STUDIES OF A HUMAN PROSTATIC GENE

AND ITS PRODUCT:

RELATIONSHIP WITH RAT PROBASIN

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© Teresa L. Miller

A thesis
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in Partial Fullfillment of the Requirements
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Abstract

Rat probasin is a major protein product of the rat dorsolateral prostate gland. Recent evidence indicates that probasin is both a nuclear protein and a component of the secretory products of the gland. Expression of the probasin gene has been shown to be regulated by both androgens and zinc. Molecular characterization of the rat probasin gene has suggested translational regulation as a mechanism for channelling probasin to either the nucleus or the endoplasmic reticulum. Sequence analysis of the probasin gene and protein indicate that it is a member of a family of ligand carrier proteins although its specific ligand remains unknown. Preliminary analysis of human prostatic tissues indicated the presence of a probasin-related gene product. Because of the unusual regulation of the rat probasin gene and the potential importance of probasin as a novel marker for human prostatic function, the characterization of the human probasin-related gene product was initiated.

Using the rat probasin cDNA as a probe, hybridization studies were performed with total RNA and genomic DNA isolated from human prostate tissues. The data indicated that there is limited sequence homology between the rat probasin cDNA and putative probasin sequences in the human. Studies of human prostate cDNA binding to the nucleotide sequence of the rat probasin gene complement these findings by indicating possible regional similarities. The expression of this related gene in the human prostate seems to be significantly lower than the levels reported for the lobes of the rat prostate. Screening of a human prostate cDNA

library with the rat probasin cDNA under conditions of reduced stringency failed to identify an homologous human cDNA clone.

Using antiserum raised against purified rat probasin protein, immunodetection of human prostatic proteins by the technique of Western blot analysis was performed. Proteins immunologically related to rat probasin were identified in preparations of nuclear proteins from the human prostate. In addition to antibody recognition, the human probasin-related protein is of a similar molecular weight by gel electrophoresis, and shares the biochemical quality of acid solubility with rat probasin.

These studies have demonstrated that, although nucleic acid homology between human sequences and rat probasin is low, the amino acid sequences may share a higher degree of similarity. Thus there may be conserved amino acid sequence or conserved epitope(s) of the probasin gene which is not reflected at the nucleotide level. Future efforts to isolate the human probasin gene could utilize antibody screening instead of molecular hybridization, although cross-reactivity with other members of the ligand carrier family would have to be considered.

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Abbreviations

BPH	benign prostatic hyperplasia
CaP	prostatic carcinoma
pM-40	probasin cDNA in pAT153
rM-40	probasin cDNA insert
SVS-II	seminal vesicle protein two cDNA
P53	nuclear protein 53 Kd cDNA
pAT153, pUC19, pGem3Z	plasmid vector
MA150, MV1193, JM109	Eschericia coli bacterial stains
EcoR1, Pst1, BamH1, HindIII	restriction endonucleases
PC3	human prostatic cell line
DU145	human prostatic cell line
λgt10	lambda bacteriophage cloning vector
cDNA	complementary DNA
mRNA	message RNA
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
pfu	plaque forming units
IgG	gamma immunoglobulin
R23	rabbit antiprobasin polyclonal serum
NRS	normal rabbit serum
dCTP	deoxycytosine triphosphate
LB	Luria Bertani medium
TBE	Tris borate EDTA buffer
EDTA	ethylene diamine tetra acetic acid
SSPE	standard saline phosphate EDTA
SCP	standard citrate phosphate
SSC	standard saline citrate
SDS	sodium dodecyl sulfate
DH	Denhardt's solution
GRB	gel running buffer
PEG	polyethylene glycol 8000
XGAL	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
IPTG	isopropyl-β-D-thiogalactopyranoside
PAGE	polyacrylamide gel electrophoresis
AP	alkaline phosphatase
NBT	nitroblue tetrazolium

BCIP----------5-bromo-4-chloro-3-indolyl phosphate
WS ----------Wray and Stubblefield buffer
RSB----------reticulocyte standard buffer
EtBr ----------ethidium bromide
^{32}P ----------radiolabelled phosphorous
^{35}S ----------radiolabelled sulfur

Compounds
NaCl ----------sodium chloride
K_2HPO_4-------dipotassium phosphate
KH_2PO_4-------monopotassium phosphate
$(NH_4)_2SO_4$ ----ammonium sulfate
Na citrate-----sodium citrate
$MgSO_4$--------magnesium sulfate
NaH_2PO_4------sodium phosphate monobasic
Na_2HPO_4------sodium phosphate dibasic
NaOH ---------sodium hydroxide
NH_4Acetate---ammonium acetate
GITC----------guanidinium isothiocyanate
CsCl----------cesium chloride
MOPS---------morpholinopropane sulfonic acid
TCA ----------trichloroacetic acid
$MgCl_2$---------magnesium chloride
PMSF---------phenyl methyl sulfonyl fluoride
Tris/Cl--------Tris buffer pH with HCl
HCl ----------hydrochloric acid
H_2SO_4---------sulfuric acid
ddH_2O --------distilled deionized water

Units

g ----------gram	^oC --------degrees celsius
mg --------milligram	µCi--------microcurie
µg ---------microgram	cpm ------counts per minute
ng---------nanogram	dpm------disintigrations per minute
L ----------litre	mAmp----milliamperes
ml---------millilitre	V---------volts
µl----------microlitre	bp--------base pair
M----------molar	Kb--------kilobase pair
mM--------millimolar	Kd--------kilodaltons
µM --------micromolar	rpm ------revolutions per minute
N----------normal	Xg--------times the force of gravity

1. Introduction

1.1 Incidence and epidemiology of prostate disease:

The adult prostate is the site of two distinct types of cellular proliferation. The non-malignant enlargement of the gland is known as benign prostatic hyperplasia (BPH) and is the most common benign neoplasm detected in an internal organ (1). The malignant condition is referred to as prostatic carcinoma (CaP) and is the second most common type of cancer in males (2). These two prostatic growths share an extremely significant correlation with advancing age, rarely occurring under the age of forty (3,4).

It is estimated that eighty percent of males over fifty have symptoms arising from BPH (4). The prevalence of pathological BPH increases from eight percent in the fourth decade, to fifty percent in males aged fifty to sixty years, and peaking at eighty-eight percent by the age of ninety (5). Although the development of BPH in aging men is common, an understanding of its etiology is still lacking.

Cancer of the prostate was established as a leading cause of cancer death in men through the first recordings of mortality data in the 1930's (6). Since then, epidemiological data have reflected a significant correlation with patient age. Although the death rates for men aged 45 through 74 has decreased or stabalized for white and nonwhite men, the death rate for age group 75 through 84 has increased slowly but steadily (6). Presently, fifty percent of malignant tumors occur in men aged seventy years and older (6). Even though the majority of CaP never become clinically apparent in

the patient's lifetime, the mortality from this cancer is significant, accounting for approximately ten percent of cancer deaths in men (7). The incidental detection of CaP when performing biopsies of prostatic tissue accounts for twenty to thirty percent of diagnosis prior to metastasis (8). Two thirds of patients presenting have tumors localized within the prostate capsule, nine percent remain in the region of the prostate, and twenty three percent are diagnosed with distinct metastases (6).

Ninety five percent of prostate cancers are adenocarcinomas arising from glandular epithelium, usually in the periphery of the prostate (9). The benign disease generally develops in the periurethral region of the gland, constricting the urethra as the disease progresses (10). The benign cells continue to express prostatic proteins and perform their secretory function. The glandular appearance of the prostate remains in BPH tissue, although the cell number and often cell size is increased. In the cancerous state the prostate tissue involved loses its differentiated function as well as its glandular appearance.

1.2 Prostatic Anatomy

In 1912 Lowsley (11) described the anatomy of the human prostate based on his studies of the embryonic and fetal glands (12). Since this report there have been many varied descriptions of the anatomy of the human prostate (13) which have culminated in an acceptance of the descriptions by McNeal (10,14) and Tisell (12,15). These two anatomical representations agree upon the very

distinctive histological appearance of the medial lobe (15) or central zone (14), from that of the rest of the epithelial portion of the gland. This distinct area is comprised of two regions surrounding the ejaculatory ducts and located in the proximal portion of the prostate. It has been suggested that because of its histological similarity to the seminal vesicles and its intimate association with the ejaculatory ducts, that this medial or central region of the prostate may actually arise from the Wolffian duct along with the seminal vesicle rather than the urogenital sinus, the fetal structure from which the prostate develops (16).

The peripheral zone (14) can be subdivided into dorsal and lateral lobes (15) and represents the remaining epithelial portion of the prostate gland. These structures radiate laterally along the distal urethral segment (12,15). It has been noted by McNeal that a region of the peripheral zone that is situated next to the preprostatic sphincter, surrounding the proximal urethral segment, and above the verumontanum, is the site of benign prostatic hyperplasia (10). This region has been termed the transitional zone (13), and may correspond to the lateral division of the peripheral zone as described by Tisell (12,15). It is the outer section of the peripheral zone (dorsal lobe) that is the site of prostatitis and carcinoma (1).

1.3 Development of the Prostate

During the ambisexual stage of sex differentiation, the developing reproductive system consists of the undifferentiated gonads, the mesonephric tubules, the Wolffian and Müllerian ducts

and the urogenital sinus (17). The Müllerian duct regresses in the male as the rudimentary gonad differentiates into the testis. The Wolffian duct develops into the seminal vesicles, the vas deferens, and the epididymis, while the urogenital sinus becomes the bladder, the urethra, the bulbourethral gland and the prostate (18,19). It is well established that specific hormonal signals arising from the fetal testis are necessary for maturation into the male phenotype (20). In addition to the implied involvement of the testicular androgens, the inductive signals from the mesenchymal component of the undifferentiated structures perform their own function (17,21).

All of the primordial reproductive structures are composed of an epithelial element surrounded by mesenchymal cells. During urogenital development, the mesenchyme stimulates and prescribes the patterns of morphogenesis, cytodifferentiation and functional activity for the epithelial cells to adopt. It has been exquisitely demonstrated that the inductive signals from urogenital sinus mesenchyme can direct the formation of histologically prostate-like structures from urogenital sinus, bladder, vagina and prostate epithelial endoderm (16,22). Biochemically these prostate-like structures are able to express prostate-specific epithelial antigens, prostate specific protein profiles, androgen receptors and to proliferate in response to androgens (17,23-26). Without the presence of a functional androgen receptor in the mesenchyme of the tissue reconstructions, the androgen receptor positive epithelial component of the urogenital sinus will be instructed to form vaginal structures in spite of the presence of androgens (16,17,22).

A reactivation of inductive activity in the stroma of the mature prostate has been proposed as a mechanism of BPH development (1). Since inductive interactions in the embryonic prostate results in the conversion of urogenital sinus epithelial cells into a glandular epithelium, McNeal has hypothesized that a reawakening of these interactions causes new ductal branching into the inductive stromal tissue (1,27). The ability of the adult prostatic epithelium to respond to the inductive forces of urogenital sinus mesenchyme (28) and the conversion of nonglandular transitional cell bladder carcinoma to a glandular adenocarcinoma in response to urogenital sinus mesenchyme induction (29) lends support to these developmental interactions playing a role in prostatic carcinoma as well.

1.4 Steroid Responsiveness

The prostate is an androgen target organ (30,31) requiring this steroid hormone for it growth and development (32). The major circulating androgen is testosterone, of which ninety percent is produced by the testes (7). Through studies of steroid metabolism it has become apparent that plasma testosterone is reduced to 5α-dihydrotestosterone (DHT) at the target tissue by the action of 5α-reductase (7,33-35). This reduced form of testosterone is the active hormone (34,36-38), binding to nuclear androgen receptors in responsive cells (36,39,40).

The testicular and adrenal production of androgens is regulated by the hypothalamic-pituitary axis. Stimulation of androgen secretion from the adrenal gland is mediated by pituitary

adrenocorticotrophic hormone (ACTH), while testicular androgen release is induced by pituitary luteinizing hormone (LH) (7). The median eminence of the hypothalamus controls the release of ACTH and LH from the anterior pituitary through the action of corticotrophic releasing hormone (CRH) and LH releasing hormone (LHRH, GnRH), respectively. There are two negative feedback mechanisms monitoring the hypothalamic-pituitary axis, one acting through cortisol from the adrenal gland, the other acting through testosterone from the testes (7).

The dog and the rodent are common models for human prostatic disease (41). Although hyperplastic disease does not naturally occur in the rat, the spontaneous development of rat prostatic carcinoma has resulted in the Dunning tumor model (42). The beagle spontaneously develops BPH with advancing age but rarely has carcinoma been detected (41). Man and canine have similar profiles, in that castration will prevent prostate disease and normal testicular function is required for its development (43,44). Prostatic hyperplasia has been induced through hormone treatment of the dog (45-47), and will regress following castration (48). These hyperplastic changes in the canine prostate are characterized by an increase in nuclear androgen receptor content (49) which can be experimentally induced with estradiol in combination with androstanediol (49), or DHT (46) administered to the castrated animal.

The level of peripheral testosterone decreases gradually with advancing age (50-51), however the androgen concentration in the

prostate itself has been reported to rise dramatically after the age of sixty (52). The level of DHT has been indicated to be higher in the periurethral region of the human prostate, while the outer region of the gland accumulates a higher testosterone content (52). In both canine and man, the DHT level of the hyperplastic prostate has been reported to be five times that of the normal gland (52,53). However, Walsh has indicated that the DHT levels of normal prostate tissue reported previously are artificially low due to this steroid's degradation prior to autopsy (4). Walsh's findings, that normal and hyperplastic prostate tissue removed surgically contain identical DHT levels, questions the role of this steroid in BPH development (4). It has been observed that the concentration of androstanediols are lower in BPH (54); whereas, high levels of testosterone and androstanedione have been found in untreated carcinomatous tissue (55,56). The presence of these various testosterone metabolites in the aging prostate and their relationship with disease has been the focus of recent research and may be indicative of alterations in the activities of the enzymes involved in this metabolic pathway (57).

The changing steroid environment as the male ages coincides with an increase in BPH diagnosis (50,52). As well, the observation that most CaP exhibit some degree of androgen dependence indicates there is an important role for these hormones (58). This common androgen requirement for these two prostatic diseases is evidenced by the lack of either condition in eunuchs (48,58-60). It is unusual, however, that the seminal vesicles and the bubulbourethal gland, both of which are androgen responsive reproductive tissues, are

relatively immune to neoplastic change (61). The unchanged estrogen levels in conjunction with the observed changes in androgens may alter the estrogen/testosterone ratio and become the environment in which neoplastic changes in the prostate can occur (51,60). It has not been determined however if the action of these steroids is to induce pathogenesis or to permit its development (4).

The development of antiandrogen treatment for prostatic disease, especially CaP, has evolved from many endocrine studies (48,58,62). Originally, orchiectomy (7), adrenalectomy (63) and hypophysectomy (7) were the radical therapies in use. More recently the suppression of pituitary release of LH by estrogen (64) and progestin (7) therapies has been replaced with LHRH analogues (65), often combined with antiandrogens (66,67). This combination therapy is believed to hormonally castrate the patient by inhibiting the release of LH, and blocking androgen binding at the cellular receptor (66,67). Androgens produced by the adrenal gland can be converted to an active metabolite at the site of action, but their functions are blocked through the actions of flutamide or cyproterone acetate (7,66). Flutamide is a nonsteroidal analogue that acts solely by competitively inhibiting DHT binding to the androgen receptor; whereas, cyproterone acetate can inhibit LH release and block testosterone synthesis in addition to binding the DHT receptor (7). These therapies remain controversial, although they do address the possibility that prostatic tumors may acquire an exquiste sensitivity to steroids thus enabling the adrenal gland to support tumor growth (68). However, this has not been established, leaving

the possibility that prostatic tumors may be able to progress to an androgen insensitive state (69).

1.5 Zinc

Zinc is a ubiquitous element in biological fluids and tissues, reported to participate in the structures and activities of a variety of proteins (70). One such example of its structural importance is the DNA-binding zinc finger (71), also described as an antiparallel β-ribbon followed by an α helix (72). This motif, originally demonstrated for the transcription factor TFIIIA (71), is found in all members of the steroid hormone receptor family (73), as well as other proteins implicated as transcriptional regulators (74). Metalloenzymes, such as zinc-copper superoxide dismutase (erythrocuprein) and aspartate transcarbamylase also require zinc for their structural integrity, while carbonic anhydrase and carboxypeptidase A seem to require zinc for their catalytic function (70). The metallothionein family of proteins, rather than requiring zinc for their function, act as zinc binding proteins for the regulation of heavy metal homeostasis (75,76).

The concentration of zinc in the male reproductive organs is high, possibly for supplying zinc to spermatocytes (77). Of these organs, the prostate contains the highest zinc concentration (70) and is in fact the most highly concentrated source of zinc in the body (70,78-80). The only cell type with zinc levels exceeding those found in prostatic cells is sperm (79). The largest pool of zinc, however, resides in the bone and muscle (approximately 90%), due to mass as

well as concentration. Zinc levels in these three sites have been reported to be 87, 66 and 48 μg per gram of wet weight for prostate, bone and muscle respectively (70). The high zinc contents of the prostate gland and bone tissue may be one reason that many patients with cancer eventually succumb to bone cancer. Prostatic cells metastatic origin may more readily establish in the zinc-rich environment of the bone than in other tissue of low zinc content.

The androgen dependence of prostatic zinc is suggested by a variety of observations, the most obvious being low prostatic zinc prior to the onset of androgen secretion from the testis (79). Experimental observations of decreased zinc in prostatic tissue following orchiectomy, the restoration of precastration zinc levels by testosterone administration, and the hormonal depression of prostatic zinc with estrogen treatment all support the involvement of androgens for regulating zinc concentrations in the prostate (79). Further corroborating evidence is the impaired uptake of ^{65}zinc in estrogen treated animals (81). Conversely, zinc has been shown to influence many areas of the endocrine system, including secretion within the hypothalamic-pituitary axis, the release of pituitary gonadotropins, and the binding of endocrine factors at the target organ (70).

There have been suggestions in the literature that zinc levels are altered in the presence of prostatic disease (82-84). Reports indicate that zinc is increased in BPH and decreased in CaP (82), however, the <u>in vitro</u> uptake of ^{65}zinc is higher in cancer and lowest in prostatic hyperplasia (83,84). Other investigations have led to the

conclusion that there is a certain degree of intraprostatic variation in zinc concentration (85). Two independent studies have revealed that the concentration of zinc in the human prostate increases from the region closest to the bladder towards the apex of the gland (85,86). These findings correlate with the present anatomical definition of the human prostate (10,12), and adds some confirmation to the concept of morphologically distinct zones (85).

In the rat dorsolateral prostate zinc has been histochemically associated with the luminal border organelle of the epithelial cells and the sub-epithelial stroma of the lateral lobe (87). Both the dorsal and lateral lobe contain zinc in the nucleoli of their epithelial cells (87). The intracellular localization of zinc also has been reported to include secretory vacuoles in the human hyperplastic prostate (83,88). In the canine prostate zinc was located in the nucleolus, nuclear chromatin, lysosomes, secretory granules, and luminol secretions (89). Together these observations have prompted Chandler et al. to suggest that zinc is taken into the nucleus of the prostate through the stroma, condensed in the golgi apparatus, and transferred to secretory vacuoles for secretion from the cells to the lumen (89).

The presence of zinc binding proteins in the prostate has been reported previously. Binding of zinc-65 to low molecular weight proteins separated by gel filtration has been noted (90). The zinc ions bound to this protein remain intact during ion exchange chromatography and electrophoresis, but are removable by dialysis (90). The amino acid content has been found to be very rich in

histidine residues, while low in phenylalanine, tyrosine, arginine, lysine and methionine, and containing no detectable tryptophan residues (91). In comparison to the metallothionein family of proteins, this prostatic zinc binding protein has the potential to bind a large number of zinc ions through an interaction with histidine and cysteine residues (75,76,91). Zinc binding proteins found in other male reproductive tissues (testis, epididymis) (92,93) and a zinc binding protein from liver reported to be immunologically detected in most other tissues of the rat (94) appear to be nonhomologous to the metallothioneins (75,76), as well as unrelated to the above prostatic zinc binding protein (90,91). Metallothionein gene expression has been detected in the human prostate (Dodd, J.G., and Engel, J.F. unpublished observations) as well as in the rat prostate (Mullin, C., and Matusik, R.J. in preparation). The induction of metallothionein mRNA by zinc and cadmium in the dorsal lobe, but only by zinc in the lateral lobe suggests alternate regulating proteins for zinc levels in the lateral lobe of the rat prostate (Mullin, C., and Matusik, R.J. in preparation).

1.6 The Rat Prostate

1.6.1 Anatomy and biochemistry

The rodent prostate consists of three types of lobes; ventral, dorsal and lateral (95), although the coagulating gland is sometimes referred to as the anterior prostate in these animals. The ventral prostate is a bilobed structure which constitutes the major portion of the rat prostate; whereas, the lateral prostate can be described as

two small, separated lobes projecting off the dorsal prostate. The dorsal and lateral lobes of the rat prostate are often studied together as the dorsolateral prostate, but in fact these lobes are anatomically and biochemically distinct (16,96-99).

The rat ventral prostate has been utilized as a model system for studying the androgen regulation of genes, however there is no analogous region in the human prostate (100). The dorsolateral prostate of the rat is the site of prostatic cancer and the region of origin for the Dunning tumor model (42,101). Therefore, the dorsolateral prostate has been described as corresponding to the outer section of the peripheral zone where human prostatic carcinoma is known to develop (1). Benign hyperplasia does not spontaneously occur in the rat prostate, although the prostates of both the rat and the mouse undergo atrophy with aging (30). Experimental attempts to hormonally induce BPH in rodents causes prostatic growth but does not seem to reproduce the type of enlargement that is characteristic of human BPH (41).

Zinc uptake is highest in the dorsolateral prostate (98,102) and results in a lateral lobe content in the lateral lobe that is twenty-five times that of any other tissue (70). The zinc concentration is greatest in the lateral lobe, which has two and a half times the zinc content of the dorsal lobe (70). Depletion of zinc causes atrophy of the germinal epithelium of the testes (103) and spermatogenic arrest, in addition to degenerative changes seen in the prostate and seminal vesicles (70). The effects on the prostate and seminal vesicles can be reversed with the administration of androgens, chorionic

gonadotropins or zinc supplements (70). This hormonal dependence of zinc levels in the prostate can be demonstrated in the cytosol of the dorsolateral prostate of the rat, where the zinc levels are returned to normal in castrated animals treated with testosterone propionate (104).

The androgenic effects on the mature prostate maintain the morphology and functional activity of the gland (16). Castration of the rat results in an eighty percent decrease in prostatic DNA content (105,106). Most of the research on androgen regulation of gene expression has been derived from studies in the rat ventral prostate (16). The major secretory protein of this portion of the gland is prostatic binding protein (PBP) (107), which is constructed from two subunits; one containing the glycopeptides C3 and C2, the other containing the glycopeptides C3 and C1 (108-110). The mRNAs coding for C1, C2 and C3 are regulated by androgens (111), and coordinately decrease to one thousandth their normal levels upon castration of the animal. Only the steroids testosterone and dihydrotestosterone are able to restore PBP synthesis to its precastration level (112).

1.6.2 Dorsolateral Prostatic Proteins

A number of major protein products of the rat dorsolateral prostate have been described using biochemical and molecular biological techniques. These include a 20 Kd nonhistone protein (113), two androgen responsive mRNAs (M-40, SVS-II) (114-117), and the proteins DP-I and DP-II (118). The clone pM-40 has been

shown to hybrid-arrest the in vitro translation of two proteins expressed in the dorsolateral prostate (114). Further characterization has revealed two sites for the initiation of translation, resulting in in vitro translated proteins of approximately 20 Kd and 18 Kd (114, Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation). The two reading frames of the mRNA initiate at +1 and +52 of the coding sequence to generate proteins of 177 and 160 amino acids, respectively (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation). The difference between the two sequences is a 17 amino acid hydrophobic signal peptide (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation,119). Synthetic M-40 mRNA containing 5' deletions extending 3' into the presumptive signal sequence initiate translation at the second set of AUG codons to produce a protein of 160 amino acids (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation). This confirms that the two proteins can be coded for by one mRNA species in vitro and may be translationally regulated to yield an intracellular and secreted form of the protein (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation).

The amino acid composition of a 20 Kd nonhistone protein (20K-NHP) isolated from the nuclei of the rat dorsolateral prostate (113,120) is identical to the predicted amino acid composition of the pM-40 gene product. The isoelectric point and molecular size are further criteria supporting this assumption (113,114,Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation,120). Protein sequencing of the N-terminal amino acids has confirmed this

relationship (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation). This prostatic basic protein, with its corresponding gene, has been named probasin (121).

1.6.3 Probasin is a Nuclear and Secreted Protein

Matuo and coworkers had originally reported that probasin was a nuclear protein (113) and proceeded to isolate this salt soluble protein from nuclear protein preparations (120). Using monoclonal antibodies directed against probasin in immunofluorescent histology experiments they were able to demonstrate extensive binding to the luminal and acinar epithelium, but hardly any to the nuclei (121). Therefore, they concluded that the probasin found in their nuclear preparations was a contaminant from the secretory fluid and demonstrated a 60% reduction of nuclear probasin with the extraction of prostatic secretory fluid prior to preparation (121).

Matusik et al. have demonstrated probasin synthesis in both highly convoluted and less convoluted acinar epithelium of the lateral prostate using in situ hybridization histochemistry with their pM-40 probasin clone (116). They have demonstrated a low level of probasin mRNA in the dorsal lobe, and a clearly defined boundary between the two epithelial cell types of the dorsal and lateral lobes (122). The use of polyclonal and monoclonal antibodies to rat probasin (Spence, A.M., Sheppard, P.C., Davie, J.R., Matuo, Y., Nishi, N., McKeehan, W.L., Dodd, J.G., Matusik, R.J. submitted for publication) has shown staining in the lateral lobe epithelium along the cellular borders, in the ductal secretions and within the nuclei. Probasin was

demonstrated to be a nuclear protein in the highly convoluted acini, and both a nuclear and secreted protein in the cells lining the ducts of less convoluted acini. The dorsal and ventral lobes appeared to synthesize only the nuclear form of probasin.

1.6.4 Androgen Regulation of Probasin mRNA

When the effects of androgens are removed by castration the prostate gland becomes atrophied. It is not known if the dorsolateral prostate exhibits a lag period prior to cell division induction by androgen treatment of castrated animals, as described for the rat ventral prostate (123). The induction of probasin mRNA by androgen replacement is sixteen fold in the dorsolateral prostate (114); however, subsequent experiments revealed only a two fold induction (116).

The expression of the probasin gene is the highest in the lateral lobe of the rat prostate (116). In the dorsal and ventral lobes the expression of this gene is 33% and 4% relative to the lateral lobe expression (116). The probasin protein is decreased in the dorsolateral prostate in response to castration, and can be restored to normal amounts with the administration of androgens (113). The synthesis of mRNA coding for probasin is also reduced following castration and returned to normal levels after two (116) to four (114) days of testosterone treatment. The level of this mRNA in the ventral lobe is unaltered by androgen manipulation (114), however, the mRNAs for the PBP subunits respond to androgen removal and replacement as previously reported (112,114).

The expression of the SVS-II mRNA is also highest in the lateral lobe of the rat prostate, with 24% and 1% the expression level of the lateral lobe seen in the dorsal and ventral lobes, respectively(115). This gene and its androgen response serve as an internal control for probasin gene expression. The response to androgen withdrawal in the dorsolateral prostate resulted in a 75% decrease in SVS-II mRNA concentration by seven days post castration, and the levels remained at least this low as long as the animals remained androgen deficient(115,116). The probasin mRNA decreased by 50% initially in response to androgen withdrawal, but by day twelve the probasin message had returned to precastration levels(116,122). This rebound effect is statistically significant, and implies factors other than androgens are controlling the expression of this gene(122).

1.6.5 Zinc Regulation of Probasin mRNA

During the development of the prostate there is a marked increase in the zinc content of the dorsolateral prostate between the age of four to eight weeks, then a gradual rise until week sixteen (124). This change coincides with the functional differentiation of the gland and the inception of testicular androgen production (116,124). The probasin protein content in the nuclei mimics the rise of cytosolic zinc in this lobe during the same time period (124). The level of probasin mRNA in the dorsolateral prostate at four weeks of age is only 10% of the levels that will be maintained at maturity (116). The low level of probasin expression in the ventral lobe is only 8% of the dorsolateral probasin mRNA level being expressed at three

weeks of age. As the animal sexually matures, the ventral level decreases to less than 1% of the levels seen at three weeks of age in the dorsolateral prostate (116).

To analyze the effects of zinc on the expression of probasin Matusik et al., treated seven day castrated animals with either testosterone or zinc (116). Both SVS-II and probasin genes were induced by testosterone, but only the probasin gene was induced by zinc administration (116). As mentioned earlier, the lateral lobe has the highest zinc content of the three rat prostatic lobes (70). This may account for the difference in probasin levels between the ventral and dorsolateral prostate (116). It has been demonstrated that high concentrations of zinc persist in the prostate of the castrated rat (104) and this could be involved in the mechanism of probasin progression to an androgen independent state (122).

1.6.6 Response Elements in the Probasin Gene

The revelation that probasin mRNA accumulation could be due to transcriptional regulation by androgens and zinc (114,116,122) has encouraged Matusik (personal communication), to study the 5' regulatory sequence of this gene. The precise sequence of an androgen response element (ARE) has not been determined for the regulation of a cellular gene, however, Parker has identified a putative sequence in the mouse mammary tumour virus long terminal repeat (MMTV-LTR) (125-127). Within this region of MMTV there is a glucocorticoid response element (GRE) core sequence (TGTTCT) that is repeated four times (127) and can be found in the

probasin gene at nucleotides -203 to -182 (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation). Since Parker et al. (125,126) has shown this element to respond to androgens, Matusik (personal communication), has generated constructions of the probasin 5' regulatory sequences for further mapping of this ARE. The metal response element (MRE) of the metallothionein gene has been identified (128,129), and there is a sequence between nucleotides -61 to -48 of the probasin gene that shares some homology with this MRE (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation). This may represent a zinc regulatory site for the induction of probasin, but the studies to prove this have yet to be pursued (Matusik, R.J. personal communication).

1.6.7 Probasin is Related to Ligand Carriers

Recently it has been shown that the probasin gene is a new member of a ligand carrier family. This family consists of small homologous proteins, some of which are known to bind and transport small lipophilic molecules (130-132). The members of this family share two types of similarity; the arrangement of their gene structure and their primary structure directing three dimensional folding (130-135).

The probasin gene is divided into seven exons which span more than 15 Kb of DNA. All of the proteins which are closely related to probasin [alpha 2 microglobulin (α2u-G), mouse major urinary protein (MUP), bovine β-lactoglobulin (BBLG)] divide their coding domains into the first six exons and contain only 3' non-coding

sequences in the seventh exon (130-134, Spence, A.M., Sheppard, P.C., Davie, J.R., Matuo, Y., Nishi, N., McKeehan, W.L., Dodd, J.G., Matusik, R.J. submitted for publication). The exons are very similar in size and divide the protein sequence at its structural domains, however, the introns vary in length (130-133, Spence, A.M., Sheppard, P.C., Davie, J.R., Matuo, Y., Nishi, N., McKeehan, W.L., Dodd, J.G., Matusik, R.J. submitted for publication).

The first exon of probasin contains a leader sequence and a signal peptide in addition to its unique basic domain. In this basic region four of the nine residues are lysines which may form a putative nuclear transport signal (Lys X_2 Lys Lys Lys) that is unique to this member of the family (Spence, A.M., Sheppard, P.C., Davie, J.R., Matuo, Y., Nishi, N., McKeehan, W.L., Dodd, J.G., Matusik, R.J. submitted for publication). The members of this ligand carrier family are all acidic proteins (pI<6); however, probasin is a basic protein (pI>8). Exon five of most members of this family codes for a large number of acidic amino acids. Probasin is consistent in this organization, having a predicted pI<4 for exon 5; however, the other probasin exons are all basic in charge. The conservation of this sequence is likely due to its functional importance rather than its acidic nature, since it codes for an alpha-helix which forms a chimney structure (135;136).

The best characterized proteins in this family are retinol binding protein (RBP) and β-lactoglobulin (BBLG) (134-136). The three dimensional structure of these proteins have been determined (134-136) and have served as the basis for structures of other family members (130-133, Spence, A.M., Sheppard, P.C., Davie, J.R.,

Matuo, Y., Nishi, N., McKeehan, W.L., Dodd, J.G., Matusik, R.J. submitted for publication). The major portion of the polypeptide chain is folded into an eight stranded β barrel. This area is believed to be the site of ligand binding since the interior cavity is lined with hydrophobic side chains that could interact with an apolar molecule and protect it from the surrounding solvent (132-136).

2. Experimental Methods and Materials

Preliminary evidence provided by Drs. Dodd and Matusik (personal communication) has indicated that a nucleotide sequence related to rat probasin is present in the human prostate. The intriguing regulation of rat probasin by zinc and androgens (113,114,116,122,124) suggests an important biological function for this gene and its product. The literature has already suggested a role for zinc in human prostate disease (82-86) and has provided evidence for the importance of androgens to human prostate cancer (58,65-67) and BPH (48,62). If a gene and/or gene product similar to rat probasin were conserved in the human, its expression in cells of the human prostate could perhaps provide a more useful marker for prostate function and disease in man. With the demonstration that rat probasin is a novel member of a conserved ligand carrier family (Matusik, R.J. personal communication) it was deemed important to determine if there truly was a human counterpart to rat probasin.

Thus the original strategy of this thesis work was to isolate a probasin-like clone from a human prostatic cDNA library. When these efforts proved unsuccessful, it was necessary to determine if a related sequence could be detected by Northern or Southern blot hybridization. To analyze if any similarity existed in relation to the exons of this rat gene, dot blots and a Southern blot of the subclones of the probasin genomic clone were hybridized to cDNA made from BPH mRNA. Protein analysis was also pursued to determine if there was an immunologically related protein in the human prostate. The

materials and methods followed to complete these goals are described in detail below.

2.1 Maintenance of biologicals

2.1.1 Bacterial strains

The MA150 (C600Hfl) E. coli strain, used for bacteriophage propagation, was maintained on LB agar plates (Luria-Bertani medium; 10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl, 15 g agar per liter, pH 7.5) (137). The MV1193 E. coli strain, used in transformation experiments, was maintained on minimal glucose agar plates [50 ml of 20X salts (210 g K_2HPO_4, 90g KH_2PO_4, 20g $(NH_4)_2SO_4$, 10g Na citrate per liter) made to 500ml with ddH_2O, 500 ml 3% agar; after autoclaving the 2X salts and 3% agar were combined, cooled to 60°C and sterile 1 M $MgSO_4$, 20% glucose and 1% thiamine were added to concentrations of 1mM, 0.4% and 0.0005%, respectively] (137). Both cultures were stored as glycerol stocks at -70°C and originated from the American Type Culture Collection (Rockville, MD).

2.1.2 Plasmids

The plasmid pGEM3Z and its host (Promega, Madisson, WI) were used for all subcloning of human M-40 cDNA clones. This vector is a derivative of the pUC19 recombinant plasmid (138) having a multiple cloning site, and SP6/T7 promoter regions, and is maintained in the restriction minus host E. coli JM109 on LB agar plates containing 200 μg/ml ampicillin (10 mg/ml stock of ampicillin

stored at -20°C) (137). Plasmid DNA was maintained at -20°C after isolation by large scale plasmid preparation (137) based on Clewell's amplification in chloroamphenicol (139), and DNA isolation by the alkali lysis method of Birnboim and Doly (140). Cesium chloride/ethidium bromide density gradient centrifugation (Ti75, 55000 rpm for 18 hours, 45000 rpm for 1 hour, 25°C) was used to further purify plasmid DNA from bacterial chromosomal DNA and proteins (141) employing a Beckmann (Fullerton, CA) L8 ultracentrifuge and Quick-Seal Ti75 disposable centrifuge tubes. Ethidium bromide was removed from the isolated plasmid by multiple extractions with isoamyl alcohol (142).

The plasmid pM-40 is a recombinant pAT153 vector containing 743 nucleotides of the rat probasin cDNA cloned into its Pst1 site (114). This recombinant molecule is maintained in E. coli MV1193 plated on LB agar containing 15 μg/ml tetracycline (12.5 mg/ml stock of tetracycline in 50% ethanol maintained at -20°C) (137). Plasmid DNA isolated as described above, and rM-40 insert DNA, isolated by digestion with the restriction enzyme Pst1 and electroelution from a Southern gel (137,143), are stored as DNA stocks at -20°C. The rat probasin genomic subclones isolated by A. Spence (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation) are maintained at 4°C as DNA stocks and were obtained from Dr. R.J. Matusik.

2.1.3 Cell lines

The prostatic cell lines PC3 (144) and DU145 (145) were maintained by S. Downes in Eagle's minimal essential medium with Earle's salts and L-glutamine, supplemented with 10% fetal bovine serum and 5% antibiotic/antimycotic (Gibco, Santa Clara, CA). Periodically cells were harvested by mild trypsin digestion and stored at -70°C for later use.

2.1.4 λgt10 BPH and CaP cDNA libraries

The two λgt10 human prostatic cDNA libraries were prepared by Dr. J.G. Dodd and G. Dotzlaw. After isolating poly A^+ mRNA (146,147), cDNA was synthesized by the methods of Okayama and Berg (148), then inserted into the EcoR1 site of the λgt10 vector DNA using EcoR1 linkers (149). The recombinant bacteriophage DNA was then packaged into the phage particles in vitro (150). The BPH and CaP libraries were amplified by plate lysis (137) to $5.25X10^{11}$ pfu/ml and $4.33X10^{11}$ pfu/ml, respectively, and maintained at 4°C in SM buffer (5.8 g NaCl, 2.0 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris/Cl pH7.5, 5 ml 2% gelatin per liter) with cholorform (137). Both λgt10 cDNA libraries were also stored at -70°C as glycerol stocks (151).

2.2 Screening the Library

The λgt10 BPH cDNA library was diluted to 10^{-6}, and 25 µl was used to infect each 100 µl aliquot of MA150 E. coli cells according to the methods described by Maniatis et al., (137). Recombinant plaques were grown overnight at 37°C in top agarose (LB medium as

described but with 7 g agarose per liter, containing 0.2% maltose and 10 mM $MgSO_4$) plated onto LB agar plates. The following day nitrocellulose filters (BioRad, Mississauga, Ont.) were used to transfer replicas of recombinant plaques from eighteen plates containing greater than 300 pfu/plate according to described procedures (152). The filters were prehybridized at 42°C for 3 hours in 5X SSPE (1X is equivalent to 0.18 M NaCl, 0.01 M NaH_2PO_4 and 1 mM EDTA, pH 7.4), 5X Denhardt's solution (153) (50X Denhardt's solution contains 1% Ficoll 400, 1% polyvinyl pyrrolidone and 1% bovine serum albumin fraction V), 0.1% SDS and 200 µg/ml denatured sonicated salmon sperm DNA. Filters were hybridized to 200 ng of nick translated (Amersham, Oakville, Ont.) pM-40 having a specific activity of $2X10^8$ dpm/µg (α^{32}PdCTP, 3000 Ci/mmol, DuPont, Mississauga, Ont.) for 18 to 36 hours (154,155). Filters were later washed with 2X SSC (1X SSC contains 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS four times for 10 minutes each at room temperature, followed by two washes with 1X SSC, 0.1% SDS at 42°C for 15 minutes each. Filters were then blotted dry and autoradiographed on Cronex X-ray film with a Quanta III intensifying screen at -70°C (156).

Positive phage plaques hybrizing to the pM-40 clone under the described hybridiztion conditions were isolated using a sterile pasteur pipet and eluted overnight at 4°C into SM buffer containing 1 drop of chloroform (137). Each positive recombinant underwent successive rescreenings until all plaques on the filters were positive. All purified λgt10 recombinants (137) were grown in microtiter plates in LB media overnight at 37°C using the liquid lysis method of

Love and Minton (157) for the testing of β-lactamase activity (ampicillin resistance) by the chromogenic reaction in the presence of cephalosporin(McNicol, P. personal communication).

2.3 Subcloning

Positive purified M-40 recombinants were propagated by confluent plate lysis in MA150 E. coli cells (137). The phage were harvested in SM buffer (137), precipitated with an equal volume of 20% PEG, 3 M NaCl in SM buffer (137,158), and phage DNA was isolated (137). The restriction enzyme EcoR1 (159) was used to digest the human insert DNA out of the λgt10 vector arms. The insert DNA was ligated to similarly digested pGEM3Z using the enzyme T4 DNA ligase (160,161) and the method described by King and Blakesley (162), in an attempt to subclone the human prostate sequence into the plasmid vector. The newly recombinant plasmid was then used to transform competent MV1193 cells by the calcium chloride heat shock technique (163,164). Transformants were grown overnight at 37°C on LB agar medium supplemented with 200 μg/ml ampicillin (137,165) and induced for color selection through β-galactosidase activity in the presence of 50 μg/ml Xgal and 5 mM IPTG (Promega, Madisson, WI). White colonies representing recombinant human M-40 plasmids were isolated, grown overnight at 37°C on LB agar plates containing 200 μg/ml ampicillin, and maintained at 4°C for further study.

To establish if the insert itself represented a piece of plasmid DNA containing the gene for ampicillin resistance, isolated phage

insert DNA was ligated in the absence of pGEM3Z and used to transform MV1193 cells as described (137).

2.4 Southern blots

Purified positive λgt10 recombinants were digested with EcoR1 (137,159) and electrophoresed (137) in 1X TBE (10X TBE contains 108 g Tris, 55 g Boric Acid, 40 mls 0.5 M EDTA per litre), 1% agarose (BioRad, Mississauga, Ont.) at 18 mA for 18 hours. The gels were stained with 0.5 μg/ml ethidium bromide, and insert sizes were determined by comparison to DNA markers (Pharmacia, Baie D'Urte, Que., and BRL, Gaithersburg, MD) by visualization with UV illumination. Permanent records of the insert sizes were obtained by photographing the UV illuminated gel with Polaroid Type 57 film, and a red filter.

Southern blots (166) were prepared by the alkali transfer method of Smith and Summers (167), and blotted to MSI NitroPlus 2000 membrane (Fisher, Winnipeg, Man.). Southern blots were prehybridized at 42°C in the presence of 6.6X SCP (1X SCP contains 0.1 M NaCl, 0.03 M Na_2HPO_4, 1 mM EDTA, pH 6.2), 5X Denhardt's solution (153), 1% N-lauroyl sarcosine (Sigma, St. Louis, MO) and 200 μg/ml denatured sonicated salmon sperm DNA for 3 to 18 hours. Blots were hybridized to nick translated (155) ^{32}P-rM-40 insert or whole plasmid DNA ($3.4X10^8$ and $1.2X10^8$ dpm/μg, respectively) overnight at 42°C, and washed with 1X SSC, 0.1% SDS twice at room temperature for 30 minutes, followed by 0.1X SSC, 0.1% SDS twice at 50°C for 15 minutes.

Human prostatic tissue specimens obtained from pathology (Health Sciences Centre or Seven Oaks Hospital), had been classified as predominately BPH or CaP based upon histopathological techniques. Human genomic DNA was isolated from prostate specimens by overnight incubation at 37°C with a solution of 0.1 M Tris/Cl (pH 7.5), 0.1 M NaCl, 0.005 M EDTA and 1% SDS, containing 100 μg/ml Proteinase K (Boehringer Mannheim, Mannheim, W. Germany). The genomic DNA was purified by multiple phenol/chloroform extractions (137) and precipitated with 0.2 M NaCl and 2.5 volumes of 95% ethanol at -20°C overnight. The DNA was recovered by centrifugation (12000xg, 15 minutes, 22°C), rinsed with 70% ethanol (-20°C), dryed in a Savant SpeedVac, and resuspended in an appropriate amount of TE buffer (10 mM Tris/Cl, 1 mM EDTA, pH 8.0) to obtain a concentration of approximately 1 μg/μl as determined spectrophotometrically at 260nm (137). The genomic DNA was digested with various restriction enzymes (Pharmacia, Baie D'Urte, Que., and Boehringer Mannheim, Mannheim, W. Germany), resolved by electrophoresis and blotted to NitroPlus 2000 using the alkali transfer method as previously described (142,166,167).

Southern transfers of genomic DNA were prehybridized at 35°C in a solution containing 50% deionized formamide or 42°C without formamide (137) and 6.6X SCP, 5X Denhardt's solution (153), 1% N-lauroyl sarcosine, 200 μg/ml denatured sonicated salmon sperm DNA for 3 hours. Hybidization to nick translated (161) ^{32}P labelled rat probasin cDNA sequence, or rat probasin genomic subclones (BH 3.0,

BB 2.7, BH 2.0) of at least $2.7X10^8$ dpm/μg, was carried out for 48 hours at the above temperatures in the prehybridization buffer. The blots were washed and autoradiographed as previously described.

Southern dot blots (168,169) were prepared of the rat probasin genomic clones (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation) using the Schleicher & Schuell (Keene, NH) minifold apparatus. Samples were applied to Nitrocellulose membrane (BioRad, Mississauga, Ont.) using the described method, loading 5 and 10 μg of each clone per well. The nitrocellulose filters were prehybridized, hybridized and washed as previously described for genomic Southerns, with the exception of a 55°C hybridization temperature and a hybridization solution lacking formamide. As well, the probe used was ^{32}P random prime labelled (Amersham, Oakville, Ont.) BPH cDNA ($1.8X10^9$ dpm/μg) (148).

Southern transfers of the rat probasin clones described above were prepared from DNA digested with the appropriate enzymes to resolve the cloned inserts by electrophoresis. The gels were blotted to NitroPlus 2000 (142,166,167) and hybridized to a ^{32}P random prime labelled (Amersham, Oakville, Ont.) BPH cDNA (148) probe ($1.8X10^9$ dpm/μg) as previously described, using the same hybridization and wash conditions, except for a hybridization temperature of 42°C.

2.5 Northern blots

Human BPH and CaP total RNA was isolated from pathology samples using a lysis buffer (4M Guanidinium isothiocyanate (GITC),

5mM sodium citrate, 0.1M β-mercaptoethanol, 0.5% N-lauroyl sarcosine) and a ultracentrifugation gradient (5.7M CsCl, 0.1M EDTA, pH7.0) (137,146,170). Message RNA (poly A^+ RNA) was selected by oligo dT cellulose binding by the column (147) or batch (171) methodology. Total RNA or poly A^+ RNA samples were heat denatured at 65°C for 20 minutes in the described sample buffer (137) and loaded onto formaldehyde denaturing 1.2% agarose/MOPS Northern gels (137). Electrophoresis was carried out for 18 hours at 30V in 1X running buffer (5X GRB prepared using 100 ml 1 M MOPS, pH 7.0, 8.3 ml 3 M Sodium Acetate, pH 5.2, 5 ml of 0.5 M EDTA, pH 8.0, and diluting to 500 mls with ddH_2O) (137,172). Northern blots were prepared by transferring RNA to NitroPlus 2000 membrane using 20X SSC as the transfer buffer, according to previously described techniques (137,173,174). Northern dot blots were prepared in triplicate as described previously (168,169).

All RNA blots were hybridized to pM-40 labelled as before ($>2.0X10^8$ dpm/μg) (154,155) or rM-40 insert labelled by random prime synthesis ($3.6X10^7$ dpm/μg) in 5X SSPE, 5X Denhardt's solution (153), 0.1% SDS, and 200 μg/ml denatured sonicated salmon sperm DNA. The Northern blots were hybridized at 42°C for nick translated probes and 47°C for random primed probes. The RNA dot blots were used to study the effect of varying the hybridization stringency by using hybridization temperatures of 42°C, 55°C or 65°C (175). Washes were done as for the previously described Southern hybridization procedures.

2.6 In vitro translations

Translation analysis was performed by incubating 1 μg of total human RNA from normal prostate tissue, and prostatic BPH and CaP tissues, in the rabbit reticulocyte lysate and wheat germ extract systems (Promega, Madisson, WI). Translation reactions were performed using the described method (142) with L-^{35}S-methionine (800 Ci/mmol, DuPont, Mississauga, Ont.). Following a 60 minute incubation at 30°C, 3 μl aliquots of the reactions were TCA precipitated onto GF/C filters (Whatmann). After drying briefly under a heat lamp the L-^{35}S-methionine incorporation was determined by scintillation counting (142) using Aquosol (DuPont, Mississauga, Ont.) scintilation fluid and a LKB beta-counter. An aliquot of each of the remaining translation products were electrophoresed on 12.5% Laemmli SDS-PAGE gels for 4 hours at 200 volts (176,177), stained with Coomassie Blue, destained for visualization of SDS-PAGE markers (BioRad, Mississauga, Ont.), dryed onto Whatmann 3M paper, and directly autoradiographed onto Cronex X-ray film at -20°C. A second aliquot of each sample of the translation products were immunoprecipitated with various dilutions of normal rabbit serum and rabbit antiprobasin polyclonal immunserum. Protein-antibody complexes were isolated with pansorbin (Staphylococcus aureus protein A, Calbiochem) (142,178,179). The immunoprecipitated proteins were either TCA precipitated and counted, or electrophoresed on SDS-PAGE gels and autoradiographed as before (142,176,177).

2.7 Protein Isolation

Acid soluble nuclear proteins were isolated from three human prostate samples (10982, 20922, 19343) and rat whole prostate (180). Tissue was minced with scissors, then homogenized in 40 mls WS buffer [10 mM Pipes, pH 7.0, 1 M Hexylene glycol (2-methyl-2,4-pentandiol), 2 mM $MgCl_2$, 30 mM Na butyrate, 1.0% (v/v) thiodiglycol], supplemented with 400 µl of 100 mM PMSF in a Waring blender at 4°C, for 60 seconds, at low speed. The homogenate was filtered through cheese cloth using a Buchner funnel, and the filtrate was centrifuged at 3000xg for 10 minutes at 4°C (JS-13, 4500 rpm). The pellet, which contains the nuclei, was resuspended in 20 mls WS buffer containing 200 µl 100 mM PMSF. The suspension was homogenized in a Dounce homogenizing pestle with three passages. The resulting homogenate was again centrifuged at 3000xg for 10 minutes at 4°C. The pellet was resuspended in 20 mls RSB buffer (10 mM Tris/Cl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 30 mM Na Butyrate) containing 200 µl of 100 mM PMSF.

The nuclear suspension was again homogenized in the Dounce homogenizing pestle and made 0.6 M in NaCl. It had been noted previously that rat probasin was soluble in this concentration of NaCl (Davie, J.R. personal communication). The suspension was centrifuged at 12000xg for 10 minutes at 4°C. The supernatant contains the 0.6 M salt soluble nuclear proteins. The nuclear pellet was resuspended in 12 ml ddH_2O. The supernatant and nuclei suspension were made 0.4 N in H_2SO_4, incubated for 80 minutes at 0°C, and centrifuged for

10 minutes at 12000xg at 4°C. The supernatants which were placed into Spectra/Por 6-8 Kd dialysis tubing, were dialyzed overnight against 0.1 N acetic acid. The dialysis was continued against ddH_2O for 48 hours at 4°C with several changes of dialysate. Protein suspensions were collected, frozen at -70°C and lyophilized. Samples were resuspended in 1 ml ddH_2O and then quantitated by Lowry protein assay (181). The protein profiles were analyzed by 12.5% SDS-PAGE (142,176,177), stained with Coomassie Blue, then to visualize low abundance proteins, destained with methanol:acetic acid (40:10) and silver stained (182).

2.8 Western blots

Using the BioRad miniprotein gel apparatus, 5 µg of each protein sample was electrophoretically separated on a 12.5% SDS-PAGE gel (176,183), then transferred to NitroPlus 2000 membrane using the continuous buffer system (39 mM Glycine, 48 mM Tris, 0.0375% SDS, 20% methanol) (184) on a LKB NovaBlot apparatus. The protein blots were treated according to the methods described in the ProtoBlot technical manual (185). Previously a polyclonal immune serum was raised against partially purified rat probasin protein. The protein had been isolated as described above in Section 2.7. Purification was by electrophoresis on a 6.7 M Urea/acetic acid gel for separation. This purified probasin preparation was used to raise rabbit anti-probasin polyclonal antiserum which was later titered for its reaction against the crude protein preparation (Davie, J.R., Dodd, J.G., personal communication). The anisera were used at dilutions of 10^{-2}, 10^{-3} and 10^{-4}. Bound antibodies were visualized with an

alkaline phosphatase (AP) catalyzed color reaction after binding alkaline phosphatase conjugated goat antirabbit IgG. Color was developed using the NBT and BCIP substrates in AP buffer (100 mM Tris/Cl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) (185).

3. Results

3.1 Library Analysis

It has previously been shown by Southern dot blot analysis that the rat probasin cDNA sequence (M-40), but not the sequence of three other rat prostatic clones (prostatic binding protein, 20K glycoprotein, RWB), cross-reacted at 55°C with ^{32}P-labelled cDNA synthesized from human BPH mRNA (Dodd, J.G., Matusik, R.J. personal communication). Conversely, labelled pM-40 hybridized at 42°C, but not 55°C, to a Northern dot blot of total RNA from human prostate. Neither of these temperatures allowed hybridization of pM-40 to human uterus total RNA spotted onto the same nitrocellulose filter. Based on this preliminary study, it was decided that it was likely that the human prostate does express a "probasin-like" message which might be selected from a cDNA library. From these initial observations, it was decided that a λgt10 BPH cDNA library would be screened with pM-40 at 42°C, as described (137,152-166).

The first screening resulted in twenty-three positive plaques, all of which remained positive on second screening (Figure 1). A number of these were purified by confluent plate lysis as described in experimental procedures (137). The isolated recombinant bacteriophage DNAs were restriction digested with the endonuclease EcoR1 (159) to reveal an insert estimated to be slightly greater than 3000 bp. A Southern blot (137,142,166,167) of these digested lambda clones was probed with ^{32}P pM-40 (Figure 2) and ^{32}P rM-40 insert (Figure 3) at 42°C under aqueous conditions. The

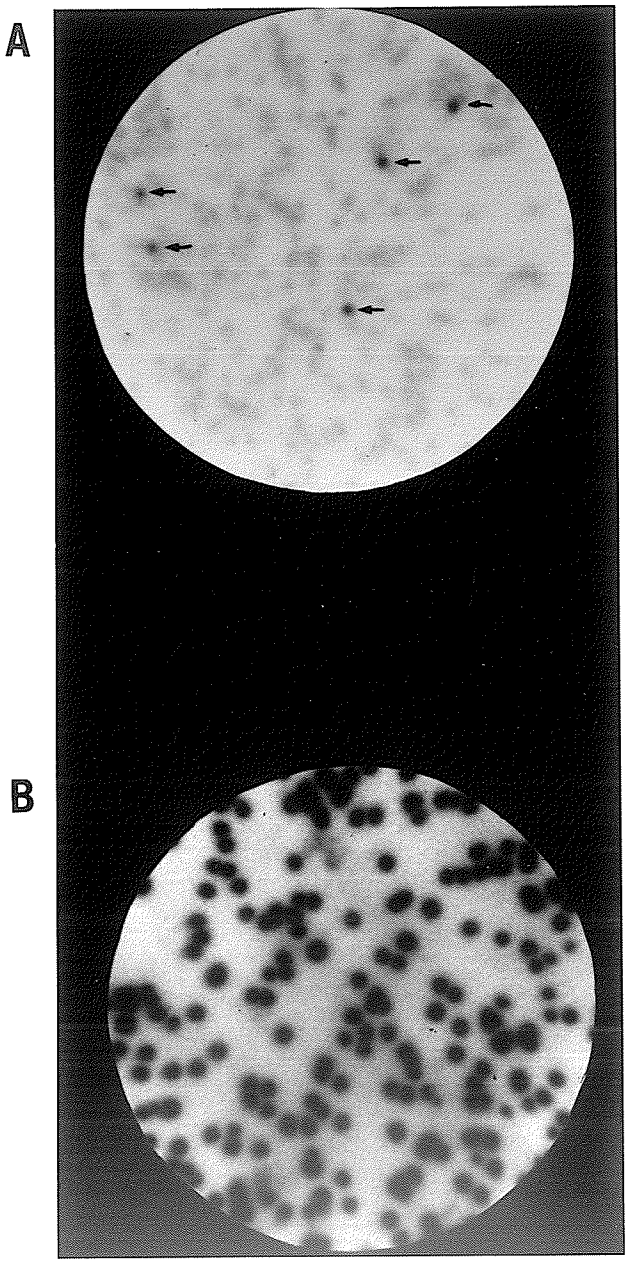

Figure 1 A. First screening of the λgt10 BPH cDNA library with pM-40.
B. Second screening of one of the positive plaques with pM-40.
The pM-40 DNA was labelled to $2X10^8$ dpm/μg and hybridized to the filters at 42°C, 5X SSPE, 5X Denhardts solution, 0.1% SDS, 200μg/ml denatured salmon sperm DNA. The exposure for A was five days, B was overnight.

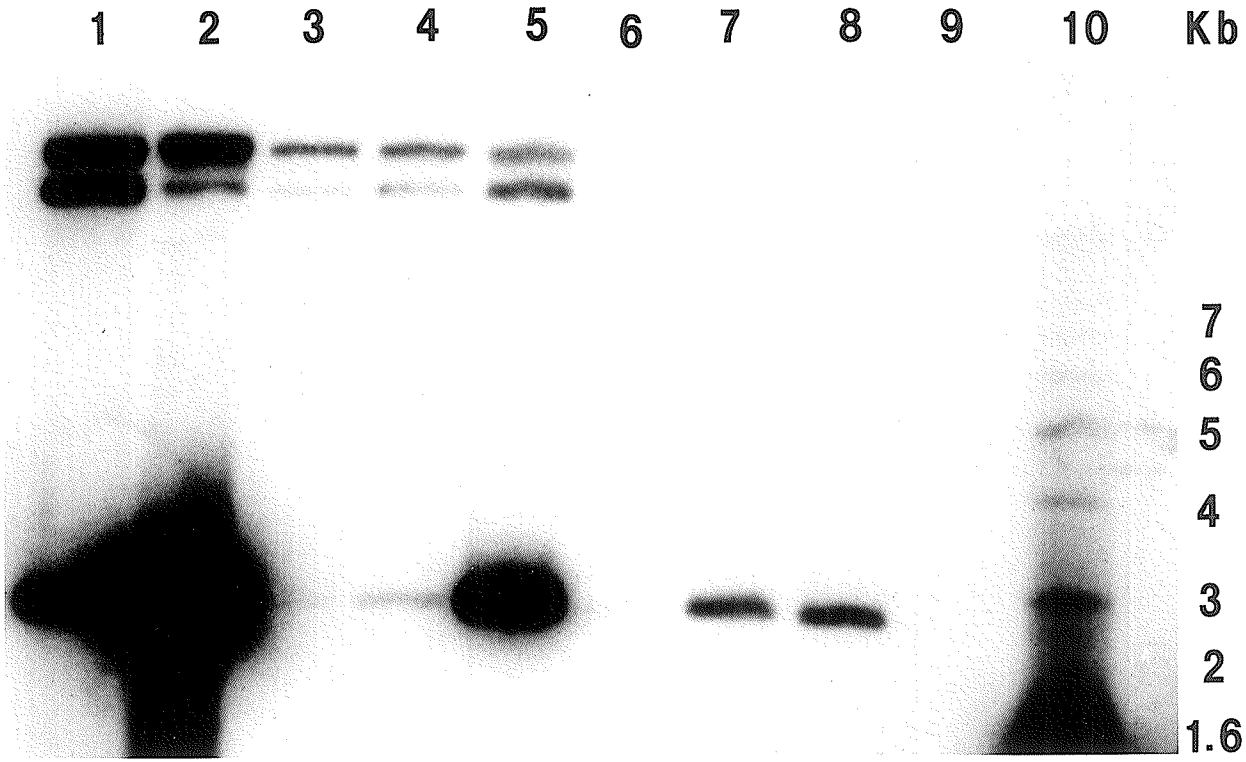

Figure 2 Lambda gt10 recombinant clones were digested with EcoR1 endonuclease, then electrophoresed on a 1% agarose gel and Southern blotted. Nick translated pM-40 ($1.2X10^8$ dpm/µg) was hybridized to phage and insert DNA at 42°C, 6.6X SCP, 5X DH, 1% n-lauroyl sarcosine, 200µg/ml denatured salmon sperm DNA. Lane 1 to 8 represent different isolated phage clones, lane 9 is blank, and lane 10 contains marker DNA (1Kb ladder) Exposure was for six days.

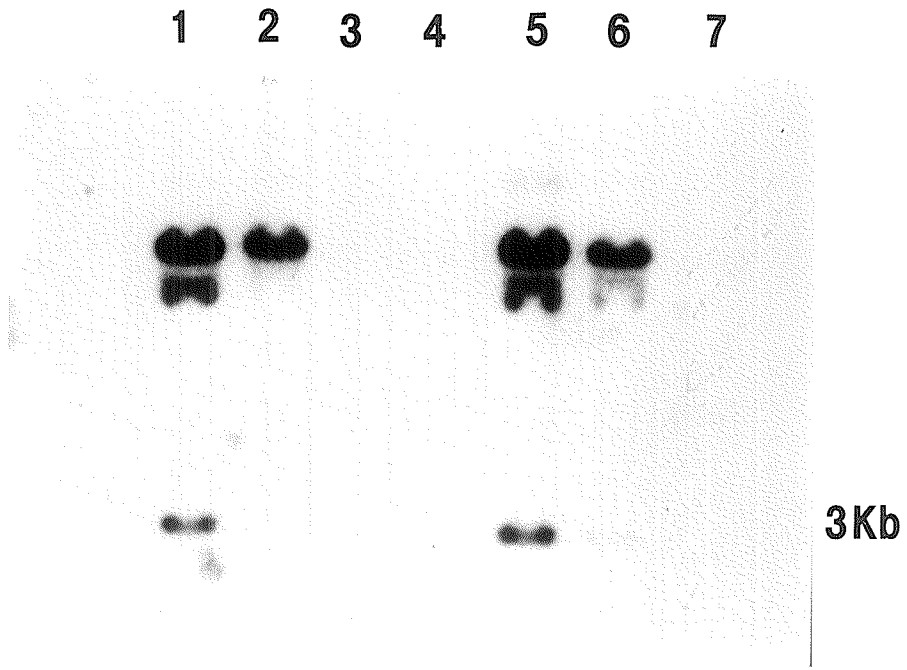

Figure 3 Lambda gt10 recombinant clones were digested with EcoR1 endonuclease, electrophoresed on a 1% agarose gel and Southern blotted. Nick translated rM-40 insert ($3.4X10^8$ dpm/μg) was hybridized to the phage and insert DNA at 42°C in 6.6X SCP, 5X DH, 1% n-lauroyl sarcosine, 200μg/ml denatured salmon sperm DNA. Lanes 1 to 6 represent different isolated phage clones, lane 7 contains marker DNA. Exposure was overnight.

hybridization to insert DNA (Figure 3) was a lot cleaner, with less cross reaction to the marker DNA than that seen with whole plasmid DNA (Figure 2). Both probes hybridized to a 3 Kb insert as well as incompletely digested recombinant phage DNA. Subsequent subcloning (137,162,164,165) into the pGEM3Z vector however proved unsuccessful.

Bacteriophage DNA containing the 3 Kb insert was cut with EcoR1 (159), religated (162) in the absence of any plasmid vector and used to transform (164,165) competent MV1193 E coli. cells. Under these conditions some insert DNA will ligate to the bacteriophage arms and some will circularize. All of the λgt10 cDNA clones used in this transformation experiment were capable of converting the MV1193 cells to an ampicillin resistant state, suggesting the presence of the β-lactamase gene. Miniplasmid preparations of randomly picked colonies revealed a piece of DNA that was 3 Kb in size, implying that the supposed cDNA insert was in fact a helper plasmid from the library kit that had been cloned into λgt10 arms.

Using a miniliquid lysis (157) method for propagation of the twenty three λgt10 BPH cDNA clones the presence of β-lactamase activity was detected by a color reaction catalyzed by the enzyme in the presence of cephalosporin (McNicol, P. personal communication). One isolated recombinant phage selected from the λgt10 CaP cDNA library under the same conditions used to screen the λgt10 BPH cDNA library also proved to contain helper plasmid as the insert. The unfortunate recombination of helper plasmid DNA into the

bacteriophage vector results in false positive plaques that will hybridize to the similar sequences in the plasmid DNA of the recombinant probe. The plasmid to plasmid hybridization seen when supposed insert DNA was utilized in the reaction is due to the presence of low levels of incompletely digested probe DNA which still has some plasmid sequences attached. This was further confirmation that the hybridization conditions were not stringent enough and needed to be determined before the selection of a human probasin-like clone would be possible.

3.2 Northern Analysis

To determine if a homologous sequence to probasin was present in human prostatic tissue, a number of blot hybridization experiments were attempted. To establish hybridization temperature parameters, a Northern dot blot containing 1, 2 and 10 μg of total RNA from rat prostate, two human prostatic cell lines (PC3, DU145) (144,145), two BPH samples (20922, 10982), and two CaP samples (19343, 519-87) with human liver and tRNA as controls, was prepared in triplicate. These were hybridized to pM-40 nick translated probe ($2\text{-}3X10^8$ dpm/μg) (154,155) under aqueous conditions in the buffer previously described at 42°C, 55°C and 65°C. These conditions permit hybridization with mismatches equivalent to 27%, 15%, and 5% of base pairs for 42°C, 55°C and 65°C, respectively (175).

After hybridization for 24 hours, the blots were washed at room temperature for one hour with two liters of 1X SSC, 0.1% SDS,

then washed twice for 15 minutes each in 0.1X SSC, 0.1% SDS and brought to the temperature of hybridization (42, 55 or 65°C). A three day exposure to X-ray film is shown in Figure 4. Here it can be seen that all samples, excluding tRNA hybridize strongly at 42°C. When the temperature is increased to 55°C, the binding to human RNA is decreased in comparison to rat RNA, however some hybridization can still be detected. In particular the two cell lines and patients 10982 (BPH) and 519-87 (CaP) appear to be hybridizing more strongly than human liver RNA or the other two patients. When the temperature is increased further to 65°C the hybridization to rat RNA remains strong, however most human RNA hybridization is lost. The probasin probe continues to recognize a sequence in the PC3 cell line RNA, as well as hybridizing weakly to 10μg of total RNA from patient 519-87 and human liver (Figure 4, Table 1).

In spite of the apparent hybridization differences between the human RNA samples analyzed, the salient feature to observe is the relative low abundance of probasin-like sequences in the human prostate as compared to the rat prostate. To calculate the percentage of probasin hybridization, relative to that of the rat prostate, each RNA spot was counted for radiolabel binding. The data was normalized for binding to tRNA, then converted to dpm/μg since the RNA spots represented 1, 2 and 10 μg of each sample. At each temperature the three dpm/μg values for each RNA sample were averaged (Table 1) and calculated as a percentage of rat prostate RNA hybridization. The histogram (Figure 5) demonstrates very clearly that human probasin-like sequences represent a smaller

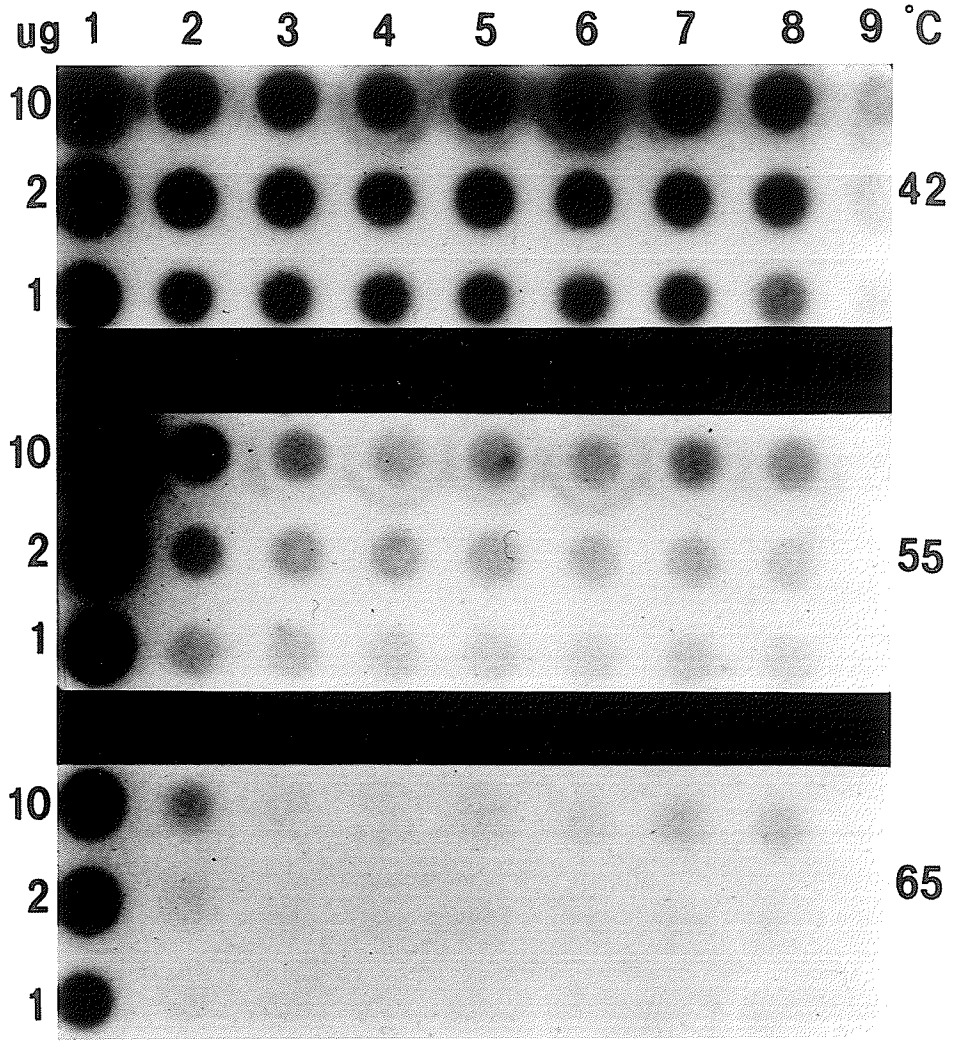

Figure 4 Total RNA was spot blotted onto nitrocellulose and hybridized to pM-40 (>2X10^8 dpm/μg) in 5X SSPE, 5X DH, 0.1% SDS, 200μg/ml denatured salmon sperm DNA. Each blot contained 10, 2 and 1μg of sample RNA and was hybridized at 42°C, 55°C or 65°C. Samples 1-9 are Rat RNA, PC3 cell line RNA, DU145 cell line RNA, BPH patient 20922 RNA, BPH patient 10982 RNA, CaP patient 19343 RNA, CaP patient 519 RNA, human liver RNA and yeast tRNA. Exposure was 3 days.

TABLE 1 The individual dots from Figure 4 were cut out and counted for binding of radiolabelled pM-40. The counts were converted to dpm/μg and averaged for each sample at each temperature. The results are listed as mean dpm/μg per hybridization temperature.

	Hybridization Temperature		
Sample	42°C	55°C	65°C
Rat	269	472	154
PC3	80	22	25
DU145	49	22	17
20922	38	11	7
10982	53	20	3
19343	39	16	12
519-87	49	12	20
hLiver	44	20	4

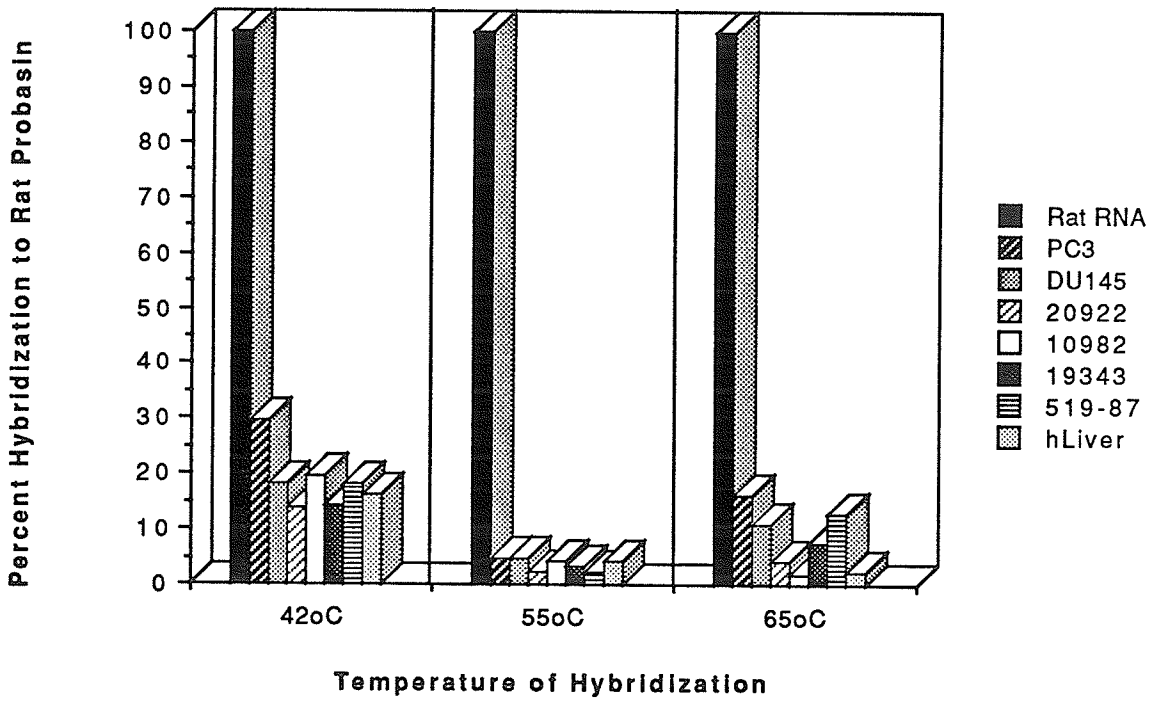

Figure 5 The data from Table 1 was plotted as a percentage of rat probasin binding at each temperature.

fraction of human prostatic total RNA than that measured for the rat prostate. If the level of hybridization to human sequences was solely a result of low homology then the amount of probasin hybridization at 42°C would be more representative of that seen with the rat prostate RNA. The levels for human RNA hybridizing are higher at 42°C than the other two temperatures indicating homology is a factor. However, the abundance estimated at this temperature remains approximately five fold lower than the rat, and reduces to twenty-five fold lower at higher temperatures. The copius quantities of rat probasin mRNA in the cells of the lateral prostate of the rat compensate for the rest of the gland, whereas the cells of the human prostate may not be expressing in sufficient levels for this to occur.

To examine the size of the hybridizing mRNA a Northern (137,146,170,174) with 50 μg of human prostate total RNA from BPH and CaP patients and 5 μg of total RNA from whole rat prostate was hybridized to the probasin cDNA clone (114). The pM-40 clone was labelledc(154,155) to a specific activity of $2.66X10^8$ dpm/μg and allowed to anneal with similar sequences on the Northern blot at 42°C under aqueous conditions. A four day exposure of this hybridization is shown in Figure 6. Strong hybridization of the rat probasin message indicates its abundance in the rat prostate, since the amount of RNA in this lane is one tenth that of the human samples. The 28s and 18s ribosomal bands in the human samples have hybridized to the labelled sequence, but this appears to be non-specific (Figure 7). Above and below the 18s ribosomal RNA there is a hybridizing signal in some lanes. It is very likely that the signals

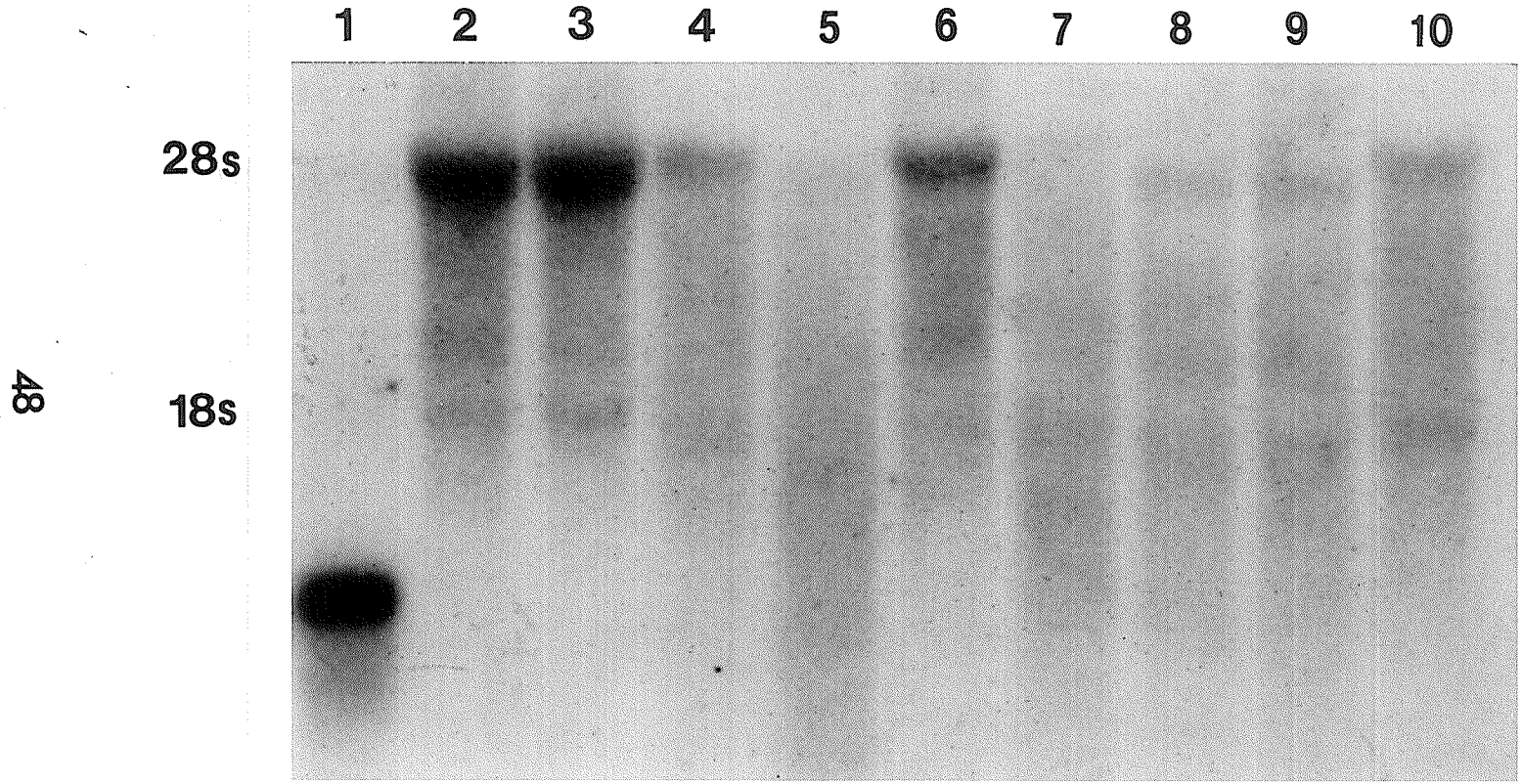

Figure 6 Fifty micrograms of total RNA from human prostate samples were electrophoresed alongside 5μg rat total RNA for Northern blotting. Lane 1 rat prostate, lane 2 PC3 cell line, lane 3 DU145 cell line, lane 4 CaP patient 519-87, lane 5 CaP patient 23355-86, lane 6 CaP patient 7076, lane 7 CaP patient 19343, lane 8 BPH patient 3611-87, lane 9 BPH patient 10982, and lane 10 normal prostate from autopsy. Samples were hybridized to pM-40 at 42°C. Four day exposure.

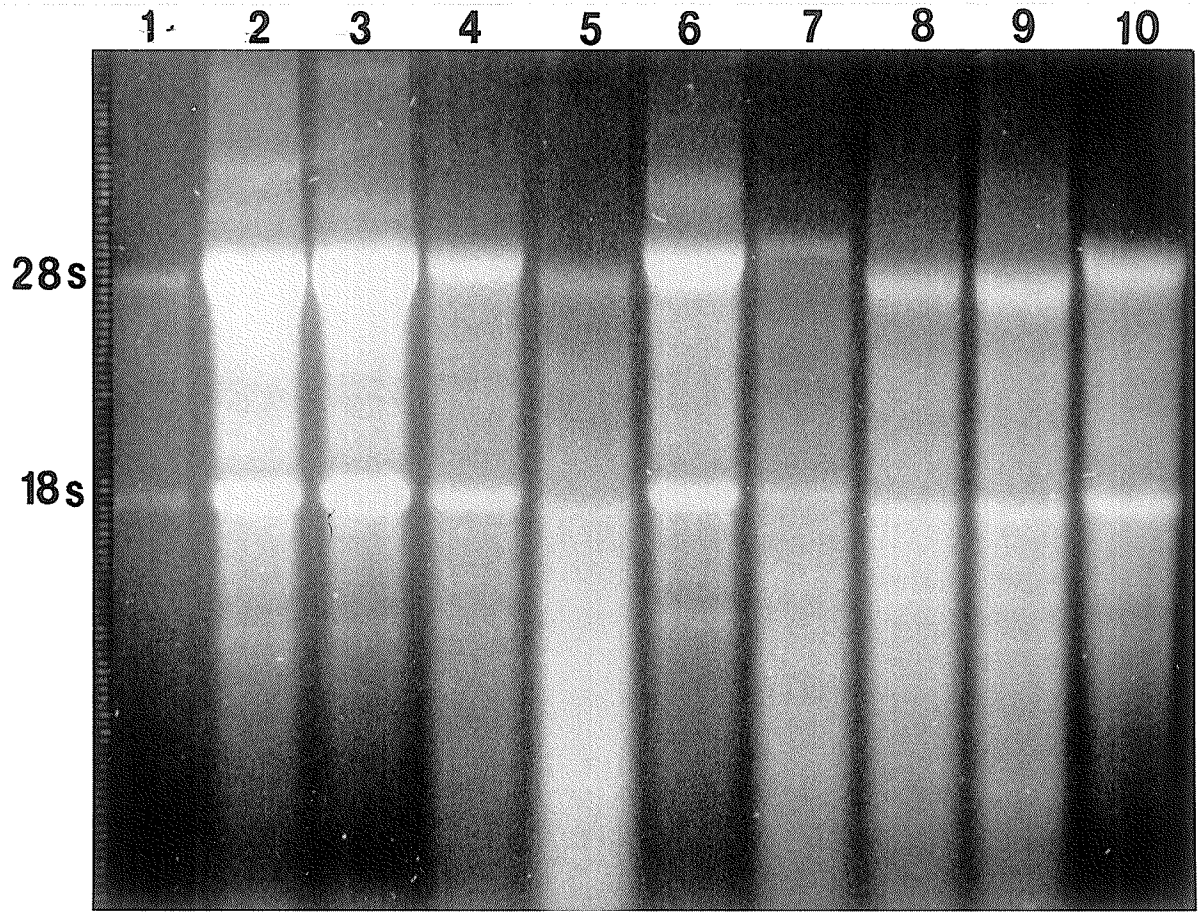

Figure 7 Ethidium bromide visualization of Northern blot in Figure 6. Lane 1 rat prostate, lane 2 PC3 cell line, lane 3 DU145 cell line, lane 4 CaP patient 519-87, lane 5 CaP patient 23355-86, lane 6 CaP patient 7076, lane 7 CaP patient 19343, lane 8 BPH patient 3611-87, lane 9 BPH patient 10982, lane 10 normal prostate from autopsy.

detected are due to the rat probasin probe hybridizing to degradation products of the 28s and 18s ribosomal RNA molecules. Whether this binding is to a specific message in these patients or is a non-specific reaction would have to be determined by enriching for message RNA. In retrospect, having a ribosomal RNA control in addition to a tRNA control on the Northern dot blot would have assisted in quantitating the specific and nonspecific hybridization.

Poly A^+ mRNA (147,171) isolated from a variety of patient total RNA samples was used to generate a number of message RNA Northern blots. Initially 12 μg and 20 μg mRNA northerns were probed with nick translated pM-40 labelled to a specific activity of $3.84X10^8$ dpm/μg at 42°C under aqueous conditions. After washing as previously described and subjecting to autoradiography, there was no hybridization detected. Repeating this experiment with a 15 μg mRNA Northern and random prime labelled (Amersham, Oakville, Ont.) rM-40 insert ($3.6X10^7$ dpm/hybridization) at 47°C aqueous was also unsuccessful. If the reacting sequences are similar mRNA species to rat probasin, they must be much lower in abundance in the human prostate than the levels seen in the rat prostate.

3.3 Homology to Probasin Genomic Clones

To resolve if there is a sequence in human prostate mRNA that shares homology only with certain regions of the probasin coding sequence an analysis of the probasin genomic clones was undertaken. As described in the introduction, the probasin gene is divided into seven exons, with coding information on all but the seventh exon

(Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation) (Figure 8). With the discovery that probasin is a member of a ligand carrier family it became necessary to distinguish if only functional domains of this gene are conserved in the human. Initially, a Southern dot blot of 5 and 10 μg of the pM-40 clone, various probasin genomic clones containing exon and/or intron sequences (Figure 9), with pAT153 and SVS-II as controls was hybridized at 55°C aqueous with cDNA labelled to a specific activity of $1.8X10^9$ dpm/μg. The cDNA was prepared from BPH mRNA (10982) (148).

Hybridization of BPH cDNA to pM-40 clone DNA was above the level detected for controls. In particular the clones pM-40, BH 3.0, BB 2.7, BH 2.0 and EE5.2 had a higher degree of hybridization (Figure 9). These subclones of the rat probasin gene correspond to the 3' end, and contain exons V, VI and VII. To ensure this hybridization was to the specific insert sequences, the rat probasin clones were digested with appropriate restriction endonucleases, electrophoresed on a 1% agarose gel, then transferred for hybridization purposes (167). Complementary DNA prepared from the same patient's mRNA and labelled to $1.8X10^9$ dpm/μg was hybridized at 42°C to these membrane bound clones. The binding pattern confirms that the hybridization is specifically bound to the insert bands and not the plasmid bands (Figure 10).

When compared to the agarose gel, ethidium bromide stained (137) prior to transfer, it is apparent that the hybridization to these bands is not simply due to the mass of DNA present since the plasmid bands contain an equal quantity of DNA to the intensely

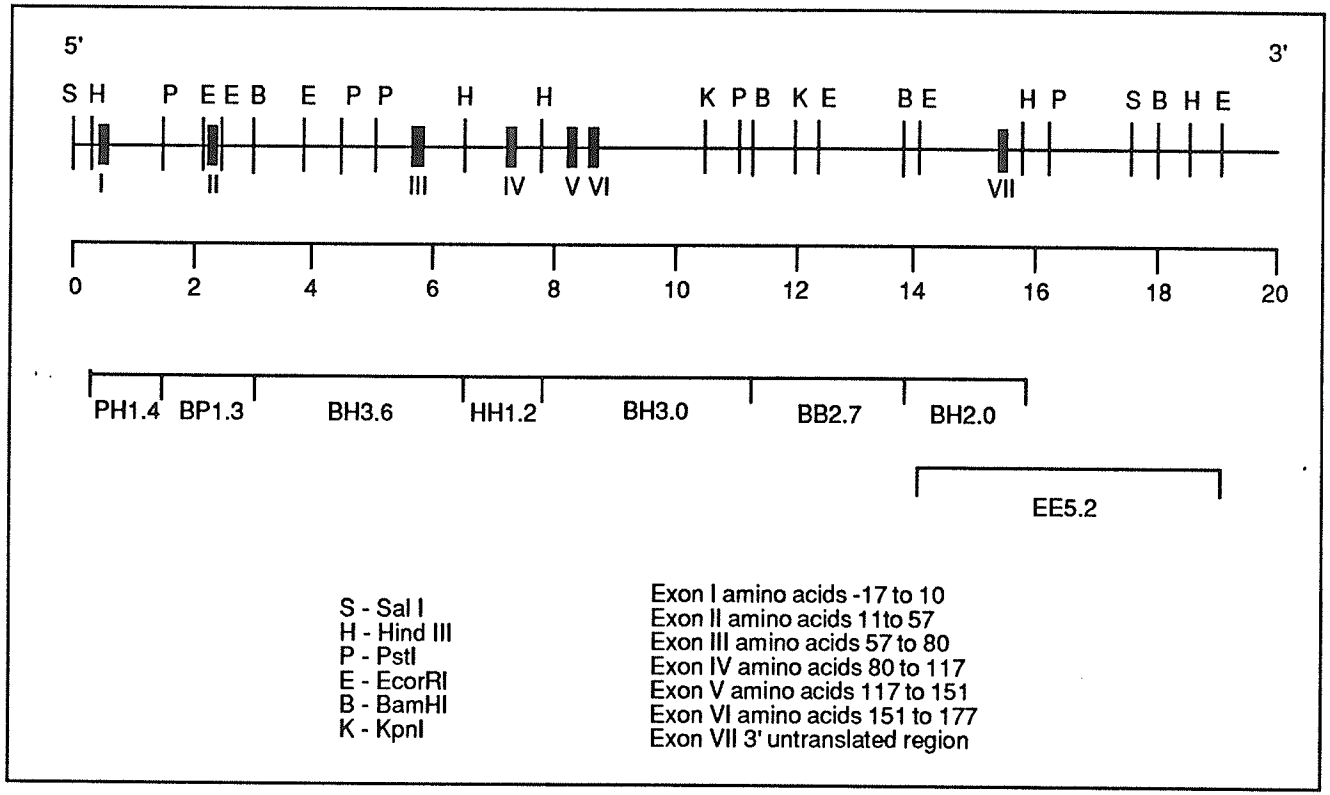

Figure 8 Probasin genomic clone restriction map showing the relations of the exons and introns. The genomic subclones used in subsequent hybridization experiments are shown below the map and are named according to the restriction sites on each side of the insert and the size of the genomic DNA fragment in kilobases.

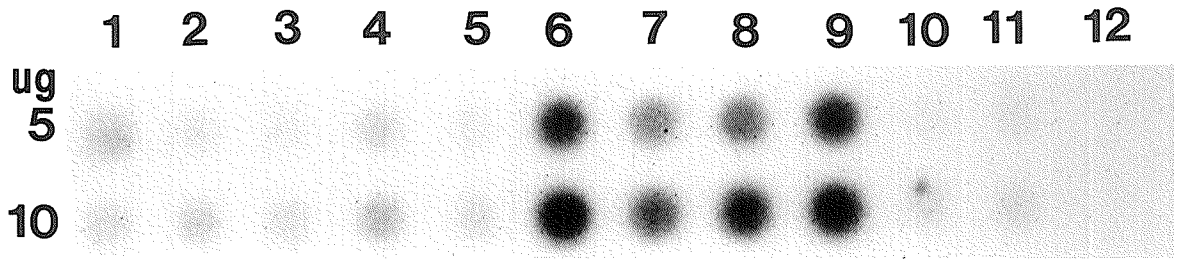

Figure 9 Five and ten micrograms of probasin clone DNA were spot blotted onto nitrocellulose and hybridized to labelled cDNA ($1.8X10^9$ dpm/µg) prepared from BPH patient 10982 mRNA. Hybridization conditions were 55°C. Lane 1 pM-40, lane 2 PH 1.4, lane 3 BP 1.3, lane 4 BH 3.6, lane 5 HH 1.2, lane 6 BH 3.0, lane 7 BB 2.7, lane 8 BH 2.0, lane 9 EE 5.2, lane 10 pAT 153, lane 11 clone RWB, lane 12 no DNA. For map of these clones refer to Figure 8.

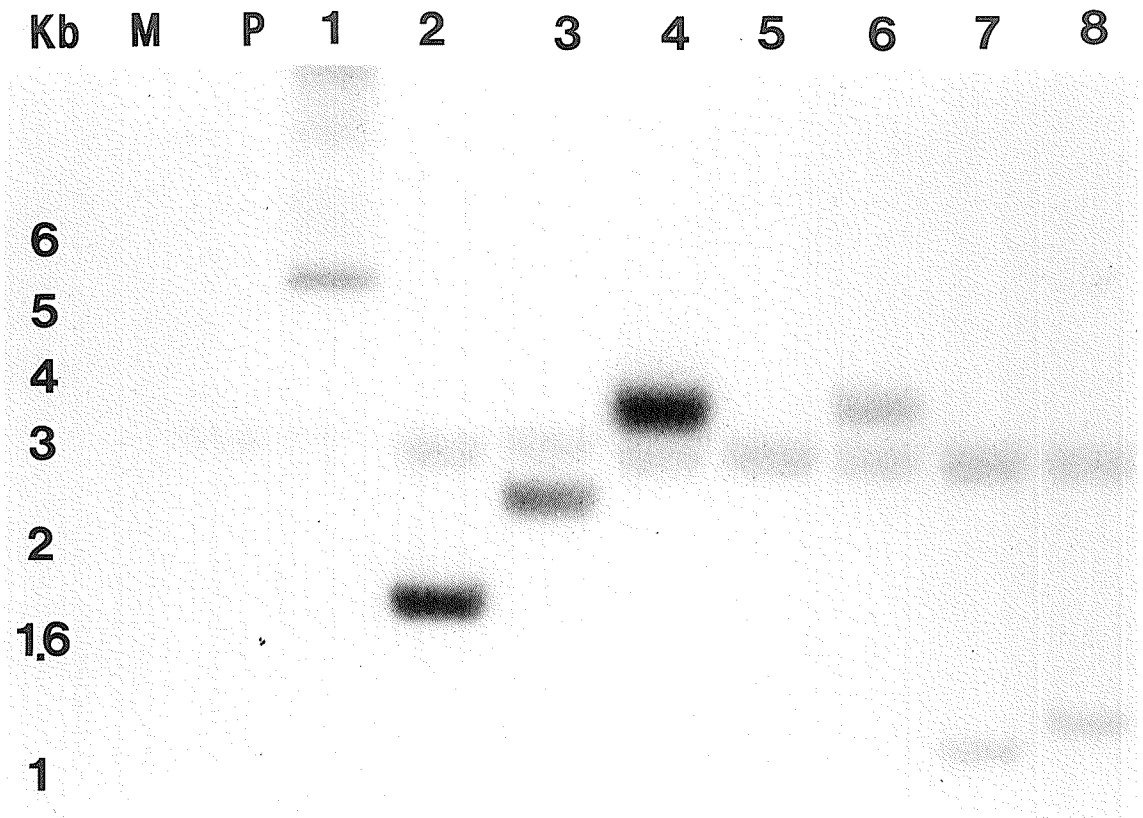

Figure 10 Restriction digested probasin genomic clones Southern blotted and hybridized to cDNA prepared from BPH patient 10982 mRNA($1.8X10^9$ dpm/μg) in 6.6X SCP, 5X DH, 1% n-lauroyl sarcosine, 200μg/ml denatured salmon sperm DNA at 42°C. Lane M marker DNA, lane P pUC119, lane 1 EE 5.2, lane 2 BH 2.0, lane 3 BB 2.7, lane 4 BH 3.0, lane 5 HH 1.2, lane 6 BH 3.6, lane 7 BP 1.3, lane 8 PH 1.4. Exposure 10 days.

binding insert bands (Figure 11). The greatest binding is to the insert bands of clones BH 3.0, BB 2.7 and BH 2.0, while weaker hybridization to BH 3.6, BP 1.3, PH 1.4 and EE 5.2 inserts is detected. The faint hybridization to the plasmid band is likely non-specific hybridization, since the control plasmid lane with a lower mass has a negligible hybridization signal. Although the binding to clones BH 3.6, BP 1.3 and PH 1.4 is a less intense hybridization than to BH 3.0, BB 2.7 and BH 2.0, it may be significant since clone HH 1.2 was not detected at all under the same hybridization conditions. These results indicate that there are human prostate cDNAs with limited homology to the rat probasin gene. Although the homology seems to be greatest at the 3' end of the gene, there are possibly regions of cross reactivity within the remaining rat probasin gene. Whether the homology corresponds to exon or intron regions of the rat gene was not determined.

3.4 Southern Analysis

The hybridization pattern of BPH cDNA to the probasin genomic clones revealed cross reactivity with exons as well as intron sequences (Figures 9-11). It was for this reason that a study of human genomic DNA was undertaken to elucidate how the banding pattern compares to the rat probasin gene. Southern blots (142) were prepared using the same BPH, CaP, normal, cell line and rat DNA for each blot, but digested with different restriction endonucleases. The patient samples used had been studied previously by Northern blot analysis (10982, 20922, 519-87) and were selected for consistency (Figures 4-7).

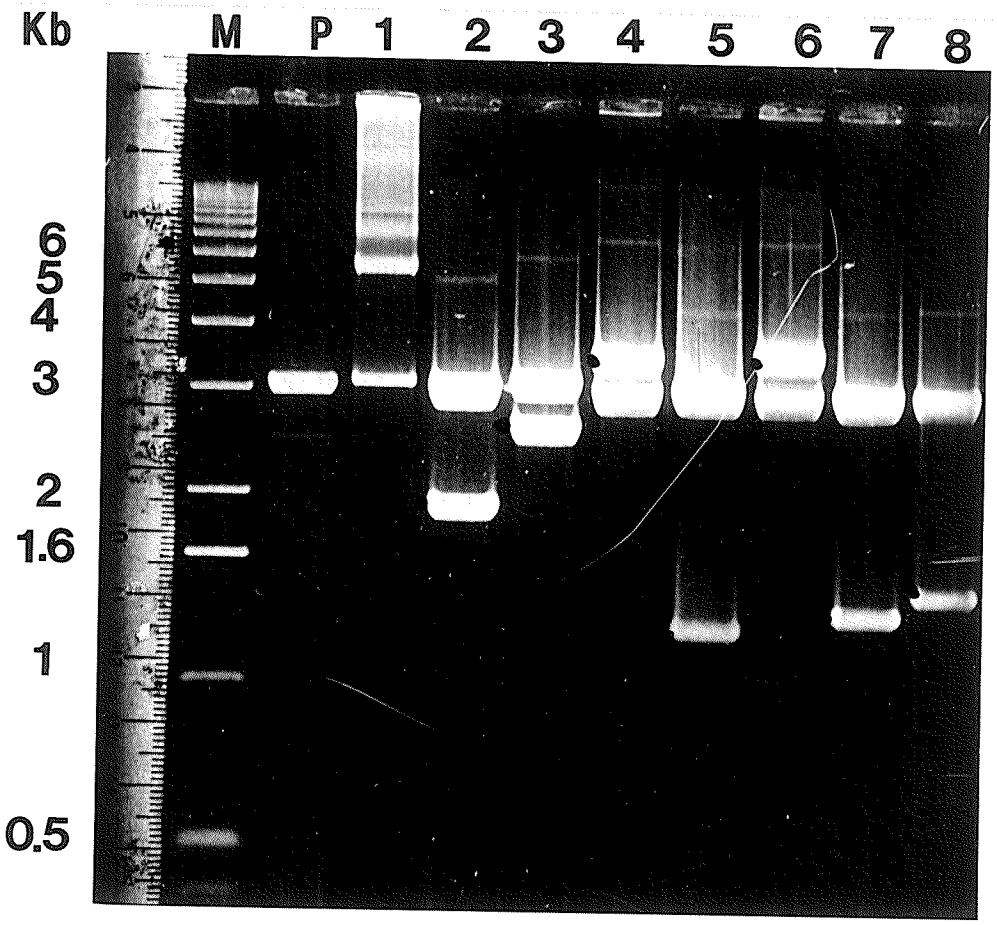

Figure 11 Ethidium bromide stained gel of the DNA transferred in Figure 10. Lanes are the same as for Figure 10. Each lane contains 2µg except lane P contains 1µg of pUC119.

Under reduced stringency (42°C aqueous conditions), genomic DNA digested with BamH1 was probed with nick translated (154,155) pM-40 (3.14X 10^8 dpm/μg), then washed as described in the methods section. These hybridization conditions were equivalent to 40% mismatched base pairs (175); however, the wash conditions (50°C, 0.1X SSC, 0.1%SDS) were equal to 25% mismatch (175). As shown in Figure 12, it is difficult to identify the high molecular weight banding pattern in the human or rat sample lanes. Although bands can be seen in both human and rat DNA samples, they are obscured by the background hybridization, particularly in the high molecular weight ranges. The high degree of background is due to the low stringency of the hybridization conditions. Increasing the stringency of these conditions to 25% mismatch (175) for the annealing reaction by hybridizing in 50% formamide at 25°C (pM-40, 4.77X10^8 dpm/μg) and washing as before does not improve the high degree of background (Figure 13).

To create a lower molecular weight banding pattern a double digest of the genomic DNA samples with the enzymes BamH1 and HindIII was used in further Southern analysis (137,142). This blot was hybridized to rM-40 insert DNA nick translated (154,155) (2.67X10^8 dpm/μg), at conditions equivalent to 12% mismatch (175) (38°C, 50% formamide), then washed as before. The hybridization conditions greatly improved the visibility of the banding pattern in the rat genomic DNA lane (Figure 14), resulting in four signals with sizes of 3.8, 3.4, 2.6 and 1.3 kilobase pairs. Although there is hybridization detected in the three patient samples, it is not in a

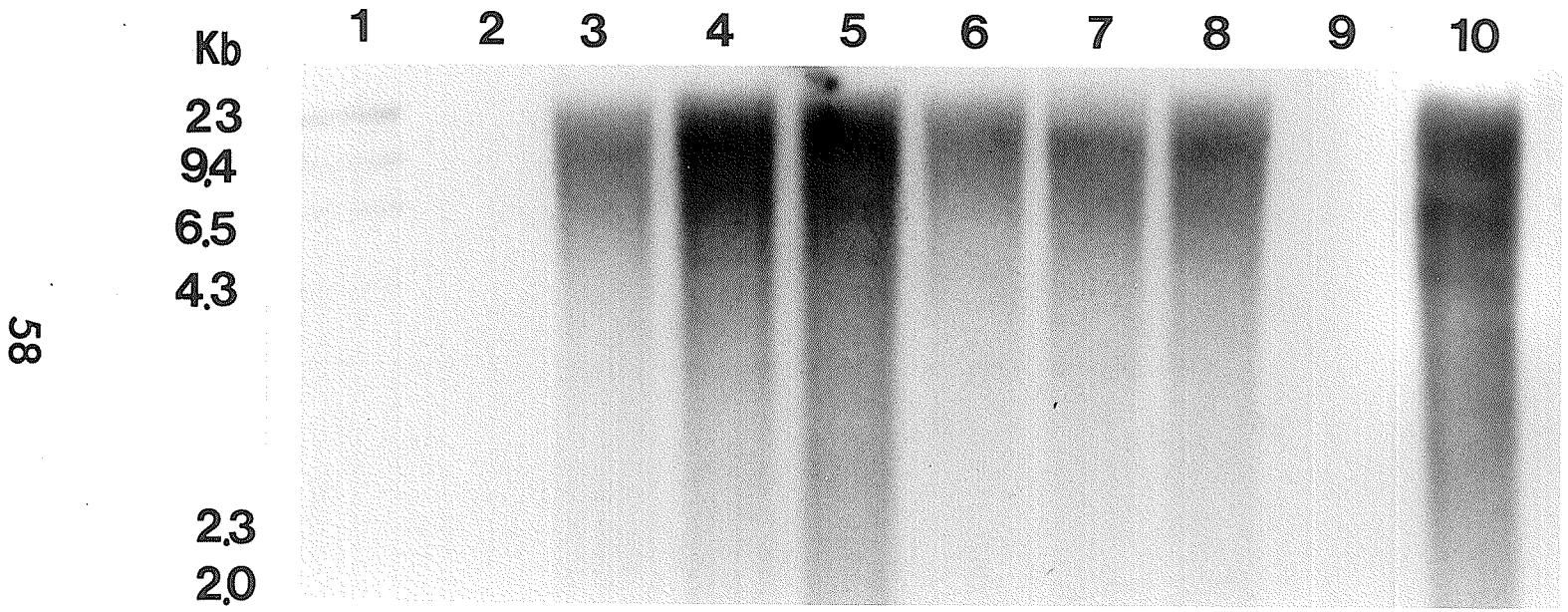

Figure 12 A Southern blot of 10µg BamH1 digested DNA hybridized to pM-40 ($3.14X10^8$ dpm/mg) at 42°C in 6.6X SCP, 5X DH, 1% n-lauroyl sarcosine, 200µg/ml denatured salmon sperm DNA. Lane 1 marker DNA, lane 2 blank, lane 3 BPH patient 10982, lane 4 BPH patient 20922, lane 5 CaP patient 519-87, lane 6 cell line DU145, lane 7 cell line PC3, lane 8 normal prostate, lane 9 blank, and lane 10 rat prostate. Exposure was 7 days.

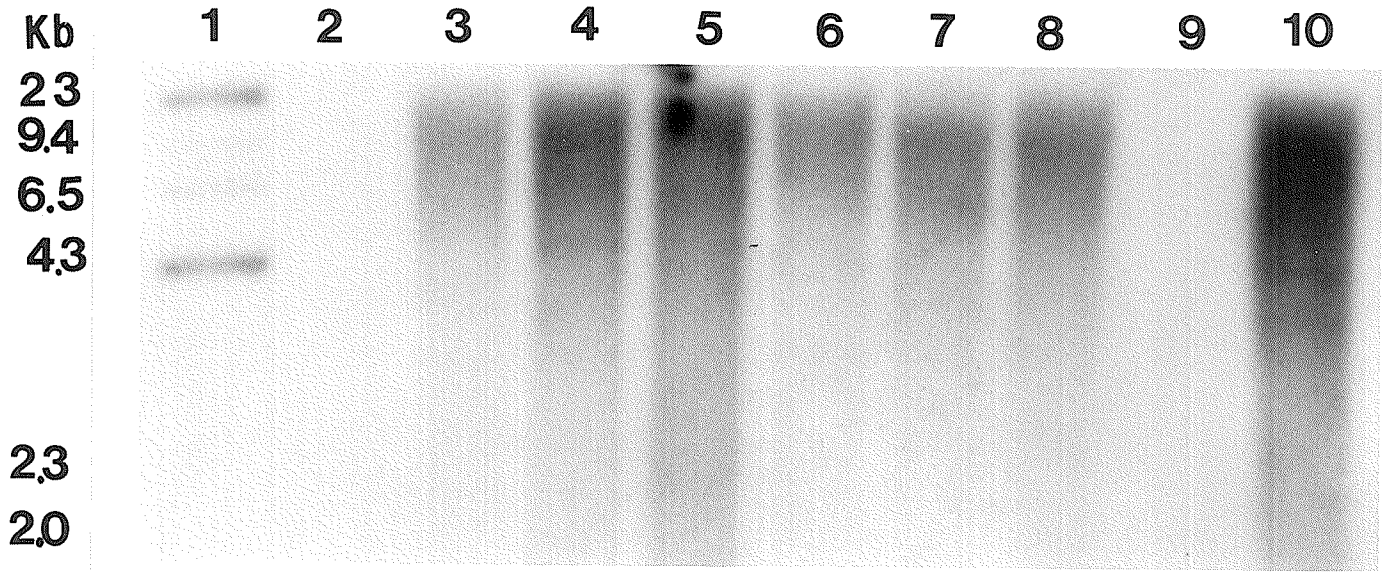

Figure 13 A Southern blot of 10μg BamH1 digested DNA hybridized to pM-40 ($4.77X10^8$ dpm/μg) at 25°C in 50% formamide, 6.6X SCP, 5X DH, 1% n-lauroyl sarcosine, 200μg/ml denatured salmon sperm DNA. Lane 1 marker DNA, lane 2 blank, lane 3 BPH patient 10982, lane 4 BPH patient 20922, lane 5 CaP patient 519-87, lane 6 cell line DU145, lane 7 cell line PC3, lane 8 normal human prostate, lane 9 blank, and lane 10 rat prostate. Exposure was for 7 days.

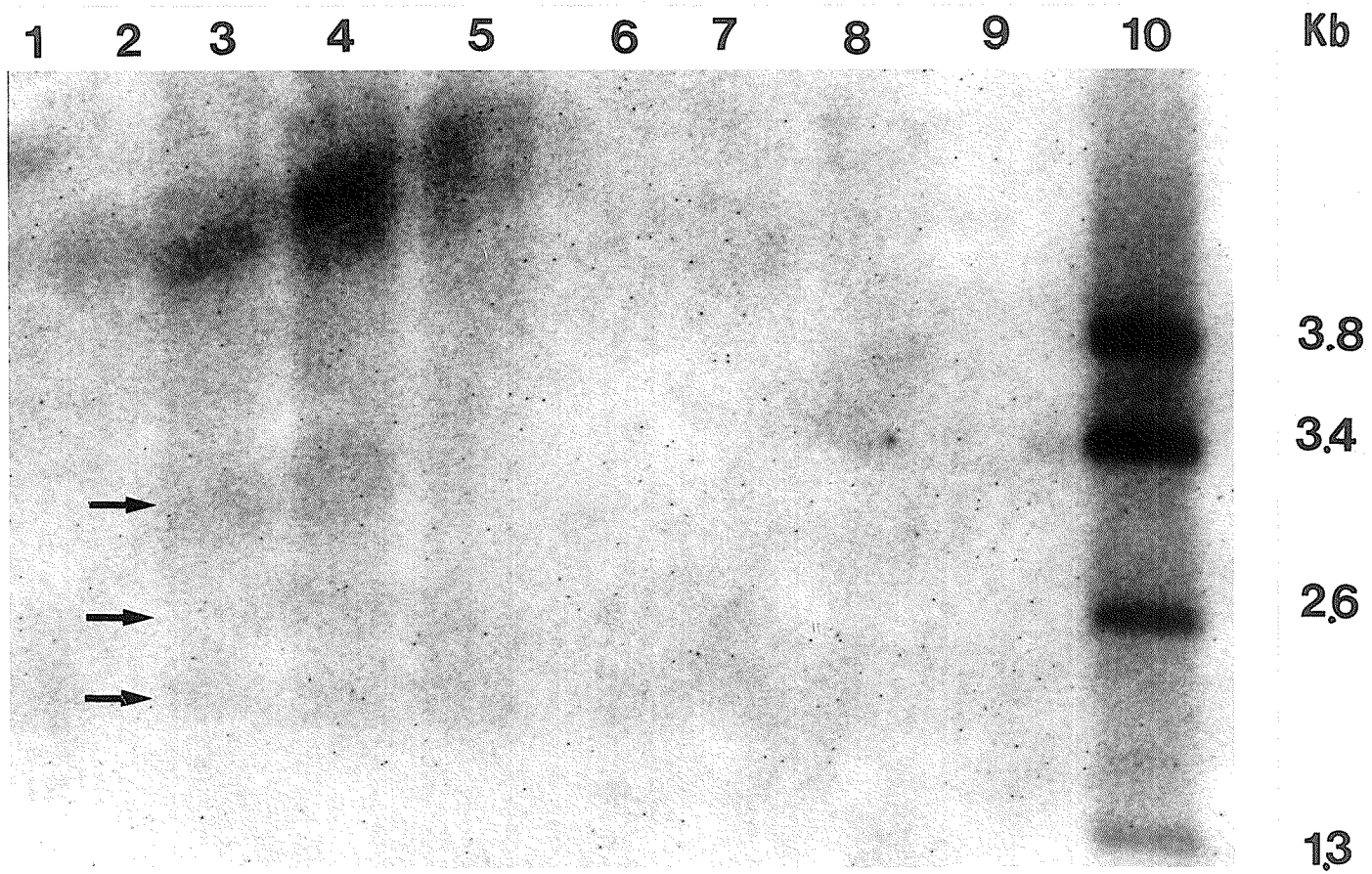

Figure 14 A Southern blot of 10µg BamH1/HindIII digested DNA hybridized to rM-40 insert DNA (2.67X10^8 dpm/µg) at 38°C in 50% formamide, 6.6X SCP, 5X DH, 1% n-lauroyl sarcosine, 200µg/ml denatured salmon sperm DNA. Lane 1 marker DNA, lane 2 blank, lane 3 BPH patient 10982, lane 4 BPH patient 20922, lane 5 CaP patient 519-87, lane 6 cell line DU145, lane 7 cell line PC3, lane 8 normal human prostate, lane 9 blank, lane 10 rat prostate. Exposure was 5 days.

crisp band pattern as seen in the rat. This could be an indication that the integrity of the human DNA, although apparently digested (Figure 15), may not be as high as that in the rat. The three human DNA signals detected may instead represent human DNA sequences that share only a low level of homology to the rat probasin coding sequence. The lack of hybridization of rat M-40 sequences to DNA in the normal and cell line samples is disturbing and could indicate an inefficient transfer. However, hybridization at 42°C, 50% formamide with nick translated human P53 insert DNA ($3.5X10^8$ dpm/μg) seems to indicate that both the transfer and integrity of the DNA samples is good (Figure 16). The hybridization of human P53 DNA to sequences in the rat genome is a further indication that cross species hybridizations are less clear than species specific reactions. Random prime labelled rM-40 insert ($3.6X10^7$ dpm/hybridization) was not successful in detecting any cross reacting human genomic DNA sequences.

An attempt at determining which rat probasin regions were represented in the hybridizing human sequences was made through the use of three of the rat probasin genomic subclones (BH3.0, BB2.7, BH2.0). These three clones were representative of the exons and introns of the 3' region of the rat probasin gene, and were previously shown to cross react to human prostate cDNAs. However, the hybridization of these subclones, which were nick translated (>$5X10^8$ dpm/μg) and allowed to anneal to human genomic DNA at 35°C, 50% formamide, was also not successful. The low homology of rat

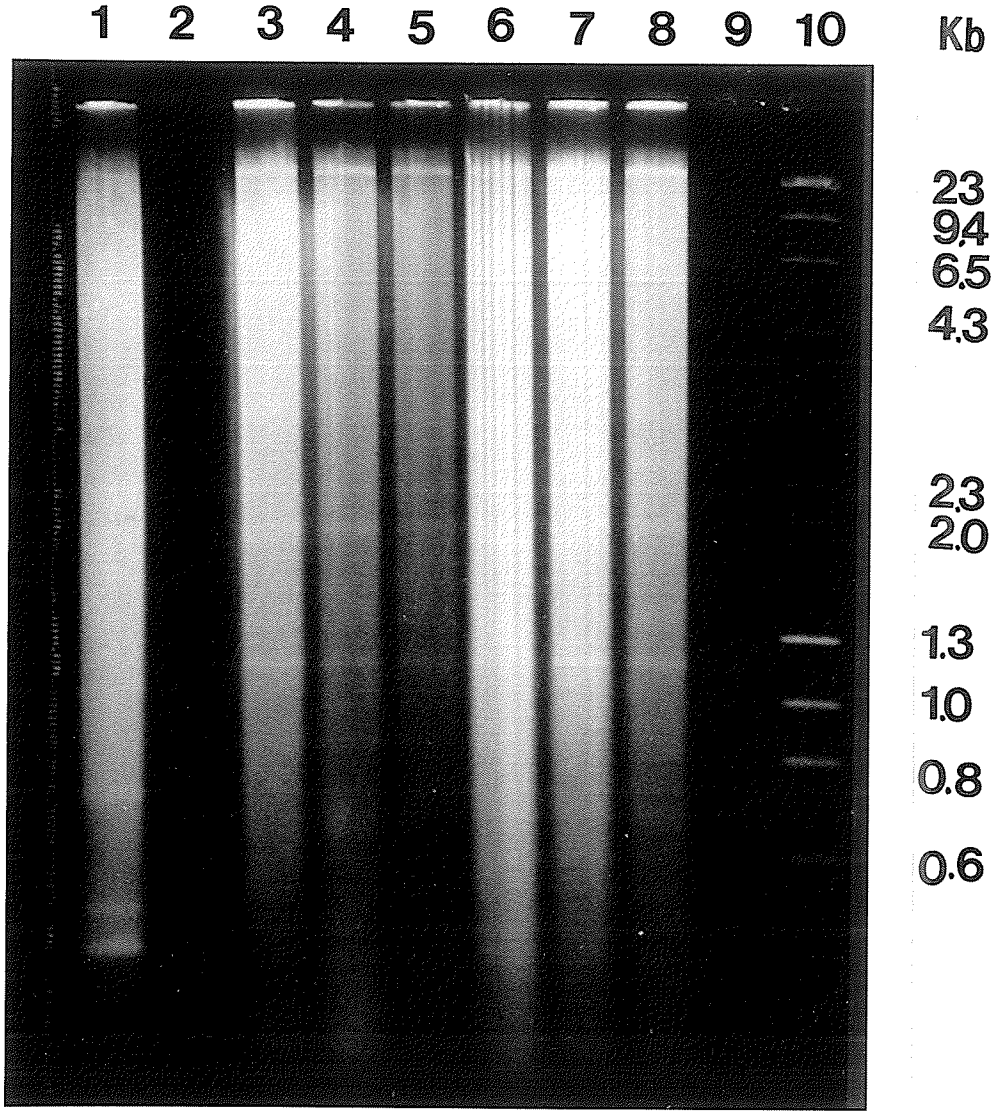

Figure 15 Ethidium bromide stained gel of blot in Figure 14. Lane 1 rat prostate, lane 2 blank, lane 3 normal human prostate, lane 4 PC3 cell line, lane 5 DU145 cell line, lane 6 CaP patient 519-87, lane 7 BPH patient 20922, lane 8 BPH patient 10982, lane 9 blank and lane 10 marker DNA.

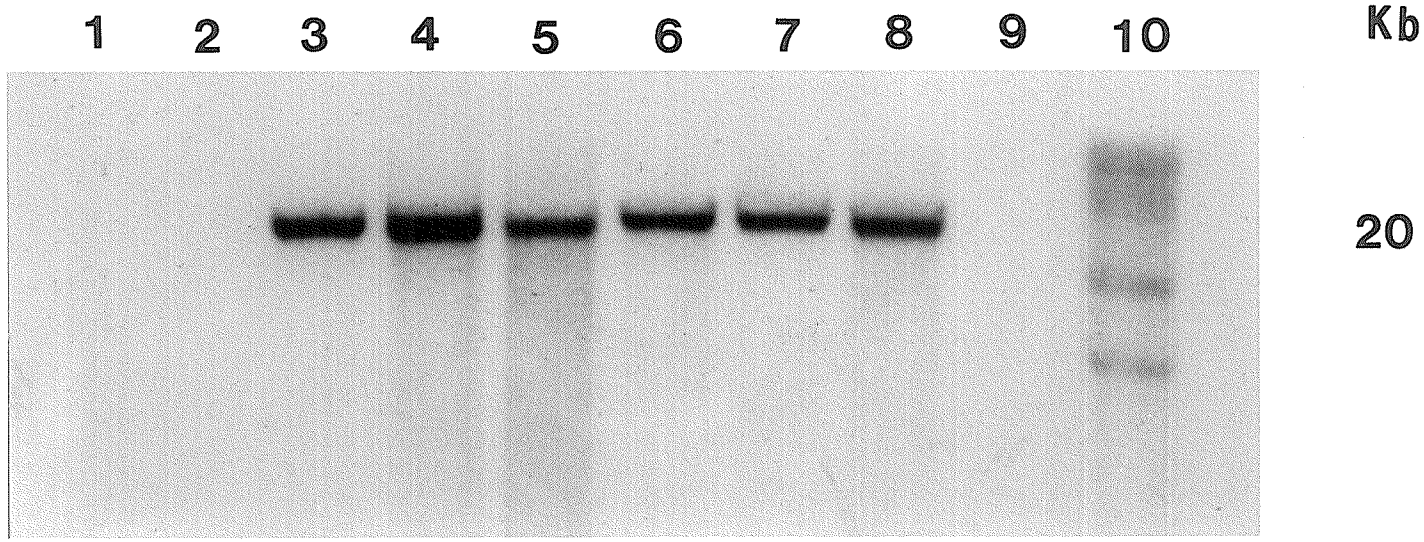

Figure 16 A Southern blot of 10μg BamH1 digested DNA hybridized to nick translated hP53 insert ($3.5X10^8$ dpm/μg) at 42°C in 50% formamide. Lane 1 marker DNA, lane 2 blank, lane 3 BPH patient 10982, lane 4 BPH patient 20922, lane 5 CaP patient 519-87, lane 6 cell line DU145, lane 7 cell line PC3, lane 8 normal human prostate, lane 9 blank, and lane 10 rat prostate. Exposure was for 3 days.

probasin nucleic acids to sequences present in the human genome is apparent from each of the Southern analysis.

3.5 In vitro translations

Since the above studies indicated limited homology between rat probasin and putative human probasin at the nucleic acid level, studies aimed at identifying human probasin-like proteins were conducted. The protein profile of the in vitro translated mRNAs from human prostate RNA samples was compared to that of the rat whole prostate. Ribonucleic acids isolated from normal prostate, the two disease states of the prostate (BPH, CaP) and whole rat prostate were in vitro translated as described under methods (141) and separated by SDS-PAGE (141,175,176). Although all samples were labelled well with ^{35}S-methionine, the rat prostate RNA seems to have translated more efficiently. As seen in the autoradiogram (Figure 17), there are obvious differences in the translation products. There are highly abundant rat prostate products present, while the human prostate products from all three tissue types show an absence of this type of profile. Although there is a product of similar molecular weight to rat probasin, there is no evidence that this protein represents the human equivalent. Immunoprecipitation of a probasin-like protein was attempted using the rabbit antiprobasin antiserum, however the reaction appeared to be inhibited by the in vitro translation cocktail since rat probasin was also not precipitated. The antibodies raised against rat probasin were useful by Western blotting analysis as described in the next section.

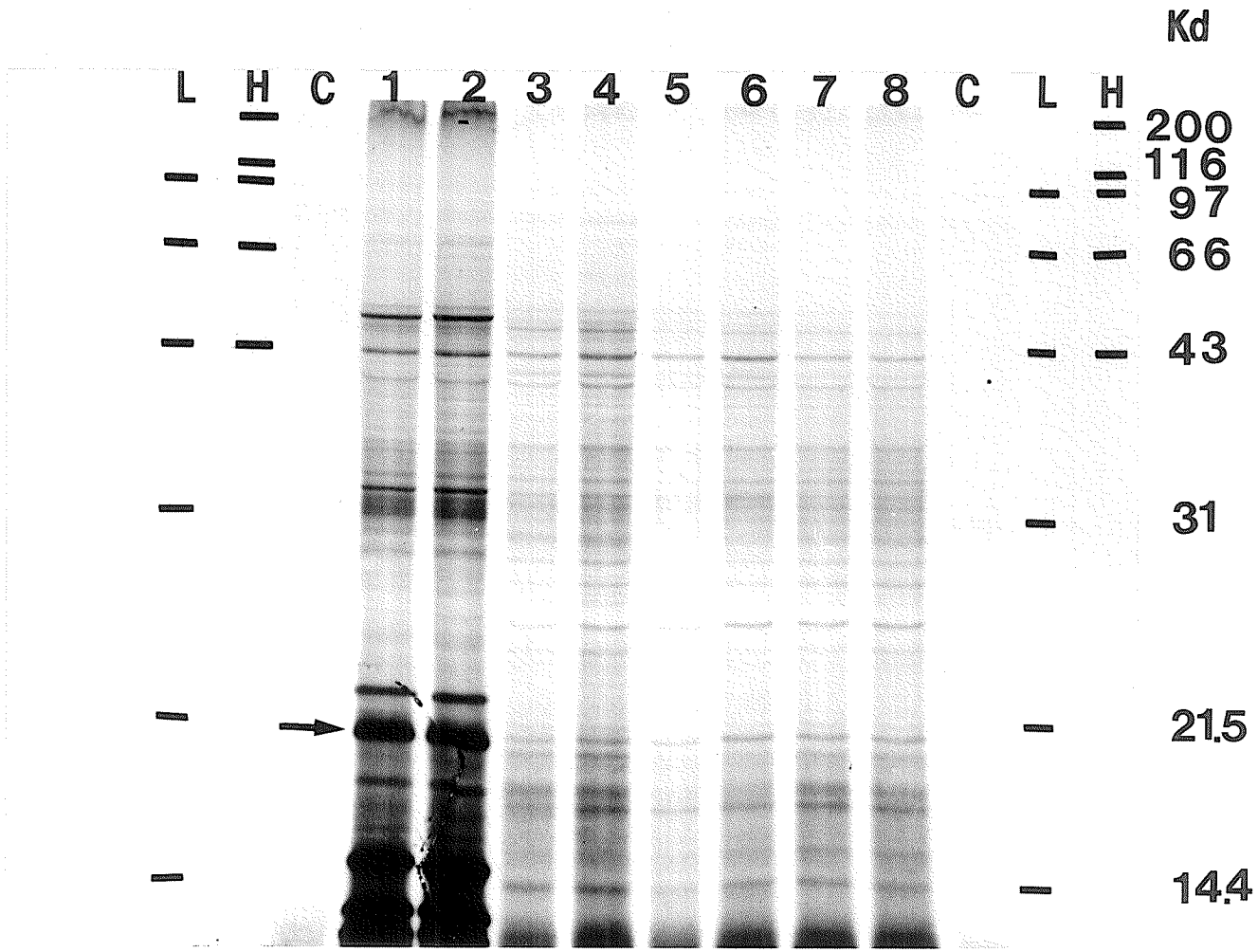

Figure 17 In vitro translated proteins labelled with L-^{35}S-methionine were electrophoretically resolved on a 12.5% Laemmli SDS-PAGE gel. Lanes L and H are low and high molecular weight markers. Lanes 1 and 2 Rat prostate, lanes 3 and 4 normal human prostate, lanes 5 and 6 CaP prostate, lane 7 and 8 BPH prostate. Exposure was for 13 days.

3.6 Protein Analysis by Immunochemical Detection

Nuclear proteins isolated from human BPH and CaP prostatic tissue were prepared as described (176) and compared to similarly prepared proteins from rat prostate. Using the known properties of rat probasin as a guide, the human prostatic nuclear proteins were enriched by fractionation into salt soluble proteins (0.6 M NaCl) and then subdivided into acid soluble and acid insoluble proteins (0.4 N H_2SO_4). Electrophoretic separation (176,177) of these proteins was necessary for analyzing the protein profiles. The rat probasin band on the silver stained gel is actually more intense than the photograph indicates (Figure 18), having incompletely destained from the previous Coomassie Blue staining. In comparison to all other protein bands the probasin protein has remained stained. The rest, having lost their Coomassie Blue stain or not visualized by Coomassie Blue staining, have now stained with silver. The sensitivity of Coomassie Blue staining is in the μg range, while silver staining can detect ng quantities (177).

The antiprobasin immuneserum was raised in rabbits, therefore normal rabbit serum (NRS) was used as a non-specific binding control. The separated nuclear proteins transferred to Nitroplus 2000 membrane were exposed to various dilutions of immune serum and NRS antibodies, which were detected by alkaline phosphatase conjugated to goat antirabbit IgG (181). A dilution of 10^{-2} detected the rat probasin doublet in both fractions of nuclear proteins (Figure 19) indicating once again the abundance of probasin in the rat prostate. Considering the high proportion of this protein in

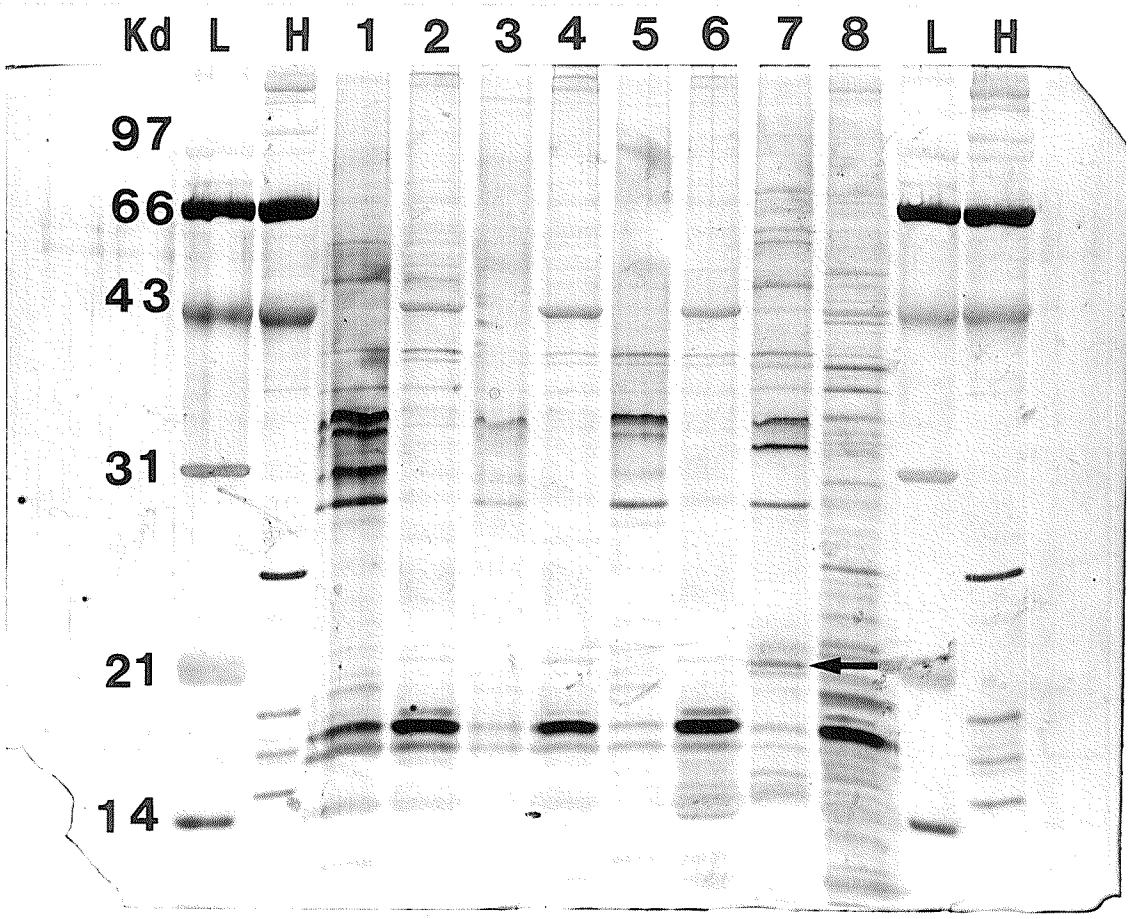

Figure 18 Silver stained nuclear protein fractions enriched as described in Section 2.7, and electrophoretocally separated on a 12.5% SDS-PAGE gel. Molecular weight markers are in Kilodaltons. Lane 1 and 2 BPH patient 20922, land 3 and 4 BPH patient 10982, lanes 5 and 6 CaP patient 19343, lanes 7 and 8 rat prostate. Lanes 1,3,5 and 7 are salt and acid soluble fractions, lanes 2,4,6 and 8 are acid soluble, salt insoluble fractions. Arrow indicates rat probasin band. 10μg protein per lane.

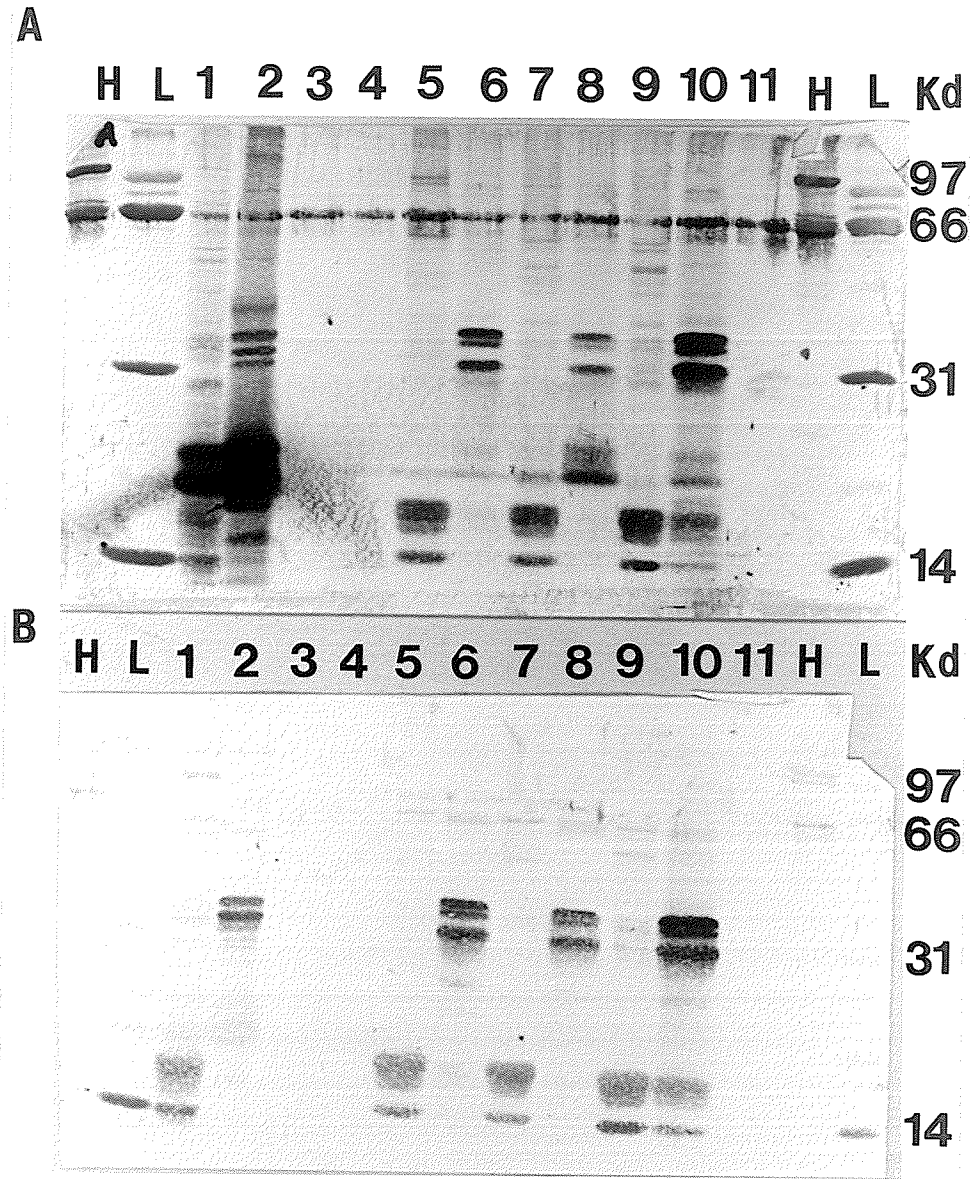

Figure 19 Western blot of nuclear proteins enriched by salt and acid fractionation then electrophoresed on mini SDS-PAGE gels. A 10^{-2} dilution of antiprobasin immune serum (A) and normal rabbit serum (B) was used and detected by alkaline phosphatase conjugated goat antirabbit IgG. Lanes for both A and B are as follows; L and H molecular weight markers, lane 1 and 2 rat prostate, lane 3 and 4 blank, lane 5 and 6 CaP patient 19343, lane 7 and 8 BPH patient 10982, lane 9 and 10 BPH patient 20922, lane 11 blank. Lanes 1,5,7,9 are salt soluble and acid insoluble fractions, lanes 2,6,8,10 are salt and acid soluble fractions. Five μg of protein was used per lane.

the rat prostate it is important to note that the NRS only slightly detects this protein which is likely due to non-specific binding (Figure 19). Although there are additional protein bands detected, some of these represent the histone proteins which are abundant nuclear proteins associated with nuclear chromatin (Davie, J.R. personal communication). These extra proteins are detected by both the immune and the NRS antisera, indicating they are being recognized through non-specific binding, or from endogenous rabbit antibodies to these proteins.

At a 10^{-2} dilution the antiprobasin antiserum clearly detects immunoreactive proteins close to the molecular weight of rat probasin in all fractions of human nuclear proteins (Figure 19). The patient material shows a more intense staining in the salt soluble samples, indicating that the properties of salt and acid solubility are probably conserved in the putative human probasin. As the dilution of this serum is increased to 10^{-3} the antiserum continues to detect probasin-like proteins in the human prostate protein samples (Figure 20). At a 10^{-4} dilution of immune serum (Figures 21,22), the doublet in patient 10982 remains detectable, however the probasin-like protein of all other human fractions is not recognized.

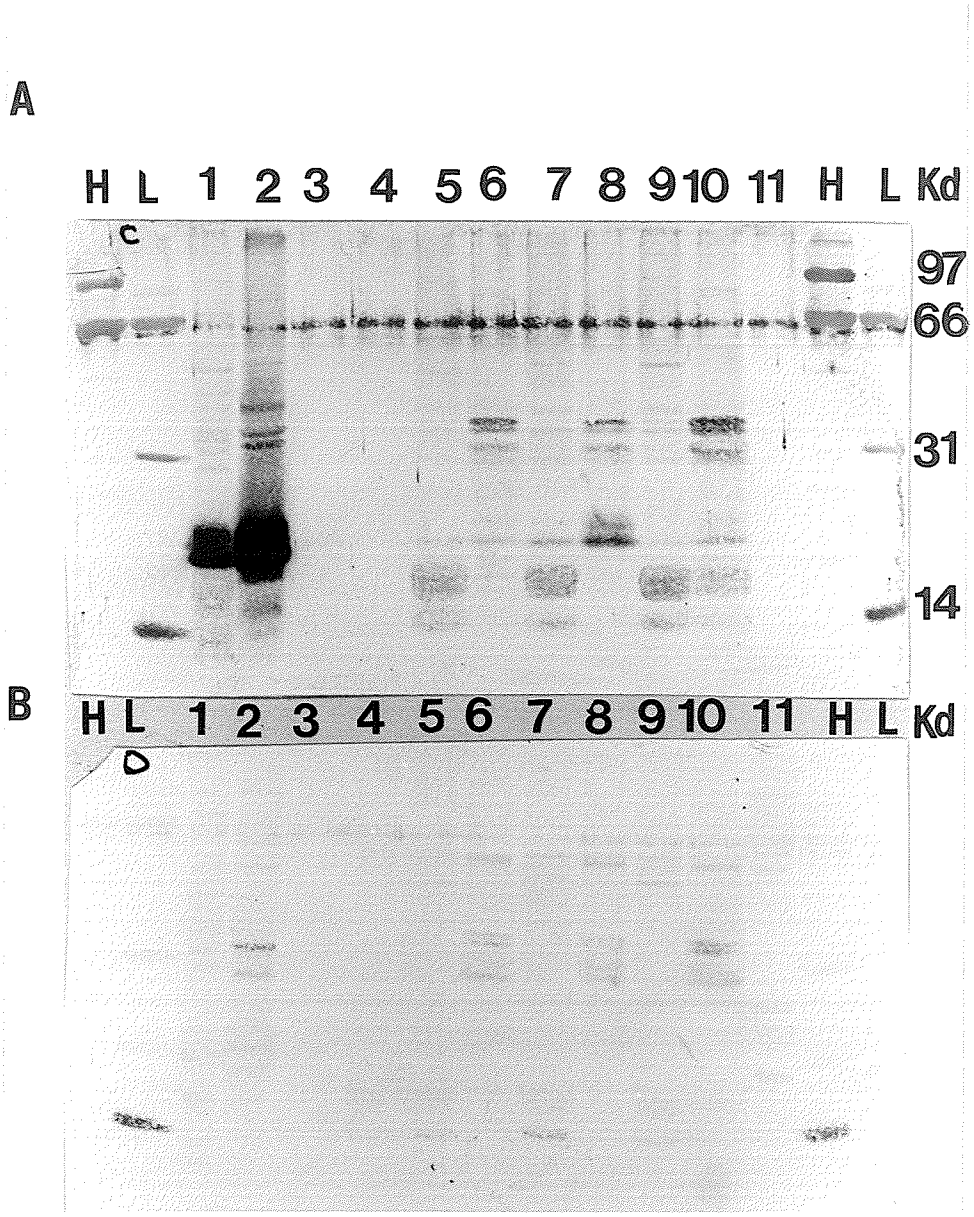

Figure 20 Western blot of nuclear proteins enriched by salt and acid fractionation, then electrophoresed on mini SDS-PAGE gels. The antiprobasin immune serum (A), and normal rabbit serum (B) were used at a dilution of 10^{-3}, and detected by alkaline phosphatase conjugated goat antirabbit IgG. Lanes for both A and B are as follows:L and H molecular weight markers, lane 1 and 2 rat prostate, lane 3 and 4 blank, lane 5 and 6 CaP patient 19343, lane 7 and 8 BPH patient 10982, lane 9 and 10 BPH patient 20922, lane 11 blank. Lanes 1,5,7,9 are acid soluble and salt insoluble fractions, lanes 2,6,8,10 are salt and acid soluble fractions. Five μg of protein was used per lane.

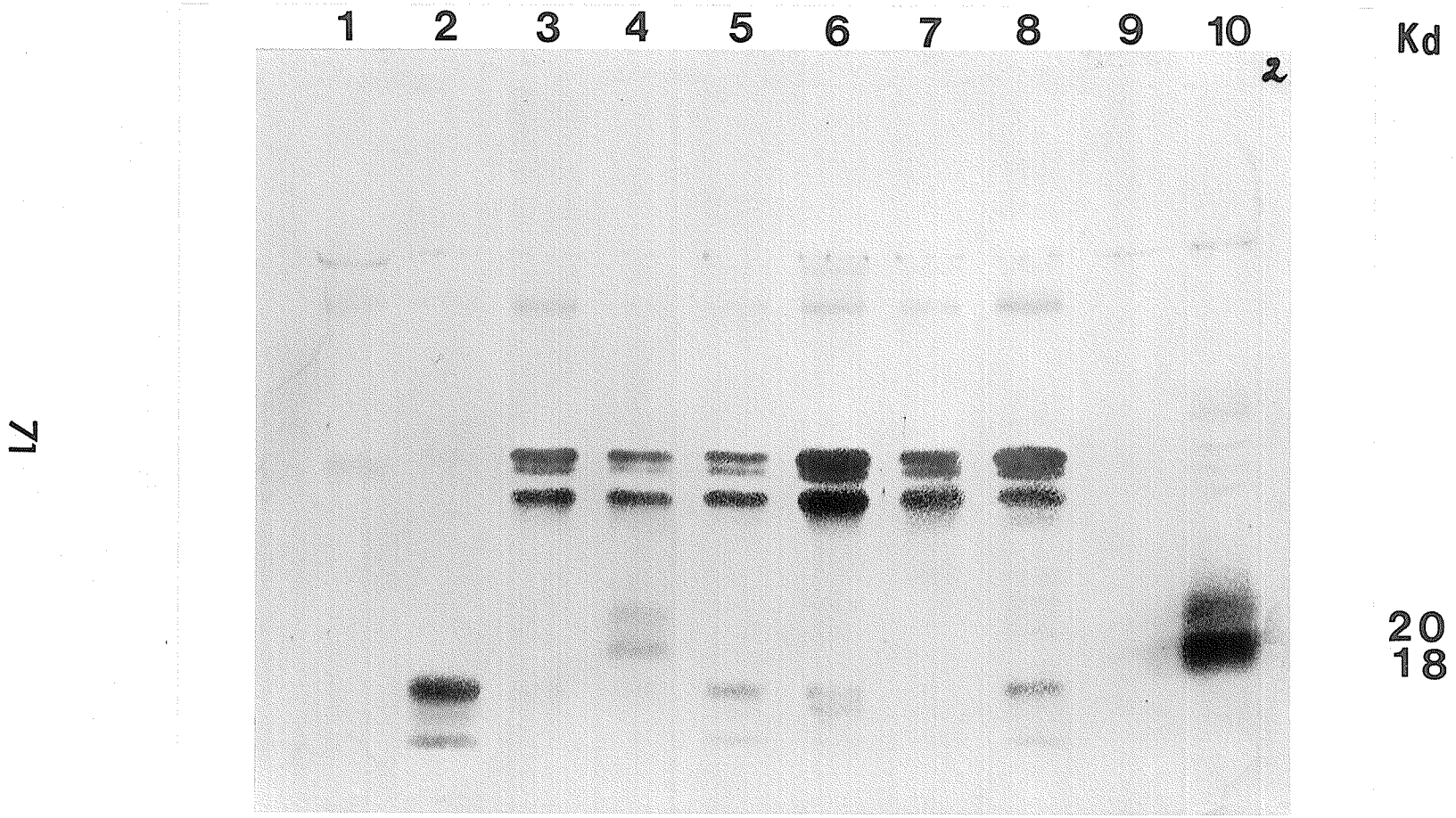

Figure 21 Western blot of 20µg human prostate, 10µg rat prostate proteins seperated by SDS-PAGE. A 10^{-4} dilution of polyclonal antiprobasin was used in the immunochemical detection. Bound antibodies were visualized by alkaline phosphatase conjugated goat antirabbit IgG. Lanes 1 to 4 BPH patient 10982, lane 5 BPH 20922, lane 6 CaP 19343, lane 7 BPH patient 3611-87, lane 8 CaP patient 519-87, lane 9 low molecular weight markers, lane 10 rat prostate. All are salt and acid soluble fractions except lane 1 cytoplasmic protein and lane 2 acid soluble salt insoluble proteins.

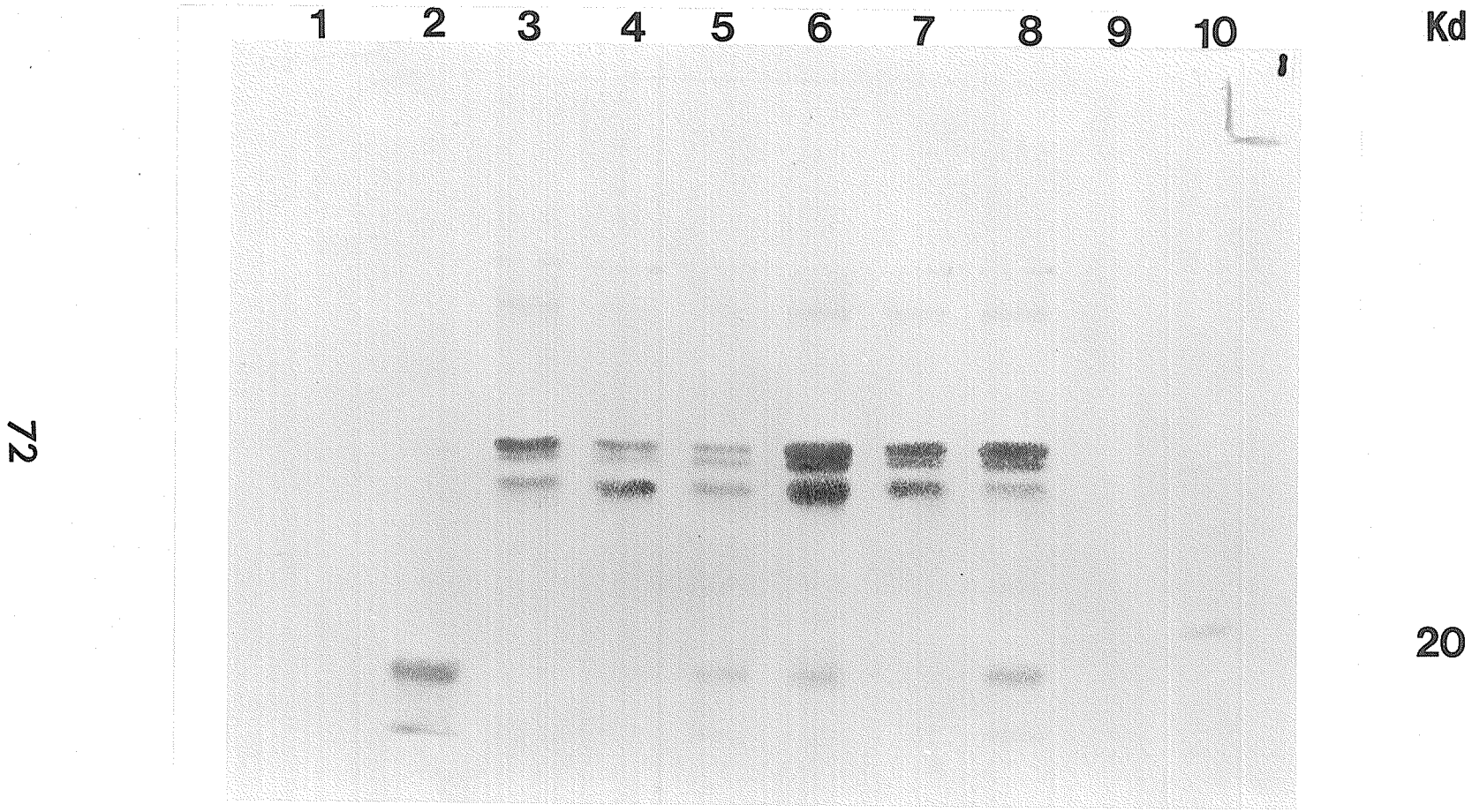

Figure 22 Western blot of 20µg human prostate and 10µg rat prostate proteins were electrophoresed on a SDS-PAGE gel. The normal rabbit serum at a dilution of 10^{-4} was used to detect non-specific proteins. Bound antibodies were visualized by alkaline phosphatase conjugated goat antirabbit IgG. Samples were loaded as described in Figure 21.

4. Discussion

Two sequences are said to be homologous if they are derived from a common ancestral sequence. Such homologous sequences may maintain equivalent functions (orthologous), or fulfill a new function (paralogous) by evolving independently after the gene duplication. The accepted dogma, that DNA codes for mRNA which in turn specifies the amino acid sequence of a protein (186,187), has resulted in the elucidation of the genetic code (188,189). The degeneracy of this code allows for the use of more than one codon to specify some amino acids (190). Some degree of nucleotide divergence is therefore allowed to occur, while still maintaining the original amino acid sequence. However, nucleotide substitutions can also result in conserved amino acid changes. The replacement of amino acids by others with very similar chemical properties (charge, size of side chain, hydrophobicity) constitutes a conservative change (191). Taken together, the nucleic acid alterations that occur may still result in the conservation of functionally relevant sequences. Consequently, the similarity of two genes should be analyzed at the nucleic acid and the amino acid level.

The thermal denaturation temperature of heteroduplexes is an indication of their degree of similarity, as represented by the guanine/cytosine content (192) and the percentage of mismatched base pairs (174). While analyzing the relationship between the probasin clone pM-40 and human prostate sequences Dodd and Matusik (personal communication) were able to detect hybridization only at 42°C (27% mismatch). The study utilized RNA pooled from

BPH patients in quantities of 0.4 μg to 1 μg. In the present analysis a variety of RNA samples, isolated from patient tissue removed by transurethral resection, were used at quantities of 1 μg to 10 μg. The apparent low level of expression of human probasin-like sequences as compared to the rat prostate message was confirmed in the present study. While there are visible differences seen between the various human RNA samples on the Northern dot blot (Figure 4), this is not the most significant observation. The more obvious difference is the binding ability of rat probasin clone DNA to sequences in the human prostate RNA samples as compared to the rat prostate RNA. The low level of hybridization in the human samples compared to the rat indicates that the abundance of human probasin-like mRNAs is very low.

Further characterization of the hybridizing sequence was undertaken using electrophoresis, to separate RNA molecules by size, and the Northern blotting technique. The probasin pM-40 sequence was able to detect an RNA species unrelated to the rRNA molecules that nonspecifically hybridized (Figure 6). The size and abundance of this human probasin-like sequence differ from the rat probasin mRNA. Although the rat RNA used for this study less than that for human samples and was from the whole rat prostate, the hybridization to the 877 bp rat probasin mRNA (114) was much stronger than to the barely discernible human prostatic sequence detected at approximately 2.0 and 1.8 Kb in some samples (Figure 6) (193). Failure to obtain hybidization signals on Northerns of poly(A^+) RNA suggests that the faint hybridizations on the previous Northerns

were non-specific. The 2.0 and 1.8 Kb bands on the total RNA Northern could represent hybridization to degradation products of the 28s and 18s ribosomal RNA, respectively. Any putative human probasin mRNA is probably of low abundance in the human prostate.

In Southern blot analysis of the rat probasin gene, four of the expected bands in the rat genome (3.8, 3.4, 2.6 and 1.3 Kb) are clearly indicated (Figure 15). The fifth band (1.8 Kb), faintly detected on longer exposure (data not shown), represents intron F and exon 7, which contains only 3' untranslated sequences (Figure 8). The hybridization pattern in the pathological tissue samples (BPH 10982, BPH 20922 and CaP 519-87) suggests there are only three detectable signals. The lack of hybridization in the cell line DNA may reflect a difference in chromosome status as these lines are known to be aneuploid. It is intriguing that the normal prostate DNA also lacks hybridization to probasin cDNA insert, however, hybridization to human p53 demonstrates that no loading error is responsible for this observation (Figure 17). The reason for this is unknown, although none of the samples demonstrate strong hybridization to pM-40, indicating low homology.

The rat probasin genomic clone has been isolated and restriction mapped for its intron/exon organization (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation) (Figure 8). This information and subclones of the genomic sequence provided a method for analyzing the relationship of the human probasin-like sequence to this gene. Using a Southern dot blot and a Southern blot of these genomic subclones, it was possible to determine which

sequences were represented in one human mRNA sample. BPH patient 10982 mRNA was converted to cDNA and used to hybridize to the probasin genomic clones (Figures 9,10). At 55°C, the Southern dot blot demonstrated considerable cross reactivity with some genomic clones and greater than control hybridization to most other genomic clones. At 42°C the Southern blot of these genomic clones demonstrated hybridization of the patient cDNA to probasin DNA, above the nonspecific hybridization to the plasmid DNA.

The probasin clones were loaded onto the agarose gel in sequence such that the 3' end of the gene is at one end of the blot (lane 1, Figures 10,11) and the 5' end is at the other (lane 8, Figures 10,11). If the probasin-like message in this BPH sample were to contain a considerable degree of homology with rat probasin, all the coding information would be expected to cross react. This seems to be the situation, with genomic subclones containing exons I, II and III (lanes 8,7,6 respectively) hybridizing to a lesser extent than those containing exons V, VI and VII (lanes 4,2,1, respectively).

Interestingly the coding information on exon IV, which is located in clone HH 1.2 (Figure 8), has not cross reacted with the BPH cDNA (Figure 10, lane 5). This fact, along with the hybridization detected to clone BB 2.7, which solely contains intron sequences except for a repeat sequence shared with exon 6, suggests there has been altered splicing in the human gene. It can be seen from the rat probasin gene restriction map (Figure 8) that the clones contain a considerable amount of intervening sequences. The conversion of heteronuclear RNA present in the mRNA sample into cDNA could

explain the cross reactivity with noncoding sequences. Conversely, the conservation of information in exon IV may not have occurred, since as compared to retinol binding protein it would code for the lower portion of the β barrel (135,136) which may require protein specific sequences to preserve the unique function of each of the family members. The pattern of hybridization indicates there is a human message which shares some regional homology with sequences of the rat probasin gene.

Proteins were isolated from human prostatic nuclei in a manner analogous to the isolation of probasin from the rat prostate. Through silver staining it is not immediately apparent that a related protein is present in the human prostate samples, however a similar sized protein is visible (Figure 18). The lower abundance of human protein is not surprising, since nucleic acid studies have demonstrated a low level expression of human probasin-like sequences. The technique of Western blotting was used to detect human prostatic proteins that would cross react with rabbit anti-probasin antiserum. It became apparent that not only was a homologous protein present, but it demonstrated a similar chemical profile to rat probasin (Figures 19-22). The protein isolation procedure had enriched for the human probasin-like protein in the salt and acid soluble nuclear fraction. The human protein profile reflects the protein size and basic nature of the amino acid sequence of rat probasin.

The specificity of antibody-antigen binding has resulted in the cross reaction of antibodies directed against a protein in one species

to a homologous protein in another species (194). The background cross reaction of this polyclonal immune serum to nonhomologous proteins could be due to the presence of similar epitopes, but is more likely explained as the binding of endogenous antibodies in the serum. This latter hypothesis can be demonstrated by the binding of normal rabbit serum to these background proteins as well (Figure 19-22). The difference in intensity of the "nonspecific" binding is due to the varying amounts of immunoglobulins present in the two serum populations, since dilutions were made by volume and were not normalized for actual protein content.

Information has been gathered on nucleic acid and amino acid similarity between rat probasin and human prostatic mRNA, genomic DNA and protein sequences. Human mRNAs related to rat probasin, if present, are either low in abundance or the percentage of homology is insufficient for stable hybridization. The similarity of genomic DNA restriction patterns and the binding of human prostatic cDNA to probasin gene clones is support for a related sequence, although until a human clone is isolated it will not be possible to conclude that the RNA and DNA sequences represent the same gene. The most promising information on the nature of human probasin is via the immuno-characterization. To determine if the immunoreactive protein in the human prostate was representative of the nucleotide sequence detected it would have been necessary to hybrid select a human message with the rat probasin clone, then immunoprecipitate the <u>in vitro</u> translated protein. This was not possible because of the limited homology detected at the nucleic acid level. An alternative

attempt aimed at immunoprecipitating a protein from the in vitro translation products of human RNA samples was unsuccessful, possibly further reflecting the low abundance of the putative human probasin-like message. The low degree of homology at the nucleic acid level and low abundance of the putative message impede the ability to link any nucleic acid hybridization to the immunoreactive protein.

To further these studies by isolating a human prostatic clone a number of variables should be considered. An expression library could be screened for detecting immunoreactive fusion proteins. The tissue selected for the creation of the library should first be screened for an immunologically related protein by Western blot analysis. It will be important to control for the possibility that the rat probasin polyclonal antiserum may recognize an epitope common to several members of the ligand carrier family. It will therefore be necessary to devise methods to screen out α2u-G, MUP, BLG and RBP proteins expressed from bacteriophage clones.

To enrich for human prostatic cells expressing this probasin-like protein it may be useful to initiate some comparative physiology. The cells of the human gland which contain the most probasin-like protein could be located by immunohistochemistry. The highest probasin expressing tissue in the rat is the lateral lobe of the prostate, although the dorsal lobe also expresses this gene to a considerable amount (114). The dorsolateral prostate of the rat has been described as corresponding to the peripheral zone of the human prostate because of the development of prostatic carcinoma in both

these regions of the gland (100). However, the human tissue received by transurethral resection consists of transitional zone cells where the development of BPH occurs (1,13). Localizing the region of the gland containing probasin in an intact prostate obtained by suprapubic prostatectomy may aid in selecting cells for an expression library.

Another physiological factor to consider is the zinc content of the rat prostate. The lateral lobe, in addition to expressing the highest levels of probasin, is the site of the highest concentration of zinc (78,87). In addition, the dorsolateral prostate has a higher rate of zinc uptake than any other tissue (98). The localization of zinc in the human prostate (83) correlates with the distinct zones found by McNeal and Tisell (10,12), increasing in concentration from the region closest to the bladder towards the apex of the prostate. The conflicting reports attempting to correlate zinc levels with prostatic disease suggests the levels in BPH are higher than CaP, but the in vitro uptake of zinc is the reverse (79,81-86). Considering that there is little evidence to suggest that BPH progresses to CaP and their site of origin differs, the zinc levels observed in these two disease states are probably due to functional differences in the gland itself. The observation that probasin is a zinc responsive gene (116,122), and possibly binds zinc (Spence, A.M., McNicol, P., Leco, K., and Matusik, R.J. in preparation) is an indication that a homologous gene in the human prostate may also be associated with the region of the gland that is highest in zinc content.

The association of probasin with epithelial cells rather than stromal tissue (122) is a third factor that could aid in the isolation of a human probasin-like clone. In the guinea pig, it is well established that castration causes a more rapid and complete regression of seminal vesicle epithelial components than their stromal counterparts (195). In the rat however, the lateral prostatic epithelium contains cells that resist the classical atrophy induced by castration (104). Although regression occurs, the lateral prostate glandular morphology remains visible even three months after castration, while the ventral lobe morphology has virtually disappeared (196). The progression of androgen sensitive cells to an insensitive state has been reported to occur without the associated loss of a functional steroid receptor (69). A recent report that androgen receptor levels decrease in all lobes of the rat prostate after castration, but return to normal levels in the lateral lobe after fourteen days of androgen deprivation (197) suggests that the lateral lobe of the rat prostate, the site of high probasin synthesis, has the potential to respond to minute levels of androgen or the ability to progress to an insensitive state. The rebound of probasin mRNA expression after prolonged castration (122) suggests its regulation correlates with the rebound of the androgen receptor (197) in this region of the rat prostate. The cells in the human prostate that perform the same function as the lateral lobe of the prostate may therefore be those cells that become overly proliferative and lose their ability to respond to hormone therapy.

5. Conclusion

The isolation of prostate tissue by transurethral resection inherently results in a tissue sample that does not reflect the heterogeneity of the human prostate. The work of McNeal and Tisell has conclusively demonstrated that the human prostate does consist of histologically different regions, in spite of the difficulty in detecting zonal differences by anatomical examination (12-15). These observations may reflect the difficulty in the immunodetection of probasin-like sequences in all tissues examined. The morphological differences in the human prostate also reflect the need to ensure by Western blot analysis that the tissue received will be useful to the isolation of probasin-like sequences.

The ability to detect an immunoreactive protein in the human prostate that shares the nuclear localization and basic charge with the rat probasin protein lends itself to alternative methods for isolating the human clone. The nucleic acid studies have demonstrated that genetic drift may have resulted in limited homology across species, while the amino acid sequence has remained relatively conserved. The resultant poor nucleotide similarity prevents the use of rat probasin DNA sequences as a method of isolating human probasin-like clones. The probasin antiserum however, could be used to screen an expression library made from a human prostate tissue that is positive by Western analysis. Conversely the protein could be isolated by gel elution or by heparin binding, since rat probasin has been demonstrated to adhere

to a heparin column (198). This isolated human prostatic protein could be characterized and the amino acid sequence used to create degenerative oligonucleotide sequences for use as a nucleic acid probe. Combining the information gathered from this present study should provide a number of paths towards the isolation of the human prostatic nucleotide sequences responsible for the probasin-like protein recognized in the human prostate.

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