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ANDROGEN INDEPENDENT EPITHELIAL CELLS OF THE RAT VENTRAL PROSTATE

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The human prostate is a secondary sexual organ that requires an uninterrupted supply of androgens to maintain structural and functional integrity of androgen dependent epithelial cells. Eventual failure of hormonal therapies for prostate cancer is often attributed to the presence of androgen independent epithelial cells in the tumor.

Using the rat ventral prostate as a model of the human prostate, we have isolated and characterized an androgen independent epithelial cell population present in the normal rat ventral prostate. These cells grow very quickly and have been termed Rapidly-Dividing Epithelial (RDE) cells. The RDE cells are completely independent of androgens for cell survival and do not secrete the androgen dependent secretory proteins, secretory acid phosphatase and prostate steroid binding protein. The epithelial cell origin of RDE cells was confirmed by cytokeratin expression, testosterone metabolism patterns and by purification parameters. Culture with various differentiation-inducing agents resulted in major morphological changes and structures reminiscent of those in the mature prostate but not in the expression of androgen-dependent secretory products. RDE cells demonstrate a very high in-vitro propensity for transformation yet no tumor growth in-vivo. None of the ten common "immortalizing" proto-oncogenes tested were expressed. RDE cells appear to be the rat counterpart to androgen-independent epithelial cells which cause renewed tumor growth in prostate cancer patients treated by hormonal therapies.

DEDICATION

This thesis is dedicated to my wife Diane, my son Adam, and my family for their unwavering support and encouragement.

PER ARDUA AD ASTRA

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Martin Tenniswood for his direction and assistance throughout these past several years. I would like to offer thanks to my fellow graduate students, especially Jocelyne Léger and Paul Wong for their smiles and commiseration at appropriate times. Three members of the Department of Microbiology, Dr. K. Dimock, Dr. T. Nicas and my friend Erling Rud also deserve special mention for the help and encouragement that they've given me; for answers to a multitude of trivial questions and often for just the opportunity to talk. Dr. Albert Clark and Mrs. Pamela Abrahams also deserve thanks for performing the acid phosphatase IEF and testosterone metabolism assays for me. I gratefully acknowledge five years of continual funding from the Cancer Research Society of Canada.

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INTRODUCTION

The prostate, an exocrine male accessory sexual gland found exclusively in mammals, produces many of the components of seminal plasma, including citrate, fructose and a number of proteins. In the human the prostate is located immediately below the urinary bladder and surrounds the urethra. During ejaculation the prostatic secretions are discharged from many glandular ducts into the prostatic urethra and are transported down the urethra by the muscular contractions of ejaculation. The prostatic ducts radiate directly from the urethra with little further organization and can be subdivided morphologically into four distinct zones, the anterior fibromuscular stroma, the peripheral zone, the central zone and the transition zone (McNeal, 1983). The clinical significance of these zonal subdivisions is that two growth-related diseases of the prostate, prostate cancer (PC) and benign prostatic hyperplasia (BPH) arise from distinct locations. Adenocarcinoma originates almost exclusively from the periurethral zone of the prostate while BPH emanates primarily from the transition zone.

A) GROWTH-RELATED DISEASES OF THE PROSTATE:

The human prostate gland undergoes very slow growth from birth until the onset of puberty, at which time a rapid increase in the size of the gland occurs until approximately age 20 when the gland enters a "maintenance phase" with little or no further development for some years. In a small percentage of males this maintenance

phase lasts until their death decades later. However, most men suffer from one of two diseases of the prostate, usually beginning some time after their fourth decade (Walsh, 1984). The most common is benign prostatic hyperplasia (BPH), which affects approximately 80% of males over 50 years of age (Walsh, 1984). As the name implies, BPH is a benign growth of the prostate which is thought to begin within the transition zone of the prostate (McNeal, 1978; 1983). Epidemiological data has not been very helpful in narrowing the range of factors able to induce BPH:

> "there are no confirmable data to suggest relationships of sociocultural variables, celibacy, specific blood groups, use of tobacco and/or alcohol, gerontologic conditions, such as coronary heart disease, cerebral vascular disease, hypertension, diabetes mellitus, and cirrhosis of the liver, to the onset of BPH" (Rotkin, 1983).

Since it is a benign growth , BPH treatment tends to be symptomatic to relieve the constriction of the urethra caused by the benign nodules. The most common treatment is a trans-urethral resection of the prostate (TURP). The growth of BPH is not unlimited. After a certain period the prostate enters a second maintenance phase and no further net growth is seen. Rotkin (1983) has also found that BPH is not a precursor of prostate cancer.

Prostate cancer (PC) affects a considerably smaller portion of the population than BPH but is still among the most prevalent forms of cancer in the male. It is present at autopsy in 10-30% of all males over 50 years of age (Coffey and Pienta,

1987), fortunately however, only a small percentage of these dormant lesions become active, accounting for some 86,000 new patients per year in the United States. Of these approximately a third will die from the disease (Byar, 1987). With an average incidence of approximately 45/100,000 North American Caucasians and the nearly logarithmic increase in incidence and mortality with age, prostate cancer currently accounts for over 1 billion dollars in surgery expenses each year in the USA (Walsh, 1984). With an 8.7% lifelong probability of developing prostatic cancer (Byar, 1987) and a 33% probability of dying from the disease, it is easy to see that increased research into the causes and treatment of prostatic cancer is of vital importance for the aging male population.

Virtually all modern therapies for prostate cancer are based upon the pioneering work of Huggins and co-workers (Huggins and Hodges, 1941; Huggins, Stevens and Hodges, 1941) who first reported that the prostate is an androgen dependent tissue and that castration and/or estrogen therapy results in the atrophy of both normal and prostate tumor tissue. In nearly all instances, metastatic prostatic cancer treated by androgen ablation responds well, a finding that has formed the basis for adoption of hormonal therapy as the treatment of choice for prostate cancer. Unfortunately, essentially all patients treated in this way eventually suffer a relapse that is unresponsive to further anti-androgen therapy, regardless of how aggressive the treatment may be (Isaacs and Kyprianou, 1987). The dismal patient survival statistical

results, despite an array of different treatments, has lead Dr. Donald Coffey to state:

> "In the past half-century (1936-1986) there has been no positive effect in diminishing the death rate from prostate cancer in the United States and we have gained only a very slight increase in the five-year survival rate. In contrast, many other forms of cancer, i.e., Hodgkins, testicular cancer and childhood acute lymphocytic leukemia, now have dramatic cure rates of 50-90%. Why has there been so little progress in prostate cancer?" (Coffey and Pienta, 1987)

B) THE FAILURE OF ANTI-ANDROGEN THERAPY:

A large number of androgen ablation therapies have been developed to prevent the synthesis or inhibit the activity of 5α-dihydrotestosterone (5α-DHT), the active androgen. These include surgical castration, the administration of estrogens, including estradiol and diethylstilbestrol, or treatment with drugs referred to collectively as anti-androgens, that interfere with the androgen steroid-receptor mechanism. The common anti-androgens being used in the clinical treatment of PC include cyproterone acetate (Neumann, 1977; Goldenberg, Bruchovsky, Rennie and Coppin, 1988) and flutamide (Neri, Florance, Koziol and Van Cleave, 1972). Other drugs that act by inhibiting steroid metabolism include ketoconazole (Trachtenberg, 1983) and 1-methyl-androsta-1,4-diene-3,17-dione (Habenicht and El Etreby, 1987). In attempts to provide a complete blockade of both gonadal and adrenal androgens and others steroids possessing low level androgenic activity, a number of combination therapies have been developed in which anti-androgens are combined with luteinizing hormone-releasing

hormone (LHRH) or LHRH analogues to obtain "complete androgen blockade" (Labrie, Dupont, Bélanger, Lacourcière, Raynaud, Hassan, Gareau, Fazekos, Sandow, Monfette, Girard, Emond and Houle, 1983; Labrie, Bélanger, Dupont, Emond, Lacourcière, Monfette, 1984; Labrie, Dupont, Bélanger, Giguère, Lacourcière, Emond, Monfette and Bergeron, 1985; Redding and Schally, 1985; Labrie, Dupont, Lacourcière, Giguère, Bélanger, Monfette and Emond, 1986; Rennie, Bruchovsky, Goldenberg, Lawson, Fletcher and Foekens, 1988a). Other forms of combination therapy use buserelin as the LHRH agonist (Schroeder, Klijn and de Jong, 1986; Schroeder, Lock, Chadha, Debruyne, Karthaus, de Jong, Klijn, Natroos and de Voogt, 1987). In general the use of androgen ablation, whether partial or complete, has been shown to provide short term improvement in tumor size, metastatic potential and general patient well being but in the longer term relapse appears to be virtually unavoidable.

C) PROSTATE MODEL SYSTEMS:

Model systems for studies of human diseases are a requirement for investigators; they permit studies into the etiology, pathogenesis, diagnosis, prevention and therapy that otherwise would be impractical or unethical. Model systems of neoplastic diseases should display the following characteristics: spontaneous appearance, organ specificity, nonsynchronous multiplication of the tumor cells, aneuploidy and metastasis

from the primary site to establish new foci of the same tumor type (Pollard, 1980).

1) Species:

Three species encompass the vast majority of model systems of prostatic disease; human, dog and rat. To select a single model for prostatic cancer may not be realistic in view of the multifaceted nature of the disease.

Since the early 1970's investigators have developed a series of human prostatic cancer sublines that can be serially passaged in nude mice. These models can be classified as androgen dependent or androgen independent. The androgen independent lines include EB-33 (Okada and Schroeder, 1974), DU-145 (Mickey, Stone Wunderli, Mickey, Vollmer and Paulson, 1977), PC-3 (Kaighn, Shakar Narayan, Ohnuki, Lechner and Jones, 1979) and LNCaP (Horoszewicz, Leong, Chu, Wajsam, Friedman, Papsidero, Kim, Chiu, Kakati, Arya, and Sandberg, 1980). The androgen dependent lines include PC-82 (Hoehn, Schroeder, Riemann, Joebsis and Hermanek, 1980), PC-EW (Hoehn, Wagner, Riemann, Hermanek, Williams, Walter and Schrueffer, 1984) and the Honda tumor (Ito and Nakazato, 1984). In addition to the lack of 5α-reductase activity PC-3 is the only one of these not to secrete prostatic acid phosphatase. The Honda tumor system is also different from all of the others in that it was generated by direct implantation of the human tumor fragment into the nude

mouse, no continuous cell line was used as an intermediate. These tumors do not generally metastasize.

Like the human prostate, the dog prostate develops spontaneous adenocarcinoma. The metastatic pattern of the human tumor is also found in the dog (lymph and bone). However the canine tumors tend to be fast growing in contrast to the human tumors. The dog prostate is used far more extensively as a model of human BPH since many dogs, beagles in particular, are prone to the development of BPH (For a review see Isaacs, 1984).

The rat prostate is the most extensively used model of the human prostate, particularly in the study of the basic biochemistry, cell biology and interactions of the prostate. Dunning isolated an androgen dependent spontaneous tumor from an aged Copenhagen rat. The Dunning tumor has since been developed into numerous sublines each with different levels of androgen dependence, metastatic potential and growth rates (Dunning, 1963; Isaacs, 1984). Some but not all of these may be cultured in-vitro rather than as transplants in syngeneic rats. Other far less diverse rat tumor models include the Pollard and ACI spontaneously appearing tumors (Pollard, 1980; Sahin, McCullough and Segaloff, 1975). A special feature of the Pollard lines is their extensive ability to metastasize, a feature enhanced or decreased by various compounds (Isaacs, 1984). The ACI tumor system is particular in its reflection of the

human development of numerous prostatic lesions relatively early in life with few of the lesions progressing to grossly manifest cancer (Ward, Reznik, Stinson, Lattuada, Longfellow and Cameron, 1980). The inbred Noble rat displays a marked susceptibility to the development of prostatic adenocarcinoma induced by long-term exposure to elevated sex steroid levels (Noble and Hoover, 1975).

2) Culture systems:

Three major systems are used for the culture of prostate cancer models: tumors transplanted into rodents (nude mice or syngeneic rats), organ culture of tissue fragments and monolayer cultures of isolated cells. As described above the human prostate tumors, with the exception of the Honda line were generated by initially obtaining monolayer cultures of the tumor cells before transplantation into nude mice. All the human lines mentioned above are currently maintained and analyzed as transplanted tumors. One advantage of this system is the ability to test systemic therapies or effects within the context of an entire animal. A second advantage is that the tumor cells are maintained in an environment that facilitates the maintenance of the properties and level of differentiation of the initial cell types. However the inability to study individual cell populations, or to accurately control certain

experimental parameters because the tumor is in an animal can pose insurmountable constraints on certain areas of investigation.

Organ cultures of normal and tumor tissue alleviates some of the problems of transplantation cultures. While the cell-cell contact and interactions are preserved within the tissue fragment being cultured the investigator has far greater control over the environment. The effects of additives or deletions in the culture media are far more readily observed while sacrificing little in the way cellular interactions and maintenance of differentiated phenotypes. However, as with the transplantation system the overgrowth of the culture by one or more cell types is difficult to control. Secondly, one has limited ability to study individual cell types within the culture.

Monolayer cultures of prostate cells offer the best ability to investigate the properties of individual cells and cell types. Cells can be obtained by dissociation of the prostate by enzymatic digestion (trypsin, collagenase, dispase) or by explants from organ culture. The very nature of the latter approach actively selects for cells that are capable of replication and thus is of limited use in the study of non-replicating fully differentiated cells (ie. secretory cells). The cell suspension achieved by dissociation of the tissue may be enriched for a given cell type by a variety of means including density gradients, selective attachment to surfaces or nutritional selection. Once separated the individual cell types may then be used for a variety of purposes. The

main disadvantage of monolayer cultures is the loss of the normal cellular environment (cell-cell interactions, extracellular matrix components) and the subsequent effect this may have on the response of the cells. The severity of these changes are both cell-type and species specific. Thus canine basal epithelial cells have been reported to be able to fully differentiate in monolayer cultures (Dionne, Chevalier, Bleau, Roberts and Chapdelaine, 1983) while rat ventral prostate basal epithelial cells are unable to differentiate and secretory epithelial cells rapidly lose their secretory capability unless cultured within collagen gels (O'Conner and Sinha, 1985).

While it is important to remember that not all information derived from animal studies may be applicable to humans the contributions of animal models to our knowledge of prostatic disease have been enormous. Selecting a single animal model for prostatic cancer is unrealistic; the advantages, disadvantages and limitations of each species and system must be weighed with particular attention to the facet of prostatic disease under investigation.

D) THE RAT VENTRAL PROSTATE:

The rat ventral prostate has served as a model system for studies into both the basic mechanism of androgen action (for review see Mainwaring, 1977; Aumüller, 1983) and for a variety of approaches to modeling human prostate cancer.

The gross differences in human and rat prostate anatomy reflect differences in the ductal morphology. Human ducts are composed of complex arrays of tubulo-acinar structures with distinct acini. The ducts are lined with simple cuboidal or columnar epithelial cells in the more distal regions of the duct, whereas transitional epithelium is found in the proximal regions as the ducts emerge from the urethra. The rodent prostate is a compound ductal gland lacking true acini, and each lobe (ventral, dorsolateral and anterior) has a distinct branching pattern (Sugimura, Cunha and Donjacour, 1986a).

1) Androgen dependence:

The classical mechanism of androgen action is described in figure 1. The vast majority of testosterone in the circulation is bound to a variety of plasma proteins. The small amount of unbound, or free, testosterone is able to enter the prostate cells, presumably by passive diffusion. Within androgen target cells, testosterone is

specifically metabolized, mainly to the active androgen, 5α-dihydrotestosterone (5α-DHT) and subsequently to other catabolites. The 5α-DHT binds to steroid-specific intra-cellular receptors causing subtle conformational changes in the receptor. As the location of the free receptor (nucleus or cytoplasm) remains the subject of considerable debate, it is sufficient to say that the 5α-DHT-receptor complex migrates from the point of androgen binding to nuclear acceptor sites within chromatin. The formation of the androgen-receptor-acceptor complex then induces, by an unknown mechanism, one or more temporal responses. The initial response includes protein phosphorylation (Ahmed, 1971; Wilson and Ahmed, 1975), ribosomal RNA synthesis (Liao, Barton and Lin, 1966; Liao and Lin, 1967; Mainwaring, Mangan and Peterken, 1971) and the induction of some protein synthesis. Mainwaring divided subsequent responses to androgen stimulation into "early" and "late" responses. The early response to androgen activation includes the synthesis of many mRNA sequences, and their subsequent translation. Late effects of androgen action on cells include the induction of cell division (Mainwaring et al., 1971; Chung and Coffey, 1971a; Chung and Coffey, 1971b; Rennie, Symes and Mainwaring, 1975).

A number of indicators of androgen action in the rat ventral prostate have been developed beginning with the most general of parameters, prostate weight and cellular morphology, as well as total protein, RNA and DNA levels. As research into androgen action became more biochemical several marker proteins involved in the actual

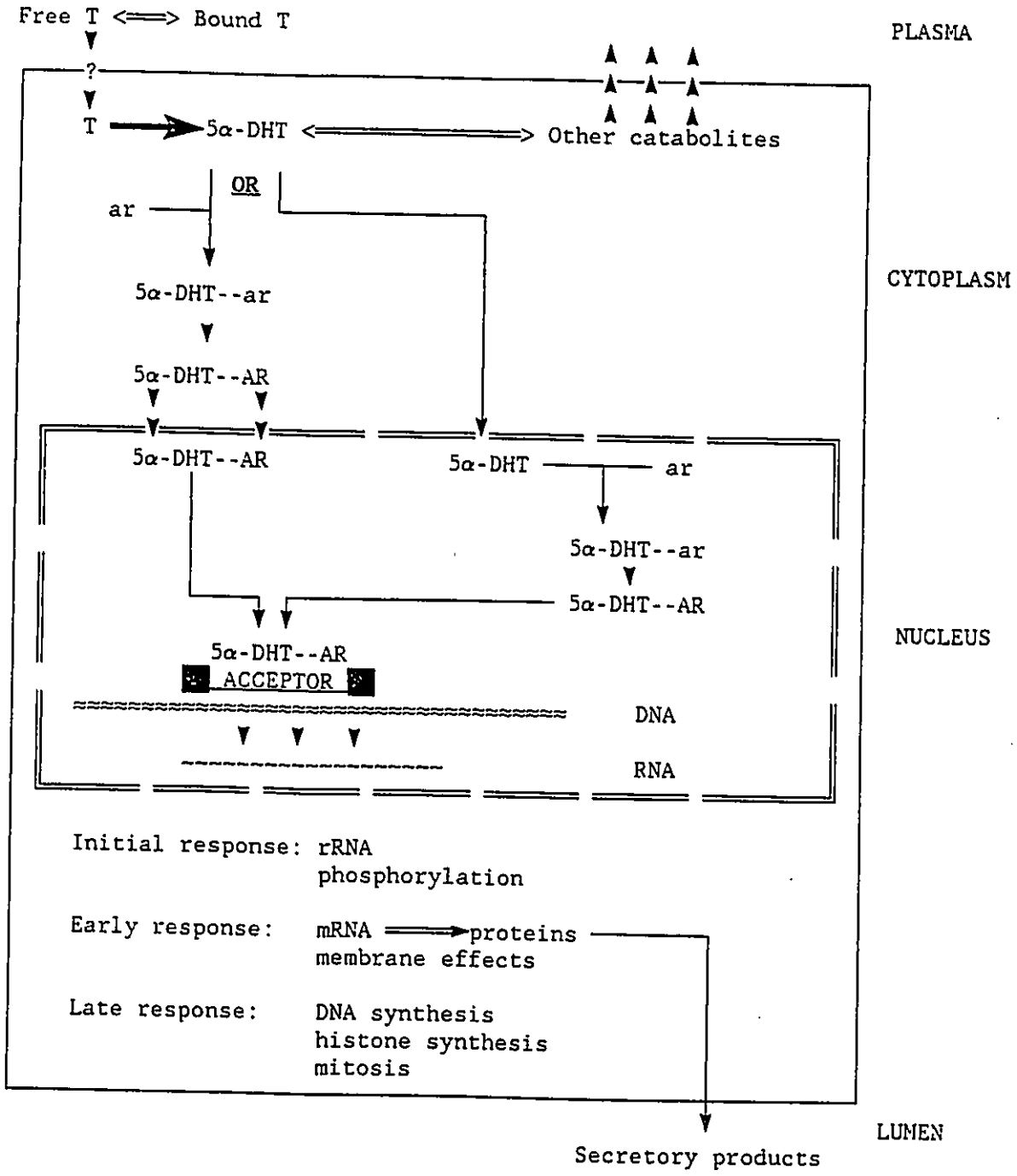
mechanism of androgen action were characterized including the receptors for 5α-DHT and the various enzymes involved in the metabolism of testosterone. In addition another group of marker proteins, which respond to androgens, were also characterized. These included the proteins involved in synthesis and transport, the prostate steroid-binding proteins and secretory acid phosphatase.

From a historical perspective the turning point in the study of the androgen dependence of the prostate on a biochemical rather than morphological basis came from the work of Mann and co-workers in the study of citric acid in rat prostate secretions. Citric acid levels were maintained only when the androgenic status of the animals was fully preserved (Humphrey and Mann, 1949; Mann and Parsons, 1950). Mann later found that the prostate is among the richest sources of polyamines (Mann, 1964). The intracellular concentrations of spermine, spermidine and putrescine, together with their synthetic enzymes, ornithine decarboxylase and S-adenosyl methionine decarboxylase are rigorously regulated by androgens. (Pegg and Williams-Ashman, 1969; Pegg, Lockwood and Williams-Ashman, 1970). The rapid increase in polyamine levels upon renewal of androgens to castrated rats was initially thought to be involved in the proliferative response of the prostate. However it was later suggested that rather than playing a role in the proliferation of the prostate, polyamines were involved in the secretory functions of the gland (Fuller, Donaldson and Thomas, 1975; McKeehan, Glass, Rosser and Adams, 1982).

FIGURE 1: Classical mechanism of androgen action (Adapted from Mainwaring, 1977)

T = Testosterone
5α-DHT = 5α-dihydrotestosterone
ar = Androgen receptor
5α-DHT--ar = 5α-DHT - androgen receptor complex
5α-DHT--AR = Activated 5α-DHT--ar
ACCEPTOR = Nuclear acceptor protein

Figure 1


Free T ⟺ Bound T
PLASMA
?
T
5α-DHT ⟺ Other catabolites
OR
ar
5α-DHT--ar
CYTOPLASM
5α-DHT--AR
5α-DHT--AR
5α-DHT
ar
5α-DHT--ar
5α-DHT--AR
NUCLEUS
5α-DHT--AR
ACCEPTOR
DNA
RNA
Initial response: rRNA
phosphorylation
Early response: mRNA ⟹ proteins
membrane effects
Late response: DNA synthesis
histone synthesis
mitosis
LUMEN
Secretory products

With the development of ornithine decarboxylase and polyamines as easily assayed markers of prostate androgen dependence, other groups sought to find additional functional markers. One of the best known and most useful of these markers is the prostate steroid-binding protein (PSBP) (Heyns and De Moor, 1977). The function of this protein is not yet known, however its synthesis is absolutely dependent upon androgens (Parker, Scrace and Mainwaring, 1978). Since PSBP is the major protein secreted by the prostate, the synthesis and responsiveness of PSBP has been studied extensively both in-vivo and in-vitro (Heyns, Peeters and De Moor, 1977; Heyns, Van Damme and De Moor, 1978; McKeehan, Rosser, Glass and Fast, 1980; Parker, White and Williams, 1980; White and Parker, 1983; Zhang and Parker, 1985). These studies have shown that PSBP is synthesized and secreted by the androgen dependent epithelial cells of the rat ventral prostate. The availability of cDNA and genomic clones has resulted in the widespread use of PSBP to study both general hormonal control of gene expression and the specific interactions involved in control of involution and renewal of the rat prostate.

In addition to PSBP the level of acid phosphatase (AP) activity has also been used as an indicator of the androgenic status of the rat ventral prostate. The ventral prostate is the only organ in the rat that expresses both lysosomal (LAP) and secretory acid phosphatases (SAP) (Vanha-Perttula, Niemi and Helminen, 1972; Tenniswood, Bird and Clark, 1976). The two forms of enzyme may be readily distinguished by the

susceptibility of LAP to inhibition by tartrate (Paul and Richardson, 1969) and by the androgen dependence of SAP (Tenniswood et al., 1976). Unlike PSBP, SAP activity changes slowly after androgen ablation and the analysis of experimental data is complicated by incomplete inhibition of LAP by tartrate. Androgenic regulation of AP activity occurs at several levels including the synthesis and glycosylation of the enzyme. This has limited the usefulness of SAP as a marker enzyme to the qualitative rather than quantitative analyses of androgen action, but the presence of the secretory form of acid phosphatase is used as an unequivocal marker of androgen dependence of the prostate.

2) Anatomical considerations

Until it was demonstrated that the response of the epithelial cell to androgens required the regulatory influence of the stromal cell component of the gland the androgen dependence of the prostate was thought to occur exclusively via the classical androgen action receptor mechanism (Cunha, 1972; Cunha and Lung, 1978; Cunha, Shannon, Taguchi, Fujii, and Chung, 1982; Cunha, Fujii, Neubauer, Shannon, Sawyer and Reese, 1982; Cunha, 1984; Cunha, Donjacour, Cooke, Mee, Bigsby, Higgins and Sugimura, 1987). Using a number of different epithelial and stromal tissues derived from the embryonic urogenital sinus these researchers have provided compelling evidence demonstrating that the androgen dependence of the secretory epithelial cells

of the prostate is dependent on the integrity of the stromal androgen receptor. This has lead to the search for the mediators of the intercellular communication including soluble growth factors (Story, Jacobs and Lawson, 1984), growth inhibitory factors (König, Romijn and Schröder, 1987), proteins associated with cell death (Léger, Montpetit and Tenniswood, 1987), and the development of extracellular matrix cell culture systems (O'Connor and Sinha, 1985; Thornton, Frederickson, Mata and Mawhinney, 1985).

Dissection of the rat prostate into a two-dimensional array demonstrates the distinctive branching pattern of the rat ventral prostate. The ventral prostate begins branching very near the urethral opening, the proximal end, and each branch then undergoes several other divisions before encountering the distal tip, producing an arborized structure.

The ducts are lined with epithelial cells around which the prostatic stroma forms a sheath-like structure composed of fibroblasts, muscle cells, nerve cells and the specialized cell structures of the circulatory system. Uniting the structural units of epithelial-stromal cells forming an individual duct are other muscle, fibroblast and connective cells that act together to preserve the global architecture of the prostate while allowing the unimpeded growth and development of the gland (Figure 2).

3) Stromal cells of the rat ventral prostate:

The stroma of the prostate is composed of several cells types, most of which play the same specifically defined roles in many tissues (eg. muscle contraction and innervation). These cells are generally considered to be distinct from the fibroblastoid cell component of the prostatic stroma. As a result the term "stroma" is conventionally used to designate the fibroblast component of the prostate alone. This convention will be maintained in this thesis. Thus the term "stroma" will refer to the fibroblasts as a tissue unit, while the dissociated cells will be termed fibroblasts.

The stromal component of the prostate was originally thought to be responsible solely for the maintenance of the structure of the gland. The importance of the stroma was greatly expanded by the demonstration by Cunha and co-workers that in the developing prostate the epithelial cells are devoid of androgen receptors and thus the response to androgens (growth and proliferation) must be mediated by the stromal component which was shown to specifically bind the androgen. To further this relationship, Cunha has also demonstrated that the prostatic stroma is in fact the controlling element in the development of prostatic epithelium in terms of cell number, differentiation and secretion (Cunha, 1984).

FIGURE 2: Schematic representation of the cell types located along the ducts of the rat ventral prostate.

NOTE: Scale has not been preserved in this diagram.

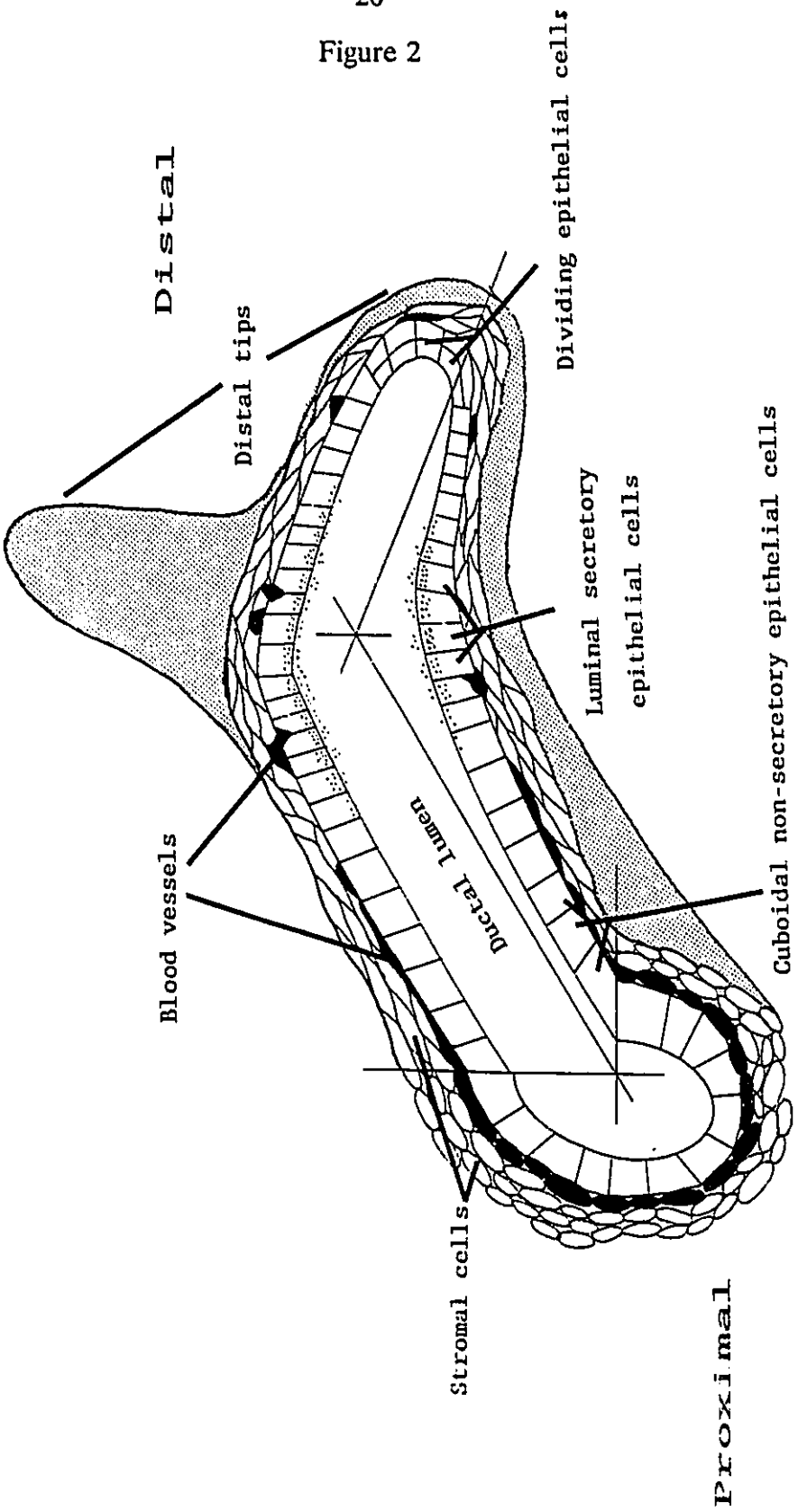
Luminal epithelial cell

Basal epithelial cell

Stromal cell

Blood vessel

Figure 2


Distal
Distal tips
Dividing epithelial cells
Luminal secretory
epithelial cells
Ductal lumen
Blood vessels
Cuboidal non-secretory epithelial cells
Stromal cells
Proximal

The stroma of the prostate does not require androgens for either cell survival or function. Castration results in the atrophy of the prostate epithelium alone, the stromal component remains virtually unaffected and as a result the normally high epithelial-stromal ratio is reversed with time. With the renewal of androgens the number of stromal cells remains unaffected while the epithelium proliferates and returns the prostate to its normal size and function. This cycle of androgen ablation and restoration can be repeated several times with full epithelial cell re-population of the atrophied prostate with each renewal of androgens (Lesser and Bruchovsky, 1973; Berry and Isaacs, 1984). The androgen-independence of the stroma has been used to suggest that it serves as the basal stem cell population for renewal of the androgen-dependent glandular epithelial cells by terminal differentiation of the undifferentiated stromal cell, much in the same manner as the renewing epithelial system of epidermal tissue (Stoningten and Hemmingsen, 1971; Bazer, 1980; Battersby, Chandler, Harper and Blacklode, 1977) with the possible "intermediate" cell type being androgen-independent epithelial cells.

Despite the attractiveness of the stromal stem cell hypothesis it has not been supported by recent results. Direct examination of the cell types involved in the regeneration of the prostate has demonstrated that the stromal cells do not serve as stem cells for the epithelium (Evans and Chandler, 1987a; Evans and Chandler, 1987b; English, Santen and Isaacs, 1987). Using [^{3}H]-thymidine uptake kinetics, labeling

indices and light microscope autoradiography the epithelial and stromal cells have been shown to act as distinct self-replicating populations, both in normal growing tissue and in the regenerating prostate of castrated rats receiving exogenous testosterone.

4) Epithelial cells of the rat ventral prostate:

The epithelial cell population of the prostate is limited to a thin layer a few cells thick surrounding the lumen of each prostate duct (Figure 2).

a) Basal epithelial cells

Interspersed among the luminal secretory epithelial cells are a small number of basal epithelial cells that are separated from the lumen by one or two layers of secretory cells. The basal epithelial cells of rat, dog and man show a variety of characteristics of undifferentiated epithelial cells: androgen independence, poorly developed Golgi complexes, short segments of rough endoplasmic reticulum, few small mitochondria and the abscence of secretory granules (Timms, Chandler and Sinowatz, 1976; Mao and Angrist, 1966). Rat prostate organ cultures and both organ and cell cultures of dog prostate have shown differentiation of the basal epithelial cells into secretory epithelium (Chandler and Timms, 1976; Chandler, Timms, Morton and

Groom, 1976; Chevalier, Bleau, Roberts and Chapdelaine, 1981). Epithelial cells differentially intermediate between basal and luminai epithelial cells are found in the dog but not rat prostate (Timms et al., 1976). However during the regeneration of the prostate induced by exogenous androgen administration to castrated rats Verhagen and co-workers have found epithelial cells co-expressing various combinations of basal and luminal cytokeratins (Verhagen, Aalders, Ramackers, Debruyne and Schalken, 1988). The basal cells of the RVP are sparsely distributed throughout the distal region of the duct but form a layer that surrounds the luminal epithelial cells in the proximal region of the ducts (Rouleau, Léger and Tenniswood, 1990).

b) Luminal epithelial cells

The luminal epithelial cells are cuboidal or columnar in morphology depending upon their location along the duct and whether or not the animal has been castrated. The vast majority of the approximately 90 million epithelial cells in the adult ventral prostate are androgen-dependent for both cell survival and function (Bruchovsky, Lesser, Van Doorn and Craven, 1975). Castration of the animal results in involution of the prostate as approximately 90% of epithelial cells die within the first week following castration while the stromal cells are reduced by less than 5% during the same period (DeKlerk and Coffey, 1978; English et al., 1987). The location of the epithelial cells along the duct (Figure 2) determines a number of the characteristics

of the epithelial cells including sensitivity to androgen ablation, morphology and secretory activity. The epithelial cells at the distal tip of a prostate duct are exquisitely sensitive to reductions in androgen levels and are the first cells to die following androgen ablation. These cells are cuboidal in morphology and have the greatest ability to proliferate as demonstrated by thymidine uptake in-vivo (Cunha, 1984). By far the most numerous cells within the prostate, the secretory cells have a tall columnar morphology under normal hormonal conditions. These cells are polarized, that is to say that the nucleus is found in the region of the cell furthest from the lumen and the organelles involved in synthesis and secretion are located closer to the luminal border of the cell. These cells secrete the marker enzymes (SAP, PSBP) and other androgen dependent components of the seminal plasma.

The last category of epithelial cells found in the prostate are in the most proximal region of the duct. These cells are cuboidal in morphology and are not involved in secretory activity (Sugimura et al., 1986a; Sugimura, Cunha and Donjacour, 1986b; Cunha, et al., 1987; Rouleau, 1989). The hormonal status of these cells is of considerable interest and controversy since they too could act as epithelial stem cells with the basal epithelial cells. These luminal, non-secretory cells survive after castration.

Cunha and co-workers (Cunha, Donjacour and Sugimura, 1986) have likened the effect of castration on the cells along the ducts to pruning the top branches of trees. The distal tips are the most sensitive cells to androgen ablation and hence are the first to die. The secretory cells cease their secretory activity within a few hours of castration and survive for a limited time. After castration, the remnants of the ducts are lined with proximal cuboidal epithelial cells and the basal epithelial cells. Upon renewal of androgens there is a spurt of growth as the epithelial cells that remained after androgen depletion proliferate rapidly at the tip of the regressed duct. Although the experiments have not determined the cell types involved in the initial proliferation, once initiated, the cells at the tip of the branch proliferate and extend the duct into the stromal cells which had survived castration. Terminal differentiation of the cells (as judged by evidence of secretory activity) to form secretory cells does not appear to commence until the prostate has nearly regained its full intact size.

5) Stromal-epithelial cell interactions:

Maintenance and growth of prostatic epithelium depends upon the presence of both extrinsic and intrinsic factors. While testosterone and its metabolites are essential extrinsic elements for the maintenance of the prostate they are not sufficient to elicit growth of the prostate since the adult prostate does not continue to enlarge in mature intact animals despite the presence of high concentrations of androgens. Experiments

with tissue recombinants of epithelial and stromal tissue from mice has demonstrated the importance of the stroma in epithelial differentiation during the development of the prostate (Cunha, Chung, Shannon and Reese, 1980). In addition to regulating the differentiation of the prostatic epithelium, the stroma also appears to determine the final size of the prostate by inducing epithelial cell growth until a pre-determined stromal-epithelial cell ratio is attained. Using different ratios of stroma and epithelium in tissue recombinants, it has been shown that that the final size of the implanted tissue is directly proportional to the amount of stromal tissue present (Chung and Cunha, 1983). Thus the constant size of prostate arising from repeated androgen ablation and replenishment experiments may be explained by the maintenance of a constant number of the androgen independent stromal cells following androgen ablation.

In addition to promoting the growth of the epithelium, prostate stroma is also involved in the repression of excess epithelial cell growth by secretion of at least one epithelial cell inhibitory factor (König et al., 1987). These findings support the hypothesis that a complex series of feedback mechanisms between the prostatic stroma and epithelium act as a unit to mutually control proliferation and differentiation (Tenniswood, 1986).

6) The role of cell death in the prostate:

The interplay between stroma and epithelium results in the maintenance of a defined gland size under normal hormonal conditions. As described earlier, several cycles of androgen ablation and renewal may be undertaken with the final result in each cycle being the formation of a mature prostate with a well defined size. This is the result of the complex interplay between stroma and epithelium being maintained as the tissue regains normal levels of androgens by exogenous administration. This interplay is short-circuited by the removal of androgens, resulting in involution of the prostate and cell death. The rapid rate of this involution and loss of epithelial cells is considerably greater than that expected if the epithelial cells were simply dying as a result of androgen depletion and epithelial cell turnover. Reports of the synthesis of novel mRNA transcripts and their corresponding proteins in response to androgen ablation have been interpreted as evidence of the active degradation of epithelial cells by apoptosis (Montpetit, Lawless and Tenniswood, 1986; Léger et al., 1987; Wyllie, 1987; Montpetit and Tenniswood, 1989a). The highly controlled destruction of epithelial cells has stimulated a number of research groups to study similar activities in other hormonally dependent systems such as the Shionogi tumors (Giles, Rennie and Bruchovsky, 1986; Rennie, Bruchovsky, Buttyan, Benson and Cheng, 1988b). These studies have demonstrated that tumor growth is a combination of increased

proliferation and decreased cell death and that alteration of either parameter has a dramatic effect on tumor growth (Isaacs, 1987).

7) Adaptation versus selection:

The source of renewed tumor activity within castrated or otherwise hormonally treated patients remains a mystery. Two hypotheses for the androgen-independent regeneration and growth of prostate cancer have been proposed. Isaacs and colleagues (Isaacs and Coffey, 1981; Isaacs and Kyprianou, 1987) have proposed that the androgen-independent relapse of prostate cancer arises either from adaptation or selection processes, and they have pointed out that:

> "The importance of resolving whether adaptation versus clonal selection is the mechanism responsible for relapse is that the optimal therapy for prostatic cancer is very different depending upon the answer." (Isaacs and Kyprianou, 1987).

The adaptation model hypothesizes that a prostate tumor is initially composed of a homogeneous population of androgen dependent cells that require androgens for maintenance and growth. Thus following castration or anti-androgen therapy most of the androgen-dependent cancer cells cease proliferation and die, producing the initial response to androgen ablation. However, under the selective pressure of the diminished levels of androgen a number of cells randomly adapt to become androgen-

independent. Once formed, these androgen-independent cells repopulate the tumor, resulting in the relapse typical of most ablative therapies. This hypothesis forms the basis for the "combination therapies" being developed by several groups, since the rapid and total elimination of circulating androgens through "total androgen blockade" should be sufficiently rapid to prevent adaptive response of the epithelial cells to the changing androgen levels, resulting in maximum cell killing.

The selection hypothesis, on the other hand, presumes that prostate tumors are heterogeneous, containing a mixture of androgen dependent and androgen independent cells. Following anti-androgen therapy the initial therapeutic response is the loss of tumor volume and/or metastases as the androgen-dependent cells are killed. However, the androgen-independent cells in the tumor remain unaffected by the diminished androgen levels and continue to proliferate slowly regardless of the hormonal status of the patient resulting in the growth and subsequent metastasis of an androgen-independent tumor. The outcome of anti-androgen therapy on a theoretical tumor composed of androgen-dependent and androgen-independent cells can be predicted based on three variables: the number of androgen-dependent cells in the tumor; the number of androgen-independent cells in the tumor; and the rate of cell proliferation (Coffey and Pienta, 1987).

The phenotypic diversity of cells within prostate tumors has been described for over a decade (Byar and Mostofi, 1972; Sinha, Blackard and Seal, 1977; Kastendieck, 1980). These reports and other studies involving the evolution of the Dunning tumor family of rat prostate tumors (Dunning, 1963; Smolev, Heston, Scott and Coffey, 1977; Isaacs and Coffey, 1981; Isaacs, 1982; Isaacs, Wake, Coffey and Sandberg, 1982; Wake, Isaacs and Sandberg, 1982; Thompson, Johnson, Heidger and Lubaroff, 1985) suggest that prostatic cancers are indeed heterogeneous in their composition.

While the selection model appears to be able to reconcile the biological data better than the adaptation model, it poses three additional questions:

1) What is the origin of the androgen independent cells?

2) How can these cells be killed selectively?

3) What new therapies are needed to induce the death of these cells?

In order to answer these questions we must turn to animal model systems. To answer the first question in particular, it is necessary to examine the normal prostate in model systems, and in particular, the rat ventral prostate, which has served as a model of the normal prostate for over 20 years (Bruchovsky and Wilson, 1968; Mainwaring, 1977).

E) PRIMARY CULTURE OF PROSTATE CELLS:

Primary cultures of rat and human prostate cells have been developed by several groups in order to isolate the individual cell types within the prostate, and to gain a better understanding of the roles of the individual cell type in normal tissues. One of the first difficulties with this approach is the need to separate and purify the different cell types of the prostate. A number of different methods have been developed including explant cultures (Schroeder, Sato and Gilles, 1971; Douglas, Terracio and Glass, 1980; Merchant, Clark, Ives and Harris, 1982), mechanical separation (Bruchovsky, McLoughlin, Rennie and To, 1981) and a variety of density gradient systems using Ficoll (Dow and Pretlow, 1975; Helms, Brazeal, Bueschen and Pretlow, 1975) or Percoll (Chevalier, Bleau, Roberts, Chapdelaine, 1980; Orlowski, Bird and Clark, 1982; Pertloft and Laurent, 1982; Cooke and Littleton, 1985; Montpetit and Tenniswood, 1989b). The initial studies of the hormonal sensitivity and secretory products of the epithelial and stromal cells were performed with cells grown on plastic or glass culture surfaces. The loss of androgen response and rapid cell senescence in-vitro has hindered more detailed analysis of epithelial and stromal cell biology. Recently the importance of the extracellular matrix in maintaining normal secretory epithelial cell morphology has been demonstrated in mammary glands and in the prostate (Thornton et al., 1985; O'Connor and Sinha, 1985; Kawamura and Ichihara, 1987; Kleinman, Luckenbill-Edds, Cannon and Sephel, 1987). These matrices allow

the epithelial cells to adopt morphologies that are more closely related to those seen in-vivo.

A number of groups have reported the isolation of androgen independent epithelial cell populations by nutritional selection (McKeehan, Adams and Rosser, 1984; McKeehan, Adams and Fast, 1987; Peehl and Stamey, 1986). These cells were isolated from primary cultures of rat ventral prostate epithelial cells in a serum-free medium before clonal isolation of the most rapidly dividing epithelial subpopulation. In order to maintain optimal growth these cells require elevated levels of epidermal and other growth factors.

F) AIM OF THE RESEARCH:

The aim of this research was to analyze androgenic regulation of prostate gene expression and to determine the origin of the androgen independent cells of the gland. To this end, I have developed methods to isolate androgen independent epithelial cells and have characterized the growth rate and markers of the androgen independent cells. In the course of this research I have attempted to answer several questions:

1) Can the androgen independent cells act as prostate epithelial stem cells?

2) Can these cells be induced to differentiate?

3) Can these cells be induced to undergo regulated cell death?

This thesis describes the initial isolation and characterization of a series of cell lines that are termed Rapidly Dividing Epithelial (RDE) cells. I suggest that these cell lines may prove to be a valuable model system for understanding androgen independent cell growth and division in the rat ventral prostate.

METHODS

A) Cell Biology

1) Animals:

Mature male Sprague-Dawley rats (250-300 g) were housed under 14 h light/ 10 h dark conditions and fed Purina Rat Chow and water ad libitum. Castrations were performed via the scrotal route under light Innovar-Vet (fentanyl citrate/droperidol) or Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) anaesthesia. The animals were sacrificed by cervical dislocation. All animal experimentation was approved by the University Animal Care Committee.

2) Tissue dissociation:

The dissociation of the rat ventral prostate and the separation of the cells on continuous Percoll gradients were performed according to Orlowski and co-workers (1982) with minor modifications (Montpetit and Tenniswood, 1989b). Mature male rats were sacrificed by cervical dislocation and the prostates were removed aseptically into cold Hank's Balanced Salt Solution (HBSS) (25mM HEPES, 130mM NaCl, 4mM glucose, 3mM KCl, 1mM monosodium phosphate, 3.3μM Phenol Red, pH7.4) (Hanks and Wallace, 1949). The prostates were minced finely (1-3 mm^3) and transferred to 75 cm^2 T-flasks and rinsed 3 times (5 min. each, on ice) with HBSS. The tissue

fragments were allowed to settle and the supernatant removed. The tissue was dissociated by digestion in 10 ml 1% collagenase, 1% trypsin, 1% chicken serum (CTC) in HBSS at 37°C for 20 min. with slow agitation. 100 μl 0.4% DNase 1 in HBSS was added to dissociate the DNA matrix formed from damaged cells and the incubation was continued for 5 min.. The tissue fragments were allowed to settle for 5 min. at 0°C before the supernatant, containing single cells, was transferred to centrifuge tubes stored on ice. The tissue fragments were washed with 10 ml HBSS and the wash supernatant was pooled with the CTC supernatant. This cycle was repeated 7 times. The pooled supernatants of the first CTC digestion/ HBSS wash were discarded because they contained mainly damaged and non-viable cells. The remaining pooled digests/washes were centrifuged at 1,000 xg for 10 min. at 4°C to pellet the cells. The cells were resuspended in a total of 5 ml HBSS. To disrupt the DNA matrix that may effect migration in the Percoll gradients 0.5 ml DNase was added to the resuspended cells and the suspension left for 15 min. on ice.

3) Continuous gradient cell separation:

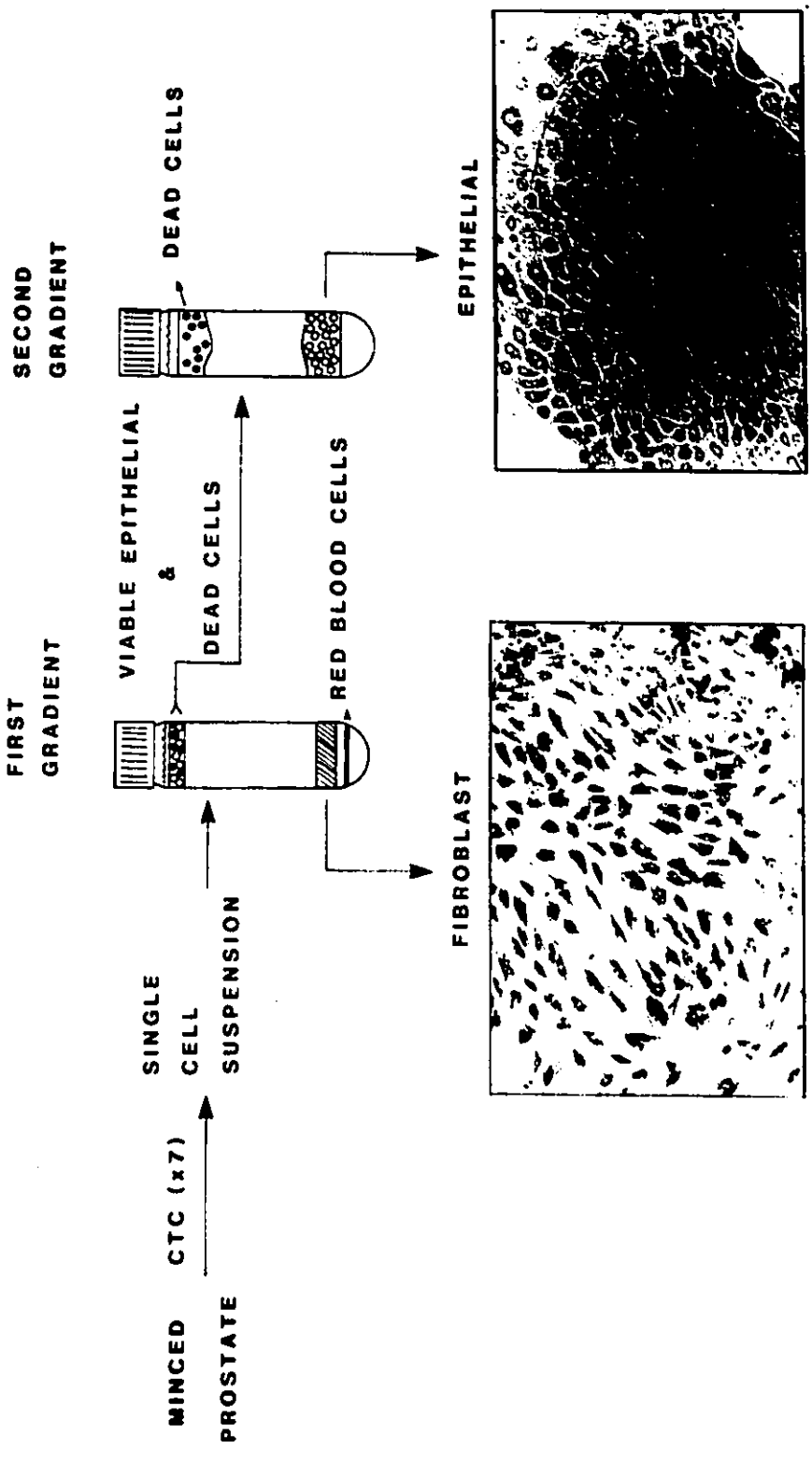
During the CTC digestions an iso-ionic solution of Percoll was prepared using 12.5 volumes Percoll to 1 volume 10xHBSS (Vincent and Nadeau, 1987). This solution was divided into a first gradient solution (47% iso-ionic Percoll, 53% HBSS) and a second gradient solution (26% iso-ionic Percoll, 74% HBSS). To avoid overloading the

separatory capacity of the gradients, two 40 ml tubes of each gradient were prepared for each excised prostate. The gradients were pre-generated immediately prior to use by centrifugation at 21,000xg (Beckman JA-20 rotor, 13,000 rpm.) for 60 min. at 2°C. Aliquots of the cell suspension were loaded onto the first pre-generated gradient and centrifuged at 1,250xg for 45 min. at 2°C. The upper band of cells (Figure 3) was removed, applied to the second pre-generated gradient and centrifuged at 2,000xg for 90 min. at 2°C. The lower band from the first gradient contained viable fibroblasts and the lower band from the second gradient consisted of viable epithelial cells respectively. These were collected by dilution of the Percoll gradient solution with HBSS (9 volumes HBSS for fibroblast cells, 4-5 volumes HBSS for epithelial cells) and centrifugation at 20,000xg for 15 min. at 2°C. Both cell types were resuspended in 2 ml HBSS and viability and cell number were determined by Trypan Blue exclusion using a hematocytometer or Model Z_B Coulter counter. Density calibration of the gradients was performed by refractive index correlation according to Orlowski et al. (1982) using an American Optical refractometer.

FIGURE 3: Percoll continuous density gradient separation of rat ventral prostate epithelial and fibroblast cells.

The prostates were excised, freed from fat and connective tissue and minced into 1 mm^3 fragments. The tissue fragments were subjected to 7 digestion cycles with CTC. The single cell suspension of cells was applied to a pre-generated gradient (47% iso-ionic Percoll, 53% HBSS) and centrifuged for 45 minutes at 1,250xg. The upper band was harvested and applied to the second pre-generated gradient (26% iso-ionic Percoll, 74% HBSS) and centrifuged for 90 min. at 2,000xg. Fibroblast and epithelial cell fractions were collected as described in Methods and the cells were plated at a density of $5x10^5$ cells/60 mm dish that had been coated with fibronectin (fibroblasts) or collagen and fibronectin (epithelial).

Figure 3


MINCED PROSTATE
CTC (x7)
SINGLE CELL SUSPENSION
FIRST GRADIENT
VIABLE EPITHELIAL & DEAD CELLS
RED BLOOD CELLS
SECOND GRADIENT
DEAD CELLS
EPITHELIAL
FIBROBLAST

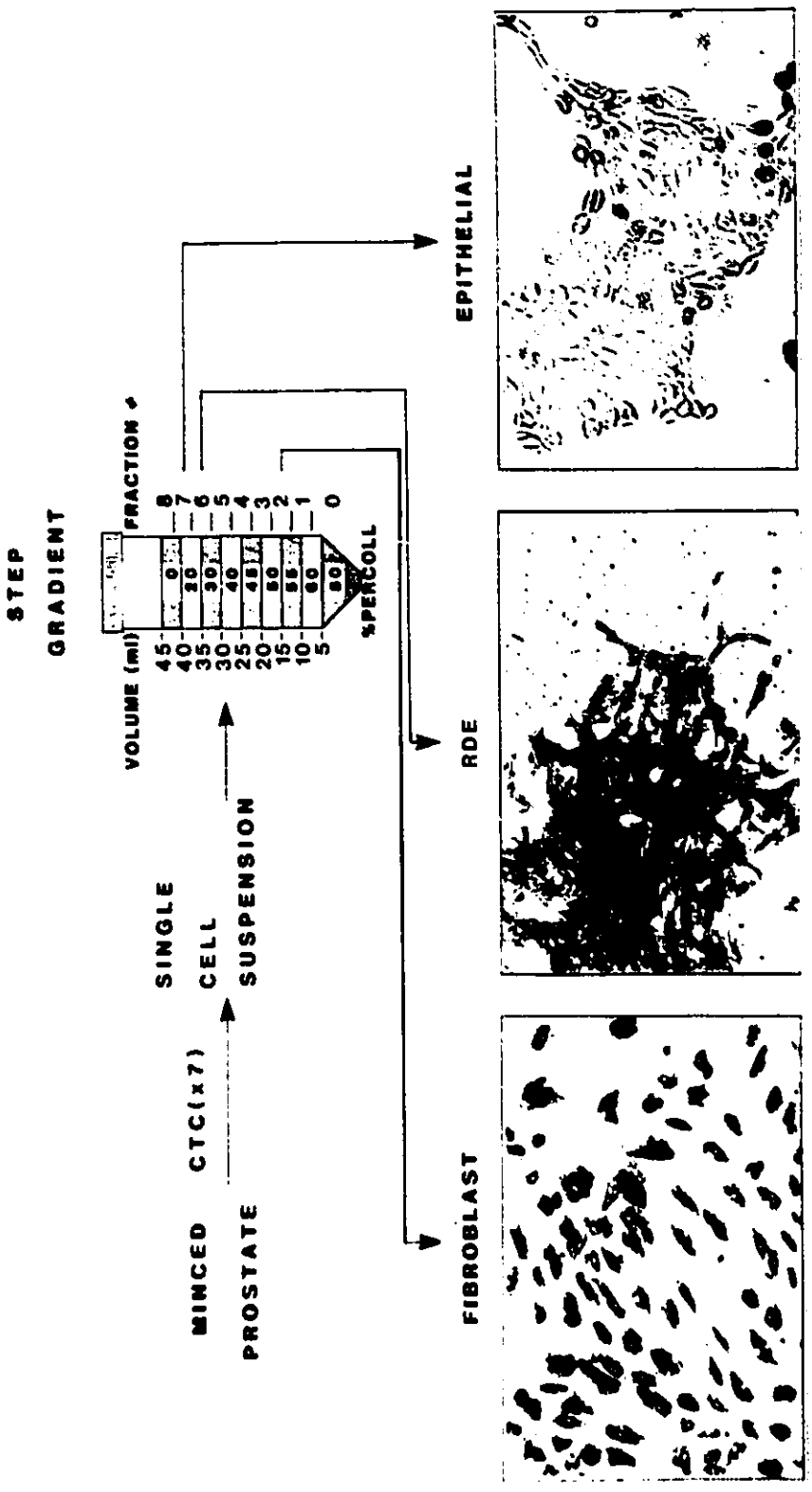
4) Step gradient cell separation:

Separation of freshly dissociated rat ventral prostate cells by step gradient centrifugation was performed as described by Montpetit and Tenniswood (1989b). The iso-ionic Percoll solution (12.5 Percoll:1 10xHBSS) was diluted with HBSS to give solutions of 60%, 55%, 50%, 45%, 40%, 30% and 20% iso-ionic Percoll (v/v). 5 ml of each density solution was sequentially underlayed beneath a 5 ml aliquot of HBSS (0% Percoll). Two gradient tubes were used per prostate to ensure good resolution of the gradient. The gradient tubes were left for 1-3 h to allow the interfaces to diffuse slightly to improve the migration of cells through the steps. Iso-ionic Percoll was added to the suspension of single cells to yield an 80% Percoll solution, of which 5 ml was used to underlay each gradient. The gradients were centrifuged at 2,500xg (Beckman J-6B centrifuge, 3,000 rpm. in JS-4.2 rotor) for 30 min. at 2°C (no brake). The cells were collected by removal of an initial 7.5 ml fraction and 5 ml fractions thereafter, thus assuring the collection of density interfaces rather than fractions of constant density (Figure 4). Each fraction was diluted with HBSS and the cells were pelleted by centrifugation for 15 min. at 6,000xg. The cells were resuspended in 2 ml HBSS for counting and viability assay as described above.

FIGURE 4: Percoll step density gradient separation of rat ventral prostate epithelial and fibroblast cells.

Single cell suspensions from freshly dispersed prostates were applied to gradients made by underlaying 5 ml HBSS with 5 ml aliquots of successively greater density Percoll solutions. The gradient was centrifuged for 30 min. at 2,500xg. Cells were harvested from the bottom of the gradient in 5 ml fractions after an initial 7.5 ml fraction. The cells were plated at a density of $5x10^5$ cells/60 mm dish onto collagen or fibronectin coated plates.

Figure 4


MINCED PROSTATE
CTC(x7)
SINGLE CELL SUSPENSION
STEP GRADIENT
VOLUME (ml)
FRACTION #
%PERCOLL
45 40 35 30 25 20 15 10 5
8 7 6 5 4 3 2 1 0
0 20 30 40 50 55 80
EPITHELIAL
RDE
FIBROBLAST

5) Cell culture medium

Primary cultures (from the gradients and subcultures) were seeded onto plastic culture dishes or extracellular matrix (ECM) coated plates in the "plating medium". This is a serum-free, retinoic acid-free, ß-mercaptoethanol- supplemented derivative of the "growth medium" (Montpetit and Tenniswood, 1989b)(Table I). The use of plating medium was adopted specifically to enhance the plating efficiency of rat ventral prostate epithelial cells in primary cultures (M. McBurney, personal communication). All sera tested (fetal and adult bovine, horse) were found to inhibit the attachment of normal epithelial cells (both freshly dissociated and subcultures) presumably due to elevated soluble fibronectin levels in the serum which saturated the cell attachment sites, thus preventing attachment to the culture vessels. Retinoic acid was removed from the plating medium to alleviate the differentiation pressure on the cells during the attachment period. ß-mercaptoethanol was included in the plating medium to prevent oxidation of the cellular attachment receptors (M. McBurney, personal communication).

TABLE I. CELL CULTURE MEDIUM

Component	Plating medium	Growth medium
F12 medium	42.5%	42.5%
DME medium	42.5%	42.5%
Glutamine	50 μg/ml	350 μg/ml
Penicillin	50 I.U./ml	50 I.U./ml
Streptomycin	50 μg/ml	50 μg/ml
Gentamycin	50 μg/ml	50 μg/ml
Transferrin	25 μg/ml	25 μg/ml
Ascorbic acid	15 μg/ml	15 μg/ml
Insulin	12.5 μg/ml	12.5 μg/ml
Testosterone	10 μg/ml	10 μg/ml
Fungizone	1 μg/ml	1 μg/ml
Dexamethasone	20 ng/ml	20 ng/ml
ß-mercaptoethanol	315 μg/ml	0
Retinoic acid	0	50 ng/ml
Horse serum	0	15%

6) Extracellular matrix preparation

Thin (<1 mm depth) or thick (>1 mm depth) collagen gels were prepared essentially as described by Michalopoulus and Pitot (1975). Eight volumes of rat tail collagen (3 mg/ml) in 0.1% acetic acid were mixed with 1 volume 10x HBSS and 1 volume 0.1N NaOH. The cold solutions were kept on ice to prevent gelling. Dishes were coated with the collagen solution (2 ml/ 60 mm dish) followed by incubation at

37°C for 20 min. to allow the collagen to gel. To coat bare plastic or the solidified collagen gels with fibronectin, 3 ml 10 μg/ml fibronectin in HBSS was applied to the dish (with or without collagen) and incubated for 2 min. at room temperature. The fibronectin-coated bare plastic was left to air dry completely while the collagen + fibronectin coated dishes were left to dry only long enough to eliminate a surface build-up of liquid. Laminin coated plates were prepared using the same procedure.

7) Subculture

Cells were harvested by digestion with 0.5% trypsin, 1mM EDTA for 10 minutes at 37°C, except for cells grown in fibronectin coated plates which required 20 minutes of digestion to release cells quantitatively. The cells were collected by centrifugation (1,000xg, 5 min., 4°C) and were resuspended in 5 ml of pre-warmed growth medium (37°C). Cells were routinely subcultured with a split ratio of 1:50 for RDE cells or 1:10 for primary cultures of rat ventral prostate fibroblast and epithelial cells.

8) Rate of cell division:

Growth rates were measured by plating triplicates of 5,000 cells/ 60 mm culture dish in the growth medium supplemented with the compounds under investigation. The cells were harvested at the appropriate times by trypsinization and triplicate

determinations of each plate were counted using a Coulter Counter Model Z_B or Model Z_F and Channelyzer. Counts were corrected for coincidence as suggested by the manufacturer before the cell numbers were averaged.

9) Clonal selection of RDE cells:

Foci of RDE cells in the primary cultures of normal epithelial cells from rat ventral prostate were isolated from the general epithelial cell population by gentle trypsinization within cloning rings. The foci were digested further with the trypsin/EDTA solution and clonally selected by 5 fold serial dilutions into 96 well culture dishes until single cells were isolated.

10) Histology:

RDE and normal RVP epithelial cells and fibroblasts were cultured on a variety of matrices in media of differing composition. Cultures to be examined histologically were washed with HBSS and fixed with citrate buffered-acetone for 30 seconds or acetone at -20°C for 10 min.. The cells were stained with acid hematoxylin for 3 min., washed in running water for 5 minutes and counterstained with 1% eosin for 3 minutes before washing for 5 minutes in running water. The cultures were air dried

and photographed using Kodak Panatomic-X film (32 ASA) or Fujichrome (100 ASA) with a blue filter for tungsten colour correction.

11) Acid phosphatase analysis:

Cells were homogenized in 100mM sodium acetate buffer (pH 4.5) and were assayed for the presence of acid phosphatase activity according to Downey, Mahan, Flynn, Bird and Clark (1983). Isoelectric focusing of equal total acid phosphatase activities per well was performed in 7.5% polyacrylamide gels for 2 .. at a constant power of 22 W with cooling to maintain the gel temperature below 10°C. Localization of acid phosphatase activity in the isoelectric focusing gels was achieved by using α-naphthylphosphate as the initial substrate with Fast Garnet GBC as the coupling dye. The incubations were carried out for two hours at pH 4.85 in 2.7mM citrate buffer. Tissue homogenates of total intact rat prostate were used for control standards. Acid phosphatase activity was demonstrated histochemically using the Sigma acid phosphatase reagent kit, using α-naphthylphosphate as the initial substrate with Fast Garnet GBC as the coupling dye as for IEF gels. Where indicated secretory acid phosphatase activity was distinguished from lysosomal acid phosphatase activity by the inclusion of 27mM sodium tartrate to selectively inhibit the lysosomal enzyme (Tenniswood, Abrahams, Bird and Clark, 1978).

12) Metabolism of testosterone:

Triplicate cultures of each cell line and primary cultures of both normal rat ventral prostate epithelial and fibroblast cells were allowed to grow to approximately 80% confluence in growth medium containing 34nM testosterone (T^+). The medium was removed and replaced with growth medium supplemented with $8x10^5$ dpm [1,2,^{3}H]-testosterone. After 4, 8 and 24 hours the medium was recovered and the cells harvested. ^{14}C labeled standards (testosterone, 5α-DHT, Δ^4-androstenedione, 3α-androstanediol, 3ß-androstanediol and 5α-androstanedione) were added before the medium and cells were extracted with 10 ml methylene chloride. The samples were processed for HPLC analysis of testosterone metabolites as described by Orlowski and Clark (1989). The steroids in methylene chloride were evaporated to dryness and dissolved in 200 μl HPLC grade methanol. Aliquots (20 μl) were injected into a Beckman HPLC system with a reverse phase C-18 column using a degassed mobile phase (flow rate of 1 ml/min) of acetonitrile:methanol:water (1:3:3). The effluent was monitored by in-line spectrophotometry (254 nm) and a radioactive flow detector using a 250 μl flow cell.

13) Immunohistochemistry:

Cells and whole mounts of prostatic ducts were fixed onto poly-L-lysine coated coverslips with ice-cold acetone for 20 min.. After 3 washes with HBSS the slides were incubated with diluted primary antibody (Appendix 3) for 1 h at 37°C in a humidity-saturated environment. The slides were washed with HBSS and incubated with 1:1000 diluted peroxidase-linked secondary antibody for 1 h as described above. Control samples were incubated with the secondary antibody alone. After washing, the samples were incubated in 0.3% 4-chloro-1-naphthol, 0.03% hydrogen peroxide in 50mM Tris pH 7.6 for 10 min. at room temperature. The slides were then washed for 5 min. with water and optionally counterstained for 3 min. with 1% eosin.

14) Growth in soft agar:

A single-cell suspension of RDE cells was diluted to a concentration of 200 cells/ml and dispersed in a mixture of warm medium and 0.5% agar. The cells were aliquoted onto base agar prepared in growth medium and subsequently covered with 0.3% agar in growth medium. The dishes were incubated at 37°C and scored on days 5 and 12 for colonies containing more than 20 cells (Suzuki, Suzuki and Nikaido, 1983).

15) In-vivo tumorigenicity:

10^6 RDE cells in 0.1 ml HBSS or 0.1 ml HBSS alone (as a control) were each injected into three 250 g male Sprague-Dawley rats under inhaled anaesthesia. The cells were injected in one of the following locations: intradermally into the dorsal area, intravenously into the tail vein, intraperitoneally, into the prostatic capsule of intact rats and into the prostatic capsule of castrated rats. Intradermally and intraperitoneally injected animals were checked weekly for 6 weeks for externally visible tumor growth. Animals were sacrificed at 6, 12 and 24 weeks after injection and necropsies were performed to locate metastatic tumor growth. In addition to gross observation of each organ, multiple thick sections of brain, heart, liver, kidney, bladder, muscle, prostate, and in intact animals, testis were examined microscopically for the presence of nodal growths.

16) Growth factor and growth inhibitor effects:

RDE cells were cultured in duplicate into 60 mm tissue culture dishes ($5x10^4$ cells) in T^+ growth medium. The specific growth factors were added at the concentration determined by the manufacturer to induce maximal stimulation of the appropriate reference cell type. The cells were left to divide for 2, 3 or 4 days with the medium being replaced on day 3 with new growth factor supplemented growth

medium. The cells were harvested on the appropriate day and processed for counting by Coulter Counter or for gene expression analysis by in-situ dot blotting. For growth inhibitors the inoculated cells were supplemented with a range of growth inhibitor concentrations, incubated 3 days and processed for counting and gene analysis on day 5 (as described above).

17) Thymidine and uridine incorporation:

Duplicate 60 mm dishes of RDE cells were cultured under the test conditions for 36 hours before the addition of 10 μCi [^{3}H]-thymidine (82 Ci/mmol) or [^{3}H]-uridine (53 Ci/mmol)(final concentration of labeled nucleoside 122pM and 189pM respectively). The cells were incubated for a further 2 hours before the medium was removed and the monolayer was washed twice with HBSS before harvesting. Cell numbers were determined by hemacytometer before the cells were homogenized and sonicated. The nucleic acids were precipitated with trichloroacetic acid (TCA) and bound to Whatman glass fibre filters. After two washes with 10% TCA the filters were dried and the incorporated radioactivity determined by liquid scintillation counting.

18) Karyotyping:

Non-confluent cultures of RDE cells were exposed to 0.1 μg/ml colcemid in T^+ growth medium for 3 hours. The cells were trypsinized and resuspended in 75mM KCl for 30 minutes before fixing for 24 hours with Carnoy's fixative (3:1 methanol:acetic acid). The cells were dropped onto cold slides and dried before staining and examination.

B) Molecular Biology

1) Extraction of RNA and "Northern" hybridizations:

Total RNA was extracted from prostates or cultured cells by the LiCl/urea method (Auffray and Rougeon, 1979) with minor modifications (Tenniswood and Simpson, 1982). Poly(A)$^+$ RNA was prepared using oligo(dT)-cellulose chromatography (Aviv and Leder, 1972). RNA preparations were electrophoresed through 1.5% agarose slab gels under denaturing conditions (McMaster and Carmichael, 1977) using rat rRNA and wheat germ tRNA as size markers. The RNA was transferred to nylon membranes essentially as described by Thomas (1980) for nitrocellulose filters. The filters were prehybridized for a minimum of 8 h at 42°C in

a buffer containing 50% formamide, 5x SSC (20x SSC is 3M NaCl, 0.3M sodium citrate, pH 7.0), 50mM phosphate buffer pH 6.8, 5x Denhardt's solution (0.02% albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone) (Denhardt, Dressler and Ray, 1978) and 250 μg/ml sheared calf thymus or salmon sperm DNA. The filters were hybridized in a fresh quantity of the same buffer with radiolabeled probe for 48-72 h at 42°C. The filters were washed twice with 2x SSC, 0.1% SDS for 20 minutes at room temperature followed by 2 washes with 0.1x SSC, 0.1% SDS at 42°C for 30 minutes. The filters were covered with polyvinylchloride film and autoradiographed at -70°C using Dupont Cronex-4DC film between intensifying screens (Bonner and Laskey, 1974).

2) Radiolabeling probes:

Nick-translation was used to label 200 ng of plasmid with [α-^{32}P-dCTP] to a specific activity of approximately 10^8 cpm/μg (Rigby, Dieckmann, Rhodes and Berg, 1977). Radiolabeled RNA probes for in-situ cell blotting were transcribed by SP6 or T7 RNA polymerase primed from the anti-sense strand promoter in the pGEMINI series of plasmid vectors (Melton, Krieg, Reblagiati, Maniatis, Zinn and Green, 1984).

3) Cell-free translation and in-vivo protein labeling:

Poly(A)$^+$ RNA and total RNA samples were translated in a methionine-free micrococcal-nuclease treated reticulocyte cell-free translation system (Pelham and Jackson, 1976; Tenniswood and Simpson, 1982) using ^{35}S-methionine (>800 Ci/mmol) as the radiolabel. Translations initiated without adding exogenous RNA were used to determine the size and abundance of endogenous translation products.

In-vivo labeling of cellular proteins with ^{35}S-methionine was performed by incubating 10^6-10^7 cells in 5 ml methionine-free Dulbecco's Modified Eagle's Medium (DMEM) for 12-24 h in the presence of 50 μCi ^{35}S-methionine (>800 Ci/mmol). The cells were then washed with HBSS, harvested with trypsin/EDTA and lysed by freeze-thawing in 0.01% NaCl.

4) SDS-Polyacrylamide gel electrophoresis:

Proteins from in-vitro translations and in-vivo labeling experiments were processed for 15% polyacrylamide gel electrophoresis according to Newbold, Boyle, Smith and Brown (1982). A 3% w/v stacking gel and 15% w/v acrylamide separating gel were used with a ratio of acrylamide to bisacrylamide of 30:0.4. Samples (volume of sample varied in order to obtain a constant 10^5 cpm/lane) were boiled for 2 min.

in a buffer containing 1% SDS, 5% ß-mercaptoethanol, 10mM EDTA, 20mM PMSF, 50mM Tris-HCl pH 7.8 and 10% glycerol before application to the gel. After electrophoresis the samples were dried and fluorographed between Kodak X-Omatic intensifying screens at -70°C using DuPont Cronex-4DC film (Bonner and Laskey, 1974).

5) Cloning and sequencing RDE sequences:

Double stranded cDNA (ds-cDNA) was prepared from RDE cell poly(A)$^+$ RNA using a modification of the method of Rutledge, Seligy, Coté, Dimock, Lewin and Tenniswood (1988). The cDNA molecules were ligated into Sma-1 digested, alkaline phosphatase treated pUC 19 prior to transformation of E. coli DH-5α cells. Plasmids of interest were initially identified by differential screening (Grunstein and Hogness, 1975) using cDNA reverse transcribed from poly(A)$^+$ RNA of RDE and normal rat ventral prostate cells. Clones demonstrating greater signal strength with RDE cDNA were isolated using a modification of the method of Birnboim and Doly (1975) and subjected to more rigorous screening by Northern hybridization. The cDNA of clones determined to be specific for RDE cells (relative to normal rat ventral prostate epithelial and fibroblast cells) were subcloned into the M13mp10 and M13mp11 vectors (Messing, 1983) to permit sequencing of both strands of the inserted DNA. Single strand phage template was prepared and annealed to a 17 base universal M13

sequencing primer for dideoxy-chain terminator sequencing (Sanger, Nicklen and Coulson, 1976; Tabor and Richardson, 1987) with ^{35}S-dATP. The sequencing reactions were heat denatured, applied to 8% polyacrylamide, 8M urea gels and electrophoresed at 2,500 volts, 40 mA and 50 watts for 1.5 to 8 hours. The DNA was fixed within the gel with 5% methanol, 5% acetic acid prior to drying. Autoradiography was performed for 18-36 hours at room temperature using Cronex-4DC film and a single intensifying screen. Computerized sequence analysis of restriction sites and comparison with the GENBANK nucleic acid and NBRF protein databases was performed using Microgenie(TM) computer software (Queen and Korn, 1984).

6) Cell blotting:

Direct blotting of glutaraldehyde-fixed cells and of T7 RNA polymerase standard transcripts was performed according to Montpetit and Tenniswood (1990). Cells (RDE, primary cultures of normal epithelial or stromal cells, or freshly isolated cells) were fixed for 1 h in 2% glutaraldehyde in HBSS. The cells were then dehydrated in 50% and 70% ethanol before storage at -20°C in 70% ethanol. The cells were blotted to poly-L-lysine coated nylon membranes before digestion with proteinase K to expose the RNA. The basic proteins were acetylated (0.25% acetic anhydride in 100mM triethanolamine) to prevent non-specific binding of probe. The filters were prehybridized for a minimum of 8 h at 42°C in a buffer containing 50% formamide,

5x SSC, 50mM phosphate buffer pH 6.8, 5x Denhardt's solution and 250 μg/ml sheared calf thymus or salmon sperm DNA. The filters were hybridized in a fresh quantity of the same buffer with radiolabeled probe for 48 h at 42°C. The filters were washed twice with 2x SSC, 0.1% SDS for 20 min. at room temperature followed by 2 washes of 30 min. each with 0.1x SSC, 0.1% SDS at 50°C. The filters were covered with polyvinylchloride film and autoradiographed at -70°C using Dupont Cronex-4DC film between intensifying screens.

7) Effects of calcium ionophore

Calcium ionophore, A23187, dissolved in 50% DMSO/50% ethanol was added at various concentrations to duplicate plates of RDE cells in 5 ml T^+ growth medium. Dilutions of A23187 were chosen to prevent solvent concentrations from exceeding 0.5% while covering a concentration range of 0 to $5x10^{-6}$M. After 16 h the dose response curve was determined by harvesting adherent RDE cells and counting on a model Z_B Coulter Counter. From this curve the concentration of A23187 required to kill 50% of cells in 16 h was determined and used for the analysis of the induction of TRPM-2 gene expression. RDE cells (3/4 confluent) were incubated with $2.5x10^{-7}$M A23187 for 0, 2, 4, 6 or 24 hours before being processed for in-situ cell blotting as described above, modified so that all cells, (both adherent and no longer adherent) were included. 25,000 cells were blotted, processed and hybridized to equal counts of

^{32}P-labeled probes. The blots were probed with each of the RDE clones (pRDE-0.25, pRDE-1.5 and pRDE-2.3) as well as p21-04, a cDNA clone of a cell death associated mRNA (Léger, Montpetit and Tenniswood, 1987). After hybridization and washing the blot was exposed for 6 and 72 hours between intensifying screens at -70°C using Dupont Cronex-4DC film.

RESULTS:

A) CELL BIOLOGY

1) Isolation of RDE cells

The separation of RVP epithelial and fibroblast cells by continuous Percoll isopycnic density gradient as outlined by Orlowski (1982) was originally optimised for the separation of cells from immature rats. The method was modified for use with mature rats by increasing the number and duration of CTC digestions (from 5 to 7). This increased the cell yields from the larger and more fibrous mature prostate. The pre-generation of the Percoll gradients was also modified by extending the duration of the centrifugation to lessen the slope of the linear portion of the density curve (Figure 5). This resulted in a greater separation of the fibroblast cells (density 1.10-1.12 g/cm^3) from the viable epithelial cells (density 1.04-1.05 g/cm^3), thereby increasing the resolution, purity and yield of cells from the gradients.

While these changes in the protocol greatly improved the separation of epithelial and fibroblast cells from mature animals, the length of the procedure (8-10 hours) was detrimental to the survival of the cells in culture. In particular it required that the cells be kept at 0°C in the Percoll/HBSS solutions for extended periods of time. The reproducibility and purity of separations and the yields of cells were too variable for

our needs, therefore we developed a simpler and more rapid step gradient isolation procedure.

Based on the banding of the fibroblast and epithelial cells within the density ranges of the two continuous Percoll gradients, a series of 5% and 10% Percoll density steps were assembled to separate the fibroblast and epithelial cells in a single centrifugation step without the need for pre-generated density gradients (Figure 6). Basal and non-secretory epithelial cells were isolated from fraction 6 (d=1.04-1.06 g/cm^3), secretory epithelial cells were found in fraction 7 (d=1.06-1.08 g/cm^3) and fibroblasts were harvested from fractions 1-3 (d=1.10-1.17 g/cm^3). The purified cell fractions were isolated with greater reproducibility and yield without sacrificing purity of separation. In addition, this method allowed for the gradient subfractionation of the epithelial cells into secretory and non-secretory epithelial cell populations. Cell type classification was based on morphology and buoyant density as described by Cooke and Littleton (Cooke and Littleton, 1985). The buoyant densities between our methods and that of Cooke differ slightly due to the difference in dilution of Percoll stock solutions (12.5:1 versus 11.5:1 respectively) to achieve iso-osmotic gradients (Vincent and Nadeau, 1984). Beyond the density differences the classification of cell types by light microscopy examination of morphology, secretory granules and lysosomal particles were all in agreement.

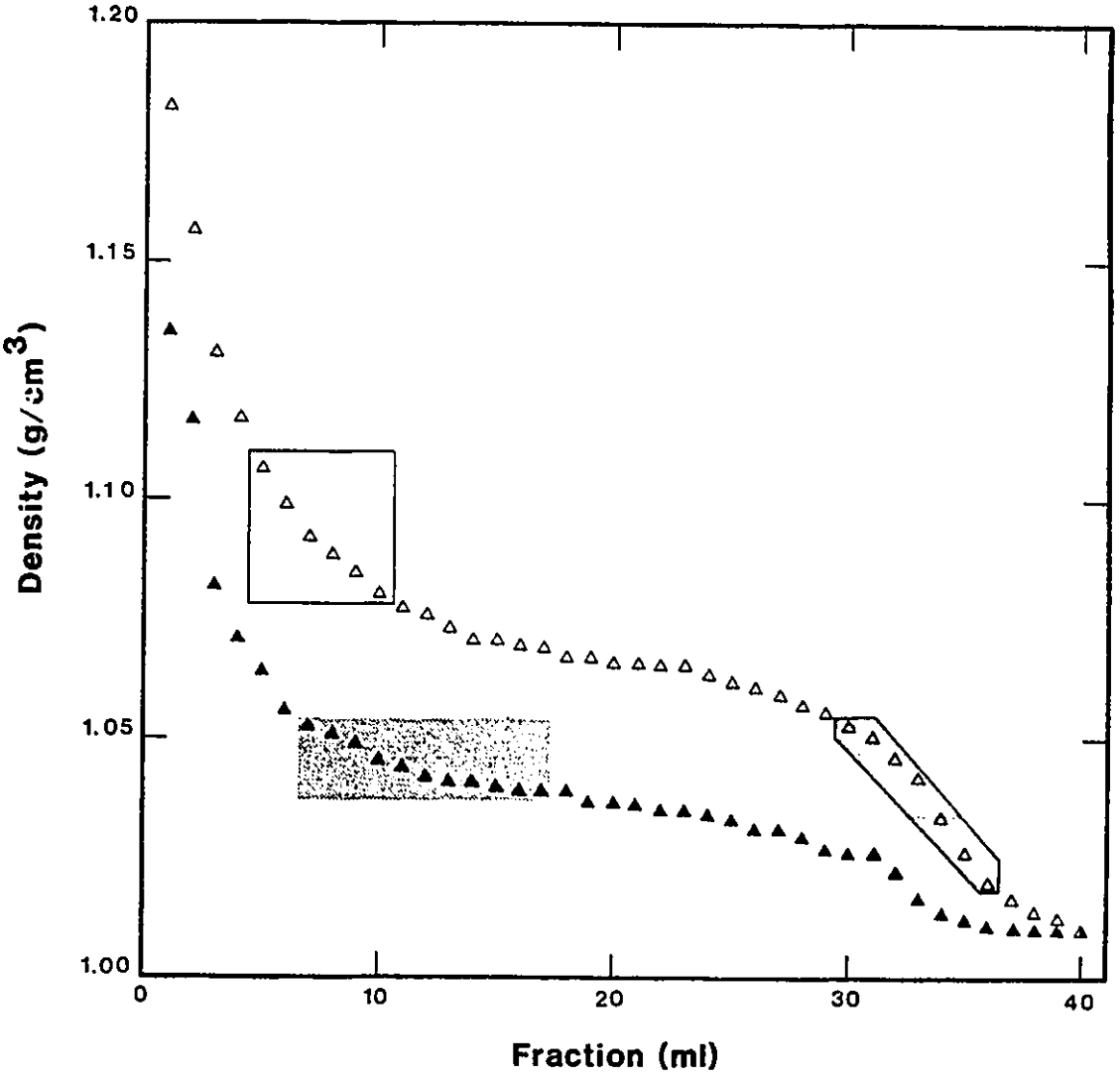
FIGURE 5: Density profile of continuous Percoll gradients

Density gradient solutions were prepared and the gradient generated by centrifugation for 1 h at 13,000 rpm, 4°C. Aliquots (1 ml) of the gradients were removed and the density determined by refractive index relative to standardized saline solutions.

△ = Fractions from first gradient (47% Percoll, 56% HBSS)
▲ = Fractions from second gradient (26% Percoll, 74% HBSS)

□ = Volume removed for fibroblast harvest
□ = Volume removed for application to second gradient
= Volume removed for epithelial cell harvest

Figure 5



Pure primary cultures of the epithelial cells and fibroblast cells were prepared from cells purified on the step gradient. On both collagen-coated and untreated polystyrene the primary cultures of epithelial cells (Figure 7, panel A) appeared as a characteristic cobblestone array of cuboidal cells with tight cell-cell interactions. The fibroblasts on the other hand were typically elongated cells which oriented themselves along an axis in culture (Figure 7, panel B).

When primary cultures of epithelial cells were grown on polystyrene, fibronectin-coated or collagen-coated plates for two or three weeks foci of very rapidly dividing cells developed in the cultures. The cells that formed the foci had morphologies distinct from either the normal epithelial cells (NE) or normal fibroblasts (NF) and were named Rapidly-Dividing Epithelial (RDE) cells (Figure 7, panel C).

The cells of the foci divided faster than the migratory rate of the cells which gave rise to multiple layers of cells at the centre of the focus. Without disaggregation the central area of the focus became necrotic as the thickness of the several layers of RDE cells exceeded the 1 mm permeability limit of nutrients and gases (Dr. J-M. Trifaro, Personal communication).

FIGURE 6: Density profiles of step Percoll gradients

The step gradient was prepared and centrifuged at 2500xg for 30 min at 4°C. One millilitre fractions were removed from the bottom of the tube and the density determined by refractive index as for the continuous gradient. Tubes with cells were run in parallel and the cell type and number were determined by morphology using a hemacytometer.

----- = Density profile in g/cm^3 (outer left vertical axis) and in % iso-ionic Percoll (inner left vertical axis).


= Fibroblast cell number.
= Non-secretory epithelial cell number.
= Secretory epithelial cell number.

Fractions to which these cell numbers refer are expressed on the lower horizontal axis, the corresponding absolute volumes are expressed on the upper horizontal axis.

Figure 6

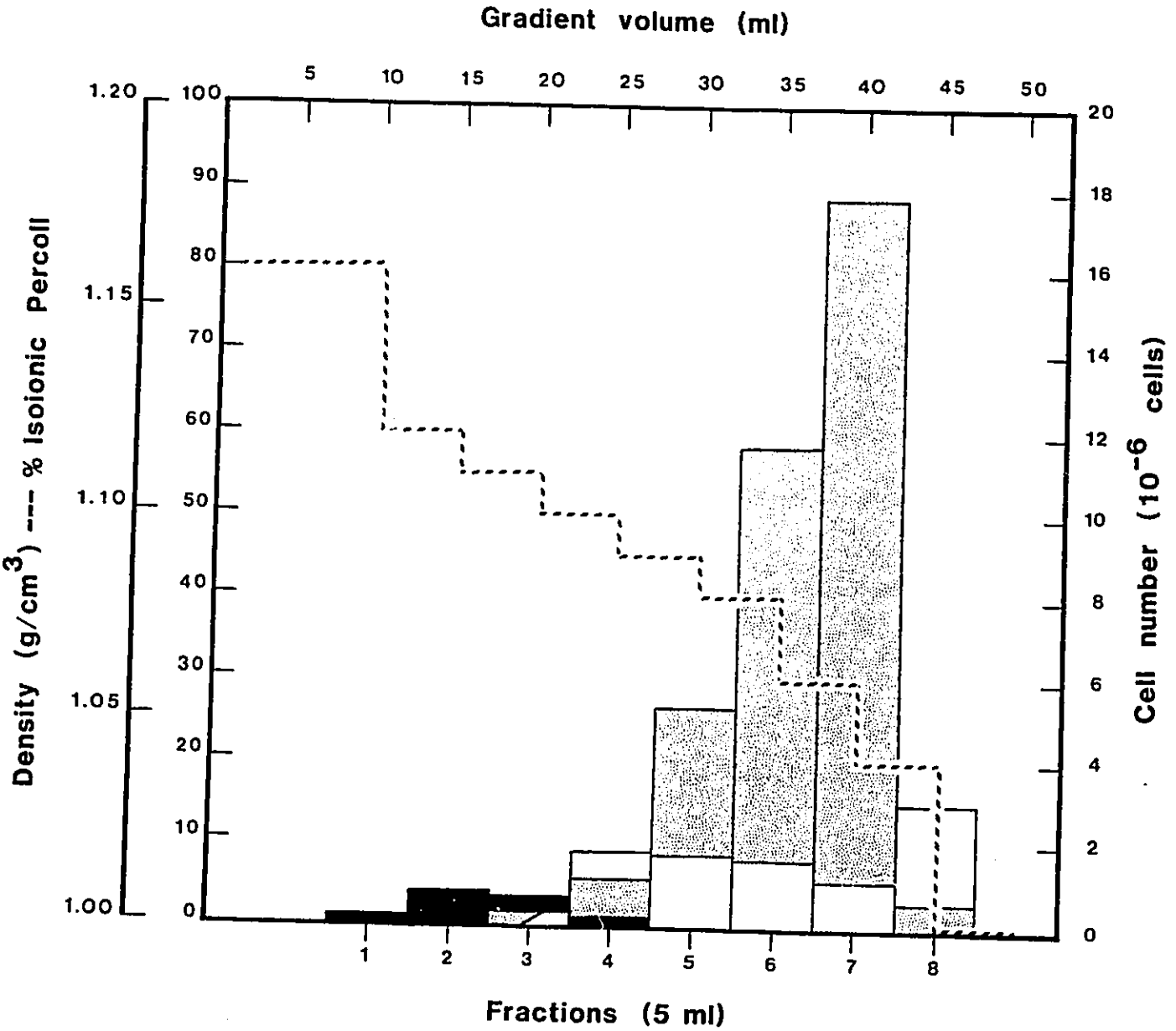
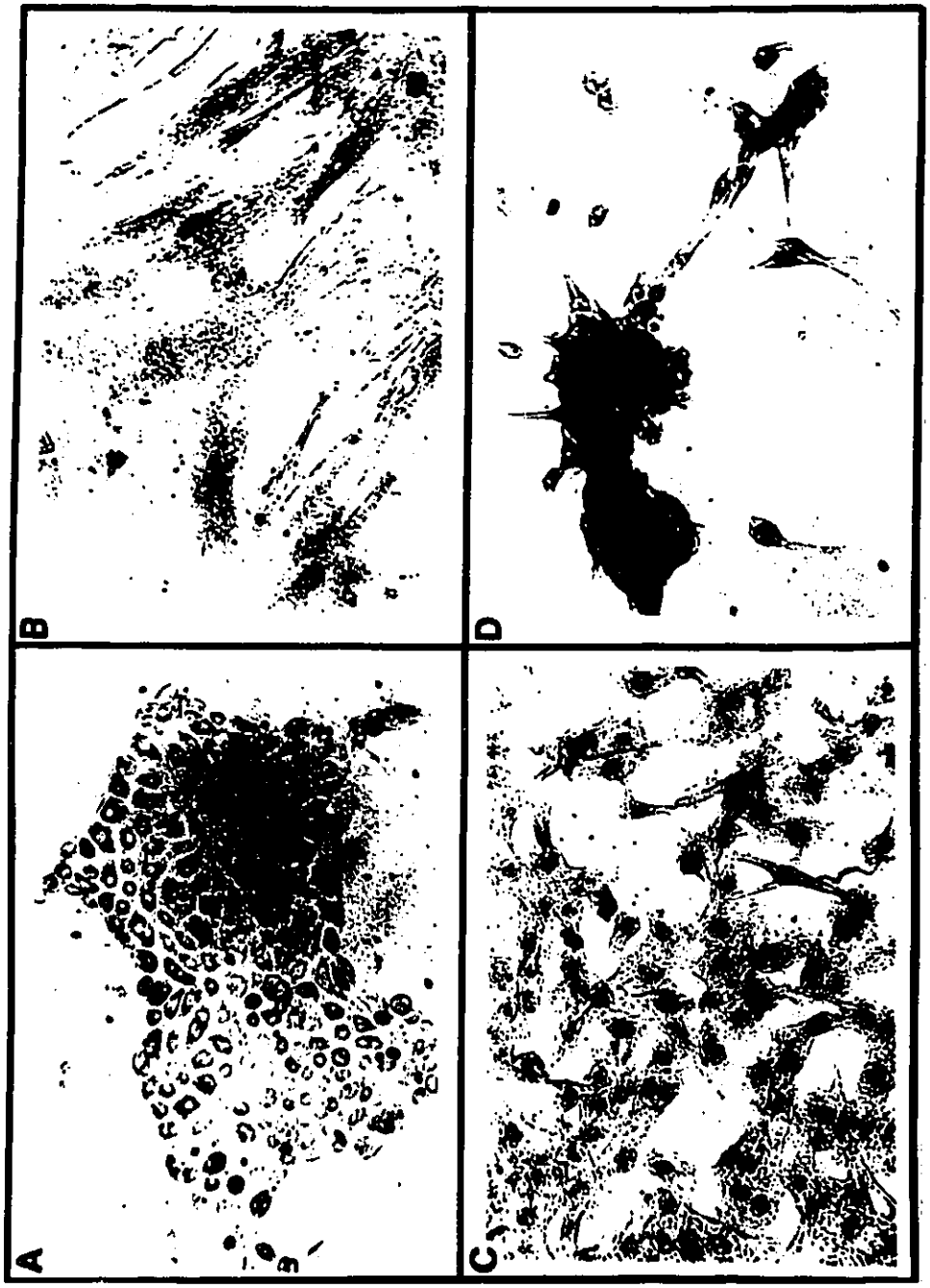

Gradient volume (ml)
5
10
15
20
25
30
35
40
45
50
1.20
1.15
1.10
1.05
1.00
100
90
80
70
60
50
40
30
20
10
0
Density (g/cm3) --- % Isoionic Percoll
20
18
16
14
12
10
8
6
4
2
0
Cell number (10-6 cells)
1
2
3
4
5
6
7
8
Fractions (5 ml)

FIGURE 7: Hematoxylin/eosin stained normal epithelial and fibroblast cells from rat ventral prostate and RDE cells grown on plastic or a collagen matrix.

Panel A: Primary monolayer culture of epithelial cells grown on plastic (x 80).
Panel B: Primary monolayer culture of fibroblasts grown on plastic (x 80).
Panel C: Primary colony of RDE cells grown on plastic (x 80).
Panel D: Subcultured RDE cells (1 passage) grown on a collagen matrix (x 80).

Figure 7



Foci of RDE cells from 4 separate culture preparations (3 from continuous gradients, 1 from step gradients), were individually trypsinized within cloning rings to obtain pure cultures of RDE cells. The trypsin digested foci were serially diluted and seeded into 96 well dishes. In this way a total of 43 separate clonally-selected cell lineages have been established.

When any of the RDE cell lines were grown on a collagen or fibronectin / collagen matrix a three-dimensional growth pattern was observed (Figure 7, panel D). When initially plated on the collagen matrix the single cells had an elongated shape with extensive cytoplasmic processes. As the cells divided, they clustered together, and formed spheres of cells, (containing 10-200 cells) which remained anchored to the culture vessel by 5-20 cells at a single anchorage site. This three dimensional growth pattern has not been observed in normal epithelial or fibroblast cell cultures grown on the same matrices.

In addition to the morphological differences, the RDE cell lines adhered much more rapidly to the culture vessels than normal androgen dependent prostatic epithelial cells.

TABLE II. EPITHELIAL CELL PLATING EFFICIENCIES

Substrate	Epithelial cells		Fibroblast cells	
	Serum +	Serum -	Serum +	Serum -
Polystyrene	≤10%	60%	70%	85%
Collagen type IV	≈20%	80%	≤ 5%	≤ 5%
Fibronectin	≈30%	>90%	70%	100%
Collagen + fibronectin	40%	>90%	30%	40%

Freshly isolated prostate epithelial and fibroblast cells were seeded in duplicate in 3 ml serum-free or serum-supplemented medium into 35 mm dishes as described in Materials. After 16 hours the medium was removed and the adherent cells harvested and counted. Values expressed are the percentage of cells adhering after 16 hours relative to the number of cells initially plated.

The normal epithelial cells did not adhere well to untreated plastic or glass culture surfaces. They required the presence of one or more of the following: fibronectin, collagen (either thick or thin layers) or poly-lysine, to achieve greater than 5% adherence. Prostate fibroblasts had considerably different attachment requirements; they did not require, but would attach to fibronectin or poly-L-lysine coated surfaces in addition to naked glass and polystyrene. Collagen inhibited the attachment of fibroblasts to a considerable degree. Primary cultures of the normal epithelial cells (0-2 passages) required the presence of exogenous fibronectin and/or collagen for attachment within 12 hours (Table II). The RDE cells on the other hand

adhered almost quantitatively to all surfaces, even bare plastic, within 30 minutes either with or without serum in the medium.

2) RDE Androgen-independence for growth

Four of the 43 RDE lines were chosen randomly for further characterization. Figure 8 demonstrates the effects of testosterone supplemented growth medium on normal epithelial and 4 RDE cell lines. Normal primary epithelial cells (NE) grown in the presence of 34nM testosterone proliferated slowly with a doubling time of approximately 70 hours. In the absence of testosterone the normal epithelial cells rounded up and lifted off the culture vessels (50% decrease in cell number in 60 hours), demonstrating the absolute requirement of androgens for both growth and maintenance of normal rat ventral prostate epithelial cells. RDE cells on the other hand displayed virtually identical growth curves in the presence or absence of androgens in the growth medium. Table III summarizes the calculated doubling times for each of the four cell lines and for primary cell cultures of normal androgen dependent prostatic epithelial cells. As predicted by the kinetics demonstrated in figure 8, the presence or absence of testosterone did not alter the doubling times for any of the RDE cell lines but dramatically affected the doubling time of the androgen dependent epithelial cell cultures.

TABLE III. DOUBLING TIMES OF RDE CELL LINES AND NORMAL EPITHELIAL CELLS IN THE PRESENCE AND ABSENCE OF TESTOSTERONE

Cell Line	Doubling Times (hours)	
	(T^+)	(T^-)
RDE 11.1	5.6	7.2
RDE 11.4	8.0	9.6
RDE 11.5	9.6	9.6
RDE 11.12	12.0	12.0
Mean RDE doubling time	8.8	9.6
NE	70	(-)60.0*

* Time to lose 50% of initial population

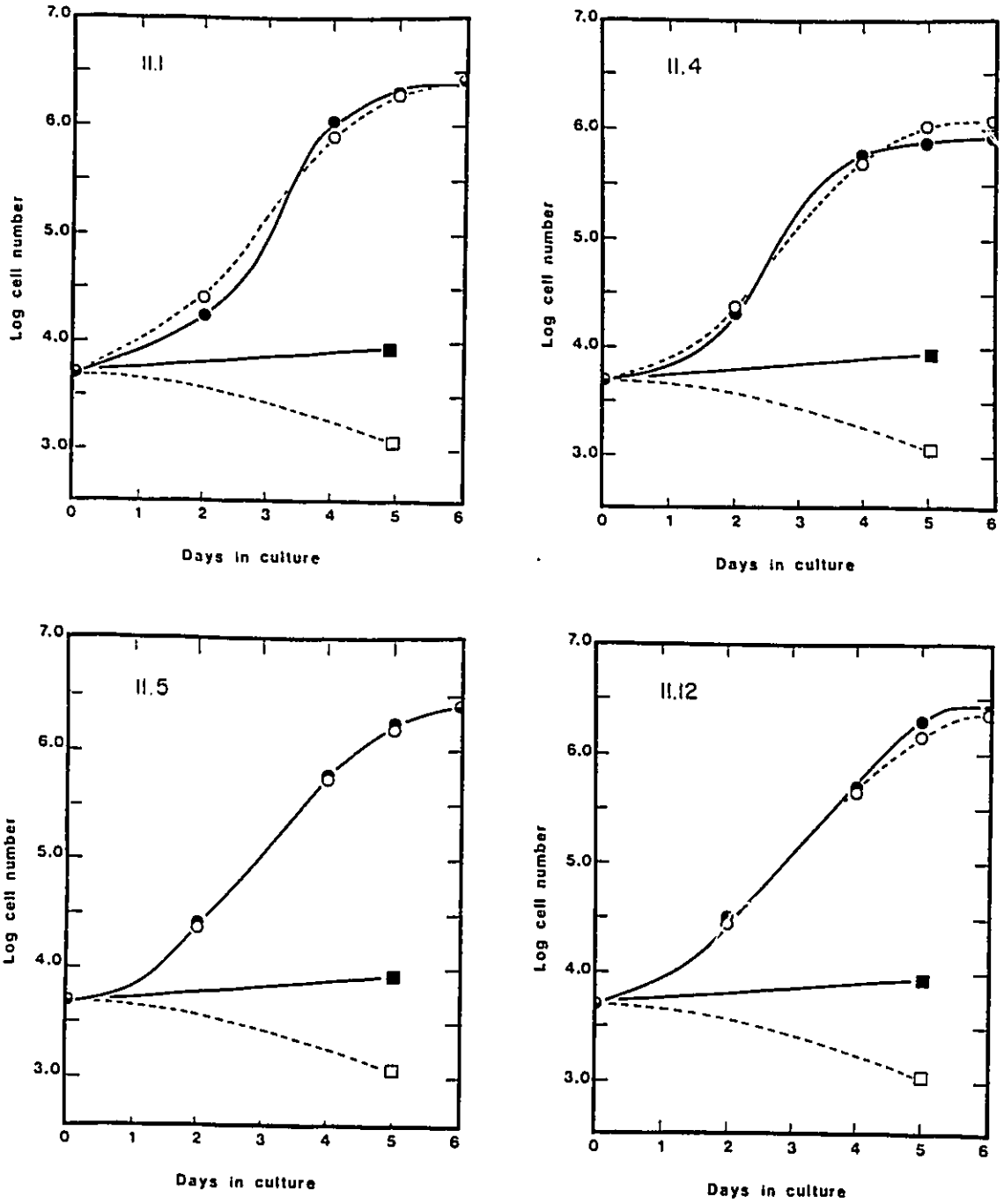
$5x10^3$ RDE or normal primary epithelial (NE) cells were plated onto plastic or fibronectin coated plates (in the case of normal epithelial cells) and cultured in the presence and absence of testosterone. On days 0, 2, 4 and 5 the adherent cells were harvested and counted on a model Z_B Coulter Counter. Doubling times are defined as the time required to double cell numbers during the linear phase of logarithmic growth or decline, calculated using the cell numbers measured on days 2 and 4.

While we have not determined if androgens are required to isolate RDE cells Miesfeld has found that isolation of RDE-like cells, using the protocols described herein, is unaffected by the absence of androgens. Under androgen-free conditions the epithelial cell population dies within 4 days and the RDE-like cells appear as foci shortly thereafter (R. Miesfeld, personal communication).

FIGURE 8: Growth kinetics of normal primary epithelial and RDE cells in the presence and absence of 34nm testosterone.

$5x10^3$ RDE cells (RDE cell lines 11.1, 11.4, 11.5, 11.12) were seeded in 60 mm diameter culture dishes and cultured in the presence (●) or absence (○) of testosterone in growth medium. On days 0, 2, 4, and 5 attached cells were harvested by trypsinization and counted using a model Z_B Coulter Counter. Each point represents the average of triplicate determinations of each of 3 plates/day. Control cultures of normal primary epithelial cells were also cultured in the presence (■) or absence (□) of testosterone. All cells were initially seeded in plating medium containing 34nM testosterone. After 16 hours, growth medium (with or without testosterone as indicated) was substituted. The growth medium was replenished every 48 hours therafter. Day 0 refers to the time of change from plating to growth medium.

Figure 8


II.1
II.4
II.5
II.12
Log cell number
Days in culture
7.0
6.0
5.0
4.0
3.0
0
1
2
3
4
5
6

3) Immunohistochemistry

To demonstrate that the RDE cells are of epithelial origin despite their rapid growth rate and androgen independence we analyzed the cytoskeletal protein content of the cells by immunohistochemistry. Normal epithelial and normal fibroblast cells were isolated by step density gradient. Both normal and RDE cells were cultured on fibronectin or poly-L-lysine coated glass coverslips in T^+ medium before being fixed in -20°C acetone and processed for immunohistochemical analysis (Table IV).

TABLE IV. IMMUNOHISTOCHEMICAL ANALYSIS OF RDE, NORMAL EPITHELIAL AND NORMAL FIBROBLAST CELLS

Antibody Specificity	Result BE*	LE	RDE	NF
EGF-R	ND	+	+	+
PKK1 (cytokeratins 8, 18, 19)	-	+	+	-
PKK2 (cytokeratins 7, 16, 17, 19)	+	-	-	-
PKK3 (cytokeratin 18)	-	+	+	-
EAB903 (cytokeratins 1, 5, 10, 11)	+	-	+	-

Freshly isolated luminal epithelial (LE) and normal fibroblast (NF) cells (cultured on fibronectin coated glass coverslips) and RDE cells cultured on poly-L-lysine coated glass coverslips in T^+ growth medium were fixed to the coverslips with acetone (-20°C, 10 minutes). Primary and peroxidase-linked secondary antibody incubations were performed for 1 h in 37°C humidity saturated environments prior to reaction with 4-chloro-naphthol. Control samples were incubated with secondary antibody alone and were all negative.

* Results for basal epithelial cells (BE) were taken from Rouleau et al., 1990.

The expression of cytokeratins 8 and 18 in the RDE cells indicates that the RDE cells are of luminal epithelial origin while the staining with EAB 903 suggests that these cells are of basal epithelial cell origin (Rouleau et al., 1990). These results reflect other reports describing the co-expression of both basal and luminal cytokeratins in a small population of epithelial cells (Verhagen et al., 1988) restricted to the proximal region of the prostatic ducts (Rouleau et al., 1990).

4) Metabolism of Testosterone

One possible explanation for the inability of the RDE cell lines to respond to androgens may be that these cells are unable to metabolize testosterone to the active androgen, 5α-dihydrotestosterone (5α-DHT). To determine if the RDE cells are capable of metabolizing the testosterone supplied in the medium we cultured the cells in testosterone-free medium supplemented with radiolabeled testosterone. HPLC radiodetector analysis of the metabolism of [^{3}H]-testosterone (Figure 9 and table V) illustrates that RDE cells metabolized testosterone into the active androgen, 5α-DHT (retention time 36 min.) and subsequently to both 3α- and 3ß-androstanediol (retention times 52 and 28 minutes respectively). Absent from the metabolism of testosterone was 5α-androstanedione (corresponding to peak 4).

Analysis of earlier time points (results not shown) suggested that Δ^4-5α-reductase, the enzyme responsible for the conversion of testosterone to 5α-DHT, was probably not the rate limiting enzyme in the metabolic pathway since considerable amounts of 5α-DHT were found in both the 4 and 8 hour metabolism assays.

TABLE V. 24-HOUR METABOLISM OF TESTOSTERONE BY RDE CELLS

	DPM RECOVERED				
RDE cells	T	5α-DHT	3α-Adiol	3ß-Adiol	Δ^4-Adione
11.1 *	367,900	46,360	10,360	36,320	10,120
11.4 *	502,880	78,720	ND	75,440	27,360
11.5 *	500,360	43,680	ND	39,680	18,760
Average % total dpm recovered	78.2	9.53	0.73	8.44	3.08

ND = None detected

5α-DHT = 5α-dihydrotestosterone
3α-Adiol = 3α-androstanediol
3ß-Adiol = 3ß-androstanediol
Δ^4-Adione = Δ^4-androstenedione

[^{3}H]-testosterone was added to RDE cells in T$^-$ medium. After 24 hours, the medium was removed and ^{14}C standards added before the medium was extracted with methylene chloride. HPLC radiodetector analysis indicates the relative abundance of ^{3}H sample metabolites after correction for lessened background signals.

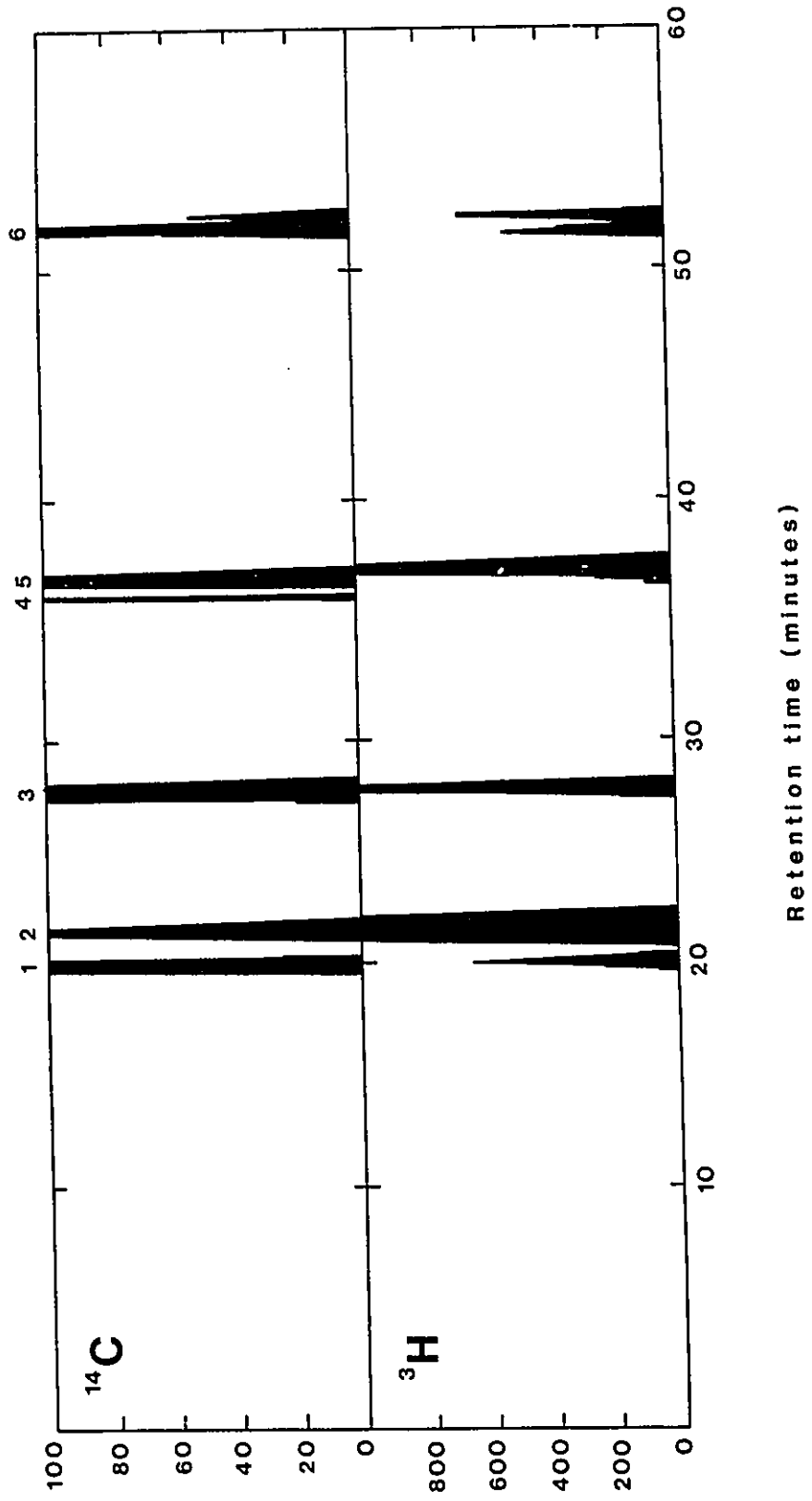
FIGURE 9: Metabolism of [^{3}H]-Testosterone by RDE Cells.

[^{3}H]-testosterone ($8x10^5$ dpm) was added to RDE cells in T- medium. After 24 hours, the medium was removed and ^{14}C standards added before the medium was extracted with methylene chloride. The tracing of the HPLC detector analysis indicates the relative abundance of ^{3}H sample metabolites and ^{14}C standards on two different scales after correction for background signals.

^{14}C standard peaks:

1 Δ^4-androstenedione
2 Testosterone
3 3ß-androstanediol
4 5α-androstanedione
5 5α-dihydrotestosterone
6 3α-androstanediol

Figure 9


^{14}C
^{3}H
100
80
60
40
20
0
800
600
400
200
0
1 2
3
4 5
6
10
20
30
40
50
60
Retention time (minutes)

5) Androgen Dependent Marker Proteins

To determine whether the RDE cell lines express androgen dependent genes, two markers of prostate function were examined. Acid phosphatase activity (Tenniswood et al, 1976) and the level of PSBP mRNA (Parker et al, 1980) are known to be androgen dependent. The isoelectric-focusing pattern of acid phosphatase activity isolated from each of the RDE cell lines is shown in figure 10. Lanes 1 and 6 represent the normal pattern of acid phosphatase activity found in homogenates of intact rat ventral prostate. Lysosomal acid phosphatase, which is androgen independent, has a pI of approximately 7.3; the pI of the androgen dependent secretory acid phosphatase is 5.0-5.8, while the androgen sensitive acid phosphatase has a pI of 6.3 (Downey et al, 1983). These same patterns have been observed in the normal androgen dependent primary cell cultures (Orlowski and Clark, 1986). In contrast to these controls, the acid phosphatase in the RDE cell lines appeared to have two active forms: the major component with a pI of 7.6-8.5, and minor components with pIs in the range 4.4-4.6. There were also minor qualitative differences in the isoelectric focusing profiles between the RDE cell lines (band at pI 4.8 in lanes 2 and 5 only), however the significance of these differences is not known. These profiles are unaltered when the RDE lines were grown in the absence of androgens (results not shown), suggesting that these forms of acid phosphatase are not androgen dependent, at least in the RDE cell lines.

Figure 11 is a Northern biot of RNA isolated from the RDE cell lines, probed with [^{32}P]-labelled pA34, a plasmid specific for the C3 component of the prostate steroid-binding protein (PSBP) (Parker et al., 1980). PSBP is known to be an androgen dependent protein synthesized by the secretory epithelial cells of the rat ventral prostate (Heyns and De Moor, 1977). Lanes 1-3 show the hybridization of the probe to poly(A)$^+$ RNA from the ventral prostate of intact rats (lane 1), and rats castrated 4 and 11 days previously (lanes 2 and 3 respectively). The decrease in signal from lane 1 to lane 3 reflects the decline in the abundance of PSBP as the level of circulating androgens decreases following castration. Lane 4 acted as an internal negative hybridization control. By comparison to these controls, it is clear that the RDE cell lines (lanes 5-8), grown to confluence in the presence of testosterone, did not express the C3 PSBP gene. No signal was found even after prolonged overexposure of the autoradiogram.

To determine whether the lack of androgen stimulation of PSBP gene expression grown in the presence and absence of testosterone was a general phenomenon we examined the in-vivo synthesis of proteins in the RDE cell lines, and the in-vitro translation products of the poly(A)$^+$ RNA extracted from the cells grown in the presence and absence of testosterone. Figure 12, panel A, shows the in-vitro translation products of poly(A)$^+$ RNA from an intact rat ventral prostate (lane 1), and poly(A)$^+$ RNA extracted from RDE 11.1 grown to confluence in the presence (lane

FIGURE 10: Iso-electric focusing profiles of acid phosphatase from RDE cell lines.

Tissue and cell homogenates (equal volumes of approximately equal numbers of normal epithelial and RDE cells) were subjected to isoelectric focusing in 7% polyacrylamide gels over a pH range of 4-9. The gels were run at a constant power of 22 W for 2 hours at a temperature of 0-4°C. Acid phosphatase activity was localized by incubating the gel in 7.4 mM α-naphthylphosphate and Fast Garnet GBC.

Lane 1: homogenate of intact rat ventral prostate.
Lane 2: homogenate of RDE 11.1.
Lane 3: homogenate of RDE 11.4.
Lane 4: homogenate of RDE 11.5.
Lane 5: homogenate of RDE 11.12.
Lane 6: homogenate of intact rat ventral prostate.

Figure 10

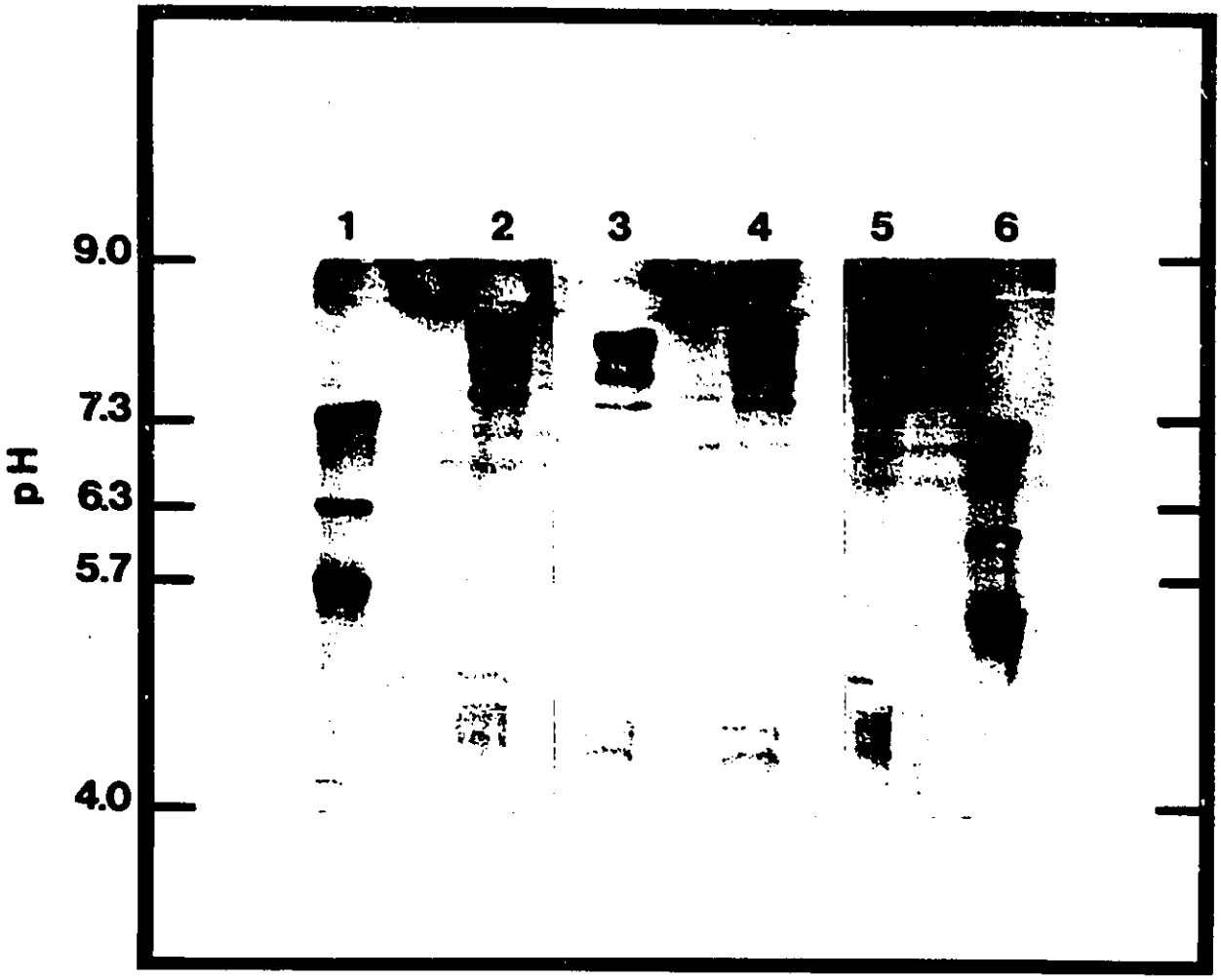
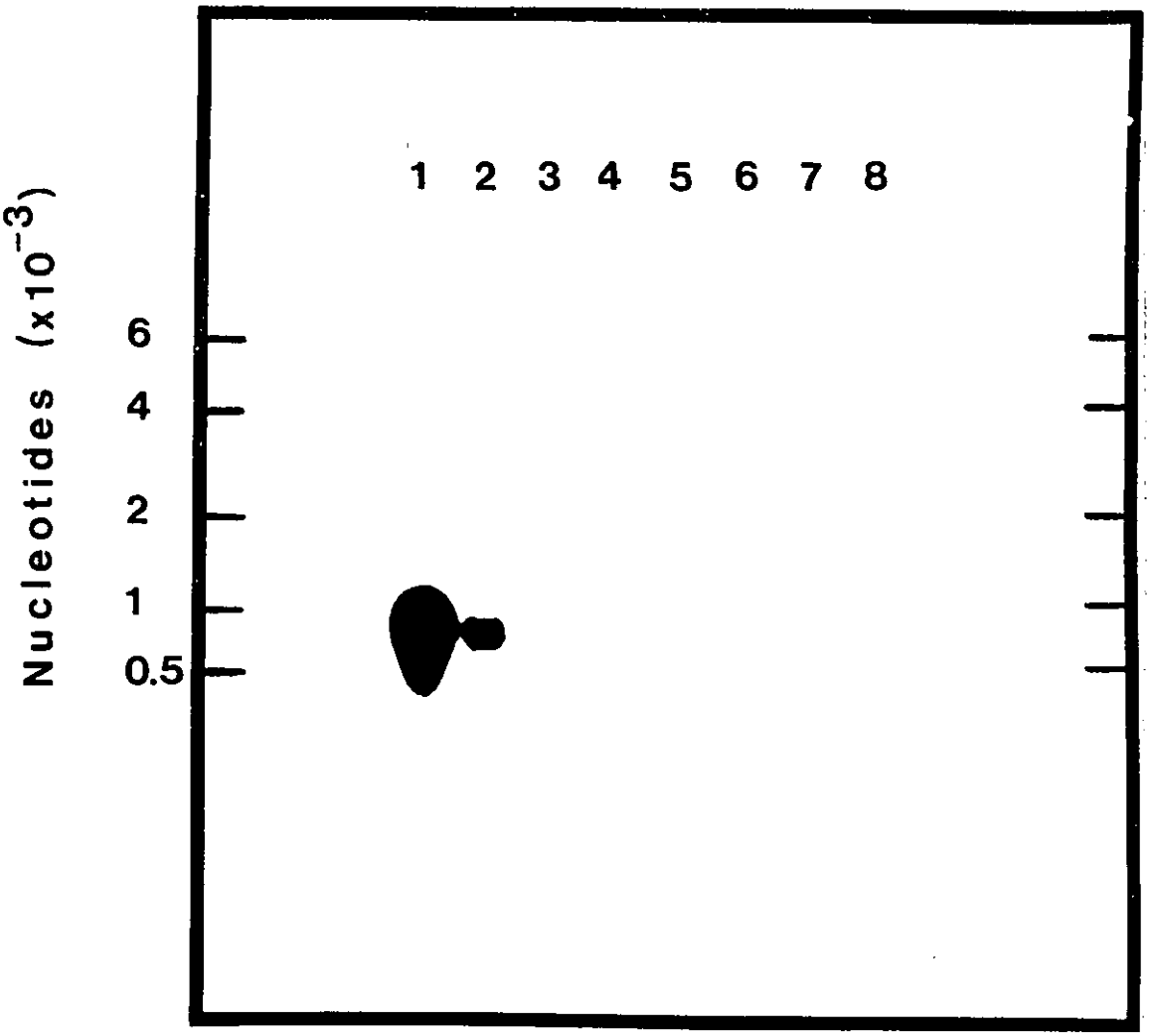


FIGURE 11: Northern hybridization analysis of RNA from RDE cells.

Poly(A)$^+$ RNA (3 μg/lane) was denatured with DMSO/glyoxal, electrophoresed through a 1.5% agarose gel and transferred to a nylon filter. The filter was hybridized to [^{32}P]-pA34, the cDNA probe specific for prostate steroid-binding protein C3 subunit (200 ng probe; specific activity 30×10^6 cpm/μg) The autoradiogram was exposed for 96 h at -70°C.

Lane 1: poly(A)$^+$ RNA from the prostate of intact rats
Lane 2: poly(A)$^+$ RNA from the prostate of rats castrated 4 days prior to RNA isolation
Lane 3: poly(A)$^+$ RNA from the prostate of rats castrated 11 days prior to RNA isolation
Lane 4: poly(A)$^-$ RNA from the prostate of intact rats
Lane 5: poly(A)$^+$ RNA from RDE 11.1 (grown in the T$^+$ medium)
Lane 6: poly(A)$^+$ RNA from RDE 11.4 (grown in the T$^+$ medium)
Lane 7: poly(A)$^+$ RNA from RDE 11.5 (grown in the T$^+$ medium)
Lane 8: poly(A)$^+$ RNA from RDE 11.12 (grown in the T$^+$ medium)

Figure 11



2) and absence (lane 3) of androgens. There are several striking differences between the translation profiles of the RNA from the intact prostate and those from the RDE cells. In particular, the PSBP proteins, which constitute the most abundant proteins in the epithelial cells of the prostate, were absent in the in-vitro translation products of the RDE cell line shown here even in the continued presence of testosterone. There were a number of other differences between the translation products of the control and the RDE cell lines. Comparison of lanes 2 and 3 shows that the translation profiles of the RDE cell lines are not altered significantly when androgens are omitted from the medium. Similar profiles were found with other RDE cell lines, in particular RDE 11.4, 11.5 and 11.12. Figure 12, panel B also demonstrates that there were virtually no differences between the proteins synthesized by the RDE cell lines in-vivo in the presence (lanes 1 and 3) and absence (lanes 2 and 4) of androgens. The differences in the in-vitro translation (Panel A) and in-vivo labeling (Panel B) profiles were probably due to the differences in translational efficiency of individual mRNAs in-vitro and in-vivo, and/or in-vitro modifications such as glycosylation, sulfation and proteolytic cleavage.

FIGURE 12: Autoradiograph of SDS-15% Polyacrylamide gel electrophoresis of [^{35}S]-methionine labeled translation products and in-vivo labeled products.

Panel A: In-vitro translations:

Samples of poly(A)$^+$ RNA (1 μg) were incubated in 25 μl micrococcal nuclease-treated rabbit reticulocyte lysate and the translation products analyzed as described in Methods. A constant volume of translation mix (90,000-130,000 cpm/well) was loaded into each well. The dried gel was fluorographed for 48 hours. Arrows indicate the position of the subunits of prostate steroid binding protein.

Lane 1: RNA from normal, androgen dependent rat ventral prostate
Lane 2: RNA from RDE 11.1 grown in T$^+$ medium.
Lane 3: RNA from RDE 11.1 grown in T$^-$ medium
Lane 4: Translation blank (no exogenous RNA)

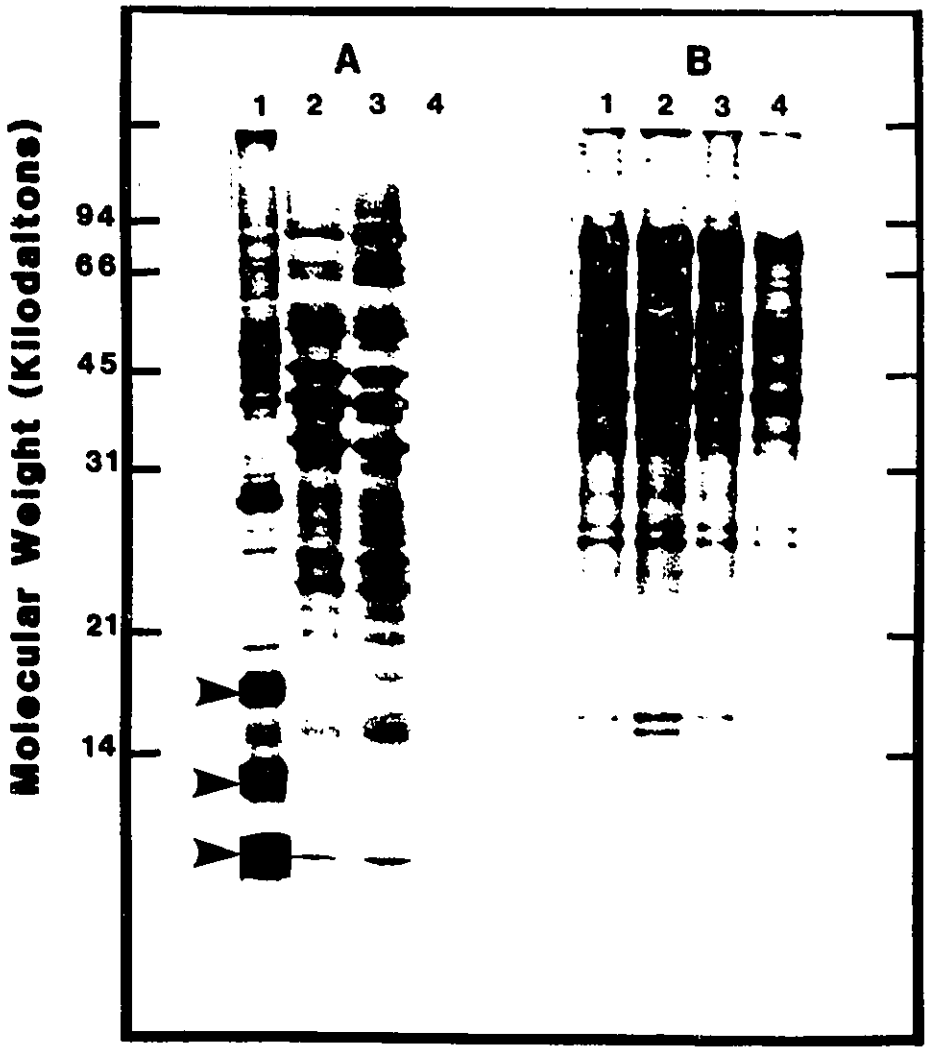
Note: This is a composite photograph of separate lanes from the same gel.

Panel B: In-vivo labelling of RDE cells.

10^6-10^7 cells were incubated for 24 hours in 5 ml of methionine and serum free medium supplemented with 50 μCi [^{35}S]-methionine (1200 Ci/mmol). After incubation, the cells were harvested, lysed and analyzed as described in Methods. Each lane contained 10^5 cpm of cell lysate. The dried gel was exposed for 48 hours.

Lane 1: RDE 11.1 cell line; T$^+$ medium
Lane 2: RDE 11.1 cell line; T$^-$ medium
Lane 3: RDE 11.12 cell line; T$^+$ medium
Lane 4: RDE 11.12 cell line; T$^-$ medium

Figure 12



6) Extracellular matrix effects on RDE cells

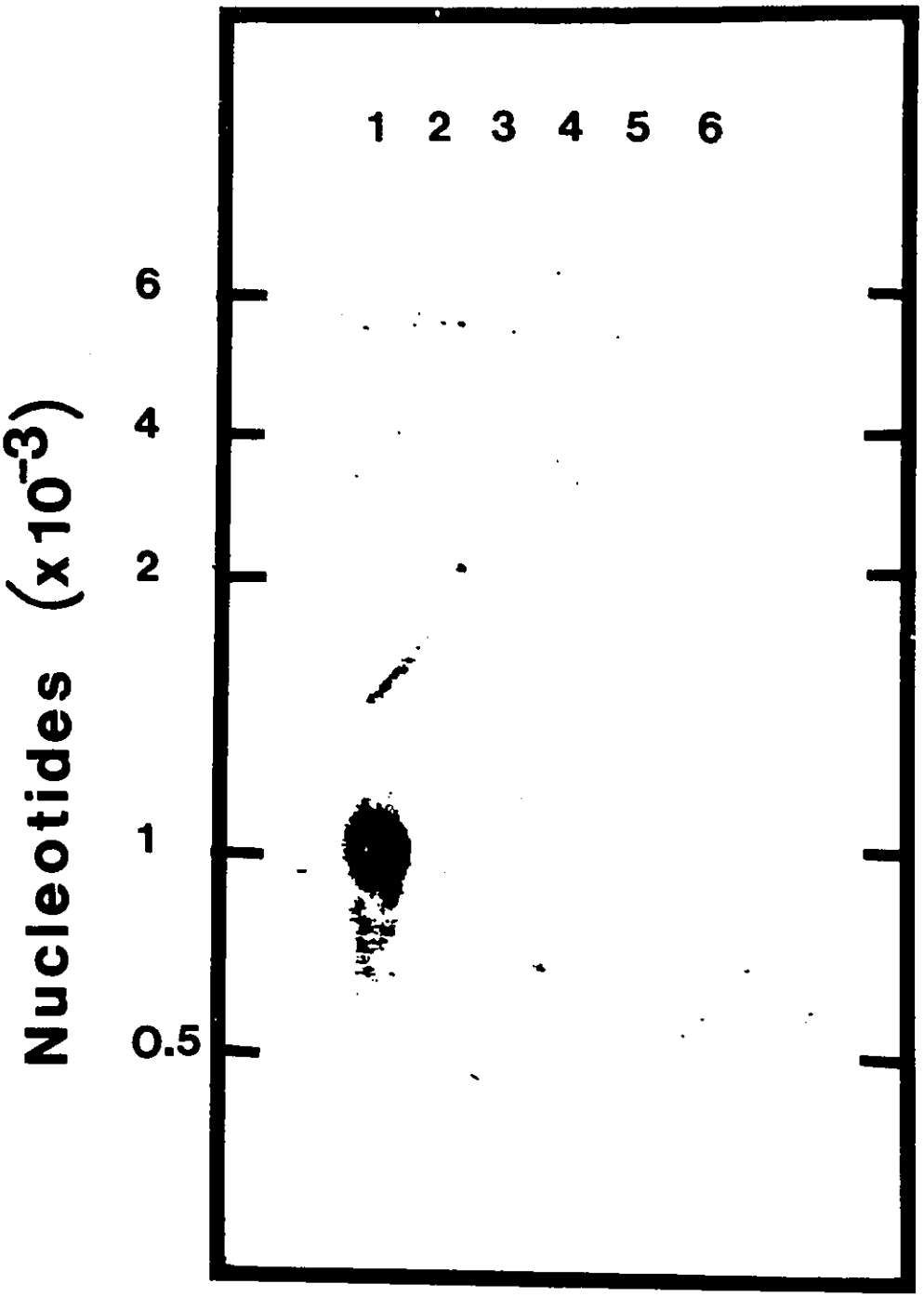
RDE cells grown on collagen adopted 3-dimensional growth patterns reminiscent of prostatic acini (Figure 7, panel D). Similar morphological effects have been reported (O'Connor and Sinha, 1985; Kawamura and Ichihara, 1987). The similarity of the resulting morphology raised the possibility that components of the extracellular matrix (ECM) might induce the RDE cells to differentiate. Figure 13 shows the Northern blot of RNA from purified epithelial cells cultured 4 days in T^+ medium (lane 1) and from RDE cells grown on plastic (lanes 2 and 3), on normal fibroblasts embedded in a collagen matrix (lane 4), or on collagen matrices in the presence (lane 5) or absence of testosterone (lane 6). The filter was hybridized with [^{32}P]-labelled pA34, the clone specific for the C3 subunit of PSBP. The PSBP mRNA was abundant in the RNA from the normal epithelial cells (lane 1), while when the RDE cell lines were grown on plastic they formed a monolayer but did not synthesize PSBP mRNA either in the presence or absence of testosterone. PSBP mRNA sequences were also absent in the RDE cell lines grown on collagen or on prostate fibroblasts embedded in collagen even though greater than 90% of the cells were organized into three dimensional dome structures.

Figure 13: Northern Hybridization Analysis of RNA from RDE Cells Grown on Different Substrata.

Total RNA (5 μg/lane) was denatured with DMSO/glyoxal and electrophoresed through a 1.5% agarose gel and transferred to a nylon filter. The filter was hybridized to [^{32}P]-pA34, the cDNA probe specific for the C3 subunit of the prostate steroid-binding proteins (200 ng probe; specific activity 2.8×10^8 cpm/μg) the autoradiogram was exposed for 120 h at -70°C.

Lane 1: RNA from normal epithelial cells (4 days post-plating, T^+growth medium)
Lane 2: RNA from RDE 11.1 grown on plastic in T^+ medium
Lane 3: RNA from RDE 11.1 grown on plastic in T^- medium
Lane 4: RNA from RDE 11.1 grown on a collagen/fibroblast matrix in T^+ medium
Lane 5: RNA from RDE 11.1 grown on a collagen matrix in T^+ medium
Lane 6: RNA from RDE 11.1 grown on a collagen matrix in T^- medium.

Figure 13



The lack of PSBP induction in RDE cells by collagen or entrapped stromal cells indicated that the 3-dimensional growth of RDE cells on the ECM components did not represent terminal differentiation of the RDE cells, as had been shown in other RVP epithelial cells with the induction of acid phosphatase in the collagen gel cultures of O'Connor and Sinha (1985).

The possibility that the formation of domes, spheres and mounds represented an earlier stage of differentiation was investigated by analyzing the ECM effects on growth rates and RNA synthesis. The results of [^{3}H]-thymidine and [^{3}H]-uridine incorporation assays on RDE cells grown on a variety of ECM components are shown in figure 14. Thymidine incorporation is a reflection of the rate of cellular DNA synthesis and hence cell division. RDE cells grown on bare plastic, fibronectin or laminin maintain a relatively constant rate of DNA synthesis. Growth of RDE cells on a fixed layer of human collagen (CH) or rat tail collagen (CR) (mostly collagen IV) occured at approximately 1.4 times the rate of those grown on bare plastic.

While growth of RDE cells on collagen-based ECM resulted in an increase in [^{3}H]-thymidine incorporation the amounts of [^{3}H]-uridine incorporated declined on all of the ECM surfaces when compared to polystyrene alone. Again the differences between plastic, fibronectin and laminin surfaces were minimal while the collagens reduced uridine incorporation by approximately 25%.

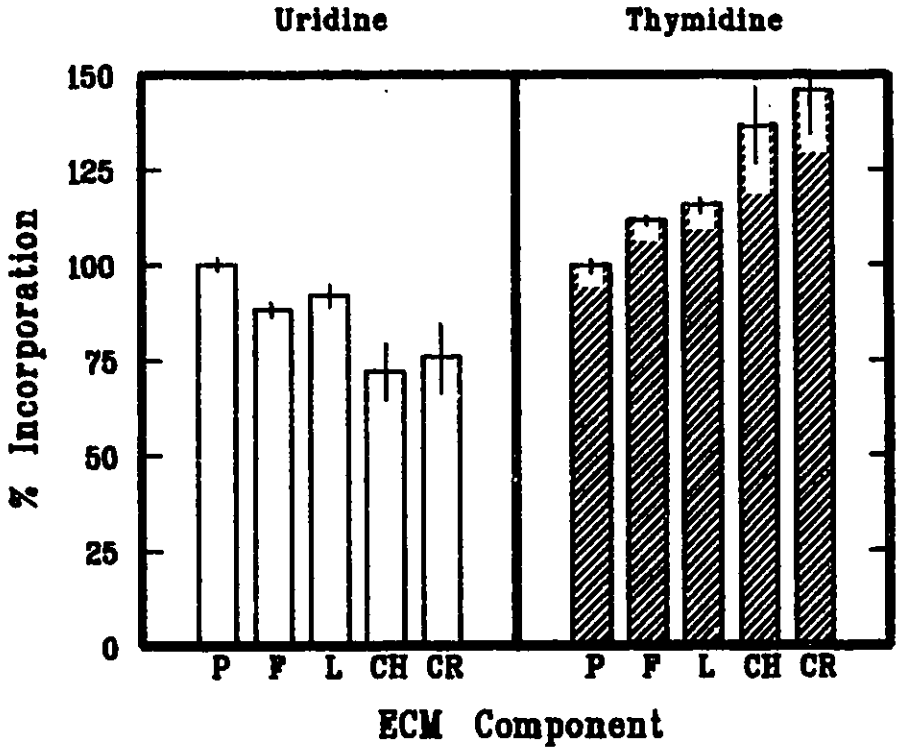
Figure 14: [^{3}H]-thymidine and [^{3}H]-uridine incorporation by RDE cells grown on various substrata.

Duplicates of 25,000 cells were plated in T^+ growth medium onto plastic plates with various ECM components. After 36 hours of culture 10 μCi of [^{3}H]-uridine or 10 μCi [^{3}H]-thymidine was added to the culture flasks. The cells were incubated a further 2 hours before the medium was removed and the cells were washed twice with HBSS. The cells were harvested by trypsinization and cell numbers determined before TCA precipitation and liquid scintillation counting.

Values are expressed as the mean ± the range of the percentage of radioactivity incorporated by RDE cells grown on bare plastic. This percentage has been corrected for cell number variations.

P	Bare plastic dishes
L	Laminin supplemented medium (5 μg/ml)
F	Fibronectin coated
CH	Human collagen
CR	Rat tail collagen

Figure 14


Uridine
Thymidine
150
125
100
75
50
25
0
% Incorporation
P F L CH CR
P F L CH CR
ECM Component

7) Growth factor and growth inhibitor effects

It has been reported that epidermal growth factor (EGF) and an unknown pituitary factor are required for in-vitro division of androgen-independent RVP epithelial cells (McKeehan et al., 1984). Recently a number of groups have isolated growth factors, collectively termed "prostatrophins", (Crabb, Armes, Johnson and McKeehan, 1986a). Of these, one has been purified (Crabb, Armes, Johnson, Roberts, Bordoli and McKeehan, 1986b) and has been shown to be homologous to acidic fibroblast growth factor (aFGF).

We have analyzed the exogenous growth factor requirement of RDE cells for division. We have also determined whether or not the addition of exogenous growth factors increased the already rapid rate of RDE cell division. Duplicate dishes of 50,000 RDE cells were plated in growth medium supplemented with maximal stimulatory doses (according to manufacturers) of mouse epidermal growth factor (EGF), bovine acidic fibroblast growth factor (FGF), bovine pituitary extract (BPE), bovine endothelial cell growth factor (ECGF) or human platelet derived growth factor (hPDGF). Cells were cultured in both the control medium and control medium supplemented with growth factor for 2, 3 or 4 days. The medium was replenished on day 3. As shown in table VI, at the concentrations used, none of the growth factors with the possible exception of BPE, increased the rate of RDE cell division beyond

the 25% change that we consider to be biologically significant. But this does not rule out that smaller changes may be biologically significant.

Table VI. GROWTH FACTOR EFFECTS

Medium supplement	Conc.	Cell number (% of T^+ control) 48 hours	72 hours	96 hours
T^+	34nM	100	100	100
T^-		116	113	100
BPE	50 μg/ml	116	105	125
ECGF	4 ng/ml	52	84	98
EGF	50 ng/ml	109	99	113
FGF	100 ng/ml	63	69	88
hPDGF	1 U/ml	98	100	109

BPE = Bovine pituitary extract
ECGF = Endothelial cell growth factor
EGF = Epidermal growth factor
FGF = Fibroblast growth factor
hPDGF = Human platelet derived growth factor

50,000 RDE cells were plated in growth medium supplemented with fully stimulatory doses of the indicated hormone or growth factor. The cells were cultured for 2, 3 or 4 days with medium replacement on the third day. The cells were harvested and cell numbers were determined by Coulter counter. Unless otherwise indicated, all cultures contained 34nM testosterone. Values are expressed as a percentage of the cell numbers obtained for cells grown on plastic in T^+ medium.

Whether the lack of EGF stimulation despite the presence of receptor is an established characteristic of RDE cells or the result of a defect or other blockage in the activation pathway has not been pursued.

RDE cells cultured in reduced-serum derivatives of growth medium showed a decline in growth rate as serum concentrations fell below 10 percent. Serum-free cultures continued to grow at approximately 20% of the rate found with complete growth medium. The presence or absence of androgens and/or EGF and/or cholera toxin in these studies did not influence the rate of replication (results not shown). Reduction or elimination of serum from the growth medium greatly increased the extent and complexity of 3-dimensional growth.

Since the common growth factors did not appear to have an effect on the rate of RDE cell growth, the effects of several growth inhibitory factors including estradiol, transforming growth factor-ß1 (TGF-ß1) and tumor necrosis factor (TNF) on RDE cell growth were studied. These results are summarized in Table VII. Over the range of concentrations tested TGF-ß1 displayed no growth inhibitory activity, on the contrary a small stimulatory activity was noted. In the absence of actinomycin D, TNF also failed to slow RDE cell division. In the presence of actinomycin D the RDE cells died but this occurred at the same rate as cell death induced by actinomycin D alone (results not shown). It is interesting that even in the androgen-independent RDE cells

estradiol in the culture medium had a significant growth inhibitory activity at early time points but not thereafter, regardless of the replenishment of the medium at 72 hours.

Table VII GROWTH INHIBITOR EFFECTS

Medium supplement	Cell number (% of T^+ control) 48 hours	72 hours	96 hours
T^+ (34nM)	100	100	100
T^-	116	113	100
Estradiol (5 μg/ml), T^+	72	97	106
Estradiol (5 μg/ml), T^-	72	87	98
TNF (5 units/ml)		100	
(50 units/ml)		99	
(200 units/ml)		109	
(1,000 units/ml)		102	
TGF-ß1 (2 ng/ml)		114	
(5 ng/ml)		119	
(10 ng/ml)		112	

TNF = Tumor necrosis factor
TGF-ß1 = Transforming growth factor ß1

50,000 RDE cells were plated in growth medium supplemented with the indicated doses of hormone or growth inhibitor. The cells were cultured for 2, 3 or 4 days with medium replacement on the third day (testosterone and estradiol). The cells were harvested and cell numbers were determined by Coulter counter. Unless otherwise indicated, all cultures contained 34nM testosterone. Values are expressed as a percentage of the cell numbers obtained for cells grown on plastic in T^+ medium. Note that the controls are the same as in Table VI since the assays were performed in parallel.

8) Tumorigenic potential of RDE cells

The rapid rate of RDE cell proliferation in both the presence and absence of androgen suggested that these cells were no longer governed by the normal growth regulatory system. A series of in-vitro and in-vivo assays were performed to determine if this loss was sufficient to render the cell tumorigenic. The tumorigenic potential of a given cell is often initially assayed by testing its ability to grow in soft agar (Hamburger and Salmon, 1977).

TABLE VIII IN-VITRO SOFT AGAR ASSAY

Cell type	Cells/dish	Number of colonies >20 cells		% Growth	
		Day 5	Day 12	Day 5	Day 12
NE	200	0	0	0	0
NF	200	0	0	0	0
RDE (1 passage)	200	162	200	81	100
RDE (25 passages)	200	200	200	100	100

Single-cell suspensions of normal primary epithelial (NE), normal primary fibroblast (NF) or RDE 11.4 cells (passages 1 and 25) at a concentration of 200 cells/ml were dispersed in a mixture of warm medium and 0.5% agar. The cells were aliquoted onto base agar prepared in growth medium and subsequently covered with 0.3% agar in growth medium. The dishes were incubated at 37°C and scored on days 5 and 12 for colonies containing more than 20 cells (Suzuki et al., 1983).

Results in Table VIII demonstrate that normal epithelial and fibroblast cells did not grow in soft agar. In contrast to this, RDE cells grew very well in soft agar. Newly derived RDE cells grew more slowly than RDE cells that had been passaged repeatedly, but both showed that 100% of the single cells initially seeded develop into colonies of >20 cells within the 12 day assay period.

The success of RDE cell growth in soft agar suggested that the RDE cells have a very high tumorigenic potential. In-vivo tumorigenic assays were conducted by injecting 10^6 RDE or normal epithelial cells into three syngeneic rats in each of a number of locations (see Methods). The animals were examined weekly for external indications of tumor growth for a period of 6 weeks. One animal from each sample group and injection site was sacrificed 6 weeks after inoculation, one at 12 weeks and the remaining animal was sacrificed 26 weeks after inoculation. Necropsies were performed on all sacrificed animals immediately following death. Despite the indications of very strong tumorigenic potential provided by the in-vitro soft agar assay, none of the RDE cells, nor any of the NE cells, showed any indication of tumor growth even after 26 weeks.

9) Karyotype:

RDE cells at early passages displayed a diploid number of chromosomes. Late passage number RDE cells maintained a predominantly diploid number of chromosomes but showed a slow increase in chromosome numbers with a greater number of passages.

B) MOLECULAR BIOLOGY:

The rapid rate of androgen independent proliferation of RDE cells could have resulted from a transformation event and/or the prevention of cell death. To determine if the RDE cells had been transformed we assayed the presence of 10 proto-oncogene sequences.

1) Proto-oncogene screening of RDE cells

Proto-oncogenes are sequences normally present in a cell that have the potential to cause transformation of the cell if expressed at inappropriate times, locations or abundance. These sequences have been incorporated into the genome of a variety of viruses, thereby conferring upon the virus the ability to transform an infected host cell

through viral expression of the sequence, then refered to as an oncogene. Using viral oncogene probes we assayed the RDE cells for the expression of elevated levels of either cellular or viral oncogene RNA sequences. Cell blots of RDE cells grown to 75% confluence in T^+ medium were hybridized to 10 commercially available oncogene probes. No RDE cell dots gave a signal even after 3 days of exposure while the control dots consisting of 1 ng unlabeled probe saturated the film with a 4 h exposure. Details of the oncogene probes (v-abl; v-erbA; v-erbB; v-mos; N-myc; v-myc; N-ras; v-Ha-ras; v-sis; v-src) are presented in appendix 6.

2) Calcium ionophore induction of TRPM-2

TRPM-2 is a gene associated with cell death which appears to code for a protein presumably involved in the apoptotic regression of the prostate after hormonal ablation or anti-androgen therapy (Léger et al, 1987, Léger, Le Guellec and Tenniswood, 1988). To determine if this mRNA is expressed during RDE cell death we analyzed the expression of RDE cells in response to the calcium ionophore A23187. Others have found that this compound induced the synthesis of TRPM-2 in normal epithelial cells even in the absence of hormonal treatment, and thus might be expected to induce expression in the androgen-independent RDE cells (Isaacs, Personal communication). Exposing RDE cells to $2.5x10^{-7}$M A23187 (a concentration found to cause 50% cell death over a 16 hour incubation) for 0, 2, 4, 6 and 24 hours

and performing in-situ cell blots on 25,000 cells gave no signal after exposing the autoradiogram for 72 hours (Figure 15). Control hybridizations with the pRDE specific clones were positive within 6 hours of autoradiographic exposure.

3) cDNA cloning of RDE-specific sequences

Since the RDE cells did not express any of the common markers of prostate secretory epithelial cells (SAP, PSBP) it was necessary to develop new markers to identify the non-secretory RDE cells. By cloning the mRNA sequences expressed by the RDE cells and eliminating messages common to both normal and RDE cells we were able to isolate a series of cDNA clones corresponding to messages expressed only in RDE cells.

The cloning methodology used gave the equivalent of 10^4 independent clones of which 750 were screened initially by differential hybridization using cDNAs reverse transcribed from RVP poly(A^+) RNA and from RDE cell poly(A^+) RNA.

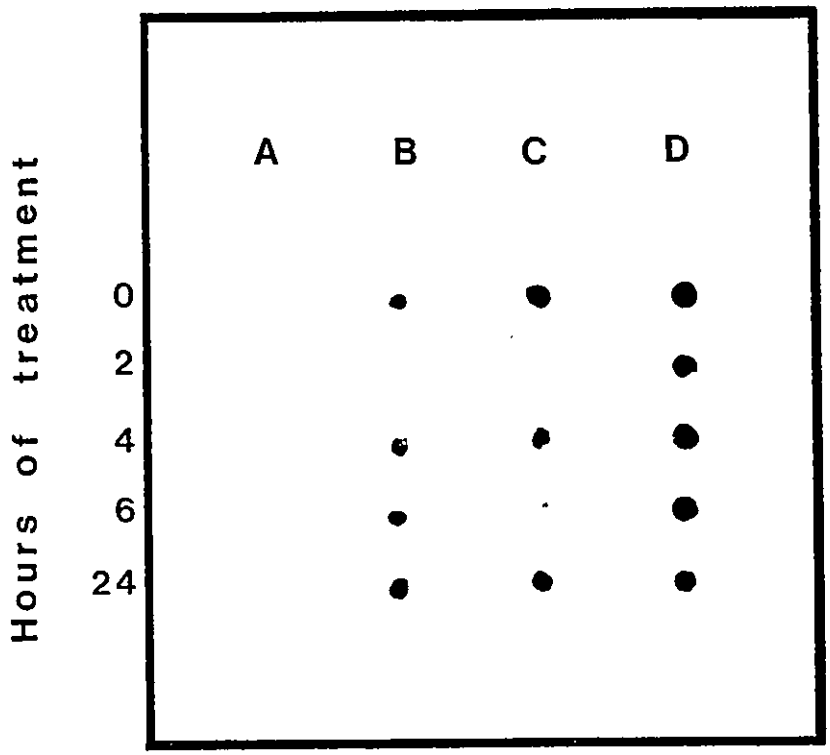
Figure 15: Calcium Ionophore A23187 Induction of TRPM-2

10^6 RDE cells grown to near confluence in growth medium were cultured for 0, 2, 4, 6 and 24 hours in medium supplemented with $2.5x10^{-7}$M A23187. The cells were processed for in-situ cell blotting. 25,000 cells were blotted at each point and hybridized for 16 h to [^{32}P]-labeled p21-04 (TRPM-2 specific sequence; $5x10^8$ cpm/μg) or pRDE clones ($2\text{-}3x10^7$ cpm/μg). The autoradiogram was exposed for 6 and 72 hours at -70°C.

Dot 1: 0 hours exposure to A23187
Dot 2: 2 hours exposure to A23187
Dot 3: 4 hours exposure to A23187
Dot 5: 6 hours exposure to A23187
Dot 6: 24 hours exposure to A23187

Series A: p21-04 (TRPM-2)
Series B: pRDE-0.25
Series C: pRDE-1.5
Series D: pRDE-2.3

Figure 15



This preliminary screening established three categories of sequence expression: A) 20% of sequences which were considerably more abundant in rat ventral prostate cells, B) 20% which were considerably more abundant in RDE cells, and C) the remaining 60% were equally expressed in both cell types. Clones giving the 8 strongest signals in group B were isolated and further screened by Northern hybridization. Of these, three sequences appeared to be expressed only in RDE cells (Figure 16). These clones have been named to reflect their RDE cell-specificity and mRNA size: pRDE-0.25 codes for a 250 bp sequence, pRDE-1.5 hybridizes to a 1.5 Kbp sequence, while pRDE-2.3 contains a portion of a 2.3 Kbp message (Montpetit and Tenniswood, 1989a). Control hybridizations to verify the presence of poly(A^+) RNA in the other (intact and castrate) lanes of these filters were performed using [^{32}P]-labeled pA34, a clone specific for the C3 component of prostate steroid binding protein and p21-04, a clone for the androgen-repressed TRPM-2 sequence, respectively (results not shown).

The distribution of PSBP and pRDE-0.25 expression in different cell types within the prostate was analyzed using a sensitive cell blot methodology on the disaggregated cells of the rat ventral prostate separated on step gradients, RDE cells and normal androgen-dependent primary cultures of epithelial cells after 2 weeks in culture. As shown in figure 17 the gradient effectively separates rat ventral prostate fibroblasts from epithelial cells as determined morphologically (panel B) and by expression of the

PSBP (panel A, dot series "a"). RDE cells grown in testosterone supplemented medium (34nM) did not express detectable levels of PSBP expression (dot 9) nor did 2 week old primary cultures of normal slowly-dividing RVP epithelial cells (dot 10), despite the continued androgen-dependence of the latter for survival. By reducing the autoradiographic exposure time the apparent signal in dot 3, panel "a" was determined to be the result of non-specific hybridization rather than a valid signal.

Panel A, dot series "b" demonstrates the expression of pRDE-0.25 expression in only one of the cell fractions. In gradient fraction 6 there was a signal of approximately 10% that found for RDE cells (dot 9). The unequal signal intensities between the duplicate dots #6 is the result of clumping of the glutaraldehyde-fixed cells thereby reducing the number of cells avaliable for hybridization and the surface area they occupy. There was no evidence of pRDE-0.25 expression in the primary cultures of epithelial cells.

FIGURE 16: Northern Hybridization of Putative RDE Specific Clones

Poly(A)$^+$ RNA (5 μg/lane) was electrophoresed through agarose gels under denaturing DMSO/glyoxal conditions and transferred to nylon filters. The filters were hybridized with ^{32}P-labeled cDNA clones suggested by Grunstein-Hogness hybridization to be RDE cell-specific. All three probes were adjusted to have 14.3×10^6 cpm in the hybridization mixture (approximate specific activity 10^8 cpm/μg) The autoradiogram was exposed for 48 h at -70°C.

Lane 1: poly(A)$^+$ RNA from intact rat ventral prostate
Lane 2: poly(A)$^+$ RNA from rats castrated 4 days prior to sacrifice
Lane 3: poly(A)$^+$ RNA from RDE cells grown in the presence of 34nM testosterone.

Panel A: clone pRDE-0.25
Panel B: clone pRDE-1.5
Panel C: clone pRDE-2.3

Figure 16

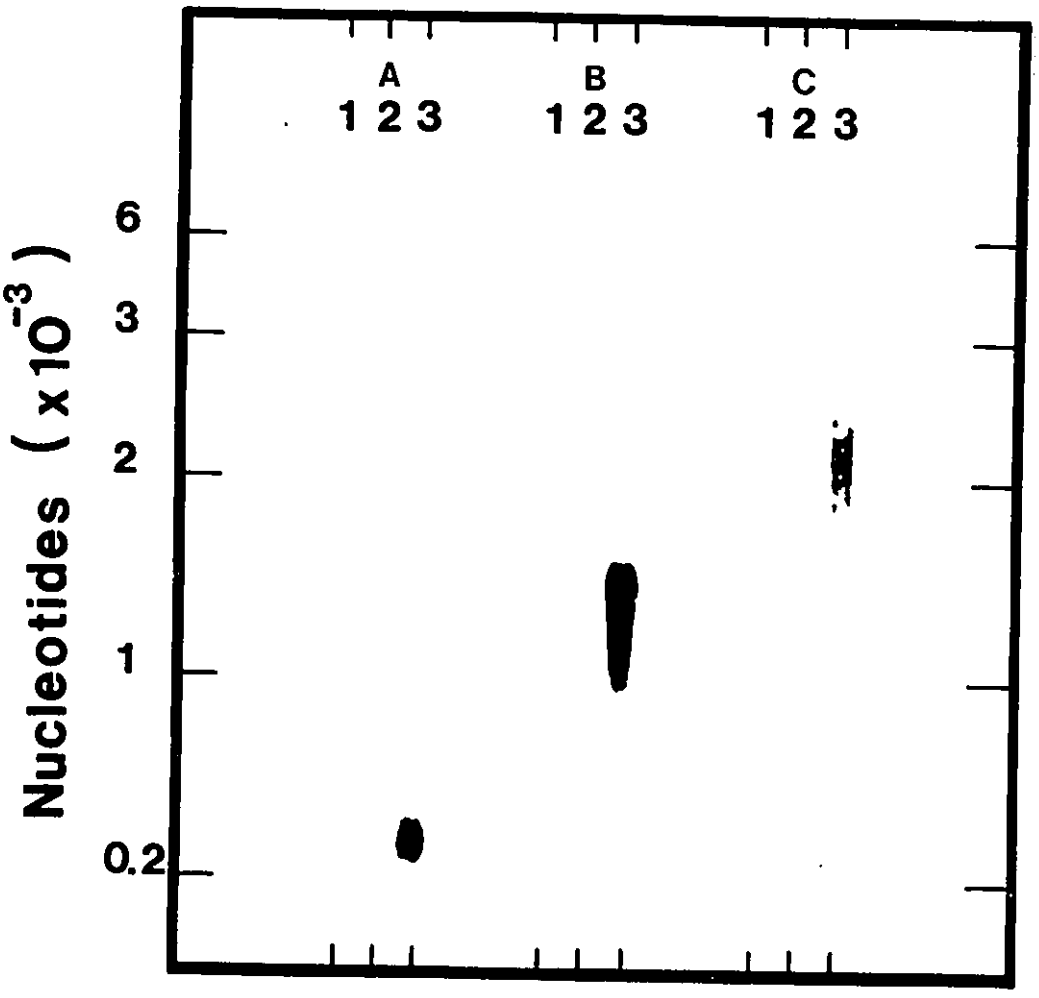


Figure 17: Cell blot hybridization of rat ventral prostate cells and RDE cells.

Panel A:

25,000 cells from fractions of a Percoll step gradient fractionation of ventral prostate cells were fixed in 3% glutaraldehyde prior to being dot blotted in quadruplicate onto a poly-L-lysine coated nylon filter. Duplicate blots were hybridized for 16 h to [^{32}P]-plasmid. The autoradiogram was exposed for 8 h at -70°C.

Series a: Plasmid pA34; 1.26×10^8 cpm/μg
Series b: Plasmid pRDE-0.25; 1.23×10^8 cpm/μg

Dots 1-8: Epithelial and fibroblast cells in fractions of the step gradient.
Dot 9: RDE cells grown in T^+ medium.
Dot 10: 2 week primary cultures of androgen-dependent RVP epithelial cells (1 passage).

Panel B:

Relative proportions of epithelial and fibroblast cells in each step gradient fraction (based on morphology):

Fractions 1-8: Epithelial and fibroblast cell proportions in step gradient fractions 1-8
= fibroblast
= epithelial
Fraction 9: RDE cells grown in T^+ medium =
Fraction 10: 2 week primary cultures of androgen-dependent RVP epithelial cells (1 passage) =

Figure 17

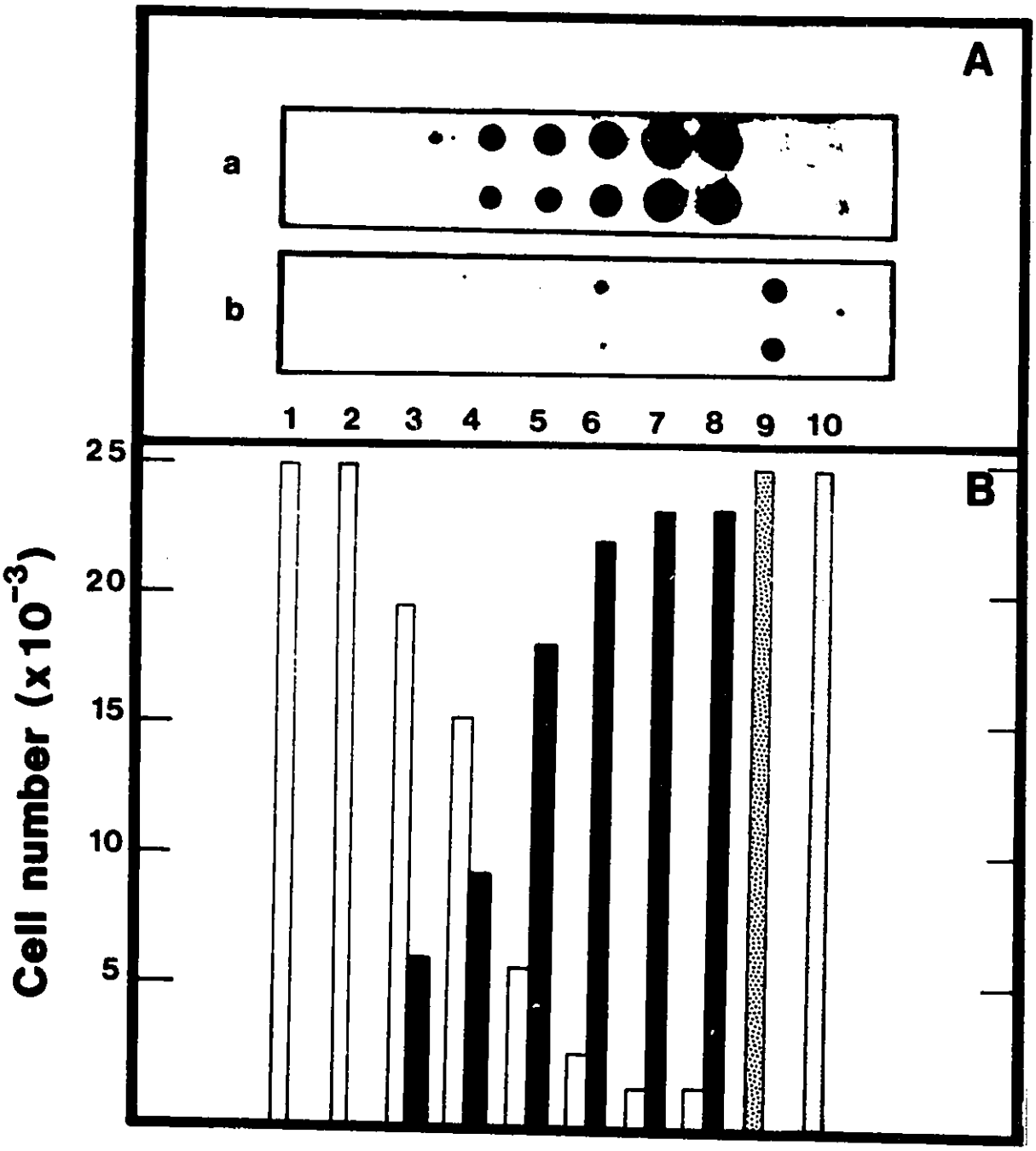


Figure 18 shows the sequence of the 3' end of the pRDE-0.25 cDNA (coding strand) and the corresponding translation of the single extended open reading frame. The sequences of the rat growth hormone (GH) gene (Barta, Richards, Baxter and Shine, 1981), the mouse **pim-1** (Zakut-Houri, Hazum, Givol and Telerman, 1987) and **c-abl II** (Ben-Neriah, Bernards, Paskind, Daley and Baltimore, 1986) genes are also shown. pRDE-0.25 has greater than 80% DNA sequence homology to these sequences. Homology between pRDE-0.25 and both **pim-1** and **c-abl II** is with the complementary sequence of the proto-oncogenes and no appropriate open reading frame exists within the portion of oncogene sequence homologous to the pRDE sequence shown. The homology with **pim-1** is to a portion of the genomic DNA beyond the 3' end of the **pim-1** mRNA (Zakut-Houri et al, 1987). The 3' end of the pRDE-0.25 sequence bears strong resemblance to a central portion of the 5' exon of **c-abl II.** The sequence homology to the rat GH genes on the other hand is with the message strand of DNA within each of two tandemly repeated sequences in the second intron of the gene (Barta et al, 1981).

Despite the high level of DNA sequence homology between pRDE-0.25 and mouse pim-1, c-abl II and the rat GH genes it is apparent that the small RDE-0.25 transcript (about 250 bases) detected by Northern blotting was not directly related to the expression of any of these larger sequences. On the other hand, figure 19 shows

the homology between pRDE-0.25 and the consensus sequence of a member of the Alu repeated sequence family, the murine B2 moderately repetitive sequence (Krayev, Markusheva, Kramerov, Ryskov, Skryabin, Bayev and Georgiev, 1982a). Transcription of the B2 repeated sequences by RNA polymerase III yields poly(A)$^+$ RNA with a narrow range of lengths; 190 ± 30 bases, depending on the length of the poly(A) tail (Krayev et al., 1982a; Kramerov, Lekakh, Samarina and Ryskov, 1982b). Rat and mouse tumors, virally transformed mouse cell lines and a few rapidly regenerating normal tissues have been found to express elevated levels of these small poly(A)$^+$ RNA transcripts (Yamamoto, Maehara, Takahashi and Endo, 1983; Ryskov, Ivanov, Tokarskaya, Kramerov, Grigoryan and Georgiev, 1985; Singh, Carey, Saragosti and Botchan, 1985; Grigoryan, Kramerov, Tulchinsky, Revasova and Lukanidin, 1985; Suzuki, Fujiyoshi, Maehara, Takahashi, Yamamoto and Endo, 1986).

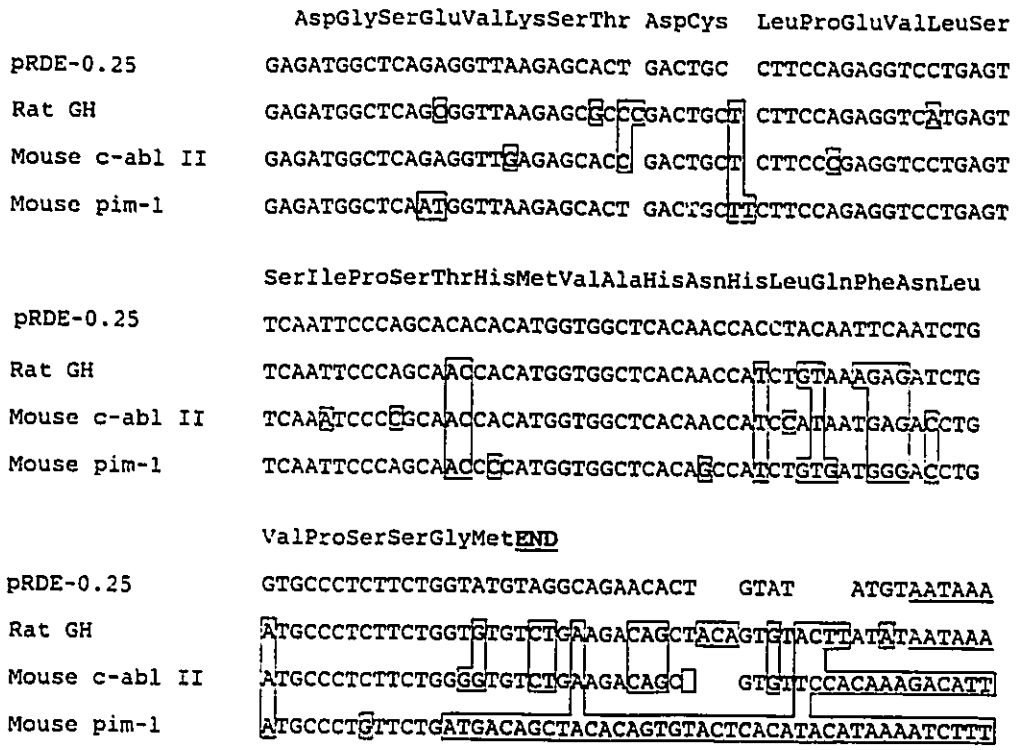
The DNA and putative amino acid sequences for all pRDE clones are shown in appendix 5. Even though open reading frames existed in both pRDE-1.5 and pRDE-2.3, no significant homology was found between pRDE-1.5 or pRDE-2.3 and sequences deposited in the GENBANK database.

Figure 18: DNA Sequence of pRDE-0.25 and Homologous Rodent Sequences

The pRDE-0.25 insert was subcloned into M13 mp10 and M13 mp11 and sequenced by [^{35}S]-dATP labeled dideoxynucleotide chain termination using a 17 base primer sequence. The samples were electrophoresed in 8% polyacrylamide gels at 2500V, 40 mA and 50 W. The gels were fixed with 5% methanol, 5% acetic acid then dried and autoradiographed for 18 hours at room temperature.

The boxed nucleotides represent non-homology relative to pRDE-0.25.
Underlined bases form the polyadenylation signal.
END indicates the termination codon of the ORF.

Figure 18



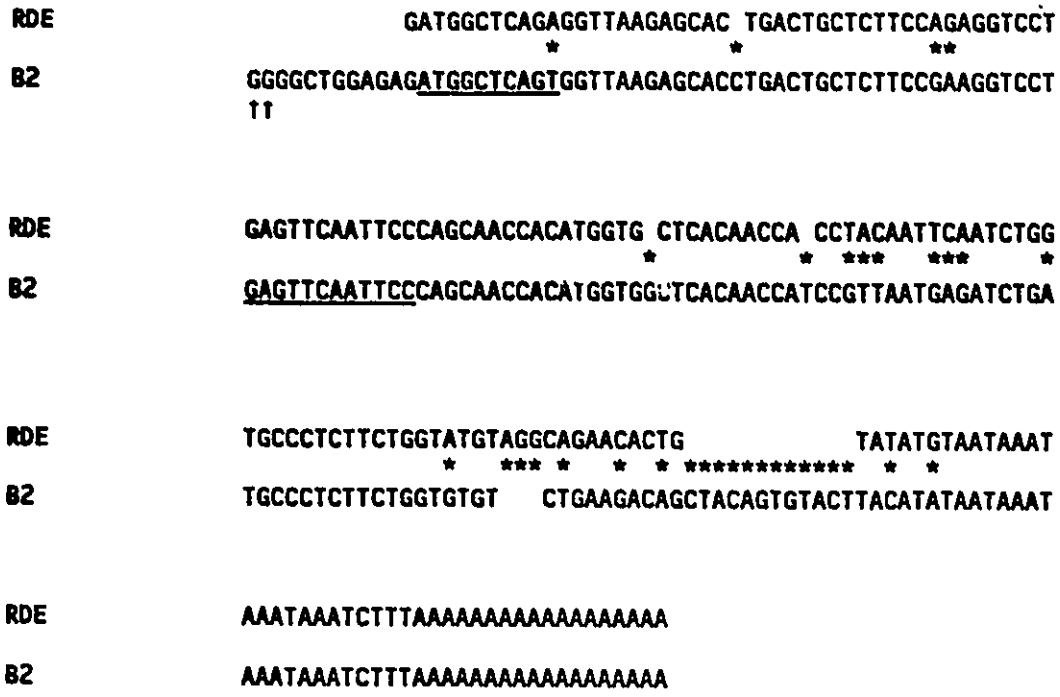
Boxed nucleotides represent non-homology relative to pRDE-0.25 sequence.

Figure 19: DNA Sequence of pRDE-0.25 and Mouse B2 Repeat Consensus Sequence

The pRDE-0.25 insert was subcloned into M13 mp10 and M13 mp11 and sequenced by [^{35}S]-dATP labeled dideoxynucleotide chain termination using a 17 base primer sequence. The samples were electrophoresed in 8% polyacrylamide gels at 2500V, 40 mA and 50 W. The gels were fixed with 5% methanol, 5% acetic acid then dried and autoradiographed for 18 hours at room temperature.

* Mismatched bases
† Transcription initiation site
Underlined bases indicate split RNA polymerase III promoter sequences.

Figure 19


RDE
GATGGCTCAGAGGTTAAGAGCAC TGACTGCTCTTCCAGAGGTCCT
B2
GGGGCTGGAGAGATGGCTCAGTGGTTAAGAGCACCTGACTGCTCTTCCGAAGGTCCT
RDE
GAGTTCAATTCCCAGCAACCACATGGTG CTCACAACCA CCTACAATTCAATCTGG
B2
GAGTTCAATTCCCAGCAACCACATGGTGGCTCACAACCATCCGTTAATGAGATCTGA
RDE
TGCCCTCTTCTGGTATGTAGGCAGAACACTG TATATGTAATAAAT
B2
TGCCCTCTTCTGGTGTGT CTGAAGACAGCTACAGTGTACTTACATATAATAAAT
RDE
AAATAAATCTTTAAAAAAAAAAAAAAAAAA
B2
AAATAAATCTTTAAAAAAAAAAAAAAAAAA

DISCUSSION

We have established a number of androgen independent epithelial cell lines from cells isolated from the rat ventral prostate. These cells were originally identified as rapidly dividing foci that appeared in primary epithelial cell cultures maintained in the presence of androgens. While the parent cultures retained their androgen dependent characteristics, the rapidly dividing epithelial (RDE) cells were androgen independent for both cell survival and proliferation. Clonal cell lines from 43 different foci were isolated from 4 separate primary cultures of androgen dependent prostate epithelial cells. All of the RDE cells possess very similar characteristics including morphology, equal growth rates in both the presence and absence of androgens, and the ability to grow in soft agar. RDE cells do not secrete the marker proteins secretory acid phosphatase (SAP) and prostate steroid-binding protein (PSBP) yet retain the ability to metabolize testosterone to 5α-dihydrotestosterone and other metabolites.

The RDE cells appear to be maximally stimulated for proliferation since supplementation of the culture medium with epidermal growth factor (EGF) had no effect on the rate of proliferation despite the presence of EGF receptors in the cells. A variety of other growth factors were equally ineffective in stimulating RDE cell proliferation. Similarly a variety of growth inhibitory factors also failed to exert any significant effects. A cDNA clone library yielded a number of RDE-specific probes

of which 3 were characterized by sequencing. These clones were used to detect the RDE cells in-vivo (Figure 17).

A) ARE THE RDE CELLS OF EPITHELIAL ORIGIN?

In the absence of prostate marker protein expression it is difficult to prove that the RDE cells are androgen independent prostatic epithelial cells, and not a contaminating cell type that co-purified with the epithelial cells or that were introduced during the cell culture. Cross-contamination of primary cell cultures with cultures of transformed cells has occurred repeatedly in the history of cell biology and remains a major concern for all investigators. The absence of prostatic markers and the partially transformed phenotype of the RDE cells upon isolation from normal tissue was cause for concern about contamination from the very beginning of our characterization of RDE cells.

However, the laboratory conditions at the time of the initial RDE cell isolation and the results of subsequent confirmatory experiments convincingly demonstrate that the RDE cells are not the result of contamination. Listed chronologically these conditions are:

a) Lack of possible contaminating cell types:

During the initial isolation of the RDE cells, and for several months therafter, no other cells of any type or species were being cultured in our laboratory.

b) Reproducible timing of RDE appearance:

In all experiments undertaken to reproduce the isolation of the RDE cells the foci of rapidly-dividing cells always appeared at the same time relative to the conditions of the primary cultures (ie. during the last third of the death phase for the primary epithelial cells; 10-12 days after plating). Had the RDE cells been the result of contamination they would be expected to appear at random times whenever the media or other contaminated entity was introduced into the cultures.

c) Constant frequency of RDE cell appearance:

Regardless of the initial plating density used to isolate RDE cells one finds that RDE foci appear with a constant frequency (10-15 foci per million cells). It is difficult to conceive that cross-contamination would produce this constant frequency over three different plating densities.

d) Gradient-free isolation of RDE cells:

RDE cells were also isolated directly from dissociated prostates without passage through Percoll gradients. In these experiments the constancy of both the time of foci appearance (relative to the epithelial cell death phase) and the frequency of appearance were maintained. Thus the gradient systems may be cleared of responsibility for contamination of the cultures or the transformation of the epithelial cells.

e) Isolation by other investigators:

The burden of proof in science has always rested on the reproducibility of a phenomenon by independent researchers. Thus our final and strongest argument that RDE cells are not contaminants comes from the isolation and characterization of very similar cells by others using the protocol that we supplied (R. Miesfeld; P. Martikaanen; personal communications).

The reproducible isolation of RDE cells from 4 separate primary cultures (using two different separation methods) indicated that any potential contaminating cells must have originated from within the prostate rather than from external sources (ie. blood, bladder, striated muscle, or intestine). Two other characteristics of RDE cells were analyzed to demonstrate that the RDE cells are of epithelial origin.

1) Metabolism of testosterone:

The rat ventral prostate has been used for over fifty years to study the mechanism of androgen action. In the course of this work the metabolism of androgens within androgen target tissues and at the cellular level has been thoroughly characterized, both in-vivo and in-vitro in epithelial cells in short term cultures (10 days) (Mainwaring, 1977). The pattern of testosterone metabolism has been established for both rat prostate fibroblast and epithelial cells (Orlowski, Bird and Clark, 1983). Orlowski and Clark have demonstrated that prostate epithelial cells and fibroblasts in culture have distinctly different metabolic patterns although both cell types actively metabolize testosterone to 5α-DHT, 5α-androstane-3α,17ß-diol (3α-adiol) and 5α-androstane-3ß,17ß-diol (3ß-adiol) and subsequently to the further catabolites of androgens (Orlowski and Clark, 1986).

TABLE IX. COMPARISON OF TESTOSTERONE METABOLISM PATTERNS

Steroid	RDE cells	Epithelial 1	Epithelial 2	Fibroblasts 1	Fibroblasts 2
Testosterone	70.1	2.4	57	74.7	86
5α-DHT	9.8	10.8	23	7.0	4
3α-adiol	2.2	2.8	2	1.0	2
3ß-adiol	7.7	6.6	3	0.6	1
Δ^4-androstanedione	2.2	1.6		1.7	
5α-androstanedione	0	24.7		0.7	

Comparison of the pattern of metabolism of testosterone in epithelial and fibroblast primary cultures ([1] Orlowski and Clark, 1986, [2] Ofner, Vera, Terracio and Dougls, 1982) and RDE cells. All values are expressed as a percentage of the initial [^{3}H]-testosterone added to the culture medium.

Figure 9 and tables V and IX show that the RDE cells actively metabolize testosterone to a full range of metabolites. This indicates that the RDE cells are androgen target cells within the prostate, capable of metabolizing testosterone. Comparison of the testosterone metabolism by the normal fibroblasts and epithelial cells to that of the RDE cells clearly demonstrates the RDE cells metabolize testosterone in a manner that is different from the bulk of the epithelial cells and the fibroblasts. RDE cells show considerable 3α-adiol and 3ß-adiol formation similar to the epithelial cells. However the low level of testosterone reduction and the low level of 5α-androstanedione synthesis is more characteristic of the fibroblast population. As

discussed by Orlowski and Clark (1986) the maturity of the animals and the degree of confluency in the culture may play an important role in both the amount of testosterone metabolized and the 5α-androstanedione levels. Rather than use confluent cultures from immature rats as was done by Orlowski and Clark (1986), Ofner and co-workers, using 3nM steroid in semi-confluent cultures of epithelial cells from mature animals, found that the amount of testosterone metabolism is considerably reduced in epithelial cells (Ofner, Vera, Terracio and Douglas, 1982), and appears to be more in line with the reduced efficiency of testosterone metabolism seen in the RDE cells using 1nM labeled steroid. The similarities and differences between RDE and both epithelial and fibroblast metabolic patterns further demonstrate that RDE cells are different from the bulk of the members of each of these cell types.

2) Immunochemical analysis of cytokeratins:

The cytoskeleton is composed of intermediate filaments, microfilaments, microtubules and a number of associated regulatory proteins. Alteration of any single component is capable of effecting normal cellular functions including the regulation of cell shape, movement, secretion and cell division (Trump, Heatfield and Phelps, 1981). One of the intermediate filament group of proteins are the cytokeratins. The cytokeratin "signature" can be used to identify specific cell types within tissues and may

be used to identify the origin of particular cells. Analysis of the cytokeratins expressed in the human prostate has shown that the different cell types within the prostate (fibroblast, epithelial and non-fibroblast stromal cells) can be readily distinguished and identified by analysis of the cytokeratins that are expressed (Achstätter, Moll, Moore and Franke, 1985).

The RDE cells express cytokeratins 8 and 18. They do not express cytokeratins 7, 16 and 17 (Table IV). The presence of cytokeratins 8 and 18 has been determined to be characteristic of the luminal epithelial cell component of the human prostate, whereas cytokeratins 5 and 19 are found only in the basal epithelial component of the human prostate (Achstätter et al., 1985; Wernert, Seitz and Achstätter, 1987). Cytokeratins 8 and 18 are well characterized markers of "simple" epithelium and clearly establish the RDE cells as being of epithelial rather than fibroblastoid origin. The simultaneous expression of secretory cytokeratins and one or more basal cytokeratins recognized by antibody EAB903 (cytokeratins 1, 5, 10, 11) (Purnell, Heatfield, Anthony and Trump, 1987) has recently been reported in-vivo in a small subset of basal epithelial cells (Verhagen et al.,1988; Rouleau et al., 1990). Both these groups found basal cells demonstrating mixed cytokeratin types in normal prostates with no reactivity to the stromal cells. The small number of mixed pattern basal cells reflects the expected low abundance of RDE cells as a putative stem cell population.

B) OTHER ANDROGEN INDEPENDENT EPITHELIAL CELLS ISOLATED FROM THE RAT VENTRAL PROSTATE:

RDE cells are not the first rapidly-dividing androgen-independent epithelial cells isolated from the rat ventral prostate. The rapid growth of epithelial cells isolated from rat ventral prostate primary cultures has been reported previously (McKeehan et al., 1984, 1987). The maximal growth of these androgen independent cells in serum-free medium requires the collective presence of cholera toxin, insulin, dexamethasone, epidermal growth factor and prostatrophins isolated from bovine pituitary (McKeehan et al., 1984). For a period of 9-12 days after initiation of the cultures, these cells proliferated exponentially on bare plastic with a 35-50 h doubling time. By assuming that the measured growth rates were representative of a constant rate of division of progeny from single cells McKeehan estimated that approximately 4% of the prostate cells give rise to the proliferative population.

At first glance it might appear that the RDE cells are the same sub-population of androgen-independent cells identified by McKeehan. However, analysis of three different criteria (nutritional requirements, rate of proliferation and morphology), strongly suggests that these two cell populations are quite distinct.

1) Nutritional requirements of the androgen independent cells:

The androgen independent cells described by McKeehan have an absolute requirement for cholera toxin, insulin, bovine pituitary extract, dexamethasone and EGF for proliferation (McKeehan et al., 1984). The absence of cholera toxin in the WAJC 404 medium used for the culture of the androgen independent cells reduces the rate of cell division by 75%. These cells require 0.05nM to 10nM epidermal growth factor (EGF) for proliferation to reach even a fraction of the rate achieved with complete WAJC 404 medium (McKeehan et al., 1984). In contrast, cholera toxin has no noticeable effects on the RDE cells and they divide very rapidly in the absence of EGF. Supplementing the medium with EGF has no noticeable effect on the rate of RDE cell division. While the addition of horse serum to the RDE cell culture medium may mask a requirement for prostatrophins, the RDE cells still proliferate at 20% maximal rate in serum-free, growth factor-free medium.

2) Proliferation characteristics of androgen independent cells:

The androgen independent cells described by McKeehan have a 35-50 h doubling time. This is considerably different from the doubling time of 8-10 h found with RDE cells grown in the presence or absence of testosterone (Table III). The androgen independent growth of both cell types is in stark contrast to the normal

epithelial primary cultures which retain the requirement for androgens for survival and growth. By far the greatest difference between RDE and the androgen independent cells characterized by McKeehan is the limited proliferative capacity of the latter. These cells display a rapid growth rate from approximately 3 days after initiation of the primary culture until 8-10 days of culture (4-5 population doublings) at which point proliferation ceases completely and the cells die within 2 days. RDE cells do not begin to proliferate in primary cultures of the ventral prostate epithelial cells until 10-14 days after initiation of the culture. Once the RDE cells have begun to proliferate they appear to be immortal, with some cell lines being maintained for more than 200 passages over 2 years.

3) Morphological comparison of androgen independent cells:

The other main difference between the androgen independent cells described by McKeehan and RDE cells described here is based on the morphological characteristics of these cells. RDE cells have a dysplastic morphology and poor cell-cell contact patterns unless near confluence. RDE cells will readily adopt complex 3-dimensional growth patterns under a variety of conditions while primary cultures of androgen dependent epithelial cells remain in monolayer culture regardless of ECM or other factors. The cells described by McKeehan display the characteristic epithelial

cobblestone pattern with tight cell-cell junctions with only a small amount of localized dissociation of cell-cell contacts (McKeehan et al., 1984).

These results suggest that the RDE cells and the androgen independent cells described by McKeehan are two very different and distinct sub-populations of prostatic cells. The relative numbers of the androgen independent cells described by McKeehan (approximately 4% of the epithelial cell population) and RDE cells (probably less than 0.1% of epithelial cells) and the differences in the lifespans of the two cell populations suggest that these two cell populations play different roles in-vivo.

C) ROLE OF THE RDE CELLS: AN HYPOTHESIS

Stem cells are defined as cells that are capable of extensive self-renewal in spite of physiological or accidental removal or loss of cells from the population (Lajtha, 1979). In his discussion of a stem cell model for the proliferation of epithelial cells in prostate cancer and benign prostatic hyperplasia, Isaacs has demonstrated that, under certain conditions, an increase in the proliferation rate of epithelial stem cells, or a decrease in the death rate of these cells will result in the observed kinetics of development of these diseases (Isaacs, 1987).

Androgen cycling experiments conducted in castrated rats demonstrate that a very small fraction of prostatic cells are stem cells and that these cells are responsible for the complete renewal of the prostate even after 30 involution/renewal cycles (60 population doublings)(Isaacs, 1987). This is in contrast to the limited proliferation of normal rat epithelial cells that can undergo less than 10 population doublings in-vitro before entering senescence (Evans and Chandler, 1987a). To resolve this apparent problem it is necessary to suggest that more than one class of cells are responsible for the renewal of the prostate.

Isaacs suggests:

> "... additional subclasses of amplifying cells capable of limited proliferation must also be present along with stem cells in the involuted prostate. While these amplifying cells originate from the stem cells and can proliferate for only limited numbers of cell division, these proliferations result in a major amplification of the total number of cells present. This amplification can be extensive, for example, if the amplifying cells can divide five times, this produces a 32-fold amplification in total cell number; if they can divide 10 times, this produces a 1,000-fold amplification. Such amplification results in the stem cells being a minority population in the tissue. Thus restoration of the involuted prostate after castration by exogenous androgen probably involves only a small, if any, increase in the rate of stem cell renewal, the major restoration in cell number being due to the increased proliferation of the pool of preexisting amplifying cells. ... In this model attention is drawn to the fact that the amplifying cells are androgen-independent since they are able to exist even in long-term castrated animals. ... both stem cells and amplifying cells are able to maintain themselves (i.e. renew themselves) during long term (1 year) androgen withdrawal (i.e. they are androgen-independent)."
>
> (Isaacs, 1987)

The theoretical considerations outlined by Isaacs strongly suggest that the androgen independent cells described by McKeehan serve as amplifying cells within the prostate whereas the RDE cells are the prostatic stem cells.

1) Characteristics of amplifying cells:

Androgen independent cells isolated from monolayer cultures of dissociated rat ventral prostate epithelium by McKeehan, and also identified in explants of human organ cultures (Merchant et al., 1983) account for approximately 4% of the epithelial cell population and are capable of rapid but limited proliferation (McKeehan, 1984; Merchant et al., 1983). The human cells attempt to terminally differentiate into androgen dependent secretory cells at the end of their proliferative life span as judged by morpholgical change and the induction of acid phosphatase and prostate specific antigen expression (Merchant et al., 1983).

The requirement for exogenous growth factors noted by McKeehan for maximal cell proliferation may be the result of one of several conditions. First, culturing androgen independent cells on bare plastic is known to inhibit the maintenance of characteristic cell traits such as replication and gene expression (O'Connor and Sinha, 1985). In addition, stromal or even basal cell factors may normally regulate the rate of proliferation or degree of differentiation. For example, the androgen independent

cells described in human explant cultures can differentiate into secretory cells if these cells are able to interact with other cell types in the explant cultures (Merchant et al., 1983).

2) Characteristics of stem cells:

The characteristics of the RDE cells reported here strongly suggest that the RDE cells are the epithelial stem cells of the rat ventral prostate. As put forth by Isaacs, the stem cells must be androgen-independent, have an unlimited potential for proliferation and occupy only a small fraction of the total cell number. RDE cells are completely androgen-independent, have apparently unlimited potential for proliferation and, as judged by cell blotting with RDE-specific cDNA probes, account for a very small percentage of the epithelial cell population. As suggested by Isaacs, the stem cells are related to but distinct from the amplifying cells. Thus the RDE cells should bear some resemblance to androgen independent amplifying cells but should not, for example, differentiate into secretory cells even when grown on appropriate substrates such as collagen or prostate fibroblasts. The paradox of the rapid growth of RDE cells in soft agar but the absence of in-vivo tumorigenesis further reinforces the belief that RDE cells are "normal" stem cells and not the result of a transformation event.

D) DIFFERENTIATION:

With this hypothesis in mind we decided to see if RDE cells could be forced or encouraged to differentiate into androgen dependent secretory cells. We have also analyzed the effects of exogenous growth factors on these cells. The RDE cells differ from normal rat ventral prostate cells in culture since they do not express either secretory acid phosphatase or the prostatic steroid binding protein genes. This is not surprising since the synthesis of secretory products, such as PSBP and SAP, is known to be reduced or eliminated in replicating cells (Tenniswood et al., 1976; McKeehan et al., 1982). However it seems unlikely that the only difference between the RDE cells and the normal androgen dependent epithelial cells is the presence or absence of the secretory processes for two reasons. First, the acid phosphatase activity detected in the RDE cells does not resemble that seen in intact RVP (Orlowski and Clark, 1986). Alteration in the constitutively expressed lysosomal acid phosphatase indicates that the differences between the RDE cells and the normal epithelial cells are more complex than just the absence of the androgen dependent secretory acid phosphatase. Secondly, a comparison of the in-vivo and in-vitro translation profiles of the RDE cell lines and normal androgen dependent epithelial cells shows numerous differences. These differences clearly encompass more than the simple loss of the androgen dependent secretory proteins and suggest that the cells have not arisen from the dedifferentiation of the androgen dependent epithelial cells in the same cultures, since

it is unlikely that such a process would consistently yield the same cell type. The preservation of epithelial cell cytokeratins in the RDE cells also makes it unlikely that the RDE cells represent a population of dedifferentiated epithelial cells. Jamieson and co-workers (Jamieson, Dunnington, Ormerod, Warburton and Rudland, 1986) analyzed the dedifferentiation of rat mammary epithelial and myoepithelial cells resulting from exposure to 7,12-dimethylbenzanthracene (DMBA). In this model system the process of dedifferentiation invariably lead to the complete loss of reactivity with anti-keratin antibodies. The preservation of the epithelial cell markers (cytokeratins 8 and 18) in the RDE cells indicates that rather than being the product of dedifferentiation of epithelial cells the RDE cells are epithelial cells with an as yet undetermined role in prostate biology. It is therefore more likely that these cell lines represent the selection of an already existing androgen independent cell type which co-purifies with the androgen dependent epithelial cells on Percoll gradients.

1) Extracellular matrix effects on differentiation:

The role of the extracellular matrix in inducing and maintaining differentiation of cells has been recognized for several years (reviewed by Kleinman et al., 1987). The use of collagen in primary cultures of rat ventral prostate epithelial cells has been found to induce the formation of three dimensional structures reminiscent of prostatic buds, and the accumulation of secretory products within the cells and lumen of these

structures (O'Connor and Sinha, 1985; Thornton et al., 1985; Kawamura and Ichihara, 1987). Growth of the RDE cells on and in collagen matrices leads to the formation of extensive 3-dimensional structures. Despite the involvement of >90% of the RDE cells in these structures we were unable to detect the appearance of PSBP gene expression as a marker of terminal differentiation of the RDE cells.

In contrast to the expected decline in thymidine uptake that would be expected to accompany differentiation we observed a 40%-50% increase when the RDE cells were cultured on collagen as opposed to bare plastic, laminin or fibronectin. At first sight these results would appear to be contrary to the established role of ECM on epithelial cell populations. However the epithelial cells that form in-vitro prostatic acini under the influence of collagen are secretory epithelial cells. If the RDE cells are the stem cell of the prostate, then they should demonstrate increased proliferation when cultured on collagen. Since both the stromal and epithelial cells produce an extracellular matrix in-vivo that serves both as anchor and control system for the epithelial cells, stem cells should interpret the expanse of collagen as an area denuded of the normal epithelial cell population and should be prompted to proliferate to establish a layer of undifferentiated epithelial cells. These undifferentiated cells should be amplifying cells if Isaacs' hypothesis is correct (ie. if they are to differentiate further) since the RDE (or stem cells) would not be expected to differentiate further.

2) Stromal-epithelial interactions in differentiation:

Collagen alone is insufficient to cause differentiation, even if it does cause the anticipated proliferation of the RDE cells. We sought to determine if a signal was missing through co-culture experiments. The critical role of the stromal cells in the development, differentiation and control of the secretory epithelium of the prostate has been elegantly demonstrated by tissue recombination experiments (Cunha, 1972; Cunha et al., 1982; Cunha, 1984; Cunha et al., 1986; Sugimura et al., 1986a, 1986b). To determine if the stromal elements would contribute the factors required for complete differentiation we cultured RDE cells on collagen matrices containing trapped rat ventral prostate fibroblasts freshly purified on Percoll gradients. The induction of secretory activity in the RDE cells grown on collagen or collagen/fibroblast matrices would clearly demonstrate the ability of RDE cells to differentiate. However the negative results obtained cannot be interpreted as conclusive evidence of the inability of RDE cells to differentiate. Normal secretory epithelial cells are well known to lose their secretory functions in culture (Figure 17), even in the presence of androgens and various ECM components. Thus the conditions for the maintenance of the differentiated phenotype are not yet clearly defined, and those required for the initiation of differentiation are less well understood.

3) Growth factors:

The response of primary cultures of canine, rat and human prostate epithelial cells to a variety of non-steroidal growth factors has been described by several groups in the past five years (Chevalier, Bleau, Roberts and Chapdelaine, 1984; Tackett, Heston, Parrish, Pletscher and Fair, 1985; Nishi, Matuo, Muguruma, Yoshitake, Nishikawa and Wada, 1985; Maehama, Li, Nanri, Leykam, and Deuel, 1986; Crabb et al., 1986a, 1986b; Matuo, Nishi, Matsui, Sandberg, Isaacs and Wada, 1987; Hierowski, McDonald, Dunn, and Sullivan, 1987). Most of the growth factors, or "prostatrophins" appear to be synthesized outside the prostate, but several groups have reported the existence of a prostate derived growth factor (Nishi et al., 1985; Tackett et al., 1985; Matuo et al., 1987).

Although the proliferation of the RDE cells already appears to be maximally stimulated the cells were tested for their response to a variety of growth factors which had been reported to effect the growth rate of secretory epithelial cells. As mentioned previously, McKeehan reported that androgen independent epithelial cells appear to require the presence of epidermal growth factor (EGF), cholera toxin (CT) or bovine pituitary extract (BPE) to achieve maximal growth (McKeehan et al., 1983). While the BPE caused a minor increase in RDE proliferation we found that EGF was completely ineffective in promoting RDE cell division despite the immunochemical

demonstration of EGF receptors. Cholera toxin, fibroblast growth factor (FGF), endothelial cell growth factor (ECGF) and platelet derived growth factor (PDGF) were also found to have no discernible effect on RDE cell proliferation. We have also established that the rapid proliferation of the RDE cells in-vitro is not due to the inappropriate expression of cellular proto-oncogenic sequences.

The DNA sequence homology between pRDE-0.25 and the murine pim-1 and c-abl II proto-oncogenes as well as the rat growth hormone gene make it tempting to assign to pRDE-0.25 a role in the rapid rate of RDE cell proliferation. However, the size of the abundant RNA transcript is not related to the much larger c-abl II, pim-1 or growth hormone messages. Conversely the sequence, transcript size and abundance in rapidly dividing cells, but not in normal cells, strongly suggest that pRDE-0.25 is the rat equivalent of the mouse B2 transcript. The homology to the RNA polymerase III promoter sequences suggested that the small RNAs were RNA polymerase III transcripts. Several groups have sought to ascertain whether the abundant expression of B2 transcripts in transformed cells was the result of generalized or regulated RNA polymerase III activity. Singh found that 5S RNA gene expression is not significantly increased by SV-40 transformation while B2 transcription increases dramatically (Singh et al., 1985). Similarly, the RNA polymerase III transcription of brain specific ID sequences in rat was shown to be highly regulated (Sutcliffe, Milner, Gottesfeld and Lerner, 1984). Despite this evidence of regulated RNA polymerase III transcription

no specific role for the B2 transcripts has been found. The split RNA polymerase III promoter in the B2 and other sequences requires the presence of transcription factors B and C acting in conjunction with RNA polymerase III to yield transcription. This has prompted Singh to question whether B2 repeats, like similar highly-repeated Alu-like sequences, serve no useful function in the cell and are simply by-products of a growth-sensitive RNA polymerase III transcription system (Singh et al., 1985).

4) Growth inhibition:

TGF-ß is a potent growth inhibitor and inducer of differentiation for many epithelial cell types including hepatocytes, bronchial epithelial cells and keratinocytes (for review see Sporn, Roberts, Wakefield and Assoian, 1986). Rat and human prostate epithelial cells (both normal and from tumors) are inhibited by TGF-ß (McKeehan and Fast, 1988; Ikeda, Lioubin and Marquardt, 1987; Schuurmans, Bolt and Mulder, 1988). RDE cell proliferation appears to be unaffected by TGF-ß, a finding that again suggests that RDE cells play a unique role in the rat ventral prostate.

E) ANDROGEN INDEPENDENT CELLS IN THE PROSTATE:

As outlined in the introduction, the need to determine if androgen independent cells arise from adaptation or selection is paramount for the development of more effective therapies for human prostate cancer. Of equal importance is the isolation and characterization of the androgen independent cells so that effective therapies against them may be developed and administered to prevent the renewal of tumor growth after the successful elimination of the androgen-independent cells.

The Dunning rat prostate tumor system has provided models of the wide gamut of epithelial cell androgen dependencies present in prostatic tumors. These models have been used extensively to confirm the hormonal heterogeneity of prostate epithelial cell tumors and to demonstrate the progression of an androgen dependent tumor to androgen independence. These studies have confirmed the presence of androgen independent epithelial cells early in the progression of the tumor, but have failed to establish the presence of androgen independent cells in-vivo as the initial source of the tumors.

The androgen independent stromal cells have been suggested as possible androgen independent epithelial stem cells in-vivo. However since it has recently been demonstrated that the stroma does not serve as the source of the epithelial stem cells

(Evans and Chandler, 1987a, 1987b; English et al., 1987), the focus for a stem cell population rests on the androgen independent epithelial cells described by McKeehan and on the RDE cells described in this thesis.

As described earlier, androgen independence is a characteristic of both the amplifying and stem cell subpopulations suggested by Isaacs. This characteristic has been found both in the androgen independent cells described by McKeehan and the RDE cells. The crucial traits separating these two androgen independent epithelial cell types are their proliferative lifespan and ability to undergo terminal differentiation. The RDE cells display an unlimited proliferative capacity, presumably under autocrine control, but are unable to differentiate into mature secretory epithelial cells. On the other hand, the androgen independent rat prostate epithelial cells described by McKeehan have shown only a limited proliferative lifespan under exogenous growth factor control. The ability or inability of McKeehan's cells to differentiate, as judged by morphology or secretion, has yet to be reported.

The identification and characterization of these two cell types allows for the development of hypotheses for the maintenance of the prostate under normal conditions and the renewal of androgen independent epithelial tumor growth following androgen ablation therapy. Normal maintenance of the prostate epithelial cell population would be the exclusive domain of the androgen independent cells described

by McKeehan, playing the role of amplifying cells. The limited proliferative ability of these cells and their differentiation into secretory epithelial cells would suffice to replace the daily attrition of prostatic epithelial cells. The slow turnover rate of the prostatic epithelium under normal conditions means that the RDE cells, as the stem cells, would rarely be called upon to replace the amplifying cells.

When faced with more substantial epithelial cell loss through injury or accident, the RDE cells would undergo controlled proliferation, sufficient only to replace the number of amplifying cells which had reached the end of their proliferative capability in an effort to maintain a constant number of secretory epithelial cells in the prostate.

Androgen ablation therapy results in the involution of the prostate as approximately 90% of epithelial cells die (DeKlerk and Coffey, 1978). While the amplifying and stem cells are androgen independent for survival, and thus remain alive under these conditions, it is clear that androgens play a major role in controlling proliferation and differentiation of these cells. As demonstrated by androgen cycling experiments, the renewal of androgens induces a massive proliferation of epithelial cells. The initial proliferation would be of RDE cells to increase the number of amplifying cells so that the latter would further divide and subsequently differentiate to yield a full-size functional prostate. The lack of increased RDE and/or amplifying cell proliferation in the continued absence of androgens demonstrates the permissive

role of androgens on the proliferation of these cells that are nonetheless independent of androgens for cell survival. In humans the constant slow proliferation of the RDE cells would explain the 3-5 year period in which anti-androgen therapy appears to remain effective. Beyond this time frame the number of RDE cells would be sufficient to appear as a renewal of growth of the involuted prostate. However, the constant rate of RDE cell growth remains too slow to account for the rapid growth of renewed prostate cancer and thus the rate of cell death becomes a critical component of questions regarding the renewal of androgen independent tumors in the prostate (Isaacs, 1987).

F) CELL DEATH IN THE PROSTATE:

Castration of a rat results in dramatic metabolic changes leading to rapid tissue involution by a well coordinated and programmed active metabolic process (apoptosis) in addition to a passive declining of cellular activities (Bruchovsky et al., 1975; Lee, 1981; Tenniswood, 1986). The latter process includes the decrease and cessation of synthesis of prostate secretory products (SAP, PSBP). The rapid involution of the prostate originates from an increase in synthesis and activity of several degradative enzymes including acidic ribonuclease, cathepsin D and plasminogen activator (Engel, Lee and Grayhack, 1980; Tanabe, Lee and Grayhack, 1982; Rennie, Bouffard, Bruchovsky and Cheng, 1984). Other novel genes are also induced by the decline in

circulating androgens but play yet undefined roles in the apoptotic process; these include TRPM-2 and others (Montpetit et al., 1986, Léger et al., 1988; Lee and Sensibar, 1985; Saltzman, Hiipaka, Chang and Liao, 1987). TRPM-2 has been identified by cDNA sequencing to be virtually identical to the sulphated glycoprotein 2, a major secretory product of the Sertoli cells (Bettuzzi, Hiipakka, Gilna and Liao, 1989; Collard and Griswold, 1987). It is thought that TRPM-2 is involved in the maintenance of membrane integrity during apoptosis and protects the cell against complement induced cytolysis (Jenne and Tschopp, 1989; Kirszbaum, Sharpe, Murphy, d'Apice, Classon, Hudson and Walker, 1989).

Normal rat ventral prostate epithelial cells in-vivo or in-vitro synthesize TRPM-2 and die as a consequence of the removal of androgens from the environment. The androgen independent RDE cells do not synthesize TRPM-2 when androgens are omitted from the culture medium nor do they die or even slow their rate of proliferation in the absence of testosterone (Montpetit et al., 1986). Prostate epithelial cell death is preceded by a dramatic change in the cellular morphology from a tall columnar secretory cell to a shorter cuboidal non-secretory cell. This altered morphology is thought to occur by changes in the internal cytoskeletal components. To determine if chemically induced cell death or changes in the cytoskeleton would cause RDE cells to express TRPM-2 we initiated a series of experiments using the calcium ionophore A23187 and tumor necrosis factor. A23187 is a well characterized

divalent cationic ionophore that is capable of causing cell death in a dose-related manner, thus allowing for the potential analysis of the course of TRPM-2 induction. On the other hand, tumor necrosis factor α (TNF) was chosen to investigate cytoskeletal effects since TNF acts via specific dissolution of intermediate and microfilaments (Scanlon, Laster, Wood and Gooding, 1989). While numerous transplanted tumors and transformed cells have been shown to be sensitive to TNF, normal epithelial and fibroblast cells seem to be immune from TNF activity as are a number of transformed cells (Ruff and Gifford, 1981; Sugarman, Aggarwal, Hass, Figari, Palladino and Shepard, 1985). RDE cells also seem to be immune to the effects of TNF as judged by the lack of cell death, slowing of proliferation or the production of TRPM-2. As might be expected from these results, TNF also failed to alter the RDE cell morphology.

Dose response curves for A23187 induced cell death of RDE cells closely follow those determined for lymphocytes by Kaiser and Edelman (1978). Using a concentration of A23187 found to yield 50% cell death over 16 hours our initial results failed to detect TRPM-2 synthesis over the course of the experiment. Subsequently it was determined that with the use of a concentration of A23187 that yields 80% cell death over 16 hours a rapid and transient burst (2 h duration) of TRPM-2 expression occurs 10 hours after initiation of the treatment (L. Klein, Personal Communication). Our failure to detect the TRPM-2 expression during the first course of A23187

experiments was probably due to a combination of insufficient A23187 and inappropriate time point selection. It was unexpected that TRPM-2 would be expressed at high levels for a short period of time rather than being continually expressed as the cells succumbed to the effects of A23187. This may also explain the lack of TRPM-2 detection in the original A23187 experiments since cell death occurred at a relatively slow rate, presumably yielding constant but undetectable levels of TRPM-2 expression. At the higher dose all cell death occurred in a shorter time span, resulting in a sudden and massive increase in TRPM-2 expression that lasted only until the number of surviving cells declined below the detection threshold.

CONCLUSION:

This thesis describes the initial isolation and characterization of a series of lines of cells termed RDE (Rapidly-Dividing Epithelial) cells. We have demonstrated that these cell lines may prove to be a valuable model system for understanding androgen independent cell growth and division in the rat ventral prostate.

This isolation and characterization of rat prostate epithelial stem cells will provide the techniques and means for identification of the corresponding cells in humans. This will open a new avenue in basic and applied prostate cell biology, the complete analysis of the differentiation processes that results in a mature, androgen-dependent secretory epithelial cell. It will encompass the development of androgen response and dependence as well as the interactions between stroma and stem cells, stroma and amplifying cells and stroma and mature epithelium. In the longer term the isolation of RDE cells will also serve in the development of treatments that will be directed at the androgen-independent stem cells to prevent tumor renewal following hormonal therapy.

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APPENDIX 1

LIST OF ABBREVIATIONS

5α-A	5α-androstanedione
3α-Adiol	3α-androstanediol
3ß-Adiol	3ß-androstanediol
4-Adione	Δ^4-androstenedione
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
5α-DHT	5α-dihydrotestosterone
DHPE	Dysplastic human prostate epithelial cells
DME	Dulbecco's modified Eagle's medium
dpm	disintegrations per minute
DNA	deoxyribonucleic acid
cDNA	complementary DNA
ds-cDNA	double-stranded cDNA
ss-cDNA	single-stranded cDNA
DTT	dithiotrietol
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor

EGFR	epidermal growth factor receptor
FGF	fibroblast growth factor
GF	growth factor
h	hours
HBSS	Hanks buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
3α-HSOR	3α-hydroxy-steroid oxidoreductase (EC 1.1.1.50)
3ß-HSOR	3B-hydroxy-steroid oxidoreductase (EC 1.1.1.51)
17ß-HSOR	17ß-hydroxy-steroid oxidoreductase (EC 1.1.1.63)
ID	intradermal
IgG	immunoglobin G
IP	intraperitoneal
IV	intravenous
5α-R	Δ^4-3-ketosteroid-5α-reductase (EC 1.3.1.4)
5α-reductase	Δ^4-3-ketosteroid-5α-reductase (EC 1.3.1.4)
LAP	lysosomal acid phosphatase
MAI	McKeehan's androgen independent RVP epithelial cells
mRDE	RDE cell specific mRNA
mRNA	messenger RNA
NE	normal rat ventral prostate secretory epithelial cell
NF	normal rat ventral prostate fibroblast cell

PAGE	polyacrylamide gel electrophoresis
PDGF	platelet-derived growth factor
pH	$\log_{10} [H^+]$
pI	isoelectric point
PMSF	phenymethylsulfonyl fluoride
poly(A)$^+$ RNA	polyadenylated RNA
poly(A)$^-$ RNA	RNA not retained on oligo(dT)-cellulose column
PSBP	prostate steroid-binding protein
pRDE	plasmid clone of mRDE
RDE	rapidly-dividing epithelial
RNA	ribonucleic acid
rRNA	ribosomal RNA
RVP	rat ventral prostate
SAP	secretory acid phosphatase
SDS	sodium dodecyl sulfate
SSC	0.15 M NaCl, 0.015 M sodium citrate
T	testosterone
T^+	testosterone supplemented
T^-	testosterone depleted
TE buffer	10 mM TRIS-HCl pH 7.5, 1 mM EDTA
TGF-ß	transforming growth factor-ß

TNF	tumor necrosis factor
Tris	Tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
TRPM-2	testosterone repressed prostatic message -2
TTP	thymidine 5'-triphosphate
UTP	uridine 5'-triphosphate

APPENDIX 2

MATERIALS

Amersham Corp. (Oakville, Ontario)

α-[^{32}P]-dATP (3,000 Ci/mmol), α-[^{32}P]-dCTP (3,000 Ci/mmol), α-[^{32}P]-UTP (3,000 Ci/mmol), α-[^{35}S]-dATP (>1,000 Ci/mmol), ^{35}S-methionine (>800 Ci/mmol), [methyl-^{3}H]-thymidine (82 Ci/mmol), [5,6-^{3}H]-uridine (53 Ci/mmol), nick translation kits, restriction endonucleases, DNase I, peroxidase-linked anti-mouse IgG antibody, peroxidase-linked anti-rabbit IgG antibody, M13 sequencing primer.

BIO/CAN Scientific (Mississauga, Ontario)

Rabbit reticulocyte lysate

Boehringer Mannheim Canada (Dorval, Québec)

HEPES, TRIS, calf intestinal phosphatase, oligo(dT cellulose),

Charles River Breeding Company (Montréal, Québec)

Male Sprague-Dawley rats, 250-300 g

Collaborative Research (Lexington, Massachusetts)

Fibronectin, laminin, matrigel, EGF, endothelial cell GF, FGF, PDGF, pituitary extract.

FLOW laboratories (Mississauga, Ontario)

Penicillin, streptomycin, gentamycin, fungizone, horse serum.

GIBCO/BRL (Mississauga, Ontario)

Ham's F12 medium, DME, glutamine, insulin, trypsin, chicken serum, tissue culture dishes (Nunc), RNase H.

International Biotechnologies Incorporated (Toronto, Ontario)

Polyacrylamide, SDS, N',N'-methylene-bisacrylamide, urea, agarose, N,N,N',N'-tetra-methylenediamine, ammonium persulfate.

ICN Immunobiologicals (Montréal, Québec)

Mouse monoclonal anti-EGFR IgG, mouse monoclonal anti-desmin IgG, rabbit polyclonal anti-EGF IgG, TGF-ß, TNF.

New England Nuclear (Montréal, Québec)

[1,2-^{3}H]-testosterone.

Oncor (Gaithersburg, Maryland)

Oncogene probes

Pharmacia (Dorval, Québec)

Percoll, RNase A, Sephadex G-50.

Picker International (Ottawa, Ontario)

Dupont Cronex-4DC X-ray film.

SIGMA chemicals (St. Louis, Missouri)

Rat tail collagen, collagenase, dexamethasone, transferrin, ascorbic acid, retinoic acid, testosterone, 17ß-estradiol, acid hematoxylin, eosin, poly-L-lysine.

United States Biochemicals (Cleveland, Ohio)

Sequenase (modified T7 DNA polymerase).

APPENDIX 3

ANTIBODIES

Primary Antibodies

Antibody	Animal of origin	Type (*)	Dilution	Recognition
Desmin	Mouse	IgG (M)	1:500	Human, mouse, rat
EGF-R	Mouse	IgG (M)	1:1,000	Human, Mouse, rat
Vimentin	Mouse	IgG (M)	None	Pig, human, mouse, rat
PKK1	Mouse	IgG (M)	None	Pig, human, mouse, rat
PKK2	Mouse	IgG (M)	None	Pig, human, mouse, rat
PKK3	Mouse	IgG (M)	None	Pig, human, mouse, rat
EAB903	Mouse	IgG (M)	1:4,000	Human, mouse, rat

(*) M=monoclonal antibody P=polyclonal antibody

Secondary antibodies

Target	Animal of origin	Type (*)	Conjugated enzyme
Rabbit	Donkey	IgG (P)	Peroxidase
Mouse IgG	Sheep	IgG (P)	Peroxidase

(*) M=monoclonal antibody P=polyclonal antibody

APPENDIX 4

GROWTH FACTORS AND GROWTH INHIBITORS

Growth Factors

Bovine pituitary extract: Lot report (Collaborative Research)

37.5 μg/ml BPE supports the growth of fetal bovine heart endothelial cells 8.47 fold over no BPE in 7 days.

Concentration used: 50 μg/ml

Endothelial cell growth factor: Lot report (Collaborative Research)

4 ng/ml ECGF is mitogenic for fetal bovine heart endothelial cells. Heparin (1-5 units/ml) enhances mitogenicity.

Concentration used: 4 ng/ml **Heparin concentration:** 5 units/ml

Epidermal growth factor: Lot report (Collaborative Research)

4 ng/ml EGF caused a 2.0 fold increase in foreskin fibrblast cell number over control cultures without EGF after 7 days in culture.

Concentration used: 50 ng/ml

Fibroblast growth factor: Lot report (Collaborative Research)

100 ng/ml FGF supported the growth of fetal bovine heart endothelial cells 17 fold over no FGF in 7 days.

Concentration used: 100 ng/ml

Human platelet derived growth factor: Lot report (Collaborative Research)

1 to 5 half-maximal units of PDGF will significantly stimulate responsive mesenchymal cell types. [The biological activity of half-maximal units of PDGF is the recipricol of that dilution of a PDGF solution that stimulates Balb/c-3T3 cells to incorporate half the amount of tritiated thymidine that they would maximally incorporate under the influence of unlimited PDGF.

Concentration used: 1 half-maximal unit/ml

Growth Inhibitors

Human transforming growth factor-ß: Lot report (Collaborative Research)

Normal rat kidney cells cultured in soft agar demonstrate a half-maximal colony forming response with 1.35 ng/ml TGF-ß. The response range extends from 0.01-10 ng/ml.

Concentrations used: 2, 5, 10 ng/ml

Human recombinant tumor necrosis factor: Lot report (ICN Biomedicals)

Specific activity $2x10^7$ units/mg. 50% viability of MCF-7 cells, 20% viability of BT-20 cells after 72 h in medium supplemented with 500 units TNF-α. (Sugarman, et al, 1985)

Concentrations used: 5, 50, 200, 1,000 units/ml

APPENDIX 5

pRDE DNA AND AMINO ACID SEQUENCES

pRDE-0.25

End indicates termination codon, underlined sequence represents polyadenylation signal sequence. Restriction enzyme abbreviations are printed vertically.

```
                .         .         30        .         .         60
GATGGCTCAGAGGTTAAGAGCACTGACTGCTCTTCCAGAGGTCCTGAGTTCAATTCCCAG
AspGlySerGluValLysSerThrAspCysSerSerArgGlyProGluPheAsnSerGln

                .         .         90        .         .        120
CAACCACATGGTGCTCACAACCACCTACAATTCAATCTGGTGCCCTCTTCTGGTATGTAG
GlnProHisGlyAlaHisAsnHisLeuGlnPheAsnLeuValProSerSerGlyMetEnd

                .         .        150        .         .
GCAGAACACTGTATATGTAATAAATAAATAAATCTTTAAAAAAAAAAAAAAAAAA
```

```
        10          20          30          40          50          60
GATGGCTCAG AGGTTAAGAG CACTGACTGC TCTTCCAGAG GTCCTGAGTT CAATTCCCAG
     D   M         B           M       MPA     D             T
     D   N         S           B       NPV     D             I
     E   L         P           O       LUA     E             H
     1   1         6           2       112     1             2

        70          80          90         100         110         120
CAACCACATG GTGCTCACAA CCACCTACAA TTCAATCTGG TGCCCTCTTC TGGTATGTAG
    D N     B                          HB     M M
    R L     S                          GS     N B
    A A     P                          IP     L O
    3 3     6                          16     1 2

       130         140         150         160         170
GCAGAACACT GTATATGTAA TAAATAAATA AATCTTTAAA AAAAAAAAAA AAAAA
                                       A
                                       H
                                       A
                                       3
```

pRDE-1.5

End indicates termination codon, underlined sequence represents polyadenylation signal sequence.

```
                                30                            60
ATTCCGACCAAGCTGCAGAATAAGGAACATGTGATTGAGGCTCTTCGTAGAGCCAAGTTC
IleProThrLysLeuGlnAsnLysGluHisValIleGluAlaLeuArgArgAlaLysPhe

                                90                           120
AAGTTCCCTGGCCGCCAGAAGATCCACATCTCCAAGAAATGGGGCTTCACCAAATTTAAT
LysPheProGlyArgGlnLysIleHisIleSerLysLysTrpGlyPheThrLysPheAsn

                               150                           180
GCAGATGAATTTGAGGACATGGTTGCTGAGAAACGGCTCATTCCTGATGGCTGTGGGGTC
AlaAspGluPheGluAspMetValAlaGluLysArgLeuIleProAspGlyCysGlyVal

                               210                           240
AAATATATCCCTAATCGTGGCCCCCTGGACAAGTGGCGAGCCCTGCACTCCTGAGAGCCT
LysTyrIleProAsnArgGlyProLeuAspLysTrpArgAlaLeuHisSerEnd

                               270                           300
CCACAGTACTCTCCTGTACCCTACCAAATCATGTTCAGTAATAAATCTCACATCCAGAAT

                          320
GCTTAAAAAAAAAAAAAAAA
```

pRDE-1.5

Restriction enzyme abbreviations are printed vertically.

```
        10          20          30          40          50          60
ATTCCGACCA AGCTGCAGAA TAAGGAACAT GTGATTGAGG CTCTTCGTAG AGCCAAGTTC
           ABP                 AN          M     M
           LBS                 FL          N     B
           UVT                 LA          L     O
           111                 33          1     2

        70          80          90         100         110         120
AAGTTCCCTG GCCGCCAGAA GATCCACATC TCCAAGAAAT GGGGCTTCAC CAAATTTAAT
      B GH F      M X B           P               H
      S DA N      B H I           F               P
      T IE U      O O N           L               H
      1 23 1      2 2 1           1               1

       130         140         150         160         170         180
GCAGATGAAT TTGAGGACAT GGTTGCTGAG AAACGGCTCA TTCCTGATGG CTGTGGGGTC
            M     N         D
            N     L         D
            L     A         E
            1     3         1

       190         200         210         220         230         240
AAATATATCC CTAATCGTGG CCCCCTGGAC AAGTGGCGAG CCCTGCAGTC CTGAGAGCCT
                  N     B               H               D     M
                  L     S               G               D     N
                  A     T               I               E     L
                  4     1               2               1     1

       250         260         270         280         290         300
CCACAGTACT CTCCTGTACC CTACCAAATC ATGTTCAGTA ATAAATCTCA CATCCAGAAT
    SR            R               N                       F     B
    CS            S               L                       O     S
    AA            A               A                       K     M
    11            1               3                       1     1

       310         320
GCTTAAAAAA AAAAAAAAAA
```

pRDE-2.3

End indicates termination codon, underlined sequence represents polyadenylation signal sequence.

```
                    .         .         30        .         .         60
ACATGTGACTTATTAGAGGTGCATACAGATGAGGTAATAAAAGGGCGGTTTACCACACCG
ThrCysAspLeuLeuGluValHisThrAspGluValIleLysGlyArgPheThrThrPro

                    .         .         90        .         .        120
CTAACCACACCCACTTTAGGCAGGACTAAAGACGTATACGTACGTAGGCGGAACTACGTC
LeuThrThrProThrLeuGlyArgThrLysAspValTyrValArgArgArgAsnTyrVal

                    .         .        150        .         .        180
ACTAGCCAGGCCCACTTTTGGCATCTGAGGGGCGTTACGGGGGAAGGCATAAAAGACAGC
ThrSerGlnAlaHisPheTrpHisLeuArgGlyValThrGlyGluGlyIleLysAspSer

                    .         .        210        .         .        240
ACACAGGAAGTCTCGGTCTCTTTTCCTGGAGATCAGCTTCCGGTTCAGCCACTGAAAACT
ThrGlnGluValSerValSerPheProGlyAspGlnLeuProValGlnProLeuLysThr

                    .         .        270        .         .        300
GCTCCTCTGGGTTTGGTACCTTGTGAGTAATTTTCTACATTAAATACTTAGCTTTACTTT
AlaProLeuGlyLeuValProCysGluEnd

                    .         .        327
CAGTAAAAAAAAAAAAAAAAAAAAA
```

pRDE-2.3

Restriction enzyme abbreviations are printed vertically.

```
        10          20          30          40          50          60
ACATGTGACT TATTAGAGGT GCATACAGAT GAGGTAATAA AAGGGCGGTT TACCACACCG
AN              M               M
FL              N               N
LA              L               L
33              1               1

        70          80          90         100         110         120
CTAACCACAC CCACTTTAGG CAGGACTAAA GACGTATACG TACGTAGGCG GAACTACGTC
                                    S  R S
                                    N  S N
                                    A  A A
                                    1  1 1

       130         140         150         160         170         180
ACTAGCCAGG CCCACTTTTG GCATCTGAGG GGCGTTACGG GGGAAGGCAT AAAAGACAGC
     B  S            S   D M
     S  A            F   D N
     T  U            A   E L
     1  6            1   1 1

       190         200         210         220         230         240
ACACAGGAAG TCTCGGTCTC TTTTCCTGGA GATCAGCTTC CGGTTCAGCC ACTGAAAACT
                        B       S   A    H
                        S       A   L    P
                        T       U   U    A
                        1       A   1    2

       250         260         270         280         290         300
 CTCCTCTGG GTTTGGTACC TTGTGAGTAA TTTTCTACAT TAAATACTTA GCTTTACTTT
   M           KR                                  D  A
   N           PS                                  D  L
   L           NA                                  E  U
   1           11                                  1  1

       310         320     327
CAGTAAAAAA AAAAAAAAAA AAAAAAA
```

APPENDIX 6

ONCOGENE PROBE DETAILS

Oncogene	Source	Reference
v-abl	Abelson murine leukemia virus	Abelson and Rabstein, 1970
v-erbA	Avian erythroblastosis virus	Lai, Hu and Vogt, 1983
v-erbB	Avian erythroblastosis virus	Privalsky, Sealy, Bishop, McGrath and Levinson, 1983
v-mos	Moloney murine sarcoma virus	Tronick, Robbins, Canaani, Devare, Anderson and Aaronson, 1979
N-myc	Human neuroblastoma	Stanton, Watt and Marcu, 1983
v-myc	Avian myelocytomatosis virus	Stanton, Watt and Marcu, 1983
v-Ha-ras	Harvey murine sarcoma virus	Ellis, Defeo, Masyak, Young, Shih, Chang, Lowy and Scolnick, 1980
N-ras	Human promyeloctic leukemia	Hall, Marshall, Spurr and Weiss, 1983
v-sis	Simian sarcoma virus	Robbins, Devare, Reddy and Aaronson, 1982
v-src	Avian sarcoma virus	Schwarz, Tizard and Gilbert, 1983

APPENDIX 7

CURRICULUM VITAE

Curriculum Vitae

NAME: Michael L. Montpetit

DATE OF BIRTH: 14 March, 1959

PLACE OF BIRTH: No. 2 (Fighter) Wing, Royal Canadian Air Force Station, Grostenquin, France

CITIZENSHIP: Canadian

EDUCATION: Ecole Secondaire Charlebois
Ottawa, Ontario (Grades 9-13)

University of Ottawa
Ottawa, Ontario
B.Sc. (Hons) Biochemistry, 1983

University of Ottawa
Ottawa, Ontario
M.Sc. (Biochemistry) 1983-1986 Transferred to Ph.D. program

University of Ottawa
Ottawa, Ontario
Ph.D. (Biochemsitry) registered 1986-present

AWARDS: Ontario Scholar 1978
Secretary of State Post-Secondary Study Scholarship 1978
Cancer Research Society Studentship 1984-1989

PUBLICATIONS AND ABSTRACTS:

A) Full Papers:

Montpetit ML, Lawless KR, Tenniswood M; Androgen-Repressed Messages in the Rat Ventral Prostate; The Prostate 8, 25-36,1986

Léger JG, Montpetit ML, Tenniswood M; Characterization and Cloning of Androgen-Repressed mRNAs From Rat Ventral Prostate; Biochemical and Biophysical Research Communications 147, 196-203, 1987

Montpetit ML, Abrahams P, Clark AF, Tenniswood M; Androgen-Independent Cells of the Rat Ventral Prostate; The Prostate 12, 13-28, 1988

Montpetit ML, Tenniswood M; Does the Lack of Regression Associated mRNA Expression Render A Rat Ventral Prostate Epithelial Cell Line Androgen Independent?; Journal of Cellular Biochemistry 39, 285-292, 1989

Montpetit, M.L. and Tenniswood, M; Methods for the Separation and Culture of Mature Rat Ventral Prostate Epithelial and Fibroblast Cells; The Prostate 15, 315-325, 1989.

B) Abstracts

Montpetit ML, Tenniswood M; Evidence for an Androgen-Repressed mRNA in the Rat Ventral Prostate. (Abstract of poster presented at the International Symposium on Androgen Action, June 29-July 1, 1984, Montreal, Canada)

Montpetit ML, Tenniswood M; Characterization of a Fast-Growing Rat Ventral Prostate Epithelial Cell Line (Abstract of poster presented at the 13th Annual Southern Ontario Reproductive Biology Meeting, May 13, 1985, Kingston, Canada)

Léger JG, Montpetit ML, Tenniswood M; Cloning, Characterization and Localization of Androgen-Repressed and Constitutively Expressed Genes in the Rat Ventral Prostate (abstract of poster presented at the 13th International Congress of Biochemistry, August 30, 1985, Amsterdam, The Netherlands)

Montpetit ML, Tenniswood; Does The Lack of Androgen-Repressed Cell Death Associated mRNA Expression Render a Rat Ventral Prostate Epithelial Cell Line Androgen Independent? (Abstract of poster presented at the UCLA Symposia on Growth Inhibitory & Cytotoxic Polypeptides, January 30, 1988, Keystone, Colorado, USA

ABSTRACT

The human prostate is a secondary sexual organ that requires an uninterrupted supply of androgens to maintain structural and functional integrity of androgen dependent epithelial cells. Eventual failure of hormonal therapies for prostate cancer is attributed to the presence of androgen independent epithelial cells in the tumor.

Using the rat ventral prostate as a model of the human prostate, we have isolated and characterized an androgen-independent epithelial cell population present in the normal rat ventral prostate. These cells grow very quickly and have been termed Rapidly-Dividing Epithelial (RDE) cells. The RDE cells are completely independent of androgens for cell survival and do not secrete the androgen-dependent secretory proteins, secretory acid phosphatase or prostate steroid binding proteins. The epithelial cell origin of RDE cells was confirmed by cytokeratin expression and testosterone metabolism patterns and by purification parameters. Culture with various differentiation-inducing agents resulted in major morphological changes and structures reminiscent of those in the mature prostate but not in the expression of androgen-dependent secretory products. RDE cells demonstrate a very high in-vitro propensity for transformation yet no tumor growth in-vivo. None of the ten common "immortalizing" oncogenes tested were expressed. RDE cells may be the rat counterpart to androgen-independent epithelial cells causing renewed tumor growth in prostate cancer patients treated by hormonal therapies.