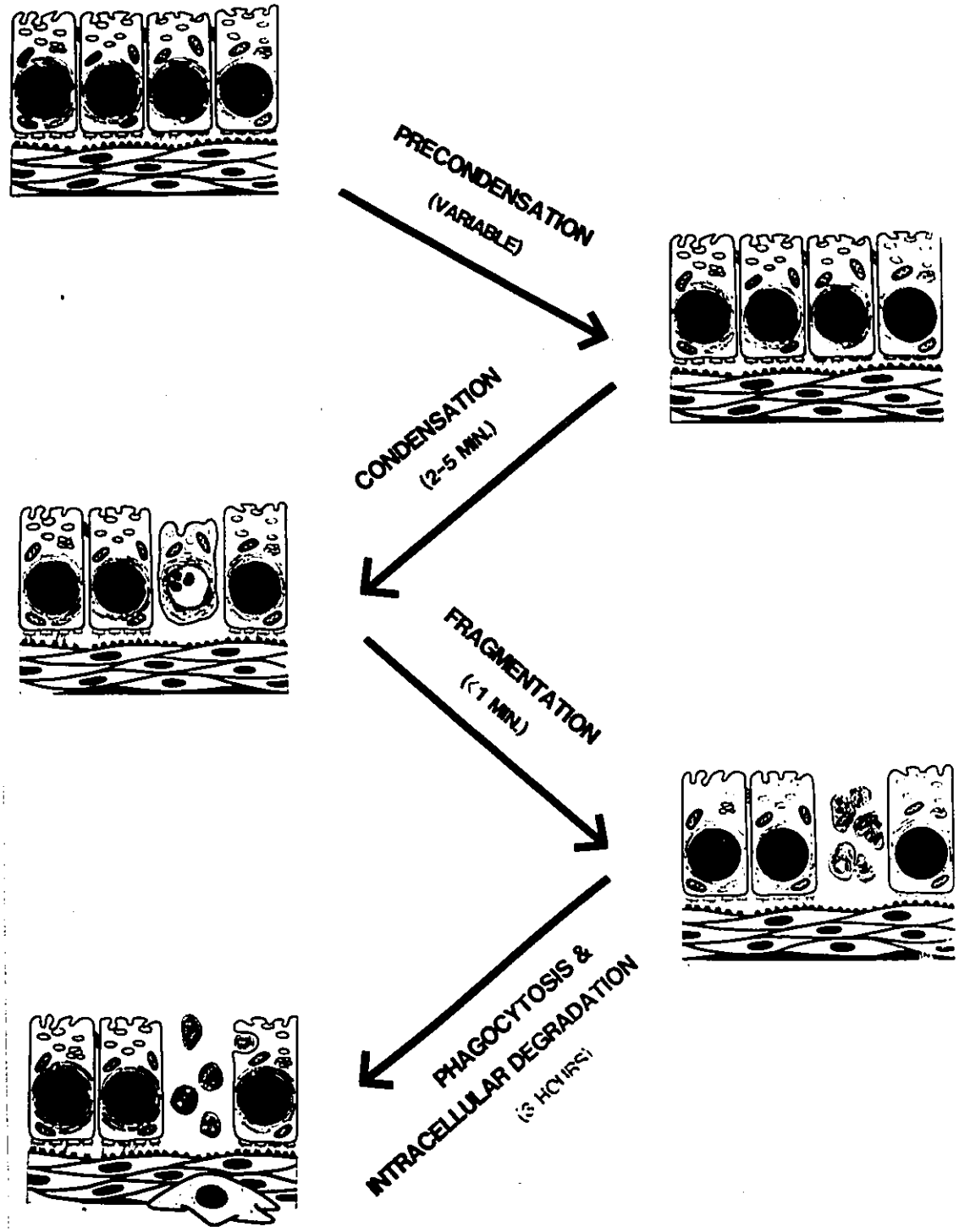

:Mitochondria


:Secretory vesicle


:Desmosomes


:Basement membrane

Adapted with permission from Bursch, Kleine & Tenniswood.


PRECONDENSATION
(VARIABLE)
CONDENSATION
(2-5 MIN.)
FRAGMENTATION
(<1 MIN.)
PHAGOCYTOSIS &
INTRACELLULAR DEGRADATION
(3 HOURS)

recognition and phagocytosis by adjacent cells (Wyllie, Kerr & Currie, 1980; Wyllie, 1981). Following a stimulus for induction of apoptosis, there is also a precondensation stage, which shows no histological changes. This phase is variable in length, from only a few minutes to several days, depending on the tissue type and the stimulus (Bursch, et al., 1990). During this period, the biochemical events necessary for the apoptotic process occur. The first visible sign of apoptosis is chromatin condensation. This has been shown to result from the activation of a Ca^{2+}/Mg^{2+}-dependent endonuclease (Wyllie, 1980; Duke, Chervenak & Cohen, 1983; Kyprianou & Isaacs, 1988). At this stage, the intercellular junctions, such as desmosomes, are destroyed, and the dying cell detaches from the basement membrane. The intermediate filaments condense around the nucleus. These condensation processes (nuclear and cytoplasmic) occur within a few minutes (Bursch, Taper, Lauer & Schulte-Hermann, 1985). This is followed by fragmentation of the cell into apoptotic bodies, which contain fragments of the nucleus, and intact cytoplasmic organelles. This occurs as fast or even faster than the condensation process (Russell, Rosenau & Lee, 1972). The apoptotic bodies are then phagocytosed by the neighbouring normal cells, and degraded by the lysosomes of these recipient cells. If the tissue regression is very pronounced, such as prostate regression following androgen depletion, the macrophages will phagocytose the excess cellular debris left behind by the normal cells. The phagocytosis of the apoptotic bodies requires several hours.

Apoptosis differs from necrosis in many ways. First, it does not involve cytolysis, since the plasma and cytoplasmic membranes are well preserved. Secondly, it affects single cells, and, as mentioned above, occurs under various physiological conditions. Many endocrine-dependent tissues, including the prostate (Kerr & Searle, 1973; Sandford et al., 1984), the mammary gland (Ferguson & Anderson, 1981a; 1981b), the uterus (Sandow, West, Norman & Brenner, 1979), and the adrenal cortex (Wyllie, Kerr & Currie, 1973; Wyllie, Kerr, Macaskill & Currie, 1973), undergo atrophy by apoptosis following hormonal depletion. Glucocorticoids have also been found to induce apoptosis of thymocytes (Munck & Crabtree, 1981; Wyllie & Morris, 1982). Apoptosis is also involved in various stages of embryonic development, when elimination of certain populations of cells is necessary for normal morphology (Wyllie, Kerr & Currie, 1980). One example is the regression of the Mullerian duct during sexual differentiation (Newbold, Suzuki, & McLachlan, 1984). This process of cell death is also important for morphogenesis, as in the loss of the tadpole tail (Kerr, Harmon & Searle, 1974; Decker, 1976). Apoptosis has been observed in the regressing liver following starvation (Schulte-Hermann, Bursch, Fesus & Kraupp, 1988) and during elimination of cyproterone acetate-induced hyperplasia (Bursch, Duesterberg & Schulte-Hermann, 1986).

1.5.3. Biochemistry of apoptosis

The biochemical characterization of apoptosis has so far been very limited, mainly because of the asynchrony of the process in any given tissue. At any given time during tissue regression, only a small percentage of the cells are in the apoptotic state. It is thus very difficult to analyse the regressing tissue for apoptotic characterization, since most of the cells are still in their normal biochemical state. It is also difficult to study gene expression associated with apoptosis, since RNAse is active during the degradation of apoptotic bodies.

It is known that apoptosis requires active protein synthesis (Wyllie, Morris, Smith & Dunlop, 1984; Cohen & Duke, 1984; Duke & Cohen, 1986; Sellins & Cohen, 1987). The chromatin condensation, which is one of the first visible signs of apoptosis, has been shown to result from DNA cleavage at internucleosomal sites by a Ca^{2+}/Mg^{2+}-dependent nuclear endonuclease (Wyllie, 1980; Duke, et al., 1983).

Another enzymatic process associated with apoptosis is an increase in transglutaminase activity. The apoptotic cells in the liver appear to specifically express this enzyme, as demonstrated by immunocytochemistry and enzymatic activity (Fesus, Thomazy & Falus, 1987; Schulte-Hermann et al., 1988). Transglutaminase is known to cross-link lysine and glutamyl residues, and its role

in apoptosis is thought to involve the production a cross-linked protein scaffold, thus maintaining the integrity of the intracellular membrane structures, and compartmentalizing intracellular enzymes. It may also ensure that the DNA remains tightly packed until phagocytosis and final degradation of the apoptotic bodies occurs (Fesus, Thomazy, Autuori, Ceru, Tarsca & Piancentini, 1989).

There are also some changes which occur at the cell surface of the apoptotic cell. It has been shown that there are changes in the sugar residues on the outer membrane of apoptotic thymocytes, which are thought to facilitate recognition by the phagocytic cells (Yamada & Ohyama, 1980; Morris, Hargraves, Duvall & Wyllie, 1984; Duvall, Wyllie & Morris, 1985).

1.5.4. Morphological changes during prostatic regression

The loss of cells in the prostate during hormonal depletion does not occur uniformally along the prostatic ducts (Fig. 7). The majority of the epithelial cells that are lost disappear from the distal region as the tips "melt back" and the number of tips and branch points is dramatically reduced (Fig. 7C). The cuboidal epithelial cells of the proximal region survive with only very slight signs of atrophy (Cunha et al., 1985; Cunha et al., 1987). The epithelial cells that do remain are considerably smaller and contain smaller nuclei, reduced numbers of secretory granules and Golgi apparatus, and much less rough endoplasmic reticulum

FIGURE 7: Changes in morphology of rat ventral prostate after castration.

Panel A: Random section of rat ventral prostate.

Scale bar: 45 μm

Panel B: Random section of rat ventral prostate 6 days after castration.

Scale bar: 45 μm

Panel C: Schematic representation of a cross-section of intact rat ventral prostate.

Panel D: Schematic representation of a cross-section of rat ventral prostate after castration.

Panel E: Schematic representation of a longitudinal section of intact rat ventral prostate.

Panel F: Schematic representation of a longitudinal section of rat ventral prostate after castration.

 : fibroblasts

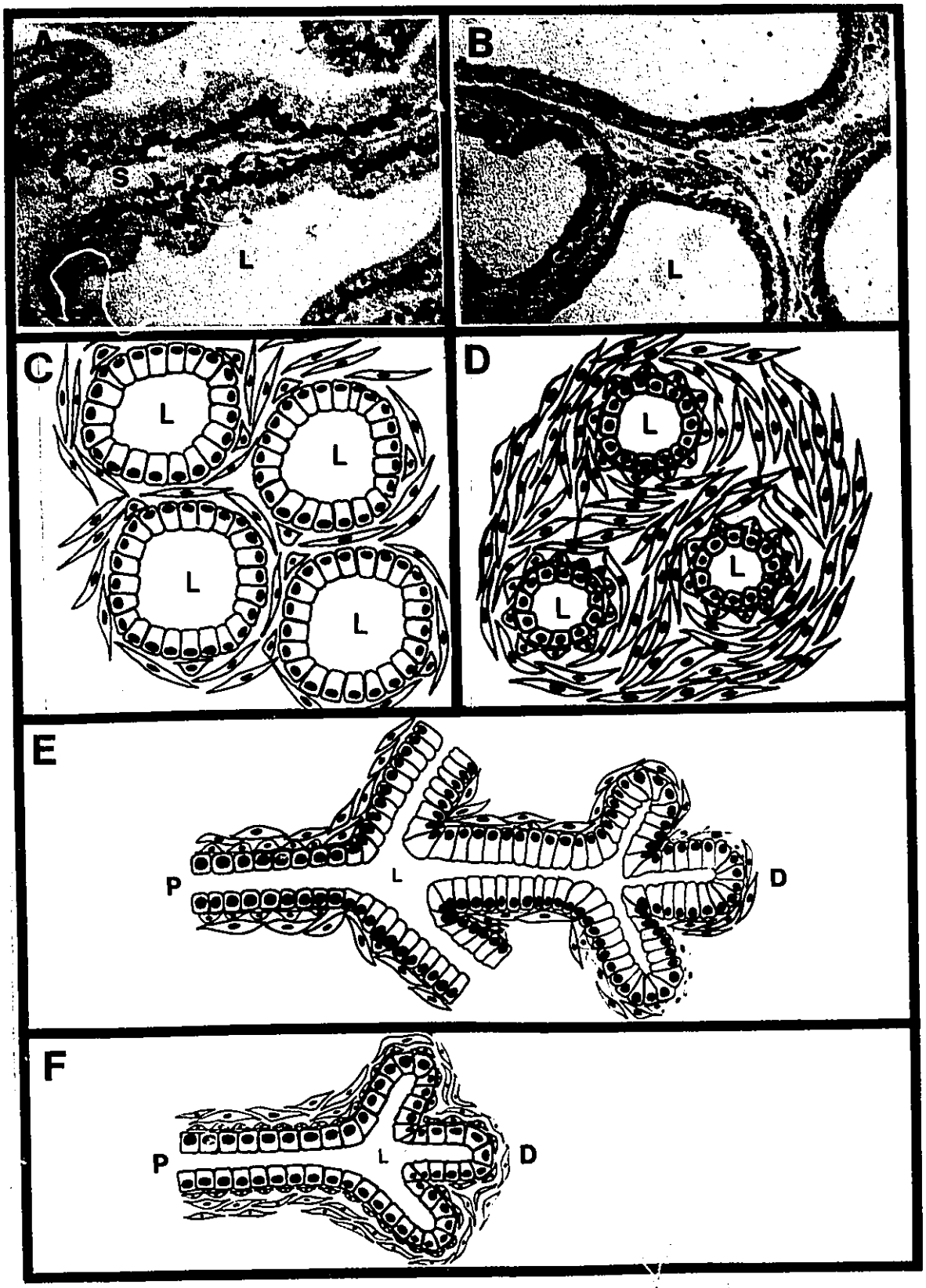
 :columnar secretory epithelial cells

 :cuboidal non-secretory epithelial cells

 : basal cells

P: proximal region; D: distal region; L: Lumen; S: Stroma.

NOTE: Panel A same as Fig. 3A, page 16, and Panel B same as Fig. 5D, Page 28.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(Brandes, 1966; Helminen & Ericsson, 1971). These remaining epithelial cells resemble the cells in the proximal region of normal ducts.

1.5.5. Prostatic gene expression during cell death

It has been shown that androgens not only have an agonistic effect on prostatic cell proliferation, but also have an antagonistic effect on cell death. The involution following castration results mostly from the loss of the antagonistic (or repressive) effect of androgen on prostatic cell death (Isaacs, 1984).

Cell death in the prostate is known to be an active process that requires protein synthesis (Bruchovsky, et al., 1975; Stanisic, Sadlowski, Lee & Greyhack, 1978). There is an increase in degradative enzymes, such as ribonuclease (Engel, Lee & Grayhack, 1980), cathepsin D (Tanabe, Lee & Grayhack, 1982), plasminogen activator (Rennie, Bouffard, Bruchovsky & Cheng, 1984), and Ca^{2+}/Mg^{2+}-dependent endonuclease (Kyprianou & Isaacs, 1988). These activities increase after castration and reach maximum levels in the prostate when involution of the tissue is at its peak (between day 3 and 7 after castration). Protein kinase C activity also increases significantly after castration (Durham, Butcher, Teoh, Miller & Mawhinney, 1986). In addition, a number of as yet unidentified proteins appear after castration (Anderson, Baranowski, Economou & Rubenstein, 1983; Lee, Tsai, Harrison & Sensibar, 1985; Lee & Sensibar, 1987).

In accordance with the requirement for protein synthesis, there are a number of "castration-induced" mRNA species being synthesized during involution of the prostate. The first ones identified were named TRPMs, for "testosterone repressed prostate messages". The most abundant, referred to as TRPM-2, is approximately 2000 nucleotides in size (Montpetit, Lawless & Tenniswood, 1986). Another castration induced mRNA was found to code for a 29 kDa protein, which was later identified as the Yb1 subunit of glutathione S-transferase (Saltzman, Hiipakka, Chang & Liao, 1987; Chang, Saltzman, Sorensen, Hiipakka & Liao, 1987). The mRNAs that are expressed after castration may code for proteins actively involved in the apoptotic processes.

1.6. AIMS OF THIS RESEARCH PROJECT

Since all therapies of prostatic diseases are designed to block androgenic effects in order to provoke tissue regression, it is important to understand the process of involution, and characterize the various components which may be involved.

The aims of this research project are:

1. To confirm that androgen-repressed gene expression exists in the rat ventral prostate.

2. To clone and characterize the most abundant of the androgen-repressed mRNAs (TRPM-2) in the rat ventral prostate.

3. To sequence the cDNA and compare the sequence to other mRNA and protein sequences in Genbank and NBFR.

4. To characterize the time course of gene expression of TRPM-2 after castration and anti-androgen therapy.

5. To determine the cell type specificity of TRPM-2 gene expression in the prostate.

6. To determine the ductal heterogeneity of TRPM-2 gene expression in the prostatic ducts.

2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Animals

Male Sprague-Dawley rats (250-300g) were ordered from Charles River Inc. (Montréal, Québec). They were housed in groups of 2 or 3, and maintained in a controlled environment (14 h light, 10 h dark). They received Purina Rat Chow and water ad libitum. Castrations were performed via the scrotal route under light Innovar-Vet (fentanyl citrate-properidol) or halothane anaesthesia. Hormonal treatments were performed by intraperitoneal injections, using sesame oil as vehicle.

The rats were sacrificed by cervical dislocation, and the tissue excised and processed immediately.

2.1.2. Materials

Innovar-Vet was obtained from MTC Pharmaceuticals (Mississauga, Ontario). Halothane was ordered from Ayerst Laboratories (Montréal, Québec). Random-sequence hexadeoxyribonucleotides, oligo-(dT) cellulose, Sephadex G-50 and Sepharose 4B were purchased from Pharmacia (Dorval, Québec). AMV reverse transcriptase was obtained from Life Sciences (St. Petersburg, FL). Rabbit reticulocyte lysate, [^{35}S]-methionine (800 Ci/mmole), [^{32}P]-dCTP (3000 Ci/mmole), [^{35}S]-dATP (>1000 Ci/mmole), [^{35}S]-dCTP (>1000 Ci/mmole), nick translation kits,

multiprime labelling kits, Hybond™ nylon membranes and restriction enzymes were obtained from Amersham Corp. (Oakville, Ontario). PALL™ nylon membranes were ordered from International Biotechnologies Inc. (Toronto, Ontario). Ampholytes, RNAse H, and vanadyl sulfate ribonucleoside complex were purchased from Bethesda Research Laboratories (Burlington, Ontario). Acrylamide,N'N'-methylene-bisacrylamide, N,N,N',N'-tetramethylenediamine, Coomassie blue, and protein molecular weight standards were obtained from Bio-Rad Laboratories (Canada) Ltd. (Mississauga, Ontario). Agar, tryptone and bacto-yeast extract were purchased from Oxoid Canada (Nepean, Ontario). Ampicillin, T4 ligase, DNA polymerase I, calf intestinal alkaline phosphatase, non-radioactive nucleotides (dNTPs), RNAse A, E.coli tRNA, yeast tRNA and calf thymus DNA were ordered from Boehringer-Mannheim Canada (Dorval, Québec). Gene clean™ kits, pGEM vectors, T7 & SP6 primers were obtained from Bio-Can Scientific Inc. (Mississauga, Ontario). DEAE paper was purchased from Mandel Scientific Corp. (Rockwood, Ontario). Sequenase™ DNA sequencing kits were ordered from US Biochemicals Corp. (Cleveland, Ohio). Synthetic oligonucleotide primers were obtained from the University of Ottawa Biotechnology Research Institute (Ottawa, Ontario). Gene amplification-DNA amplification kits were purchased from Perkin-Elmer Cetus (Nepean, Ontario). 5α-dihydrotestosterone proprionate was obtained from Steraloids Inc. (Wilson, NH). Cyproterone acetate was a gift from Schering AG (Berlin, FRG) and flutamide was a gift from Schering Corporation (Bloomfield, NJ). Collagenase type IV and 3-aminopropyltriethoxysilane were purchased from Sigma

Chemical Co. Ltd (St-Louis, MO). Cronex X-ray film and other photographic material were obtained from Picker International (Ottawa, Ontario). Nuclear track emulsion Type 2 was ordered from Kodak Canada Inc. (Toronto, Ontario). All other chemicals, of reagent grade, were purchased from Fisher Scientific Co. Ltd (Ottawa, Ontario) or BDH (Toronto, Ontario).

2.2. METHODS

2.2.1. Animals

2.2.1.a. Expression of TRPM-2 in rat ventral prostate after castration

Male Sprague-Dawley rats were castrated via the scrotal route under light Innovar-Vet (fentanyl citrate-roperidol) or halothane anaesthesia. They were sacrificed by cervical dislocation on indicated times after castration. For the time course, 10 rats were sacrificed on day 2, 10 on day 4, 12 on day 6 and 18 on day 8. The ventral prostates were excised and processed immediately.

For time course studies, organ weight/body weight ratios (OW/BW) were calculated as described previously (Jackson, Tenniswood, Bird & Clark, 1977).

2.2.1.b. Expression of TRPM-2 in rat ventral prostate after anti-androgen treatment

Rats were castrated and randomly divided into groups. Hormone and anti-androgens were dissolved in sesame oil, and injected intraperitoneally. One group of eighteen rats received a maintenance dose of 250 μg 5α-DHT proprionate/day. The rats to be treated with anti-androgens also received the maintenance dose. It has been shown that the effects of anti-androgens cannot be demonstrated in intact rats due to the high and variable levels of endogenous testosterone. A maintenance dose of 250 μg/day has been shown to maintain the size and function of the prostate, and to permit the assessment of the effects of anti-androgens on the androgen-dependent processes in the ventral prostate (Neumann, 1977; Tenniswood, Abrahams, Bird & Clark, 1981). A second group (36 rats) received 10 mg/day cyproterone acetate in addition to the maintenance dose of 5α-DHT; a third group (36 rats) received 15 mg/day flutamide in addition to the maintenance dose. On the indicated days, the rats were sacrificed in groups of three in the case of the control animals, and groups of six for the treated animals. The prostates were pooled, the RNA extracted, and analyzed by Northern and dot blot hybridization as described below.

2.2.2. Extraction of RNA

Total RNA was extracted by the LiCl/urea procedure (Auffray & Rougeon, 1979) with minor modifications (Tenniswood & Simpson, 1982). Polyadenylated RNA (poly $(A)^+$RNA) was isolated using oligo-(dT) cellulose chromatography (Aviv & Leder, 1972). The samples were quantitated by measuring the optical density at 260 nm wavelength. The ratio of O.D. 260/O.D. 280 was used to assess purity. Ratios of 1.5-1.8 were considered to indicate negligible protein or phenol contamination. The quality of the RNA was also assessed by electrophoresis on 1.5% denaturing agarose slab gels (McMaster & Carmichael, 1977) and staining of the ribosomal RNA bands. It was shown that the quality of the RNA was maintained throughout the time course studies. The amount of extractable poly $(A)^+$ also remained constant (range of 1-1.5% of total RNA extracted).

2.2.3. Cell-free translations and analysis of products

In vitro translations using 1 μg poly(A)+RNA and 10 μCi [^{35}S]-methionine were performed in a rabbit reticulocyte cell-free system for 60 min. at 30°C (Pelham & Jackson, 1976). Aliquots of 1 μl were removed, spotted onto GF/C filters, and the amount of [^{35}S]-methionine incorporated was determined by trichloroacetic acid precipitation. The translation products were analyzed by two-dimensional gel electrophoresis (O'Farrell, 1975) using a pH gradient of 3.5-9.0 in the first

dimension and an SDS/15% polyacrylamide gel with acrylamide:bisacrylamide of 30:0.4 in the second dimension (Newbold, Boyle, Smith & Brown, 1982). To determine the pH gradient of sample gels, gels with unlabeled reticulocyte lysate were run concurrently, and cut into 1 cm segments. Each of these segments was placed in 1 ml double-distilled water, and the pH measured. The gels were stained with Coomassie blue for visualization of low molecular weight SDS-PAGE markers. They were then prepared for fluorography (Bonner & Laskey, 1974), and dried down under vacuum. The dried gels were fluorographed between Kodak X-Omatic intensifying screens, using Cronex X-ray film.

2.2.4. Preparation of cDNA clone library from castrated rat prostate

Double stranded cDNA was prepared from poly(A)+RNA extracted from rats castrated 8 days previously as recently described (Rutledge, Seligy, Côté, Dimock, Lewin & Tenniswood, 1988). A schematic diagram of the procedure is presented in Fig. 8. The ss-cDNA was synthesized using 10μg of poly(A)$^+$RNA, in a buffer containing 100mM Tris-HCl, pH 8.3, 130mM KCl, 10mM $MgCl^2$, 2.5mM DTT, 10μg oligo$(dT)_{12\text{-}18}$, 1mM of each of the four dNTPs, and 50μCi [α-^{32}P]dATP or [α-^{32}P]dCTP. Random hexadeoxynucleotides were dissolved in 10mM Tris-HCl, 1mM EDTA pH 8.0 to a final concentration of 10mg/ml, and used without further purification. Primers were added to the reaction in a 1:1 (w/w) ratio to the template RNA. After heating to 60°C for 2 min., the reaction was initiated by the addition of

FIGURE 8: Schematic diagram of cloning strategy

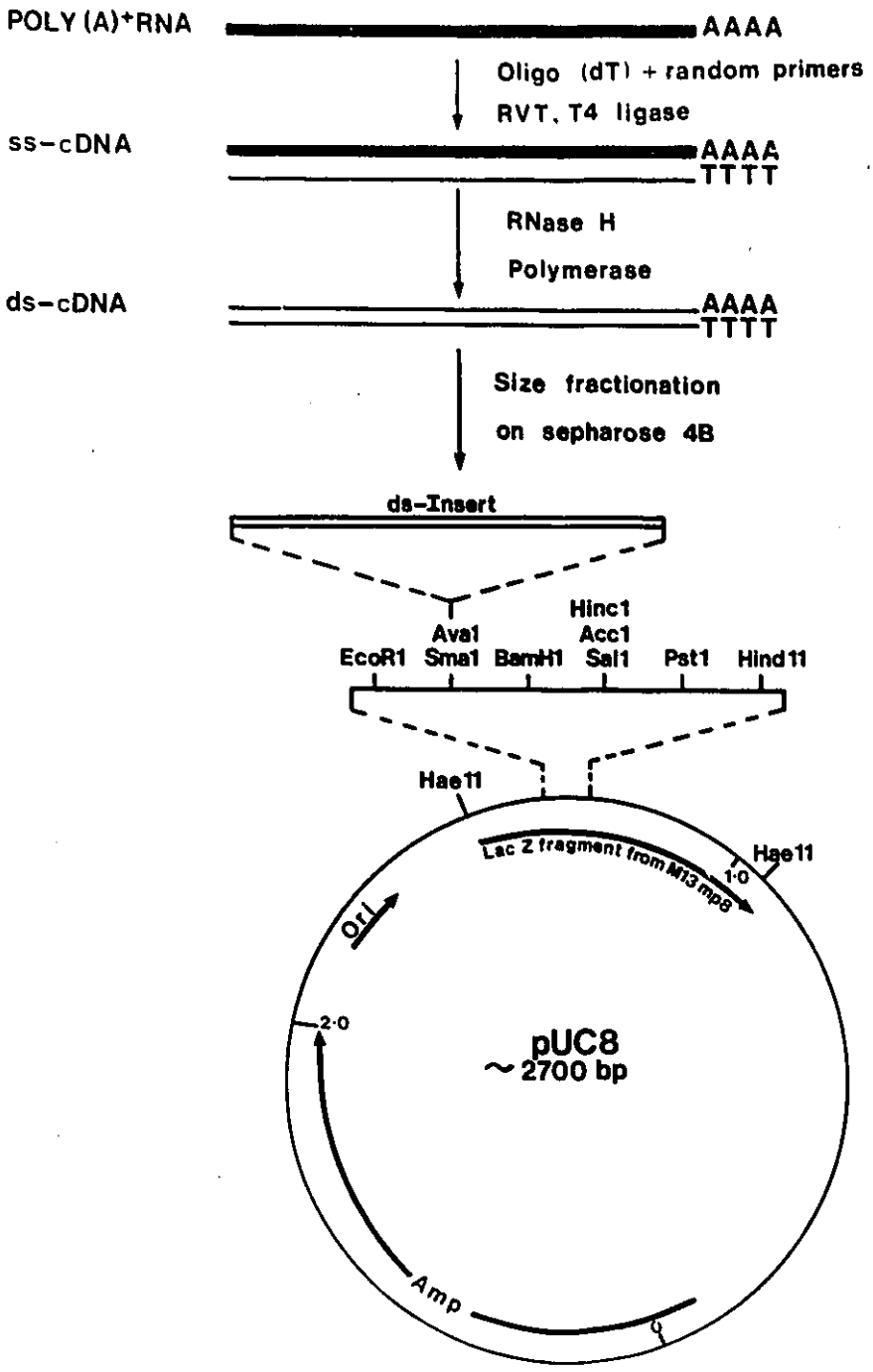
 : mRNA

 : cDNA

ss-cDNA : single-stranded cDNA

ds-cDNA : double-stranded cDNA

RVT : reverse transcriptase


POLY (A)+RNA
AAAA
Oligo (dT) + random primers
RVT, T4 ligase
ss-cDNA
AAAA
TTTT
RNase H
Polymerase
ds-cDNA
AAAA
TTTT
Size fractionation
on sepharose 4B
ds-Insert
Hinc1
Ava1
Acc1
EcoR1
Sma1
BamH1
Sal1
Pst1
Hind11
Hae11
Lac Z fragment from M13 mp8
Hae11
1·0
Ori
2·0
pUC8
~2700 bp
Amp
0

AMV reverse transcriptase to a final concentration of 800 units/ml. The reaction mixture was incubated for 45 min. at 42°C. The cDNA/RNA hybrid resulting from first-strand synthesis was incubated at 15°C for 1 h with 4 Weiss units of T4 DNA ligase to ligate the single-stranded cDNA in these hybrids. After incubation, the samples were heated to 65°C for 15 min. to inactivate the ligase. RNase H (2 units) and E. coli DNA polymerase I (20 units) were added to initiate second strand synthesis and the reaction was continued in the same tube for 3 h at 15°C. At the end of the incubation period the sample was extracted with phenol/chloroform/isoamyl alcohol and passed through a 1 ml G-50 spun column to remove unincorporated dNTPs (Maniatis, Fritsch & Sambrook, 1982). The void volume eluant (100 l) from the Sephadex G-50 column was passed through Sepharose 4B to size select for the larger cDNA fragments.

The size fractionated cDNA fragments (size range 0.8-1.5 kb) were ligated into pUC8 vector which had been previously linearized with Sma I to yield blunt ends, and treated with calf intestinal alkaline phosphatase to prevent religation. The ds-cDNA and vector were mixed in an equimolar ratio, precipitated at -20°C for 1 h, redissolved in 20 l ligation buffer (50mM Tris-HCl, pH 7.8, 10mM $MgCl_2$, 1mM ATP, 20mM DTT) and ligated using 5 Weiss units of T4-DNA ligase overnight at 15°C. Aliquots of the ligation mixture containing recombinant plasmids were used to transform E. coli DH1 (Hanahan, 1983) which were then grown on Luria agar

containing 100 g/ml of ampicillin. Transformation efficiency was $5x10^4$ cfu/ g of ds-cDNA.

2.2.5. Identification of TRPM-2-specific clones

Ampicillin-resistant colonies were screened by colony hybridization (Grunstein & Hogness, 1975). Transformed colonies, fixed in duplicate on nylon membranes, were hybridized to [^{32}P]-labeled ss-cDNA prepared essentially as described by Williams and Penman (1975), with minor modifications (Smith, Searle & Williams, 1979), using the prostatic poly(A)$^+$RNA from normal or castrated rats as templates. The colonies hybridizing only with the labeled cDNA from RNA from castrated rats were assumed to harbour cDNA sequences corresponding to RNA species induced after castration. Plasmids of interest were prepared by alkaline extraction (Birnboim & Doly, 1979), and were further characterized by differential Northern hybridization and by digestion with restriction endonucleases (described below).

2.2.6. Restriction digestions

Restriction digestions were performed under the conditions suggested by the enzyme supplier. When double digestions were performed, the plasmids were

first digested with the enzyme requiring the lower salt concentration for optimal activity. The salt concentration was then increased and the second enzyme added. The digestion products were analyzed by electrophoresis through non-denaturing Tris-acetate agarose gels, using Hind III digest of lambda DNA &/or Taq I digest of pBR322 as size markers. The gels were stained with ethidium bromide (1 g/ml), and photographed on a 306 nm ultra-violet transilluminator with an orange filter, using Ilford FP4 film.

2.2.7. Differential Northern hybridization

Replicate samples of prostatic poly(A)$^+$RNA from intact rats and rats castrated 8 days previously were electrophoresed on 1.5% denaturing agarose slab gels (McMaster & Carmichael, 1977). Total RNA was run in parallel as a size marker. The electrophoresed RNA samples were transferred to PALL^{TM} nylon filters (Thomas, 1980). The plasmids of interest were radioactively labeled by nick translation (as described in section 2.2.9), and used individually as probes for Northern hybridization analysis of the above filters. Conditions for Northern analysis are described in section 2.2.10.

2.2.8. Insert isolation for labeling and subcloning

Plasmids of interest were digested with the appropriate restriction enzymes [for TRPM-2 specific clones, Eco RI and Bam HI, and for pA34 (PSBP), Pst I (Parker, White, Hurst, Needham & Tilly, 1983)]. The digestion reaction mixes were run on 0.8% agarose slab gels. The inserts were isolated from the gels by one of two methods. For the first method, the inserts were electrophoresed onto Schleicher & Schuell DEAE membrane, and then eluted in a high salt buffer (1M NaCl, 0.1mM EDTA, 20mM Tris, pH 8) at 65°C (Dretzen, Bellard, Sassone-Corsi & Chambon, 1981). For the second method, the inserts were cut out of the gel and retrieved by binding the DNA to glass beads in the presence of NaI, followed by elution in 10mM Tris, 1mM EDTA, pH 8 (Vogelstein & Gillespie, 1979).

2.2.9. Preparation of radiolabeled probes

Plasmids were labeled by nick translation in the presence of [^{32}P]-dCTP following a standard procedure (Rigby, Dieckman, Rhodes & Berg, 1977). Isolated inserts were labeled by the oligo-nucleotide labeling technique (Feinberg & Vogelstein, 1983). Reactions were performed on 200ng plasmid DNA for nick translations, and on 50-200ng insert DNA for multiprime labeling.

2.2.10. Northern hybridization analysis

Poly(A)$^+$RNA was electrophoresed and transferred to PALL^{TM} nylon filters as described above (section 2.2.7). The filters were prehybridized at 42°C for a minimum of 6 h in 50% formamide, 5X SSC (0.75M NaCl, 0.075M Na citrate, pH 7), 5X Denhardt's solution (0.1% albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), and 100 g/ml single-stranded calf thymus DNA. The filters were hybridized with [^{32}P]-labeled probe (50-200ng) in the same buffer for 48 h in the case of TRPM-2, and for 16-20 h for the PSBP probe. They were washed twice in 2X SSC, 0.1% SDS for 5-10 min. at room temperature, then twice for 5-10 min. in 0.1X SSC, 0.1% SDS at 42°C. Filters were sealed in Saran Wrap, and autoradiographed between intensifying screens using Cronex X-ray film in Kodak X-Omatic cassettes. Exposure times varied depending on the specific activity of the probes and the abundance of the specific sequences in the poly(A)$^+$ RNA.

2.2.11. Dot blot hybridizations

Dot blots of the poly(A)$^+$RNA were performed essentially as described by Thomas (1983). Triplicate 1 g samples from each time point were blotted onto PALL^{TM} membrane. Isolated plasmid inserts were used as standards, and 10 g aliquots of yeast tRNA were used as controls for non-specific hybridization. The filters were prehybridized at 42°C for a minimum of 6 h in 50% formamide, 5X SSC,

5X Denhardt's solution, and 100 g/ml single-stranded calf thymus DNA. The filters were hybridized with [^{32}P]-labeled probe (50-200ng) in the same buffer for 48 h in the case of TRPM-2, 16-20 h for other probes specific for more abundant mRNAs. They were washed twice for 5-10 min. in 2X SSC, 0.1% SDS at room temperature, then twice for 5-10 min. in 0.1X SSC, 0.1% SDS at 42°C. Following the washes, the membranes were cut into individual dots and air dried, placed in scintillation vials with 5ml Beckman Ready-Solv ET scintillation fluid and the bound radioactivity determined in a Beckman LS1801 scintillation counter. The average of the determinations obtained were compared to the standard curve to estimate the relative levels of each message. The result were calculated and reported as ppm of poly(A)$^+$RNA, as shown below:

x cpm = cpm on sample - cpm on yeast tRNA

x cpm = y ng poly(A)$^+$RNA

(as determined from standard curve)

$$\frac{\text{y ng poly(A)}^+\text{RNA}}{\text{mg poly(A)}^+\text{RNA blotted}} = \text{ppm}$$

2.2.12. Subcloning and sequencing

To sequence the cDNA inserts of interest (22-12 & 21-04), they were isolated as described above following BamHI-EcoRI digestion. The inserts were

ligated into the vectors pGEM-3 and pGEM-4 that had been prelinearized with BamHI and EcoRI, using 2 units T4 DNA ligase. These vectors contain the T7 & SP6 polymerase promoter regions flanking the multiple restriction cloning site, therefore the T7 & SP6 primers can be used for the sequencing reactions. The ratio of vector:insert was 10:1 (w/w). E.coli DH5α cells were transformed with the recombinant plasmids. The transformed cells were grown on Ψ agar (0.5% bacto yeast extract, 2% bacto tryptone, 0.5% $MgSO_4$, 1.4% agar, pH 7.6) plates + 100μg/ml ampicillin and the recombinant plasmids isolated as described previously (section 2.5).

Sequencing was performed by the chain termination method (Sanger, Milken & Coulson, 1977) in the presence of [^{35}S]-dATP and T7 & SP6 primers, using Sequenase™, the modified version of T7 DNA polymerase (Tabor & Richardson, 1987). To obtain the full sequence of the TRPM-2 clones, synthetic oligonucleotides (18mers) complementary to determined regions were used. Computerized analysis of the sequences obtained and comparison with the GenBank database were performed using Microgenie™ computer software (Queen & Korn, 1984)(Beckman, Mississauga).

2.2.13. Polymerase chain reaction amplification of inserts

In order to obtain large quantities of insert necessary for preparing probes for in situ hybridizations, polymerase chain reaction amplifications (Mullis & Faloona, 1987) were performed on 3ng plasmid, using the T7 & SP6 primers. The following cycle was repeated 25 times: denaturation at 94°C for 1.5 min., annealing at 50°C for 2.5 min. and elongation at 70°C for 4 min.. During the last cycle, the elongation step was prolonged to 15 min.. Yields varied between 1 to 3μg of insert. The size of the resulting insert was verified by electrophoresis.

2.2.14. In situ hybridization

In situ hybridizations were performed as described by Lawrence & Singer (1986), with minor modifications. Freshly excised prostate pieces were embedded in Tissue-Tek O.C.T. embedding compound, and frozen in isopentane at -70°C. They were randomly sectioned to a thickness of 5μm in a Damon microtome at -20°C, and placed on silane treated slides for better adhesion (Rentrop, Knapp, Winter & Schweizer, 1986). For the localization of hybridization along the prostatic ducts, prostatic tissues were micro-dissected (Sugimura, Cunha & Donjacour, 1986b). Freshly excised prostates were treated with 1% collagenase in Ca^{2+}/Mg^{2+}-free Hank's buffer (0.14M NaCl, 5.4mM KCl, 0.44mM KH_2PO_4, 4.2mM $NaHCO_3$, 0.42mM Na_2HPO_4, 5.6mM glucose) at room temperature for 10 min., during which

time the micro-dissection was started. Following this incubation period, the tissue was rinsed in Ca^{2+}-Mg^{2+}-free Hank's solution and the micro-dissection was completed. Tissue pieces corresponding to the distal and proximal regions of the prostatic ducts were mounted and sectioned as described above for the random sections.

All the sections were fixed in 4% paraformaldehyde/PBS + 5mM $MgCl_2$, and stored at 4°C in 70% ethanol until use (normally overnight). The sections were rehydrated for 10 min. in PBS + 5mM $MgCl_2$, and then for 10 min. in 0.1M glycine, 0.2 M Tris, pH 7.4. Negative controls were treated with 100μl 100 μg/ml RNAse A/2X SSC for 1 h at 37°C prior to following steps. All the sections (negative controls and experimental) were prehybridized with 200μl 50% formamide, 2X SSC for 10 min. at 60°C prior to hybridization. The slides were hybridized for 3 h at 37°C with 20μl of the following mix: 2X SSC, 0.2% BSA, 10% dextran sulfate, 25mM DTT, 10mM vanadyl sulfate ribonucleoside complex, 1mg/ml E.coli tRNA, 1mg/ml calf thymus DNA and 1 μg/ml [^{35}S]-labeled probe (specific activity 1-3 X 10^8 cpm/μg). The slides were washed once in 2X SSC, 50% formamide at 37°C for 30 min., twice in 1X SSC, 50% formamide at 37°C for 30 min., and once in 1X SSC at room temperature for 30 min.. The sections were dehydrated by rinsing in ethanol, air dried, and dipped in nuclear track photographic emulsion. The sections were exposed for 2 to 3 days, and were processed at 15°C. The slides were developed for 5 min. in Kodak D-19 developer, rinsed for 30 sec. in 2% acetic acid,

fixed for 5 min. in Kodafix, and rinsed for 30 min. in running water. The slides were lightly counterstained for 2 min. with haematoxylin and 12 sec. with eosin. They were photographed using Kodak Pan-X or Kodak Ektachrome 400 film.

3. RESULTS

3.1. CHANGES IN OW/BW RATIOS AND TOTAL RNA LEVELS; AFTER CASTRATION

The most obvious effect of castration on the prostate is a decrease in the size of the gland. Figures 9 & 10 show a profile of the decrease in OW/BW ratios and RNA levels which occur following castration. Following castration, the OW/BW ratio decreases from initial values of approximately 1 to 0.56 by the fourth day, and to 0.23 by day eight (Fig. 9). Similar results have been reported previously, and the OW/BW ratio does not decrease significantly between day 8 and day 20 following castration (Tenniswood et al., 1978a; 1978b).

In parallel with the decrease in OW/BW, the total RNA levels in the prostate are also greatly reduced following castration (Fig. 10). Between day zero and four after castration, the total RNA levels are decreased from over 1 mg/prostate to 0.3 mg/prostate. By day eight the levels decrease to less than 0.1 mg/prostate.

These results demonstrate that castration has two superimposed effects on the rat ventral prostate: the loss of tissue mass, presumably due to cell loss, and a significant decrease in RNA levels.

FIGURE 9: Effects of castration on the OW/BW ratio.

Rats were sacrificed and the ventral prostates excised and pooled on indicated days after castration. The average prostate weights and average body weights were determined, and the OW/BW ratios were calculated as described previously (Jackson et al., 1977).

 : normal range

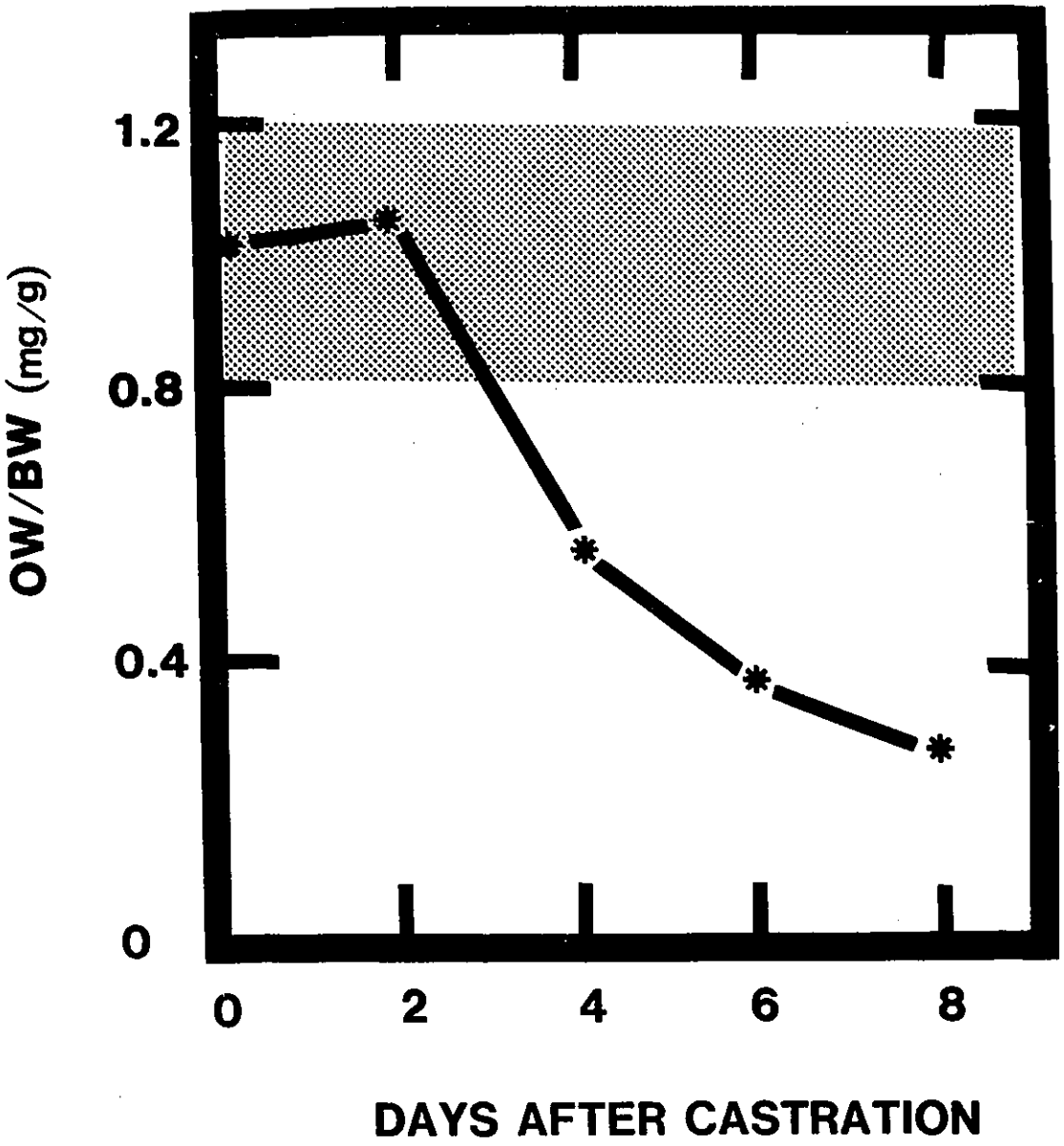
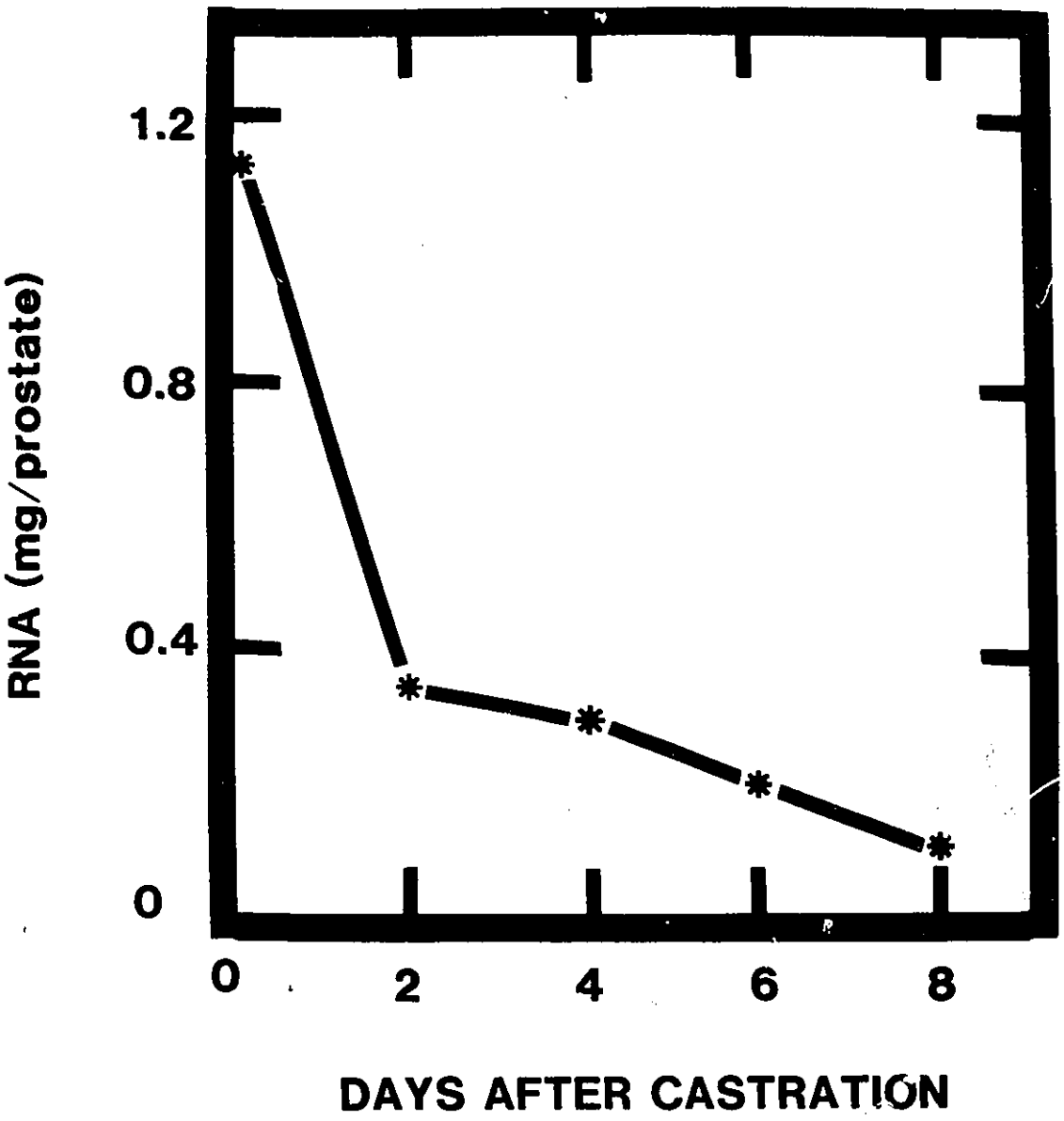

OW/BW (mg/g)
1.2
0.8
0.4
0
0
2
4
6
8
DAYS AFTER CASTRATION

FIGURE 10: Effects of castration on total prostatic RNA levels.

Rats were sacrificed and the ventral prostates excised and pooled on indicated days after castration. Total RNA was extracted and the concentration determined as described in section 2.2.


RNA (mg/prostate)
1.2
0.8
0.4
0
0
2
4
6
8
DAYS AFTER CASTRATION

3.2. IN VITRO TRANSLATION PRODUCTS BEFORE AND AFTER CASTRATION

The in vitro translation products of the poly $(A)^+$ RNA extracted at various times after castration were analyzed by 2 dimensional gel electrophoresis to characterize changes in the mRNA population, thus possible changes in the protein composition of the prostate. The comparison of the in vitro translation products of the poly$(A)^+$RNA extracted from the prostates of intact rats (Fig. 11) and of rats castrated 8 days previously (Fig. 12) reveals a number of striking differences. The PSBP subunits are the most abundant androgen dependent proteins in the prostate (Parker et al., 1978), and are clearly visible in the translation products of the poly$(A)^+$RNA extracted from the prostate of intact rats (Fig. 11). Although this gel system does not adequately resolve proteins with molecular weights less than 18,000 daltons, the prostate binding proteins are visible as an unresolved group at an apparent molecular weight of approximately 14,000 daltons with isoelectric points ranging from 5.5 to 6.8. These proteins are conspicuously absent in the translation products of the RNA extracted from the prostate after castration (Fig. 12), which makes them an excellent control for androgen dependent gene expression in the prostate (Parker et al., 1983). There are also several unidentified proteins which show a similar degree of androgen dependence, for example proteins of 31,000 daltons, pH 6-8. Constitutively expressed proteins, such as actin (45,000 daltons, pI 5.5-5.9) are present both before and after castration (Fig. 11 & 12). There are a number of proteins which

FIGURE 11: Two dimensional gel electrophoresis of in vitro translation products from intact rat ventral prostate.

45,000 cpm (TCA precipitable) of [^{35}S]-labeled translation products from 1μg prostatic poly(A)$^+$ RNA were run on a two dimensional gel, as described in section 2.3, prepared for fluorography, and exposed for 3 days. The position of molecular weight standards is shown on scale. The position of prostate steroid binding protein (PSBP), actin and the presumed position of TRPM-2 are indicated on the figure.

Molecular weight standards:

97.4 kDa: phosphorylase B
66.2 kDa: bovine serum albumin
45.0 kDa: ovalbumin
31.0 kDa: carbonic anhydrase
21.5 kDa: soybean trypsin inhibition
14.4 kDa: lysozyme

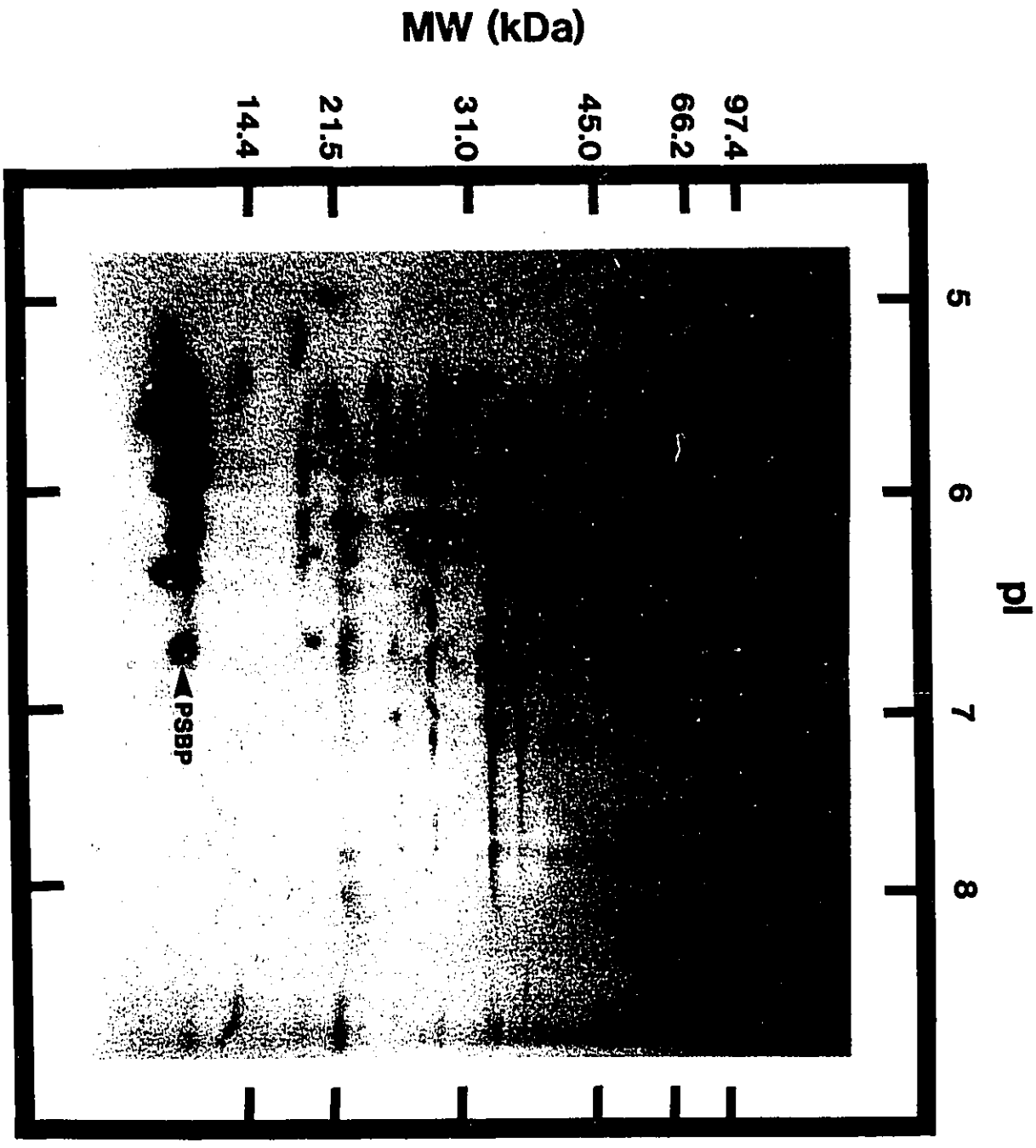
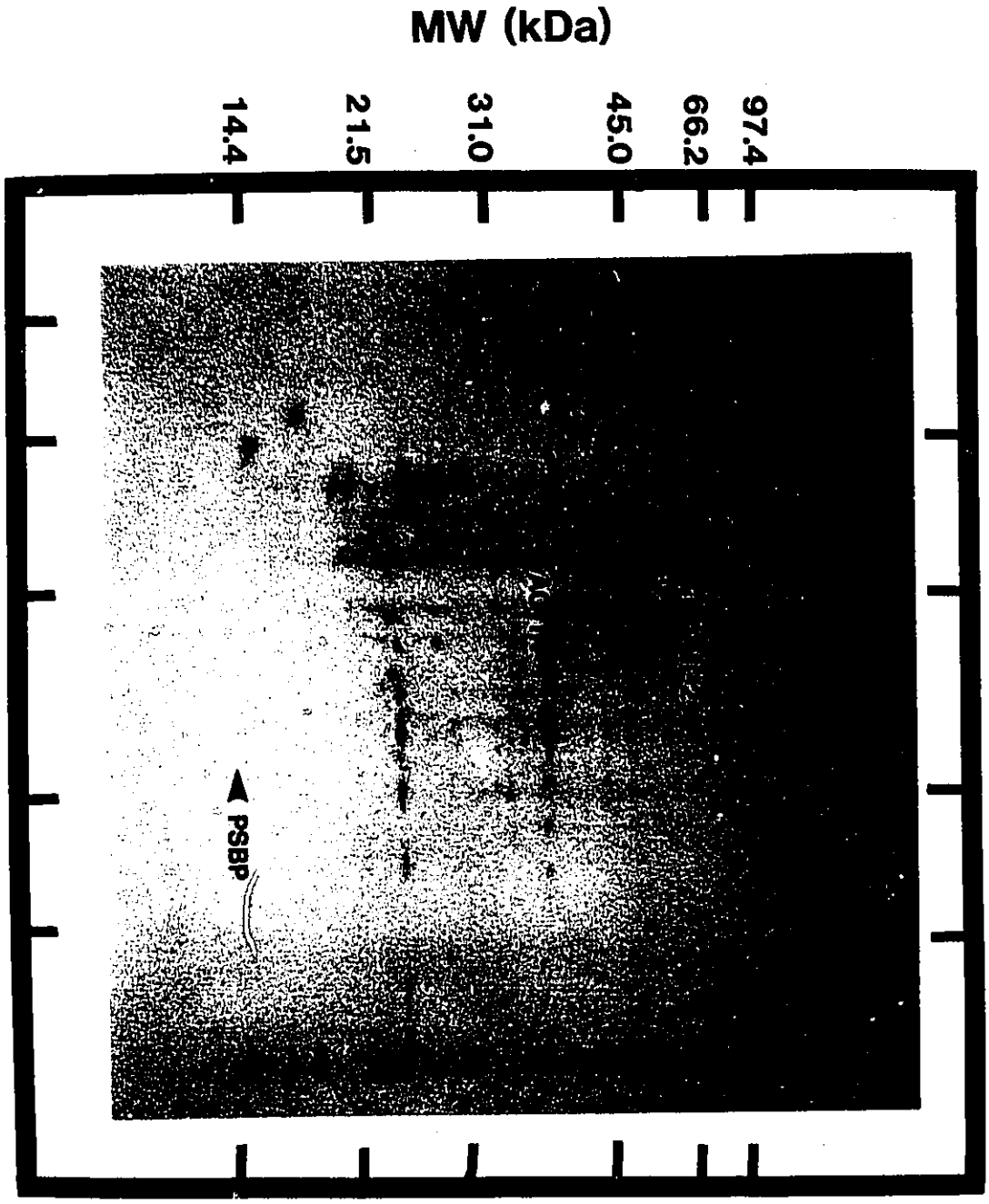

MW (kDa)
97.4
66.2
45.0
31.0
21.5
14.4
PSBP
5
6
7
8
pI

FIGURE 12: Two dimensional gel electrophoresis of in vitro translation products from rat ventral prostates 8 days after castration.

45,000 cpm (TCA precipitable) of [^{35}S]-labeled translation products from 1μg poly(A)$^+$ RNA from prostates of 8 day castrates were run on a two dimensional gel, as described in section 2.3, prepared for fluorography, and exposed for 3 days. The position of molecular weight standards is shown on scale. The position of TRPM-2, actin and the presumed position of PSBP are shown on the figure.

Molecular weight standards:

97.4 kDa: phosphorylase B
66.2 kDa: bovine serum albumin
45.0 kDa: ovalbumin
31.0 kDa: carbonic anhydrase
21.5 kDa: soybean trypsin inhibition
14.4 kDa: lysozyme


MW (kDa)
97.4
66.2
45.0
31.0
21.5
14.4
PSBP
5
6
7
8
pI

are more prominent in the translation products of prostatic poly(A)$^+$RNA extracted from rats castrated 8 days previously, most notably a protein band slightly larger and more basic than actin, at a molecular weight of approximately 50,000 daltons and an isoelectric point between 5.7 and 6.0 (Fig. 12). This protein is not present in the translation products of intact rat prostate poly(A)$^+$RNA (Fig. 11). It appears to correspond to the translation product of the most abundant mRNA species which have been previously described in the rat ventral prostate after castration (Montpetit et al., 1986). This mRNA species has been named TRPM-2, as it is a Testosterone Repressed Prostate Message of approximately 2000 nucleotides in length.

The identification of TRPM-2, a testosterone repressed prostate message, is very significant since this represents the first mRNA species to be identified in the prostate which is repressed in the normal state of the gland.

3.3. ISOLATION OF TRPM-2 SPECIFIC cDNA CLONES

In view of the potential importance of TRPM-2 in the normal growth and involution processes in the prostate, it was of interest to isolate cDNA clones specific for TRPM-2 and to study the expression of this message. To obtain these specific clones, its cDNA was prepared using prostatic poly(A)$^+$RNA from castrated rats as a template. It was ligated into pUC8, and the recombinant plasmids were

used to transform E. coli DH1 cells. Of the approximately 500,000 clones in the resulting library, 4400 were screened by Grunstein-Hogness hybridization. The results of the screening show that TRPM-2 is a relatively rare message. Out of 4400 clones from a cDNA library prepared from poly(A)$^+$RNA from 8 day castrated rats, only 35 sequences (or less than 0.8% of the clones) were found, by Grunstein-Hogness analysis, to represent androgen repressed sequences. Eight of these have been shown to hybridize to an RNA species of 2000 nucleotides present in the poly(A)$^+$RNA from castrated rats, as represented in figure 13a, lane 2, for two of the cDNA clones, p22-12 & p21-04. No signal was detected in the lane containing poly(A)$^+$RNA from intact rats (Fig. 13a, lane 1). The PSBP mRNA sequences are the most abundant mRNA species in the prostate. Since they are completely androgen dependent they are not represented in the poly(A)$^+$RNA used to prepare the clone bank. This suggests that the TRPM-2 mRNA sequence represents approximately 0.2% of the poly(A)$^+$RNA (8 of 4400 clones) in the prostate of rats castrated 8 days previously, and if TRPM-2 sequences are expressed at all in the normal prostate, they must be present at significantly lower levels.

The restriction maps of the TRPM-2 specific plasmids show many similarities, including common Hae III, Msp I and Pst I sites (Fig. 13b). A number of restriction enzymes did not cut the insert, notably Bam HI, Eco RI, Eco RV, Hind III, Kpn I, Sma I & Sph I. The largest clone isolated, p21-04, is 1300 bases. This

FIGURE 13: Characterization of TRPM-2 plasmids by Northern hybridization analysis and restriction mapping.

Panel A: Replicate samples of 2μg poly(A)$^+$ RNA from intact (lane 1) and castrated (lane 2) rat prostate were electrophoresed on 1.5% agarose gels, and transferred to nylon membrane filters by Northern transfer, as described in section 2.7. Individual plasmids were nick translated to an average of 4 X 10^7 cpm/μg and hybridized to individual replicates of the Northern blots. The blots were washed and exposed overnight.

Panel B: Restriction digests were performed as described in section 2.6.

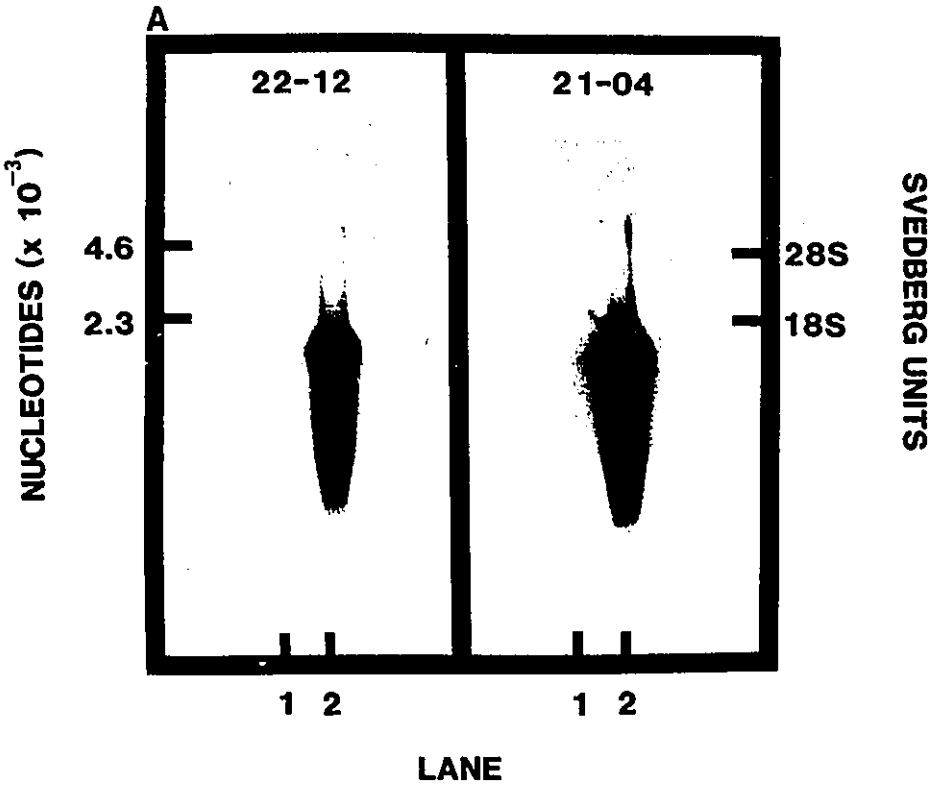
B : Bam HI

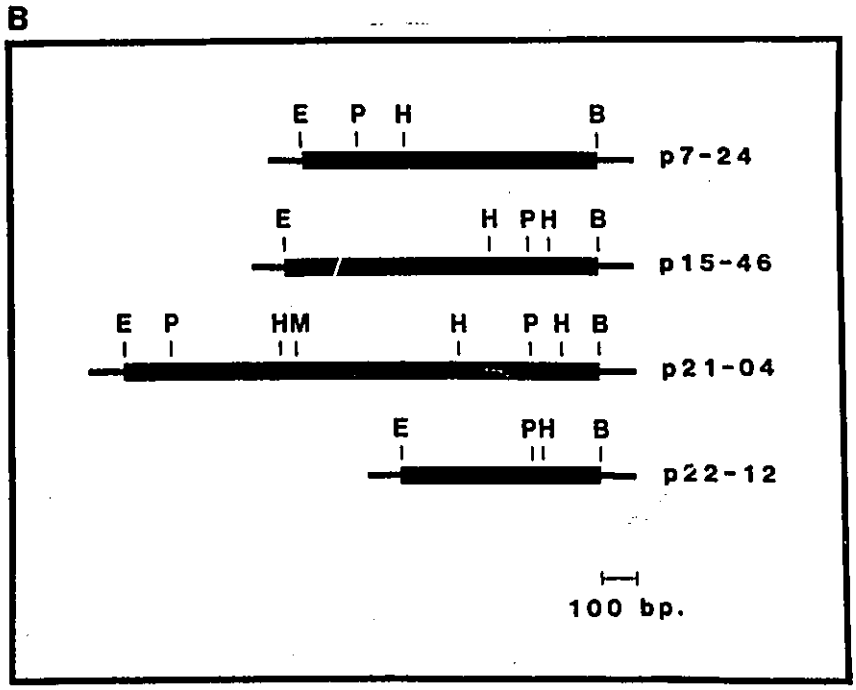
E : Eco RI

P : Pst I

H : Hae III

M : Msp I


A
22-12
21-04
NUCLEOTIDES (x 10^{-3})
4.6
2.3
28S
18S
SVEDBERG UNITS
1 2
1 2
LANE


B
E P H B
p7-24
E H PH B
p15-46
E P HM H P H B
p21-04
E PH B
p22-12
100 bp.

does not represent a full length clone, since TRPM-2 is approximately 2000 nucleotides in size. Clone p21-04 therefore represents about 65% of the TRPM-2 sequence.

3.4. SEQUENCE OF 21-04

The sequence of 21-04 is shown in Fig. 14 (clone 22-12 was also sequenced, and found to be identical to 21-04, therefore only the latter is presented). The presence of the poly (A) tail demonstrates that the 3'end of the gene was cloned. Initial sequence analysis of this TRPM-2 clone using Microgenie™ software demonstrated no sequence homology with registered sequences in GENBANK or NBRF. More recently, the reported sequence of the cDNA clone for sulfated glycoprotein-2 (SGP-2) (Collard & Griswold, 1987; Bettuzzi, Hiipakka, Gilna & Liao, 1989), the most abundant secretory protein of the Sertoli cells in the testis (Kissinger, Skinner & Griswold, 1982; Sylvester, Skinner & Griswold, 1984), and the clusterin protein sequence (Cheng, Chen, Feng, Marshall & Bardin, 1988) were shown to be highly homologous to TRPM-2 (appendix 2). Of the 1337 bases compared, only 8 were mismatched. Clusterin, or SGP-2, is a disulfide-linked heterodimer whose reduced subunits migrate with mobilities of 47 and 34 kDa in SDS-PAGE. It contains 23.7% carbohydrate, and the N-linked oligosaccharides are highly sulfated (Griswold, Roberts & Bishop, 1986). In vitro translation of immunopurified SGP-2 mRNA results in a 50 kDa non-glycosylated

FIGURE 14: Nucleotide and presumed amino acid sequence of 21-04.

Position of termination codon, poly (A) addition signal and poly (A) tract are underlined. Mismatches with the sequence of SGP-2 indicated by dots.

```
      10        20        30        40        50        60        70        80        90       100       110       120
CTGCTCAACAGTTTAGAGGAAGCCAAAAAGAAGAAAGAGGGTGCTCTAGATGACACCAGGGATTCTGAAATGAAGCTGAAGGCTTTCCCCGAAGTGTGTAACGAGACCATGATGGCCCTC
LeuLeuAsnSerLeuGluGluAlaLysLysLysLysGluGlyAlaLeuAspAspThrArgAspSerGluMetLysLeuLysAlaPheProGluValCysAsnGluThrMetMetAlaLeu

     130       140       150       160       170       180       190       200       210       220       230       240
TGGGAAGAGTGTAAGCCCTGCCTGAAGCACACCTGCATGAAGTTCTACGCACGCGTCTGCAGGAGCGGCTCGGGGCTGGTTGGTCGCCAGCTAGAGGAGTTTCTGAACCAGAGCTCACCC
TrpGluGluCysLysProCysLeuLysHisThrCysMetLysPheTyrAlaArgValCysArgSerGlySerGlyLeuValGlyArgGlnLeuGluGluPheLeuAsnGlnSerSerPro

     250       260       270       280       290       300       310       320       330       340       350       360
TTCTACTTCTGGATGAACGGGGACCGCATCGACTCCCTGCTGGAGAGTGACCGCCAGCAGAGCCAAGTCCTAGATGCTATGCAGGACAGCTTCACTCGGGCGTCTGGCATCATAGATACG
PheTyrPheTrpMetAsnGlyAspArgIleAspSerLeuLeuGluSerAspArgGlnGlnSerGlnValLeuAspAlaMetGlnAspSerPheThrArgAlaSerGlyIleIleAspThr

     370       380       390       400       410       420       430       440       450       460       470       480
CTTTTCCAGGACCGGTTCTTCACCCATGAGCCCCAGGACATCCACCATTTCTCCCCCATGGGCTTCCCACACAAGCGGCCTCATTTCTTGTACCCCAAGTCCCGCTTGGTCCGCAGCCTC
LeuPheGlnAspArgPhePheThrHisGluProGlnAspIleHisHisPheSerProMetGlyPheProHisLysArgProHisPheLeuTyrProLysSerArgLeuValArgSerLeu

     490       500       510       520       530       540       550       560       570       580       590       600
ATGCCTCTCTCCCACTACGGGCCTCTGAGCTTCCACAACATGTTCCAGCCTTTCTTTGATATGATACACCAGGCTCAACAGGCCATGGACGTCCAGCTCCATAGCCCAGCTTTACAGTTC
MetProLeuSerHisTyrGlyProLeuSerPheHisAsnMetPheGlnProPhePheAspMetIleHisGlnAlaGlnGlnAlaMetAspValGlnLeuHisSerProAlaLeuGlnPhe

     610       620       630       640       650       660       670       680       690       700       710       720
CCGGATGTGGATTTCTTAAAAGAAGGTGAAGATGACCCGACAGTGTGCAAGGAGATCCGCCATAACTCCACAGGATGCCTGAAGATGAAGGGCCAGTGTGAGAAGTGCCAAGAGATCTTG
ProAspValAspPheLeuLysGluGlyGluAspAspProThrValCysLysGluIleArgHisAsnSerThrGlyCysLeuLysMetLysGlyGlnCysGluLysCysGlnGluIleLeu

     730       740       750       760       770       780       790       800       810       820       830       840
TCTGTGGACTGTTCGACCAACAATCCTGCCCAGGCTAACCTGCCCCAGGAGCTAAACGACTCGCTCCAGGTGGCTGAGAGGCTGACCCAGCAGTACAACCAGCTGCTTCATTCCCTCCAG
SerValAspCysSerThrAsnAsnProAlaGlnAlaAsnLeuArgGlnGluLeuAsnAspSerLeuGlnValAlaGluArgLeuThrGlnGlnTyrAsnGluLeuLeuHisSerLeuGln

     850       860       870       880       890       900       910       920       930       940       950       960
TCCAAGATGCTCAACACCTCATCCCTGCTGGAACAGCTGAACGACCAGTTCAACGTGGGTGTCCCAGCTGGCTAACCTCACACAGGGCGATGACCAGTACCTCGGGTCTCCACAGTGACA
SerLysMetLeuAsnThrSerSerLeuLeuGluGlnLeuAsnAspGlnPheThrTrpValSerGlnLeuAlaAsnLeuThrGlnGlyAspAspGlnTyrLeuArgValSerThrValThr

     970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080
ACCCATTCTTCTGACTCAGAAGTCCCCTCTCGTGTCACTGAGGTGGTGGTGAAGCTGTTTGACTCTGACCCCATCACAGTGGTGTTACCAGAAGAAGTCTCCAAGGATAACCCTAAGTTT
ThrHisSerSerAspSerGluValProSerArgValThrGluValValValLysLeuPheAspSerAspProIleThrValValLeuProGluGluValSerLysAspAsnProLysPhe

     1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
ATGGACACAGTGGCAGAGAAAGCGCTACAGGAATACCGCAGAAAAGCCGCCATGGAATGAGACAGAAGCATCAGTTTTCTATATGTAGGAGTCTCAAGGAGGGATCTCCCAGCTTTCCGA
MetAspThrValAlaGluLysAlaLeuGlnGluTyrArgArgLysAlaAlaTrpAsnGluThrGluAlaSerValPheTyrMetEnd

     1210      1220      1230      1240      1250      1260      1270      1280      1290      1300      1310      1320
GGTTGCTGCAGACCCCTAGAGAACTCCACATGTCTCCAGCGCCTAGGCCTCCACCCCAGCAGCCTCTCCTTCCTCTGGGTTCTGTACTGTAATGCCTGCACTTGATGCTCTCGGGGAAGAA

     1330      1340      1350      1360      1370
CTGCTTCCCCCACGCAACTAATCCAATAAAGCCACCTTGCGATAAAAAAAAAAAA
```

product (Collard and Griswold, 1987). This corresponds to the migration of the in vitro translation product of TRPM-2 (Fig. 12) and to the hybrid selected translation product of 21-04 (Tenniswood, personnal communication). These results strongly suggest that TRPM-2 also codes for a membrane associated protein.

The clone p21-04 has been used in subsequent screening of a λgt-10 cDNA library made with poly(A)$^+$ RNA from castrated rat ventral prostate. A full length TRPM-2 clone has been isolated and sequenced. It is 1700 bases long, and has been named 17H (Wang, Pineault & Tenniswood, in preparation).

3.5. EXPRESSION OF TRPM-2 FOLLOWING CASTRATION

3.5.1. Northern hybridization analysis

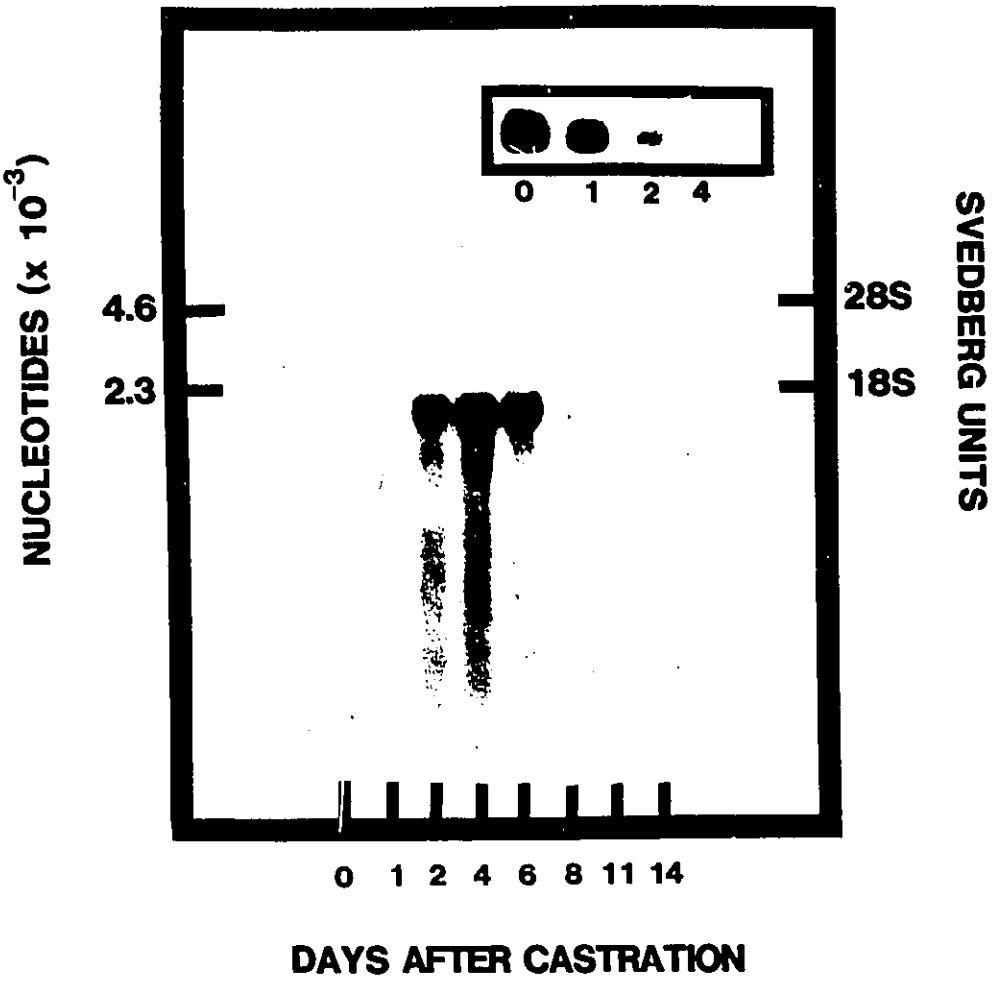
Poly(A)$^+$RNA from different time points was separated according to size by agarose gel electrophoresis, transferred to Pall™ membranes by Northern blotting, and hybridized to [^{32}P]-radiolabeled p22-12, one of the TRPM-2 specific cDNA clones (Fig. 15). The blot was subsequently hybridized to nick translated pA34, a cDNA clone specific for the C3 subunit of PSBP (Parker et al., 1983). Hybridization with [^{32}P]-22-12 demonstrates that TRPM-2 appears as soon as two days after castration, peaks between days four and six, then decreases to undetectable levels by the eighth day after castration (Fig. 15). The inset of Fig. 15 shows that the

FIGURE 15: Northern analysis of TRPM-2 induction after castration.

3μg poly(A)$^+$RNA, extracted from the prostate at the indicated times after castration, was Northern blotted as described in section 2.10. The filter was hybridized to a TRPM-2 specific probe (p22-12, $5x10^8$cpm/μg).

The inset shows results of sequential hybridization with a PSBP specific probe (pA34, $2.9x10^8$ cpm/μg).

The autoradiographs were exposed for 5 days (TRPM-2), and 18 hours (PSBP).


NUCLEOTIDES (x 10^{-3})
4.6
2.3
0 1 2 4
28S
18S
SVEDBERG UNITS
0 1 2 4 6 8 11 14
DAYS AFTER CASTRATION

levels of PSBP decrease dramatically, as has previously been demonstrated (Page & Parker, 1982; Zhang & Parker, 1985). By the fourth day after castration, the RNA is essentially undetectable.

The Northern analysis thus demonstrates that TRPM-2 is induced shortly after castration, and is expressed at maximal levels between days 4 and 6. It is interesting to note that this coincides to the time when the rate of cell death is maximal (Isaacs, 1984). This suggests that TRPM-2 plays a role in the process of regression.

3.5.2. Dot blot analysis

Dot blot analysis was performed to quantitate the relative levels of TRPM-2 poly(A)$^+$RNA at various times after castration. The results show essentially the same profile of expression as the Northern analysis: PSBP mRNA levels decrease dramatically, from 80,000 ppm, to 50,000 ppm by day two after castration, and finally to undetectable levels by the sixth day. TRPM-2 on the other hand increases from undetectable levels on day zero to reach a peak of 1440 ppm on day 6 (almost two orders of magnitude lower than the steady state levels of PSBP mRNA in the intact rat prostate) before decreasing to 6% of maximal levels by day 8 (Fig. 16).

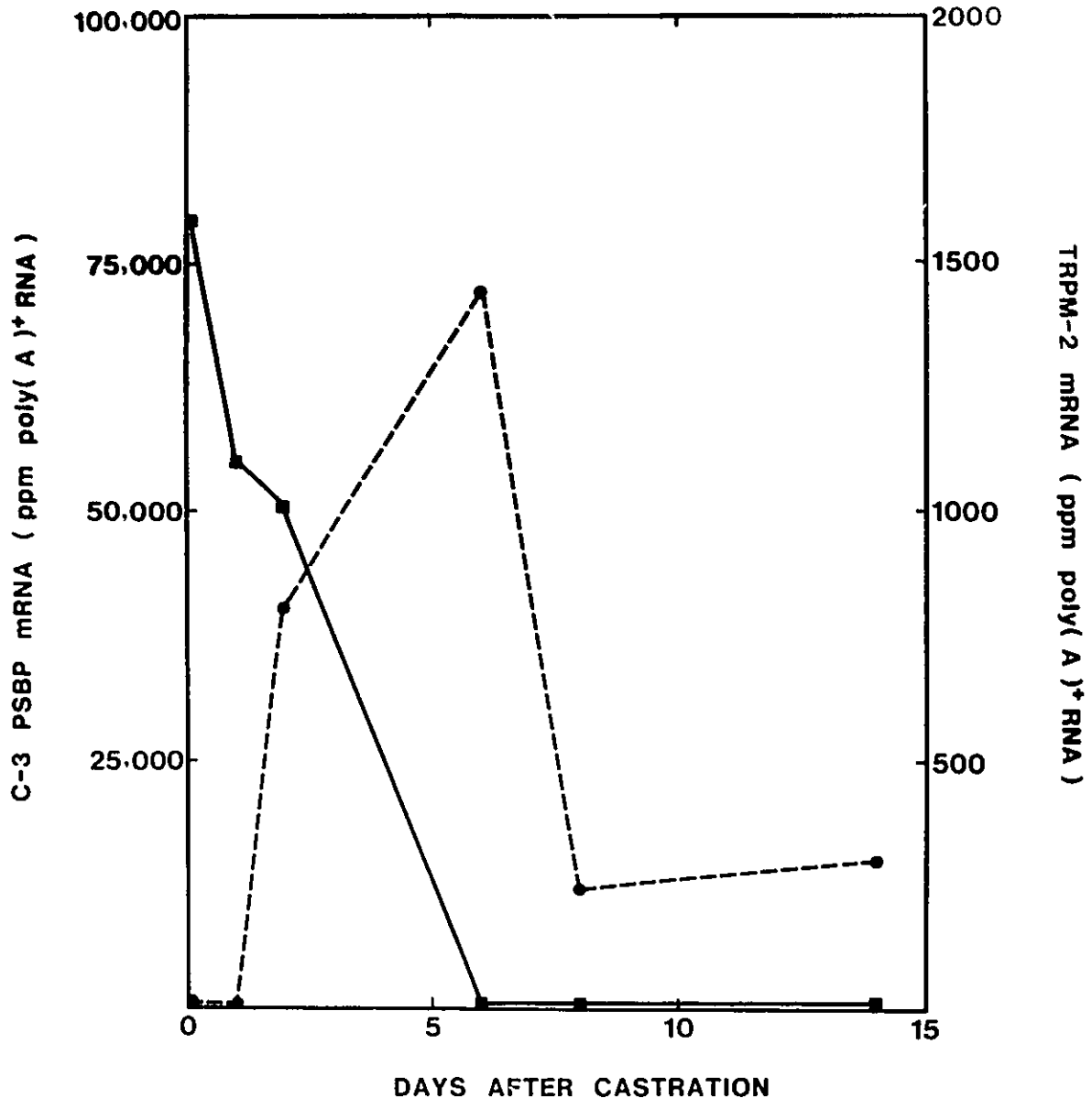
FIGURE 16: Dot blot analysis of TRPM-2 induction after castration.

One μg poly(A)$^+$RNA, extracted from the prostate at the indicated times after castration was blotted in triplicate onto duplicate nylon membranes. One membrane was hybridized to a TRPM-2 specific [^{32}P]-labeled probe (p21-04, 8 X 10^7 cpm/μg), the other to a PSBP specific [^{32}P]-labeled probe (pA34, 5 X 10^7 cpm/μg). The steady state levels of each mRNA sequence (ppm of poly(A)$^+$RNA) were calculated from standard curves established for each of the sequences, as described in section 2.11.

(Note difference in scales for the two sequences).

(■——) Steady state level of C-3 mRNA.

(●– – –) Steady state level of TRPM-2 mRNA


100,000
75,000
50,000
25,000
2000
1500
1000
500
0
5
10
15
C-3 PSBP mRNA (ppm poly(A)+ RNA)
TRPM-2 mRNA (ppm poly(A)+ RNA)
DAYS AFTER CASTRATION

These results confirm that TRPM-2 expression in the rat ventral prostate peaks between days 4 and 6 after castration. They also confirm that the steady state levels of TRPM-2 mRNA are low when compared to PSBP mRNA levels, and that TRPM-2 is expressed transiently in the regressing prostate.

3.6. EXPRESSION OF TRPM-2 FOLLOWING ANTI-ANDROGEN TREATMENTS

Since Huggins and Hodges first observed that castration resulted in regression of the prostate (Huggins & Hodges, 1941), treatments for prostatic diseases have all aimed to mimic the effects of castration. It was therefore of interest to determine whether two anti-androgens commonly used in therapy, cyproterone acetate and flutamide, induce expression of TRPM-2.

3.6.1. Changes in OW/BW ratios and total RNA levels

Fig. 17 and 18 show, respectively, the effects of anti-androgen treatments on OW/BW ratio and total RNA levels. (This time course study was repeated twice, with similar results. Only the results from one study are presented here). The maintenance dose of 250 g/day of 5α-DHT is sufficient to maintain the organ weight to body weight ratio at an average of 1.1, which is close to that seen in intact animals. It had previously been shown that this dose of androgen is adequate to maintain normal prostate size (OW/BW 0.8-1.2 mg/g) and secretory

FIGURE 17: Effects of anti-androgen treatments on the OW/BW ratio.

Rats were sacrificed and the ventral prostates excised and pooled on indicated days after treatment. The OW/BW ratios were calculated as described previously (Jackson et al, 1977). 5α-DHT treated controls were sacrificed for comparative purposes.

(◆——) castrated + 5α-DHT (250μg/day)

(■·····) castrated + 5α-DHT (250μg/day) + cyproterone acetate (10mg/day)

(▲— —) castrated + 5α-DHT (250μg/day) + flutamide (15mg/day)

: normal range

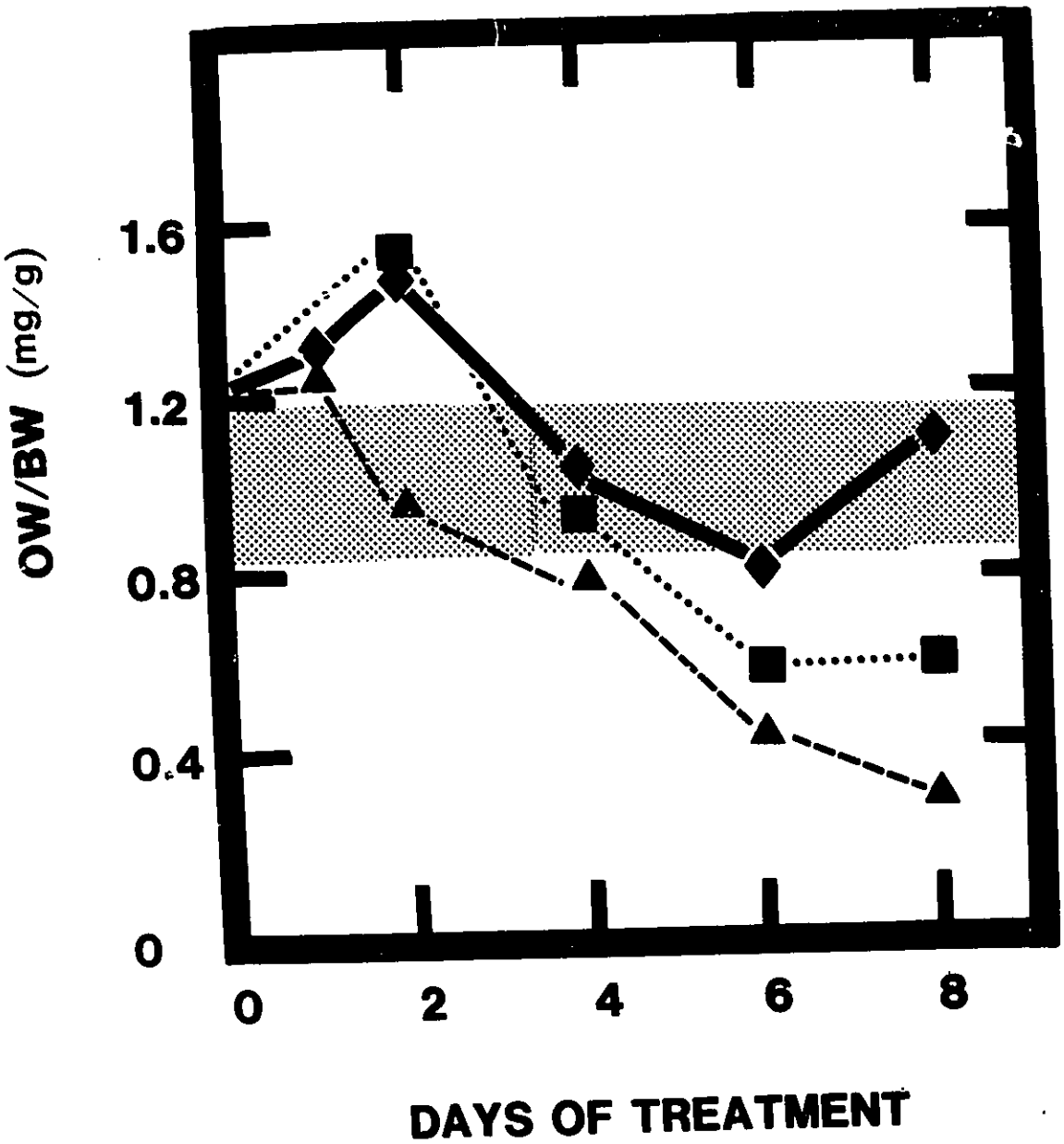

OW/BW (mg/g)
1.6
1.2
0.8
0.4
0
0
2
4
6
8
DAYS OF TREATMENT

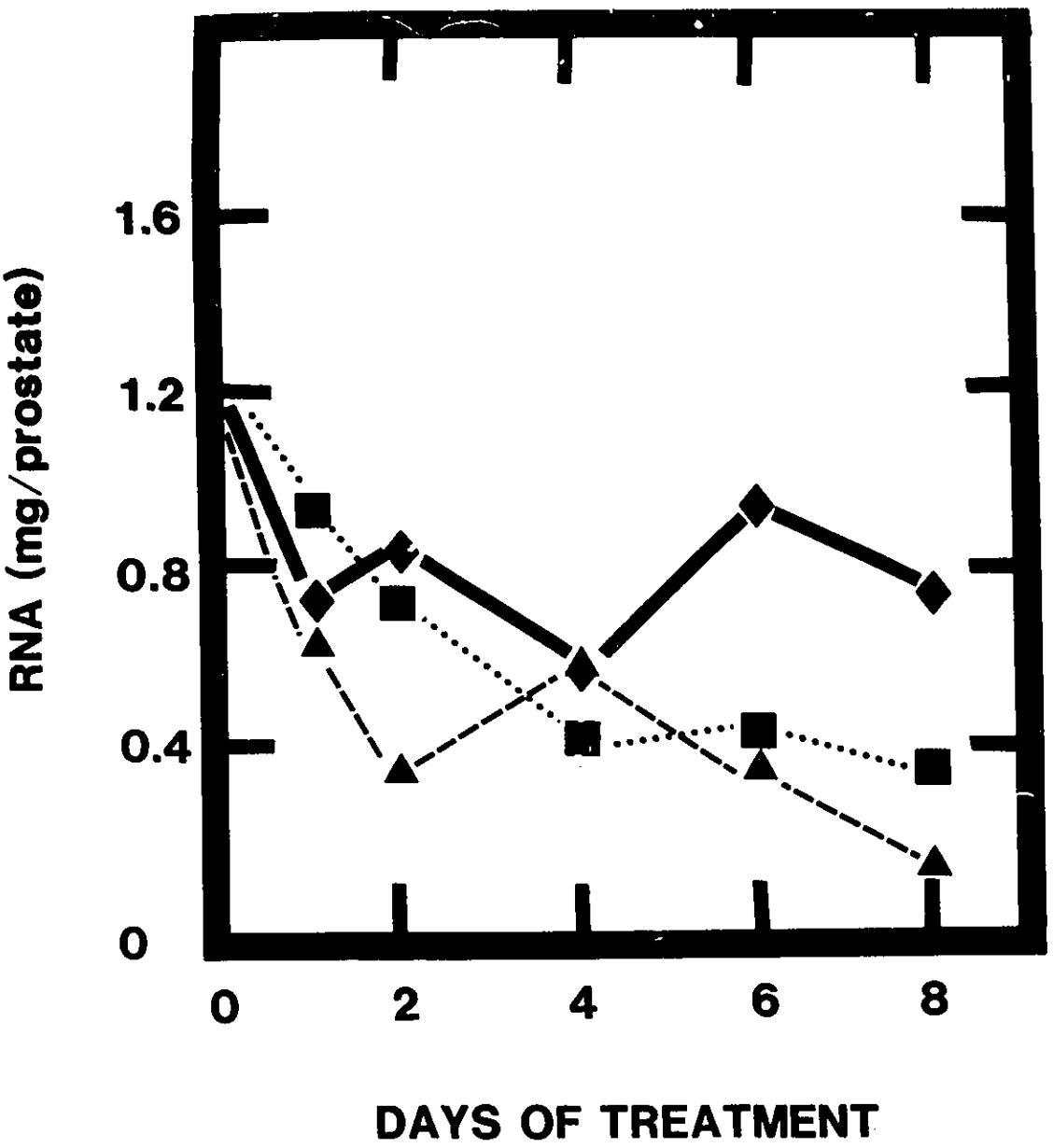
FIGURE 18: Effects of anti-androgen treatments on total prostatic RNA levels.

Rats were sacrificed and the ventral prostates excised and pooled on indicated days after treatment. Total RNA was extracted and the concentration determined as described in section 2.2. 5α-DHT treated controls were sacrificed for comparative purposes.

(◆——) castrated + 5α-DHT (250μg/day)

(■•••••) castrated + 5α-DHT (250μg/day) + cyproterone acetate (10mg/day)

(▲– – –) castrated + 5α-DHT (250μg/day) + flutamide (15mg/day)


RNA (mg/prostate)
1.6
1.2
0.8
0.4
0
0
2
4
6
8
DAYS OF TREATMENT

acid phosphatase activity (Tenniswood, Abrahams, Bird & Clark, 1981). Northern analysis also demonstrated that this dose maintains normal expression of PSBP (results not shown). Both cyproterone acetate (10mg/day) and flutamide (15mg/day) reduce organ weight to body weight ratios when administered to castrated rats supplemented with 250μg 5α-DHT/day. The very slight increase observed on the second day is probably a reflection of water imbibition which has previously been observed following castration at this time point (Lee, Bahnson, Blum, Anderson & Bockrath, 1986). The doses of cyproterone acetate and flutamide used in these experiments have previously been shown to induce substantial regression in the rat ventral prostate in a manner similar to castration (Tenniswood, Abrahams, Bird & Clark, 1984; Neri & Peets, 1975; Neumann, 1977; 1985). By the eighth day of treatment with cyproterone acetate, the OW/BW ratio has decreased to 0.5, while the administration of flutamide reduces the OW/BW to 0.3. In comparison to the results obtained following castration (Fig. 9), the reduction in OW/BW is not as rapid, nor as pronounced. For example, 4 days of treatment with cyproterone acetate reduces the OW/BW ratio to 0.8, whereas this ratio falls to 0.4 four days after castration. By the eighth day, neither flutamide nor cyproterone acetate has reduced the ratio to the level seen after castration. There is however a concomitant reduction in the amount of total RNA per prostate with these treatments (Fig. 18). The administration of 5α-DHT at a dose of 250μg/day maintains the RNA content of the prostate at 1.1mg per prostate, similar to the amounts present in normal prostate (Montpetit et al., 1986), confirming that this is

a suitable maintenance dose of 5α-DHT. The administration of cyproterone acetate or flutamide results in a decrease in the RNA content of the prostate, which by day eight is 0.35mg/prostate and 0.14mg/prostate respectively. At this time point after castration, the level has decreased to 0.13mg/prostate (Fig. 16), which is similar to the decrease shown after flutamide treatment. Treatment with these two anti-androgens thus mimics the effects of castration on the OW/BW and RNA levels, although it is obvious that the effects of the anti-androgens are not manifested as rapidly as castration.

3.6.2. Dot blot hybridization

The changes in the steady state levels of TRPM-2 and PSBP poly(A)$^+$RNA induced by cyproterone acetate treatment as measured by dot blot hybridization are shown in Fig. 19. The level of TRPM-2 mRNA increases from undetectable levels on day zero to a maximum of 520 ppm on day four before declining to approximately 250 ppm between days six and eight. During the same period the levels of PSBP mRNA decrease substantially from nearly 75,000 ppm on day zero to 12,000 ppm by day eight of treatment. The levels of TRPM-2 and PSBP mRNA do not appear to be further reduced, even after 20 days of treatment (results not shown).

FIGURE 19: Dot blot analysis of TRPM-2 induction after cyproterone acetate treatment.

One μg poly(A)$^+$RNA, extracted from the prostate at the indicated times of cyproterone acetate treatment (10mg/day) was blotted in triplicate onto duplicate nylon membranes. One membrane was hybridized to a TRPM-2 specific [^{32}P]-labeled probe (p21-04, 8 X 10^7 cpm/μg), the other to a PSBP specific [^{32}P]-labeled probe (pA34, 5 X 10^7 cpm/μg). The steady state levels of each mRNA sequence (ppm of poly(A)$^+$RNA) were calculated from standard curves established for each of the sequences, as described in section 2.11.

(Note difference in scales for the two sequences).

(■——) Steady state level of C-3 mRNA.

(●– – –) Steady state level of TRPM-2 mRNA

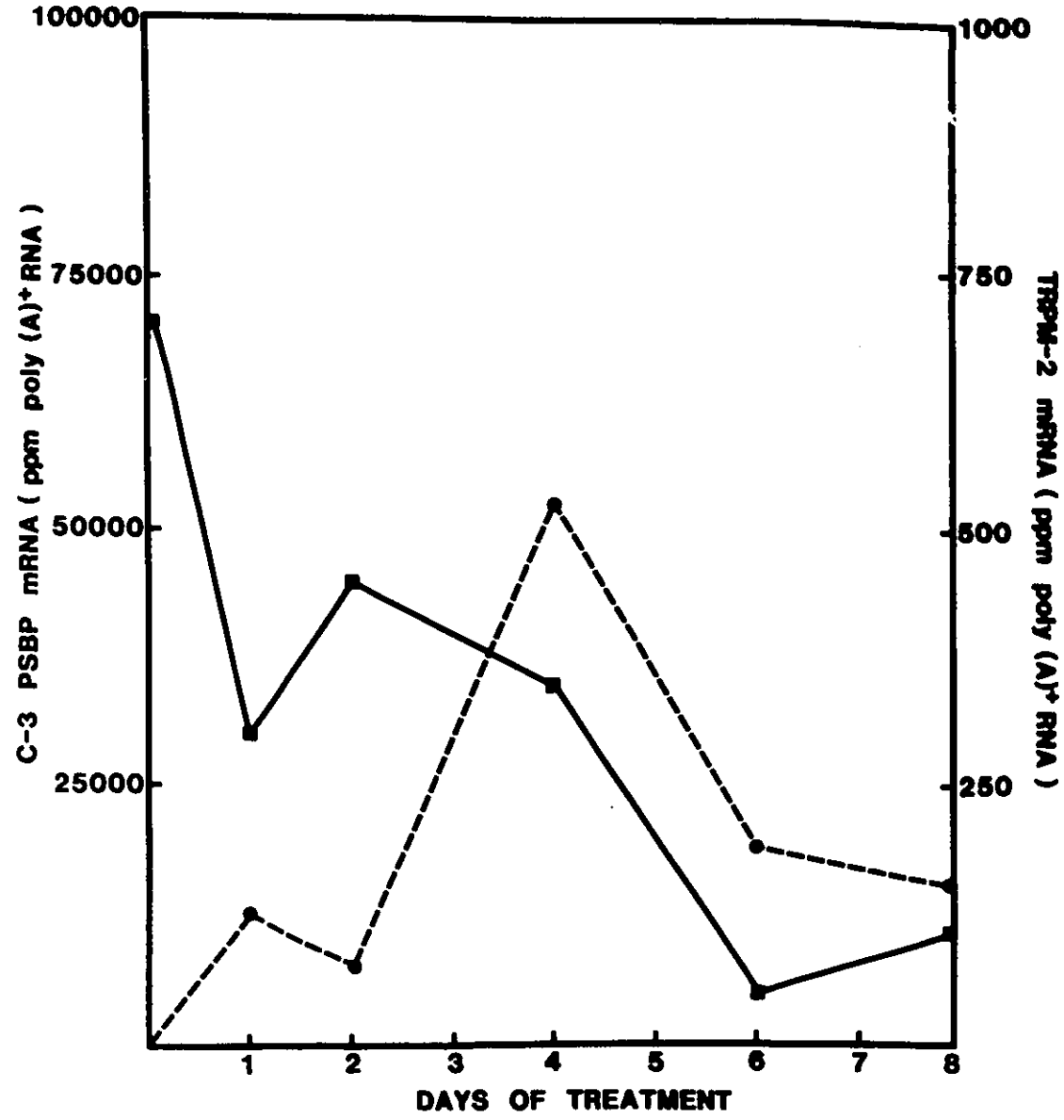

100000
75000
50000
25000
1000
750
500
250
C-3 PSBP mRNA (ppm poly (A)+ RNA)
TRPM-2 mRNA (ppm poly (A)+ RNA)
1
2
3
4
5
6
7
8
DAYS OF TREATMENT

Fig. 20 demonstrates the changes in the steady state levels of TRPM-2 and PSBP mRNA induced by flutamide. TRPM-2 mRNA increases steadily from undetectable levels on day zero to a maximum of 220 ppm on day six. PSBP mRNA levels, on the other hand, decrease over the same period from 75,000 ppm to approximately 5,000 ppm by day six. [Due to low recovery of poly $(A)^+$RNA from the day eight samples it was not possible to quantitate the levels of PSBP or TRPM-2 mRNA in this particular experiment]. The results obtained with both cyproterone acetate and flutamide treatments on PSBP mRNA expression are very similar. Both anti-androgens reduce, but do not eliminate PSBP mRNA. Cyproterone acetate appears to induce TRPM-2 more efficiently than flutamide. A maximal level of 520 ppm is reached on the fourth day after castration, whereas the maximal level attained following six days of flutamide treatment is only 220 ppm. The induction by flutamide appears more gradual, and does not peak as suddenly as the induction by cyproterone acetate. The significance of these differences between the anti-androgens is not known, but may reflect variation in the efficacy of the two drugs.

Comparison of these results to those obtained following castration (Fig. 16) demonstrates that neither anti-androgen is as effective as castration in inducing TRPM-2 or in repressing PSBP expression. This is particularly evident with the repression of PSBP expression since the anti-androgens do not fully repress PSBP steady state levels even after extended treatment.

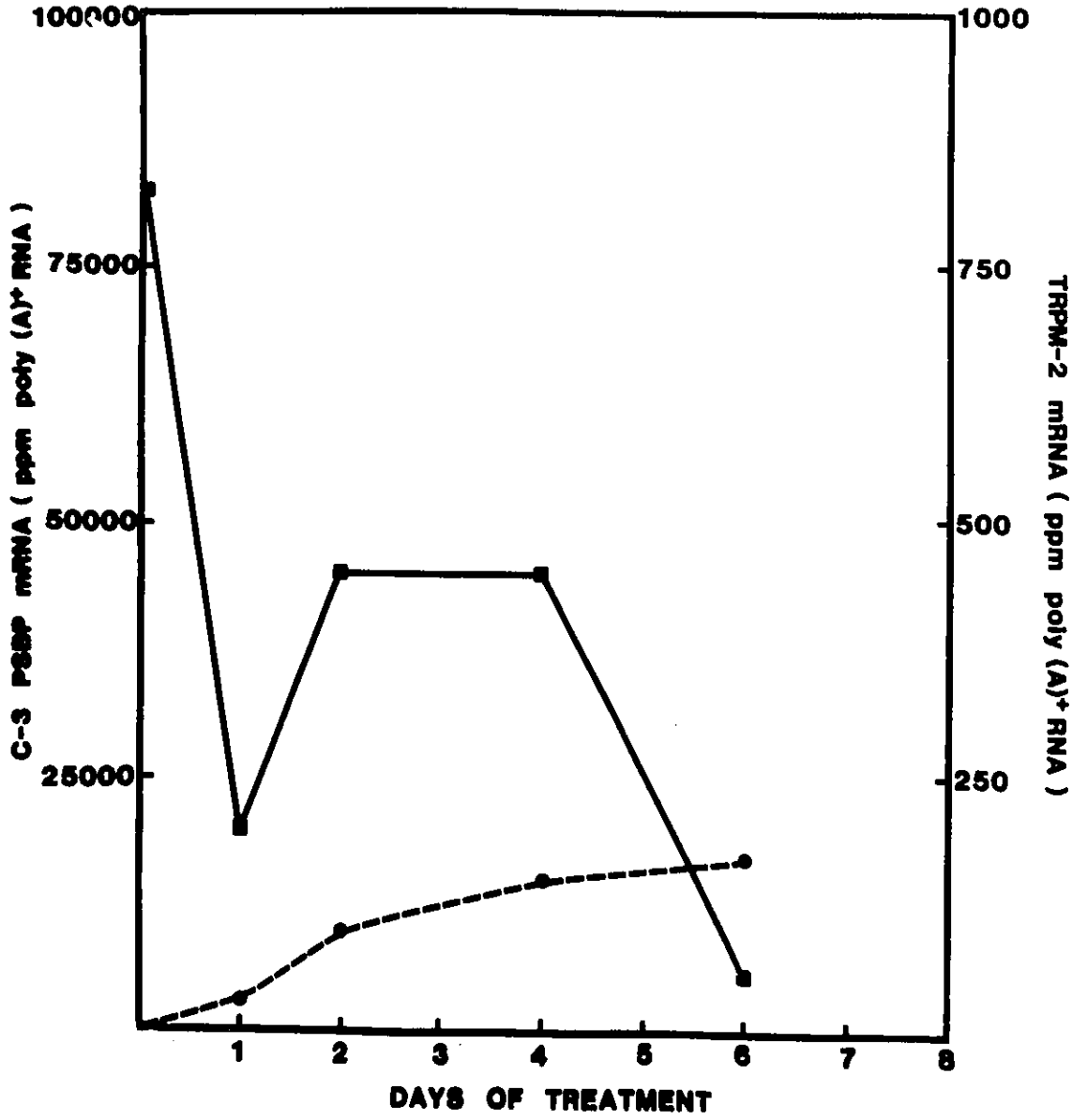
FIGURE 20: Dot blot analysis of TRPM-2 induction after flutamide treatment.

One μg poly(A)$^+$RNA, extracted from the prostate at the indicated times of flutamide treatment (15 mg/day) was blotted in triplicate onto duplicate nylon membranes. One membrane was hybridized to a TRPM-2 specific [^{32}P]-labeled probe (p21-04, 8 X 10^7 cpm/μg), the other to a PSBP specific [^{32}P]-labeled probe (pA34, 5 X 10^7 cpm/μg). The steady state levels of each mRNA sequence (ppm of poly(A)$^+$RNA) were calculated from standard curves established for each of the sequences, as described in section 2.11.

(Note difference in scales for the two sequences).

(■——————) Steady state level of C-3 mRNA

(●— — — —) Steady state level of TRPM-2 mRNA


100000
75000
50000
25000
1000
750
500
250
1
2
3
4
5
6
7
8
DAYS OF TREATMENT
C-3 PSSP mRNA (ppm poly (A)+ RNA)
TRPM-2 mRNA (ppm poly (A)+ RNA)

3.6.3. In vitro translation products

Fig. 21 shows the translation profiles of poly(A)$^+$RNA extracted from the prostates of rats treated with cyproterone acetate for twenty days, using the same in vitro translation system and gel electrophoresis protocol described earlier (section 2.3). This sample was used to determine whether prolonged treatment would retain TRPM-2 induction and repress PSBP. The individual subunits of PSBP are clearly visible among the translation products, migrating at an apparent molecular weight of 12,000 daltons with a broad pI of 4.9-7.0. In the same translation profile the TRPM-2 translation products are also evident (pI 5.5-6.3 and molecular weight approximately 50,000 daltons). Thus the proteins present in the translation products of the poly(A)$^+$RNA extracted from the prostate of animals treated with cyproterone acetate for twenty days include both androgen dependent and androgen repressed proteins. A similar result was obtained after flutamide treatment (not shown).

3.7. IN SITU LOCALIZATION OF TRPM-2 AND PSBP EXPRESSION

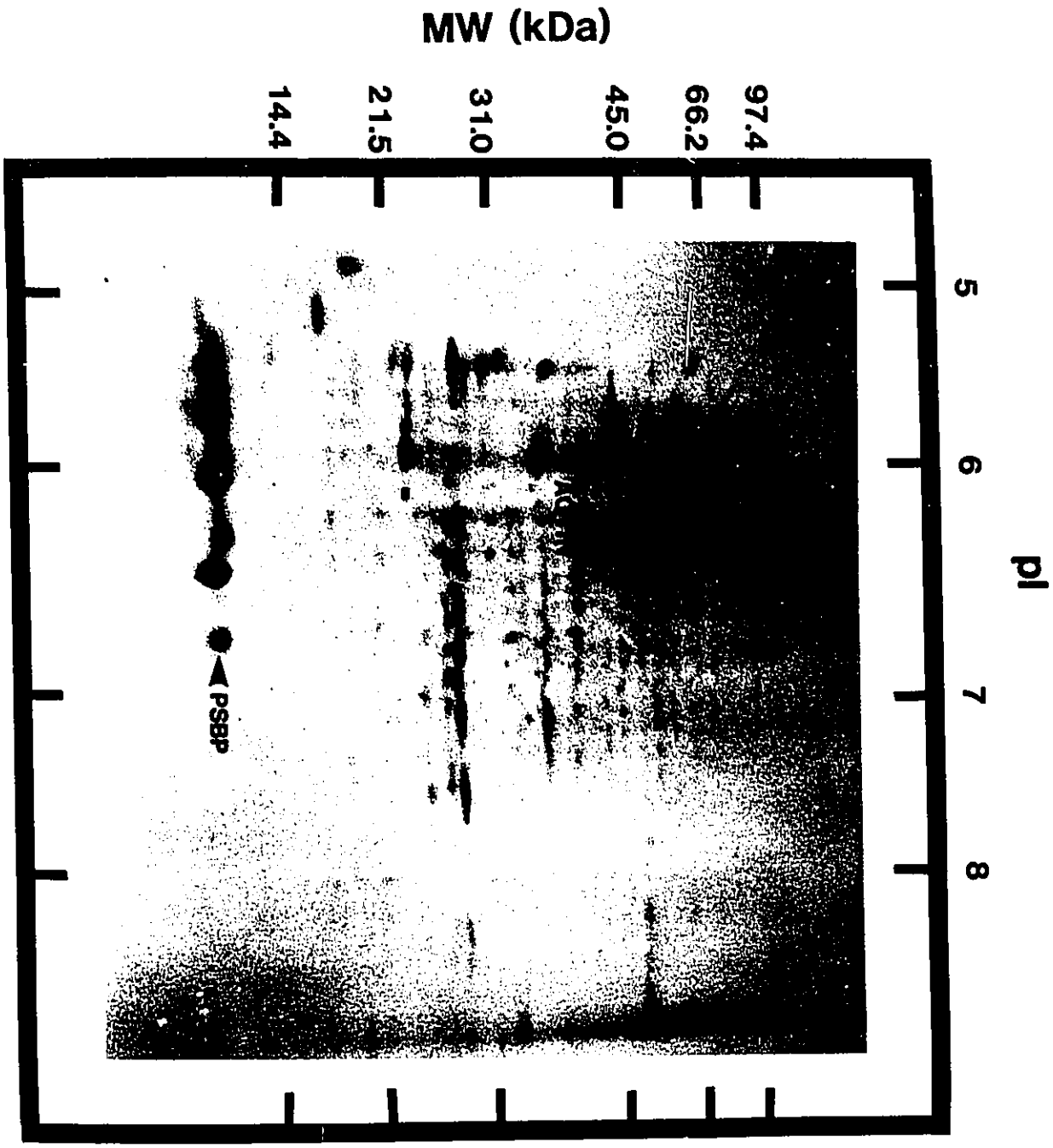
The prostate is composed of a very heterogeneous cell population. In order to determine which cell type(s) express(es) TRPM-2, in situ hybridization was performed on serial sections of the ventral prostates from normal and castrated rats.

FIGURE 21: Two dimensional gel electrophoresis of in vitro translation products from rat ventral prostate after 20 days of cyproterone acetate (10 mg/day) treatment.

45,000 cpm (TCA precipitable) of [^{35}S]-labeled translation products from 1 μg poly(A)$^+$ RNA from prostates of castrated rats treated for 20 days with 5α-DHT (250μg/day) + cyproterone acetate (10 mg/day) were run on two dimensional gels as described in section 2.3, prepared for fluorography and exposed for 8 days. The position of standard molecular weight markers is shown on scale. The position of TRPM-2, actin and PSBP are indicated on the figure.

Molecular weight standards:

97.4 kDa: phosphorylase B
66.2 kDa: bovine serum albumin
45.0 kDa: ovalbumin
31.0 kDa: carbonic anhydrase
21.5 kDa: soybean trypsin inhibition
14.4 kDa: lysozyme


MW (kDa)
97.4
66.2
45.0
31.0
21.5
14.4
5
6
7
8
pI
PSBP

3.7.1. Hybridization to random sections

In order to determine whether the epithelial or stromal cell population is responsible for the expression of TRPM-2 and PSBP, in situ hybridizations were performed on random sections of ventral prostates from normal and castrated rats.

The in situ hybridization of the TRPM-2 probe to prostate sections isolated from intact rats is shown in Fig. 22b. The hybridization level is minimal and mostly non-specific, as demonstrated by the RNAse-treated control (Fig. 22a). The PSBP probe hybridizes strongly to the luminal epithelial cells of these sections (Fig. 22d). Five days after castration, the expression of TRPM-2 is greatly increased and is localized in the luminal epithelial cells (Fig. 23b). On the other hand, the hybridization signal using the PSBP probe (Fig. 23d) is comparable to the RNAse treated control (Fig. 23c).

These results complement the data obtained from Northern and dot blot hybridizations, and demonstrate that TRPM-2 is expressed in the epithelial cells of the rat ventral prostate during the regression of the gland after castration.

FIGURE 22: In situ hybridization of TRPM-2 and PSBP to random transverse sections of the ventral prostate from intact rats.

Rat ventral prostates were excised, sectioned and fixed as described in section 2.14. The fixed sections were hybridized with 20ng of TRPM-2 specific probe ([^{35}S]-21-04 insert, 1.0 X 10^8 cpm/μg) or PSBP specific probe ([^{35}S]-A34 insert, 2.7 X 10^8 cpm/μg).

Panel a: Section hybridized to the TRPM-2 specific probe after RNAse treatment (negative control).

Panel b: Section hybridized to the TRPM-2 specific probe.

Panel c: Section hybridized to the PSBP specific probe after RNAse treatment (negative control).

Panel d: Section hybridized to the PSBP specific probe.

Scale bar: 40 μm

L: Lumen; S: Stroma.

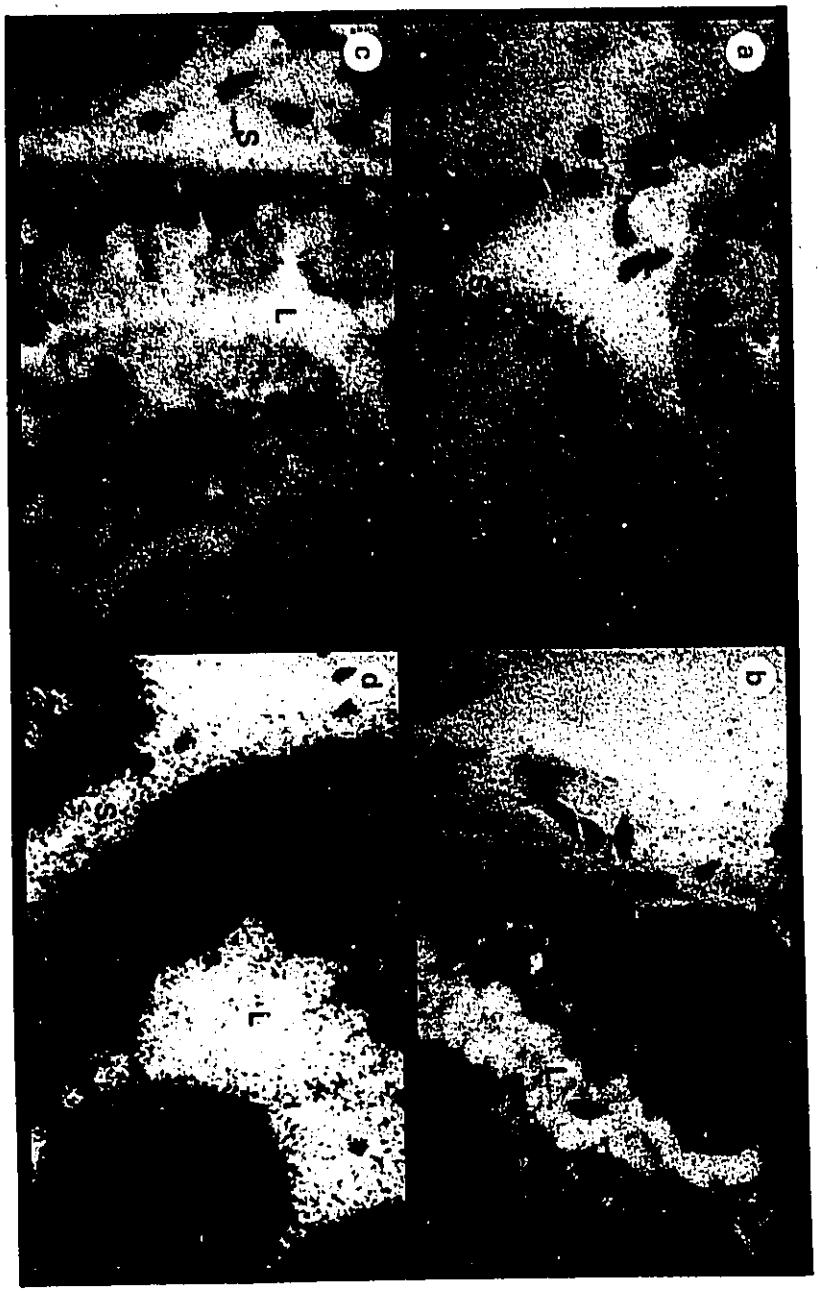

a
b
c
d
L
S
P

FIGURE 23: In situ hybridization of TRPM-2 and PSBP to transverse random sections of rat ventral prostates five days after castration.

Rat ventral prostates were excised, sectioned and fixed as described in section 2.14. The fixed sections were hybridized with 20ng of either the TRPM-2 specific probe ([^{35}S]-21-04 insert, 1.0 X 10^8 cpm/μg) or the PSBP specific probe ([^{35}S]-A34 insert, 1.5 X 10^8 cpm/μg).

Panel a: Section hybridized to the TRPM-2 specific probe after RNAse treatment (negative control).

Panel b: Section hybridized to the TRPM-2 specific probe.

Panel c: Section hybridized to the PSBP specific probe after RNAse treatment (negative control).

Panel d: Section hybridized to the PSBP specific probe.

Scale bar: 40 μm

L: Lumen; S: Stroma.


c
a
d
b

3.7.2. In situ hybridization to microdissected prostatic ducts

In view of the heterogeneity of the epithelial cell population, which is dependent on the localization of the cells along the prostatic duct (Sugimura et al., 1986a), in situ hybridizations were performed on sections of prostatic ducts microdissected from the prostates of normal and castrated rats.

The results of hybridization of the TRPM-2 probe to sections of prostatic ducts from prostates of intact and castrated animals are shown in Fig. 24. Before castration, TRPM-2 shows no hybridization in the proximal region (Fig. 24a), and negligible hybridization in the distal region of the prostatic ducts (Fig. 24b). By comparison to the RNAse controls (these are not shown, but are comparable to those presented for the random sections in Fig. 22 and Fig. 23), these grains represent background hybridization. Four days after castration, the hybridization appears to increase slightly in the proximal region (Fig. 24c). However, hybridization of TRPM-2 is greatly increased in the epithelial cells of the distal region (Fig. 24d). Eight days after castration (Fig. 24f), hybridization of TRPM-2 in the distal epithelial cells has decreased to levels comparable to the initial levels seen in Fig. 24b. Hybridization in the proximal region is also back to initial levels (Fig. 24e).

FIGURE 24: In situ hybridization of TRPM-2 specific probe to longitudinal sections of micro-dissected ducts of ventral prostates of intact and castrated rats.

Rat ventral prostates were excised, micro-dissected, sectioned and fixed as described in section 2.14. Sections were hybridized with 20ng of TRPM-2 specific probe ([^{35}S]-21-04 insert, 3.0 X 10^8 cpm/μg).

Panels a, c and e: Proximal region of the duct

Panels b, d and f: Distal region of the duct

Panels a and b: Sections before castration

Panels c and d: Sections 4 days after castration

Panels e and f: Sections 8 days after castration

Scale bar: 40 μm

S: Stroma; L:Lumen


a
b
c
d
e
f

The results of hybridization of the PSBP probe to sections of prostatic ducts from prostates of intact and castrated rats are shown in Fig. 25. Before castration, PSBP shows no hybridization in the proximal region of the prostatic ducts (Fig. 25a). However, it hybridizes very strongly in the distal epithelial cells, to the extent that the density of grains almost completely masks the morphology of the cells (Fig. 25b). Four days after castration, the hybridization profile in the proximal region remains unchanged (Fig. 25c). The hybridization of PSBP in the distal area (Fig. 25d) is much less intense than it was on day 0 (Fig. 25b), and the cells are now visible under the grains. Eight days after castration PSBP expression in the distal area has decreased to negligible levels (Fig. 25f), and hybridization in the proximal region of the ducts remains nonexistent (Fig. 25e).

These results demonstrate that the expression of both TRPM-2 and PSBP is localized in the distal region of the ducts. Very little specific hybridization is detected in the proximal regions with either probe. These results correlate with the fact that androgen dependent secretory cells are found more distally along the duct, and that cell death is also localized in this area. Therefore, in the intact rat prostate, PSBP mRNA levels are very high in the distal luminal epithelial cells. Four days after castration, the levels decrease dramatically as the luminal secretory epithelial cells are destroyed as the rate of cell death is increased. During this period of cellular destruction, TRPM-2 expression is induced. By the eighth day after castration, the epithelial cell population is greatly reduced, and the rate of cell

FIGURE 25: In situ hybridization of PSBP specific probe to longitudinal sections of micro-dissected ducts of ventral prostates of intact and castrated rats.

Rat ventral prostates were excised, micro-dissected, sectioned and fixed as described in section 2.14. Sections were hybridized with 20ng of PSBP specific probe ([^{35}S]-A34 insert, 2.7 X 10^8 cpm/μg).

Panels a, c and e: Proximal region of the duct

Panels b, d and f: Distal region of the duct

Panels a and b: Sections before castration

Panels c and d: Sections 4 days after castration

Panels e and f: Sections 8 days after castration

Scale bar: 40 μm

S: Stroma; L:Lumen


a
b
c
d
e
L
f

death decreases. At this time point, expression of PSBP and TRPM-2 is greatly reduced since the epithelial cells which expressed PSBP in the presence of androgens, and TRPM-2 in their absence, have died.

There are several questions that cannot be answered using this particular in situ hybridization technology. For example, it is not possible to determine whether expression of PSBP and TRPM-2 can occur simultaneously within the same cells. In addition, it is unclear whether TRPM-2 is expressed in the epithelial cells which die or in the adjacent epithelial cells which surround them. It seems likely that they would be expressed in the dying cells, since the hybridization is so prominent at a time when regression is most evident. However, since the adjacent cells are also involved in the apoptotic process (as they phagocytose the apoptotic bodies), the possibility that TRPM-2 is expressed in the neighboring cells cannot be eliminated. These questions will most likely be resolved by immuno-cytochemistry, once antibodies specific for both PSBP and TRPM-2 are available.

4. DISCUSSION

4.1. BRIEF SUMMARY OF PROSTATE PHYSIOLOGY

As described in the introduction, the function and growth of the prostate are under the control of androgens. The active form of the hormone, 5α-DHT, binds to the androgen-receptor, and the hormone-receptor complex enters the nucleus and binds to chromatin, where it exerts its effects on gene expression (Mainwaring et al., 1976; Nyberg et al., 1976; Wang et al., 1984). Many prostatic marker proteins are androgen dependent. One example is prostate steroid-binding protein (PSBP), which is the most abundant secretory protein in the rat ventral prostate (Heyns et al., 1978; Parker & Scrace, 1979). Because of its abundance and strong dependence on androgens, it has been a useful marker of the hormonal status of the prostate.

The prostate is made up of two cell types: stromal and epithelial. These are arranged as ductal networks, with the epithelial cells facing the lumen, and surrounded by fibroblasts of the stroma. The epithelial cells along the ducts appear to be heterogeneous both morphologically and biochemically. In the distal region of the ducts, the luminal epithelial cells are tall, columnar secretory cells. There are very few basal epithelial cells in this region (Rouleau et al., submitted). In the proximal region of the ducts, the luminal epithelial cells are cuboidal and non-secretory (Cunha et al., 1980; 1985; 1987; Sugimura et al., 1986a). Basal epithelial cells are very abundant in the proximal region, and are intercalated between the

epithelial and stromal cells (Rouleau et al., submitted). The direct interaction between the luminal epithelium and stroma is essential for growth, differentiation, and function of the prostate (Franks et al., 1970; Lasnitzki & Mizuno, 1970; 1977; Cunha et al., 1975; 1976; 1978; 1980; 1983; 1985), and is likely to involve both growth factors and the basement membrane (Tenniswood, 1986; Tenniswood et al., 1990). In the proximal region, the basal epithelial cells appear to disrupt the interaction between the luminal epithelium and stromal cells, attenuating the stromal-epithelial interaction (Rouleau et al., submitted).

After castration, there is a dramatic loss in prostatic size and secretion of marker proteins (Bruchovsky et al., 1975; Lee, 1981; Isaacs, 1984). These effects have been localized to the distal region of the prostatic ducts and the number of tips and branch points in this region decrease dramatically as the arborized network of ducts "melt-back" (Cunha et al., 1985; 1987). The cells that remain are cuboidal in shape, and appear to be the epithelial cells of the proximal region. No major changes are observed in the proximal region following androgen ablation.

This loss of cells has been shown to result from an active cell death process, termed apoptosis (Kerr et al., 1972). The morphological characteristics of this process have been well described. The four major steps involved in the apoptotic process are: contraction of cell volume, nuclear chromatin condensation,

cellular fragmentation and swift recognition and phagocytosis by adjacent cells (Wyllie, Kerr & Currie, 1980; Wyllie, 1981).

To date, only a few of the biochemical characteristics of apoptosis have been defined. It is known that the chromatin condensation results from the activation of an endogenous Ca^{2+}/Mg^{2+}-dependent endonuclease. In the prostate, the induction of this endonuclease activity has been reported following castration-induced involution (Kyprianou & Isaacs, 1988). Due to the high Ca^{2+}-dependence of the endonuclease, studies have been performed to investigate the possible involvement of calcium in castration-induced cell death. It has been shown that by disturbing the calcium homeostasis with potent calcium channel antagonists simultaneously with castration, changes associated with prostatic cell death, such as weight loss, DNA loss and DNA fragmentation, and expression of specific genes, are delayed (Kyprianou, English & Isaacs, 1988; Connor, Sawczuk, Benson, Tomashefsky, O'Toole, Olsson & Buttyan, 1988). Thus, there appears to be a role for calcium influx in the pathway leading to hormonally induced apoptosis.

The process of regression in the prostate has been shown to require active protein synthesis (Bruchovsky et al., 1975; Stanisic et al., 1978) and to involve the synthesis of new mRNA species (Montpetit et al., 1986). At the time it was originally identified, TRPM-2 was the first mRNA species to be shown to be

specifically expressed after castration (Montpetit et al., 1986). For this reason, it has been proposed that its cognate protein may be involved in the process of regression, and specifically in apoptosis. To investigate this possibility, it was necessary to isolate a cDNA clone specific for TRPM-2 and subsequently to study the time course of its expression, and to localize the expression of the gene in the luminal epithelial cells.

4.2. THE NATURE OF TRPM-2 AND THE TRPM-2 GENE PRODUCT

Analysis of the time course of induction by Northern analysis and dot blot hybridization shows that TRPM-2 is induced very rapidly following castration, and that its expression peaks on day four, coinciding with the time at which the rate of cell death is maximal in the prostate (Isaacs, 1984). These results suggest a direct involvement of TRPM-2 in this process. Indeed, the correlation between the site of expression of TRPM-2 along the prostatic ducts (as demonstrated by in situ hybridization) and the subsequent death of the luminal epithelial cells in which the gene is expressed, provides the very powerful suggestion that TRPM-2 is directly involved in (or associated with) the regression of the tissue.

It is now evident that TRPM-2 is not a "testosterone-repressed prostatic message". It is expressed in other hormone-responsive tissues. It has recently been demonstrated that TRPM-2 expression is induced after weaning in the

regressing mammary gland (Léger, Catzavelos & Tenniswood, in preparation), and TRPM-2 induction has been observed in the rat uterus during the involution stages of the estrous cycle (Léger & Tenniswood, in preparation). TRPM-2 can also be induced by non-hormonal stimuli. For example, it is induced in the rat kidney following ureteral obstruction, which leads to regression of the obstructed kidney and the death of the epithelial cells of the distal tubules and collecting ducts (Sawczuk, Hoke, Olsson, Connor & Buttyan, 1989).

TRPM-2 expression has also been observed in non-mammalian systems. Its expression is specifically induced at pupation in regressing juvenile muscle of the tomato horn worm Manduka sexta, but not in non-regressing tissue (Lockshin, personal communication). Within the plant kingdom, an example of regression occurs during the cessation of nitrogen fixation prior to the formation of the mature flower in soya bean Glycin max. TRPM-2 is not detected in root tissue or in nodules at the time of maximum nitrogen fixation. However, with the onset of flowering and nodule regression, TRPM-2 expression is induced (Gottlob & Johnson, personal communication).

With the exception of the plant systems (where it has not been studied), the tissue regression described above occurs via the process of apoptosis. The involution of the rat prostate following castration has been shown to result from apoptosis of the epithelial cells (Kerr & Searle, 1973). Apoptosis has been well

characterized in the human breast, where it occurs as a response to the decreasing hormonal levels at the end of the menstrual cycle (Ferguson & Anderson, 1981a), and may also play a role in the "resting" breast, balancing cell proliferation and cell deletion (Ferguson & Anderson, 1981b). The "cytosegresomes" and "cytosomes" that have been described by Helminen & Ericsson (1968) in the rat mammary gland following weaning correspond to apoptotic bodies. Apoptosis has also been shown to be induced by hormonal changes in the uterine luminal epithelium of hamsters and humans (Sandow et al., 1979; Hopwood & Levison, 1974). It therefore appears that there is a common mechanism of regression in these various system, and a common pathway of induction seems likely. TRPM-2 may play a role in these processes.

The analysis of the nucleotide sequence of p21-04 demonstrated that it shows significant homology with the sequence of a cDNA clone isolated from rat testes which codes for sulfated glycoprotein-2 (SGP-2) (Collard & Griswold, 1987; Bettuzzi et al., 1989), the most abundant secretory protein of the Sertoli cells (Kissinger et al., 1984). Recently, a full length cDNA clone specific for TRPM-2 (17H) has been isolated, and its sequence differs from the SGP-2 cDNA clone in the 5' untranslated region (Pineault, personnal communication). This may affect both the regulation of transcription and the function of the resulting protein, although it seems likely that the TRPM-2 protein has a similar function to that of SGP-2. SGP-2 has been shown by immunocytochemistry to be associated with the

acrosome and distal tail portion of mature spermatozoa and with the luminal surfaces throughout the epididymis and vas deferens (Sylvester, et al., 1984; Tung & Fritz, 1985). More recent evidence suggests that SGP-2/TRPM-2 is expressed by the spermatids themselves (Hoover, Zachari & Tenniswood, in preparation). If SGP-2 and the TRPM-2 protein have similar function, and since TRPM-2 has been associated with cell death, the detection of SGP-2 in the Sertoli cells can be attributed to the fact that in the cell culture conditions used, 50% of the cells died, and this would lead to TRPM-2 induction.

More recent evidence demonstrated that the DNA sequence of the human complement-associated protein SP-40,40, also known as CLI (cytolysis inhibitor), shows great homology (75%, which is significant considering the species difference) with TRPM-2 (Jenne & Tschopp, 1989; Kirszbaum, Sharpe, Murphy, d'Apice, Classon, Hudson & Walker, 1989). CLI has been shown to be a potent inhibitor of the terminal complement pathway which is involved in complement-mediated cytolysis.

Thus, although it remains to be firmly established, TRPM-2 appears to be a membrane associated protein (as homology with SGP-2 and CLI suggests) which is synthesized in terminally differentiated cells in the process of programmed cell death. The process of apoptosis is not an inflammatory process; there is no leakage of intracellular enzymes and components. TRPM-2 induction may thus be

necessary during the membrane remodelling stages of cell death to maintain the integrity of the cell membrane.

At what stage of apoptosis is TRPM-2 expressed? It is difficult to determine this from the in situ hybridization results in the prostate, since the hybridization signal is so strong that morphology of the cells and apoptotic bodies is masked. However, recent studies on liver sections seem to indicate that TRPM-2 is expressed in the very early stages of apoptosis (Bursch, Gleeson, Catzavelos & Tenniswood, in preparation). In this study, 100 mg/day of cyproterone acetate was administered by gavage to female rats in order to induce liver hyperplasia (Schulte-Hermann, Hoffmann, Parzefall, Kallenbach, Gerhardt & Schuppler, 1980). When the animals are taken off this drastic treatment, the liver regresses to its initial size since the cells undergo apoptosis (Bursch et al., 1986). The number of apoptotic cells in this model is relatively low, therefore hybridization signals are weaker, which permits more precise observation of the process. The earliest visible stage of apoptosis is chromatin condensation. It was shown that TRPM-2 is not expressed in cells showing signs of apoptosis, nor in the apoptotic bodies. However, it was observed that a low number of "normal" liver cells shows TRPM-2 expression. This implies that TRPM-2 is expressed at the very early stages of the process, presumably during the precondensation stages.

The control of the induction of TRPM-2 and other proteins involved in the regression process must be relatively simple, since the apoptotic pathway is common to all the tissues mentioned above. It most likely involves normal cellular processes such as changes in Ca^{2+} homeostasis and activation of second messenger cascades. However, it does have some complexities, since not all the cells die. In the case of the prostate, the cells in the proximal region do not express TRPM-2 and do not die following castration. Such differences between the distal and proximal luminal epithelial cells are likely to result from differences in the epithelial-stromal interactions. As described previously, the basal epithelial cells are much more abundant in the proximal region, and intercalate between the stroma and epithelial cells. There may be differences at the level of the basement membrane, of cell-cell junctions, and of growth and inhibitory factors.

Many groups working on apoptosis have been looking for a specific marker of apoptosis. The first candidate was the Ca^{2+}/Mg^{2+}-dependent endonuclease. This enzyme is involved in chromatin condensation, by cutting at internucleosomal sites (Wyllie, 1980; Duke et al., 1983). However, nucleosome formation is not necessarily a specific marker of apoptosis. Cytosolic Ca^{2+} levels can be increased by non-apoptotic processes, thus activating the enzyme (Tannock & Steel, 1969; Wyllie, 1985). Also, some degenerative changes typical of apoptosis have been observed without the usual formation of nucleosomes (Laster, Wood & Gooding, 1988).

Another enzyme which is linked with apoptosis is the transglutaminase, which produces a cross-linked protein scaffold that keeps intracellular enzymes and DNA packed until phagocytosis and final degradation of apoptotic bodies occur (Bursch et al., 1986; Fesus et al., 1989). However, it cannot be used as a specific apoptotic marker, since its detection by antibodies is complicated by the cross-linking of the enzyme upon itself, which destroys the epitopes recognized by the antibodies (Bursch & Kraupp, unpublished observation).

Since TRPM-2 is expressed in various tissues which undergo involution by apoptotic processes, it was hoped it would be a good marker of apoptosis. However, recent studies have resulted in surprising observations. Not only is TRPM-2 expressed in the regressing liver as described previously, but also in the proliferating liver after cyproterone acetate treatment (Bursch et al., in preparation). It is also continuously expressed in a prostate epithelial cell line, RDE, that has a very rapid doubling time. The RDE cells are non-secretory, and do not synthesize marker proteins such as acid phosphatase and PSBP (Montpetit et al., 1988), nor do they express TRPM-2 in the absence of androgens at confluence (Montpetit & Tenniswood, 1989). However, it was found that TRPM-2 is expressed when these cells are actively proliferating. TRPM-2 can be further induced when the cells are treated with tumor necrosis factor (TNF) to induce programmed cell death. Calcium ionophore can induce programmed cell death in the RDE cells, but without further augmenting the levels of TRPM-2 (Lakins & Tenniswood, in preparation).

These results demonstrate that TRPM-2 is expressed not only in apoptotic cells but also in proliferating cells. How are these two cellular events related? As discussed previously, TRPM-2 appears to be involved in inhibition of complement-mediated cytolysis. One common phenomenon in both proliferation and apoptosis is that many changes occur at the level of the membrane as cell-cell junctions and cell-basement membrane interactions are disrupted. In the case of apoptosis, changes in sugar residues on the outer cell membrane have been demonstrated (Duvall et al., 1985; Wyllie et al., 1984). Such changes occurring at the membrane of cells undergoing proliferation or apoptosis might elicit an undesired immune response. TRPM-2 may be important in inhibiting the complement pathway which leads to cytolysis, preventing leakage of intracellular enzymes into the intercellular space.

Another common feature of proliferation and cell death is the requirement for a Ca^{2+} influx. It has been clearly demonstrated in the regressing prostate that activation of a Ca^{2+}-Mg^{2+}-dependent endonuclease is an early event in castration-induced cell death, and that blocking calcium influx delays the changes associated with cell death (Kyprianou & Isaacs, 1988; Kyprianou et al., 1988; Connor et al., 1988). It has also been demonstrated in the RDE prostatic cells that calcium ionophore can induce cell death without further induction of TRPM-2 (Lakins & Tenniswood, in preparation). It was suggested that, since TRPM-2 appears to play a role in the early events of apoptosis, it may be involved in the Ca^{2+} influx which

leads to activation of the endonuclease responsible for chromatin fragmentation. Adding the calcium ionophore to the RDE cells circumvents the necessity TRPM-2 induction to signal endonuclease activation. TRPM-2, as mentioned earlier, is also expressed in these cell during active proliferation. It has been suggested that the absolute level of TRPM-2 may be a feature in the switch from proliferation to cell death. Since sequence analysis of TRPM-2 and SGP-2 shows differences in the 5' region, it has been speculated that the expression of TRPM-2 in proliferating and terminally differentiated cells is regulated by different promoters (Tenniswood, personal communication). One promoter would allow low levels of TRPM-2 transcription during proliferation. The expression would be silenced during terminal differentiation, and would be reinduced by a second promoter, activated during the absence of the trophic hormone, thus leading to cell death.

4.3. PROSTATIC DISEASE THERAPIES

Ever since Huggins demonstrated that castration reduces prostate size, and causes the regression of prostatic cancer (Huggins & Hodges, 1941; Huggins et al., 1941), the main aim of hormonal therapy has been to inhibit the growth of the gland by disrupting the normal mechanism of action of androgens. This has lead to the development of a number of anti-androgens, including flutamide and cyproterone acetate, which are capable of competing with 5α-DHT for the androgen receptor (Neri & Peets, 1975; Neumann, 1977; Neumann, 1985). The

effectiveness of the anti-androgens has usually been monitored by following the decrease or elimination of androgen dependent functions in the prostate, such as secretory acid phosphatase (SAP), and prostatic steroid-binding protein (PSBP) (Clark, Tenniswood, Bird, Flynn, Jacobs & Abrahams, 1980; Clark, Flynn, Downey, Bird, Mahan & Tenniswood, 1983). In the case of cyproterone acetate and flutamide it has already been demonstrated that anti-androgen therapy does not affect SAP (Tenniswood et al., 1984), possibly due to the inability of the anti-androgens to totally eliminate the nuclear accumulation of 5α-DHT (Callaway, Bruchovsky, Rennie & Corneau, 1982). The results obtained during this research project demonstrate that neither flutamide nor cyproterone acetate are able to fully eliminate C3-PSBP mRNA, presumably for the same reason. The activities of other androgen-dependent enzymes in the prostate, such as nuclear phosphokinases and phosphatases and the enzymes involved in polyamine biosynthesis, had previously been reported to be fully inhibited by anti-androgens (Mangan, Pegg & Mainwaring, 1973; Wilson, et al., 1979). However, the replenishment doses used in these experiments (50-250μg/day) is not sufficient to maintain normal prostatic function (Tenniswood et al., 1981). Thus cyproterone acetate and flutamide appear to be relatively ineffective in inhibiting the synthesis of secretory proteins in the rat ventral prostate.

On the other hand, cyproterone acetate and flutamide mimic a number of the effects of castration, most notably the induction of the androgen repressed

TRPM-2 mRNA. While the administration of either cyproterone acetate or flutamide induces TRPM-2, neither compound induces the gene to the same extent as castration. However the relative level of induction of TRPM-2 appears to correlate with the rate of involution of the prostate (as the OW/BW decreases, TRPM-2 is induced).

The expression of both androgen dependent (PSBP) and androgen repressed (TRPM-2) genes in the prostate at the same time is somewhat surprising. This may have an important bearing on the mechanism of involution, particularly if they are expressed simultaneously in the same cell. In view of the complexity of the prostatic architecture (Cunha et al., 1985), it is probable that the genes are expressed in different cells of the ductal-acinar network. TRPM-2 could be expressed in cells which are more distally localized, as these cells are the first to undergo cell death following androgen ablation. It would be more likely that TRPM-2 and PSBP are expressed by the same cell, but that their expression is dependent on the hormonal status of the gland. In this case, under normal hormonal conditions, secretion of PSBP in the cell would be maintained, whereas TRPM-2 expression is repressed. Following androgen depletion, the cells would lose their ability to express PSBP, while at the same time gaining the ability to express TRPM-2.

PSBP has been used as an androgen-dependent marker of prostatic function for many years (Heyns et al., 1978; Parker & Scrace, 1979). However, the fact that TRPM-2 is expressed in the same cell population brings into question this dependence. The decrease of PSBP may not directly be due to the lack of androgens, but to changes which are occurring in the dying cell which influence gene expression, such as changes in cell shape. Indeed, PSBP can be regarded as a constitutive androgen independent gene that is expressed exclusively in the luminal secretory cells that require androgens for survival. The fact that no androgen-receptor binding sites or androgen regulatory elements (AREs) have been identified in the 5' region of the PSBP genes supports this proposal.

The regional expression of genes such as PSBP and TRPM-2 raises another issue. If TRPM-2 is expressed in all androgen dependent cells in the prostate, and this expression results in the death of these cells, then it is reasonable to suggest that the androgen-independent cells that survive do not express TRPM-2.

The results of in situ hybridizations presented in this thesis demonstrate that TRPM-2 and PSBP are both expressed in the luminal epithelial cells of the distal region of the prostatic ducts. This region consists mainly of tall columnar secretory epithelial cells (Cunha et al., 1980; Shannon & Cunha, 1983; Rouleau et al., submitted). However, it is clear from these results that the luminal epithelial and basal epithelial cells in the proximal region of the duct do not express TRPM-2, and

do not die after androgen ablation. The stem cells required for prostatic growth on re-administration of androgens are maintained in this androgen independent cell population. These results help explain the failure of long term ablative therapies.

In order to develop new therapies for prostatic diseases, it is important to focus on the androgen-independent cells, and attempt to increase the rate of cell death in this population rather than to decrease the rate of proliferation. It thus becomes essential to fully understand the mechanisms involved in cellular regression in order to attempt to induce these mechanisms in the normally unresponsive cells, the luminal epithelial cells in the proximal region of the ducts. Studies on the androgen independent prostate RDE cells are very encouraging, since it has been shown that these cells can be induced to undergo apoptosis by TNF or Ca^{2+} ionophore. As discussed previously, alteration of Ca^{2+} homeostasis is an important factor in the process of cell death. The search for better therapies should be based on what is known of the chronology of apoptotic events, and finding the yet unknown components which could trigger apoptosis. The very early events occurring at the precondensation stage are not well characterized, but TRPM-2 induction appears to occur at this time. This is followed by an influx of Ca^{2+}, resulting in activation of the Ca^{2+}/Mg^{2+}-dependent endonuclease. It appears that once the endonuclease has been activated, the cells are committed to undergo apoptosis. The physiological signals that lead to endonuclease activation

must be well characterized in order to be able to specifically induce cell death in the diseased target tissue.

CONCLUSIONS

1. cDNA clone specific for TRPM-2 has been isolated. Several membrane associated proteins show strong sequence homology to TRPM-2.

2. The peak of TRPM-2 expression after castration and anti-androgen treatment coincides with the peak of cell death.

3. TRPM-2 was shown to be expressed in the cells which undergo apoptosis after castration, the luminal epithelial cells of the distal region of the prostatic ducts.

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APPENDIX 1:

List of abbreviations

AMV: avian myeloblastosis virus

BSA: bovine serum albumin

BPH: benign prostatic hyperplasia

cDNA: DNA complementary to RNA

cfu: colony forming units

CLI: cytolysis inhibitor

cpm: counts per minute

dATP: deoxyadenosine 5'-triphosphate

dCTP: deoxycytidine 5'-triphosphate

DEAE: diethylaminoethyl

5α-DHT: 5α-dihydrotestosterone

DNA: deoxyribonucleic acid

dNTP: deoxynucleotide 5'-triphosphate

ds-cDNA: double-stranded cDNA

DTT: dithiothreitol

EDGF: epithelially derived growth factor

EDIF: epithelially derived inhibitory factor

EDTA: ethylenediaminetetra-acetate

GnRH: gonadotrophin releasing hormone

GOT: glutamate oxaloacetate transferase

hnRNA: heteronuclear ribonucleic acid

kDa: kilo daltons

LH: luteinizing hormone

mRNA: messenger ribonucleic acid

O.D.: optical density

oligo-(dT): oligodeoxythimidylate

OW/BW: organ weight/body weight

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate buffered saline

PC: prostate cancer

PCR: polymerase chain reaction

pH: $-\log_{10}[H^+]$

pI: isoelectric point

poly(A)$^+$RNA: polyadenylated ribonucleic acid

ppm: parts per million

PSBP: prostate steroid-binding protein

RNA: ribonucleic acid

RNAse: ribonuclease

RVP: rat ventral prostate

SAP: secretory acid phosphatase

SDS: sodium dodecyl sulfate

SDGF: stromally derived growth factor

SGP-2: sulfated glycoprotein 2

SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7

ss-cDNA: single-stranded cDNA

TCA: trichloroacetic acid

Tfm: testicular feminized

TNF: tumor necrosis factor

Tris: 2-amino-2-hydroxy-methylpropane-1,3-diol

tRNA: transfer ribonucleic acid

TRPM: testosterone repressed prostate message

UGE: urogenital sinus epithelium

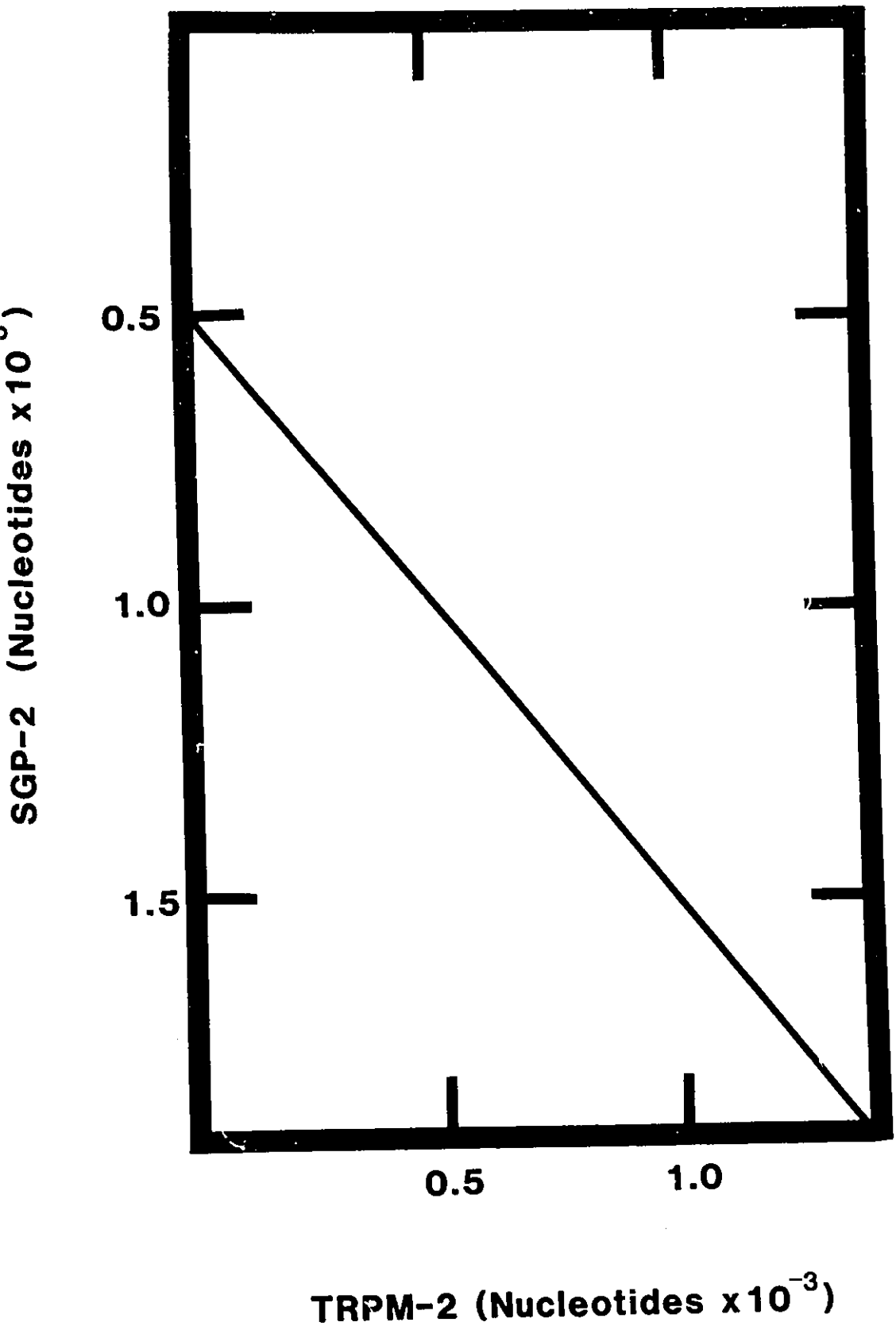
UGM: urogenital sinus mesenchyme

UGS: urogenital sinus

w/w: weight/weight

APPENDIX 2:

DOT PLOT COMPARISON OF TRPM-2 AND SGP-2


0.5
1.0
1.5
SGP-2 (Nucleotides x10^{-3})
0.5
1.0
TRPM-2 (Nucleotides x10^{-3})

APPENDIX 3:

Curriculum vitae

PERSONAL DATA

NAME:	Jocelyne G. Léger
DATE OF BIRTH:	October 24, 1961
MARITAL STATUS:	Separated
ADDRESS:	1839 Haig Drive Ottawa, Ontario CANADA K1G 2J2
TELEPHONE:	(613) 526-2761 (home) (613) 787-6419 (laboratory)
SPOKEN & WRITTEN LANGUAGES:	French and English

EDUCATIONAL BACKGROUND

PhD Biochemistry	University of Ottawa (1984-1990)

Thesis: "Characterization of TRPM-2, a gene involved in the regression of the rat ventral prostate after castration"

BSc (Hons) Biochemistry	Université de Moncton (1979-1983)

TEACHING EXPERIENCE

1990	**Lecturer** University of Ottawa -BCH 3570 Biologie Moléculaire
1985-1988	**Laboratory demonstrator** University of Ottawa -supervision and evaluation of BCH 3946 Metabolism .
1984	**Lecturer** Université de Moncton -Introduction à la Biochimie.
1980-1983	**Laboratory demonstrator** Université de Moncton -supervision and evaluation CH 1001 & 1002 , BC 3801 & 3802.

RESEARCH EXPERIENCE

1984-1989	**Graduate student** University of Ottawa (Dr. Martin Tenniswood) -studying the expression of genes in the rat ventral prostate before and after castration, and after antiandrogen treatment.
1982 & 1983	**Summer research assistant** - Université de Moncton (Dr. Alan Fraser) -assisting graduate students with tasks such as bacterial cultures, ribosome isolations, immunoprecipitations.
1981	**Summer research assistant**-Environment Canada (Water Quality Branch) -extracting organic pesticides from water, sediment and fish samples for GC and HPLC analysis.

METHODOLOGY ACQUIRED

The methods acquired mostly include molecular biology techniques, such as: DNA and RNA extractions, Southern and Northern hybridization analysis, pGEM and M13 subcloning, in situ hybridization analysis, polymerase chain reaction amplifications.

AWARDS AND SCHOLARSHIPS

Graduate awards:

1984-1988	Natural Sciences and Engineering Research Council Postgraduate Scholarship University of Ottawa
1984-1987	Entrance Scholarship University of Ottawa
1986	Boehringer Mannheim Graduate Prize for Oral Presentation at the Canadian Federation of Biological Sciences meeting, Guelph, Ontario.
1985	Boehringer Mannheim Graduate Prize (runner up) for oral presentation at the Canadian Federation of Biological Sciences meeting, Toronto, Ontario.

Undergraduate awards:

1982-1983	Natural Sciences and Engineering Research Council Summer Studentship Université de Moncton
1981-1983	Merit Scholarship Université de Moncton

PUBLICATIONS

1) PAPERS

Léger, J.G., Montpetit, M.L., & Tenniswood, M.P. (1987) Characterization and cloning of androgen-repressed mRNAs from rat ventral prostate. Biochem.Biophys.Res.Commun. 147, 196-203.

Léger, J.G., Le Guellec, R., & Tenniswood, M.P. (1988) Treatment with antiandrogens induces an androgen-repressed gene in the rat ventral prostate. The Prostate 13, 131-142.

Tenniswood, M.P., Montpetit, M.L., Léger, J.G., Wong, P., Pineault, J., & Rouleau, M. (1990) Epithelial-Stromal interactions and cell death in the prostate. In The prostate as an endocrine gland?, eds. R.J. Ablin and W.E. Farnsworth, CRC Press, Boca Raton, Florida, 187-198.

Rouleau, M., Léger. J.G. & Tenniswood, M. (1990) Ductal heterogeneity of cytokeratins, gene expression and cell death in the rat ventral prostate. Submitted.

Léger, J.G., Catzavelos, G.C. & Tenniswood, M. (1990) Expression of TRPM-2 in the regressing mammary gland, in preparation.

2) ABSTRACTS:

Rouleau, M., Léger, J. & Tenniswood, M., "Cytokeratin composition and androgen dependence of the epithelial cells in the rat ventral prostate" (poster presentation at the American Association for Cancer Research annual meeting, May 1989, San Francisco, USA).

Léger, J.G., Major, N., Gottlob-McHugh, S., Johnson, D. & Tenniswood, M., "TRPM-2: A gene involved in cellular regression" (poster presentation at the Fourth International Cell Biology Congress, August 1988, Montreal).

Léger, J.G., Gottlob-McHugh, S., Johnson, D., Tenniswood, M., "Regression associated gene expression" (oral presentation at Canadian Federation of Biological Societies meeting, June 1988, Québec).

Léger, J.G., Gottlob-McHugh, S., Johnson, D., Tenniswood, M., "Expression d'un gène impliqué dans la régression cellulaire" (oral presentation at l'Association Canadienne Française pour l'Avancement des Sciences meeting, May 1988, Moncton, N.B.).

Tenniswood, M. & Léger, J., "Expression of androgen repressed messages in the rat ventral prostate during regression" (poster presentation at UCLA conference, January 1988, Keystone, Colorado, USA).

Léger, J.G., LeGuellec, R., & Tenniswood, M.,"TRPM-2 - Un gène réprimé par les androgènes dans la prostate de rat" (oral presentation at l'Association Canadienne Française pour l'Avancement des Sciences meeting, May 1987, Ottawa, Ontario).

Léger, J.G., LeGuellec, R., & Tenniswood, M.," Anti-androgen induced expression of androgen-repressed genes in the rat ventral prostate" (oral presentation at Canadian Federation of Biological Societies, June 1986, Guelph, Ontario).

Léger, J.G., LeGuellec, R., & Tenniswood, M.,"Anti-androgens affect gene expression in the prostate" (oral presentation at Reproductive Biology Workshop, May 1986, Ottawa, Ontario).

Léger, J.G., LeGuellec, R., & Tenniswood, M.,"Anti-androgens affect gene expression in the rat ventral prostate" (poster presentation at Southern Ontario Reproductive Biology meeting, May 1986, Hamilton, Ontario).

Léger, J.G., Montpetit, M.L., & Tenniswood, M.,"Cloning, characterization and cell type localization of androgen repressed and constitutively expressed genes in the rat ventral prostate" (poster presentation at the International Congress of Biochemistry, August 1985, Amsterdam, The Netherlands).

Léger, J.G., & Tenniswood, M.,"Cloning and Identification of androgen-repressed and constitutively expressed genes in the rat ventral prostate" (oral presentation at the Canadian Federation of Biological Societies meeting, June 1985, Toronto, Ontario).

Léger, J.G., & Tenniswood, M.,"Cloning and identification of androgen-repressed and constitutively expressed genes in the rat ventral prostate" (oral presentation at the Southern Ontario Reproductive Biology meeting, May 1985, Kingston, Ontario).

REFERENCES:

Dr. Martin Tenniswood
Associate Professor
Department of Biochemistry
Health Sciences
University of Ottawa
Ottawa, Ontario
CANADA K1H 8M5
Tel.: (613) 787-6419

Dr. Yvonne Lefebvre
Director of Endocrine Research
Division of Endocrinology & Metabolism
Ottawa Civic Hospital
1053 Carling Avenue
Ottawa, Ontario
CANADA K1Y 4E9
Tel.: (613) 761-4657

Dr. Nicole Bégin-Heick
Dean of Graduate Studies
Hagen Building
115 Séraphin-Marion
University of Ottawa
Ottawa, Ontario
CANADA K1N 6N5
Tel.: (613) 564-5810