PROSTATE-SPECIFIC ANTIGEN: GENE STRUCTURE AND REGULATION OF EXPRESSION

PROSTAAT SPECIFIEK ANTIGEEN:
GENSTRUKTUUR EN REGULATIE VAN EXPRESSIE

PROEFSCHRIFT

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Tijdens dit onderzoek op de afdeling PA bestudeerde ik PA, begreep ik PA en werd ik PA, bedankt PA.
Aan: MIA, PA & MA.

CONTENTS:

LIST OF ABBREVIATIONS

aa	Amino acid
AR	Androgen receptor
ARE	Androgen responsive element
ß-MSP	ß-Microseminoprotein
bp	Basepairs
BPH	Benign prostate hyperplasia
CAT	Chloramphenicol acetyl transferase
cDNA	Copy DNA
DBD	DNA binding domain
DHT	Dihydrotestosterone
DFP	Diisopropylfluorophosphate
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
EGF-BP	EGF-binding protein
ER	Estrogen receptor
FSH	Follicle stimulating hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
hGK	Human glandular kallikrein
HMW-SV-proteins	High molecular weight seminal vesicle proteins
HRE	Hormone responsive element
kb	Kilo basepairs
kDa	Kilo Dalton
KLK1	Human tissue kallikrein
KLK2	Human glandular kallikrein-1
LHRH	Luteinizing hormone-releasing hormone
LTR	Long terminal repeat
mGK	Mouse glandular kallikrein
MMTV	Mouse mammary tumor virus
mRNA	Messenger RNA
NF	Nuclear factor
NGF	Nerve growth factor
Oct	Octamer binding protein
PA	Prostate-specific Antigen
PAP	Prostatic acid phosphatase
PBP	Prostatic (steroid) binding protein
PMSF	Phenylmethylsulfonyl fluoride
PR	Progesterone receptor
PRE	Progesterone responsive element
PS	Rat tissue kallikrein
PSP94	Prostatic secretory protein
rGK	Rat glandular kallikrein
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SMG	Submandibular glands
SSC	Standard saline citrate
T	Testosterone
TK	Thymidine kinase

The one and three letter-codes for amino acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

The (GCG)letter-code for nucleotides

A	Adenine
C	Cytosine
G	Guanine
T	Thymidine
K	G/T
M	C/A
R	G/A
S	C/G
W	A/T
Y	C/T
N	G/A/T/C

CHAPTER I

GENERAL INTRODUCTION

INTRODUCTION

The development of the prostate is a very complex process. The normal growth and development of the prostate is controlled by epithelial-stromal cell interactions and is strongly androgen-dependent. Absence of androgens or functional androgen receptors (ARs) cause a complete inhibition of prostate development. Androgens also play an important role in the maintenance of morphology and function of the adult prostate.

The growth of the majority of prostate tumors is also androgen-dependent. Several different forms of endocrine treatment can be used for therapy of infiltrated and metastasized prostate cancer. Endocrine treatment can include orchiectomy or application of anti-androgens, estrogens, and LHRH agonists. In the majority of tumors a clinical effect of the hormone therapy can be observed, however, eventually androgen-independent tumors continue to grow.

Regulation of specific gene expression will be one of the key elements in prostate development. So far, it is not known which genes or gene products are essential for normal prostate growth and development, or androgen-dependent prostate tumor growth. Similarly, the molecular mechanism of androgen-independent tumor growth is not understood.

This study was initiated in order to gain more insight in the regulation of gene expression in the prostate. The regulation of Prostate-specific Antigen (PA) expression was chosen as a model system because of its unique prostate specificity and presumed androgen-regulated expression. An additional reason for investigation of PA expression was the broad application of PA as a prostate-specific tumor marker. PA serum levels are used both for diagnosis of prostate cancer and for monitoring of prostate cancer therapy. Normally, no or only small amounts of PA can be found in the serum. However, in the case of invasive growth of prostate tumors, significantly elevated PA levels can be detected. Therefore, as a spin off of our studies, we expected to obtain information about the regulation of expression of this important prostate tumor marker, which could be of potential importance for clinical practise.

1 THE PROSTATE

1.1 PROSTATE MORPHOLOGY AND DEVELOPMENT

Prostate morphology:

The adult human prostate surrounds the urethra and is situated immediately below the bladder. It is composed of 30 to 50 compound tubuloalveolar exocrine glands. The prostate gland is exclusively present in mammals and prostate morphology can differ in various different species. In primates and dogs the prostatic ducts originate from the urethra and radiate peripherically, thereby completely surrounding the urethra. In other species the prostatic ducts are organized and encapsulated in individual lobes (Cunha et al., 1987). The prostate of rats and mice is organized in ventral, dorsolateral and anterior lobes. In earlier studies the human prostate was also subdivided in lobes, but after histological examination four distinct zones were postulated: the anterior fibromuscular zone, the peripheral zone with the main glands, the transition zone with main glands converting into periurethal with mucosal glands and the central zone with the submucosal glands (reviewed by Cunha et al., 1987). Another frequently used, more simplified classification is: inner glands (the central zone and the transition zone) and outer glands (the peripheral zone).

The acini of the inner and outer glands are both lined by a double layer of epithelial cells (see Figure 1). The inner cylindric cell layer has secretory activity. The outer or basal cell layer is composed of myoepithelial cells. The acini and ducts are surrounded by stromal tissue (see Figure 1). The prostatic stroma is composed of smooth muscle cells, fibroblasts, blood vessels, connective tissue and lymphatic cells.

Prostate development and homeostasis:

It was demonstrated in mouse and rat prostates, that the stroma plays an important role in maintaining the normal function of the epithelial cell layers and in the morphogenesis of the prostate (Chung & Cunha, 1983; Tenniswood, 1986; Cunha et al., 1987; Chang & Chung, 1989; Chung et al., 1990). The normal growth and development of the prostate is strongly androgen dependent. Androgen action is mediated by the androgen receptor (AR) in target cells (discussed more extensively in 1.3). The AR is a ligand-responsive transcription factor able to stimulate transcription of target genes after androgen-binding (see also Figure 6). In the prostate, testosterone (T) is reduced by the enzyme 5α-reductase to dihydrotestosterone (DHT), which seems to be the main ligand for the AR.

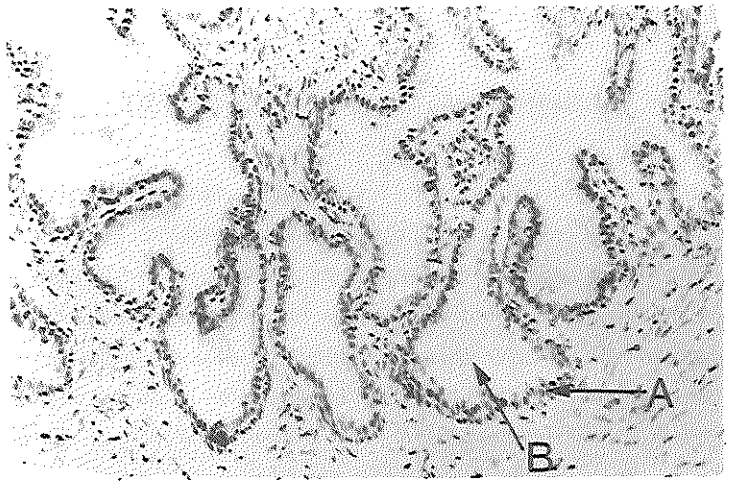

FIGURE 1: Section of prostate tissue (immunohistochemically stained with anti AR antibodies). The arrow marked A points to the double layer of epithelium, which is surrounded by the stromal tissue. The arrow marked B points into the lumen of the gland. Magnification is 25X. This photograph was kindly provided by Drs.J.A.Ruizeveld de Winter.

The prostate develops from the (endodermal) urogenital sinus as epithelial buds. During early embryogenesis the epithelial component of the endodermal urogenital sinus will differentiate in the presence of T and functional ARs to prostate epithelium, or in the absence of T or functional ARs to vaginal epithelium (Takeda et al., 1986; Cunha et al., 1987). During fetal life, the epithelial component is not directly androgen sensitive. At this stage the androgen-dependent growth and differentiation of the epithelial cells is controlled by the mesenchymal component (Cunha et al., 1980). It is presumed, that the mesenchymal component produces (growth) factors, which stimulate the epithelial cell proliferation (Tenniswood, 1986; Chang & Chung, 1989). In later stages of prostate development expression of the AR is also observed in epithelial cells, which renders these cells directly androgen sensitive (Shannon & Cunha, 1983). It is still a matter of dispute whether the growth of prostate epithelial cells can directly be stimulated by androgens. There is little doubt that cocultivation of adult prostate epithelial cells with mesenchymal cells results in DHT-stimulated proliferation (Chang & Chung, 1989). Prostate tumor cell lines can also be stimulated by mesenchymal cells (or conditioned medium of in vitro grown mesenchymal cells) in their in vitro and in vivo growth (Camps et al., 1990; Gleave et al., 1991). In addition, however, proliferation of prostate tumor cell lines incubated in vitro without stromal cells can also be stimulated

by androgens. So, at least in the latter situation stromal cell independent growth of epithelial cells has been established, although this is possibly less efficient than in the presence of the stromal compartment. In contrast, it has been documented that DHT is able to inhibit the in vitro growth of normal prostate epithelial cells in the absence of the stromal cells (Nishi et al., 1988; Chang & Chung, 1989).

The adult prostate decreases considerably in size after androgen deprivation (orchiectomy), caused by atrophy and active cell death (Lee, 1981). The cell death within the rat prostate is not homogeneous, the distal (secretory active) epithelial component is more affected than the proximal component (DeKlerk & Coffey, 1978; English et al., 1985; Lee et al., 1990; Rouleau et al., 1990). The distal epithelial component is characterized by reductions in nuclear size, secretory granula, rough endoplasmatic reticulum and the Golgi apparatus (Brandes, 1966; Leav et al., 1971; Helminen & Ericsson, 1972; Rouleau et al., 1990).

1.2 THE FUNCTION OF THE PROSTATE

The prostate is the largest of the male accessory sex glands (prostate, seminal vesicles and bulbourethal glands). The specific products of these glands probably have the common purpose to increase the efficiency of conception. Our knowledge concerning the molecular mechanisms involved in this process is very limited. The function of some of the proteins involved has been elucidated, however, the function of a large number of other proteins is still unclear (Cunha et al., 1987).

During spermatogenesis and epididymal maturation of the spermatozoa, the sperm cells are prepared for flagellar movement. During this time period and during storage in the epididymal cauda the sperm cells do not exhibit any progressive movement. At ejaculation the prostate and the seminal vesicles secrete their fluids into the urethra, where they mix with the sperm cells. In this way the semen is formed, which immediately turns into a gel-like structure or coagulum. The sperm cells are entrapped within the coagulum. The gel–like structure is predominantly formed by HMW-SV-proteins (High Molecular Weight Seminal Vesicle proteins), which are abundantly produced by the seminal vesicles (Lilja et al., 1984). The HMW-SV-proteins include as a major component semenogelin, a 52 kDa protein, and the less-abundant 71 and 76 kDa semenogelin-related proteins (Lilja, 1988; Lilja et al., 1989). These proteins and

fibronectin, which is also produced by the seminal vesicles, can form disulfide-linked high molecular weight complexes (Chaistivanich & Boonsaeng, 1983; Lilja & Laurell, 1985). The HMW-SV proteins and fibronectin are cleaved to low molecular weight proteins by proteases present in the prostatic fluid (Lilja et al., 1984; Lilja & Laurell, 1985). Cleaving of the proteins might result in the generation of new bioactive peptides (Lilja et al., 1989). Within 15-20 minutes the coagulum is liquefied and the sperm cells display vigorous progressive motility. This is mainly due to the proteolytic activity of Prostate-specific Antigen (PA), which is a major constituent of the prostatic secretion (Lilja, 1984, 1985, 1987; Lilja & Abrahamson, 1988; McGee & Herr, 1988; Lee et al., 1989).

Two other proteins, which are secreted in large amounts by the prostate are Prostatic Acid Phosphatase (PAP) and Prostatic Secretory Protein (PSP_{94}) (Lilja & Abrahamson, 1988). PSP_{94} is composed of 94 amino acid residues (Mbikay et al., 1987) and is also known as ß-microseminoprotein (ß-MSP) (Akiyama et al., 1985), or ß-inhibin (Seidah et al., 1984; Johansson et al., 1984). The physiological functions of these proteins are still unknown. PAP is a glycoprotein, composed of two disulfide-linked 50 kDa subunits (Choe & Rose, 1982), with phosphotyrosyl-protein phosphatase activity (Li et al., 1984; Lin & Clinton, 1986). The PAP cDNA has been cloned and characterized (Vihko et al., 1988; Sharief et al., 1989; Tailor et al., 1990) and the gene has been assigned to chromosome 3 (Sharief et al., 1989). PSP_{94} or ß-MSP is a protein of 14 kDa. The PSP_{94} cDNA has been cloned and its expression appeared not to be confined to the prostate alone (Ulvsback et al., 1989; Weiber et al., 1990). It has been suggested to have an inhibitory effect on the FSH secretion of the pituitary, but this was later proven to be incorrect (Kohan et al., 1986; Mbikay et al., 1987).

Another prostate product is Zn-α_2-Glycoprotein, which is produced and secreted at lower levels than PA, PAP and PSP_{94}. Zn-α_2-Glycoprotein appears to be a truncated secretory major histocompatibility complex related protein, with so far unknown physiological function. This 40 kDa protein is also found in serum of men and women, although in a 5-fold lower concentration as compared to that in prostatic fluid (Frenette et al., 1987; Araki et al., 1988).

In the rat prostate a protein called Prostatic (steroid) binding protein (PBP) is produced at high levels. It was originally identified by Heyns et al. (1976) and appeared to be specific for the rat ventral prostate. It was recognized by its low affinity steroid-binding properties (Heyns et al., 1976). In vitro it is able to inhibit binding of the androgen-AR

complexes to prostatic chromatin (Chen et al., 1979). These properties, however, are thought not to be related to its in vivo function, which is still unclear (Heyns et al., 1981). Rat PBP consists of two subunits: One contains the polypeptides C1 and C3; the other is composed of C2 and C3. The C1, C2 and C3 polypeptides are encoded by three different, but related genes. The expression of these genes is confined to the epithelial cells of the prostate (Heyns et al., 1981; Aumüller et al., 1982) and regulated by androgens (Parker et al., 1978). C3 can be coded by two non-allelic genes C3(1) and C3(2), of which the C3(2) is probably silent (Hurst & Parker, 1983). The genes encoding rat PBP are introduced here, because of their androgen-regulated and tissue-specific expression. These characteristics will be discussed in I.3 "Androgen-regulated gene expression".

1.3 PROSTATE CANCER

General aspects of prostate cancer:

The prostate has been a subject for study for many years, because it is the site of various types of inflammatory and infectious conditions as well as benign and malignant proliferative changes in aging males. Benign Prostate Hyperplasia (BPH) arises almost exclusively from the transition zone (inner glands) and prostatic adenocarcinoma from the peripheral zone (outer glands) (McNeal, 1984). Prostate cancer is discriminated from BPH by the absence of the basal mebrane, which is present in both BPH and normal prostate tissue. Both BPH and prostatic cancer can cause obstruction of the urethra (reviewed by Korman, 1989). Because prostatic tumors are mostly located in a more peripheral area than BPH, the symptoms of prostatic cancer are in general noticed much later. As a consequence, most prostatic cancers are in an advanced stage at the time of presentation.

In most Western countries prostatic cancer is the second most common cancer, after lung cancer, occurring in males (Silverberg, 1987). The incidence of prostatic cancer in the Netherlands in 1982 was 25.1 (per 100,000 male population average annual age standardized) (Zaridze et al., 1984). The incidence rate varies strongly with age. Prostatic cancer is a disease almost exclusively found in the elderly male population (Silverberg, 1987).

Molecular mechanisms of prostate cancer:

For the study of the molecular mechanisms involved in prostatic cancer a limited number of transplantable prostate tumors or in vitro growing cell lines is available. The origin and characterization of most of the prostate tumors/cell lines, with their various sublines, has been reviewed by Isaacs (1987). The human prostate tumors/cell lines, which were used for studies presented in this thesis, are listed in Table 1. Only a few of the human prostate cell lines can be propagated in vitro. Tumors/cell lines with a detectable AR expression, show androgen-responsive growth. No aberrations were found in the affinities and specificities of the AR from BPH, prostate carcinoma, and the prostate tumor lines PC 82 and HONDA compared to normal prostate tissue (van Aubel et al., 1985; Ito et al., 1985; Brinkmann et al., 1987). The AR in the human prostate tumor cell line LNCaP has an unusual ligand specificity, due to a mutation in the ligand-binding domain (Veldscholte et al., 1990a,b). The LNCaP AR is not only able to bind androgens with high affinity, but also estrogens and progestagens. The AR is undetectable in the androgen-independent prostate tumors/cell lines (Trapman et al., 1990 and Table 1). Prostate tumors/cell lines stem from different origins. These origins can be primary tumor, lymph node metastasis and bone metastasis. Not all prostate tumors/cell lines produce a detectable level of PA and/or hGK-1 mRNA. A correlation is found between the expression of PA and hGK-1 mRNA and the androgen-responsive growth or AR expression of these tumors/cell lines (see Table 1).

Two different categories of genes are considered to play a major role in tumorigenesis: oncogenes and tumor suppressor genes (Weinberg, 1989). (Proto)-oncogenes form a class of genes that upon activation can cause malignant transformation. Activation of oncogenes can occur by change in the properties or expression pattern of the protein product. The expression and structure of oncogenes in prostatic cancer is poorly studied, due to the fact that RNA and DNA samples collected from normal prostate tissue or BPH have a large stromal component, whereas RNA and DNA samples from the tumors have a much larger epithelial component. In addition, the growth pattern of many prostate tumors is heterogeneous, which may result from different modifications at the genomic level. Despite these major drawbacks, several studies concerning the role of oncogenes in prostate cancer have been published (Cooke et al., 1988; Nag and Smith, 1989, Viola et al., 1986; Davies et al., 1988; Fan, 1988; Varma et al., 1989; Ichiwaka et al., 1991; Gumerlock et al., 1991). So far,

Table 1: Human prostate tumors/cell lines

	IN VITRO GROWTH	IN VIVO GROWTH	EXPRESSION OF AR[1]	EXPRESSION OF PA AND hGK-1[2]	ORIGIN
PC 82[3]	-	+	+	+	Primary tumor
PC EW[4]	-	+	+	+	Lymph node metastasis
PC 3[5]	+	-	-	-	Bone metastasis
PC 133[6]	-	+	-	-	Bone metastasis
PC 135[6]	-	+	-	-	Primary tumor
LNCaP[7,8]	+	±	+	+	Lymph node metastasis

[1] Data from: Trapman et al., (1990)
[2] Data from: Riegman et al., (1991a)
[3] Data from: Hoehn et al., (1980)
[4] Data from: Hoehn et al., (1984)
[5] Data from: Kaighn et al., (1979)
[6] Data from: van Steenbrugge (1988)
[7] Data from: Horoszewich et al., (1980)
[8] Data from: van Steenbrugge et al., (1989)

however, the data obtained do not point to one or more of the well known oncogenes as being major contributors to development or progressive growth of prostate cancer.

Tumor suppressor genes promote tumor growth through their inactivation. Inactivation may involve deletion, point mutation or mitotic recombination (Knudson, 1989). The tumor suppressor gene known to be modified in some prostate cancer tissues is the retinoblastoma gene (Bookstein et al., 1990a,b). Also, familial aggregation of prostate cancer has been described by several authors (Woolf, 1960; Cannon et al., 1982; Meikle et al., 1985; Steinberg et al., 1990). Several families with an excess of prostate cancer have been identified. Possibly these families possess a genetic predisposition to prostate cancer (Steinberg et al., 1990). These families could form an important source of information when studying the involvement of tumor suppressor genes in prostatic cancer.

Prostate tumor markers:

The prognosis of prostatic cancer is predominantly based on histological grading and clinical staging. Determination of a prostate tumor marker in serum is one of the techniques used to assess the clinical stage of prostatic cancer (reviewed by Gittes, 1991). At present the most important prostate tumor marker is Prostate-specific Antigen (PA). PA is exclusively synthesized in the epithelial cells of the prostate. Normally, PA is only secreted in the lumen of the prostate gland, but on invasive growth of prostate tumors, it is also detectable in the serum. Since the discovery of PA as a putative prostate tumor marker (Papsidero et al., 1980; Kuriyama et al., 1980) it was thoroughly investigated and compared with the previously used marker Prostate Acid Phosphatase (PAP) (Kuriyama et al., 1982; Takeuchi et al., 1983; Chu & Murphy, 1986; Ito et al., 1986; Killian et al., 1986; Stamey et al., 1987; Barak et al., 1989; Hasenson et al., 1989; Hara & Kimura, 1989; Cooner et al., 1990; Filella et al., 1990; Babaian et al., 1991; Catalona et al., 1991). It is now generally accepted that PA is more sensitive and selective than PAP. PA also appeared to be a useful marker for monitoring prostatic cancer recurrence and treatment response (Kuriyama et al., 1981; Hudson et al., 1989; Stamey et al., 1989abc; Arai et al., 1990).

In general, patients with prostatic cancer show an increase of the mean PA serum level with advancing disease stage. However, the spread of PA serum values also increases (Kuriyama et al., 1980; 1982). PA expression is diminished in undifferentiated tumors (Epstein & Eggleston, 1984; Gallee et al., 1990; Qui et al., 1990) (discussed in I.1.4). This could have its impact on the PA serum levels in

patients with prostatic cancer. Other parameters, which will contribute to the serum level are total tumor load (Oesterling et al., 1988; Stamey et al., 1989a; Partin et al., 1990), which increases considerably with advancing stage, and the contact of prostate epithelial cells with blood or lymph fluid. Combined, the variation in these different factors might explain the observed large differences of PA serum levels in patients with far advanced prostatic cancer. PA antibodies can also be used to discriminate prostatic metastases from metastases from different origin by immunohistochemistry (Papsidero et al., 1985).

Metastatic prostatic cancer is treated by hormonal manipulation via orchiectomy, administration of estrogens, luteinizing hormone-releasing hormone analogs and anti-androgens. The majority of the treated patients initially responds to this therapy. However, endocrine therapy hardly ever leads to the cure of a patient (Menon & Walsh, 1979). Relapse is characterized by a progression of the tumor from an androgen-responsive to an androgen-independent stage. The response of the patients to the treatment can be monitored using PA serum levels. Even in the majority of D2 stage patients, anti-androgen treated patients show an, initially often dramatic, decrease in PA serum levels, but after an avarage period of about 6 months, PA serum levels of the majority of these patients begin to rise again (Stamey et al., 1989c).

2 KALLIKREINS

2.1 PROSTATE-SPECIFIC ANTIGEN

Properties of PA:

PA was first isolated and characterized as a 33-35 kDa protein by Wang et al. (1979). It has independently been characterized by other investigators and thus became known under several different names. So, PA (PSA or APS) turned out to be identical to γ seminoprotein (Schaller et al., 1987) and P-30 (Graves et al., 1990). P-30 became known in forensic science, where it is used as a marker for postcoital detection in rape investigations (Sensabaugh, 1978; Graves et al., 1985). In men without prostatic lesions, it is only detected in high amounts in the epithelial cells of the prostate, the prostatic fluid and in the semen (Wang et al., 1981; Papsidero et al., 1981; Tsuda et al., 1983; Gallee et al., 1986; Lilja & Abrahamson, 1988).

PA was found to have proteolytic activity, with an optimum at neutral pH (Ban et al., 1984). The proteolytic activity can be inhibited by Cu^{++}, Zn^{++} or Hg^{++} ions, the protease inhibitors leupeptin and aprotinin, and the serine protease inhibitors PMSF and DFP (Ban et al., 1984; Watt et al., 1986). The amino acid sequence of PA, as determined by peptide sequence analyses (Watt et al., 1986) and as deduced from the cDNA sequence (Lundwall & Lilja, 1987; Riegman et al., 1988, Chapter II) showed a high degree of homology with that of other serine proteases of the kallikrein family (discussed in I.2.3).

The mature PA protein consists of 237 amino acids, it has a signal peptide of 17 amino acids and a profragment of 7 amino acids. These moieties were identified by their homology to other kallikreins and to trypsinogen (Thomas et al., 1981; Mason et al., 1983; Lundgren et al., 1984; Fukushima et al., 1985). The hydrophobic signal peptide is split of during PA secretion. The profragment or zymogen peptide keeps PA inactive until removal. The physiological function of PA is already described in I.1.2. "The function of the prostate".

Expression of PA:

The expression of PA mRNA varies strongly between the different prostate tumors and cell lines (See also Table 1; Riegman et al., 1991a, Chapter VI). PA mRNA could not be detected in the androgen-independent prostate tumors PC 133 and PC 135 and the androgen-independent cell line PC 3. However, in the more differentiated,

androgen-dependent prostate tumors PC EW and PC 82 and in the androgen-sensitive prostate tumor cell line LNCaP, PA mRNA is present in large amounts. As compared to PA mRNA levels in BPH, decrease of PA mRNA expression was observed in malignant prostate epithelium, using in situ hybridisation techniques (Qui et al., 1990). This finding is in full agreement with the previously described diminished PA protein levels in prostate cancer tissue, especially in undifferentiated tumors, as determined by immunohistochemistry (Epstein & Eggleston, 1984; Gallee et al., 1990).

The expression of PA mRNA is androgen-responsive and can be induced within 6 hours by androgens in the androgen-sensitive prostate tumor cell line LNCaP (Trapman et al., 1988; Riegman et al., 1991a,b, Chapters VI and VII). In the androgen-dependent prostate tumors PC EW and PC 82, propagated as transplants on male nude mice, deprivation of androgens leads to a marked decrease of PA mRNA expression.

PA transcripts:

Northern blots hybridized with PA specific probes revealed seven different PA mRNAs, ranging in size from 0.5 to 5.6 kb (Riegman et al., 1988, 1991a, Chapters II and VI). The most abundant transcript has a length of 1.5 kb. The corresponding cDNA contains an open reading frame encoding a 261 amino acid preproprotein (Lundwall & Lilja, 1987; Riegman et al., 1988, Chapter II). Two other (less abundant) transcripts turned out to be the result of alternative processing. A 0.9 kb transcript is formed by intron retention and alternative polyadenylation. A 1.9 kb transcript is the result of alternative splicing (Riegman et al., 1988, Chapter II). The open reading frames in these transcripts differ at the 3'-end from that in the 1.5 kb mRNA. The function of the presumed protein products, which are probably devoid of serine protease activity, is unclear. Human BPH lysates often contain in addition to the major 35 kDa PA component a 25 kDa component (Gallee et al., 1986), which is recognized by PA antibodies. This 25 kDa protein could be a translation product from one of these less abundant PA transcripts or, alternatively, a degradation product from the 35 kDa PA protein.

2.2 HUMAN GLANDULAR KALLIKREIN-I (hGK-1)

In addition to PA, the epithelial cells of the prostate presumably express a related protein: hGK-1. The expression of hGK-1 has so far only been observed at the mRNA level; a hGK-1 protein has not yet been identified (Chapdelaine et al., 1988a; Morris, 1989; Henttu et al., 1990; Riegman et al., 1991a, Chapter VI). Expression of hGK-1

mRNA has been found in the prostate, but not in other tissues (Morris, 1989). Similar to PA, expression of hGK-1 mRNA in LNCaP cells can be stimulated by androgens within 6 hours (Riegman et al., 1991a, Chapter VI and unpublished data). The expression of hGK-1 mRNA varies strongly between different prostate tumors and cell lines (See also Table 1; Riegman et al., 1991a, Chapter VI). Similar to PA, hGK-1 mRNA could not be detected in the androgen-independent prostate tumors PC 133 and PC 135 and the androgen-independent cell line PC 3. However, in the more differentiated, androgen-dependent prostate tumors PC EW and PC 82 and in the androgen-sensitive prostate tumor cell line LNCaP, the hGK-1 mRNA level is high. The ratio of PA/hGK-1 expression varied in the different investigated prostate tumors/cell lines (Riegman et al., 1991a, Chapter VI). Varying PA/hGK-1 expression ratios were also found in different prostate cancer mRNA preparations from primary tumors (Henttu et al., 1990). Northern blots hybridized with hGK-1 specific probes showed five different hGK-1 transcripts, ranging in size from 1.5 to 5.6 kb (Riegman et al., 1991a, Chapter VI). The most abundant RNA transcript has a length of 1.5 kb. The open reading frame encodes a hGK-1 preproprotein of 261 amino acids. The predicted hGK-1 protein shows a homology of 78% to PA. Its amino acid sequence points to serine protease activity and, similar to PA, hGK-1 is a member of the kallikrein family (discussed in 1.2.3). Like PA, the hGK-1 protein is probably synthesized as a preproprotein, with a signal peptide of 17 amino acids and a propeptide of 7 amino acids.

One alternatively spliced transcript of similar size as the major 1.5 kb mRNA has been characterized. This transcript contains an extra internal fragment of 37 nucleotides. This will result in a frame shift and the synthesis of a truncated protein product. Instead of a 237 amino acid hGK-1 protein a protein of 199 amino acids will be produced (Riegman et al., 1991a, Chapter VI). The protein encoded by this second transcript is probably devoid of serine protease activity.

According to the amino acid sequence of hGK-1, the specificity of the protease activity is that of an arginyl esteropeptidase, which is different from the specificity of PA (discussed in I.2.4). Although the hGK-1 protein has not yet been identified, arginyl esteropeptidase activity was found to be present in the prostate (Paradis et al., 1989).

2.3 KALLIKREIN GENES

PA and hGK-1 are both members of the kallikrein gene family. In mouse and rat kallikreins are encoded by a large multigene family. At least 24 genes have been identified in the mouse (Mason et al., 1983; Evans et al., 1987), of which 10 are considered to be pseudogenes. Expression of 11 genes has been confirmed and physiological functions of 7 genes have been reported and will be discussed in I.4.2 (Lundgren et al., 1984; Ullrich et al., 1984; Evans & Richards, 1985; van Leeuwen et al., 1986; Blaber et al., 1987; Drinkwater et al., 1987; Drinkwater et al., 1988b; reviewed by Clements, 1989). The mouse kallikrein genes are clustered in groups of up to 11 genes on chromosome 7 (Evans et al., 1987).

The rat kallikrein gene family is somewhat smaller and has been estimated to contain up to 20 members (Gerald et al., 1986; Evans, 1987; Pritchet & Roberts, 1987; Chen et al., 1988). So far, 11 genes have been isolated (Chen et al., 1988; Wines et al., 1989) and the possible physiological function of 2 genes (rGK-1 and rGK-2) has been reported (see also I.2.4) (Swift et al., 1982; Ashley & MacDonald, 1985a; Pritchet & Roberts, 1987; Wines et al., 1989; Clements, 1989).

The human kallikrein family probably contains only 3 genes, PA, hGK-1 and KLK1 (Riegman et al., 1989b, Chapter IV). KLK1 encodes tissue kallikrein. All three genes have been assigned to chromosome 19q13.2-13.4 (Evans et al., 1988; Sutherland et al., 1988; Riegman et al., 1989b, Chapter IV; Schonk et al., 1990). The human kallikrein genes are clustered in an area of 60 kb (Chapter V). The PA gene is located only 12 kb upstream of the hGK-1 gene (Riegman et al., 1989b, Chapter IV). KLK1 is located 31 kb upsteam of the PA gene (Chapter V). The mouse kallikrein genes within one cluster are more closely linked. The distance between the different mouse kallikrein genes can be as small as 3-7 kb (Evans et al., 1987). Mouse chromosome 7 shows high conservation of a region containing 17 different loci to a region of human chromosome 19. This region has a high conservation in gene order between human and mouse (Saunders & Seldin, 1990). The kallikrein locus is located in this region.

The PA gene has a total length of 5.8 kb and is completely sequenced (Riegman et al., 1989a, Chapter III). The hGK-1 gene has a total length of 5.2 kb and is also completely sequenced (Schedlich et al., 1987; Schedlich & Morris, 1988). The PA and the hGK-1 gene show a high overall homology of 82% (Riegman et al., 1989a,

Chapter III). The sequence of the KLK1 gene has only partially been determined; the gene has a size of 4.5 kb (Evans et al., 1988).

The organization of the kallikrein genes is conserved among human, mouse, rat and hamster, they all consist of 5 exons and 4 introns. The general organization is depicted in Figure 2 (Evans et al., 1987; Wines et al., 1989, Chapter V). The positions of the open reading frames and intron/exon boundaries are conserved between the different kallikreins, a difference was found in intron lengths and the length of the 3' untranslated region, the latter resulting in (major) mRNAs with a length of about 1 kb or 1.5 kb. The clustering of the kallikrein genes, their identical organization and their structural similarity including the promoter regions (See 2.5, Figure 5) suggests, that ancestral gene duplications occurred relatively recently.

Originally, kallikreins were defined as enzymes with the ability to generate vasoactive (directly regulating local blood pressure) kinins from kininogen. At present, the term kallikrein is often used to describe all genes, which are structurally and sequentially closely related to the prototype (tissue) kallikrein. Plasma kallikrein, which is also able to generate vasoactive kinins from kininogen, and tissue kallikrein, however, are distinct enzymes with clearly differing molecular weights, amino acid sequence, structure and major physiological functions. Comparison of sequence data shows that

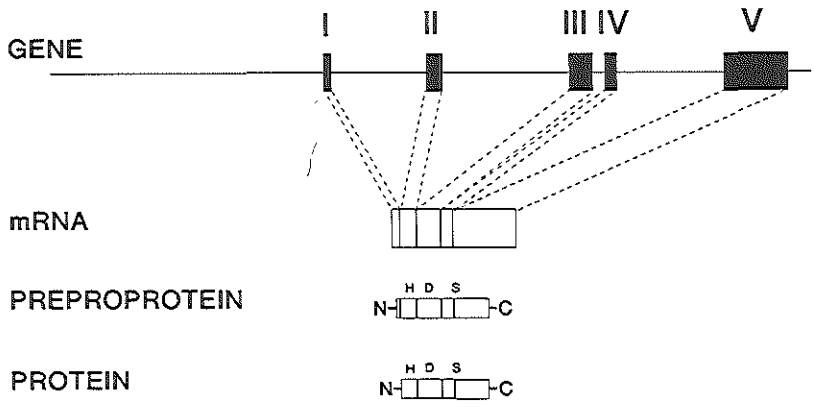

FIGURE 2: General organization of the kallikrein genes.
The genes have a length of about 6 kb. The exons are depicted as closed bars and are numbered I to V. The mRNAs have a length of 1 or 1.5 kb. The open reading frame encodes a preproprotein of about 260 amino acids. The mature protein consists of about 240 amino acids. The positions of the amino acids histine, aspartic acid and serine involved in the catalytic triad are presented in one letter code.

the limited homology to tissue kallikrein (30-40%) is restricted to the light chain of the plasma kallikrein complex (Chung et al., 1986). Therefore, one enzyme, plasma kallikrein does not belong to the kallikrein gene family. The human plasma kallikrein gene is located on chromosome 4q34-35.

2.4 FUNCTIONS OF KALLIKREINS

Kallikreins belong to the family of serine proteases. Other well known members of this family are elastase, thrombin, plasmin, chymotrypsin and trypsin. Serine proteases are characterized by the presence of a serine residue and the conserved adjacent amino acids Gly-Asp-Ser-Gly in the active catalytic site (Neurath et al., 1967). In the correct spatial configuration of the protein a catalytic triad is formed between this serine, and a histidine and aspartic acid residue in other parts of the molecule (Neurath et al., 1967; Young et al., 1978). Kallikreins show a marked substrate specificity. In general, kallikreins are involved in the selective cleavage of specific precursor proteins to their mature, bioactive forms (Drinkwater et al., 1988a; MacDonald, et al., 1988). The kallikreins have a length of approximately 237 amino acids. The amino acids involved in the catalytic triad of the different kallikreins are located at conserved positions: His at position 41, Asp at position 96 and Ser at position 189 (see Figure 3). Amino acids important for substrate recognition were identified with X-ray crystallographic studies of tissue kallikrein, which recognizes plasma kininogen as its physiological substrate (Bode et al., 1983; Chen & Bode, 1983). The amino acids in tissue kallikrein proposed to be important in the recognition of plasma kininogen are Tyr 93, Asp 183, Trp 205, Gly 206, and Ser/Ala 217 (Bode et al., 1983; Chen & Bode, 1983). Some of these amino acids are conserved in PA and hGK-1 (Trp 205, Gly 206 and Ser/Ala 217). A serine residue is found at position 93 in both PA and hGK-1 instead of tyrosine in human tissue kallikrein KLK1 (see Figure 3). Asp 183 was found to be important for the recognition of arginyl groups (Swift et al., 1982; Nakanishi et al., 1985). hGK-1 is expected to have an arginyl esteropeptidase activity, because there is an aspartate residue at position 183. PA has a serine residue instead at this position (see Figure 3) and is probably devoid of arginyl esteropeptidase activity.

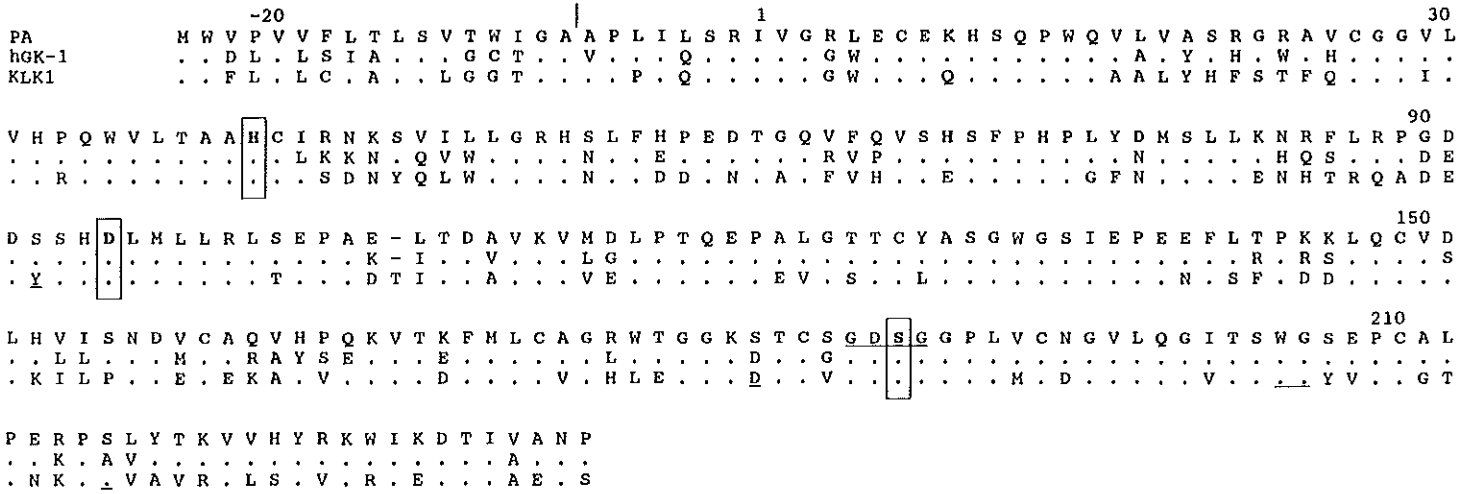

FIGURE 3: Alignment of the amino acid sequences of PA, hGK-1 and KLK1.
Amino acids are shown in the one letter code. Dots represent identical amino acids. The amino acids involved in the catalytic triad are boxed. In the PA protein, the amino acids characteristic for serine proteases are underlined. The amino acids involved in the substrate recognition of KLK1 are underlined. The asterisk represents a stop codon. The position where the signal peptide ends and the preprotein begins is given by a vertical bar above the sequence.

The physiological function of PA (previously discussed in I.1.2) is most likely the liquefacation of the gel like structure of the semen by digestion of HMW-SV proteins and fibronectin, which are secreted by the seminal vesicles (Lilja et al., 1989). The physiological function of hGK-1 is still unknown.

KLK1 is considered to play an important role in the regulation of blood pressure, local blood flow, and/or sodium balance in the renal kallikrein-renin system (Levinsky, 1979; Schachter, 1979; Margolius, 1984; Scicli & Carretro, 1986; MacDonald et al., 1988; Sharma, 1988; Clements, 1989). Expression of KLK1 in the kidney is predominantly found in the luminal cells of the distal tubules and enters the urine from the luminal or apical side and the renal blood circulation from the basal side of the cell (Yamada et al., 1981; Misumi et al., 1983). Tissue kallikrein releases kalladin by cleaving Low (LMW) and High Molecular Weight (HMW) kininogens (see Figure 4). Bradykinin is released by subsequent cleaving of kalladin by an ubiquitous arginine aminopeptidase (Erdös, 1979). Bradykinin is a nonapeptide, which induces vasodilation, increases capillary permeability and causes contraction of intestinal and bronchial smooth muscles.

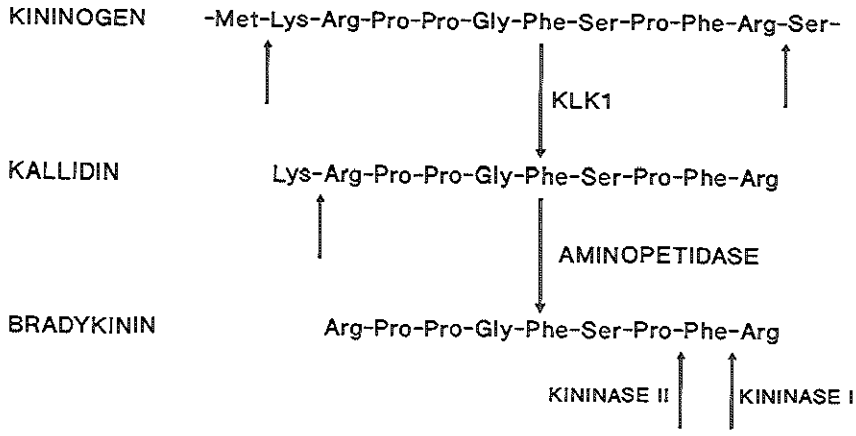

FIGURE 4: Mechanism of bradykinin release and inactivation by the kallikrein-renin system. Amino acids are shown in three letter code. Only a small part of the amino acid sequence of Low (LMW) and High Molecular Weight (HMW) kininogen is given.

Bradykinin is rapidly inactivated by kininase I and kininase II (angiotensin I converting enzyme) (see Figure 4) (Erdös, 1979; Gafford et al., 1983). Because the renal kallikrein-renin system seemed to be involved in the regulation of blood pressure, regulation of expression of tissue kallikrein became an interesting study object. In a genetic study in rats, linkage of a tissue kallikrein gene polymorphism to blood pressure was reported. A co-segregation of high blood pressure with the tissue kallikrein gene polymorphism was found in inbred strains of spontaneously hypertensive rats crossed with rat strains with normal blood pressure (Pravenec et al., 1991).

Many of the 24 or more kallikrein genes in mice are expressed. Despite the complexity of the mouse kallikrein system (previously discussed in I.2.3), it is, relative to other species, the best characterized group of kallikrein genes to date (see Table 2). Besides tissue kallikrein (van Leeuwen et al., 1986), α–Nerve Growth Factor (NGF) (Evans & Richards, 1985), γ–NGF (Ullrich, 1984; Evans & Richards, 1985), Epidermal Growth Factor–Binding Protein A (EGF-BP A) (Drinkwater et al.,1987), EGF–BP B (Lundgren et al., 1984; Drinkwater et al.,1987), EGF–BP C (Blaber et al., 1987; Drinkwater et al.,1987) and γ–renin (Drinkwater et al., 1988b) have been identified as kallikreins (see Table 2).

Mouse Nerve Growth Factor (ß-NGF) is isolated from the submandibular gland in a large complex containing the kallikreins α–NGF and γ-NGF (Stach et al., 1980). The γ subunit cleaves pro-ß-NGF to generate the mature NGF (Scott et al., 1983). The α subunit has no detectable protease activity, but is still considered to be a kallikrein because of its structural homology with kallikreins. It is probably required to stabilize a complex between 2 α-NGF, 2 γ-NGF and 2 ß–NGF proteins (Greene et al., 1968; Stach et al., 1980). Precursor EGF is cleaved by EGF-BP to produce mature EGF (Frey et al., 1979). It is not yet clear how the three EGF-BPs are involved in this process (Drinkwater et al.,1987).

The physiological function of γ-renin is so far not established. It is expressed in the submaxillary glands and is, like renin, able to cleave the peptide bond between two leupeptine residues in a synthetic renin substrate (Poe et al., 1983). Many of the here described mouse kallikreins seem to be involved in the maturation of growth factors. However, a general role of glandular kallikreins as growth factor processing enzymes is, because of their limited tissue distribution and low copy number in some species (discussed in I.2.3 and I.2.5) doubtful (Isackson et al., 1987).

TABLE 2 CHARACTERIZED GENES AND cDNAs OF THE HUMAN, MOUSE AND RAT KALLIKREIN FAMILIES

PROTEIN	GENE	SITE OF EXPRESSION	REGULATION mRNA BY ANDROGENS
HUMAN			
TISSUE KALLIKREIN	KLK1	Kidney, Pancreas, Salivary Gland	
PROSTATE-SPECIFIC ANTIGEN	APS	Prostate	+
HUMAN GLANDULAR KALLIKREIN-1	KLK2	Prostate	+
MOUSE			
NI	mGK-1	SMG	
r-NGF	mGK-3	SMG	+
a-NGF	mGK-4	SMG, Pancreas, Testis	+
NI	mGK-5	SMG	+
TISSUE KALLIKREIN	mGK-6	SMG, Pancreas, Kidney, Spleen	–
NI	mGK-8	SMG	
EGF-BP C	mGK-9	SMG	
NI	mGK-11	SMG	
EGF-BP B	mGK-13	SMG	
r-RENIN	mGK-16	SMG	
EGF-BP A	mGK-22	SMG	
RAT			
TISSUE KALLIKREIN (PS)	rGK-1	SMG, Pancreas, Kidney, Spleen, Gut, Pituitary, Brain SMG, Kidney, Brain	–
TONIN (S2)	rGK-2	SMG, Prostate	+
NI	rGK-8	SMG, Kidney	+
NI	RSKG-3	SMG, Kidney	+
K1	RSGK-7	SMG	+
S1	NI	SMG, Prostate	+
S3	NI		+

NI = NOT IDENTIFIED
SMG = SUBMANDIBULAR GLAND

In rats, the physiological function of only 2 of the protein products of the kallikrein gene family has been established (see also Table 2). Both rGK-1 and rGK-2 were identified as genes encoding tissue kallikreins, the corresponding cDNAs were designated PS and S2 or tonin, respectively (Swift et al., 1982; Ashley & MacDonald, 1985b; Pritchet & Roberts, 1987; Wines et al., 1989; Clements, 1989). S3 can act as a vasoconstrictor directly or by releasing angiotensin II, however, whether this is also its physiological function is unknown (Yamaguchi et al., 1991). Other isolated rat kallikreins are P1, K1 and S1 (Ashley & MacDonald, 1985a; Wines et al., 1989). Interestingly, P1 and S3 are expressed in the prostate (Ashley & MacDonald, 1985b; Winderickx et al., 1989; Wines et al., 1989). Although it is tempting to speculate that the physiological functions of these kallikreins are identical to those of PA and hGK-1, differences in critical amino acids suggest different cleavage specificities (Brady et al., 1989).

2.5 EXPRESSION OF KALLIKREINS

The name kallikrein is derived from the Greek word for pancreas "Kallikreas", and was originally given to a substance with hypotensive activity, which was isolated from the human pancreas. This substance is now known as KLK1. It is not only expressed in the acinar cells and the ß-cells of the pancreas (Ole-Moiyoi et al., 1979; Pinkus, et al., 1983), but also in human kidney and salivary glands (Baker & Shine, 1985; Fukushima et al., 1985). Rat tissue kallikrein was also reported to be present in the anterior pituitary, parts of the brain and the gastrointestinal tract (Powers & Nasjletti, 1983; Chao et al., 1983, 1987; Fuller et al., 1989). Expression sites of the different kallikrein genes and/or cDNAs in human, mouse and rat are summarized in Table 2. PA and hGK-1 are only detected in the human prostate (Wang et al., 1981; Papsidero et al., 1981; Tsuda et al., 1983; Gallee et al., 1986; Lilja & Abrahamson, 1988; Morris, 1989). In the rat, the kallikreins P1 and S3 are, like PA and hGK-1, expressed in the prostate. Unlike PA and hGK-1, P1 and S3 are also expressed in the submandibular gland (SMG), which is one of the large salivary glands (Ashley & MacDonald, 1985b; Brady et al., 1989; Winderickx et al., 1989; Wines et al., 1989). S1, or S2 (tonin) and K1 are expressed in the SMG, SMG/kidney/brain and SMG/kidney, respectively (Ashley & MacDonald, 1985b; Chen et al., 1988). The majority of the mouse kallikreins is

exclusively expressed in the SMG. Exceptions are tissue kallikrein and α-NGF, which are also expressed in the pancreas/kidney/spleen and pancreas/testis, respectively (Evans et al., 1985; van Leeuwen et al., 1986; Drinkwater et al., 1987; Drinkwater et al., 1988a).

The promoter regions of the kallikrein genes show a high degree of homology. So far the promoter regions of 14 kallikrein genes have been sequenced. These sequences are compared in Figure 5. The homology between promoters of mouse and rat kallikrein genes is much stronger than between those and the promoters of the human kallikreins. All genes contain a variant TATA box (TTTATA or TTTAAA) at position -28 to -23. The Sp1-binding site (or GC-box; GGGGCGGAGT), as it is postulated in the promoter region of the PA gene, is not well conserved among the kallikrein genes. In the other kallikrein genes most Sp1-like elements contain an A or T instead of a C at the central position. This might still mean interaction with Sp1, although with a lower affinity (Johnson et al., 1988; Letovsky & Dynan, 1989; Yu et al., 1991). Alternatively, other factors could bind to this sequence.

It has been reported that expression of many kallikreins is not only tissue specific, but also dependent on steroid hormones (see also Clements, 1989). Androgen-regulated mRNA expression has been reported for PA and hGK-1 in human prostate (Riegman et al., 1991a,b, Chapters VI and VII), for mGK-3, mGK-4 and mGK-5 in SMG of mice (van Leeuwen et al., 1987), for arginine esterase in dog prostate (Chapdelaine et al., 1988b) and for rGK2, rGK-8 RSGK-3 and RSKG-7 in rat SMG and prostate (Clements et al., 1988; Winderickx et al., 1989). Although PS (rat tissue kallikrein) mRNA expression was not affected by androgens in rat kidney (Clements et al., 1988), altered enzymatic kallikrein levels and tissue kallikrein protein synthesis were reported after treatment with androgens (Chao & Margolius, 1983; van Leeuwen et al., 1984; Miller et al., 1984). These data imply that there is regulation by androgens of PS, but on the level of protein synthesis and/or stability. Regulation of rat tissue kallikrein mRNA and protein levels by estrogens was found in pituitary tumors of rats (Fuller et al., 1988; Hatala & Powers, 1988). Glucocorticoids have been reported to down regulate rat tissue kallikrein mRNA levels in the rat pancreas (Rosewicz & Logsdon, 1989; Rosewicz et al., 1991). PS mRNA expression was not detectable in the SMG and kidney until early neonatal life (day 1), whereas S1, S2, S3, K1 and P1 transcripts were not detected until puberty (40 days) (Clements et al., 1990). The androgen-regulated S1, S2, S3, K1 and P1 mRNA expression correlates with the rise in serum testosterone levels at 35-45

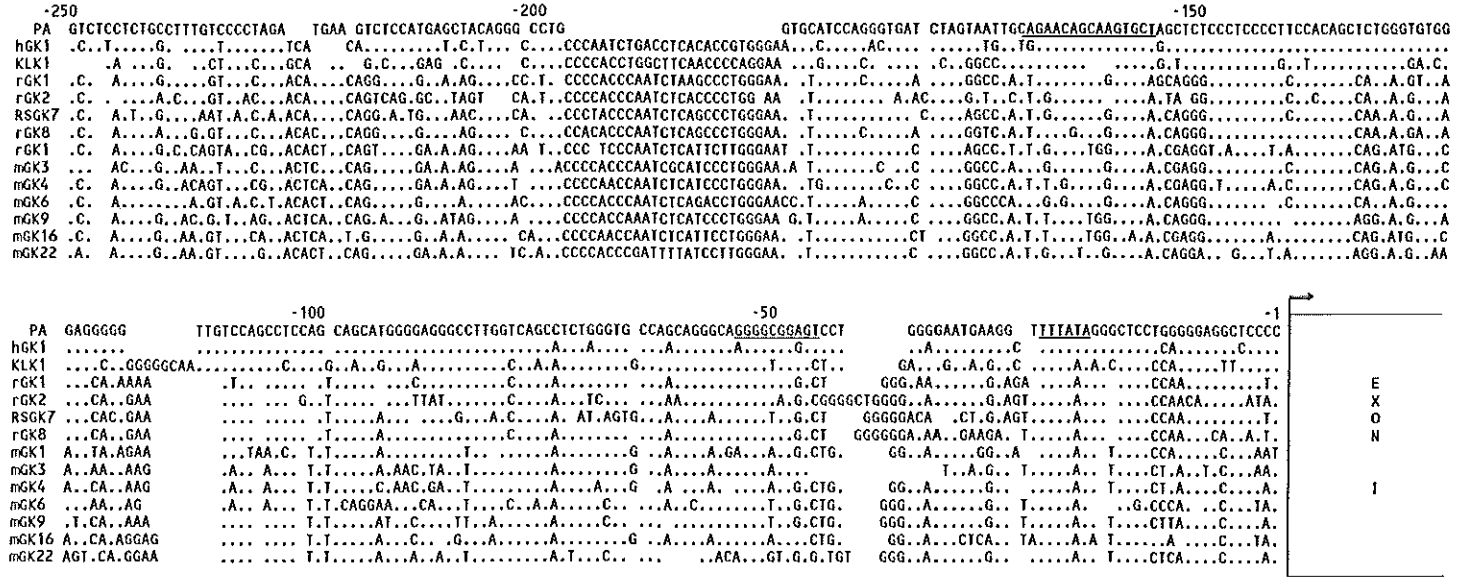

FIGURE 5: Alignment of the promoter regions of the human, mouse and rat kallikrein genes with the PA promoter region. Dots represent identical nucleotides. Regulatory elements in the PA promoter are underlined. Numbering is according to the PA gene sequence (Chapter III).

days postpartum. Similar results were found for the expression pattern of PS in the anterior pituitary, which in this tissue is estrogen dependent (Clements et al., 1990). Androgen-responsive mRNA expression in human, mouse and rat kallikreins is summarized in Table 2. Regulation of mRNA expression can be on transcription (direct or indirect), RNA processing and/or mRNA stability. Direct androgen responsive regulation of transcription is mediated by the AR and androgen responsive elements (AREs) (discussed in I.3). So far, only for PA regulation of transcription by androgens has been established (Riegman et al., 1991b, Chapter VII). A functional ARE is located in the PA promoter region at position -170 to -155 (Riegman et al., 1991b, Chapter VII). In the alignment of the promoter regions (Figure 5), sequences closely resembling the ARE in other kallikrein promoter regions are found at identical positions. It seems, however, that the ARE at -170 to -155 in the PA gene is not completely conserved among other kallikrein genes with androgen-responsive mRNA expression in different species. For instance, the androgen responsive genes rGK-2 and rGK-8 both contain ARE-like sequences at this position, however, one nucleotide is lacking in the ARE-like sequence of rGK-2, and the sequence of rGK-8 at this position is identical to that of rGK-1, which is not regulated by androgens (see Table 2). Summarizing, it can be concluded that at least part of the ARE-like sequences located at this postion in the kallikrein genes could in principle be a binding site for the AR and as a consequence be involved in androgen regulated expression. However, the simple fact of a structural relationship does not garantee that it functions as such. To establish this point, functional assays have to be done. Androgen regulated expression will not only depend on the presence of an ARE, but also on the further context of the promoter in which the element is situated, including the possibility of synergistic cooperation of the AR with other transcription factors. This is also discussed in I.3 and in Chapter VIII.

Other segments in the kallikrein promoter regions, which share a strong homology are located from −195 to −178, −149 to −132 and from -111 to −51. Remarkable is the 25 bp segment between −197 and -172 , which is conserved between all kallikrein promoters with the exception of the PA gene. Conserved areas could represent functional constrains, involved in the tissue-specific and differential transcription regulation during development.

3 ANDROGEN REGULATED GENE EXPRESSION

The expression of a number of genes, like PA, hGK-1 and rat PBP in the prostate and kidney androgen-regulated protein, ornithine decarboxylase, 908, ß-glucuronidase and RP-2 in the mouse kidney is stimulated by androgens (Parker et al., 1978; Toole et al., 1979; Watson et al., 1981; Seely & Pegg, 1983; Berger 1984; Trapman et al., 1988,1990; Riegman et al., 1991a,b, Chapters VI and VII). In contrast, down regulation of gene expression by androgens has also been reported. For instance in the prostate, TRPM-2, TGF-ß, c-Myc and AR mRNA are repressed (Léger et al., 1987; Quarmby et al., 1987; Kyprianou & Isaacs, 1989; Shan et al., 1990). Interestingly, expression of ornithine decarboxylase is stimulated in mouse kidney and repressed in rat Sertoli cells by androgens (Pajunen et al., 1982; Weiner et al., 1990).

Regulation of mRNA expression by androgens can be found at several levels: transcription (directly or indirectly), RNA processing and RNA stability. Evidence for transcriptional and post-transcriptional regulation by androgens is found for several genes. For instance, the androgen responsive rat PBP subunit genes show an increase in transcription rate of 2-3 fold, whereas the PBP mRNA level increases 30-fold (Page & Parker, 1982). Another example is found in the regulation of expression in the mouse kidney of the genes encoding ornithine decarboxylase, 908 mRNA, kidney androgen-regulated protein and ß-glucuronidase. These genes show no significant increase in transcription rate during testosterone treatment, whereas the mRNA levels increase 10-20 fold, suggesting mRNA stabilization to be the major factor in the induction (Berger, 1986). Similar results were found for the androgen-responsive regulation of secretory proteins SVS-IV and SVS-V in rat seminal vesicles (Higgins & Hemingway, 1991).

Androgen-regulated gene expression is mediated by the intracellular AR. Upon ligand binding the AR can regulate transcription of target genes by binding to its ARE in the regulatory regions of the target genes, thereby activating the transcription initiation complex (see Figure 6). Although it has been reported for many genes that expression is regulated by androgens (see above), it has been proven for only a few genes that this regulation is directly on the level of transcription.

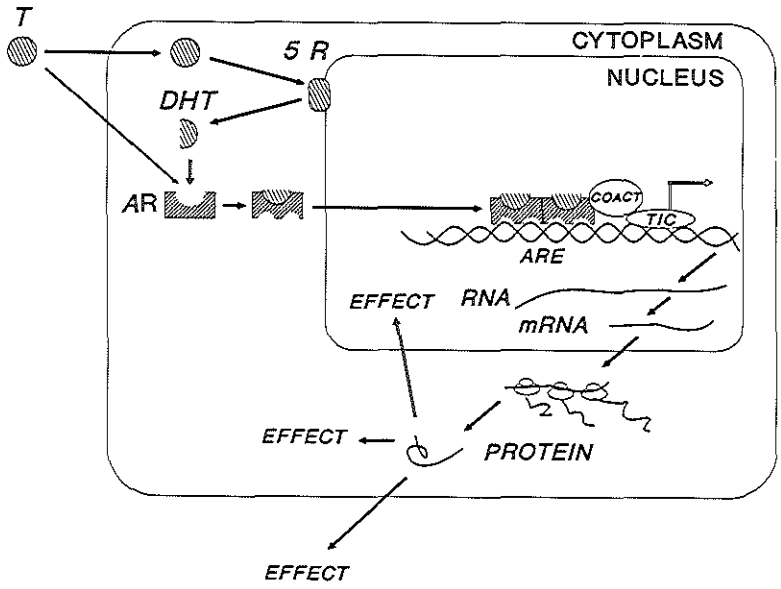

FIGURE 6: Model for androgen action in a prostate cell.
T=Testosterone, DHT=Dihydrotestosterone, 5αR=5αReductase, AR=Androgen receptor, COACT=Coactivator and TIC=Transcription Initiator Complex.

The androgen receptor:

Although the AR has many activities, this discussion will be limited to those, which directly influence DNA-binding and/or transcription activation. Recently, the human AR cDNA and its corresponding gene have been characterized (Chang et al., 1988; Lubahn et al., 1988a,b; Trapman et al., 1988; Faber et al., 1989; Kuiper et al., 1989). The AR appeared to be a member of the superfamily of ligand responsive transcription factors, which also encompasses the progesterone (PR), estrogen (ER), glucocorticoid (GR), mineralocorticoid (MR), retinoic acid, vitamin D_3 and thyroid hormone receptors (reviewed by Evans, 1988; Green & Chambon, 1988; Beato, 1989; Carson-Jurica et al., 1990). These receptors are all structurally related (see Figure 7). The N-terminal region is variable in length and amino acid composition. It is followed by a well-conserved, cysteine-rich DNA-binding domain (DBD) of 66-68 amino acids. The ligand-binding domain is located at the C-terminus of the receptor protein. DNA-binding as well as trans-activation of the ligand-responsive transcription factors is controlled by ligand-binding to the receptor. The receptors are "transformed" by hormone-binding from the inactive to the active state (Grody et al., 1982). The AR binds the androgens

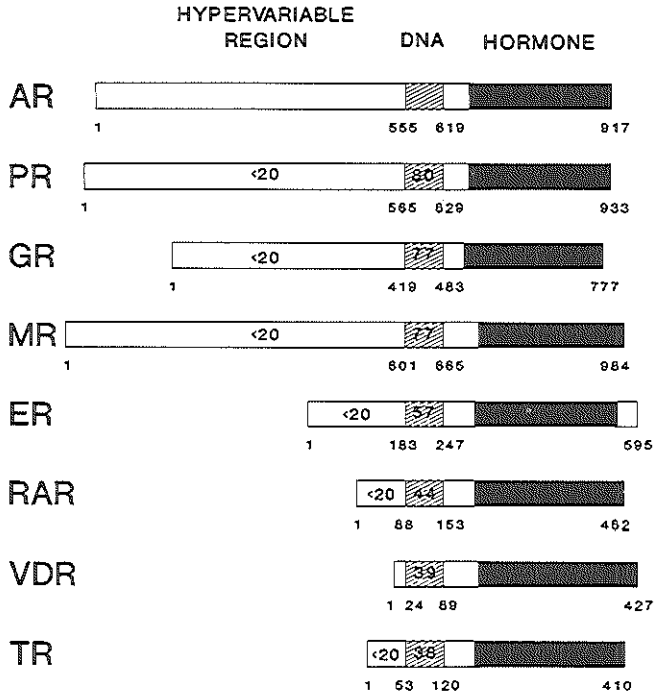

FIGURE 7: Comparison of different ligand responsive transcription factors. Numbers in the open bars represent the percentage of homology. Numbers under the bars represent the amino acid number.

DHT and T with high affinity and specificity (Murthy et al., 1984). Highest relative binding affinity was found for DHT and the synthetic androgen R1881; about 3- to 5-fold lower affinity was found for T (Asselin et al., 1976,1979; Trapman et al., 1988).

Interaction of steroid receptors with hormone responsive elements:

Activated receptors are able to bind to a DNA motif: the Hormone Responsive Element (HRE). A HRE is a specific DNA sequence found in the regulatory regions of hormone responsive genes, which are directly regulated by the hormone-receptor complex (reviewed by Schütz, 1988). HREs can act as enhancers, because they function in a relatively orientation and position independent manner (Ponta et al., 1985). The AR, GR and PR are all able to bind to similar HREs and activate transcription from these HREs (Cato et al., 1987; Ham et al., 1988; Denison et al., 1989; Claessens et al., 1989, 1990; Riegman et al. 1991b, Chapter VII). This was first demonstrated in transfection experiments with reporter gene constructs using the Mouse Mammary Tumor Virus

(MMTV) Long Terminal Repeat (LTR) as promoter. The MMTV promoter is regulated by glucocorticoids and progestins, but it is also able to confer androgen responsiveness to a reporter gene, although less efficiently (Cato et al., 1987; Ham et al., 1988; Otten et al., 1988). The consensus sequence of the HRE is the imperfect palindrome GGTACAnnnTGTTCT (Beato, 1989). This responsive element is also referred to as GRE, PRE or GRE/PRE. The important nucleotides in the GRE (underlined in the consensus sequence), were identified by generation of single point mutations at almost all positions and subsequent transcription activation analysis (Nordeen et al., 1990). The ER binds to an imperfect palindrome (ERE) with the consensus sequence GGTCAnnnTGWCC (Beato, 1989).

Originally, the DBD of steroid receptors was thought to consist of two Cys_2-Cys_2 zinc fingers with the fingers interacting with the DNA, because of the homology of this region with the DBD of TFIIIA. In the latter case such an interaction had been established for the three Cys_2-His_2 zinc fingers (Parraga et al., 1988, 1990; Lee et al., 1989). Recently, the three dimensional structures of the DBDs of the GR and the ER have been determined, which appeared to be different from the TFIIIA zinc finger structures (Härd et al., 1990; Schwabe et al., 1990). So, maybe the term zinc twist is more appropriate for the DBA of steroid receptors (Vallee et al., 1991). The structures of both the GR and ER DBD are strikingly similar. They consist of two alpha-helices perpendicular to each other. Each helix is anchored and oriented by a zinc atom at the N-terminus of the helix. The N–terminal part of the DBD is directly involved in the recognition of the HRE. This was shown by the generation of a chimeric ER, of which the N–terminal part of the DBD was replaced by the identical region of the GR. This receptor, when activated by estrogens, is able to activate transcription from a GRE, but not from an ERE. By exchanging only three amino acids in the N-terminal region of the N-terminal helix of the DBD, an "ER" with the same properties was generated (Green et al., 1988; Mader et al., 1989). Using a similar approach, a GR with a higher affinity to an ERE was constructed, but this receptor still showed some affinity to the GRE (Zilliacus et al., 1991). The N-terminal helix of the DBD is supposed to interact with the HRE in the major groove of the DNA. In this model, one DBD occupies only one half of the palindromic sequence of the HRE. The other half of the HRE is cooperatively bound by a DBD of a second receptor molecule. Binding studies of the GR DBD to its HRE located in the promoter region of the TAT gene, showed that binding of one receptor to the high affinity half site (TGTTCT) facilitates binding of a second receptor to the low

affinity half-site (Dahlman-Wright et al., 1990). Activated steroid receptors are able to form dimers in solution and bind as dimers to a palindromic HRE. A conserved region in the ligand-binding domain of the ER was demonstrated to be involved in dimer formation (Fawell et al., 1990). The amino acid sequence of this conserved region shows some similarity with the leucine zipper motif, which is involved in dimerization of various groups of transcription factors (jun/fos, C/EBP and others) (Landschutz et al., 1988). Because this region is conserved in steroid receptors, it might represent a common site of steroid receptor dimer formation. The DBDs of the GR, PR and ER were also found to have protein-protein interactions, if two DBDs occupy both half sites of the cognate palindromic HRE (Tsai et al., 1988; Green et al., 1988). Amino acids involved in the protein-protein interaction between the DBDs of two GRs were identified at the N–terminus of the second zinc twist (Dahlman-Wright et al., 1991). The model constructed from the 3-D structures of the DBD binding to a PRE/GRE is in agreement with the protein-protein interaction between two DBDs (Härd et al., 1990).

Transcription activation:

In addition to the DBD and a ligand-binding domain, steroid receptors also require specific regions to activate transcription of target genes (Hollenberg & Evans, 1988; Tora et al., 1988; 1989). These domains are referred to as transcriptional activating domains. Such transcriptional activating domains have been identified in the N-terminal region of the AR, located between amino acids 51–211 (Jenster et al., 1991) and 141–338 (Simental et al., 1991). These segments contain acidic regions. Co-transfection of an AR expression plasmid and a plasmid expressing only the AR N-terminal domain resulted in inhibition of an ARE-driven reporter gene (Simental et al., 1991). This suggests the requirement of a co-activator (Pugh & Tjian, 1990), or mediator as found in the transcription activation of GAL4-VP16 and GCN4 in yeast (Flanagan et al., 1991), binding to the N-terminal, trans-activating domain of the AR (Simental et al., 1991).

Synergism:

It has been reported that sequences outside the HRE are able to modulate the response of steroid receptors (Cato et al., 1988a). These sequences are supposed to be regulatory elements of other transcription factors, which differentially influence the trans- activating function of various steroid receptors. Possibly, certain transcription factors are able to stabilize specific receptor/HRE complexes, which can lead to synergistic effects. Synergism has been described between two HREs, and between

one HRE and a binding site for several other transcription factors (Ankenbauer et al., 1988; Cato et al., 1988b; Schüle et al., 1988a,b; Strahle et al., 1988). Both the PR and GR were found to be able to act synergistically with Sp1, Oct-1 and NF1. This was tested using reporter gene constructs containing a PRE/GRE and a binding site for these respective factors (Schüle et al., 1988a,b; Strahle et al., 1988; Allen et al., 1991). The degree of synergism appeared to be inversely related to the strength of the GRE (Schüle et al., 1988b). Binding of PR or GR to the PRE/GRE facilitates binding of Oct-1 to the octamer-binding motif in the MMTV promoter (Brüggemeier et al., 1991). Synergism was also described for the estrogen responsive chicken Vitellogenin II gene between two EREs located in the promoter region (Burch et al., 1988). Interactions between transcription factors and steroid receptors leading to down regulation of transcription have also been observed. For instance, by competition in binding of overlapping response elements (Gaub et al., 1990), or by direct protein-protein interactions, which was observed between the GR and c-jun or c-fos (Jonat et al., 1990; Lucibello et al., 1990; Schüle et al., 1990; Yang-Yen et al., 1990).

AR expression:

Expression of the AR is a prerequisite for androgen regulated expression of target genes. The AR is expressed at moderate or low levels in many tissues; high expression levels are observed in cells of the male urogenital system, including the prostate (see for review Mooradian et al., 1987). Expression of the AR is differentially regulated in the prostate epithelial cells and the stromal compartment during prostate development (discussed more extensively in I.1.1). In the LNCaP prostate cell line, androgens are able to down regulate the expression of AR mRNA (Quarmby et al., 1990; Trapman et al., 1990).

Androgen responsive elements in androgen responsive genes:

Although direct androgen-responsive transcription regulation could be studied exploiting the different MMTV promoter constructs, only recently functional androgen responsive elements (AREs) were described of natural androgen responsive target genes. The first ARE was described in the rat C3(1) gene, which encodes a subunit of prostatic (steroid) binding protein (PBP) (see also I.1.2). The expression of this gene is regulated by androgens partly on the transcriptional level and partly post-transcriptionally (Page & Parker, 1982; Bossyns et al., 1986; Zhang et al., 1988). AR-binding regions were located in the promoter region and in the first intron of the C3(1) gene (Rushmere et al., 1987; van Dijck et al., 1989). Positioning of promoter and intron

fragments containing the AR-binding regions in front of a TK (Thymidine Kinase) promoter CAT (Chloramphenicol Acetyl Transferase) reporter gene and subsequent transfection of this construct to T47D cells, showed that only the intron fragment could confer androgen-responsiveness to the TK promoter. This fragment contains a sequence similar to the PRE/GRE consensus sequence. After mutation of this sequence, the fragment was no longer able to confer androgen responsiveness to the TK promoter (Claessens et al., 1989, 1990). However, evidence that this ARE is functional in the context of the C3 promoter is still lacking. An ARE was also found in the promoter region of the PA gene (Riegman et al., 1989a, 1991b, Chapters III and VII). Run on experiments showed that at least part of the androgen responsive PA mRNA expression is regulated at the transcription level. The ARE in the promoter region of the PA gene resembles the PRE/GRE consensus sequence. It was proven to be functional because mutation of the sequence resulted in strong repression of androgen regulation of a PA promoter-CAT reporter gene construct. In addition, the ARE and ARE multimers stimulated androgen regulated expression from the TK promoter. The ARE in the PA promoter acted in synergism with a more upstream region. Also the hGK-1 promoter region, which has a putative ARE located at the identical position as in the PA gene, was found to be responsive to androgens (unpublished data).

4 SCOPE OF THIS THESIS

As a start of our study of prostate-specific and androgen-dependent expression of PA, various PA cDNAs (Chapter II) and the PA gene (Chapter III) were isolated and characterized. The PA gene turned out to be a member of a small kallikrein family, encompassing two other closely related genes [the human Glandular Kallikrein-1 gene (hGK-1), and the tissue kallikrein gene (KLK1)]. Genetic characterization of the human kallikrein genes showed clustering in a 60 kb segment on chromosome 19q13.2–13.4 (Chapters IV and V). The hGK–1 gene shows a strong homology to PA and is, similarly to PA, exclusively expressed in prostate tissue. KLK1 is mainly expressed in kidney, pancreas and salivary glands.

In addition to PA cDNA, hGK–1 cDNAs were isolated and characterized. This allowed the comparison of PA and hGK-1 mRNA expression. Using hGK-1 and PA-specific cDNA probes, androgen-stimulated mRNA expression of PA and hGK-1 could be determined (Chapter VI). Further, an androgen responsive element in the promoter region of the PA gene was defined and tested for its functional activity (Chapter VII).

The homology of the PA, hGK-1 and KLK1 genes does not only include the open reading frame, but extends into the promoter regions, although the genes are at least partially expressed in different tissues and at different levels. The above mentioned aspects, resulted in the development of a model system for the study of tissue-specific and hormone-responsive gene expression in the human prostate.

5 REFERENCES

Akiyama,K., Yoshioka,Y., Schmid,K., Offner,G.D., Troxler,R.F., Tsuda,R. & Hara,M. (1985) Biochim. Biophys. Acta 829, 288–294.

Allan,G.F., Ing,N.H., Tsai,S.Y., Srinivasan,G., Weigel,N.L., Thompson,E.B., Tsai,M-J. & O'Malley,B.W. (1991) J. Biol. Chem. 266, 5905–5910.

Ankenbauer,W., Strahle,U. & Schutz,G. (1988) Proc. Natl. Acad. Sci. USA 85, 7526-7530.

Arai,Y., Yoshiki,T., Oishi,K., Takeuchi,H. & Yoshida,O. (1990) Urol. Res. 18, 331–336.

Araki,T., Gejyo,F., Takagaki,K., Haupt,H., Scwick,H.G., Bürgi,W., Marti,T., Schaller,J., Rickli,E., Brossmer,R., Atkinsson,P.H., Putnam,F.W. & Schmid,K. (1988) Proc. Natl. Acad. Sci. USA 85, 679–683.

Ashley,P.L. & MacDonald,R.J. (1985a) Biochemistry 24, 4512-4520.

Ashley,P.L. & MacDonald,R.J. (1985b) Biochemistry 24, 4520-4527.

Asselin,J., Labrie,F., Bonne,C. & Raynaud,J.P. (1976) Steroids 28, 449–459.

Asselin,J., Melancon,R., Gourdou,Y., Labrie,F., Bonne,C. & Raynaud,J.P. (1979) J. Steroid Biochem. 10, 483–486.

Aubel,O.G.J.M. van, Bolt-de Vries,J., Blankenstein,M.A., Kate,F.J.W. ten & Schröder,F.H. (1985) Prostate 6, 185-194.

Aumüller,G., Seitz,J., Heyns,W. & Flickinger,C.J. (1982) Histochemistry 76, 497-516.

Babaian,R.J., Camps,J.L., Frangos,D.N., Ramirez,E.I., Tenney,D.M., Hassel,J.S. & Fritsche,H.A. (1991) Cancer 67, 2200–2206.

Baker,A.R. & Shine,J. (1985) DNA 4, 445–450.

Ban,Y., Wang,M.C., Watt,K.W.K., Loor,R. & Chu,T.M. (1984) Biochem. Biophys. Res. Commun. 123, 482–488.

Barak,M., Mecz,Y., Lurie,A. & Gruener,N. (1989) J. Lab. Clin. Med. 113, 598–603.

Beato,M. (1989) Cell 56, 335–344.

Berger,F.G., Szymanski,P., Read,E. & Watson,G. (1984) J. Biol. Chem. 259, 7941–7946.

Berger,F.G. (1986) Biochemistry 25, 1170–1175.

Blaber,M., Isackson,P.J. & Bradshaw,R.A. (1987) Biochemistry 26, 6742-6749.

Bode,W., Chen,Z., Bartels,K., Kutzbach,C., Schmidt-Kastner,G. & Bartunik,H. (1983) J. Mol. Biol. 164, 237–282.

Bookstein,R., Shew,J-Y., Chen,P-L., Scully,P. & Lee,W-H. (1990a) Science 247, 712–715.

Bookstein,R., Rio,P., Madreperla,S.A., Hong,F., Alfred,C., Grizzle,W.E. & Lee,W-H. (1990b) Proc Natl. Acad. Sci. USA 87, 7762–7766.

Bossyns,D., Delaey,B., Rombauts,W. & Heyns,W. (1986) J. Steroid Biochem. 3, 661-668.

Brady,J.M., Wines,D.R. & MacDonald,R.J. (1989) Biochemistry 28, 5203-5210.

Brandes,D. (1966) Int. Rev. Cytol. 20, 207–276.

Brinkmann,A.O., Bolt,J., Steenbrugge,G.J. van, Kuiper,G.G.J.M., Boer,W. de & Mulder,E. (1987) Prostate 10, 133-143.

Brüggemeier,U., Kalff,M., Franke,S., Scheideret,C. & Beato,M. (1991) Cell 64, 565-572.

Burch,J.B.E., Evans,M.I., Friedman,T.M. & O'Malley,P.J. (1988) Mol. Cell. Biol. 8, 1123–1131.

Camps,J.L., Chang,S-M., Hsu,T.C., Freeman,M.R., Hong,S-J., Zhau,H.E., Eschenbach,A.C.von & Chung,L.W.K. (1990) Proc. Natl. Acad. Sci. USA 87, 75–79.

Cannon,L., Bishop,D.T., Skolnick,M., Hunt,S. & Smart,C.R. (1982) Cancer Surv. 1, 47–69.
Carson–Jurica,M.A., Schrader,W.T. & O'Malley,B.W. (1990) Endocrine. Rev. 11, 201–220.
Catalona,W.J., Smith,D.S., Ratliff,T.L., Dodds,K.M., Coplen,D.E., Yuan,J.J.J., Petros,J.A. & Andriole,G.L. (1991) N. Eng. J. Med. 324, 1156-1161.
Cato,A.C.B., Henderson,D. & Ponta,H. (1987) EMBO J. 6, 363-368.
Cato,A.C.B., Skroch,P., Weinmann,J., Butkeraitis,P. & Ponta,H. (1988a) EMBO J. 7, 1403-1410.
Cato,A.C., Heitlinger,E., Ponta,H., Klein–Hitpass,L., Ryffel,G.U., Bailly,A., Rauch,C. & Milgrom,E. (1988b) Mol. Cell. Biol. 8, 5323–5330.
Chaistivanich,N. & Boonsaeng,V. (1983) Andrologia 15, 446–451.
Chang,C., Kokontis,J. & Liao,S. (1988) Science 240, 324–326.
Chang,S–M. & Chung,L.W.K. (1989) Endocrinology 125, 2719-2727.
Chao,J., Woodley,,C., Chao,L. & Margolius,H.S. (1983) J. Biol. Chem. 258, 15173–15178.
Chao,J. & Margolius,H.S. (1983) Endocrinology 113, 2221–2225.
Chapdelaine, P., Paradis, G., Tremblay, R.R. & Dube, J.Y. (1988a) FEBS Lett. 236, 205–208.
Chapdelaine,P., Potvin,C., Ho–Kim,M–A., Larouche,L., Bellemare,G., Tremblay,R.T. & Dubé,J.Y. (1988b) Mol. Cell. Endocrinol. 56, 63–70.
Chen,C., Hiipakka,R.A. & Liao,S. (1979) J. Steroid Biochem. 11, 401–405.
Chen,Y.P., Chao,J. & Chao,L. (1988) Biochemistry 27, 7189-7196.
Chen,Z. & Bode,W. (1983) J. Mol. Biol. 164, 283–311.
Choe,B.K. & Rose,N.R. (1982) Methods Cancer Res. 19, 199–207.
Chu,T.M. & Murphy,G.P. (1986) Urology 27, 487–497.
Chung,D.W., Fujikawa,K., McMullen,B.A. & Davie,E.W. (1986) Biochemistry 25, 2410-2417.
Chung,L.W.K. & Cunha,G.R. (1983) Prostate 4, 503–511.
Chung,L.W.K., Zhau,H.E. & Ro,J.Y. (1990) Prostate 17, 165–174.
Claessens,F., Celis,L., Peeters,B., Heyns,W. & Verhoeven,G. (1989) Biochem. Biophys. Res. Commun. 164, 833-840.
Claessens,F., Rushmere,N.K., Davies,P., Celis,L., Peeters,B. & Rombauts,W.A. (1990) Mol. Cell. Endocrinol. 74, 203-212.
Clements,J.A., Matheson,B.A., Wines,D.R., Brady,J.M., MacDonald,R.J. & FunderJ.W. (1988) J. Biol. Chem. 263, 16132-16137.
Clements,J.A. (1989) Endocrine Rev. 10, 393–419.
Cooke,D.B., Quarmby,V.E., Mickey,D.D., Isaacs,J.T. & French,F.S. (1988) Prostate 13, 263–272.
Cooner,W.H., Mosley,B.R. Rutherford,C.L., Beard,J.H., Pond,H.S., Terry,W.J., Igel,T.C. & Kidd,D.D. (1990) J. Urol. 143, 1146–1154.
Cunha,G.R., Chung,L.W.K., Shannon,J.M. & Reese,B.A. (1980) Biol. Reprod. 22, 19–42.
Cunha,G.R., Donjacour,A.A., Cooke,P.S., Mee,S., Bigsby,R.M. Higgins,S.J. & Sugimura,Y. (1987) Endocrine Rev. 8, 338–362.
Dahlman–Wright,K., Siltala–Roos,H., Carlstedt–Duke,J. & Gustafsson,J.A. (1990) J. Biol. Chem. 265, 14030–14035.
Dahlman–Wright,K., Wright,A., Gustafsson,J.A. & Carlstedt–Duke,J. (1991) J. Biol. Chem. 266, 3107–3112.
Davies,P., Eaton,C.L., France,T.D. & Phillips,M.E. (1988) Am. J. Clin. Oncol. 11 Suppl2, S1–S7.

DeKlerk,D.P. & Coffey,D.S. (1978) Invest. Urol. 16, 240–245.
Denison,S.H., Sands,A. & Tindall,D.J. (1989) Endocrinology 124, 1091–1093.
Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1987) Biochemistry 26, 6750-6756.
Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1988a) Trends Biochem. Sci. 13, 169–1722.
Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1988b) J. Biol. Chem. 263, 8565-8568.
Dijck,P. van, Winderickx,J., Heyns,W. & Verhoeven,G. (1989) Mol. Cell. Endocrinol. 64, 195-204.
English,H.F., Drago,J.R. & Santen,R.J. (1985) Prostate 7, 41–51.
Epstein,J.I. & Eggleston,J.C. (1984) Hum. Path. 15, 853–859.
Erdös,E.G. (1979) In: Handbook of experimental pharmacology, Springer, Heidelberg, Vol 25, 427–448.
Evans,B.A. & Richards,R.I. (1985) EMBO J. 4, 133–138.
Evans,B.A., Drinkwater,C.C. & Richards,R.I. (1987) J. Biol. Chem. 262, 8027-8034.
Evans,B.A., Yun,Z.X., Close,J.A., Tregear,G.W., Kitamura,N, Nakanishi,S., Callen,D.F., Baker,E., Hyland,V.J., Sutherland,G.R. & Richards,R.I. (1988) Biochemistry. 27, 3124–3129.
Evans,R.M. (1988) Science 240, 889–895.
Faber,P.W., Kuiper,G.G.J.M., Rooij,H.C.J. van, Korput,J.A.G.M. van der, Brinkmann,A.O. & Trapman,J. (1989) Mol. Cell Endocrin. 61, 257–262.
Fawell,S.E., Lees,J.A., White,R. & Parker,M.G. (1990) Cell 60, 953–962.
Fan,K (1988) J. Urol. 139, 318–322.
Filella,X., Molina,R., Jo,J., Umbert,B., Bedini,J.L. & Ballesta,A.M. (1990) Tumor Biol. 11, 289–294.
Flanagan,P.M., Kelleher III,R.J., Sayre,M.H., Tschochner,H. & Kornberg, R.D. (1991) Nature 350, 436-438.
Frenette,G., Dubé,J.Y., Lazure,C., Paradis,G., Chretien,M. & Tremblay,R.R. (1987) Prostate 11, 257–270.
Frey,P., Forand,R., Maciag,T. & Shooter,E.M. (1979) Proc. Natl. Sci. USA 76, 6294-6298.
Fukushima,D., Kitamura,N. & Nakanishi,S. (1985) Biochemistry 24, 8037–8043.
Fuller,P.J., Matheson,B.A., MacDonald,R.J., Verity,K. & Clements,J.A. (1988) Mol. Cell. Endocrinol. 60, 225-232.
Fuller,P.J., Verity,K., Matheson,B.A. & Clements,J.A. (1989) Biochem. J. 264, 133-136.
Gafford,J.T.,Skidgel,R.A., Erdös,E.G. & Hersh,L.B. (1983) Biochemistry 22, 3265-3271.
Gallee,M.P.W., Vroonhoven,C.C.J. van, Korput,J.A.G.M. van der, Kwast,T.H. van der, Kate,F.J.W. ten, Romijn,J.C. & Trapman,J. (1986) Prostate 9, 33-45.
Gallee,M.P.W., Visser–de Jong,E., Korput,J.A.G.M.van der, Kwast,T.H.van der, Kate F.J.W. ten, Schroeder,F.H. & Trapman,J. (1990) Urol. Res. 18, 181-187.
Gaub,M–P., Bellard,M., Scheuer,I., Chambon,P. & Sassone–Corsi,P. (1990) Cell 63, 1267–1276.
Gerald,W.L., Chao,J. & Chao,L. (1986) Biochim. Biophys. Acta 866, 1–14.
Gittes,R.F. (1991) N. Eng. J. Med. 324, 236-245.
Gleave,M, Hsieh,J-T, Gao,C, Eschenbach,A.C. von & Chung,L.W.K. (1991) Cancer Res. 51, 3753–3761.
Graves,H.C.B., Sensabaugh,G.F. & Blake,R.T. (1985) N. Engl. J. Med. 312, 338–343.
Graves,H.C.B., Kamarei,M. & Stamey,T.A. (1990) J. Urol. 144, 1510–1515.
Green,S. & Chambon,P. (1988) TIG 4, 309–314.

Green,S., Kumar,V., Theulaz,I., Wahli,W. & Chambon,P. (1988) EMBO J. 7, 3037-3044.
Greene,L.A., Shooter,E.M. & Varon,S. (1968) Proc. Natl. Acad. Sci. USA 60, 1383-1388.
Grody,W.W., Schrader,W.T. & O'Malley,B.W. (1982) Endocrine Rev. 3, 141-163.
Gumerlock,P.H., Poonamallee,U.R., Meyers,F.J. & VereWhite,R.W.,de (1991) Cancer Res. 51, 1632-1637.
Ham,J., Thomson,A., Needham,M., Webb,P. & Parker,M. (1988) Nucl. Acids Res. 16, 5263-5277.
Härd,T., Kellenbach,E., Boelens,R., Maler,B.A., Dahlman,K., Freedman,L.P., Carlstedt-Duke,J., Yamamoto,K.R., Gustafsson,J.A. & Kaptein,R. (1990) Science 249, 157-160.
Hara,M. & Kimura,H. (1989) J. Lab. Clin. Med. 113, 541-548.
Hasenson,M., Lundh,B., Stege,R., Carlström,K. & Pousette,A. (1989) Prostate 14, 83-90.
Hatala,M.A. & Powers,C.A. (1988) Cancer Res. 48, 4158-4162.
Helminen,H.J. & Ericsson,J.L.E. (1972) J. Ultrastruct. Res. 40, 152-166.
Henttu,P., Lukkarinen,O. & Vihko,P. (1990) Int. J. Cancer 45, 654-660.
Heyns,W., Verhoeven,G. & Moor,P.de (1976) J. Steroid. Biochem. 7, 987-991.
Heyns,W., Peeters,B., Mous,J., Bosseyns,D., Rombauts,W. & Moor,P. de, (1981) The prostatic cell (A): Structure and function, Prog. Clin. Biol. Res., Alan R. Liss, Inc., New York, 339-350.
Higgins,S.J. & Hemingway,A.L. (1991) Mol. Cell. Endocrinol. 76, 55-61.
Hoehn,W., Schroeder,F.H., Riemann,J.F., Joebsis,A.C. & Hermanek,P. (1980) Prostate 1, 95-104.
Hoehn,W., Wagner,M., Riemann,J.F., Hermanek,P., Williams,E., Walther,R. & Schrueffer,R. (1984) Prostate 5, 445-452.
Hollenberg,S.M. & Evans,R.M. (1988) Cell 55, 899-906.
Horoszewicz,J., Leong,S., Chu,T., Wasjman,Z., Friedman,M., Papsidero,L., Kim,U., Chiu,L., Kakati,S., Arya,S. & Sandberg,A. (1980) Prog. Clin. Biol. Res. 37, 115-132.
Hudson,M.A., Bahnson,R.R. & Catalona,W.J. (1989) J. Urol. 142, 1011-1017.
Hurst,H.C., & Parker,M.G. (1983) EMBO J. 2, 769-774.
Ichiwaka,T., Schalken,J.A., Ichkaw,Y., Steinberg,G.D. & Isaacs,J.T. (1991) Prostate 18, 163-172.
Isaacs,J.T. (1987) Prog. Clin. Biol. Res. 239, 513-566.
Isackson,P.J., Dunbar,J.C. & Bradshaw,R.A. (1987) J. Cell. Biochem. 33, 65-75.
Ito,H., Yamaguchi,K., Sumiya,H. & Matsuzaki,J. (1986) Eur. Urol. 12, 49-53.
Ito,Y.Z., Mashimo,S., Nakazato,Y. & Takikawa,H. (1985) Cancer Res. 45, 5058-5063.
Jenster,G., Korput,J.A.G.M. van der, Vroonhoven,C. van, Kwast,T.H. van der, Trapman,J. & Brinkmann,A.O. (1991) Mol Endocrinol. 5, 1396-1404.
Johansson,J., Sheth,A., Cederlund,E. & Jörnvall,H. (1984) FEBS Lett. 176, 21-26.
Johnson,A.C., Jinno,Y. Merlino,G.T. (1988) Mol. Cell. Biol. 8, 4174-4184.
Jonat,C., Rahmsdorf,H.J., Park,K-K., Cato,A.C.B., Gebel,S., Ponta,H. & Herrlich,P. (1990) Cell 62, 1189-1204.
Kaighn,M.E., Narayan,K.S., Ohnuki,Y., Lechner,J.F. & Jones,L.W. (1979) Invest. Urol. 17, 16-23.
Killian,C.S., Emrich,L.J., Vargas,F.P., Yang,N., Wang,M.C., Priore,R.L., Murphy,G.P, & Chu,M.T. (1986) J. Natl.Cancer Inst. 76, 179-185.
Knudson,A.G. (1989) Cancer 63, 1888-1891.

Kohan,S., Fröysa,B., Cederlund,E., Fairwell,T., Lerner,R., Johansson,J., Khan,S., Ritzen,M., Jörnvall,H., Cekan,S. & Diczfalusy,E. (1986) FEBS Lett. 199, 242-248.
Korman,L.B. (1989) Clin. Pharm. 8, 412–424.
Kuiper,G.G.J.M., Faber,P.W., Rooij,H.C.J. van, Korput,J.A.G.M.van der, Ris-Stalpers,C., Klaassen,P., Trapman,J. & Brinkmann A.O. (1989) J. Mol. Endocrinol. 2, R1-R4.
Kuriyama,M., Wang,M.C., Papsidero,L.D., Kilian,C.S., Shimano,T., Valenzuela,L., Nishuira,T., Murphy,G.P. & Chu,T.M. (1980) Cancer Res. 40, 4568–4662.
Kuriyama,M., Wang,M.C., Lee,C.L., Papsidero,L.D., Kilian,C.S., Inaji,H., Slack,N.H., Nishuira,T., Murphy,G.P. & Chu,T.M. (1981) Cancer Res. 41, 3874–3876.
Kuriyama,M., Wang,M.C., Lee,C.L., Kilian,C.S., Papsidero,L.D., Inaji,H., Loor,R.M., Lin,M.F., Nishuira,T., Slack,N.H., Murphy,G.P. & Chu,T.M. (1982) J. Natl.Cancer Inst. 68, 99–105.
Kyprianou,N. & Isaacs,J.T. (1989) Mol. Endocrinol. 3, 1515–1522.
Landschulz,W.H., Johnson,P.F. & McNight,S.L. (1988) Science 240, 1759-1764.
Leav,I., Morfin,R.F., Ofner,P., Cavazos, L.F. & Leeds,E.B. (1971) Endocrinology 89, 465–483.
Lee,C. (1981) The prostatic cell: structure and function, Part A, Alan R., Prog. Clin. Biol. Res, Liss, Inc., New York, 145–159.
Lee,C., Sensibar,J. Dudek,S.M. Hiipakka,R.A. & Liao,S. (1990) Biol. Reprod. 43, 1079–1086.
Lee,M.S., Gippert,G.P., Soman,K.V., Case,D.A. & Wright,P.E. (1989) Science 245, 635–637.
Leeuwen,B.H.van, Grinblat,S.M. & Johnston,C.I. (1984) Am. J. Physiol. 247, F760-F764.
Leeuwen,B.H.van, Evans,B.A., Tregear,G.W. & Richards,R.I. (1986) J. Biol. Chem. 261, 5529–5535.
Leeuwen,B.H.van, Penschow,J.D., Coghlan,J.P. & Richards,R.I. (1987) EMBO J. 6, 1705-1713.
Léger,J.G., Montpetit,M.L. & Tenniswood,M.P. (1987) Biochem. Biophys. Res. Commun. 147, 196–303.
Letovski,J. & Dynan,W.S. (1989) Nucl. Acids Res. 17, 2639–2653.
Levinsky,N.G. (1979) Circ. Res. 44, 441–451.
Li,H.C., Chernoff,J., Chen,L.B. & Kirschonbaum,A (1984) Eur. J. Biochem. 138, 45–51.
Lilja,H. Laurell,C.B. & Jeppson,J.O. (1984) Scand. Lab. Invest. 44, 439-446.
Lilja,H. (1984) Scand. Lab. Invest. 44, Suppl. 191, 13–20.
Lilja,H. & Laurell,C.B. (1985) Scand. J. Lab. Invest. 45, 635–641.
Lilja,H. (1985) J. Clin. Invest., 76, 1899–1903.
Lilja,H. (1987) J. Clin. Invest., 80, 281–285.
Lilja,H. (1988) Scand. J. Lab. Invest. 48, Suppl. 191, 13–20.
Lilja,H. & Abrahamson,P.A. (1988) Prostate 12, 29–38.
Lilja,H., Abrahamsson,P.A. & Lundwall,A. (1989) J. Biol. Chem. 264, 1894-1900.
Lin,M.F. & Clinton,G.M. (1986) Biochem. J. 235, 351–357.
Lubahn,D.B., Joseph,D.R, Sar,M., Tan,J., Higgs,H.N., Larson,R.E., French,F.S. & Wilson,E.M. (1988a) Mol. Endocrinol. 2, 1265-1275.
Lubahn,D.B., Joseph,D.R., Sullivan,P.M., Willard,H.F., French,F.S. & Wilson,E.M. (1988b) Science 240, 327–330.
Lucibello,F.C., Slater,E.P., Jooss,K.U., Beato,M. & Müller,R. (1990) EMBO J. 9, 2827–2834

Lundgren,S., Ronne,H., Rask,L. & Peterson,P.A. (1984) J. Biol. Chem. 259, 7780–7784.
Lundwall,A. & Lilja,H. (1987) FEBS Lett. 214, 317–322.
MacDonald,R.J., Margolius,H.S. & Erdös,E.G. (1988) Biochem. J. 253, 313-321.
Mader,S., Kumar,V., Verneuil,H. de & Chambon,P. (1989) Nature 338, 271-274.
Margolius,H.S. (1984) Annu. Rev. Physiol. 46, 309–326.
Mason,A.J., Evans,B.A., Cox,D.R., Shine,J. & Richards,R.I. (1983) Nature 303, 300–307.
Mbikay,M., Nolet,S., Fournier,S., Benjannet,S., Chapdelaine,P., Paradis,G., Dubé,J.Y., Tremblay,R., Lazure,C., Seidah,N.G. & Chrétien,M. (1987) DNA 6, 23–29.
McGee,R.S. & Herr,J.C. (1988) Biol. Reprod. 39, 499–510.
McNeal,J.E. (1984) New approaches to the study of benign prostatic hyperplasia, Prog. Clin. Biol. Res., Alan R. Liss, Inc., New York, 145, 27-53.
Meikle,A.W., Smith,J.A., & West,D.W. (1985) Prostate 6, 121–128.
Menon,M. & Walsh,P.C. (1979) Prostatic Cancer, Murphy,G.P., Publishing Comp. Inc., 175–200.
Miller,D.H., Chao,J. & Margolius,H.S. (1984) Biochem. J. 218, 37-43.
Misumi,J., Alhenc-Gelas,F., Marre,M., Marchetti,J., Corvol,P. & Menard,J. (1983) Kidney Int. 24, 58–65.
Mooradian,A.D., Morley,J.E. & Korenman,S.G. (1987) Endocrine Rev. 8, 1-27.
Morris,B.J. (1989) Clin. Exp. Pharm. Phys. 16, 345–351.
Murthy,L.R., Chang,C.H., Rowly,D.R., Scardine,P.T. & Tindall,D.J. (1984) Prostate 5, 567-579.
Nag,A. & Smith,R.G. (1989) Prostate 15, 115–122.
Nakanishi,S., Kitamura,N. & Ohkubo,H. (1985) Biotechnology 3, 1089–1098.
Neurath,H., Walsh,K.A. and Winter,W.P. (1967) Science 158, 1638–1644.
Nishi,N., Matuo,Y., Nakamoto,T. & Wada,F. (1988) In Vitro Cell Biol. Dev. 8, 778–.
Nordeen,S.K., Suh,B.J., Kühnel,B. & Hutchinson III,C.A. (1990) Mol. Endocrinol. 4, 1866-1873.
Oesterling,J.E., Chan,D.W., Epstein,J.I., Kimball,A.W., Bruzek,D.J., Rock, R.C., Brendler,C.B. & Walsh,P.C. (1988) J. Urol. 139, 766–772.
Ole-Moiyoi,O., Pinkus,G.S., Spragg,J. & Austen,K.F. (1979) N. Engl. J. Med. 300, 1289-1294.
Otten,A.D., Sanders,M.M. & McKnight,G.S. (1988) Mol. Endocrinol. 2, 143–147.
Page,M.J. & Parker,M.G. (1982) Mol. Cell. Endocrinol. 27, 343-355.
Pajunen,A.E., Isomaa,V.V., Janne,O.A. & Bardin,C.W. (1982) J. Biol. Chem. 257, 8190–8196.
Papsidero,L.D., Wang,M.C., Luis,A., Valenzuela,L.M., Murphy,G.P. & Chu,T.M. (1980) Cancer Res. 2428–2432.
Papsidero,L.D., Wang,M.C., Horoszewicz,J.S., Leong,S.S, Kuriyama,M., Valenzuela,L.M., Murphy,G.P. & Chu,T.M. (1981) The prostatic cell: structure and function, Part A, Alan R. Liss, Inc., New York, 435–443.
Papsidero,L.D., Croghan,G.A., Asirwatham,J., Geata,J., Abenoza,P., Englander,L. & Valenzuela,L.M. (1985) Am. J. Path. 121, 451–454.
Paradis,G., Tremblay,R.R. & Dubé,J. (1989) Prostate 15, 343–353.
Parker,M.G., Scrace,G.T. & Mainwaring,W.I.P. (1978) Biochem. J. 170, 115–121.
Parraga,G., Horvat,S.J., Eisen,A., Taylor,W,E., Hood,L., Young,E.T. & Klevit,R.E. (1988) Science 241, 1489–1492.
Parraga,G., Horvat,S., Hood,L., Young,E.T. & Klevit,R.E. (1990) Proc. Natl. Acad. Sci. USA 87, 137–141.

Partin,A.W., Carter,H.B., Chan,D.W., Epstein,J.I., Oesterling,J.E., Rock,R.C. & Walsh,P.C. (1990) J. Urol. 143, 747–752.
Pinkus,G.S., Maier,M., Seldin,D.C., Ole–Moiyoi,O. & Austen,K.F. (1983) J. Histochem. Cytochem. 31, 1279-1288.
Poe,M., Wu,J.K., Florence,J.R., Rodkey,J.A., Bennet,C.D. & Hoogsteen,K. (1983) J. Biol. Chem. 258, 2209-2216.
Ponta,H., Kennedy,N., Skroch,P., Hynes,N.E. & Groner,B. (1985) Proc. Natl. Acad. Sci. USA 82, 1020-1024.
Powers,C.A. & Nasjletti,A. (1983) Endocrinology 112, 1194–1200.
Pravenec,M., Kren,V., Kunes,J., Scicli,G., Carretero,O.A., Simonet,L. & Kurtz,T.W. (1991) Hypertension 17, 242–246.
Pritchet,D.B. & Roberts,J.L. (1987) Proc. Natl. Acad. Sci. USA 84, 5545-5549.
Pugh,B.F. & Tjian,R. (1990) Cell 61, 1187-1197.
Quarmby,V.E., Beckman,W.C., Wilson,E.M. & French,F.S. (1987) Mol. Endocrinol. 1, 865–874.
Quarmby,V.E., Yarbrough,W.G., Lubahn,D.B., French,F.S. & Wilson,E.M. (1990) Mol. Endocrinol. 4, 22-28.
Qui,S.D., Young,C.Y.F., Bilhartz,D.L.,Prescott,J.L., Farrow,G.M., He,W.W. & Tindall,D.J. (1990) J. Urol. 144, 1550–1556.
Riegman, P.H.J, Klaassen, P., Korput, J.A.G.M.van der, Romijn, J.C. & Trapman, J. (1988) Biochem Biophys. Res. Commun. 155, 181–188.
Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M.van der, Romijn,J.C. & Trapman,J. (1989a) Biochem. Biophys. Res. Commun. 159, 95-102.
Riegman,P.H.J., Vlietstra,R.J., Klaassen,P., Korput,J.A.G.M.van der, Romijn,J.C. & Trapman,J. (1989b) FEBS Lett. 247, 123–126.
Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M.van der, Romijn,J.C. & Trapman,J. (1991a) Mol. Cell Endocrinol. 76, 181-190.
Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M.van der. & Trapman,J. (1991b) Mol. Endocrinol. 5, 1921–1930.
Rosewicz,S. & Logdson,C.D. (1989) Gastroenterology 97, 1005-1010.
Rosewicz,S., Dertjen,C.D., Logdson,C.D., Chen,L-M., Chao,J. & Riecken,E.O. (1991) Endocrinology 128, 2216–2222.
Rouleou,M., Leger,J. & Tenniswood,M. (1990) Mol. Endocrinol. 4, 2003–2013.
Rushmere,N.K., Parker,M.G. & Davies,P. (1987) Mol. Cell. Endocrinol. 51, 259-265.
Saunders,A.M., & Seldin,M.F. (1990) Genomics 8, 525-535.
Savanger,P., Miyashita,T. & Yamada,Y. (1990) J. Biol. Chem. 265, 6669–6674.
Schachter,M. (1979) Pharmacol. Rev. 31, 1–17.
Schaller,J., Akiyama,K., Tsuda,R., Hara,M., Marti,M.T. & Rickli,E.E. (1987) J. Biochem. 170, 111–120.
Schedlich,L.J., Bennetts,B.H. & Morris,B.J. (1987) DNA 6, 429–437.
Schedlich,L.J. & Morris,B.J. (1988) Clin. Exp. Pharm. & Phys. 15, 339-344.
Schonk,D., Dijk,P.van, Riegman,P., Trapman,T., Holm,C., Willcocks,T.C., Sillekens,P., Venrooij,W.van, Wimmer,E.M., Geurts van Kessel,A., Ropers,H.H. & Wieringa,B. (1990) Cytogenet. Cell. Genet. 54, 15–19.
Schüle,R., Muller,M., Otsuka-Murakami & Renkawitz,R. (1988a) Nature 332, 87-90.
Schüle,R., Muller,M., Kaltschmidt,C. & Renkawitz,R. (1988b) Science 242, 1418-1420.
Schüle,R., Rangarajan,P., Kliewer,S., Ransone,L.J., Bolado,J., Yang,N., Verma,I.M. & Evans,R.M. (1990) Cell 62, 1217-1226.
Schütz,G. (1988) Biol. Chem. 369, 77-86.
Schwabe,J.W.R., Neuhaus,D. & Rhodes,D. (1990) Nature 348, 458-461.

Scicli,A.G. & Carretro,O.A. (1986) Kidney Int. 29, 120-130.
Scott,J., Selby,M., Urdea,M., Quiroga,M., Ball,G.I. & Rutter,W.J. (1983) Nature 302, 538-540.
Seely,J.E. & Pegg,A.E. (1983) J. Biol. Chem. 258, 2496–2500.
Seidah,N.G., Arbatti,N.J., Rochemont,J., Sheth,A.R. & Chrétien,M. (1984) FEBS Lett. 175, 349–355.
Sensabaugh,G.F. (1978) J. Forensic Sci. 23, 105–115.
Shan,L–X., Rodriguez,M.C. & Janne,O.A. (1990) Mol. Endocrinol. 4, 1636–1646.
Shannon,J.M. & Cunha,G.R. (1983) Prostate 4, 367–373.
Sharief,F.S., Lee,H., Leuderman,M.M., Lundwall,A., Deaven,L.L., Lee,C.L. & Li,S.S.L. (1989) Biochem. Biophy. Res. Comm. 160, 79–86.
Sharma,J.N. (1988) Gen. Pharmacol. 19, 177–187.
Silverberg,E. (1987) Cancer 60, 692-717.
Simental,J.A., Sar,M., Lane,M.V., French,F.S. & Wilson,E.M. (1991) J. Biol. Chem. 266, 510-518.
Stach,R.W., Pignatti,P–F., Baker,M.E. & Shooter,E.M. (1980) J. Neuro. Chem. 34, 859-865.
Stamey,T.A., Yang,N., Hay,A.R., McNeal,J.E., Freiha,F.S. & Redwine,B.A. (1987) N. Engl. J. Med. 317, 909–916.
Stamey,T.A., Kabalin,J.N., McNeal,J.E., Johnstone,I.M., Freiha,F.S., Redwine,B.A. & Yang,N. (1989a) J. Urol. 141, 1076–1083.
Stamey,T.A., Kabalin,J.N. & Ferrari,M. (1989b) J. Urol. 141, 1084–1087.
Stamey,T.A., Kabalin,J.N., Ferrari,M. & Yang,N. (1989c) J. Urol. 141, 1088-1090.
Steenbrugge,G.J.van (1988) Ph.D. Thesis: Transplantable human prostate cancer (PC82) in athymic nude mice: A model for androgen regulated tumor growth.
Steenbrugge,G.J.van, Groen,J.W., Dongen,J.W.van, Bolt,J., Korput,H.van der, Trapman,J., Hasenson,M. & Horoszewicz,J. (1989) Urol. Res. 17, 71-77.
Steinberg,G.D., Carter,B.S., Beaty,T.H., Childs,B. & Walsh,P.C. (1990) Prostate 17, 337-347.
Strahle,U., Schmid,W. & Schutz (1988) EMBO J. 7, 3389-3395.
Sutherland,G.R., Baker,E., Hyland,V.J., Callen,D.F., Close,J.A., Tregear,G.W., Evans,B.A. & Richards,R.I. (1988) Cytogenet. Cell. Genet. 48, 205–207.
Swift,G.H., Dagorn,J.C., Ashley,P.L., Cummings,S.W. & MacDonald,R.Y. (1982) Proc. Natl. Acad. Sci. USA 79, 7263–7267.
Tailor,P.G., Govindan,M.V. & Patel,P.C. (1990) Nucl. Acids Res. 18, 4928.
Takeda,H., Lasnitzki,I. & Mizuno,T. (1986) J. Endocrinol. 110, 467-470.
Takeuchi,T., Kuriyama,M, Fujihiro,S., Fujimoto,Y., Okano,M. & Nishiura,T. (1983) J. Surg. Onc. 24, 157–160.
Tenniswood,M. (1986) Prostate 9, 375–385.
Thomas,K.A., Baglan,N.C. & Bradshaw,R.A. (1981) J. Biol. Chem. 256, 9156–9166.
Toole,J.J., Hastie,N.D. & Held,W.A. (1979) Cell 17, 441–448.
Tora,L. Gronemeyer,H., Turcotte,B., Gaub,M-P. & Chambon,P. (1988) Nature 333, 185-188.
Tora,L. White,J., Brou,C., Tasset,D., Webster,N., Scheer,E. & Chambon,P. (1989) Cell 59, 477–487.
Trapman,J., Klaassen,P., Kuiper,G.G.J.M., Korput,J.A.G.M. van der, Faber,P.W., Rooij,H.C.J. van, Geurts van Kessel,A., Voorhorst,M.M., Mulder,E. & Brinkmann,A.O. (1988) Biochem. Biophys. Res. Commun. 153, 241-248.
Trapman,J., Ris–Stalpers,C., Korput,J.A.G.M.van der, Kuiper,G.G.J.M., Faber,P.W., Romijn,J.C., Mulder,E. & Brinkmann,A.O. (1990) J. Steroid Biochem. 37, 837-842.

Tsai,S.Y., Carlstedt-Duke,J., Weigel,N.L., Dahlman,K., Gustafsson,J.A., Tsai,M.J. & O'Malley,B.W. (1988) Cell 55,361-369.
Tsuda,R., Hara,M., Inoue,T. & Okabe,T. (1983) Jpn. J. Urol. 74, 1313-1319.
Ullrich,A., Gray,A., Wood,W.I., Hayflick,J. & Seeburg,P.H. (1984) DNA 3, 387-392.
Ulvsback,M., Lindstrom,C., Weiber,H., Abrahamsson,P.A., Lilja,H. & Lundwall,A. (1989) Biochem. Biophys. Res. Commun. 164, 1310-1315.
Vallee,B.L., Coleman,J.E. & Auld,D.S. (1991) Proc. Natl. Acad. Sci. USA 88, 999-1003.
Varma,V.A., Austin,G.E. & O'Connell,A.C. (1989) Arch. Path. Lab. Med. 113, 16-19.
Veldscholte,J., Voorhorst-Ogink,M.M., Bolt-de Vries,J., Rooij,H.C.J. van, Trapman,J. & Mulder,E. (1990a) Biochim. Biophys. Acta 1052, 187-194.
Veldscholte,J., Ris-Stalpers,C., Kuiper,G.G.J.M., Jentser,G., Berrevoets,C., Claassen,E., Rooij,H.C.J. van, Trapman,J., Brinkmann,A.O. & Mulder,E. (1990b) Biochem. Biophys. Res. Comm. 173, 534-540.
Vihko,P., Virkkunen,P., Henttu,P., Roiko,K., Solin,T. & Huhtala,M.L. (1988) FEBS Lett. 236, 275-281.
Viola,M.V., Fromowitz,F., Oravez,S., Deb,S., Finkel,G., Hand,P. Thor,A. & Schlom,J. (1986) N. Engl. J. Med. 314, 133-137.
Wang,M.C., Valenzuela,L.A., Murphy,G.P. & Chu,T.M. (1979) Invest. Urol. 17, 159-163.
Wang,M.C., Papsidero,L.D., Kuriyama,M., Valenzuela,L.M., Murphy,G.P. & Chu,T.M. (1981) Prostate 2, 89-96.
Watson,G, Davey,R.A., Labarca,C. & Paigen,K. (1981) J. Biol. Chem. 256, 3005-3011.
Watt,K.W.K., Lee,P.J., M'Timkulu,T., Chan,W.P. & Loor,R. (1986) Proc. Natl. Acad. Sci. USA 83, 3166-3170.
Weiber,H., Andersson,C., Murne,A., Rannevink,G., Lindstrom,C., Lilja,H. & Fernlund,P. (1990) Am. J. Path. 137, 593-603.
Weinberg,R.A. (1989) Cancer Res. 49, 3713-3721.
Weiner,K.X.B., Pentecos,B.T. & Dias,J.A. (1990) Mol. Endocrinol. 4, 1249-1256.
Winderickx,J., Swinnen,K., Dijck,P. van, Verhoeven,G. & Heyns,W. (1989) Mol. Cell. Endocrinol. 62, 217-226.
Wines,D.R., Brady,J.M., Pritchett,D.B., Roberts,J.L. & MacDonald,R.J. (1989) J. Biol. Chem. 264, 7653-7662.
Woolf,C.M. (1960) Cancer 13, 739-744.
Yamaguchi,T., Carretero,O.A. & Scicli,G. (1991) J. Biol. Chem. 266, 5011-5017.
Yamada,K., Schulz,W.W, Page,D.S. & Erdös,E.G. (1981) Hypertension 3, II-59-64.
Yang-Yen,H-F., Chambard,J-C., Sun,Y-L., Smeal,T., Schmidt,T.J., Droin,J. & Karin,M. (1990) Cell 62, 1205-1215.
Young,C.L., Barker,W.C., Tomaselli,C.M. and Dayhoff,M.O. (1978) In: Atlas of protein sequence and structure. Dayhoff,M.O., National Biomedical Research Foundation, Vol 5, 73-93.
Yu,C-Y., Motamed,K., Chen,J, Bailey,A.D. & Shen,J.C-K. (1991) J. Biol. Chem. 266, 8907-8915.
Zaridze,D.G., Boyle,P. & Smans,M. (1984) Int. J. Cancer 33, 223-230.
Zhang,Y-L., Zhou,Z.X., Zhang,Y.D. & Parker,M.G. (1988) J. Endocrinol. 117, 361-366.
Zilliacus,J., Dahlman-Wright,K., Wright,A., Gustafsson,J-A. & Carlstedt-Duke,J. (1991) J. Biol. Chem. 266, 3101-3106.

CHAPTER II

MOLECULAR CLONING AND CHARACTERIZATION OF NOVEL PROSTATE-SPECIFIC ANTIGEN cDNAs

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SUMMARY

Three different Prostate-specific Antigen cDNAs were isolated from a PC 82 prostate tumor cDNA library. PA 75 has a size of 1.4 kb and contains the almost complete information for the 35 kD Prostate-specific Antigen preproprotein. The 1.6 kb PA 525 cDNA lacks about 0.2 kb of the 3'-noncoding region and contains an additional internal 0.4 kb fragment as a result of alternative splicing. PA 424 represents a 0.6 kb variant of PA 75. It contains a 0.15 kb internal fragment and a poly(A) tail preceded by an AAGAAA motif at the 3'-end. The predicted protein products of PA 525 and PA 424 will be different from PA 75 at the C-terminal end. In RNA preparations of two human prostate tumors (PC 82 and PC EW) seven different Prostate-specific Antigen transcripts can be detected, ranging in size from 0.6 kb to 5.6 kb. PA 75 cDNA represents the major 1.5 kb mRNA. PA 424 correlates with a 0.9 kb transcript and PA 525 with a 1.9 kb mRNA species.

INTRODUCTION

Prostate Antigen or prostate-specific antigen (PA or PSA) is a glycoprotein with a molecular weight of approximately 35 kD (Wang et al., 1979). The complete amino acid and cDNA sequence have recently been determined (Watt et al., 1986; Lundwall & Lilja, 1987). The structure shows strong homology with kallikrein-like proteins. The highest homology is found with the human kallikrein hGK-1 (80%) and human pancreatic kallikrein (62%) (Schedlich et al., 1987; Fukushima et al., 1985). Similar to PA these proteins are serine proteases. The PA substrate probably is high molecular weight seminal vesicle protein also known as seminogelin, which is secreted by the seminal vesicles and causes the gel-like structure of the semen (Lilja, 1985; Lilja & Laurell 1984). PA is presumed to play a role in "dissolving" the seminal coagulum by digesting this protein into various fragments. PA is exclusively synthesized by the epithelial cells of the prostate gland (Stein et al., 1982; Gallee et al., 1986). Normally, only small amounts of PA can be found in the serum. However, in the case of invasive growth of prostate tumors significantly higher PA levels are detected (Takeuchi et al., 1983; Kuriyama et al., 1982ab; Chu, 1986; Stamey et al., 1987). Therefore, measuring of serum PA concentration is presumed to be of high value in diagnosis of prostatic cancer. Because of its tissue specific expression and its clinical importance, knowledge of the properties and expression of PA are of high interest. As part of our study of PA gene expression we molecularly cloned PA cDNAs. In this paper the characterization of two novel PA cDNAs is described.

METHODS

Isolation of total cellular RNA from the different cell lines was done by the guanidinium thiocyanate method (Chirgwin et al., 1979). Poly A^+RNA was isolated by oligo dT cellulose chromatography. In collaboration with Dr. R. Dijkema (Organon, Oss) a lambda gt 10 cDNA library of 1.5 x 10^5 independent clones was prepared from PC 82 prostate tumor poly A^+RNA using standard conditions (Huyn et al., 1985). Screening was carried out with an in vitro synthesized oligonucleotide (TTACTACACACACGGGTTCACGTGGGGGTTTTTCACTGGTTTAAGTACGA), which was deduced from amino acids 160 to 176 of the published PA sequence (Watt et al., 1986). Duplicate nitrocellulose filters were hybridized overnight at 42°C in 6x SSC containing 10x Denhardt, 0.1% SDS, and 100 μg/ml salmon sperm DNA. The filters were washed twice in 3x SSC, 0.1% SDS for 20 minutes at 37°C and once in 1x SSC for 20 minutes at room temperature. Filters were exposed to X-ray films for 16 to 48 hours at -70°C using intensifying screens. To obtain additional PA cDNA clones the library was rescreened under stringent conditions with the EcoRI - SacI fragment of PA 75 (see Figure 1). Positive clones were isolated by two cycles of purification.

DNA was isolated from these clones and cDNA fragments were subcloned into pUC9 for further analysis (Maniatis et al., 1982). Nucleotide sequences of the appropriate fragments subcloned into M13mp18/19 were determined by the dideoxy chain termination method (Sanger et al., 1977).

Northern blot analysis of poly A^+RNA was performed with glyoxal denatured RNA, separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane. The probe was labeled as described (Feinberg & Vogelstein, 1983). Hybridization was at stringent conditions in the presence of 50% formamide at 42°C. After extensive washing filters were exposed to X-ray film at -70°C using intensifying screens.

PC 82 and PC EW prostate tumors were propagated as transplants on male nude mice (Hoehn et al., 1980; van Steenbrugge et al., 1984; Hoehn et al., 1984).

RESULTS

We used a specific oligonucleotide probe to screen a PC 82 prostate tumor cDNA library for PA cDNAs (see methods section). Two PA cDNAs were isolated, PA 133 (approximately 250 bp, data not shown) and PA 525 (approximately 1600 bp). Using the 5' EcoRI - SacI as a probe the library was rescreened. Two clones PA 75 and PA 424, with a restriction map different from PA 525 were isolated (see Figure 1). The size of PA 75 was 1400 bp. This cDNA lacked the NcoI and PstI sites present in PA 525. PA 424 was about 600 bp, and contained a BstXI site which was absent in PA 525 and PA 75.

Sequence analysis demonstrated that, beginning directly at the 5'-end, PA 75 contains an open reading frame of 771 bp encoding 13 amino acids of the signal peptide and the complete PA proprotein, that consists of 244 amino acids. PA 75 differs from the earlier reported cDNA clone (Lundwall & Lilja, 1987) at two positions

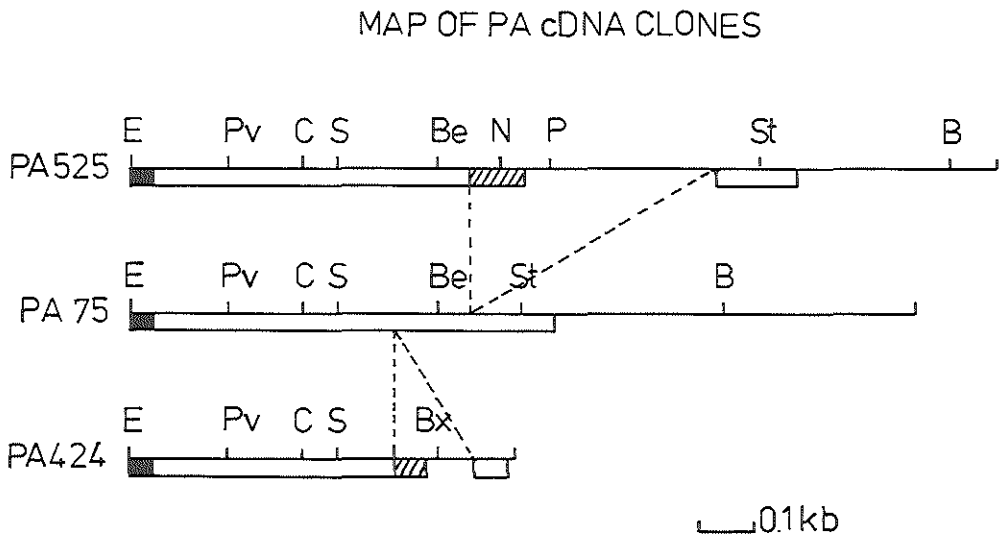

FIGURE 1: Comparison of the restriction maps of PA 75, PA 525 and PA 424. The open boxes correspond to the open reading frame of PA 75 encoding the mature protein. Closed boxes represent the sequences encoding (part of) the signal protein and the propeptide sequence. The hatched areas represent remaining parts of the open reading frame of PA 424 and PA 525.
Ba=BamHI, Be=BstEII, Bx=BstXI, C=ClaI, E=EcoRI, N=NcoI, P=PstI, Pu=PvuII, S=SacI and St=StuI.

(790 and 1033) in the 3'-noncoding region. Discrepancies observed between the PA amino acid sequence as determined by direct analysis of peptide fragments of the purified protein and as deduced from the cDNA structure (Watt et al., 1986; Lundwall & Lilja, 1987) were also found in PA 75. PA 75, 424 and 525 begin at exactly the same position as the PA cDNA isolated by Lundwall and Lilja (1987). This can be explained by the presence of a sequence in the PA mRNA, which is too difficult for reverse transcriptase or Klenow DNA polymerase to transcribe, or to a natural occurring 5'-EcoRI site in all the cDNAs.

Structural analysis of PA 424 revealed, starting at position 483, the presence of a short stretch of 145 bp, which was absent in PA 75. This sequence is followed by a

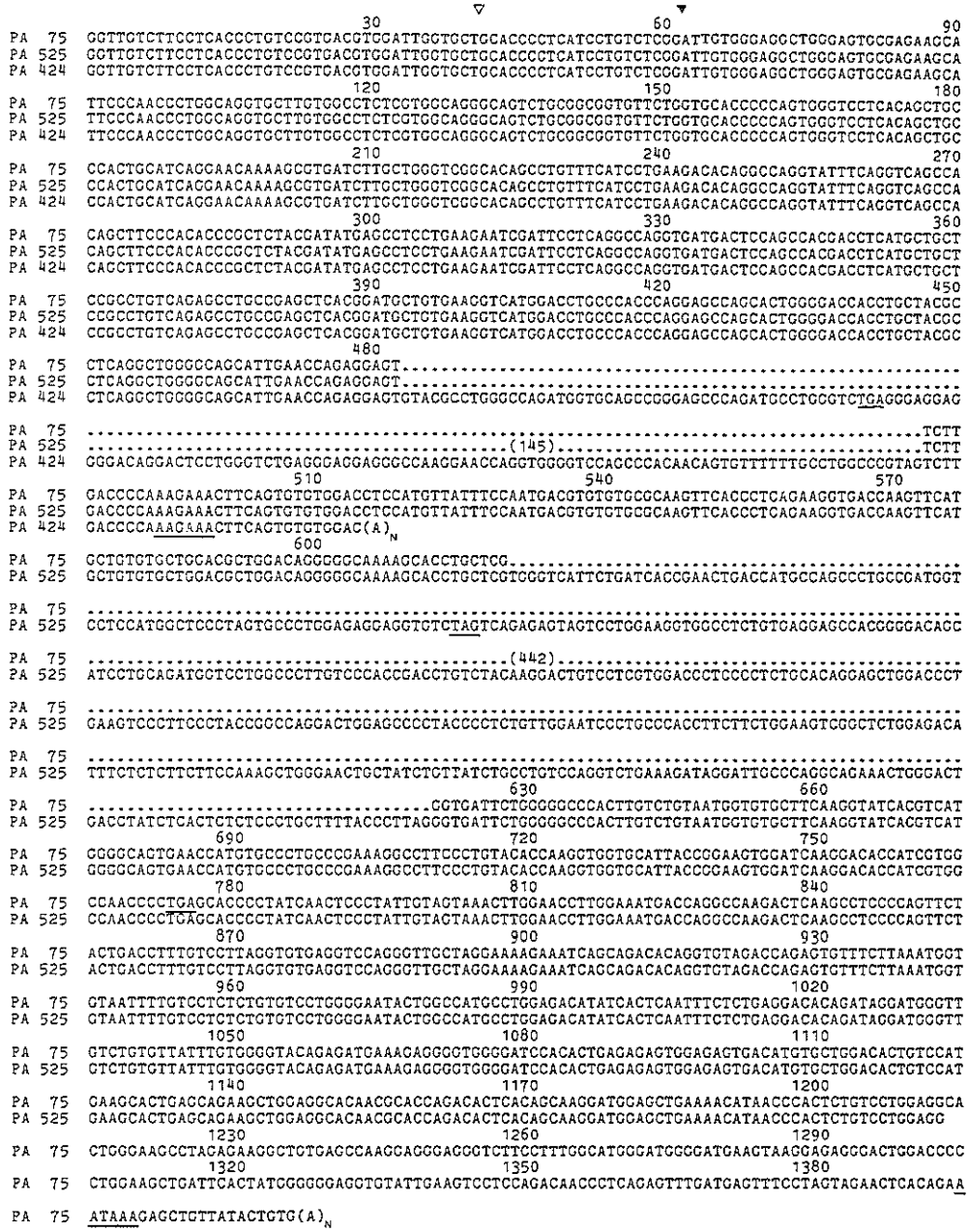

FIGURE 2: Comparison of the nucleotide sequences of PA 75, PA 525 and PA 424. The dotted lines represent open spaces. The open arrow head represents the end of the signal peptide and the beginning of the propeptide The solid arrowhead represents the sequence encoding the end of the propeptide and the beginning of the mature protein. The sequences which code for a stop codon and the poly(A) signal sequences are underlined. Poly(A) tails are given by the symbol: $(A)_n$.

```
        -20                          1                              30
PA  75....VVFLTLSVTWIGAAPLILSRIVGRLECEKHSQPWQVLVASRGRAVCGGVL
PA 525....VVFLTLSVTWIGAAPLILSRIVGRLECEKHSQPWQVLVASRGRAVCGGVL
PA 424....VVFLTLSVTWIGAAPLILSRIVGRLECEKHSQPWQVLVASRGRAVCGGVL

                                                               90
VHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG
VHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG
VHPQWVLTAAHCIRNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPG

                                                              150
DDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQCV
DDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLTPKKLQCV
DDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEECTAGPDGAAG

                                                              210
DLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPLVCNGVLQGITSWGSEPC
DLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSWVILITELTMPALPMVLHGSLVPW
SPDAWV*

ALPERPSLYTKVVHYRKWIKDTIVANP*
RGGV*
```

FIGURE 3: Comparison of the predicted amino acid sequences of PA 75, PA 525 and PA 424 proteins. The diverging sequences are underlined. The boxed and bold printed amino acids are thought to be responsible for the serine protease activity of the protein.

32 bp fragment, which is identical to PA 75 beginning at position 484 (Figure 2). PA 424 ends in 28 A-residues. The internal 145 bp fragment seems to be a retained intron. At the boundaries the splice consensus GT...AG sequence is found. The human kallikrein gene hGK-1, which has recently been sequenced, and which shows a homology to PA of about 80%, contains at exactly the same position a 113 bp intron (Schedlich et al., 1987). Especially the first 61 bp of this sequence are highly conserved between PA 424 and hGK-1. The poly(A) tail of PA 424 is preceded by an AAGAAA motif which presumably serves as the polyadenylation signal. The AAGAAA sequence is situated within the coding region of PA 75. A possible mature protein product of PA 424 will have a size of 156 amino acids with the first 140 amino acids identical to PA 75 (Figure 3).

Sequence analysis of PA 525 showed that it contains a fragment of 442 bp, in which the NcoI and PstI site are situated, which is not present in PA 75. The diversion from the PA 75 sequence begins at position 620 (Figure 2). Starting at position 1062 of PA 525 the sequence further parallels that of PA 75. PA 525 lacks the 3'-203 bp of PA 75, including the polyadenylation motif. For several reasons it is likely that PA 525 results from alternative splicing. The structure reveals it to contain a splice acceptor

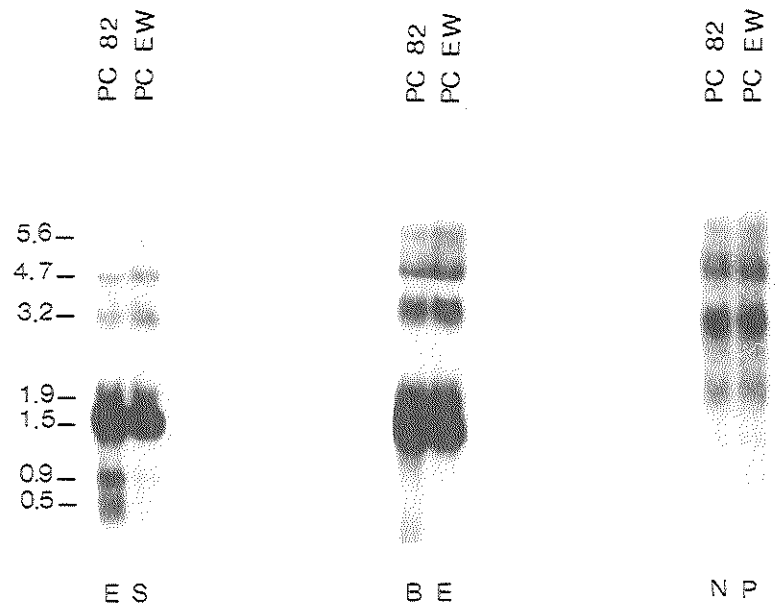

FIGURE 4: Northern blots of PC 82 and PC EW poly A⁺RNA hybridized with different PA probes. a; 5'-EcoRI - SacI fragment. b; 3'-BamHI - EcoRI fragment. c; NcoI - PstI fragment of PA 525.

site, but not the GT donor consensus sequence (Figure 2). Comparison of PA 525 with the structure of the hGK-1 gene shows that hGK-1 contains an intervening sequence at an identical position (Schedlich et al., 1987), however, this intron is much larger (1337 bp). The PA 525 internal fragment is highly homologous to the last 446 bp of the hGK-1 intron. Preliminary data from a recently isolated genomic PA clone confirm that the 442 bp fragment of PA 525 is part of a larger intervening sequence (data not shown). Translation of PA 525 mRNA will result into a mature PA protein of 214 amino acids. The terminal 28 amino acids of this protein will be different from the PA 75 protein (Figure 3).

The 380 bp EcoRI - SacI fragment common for the various PA cDNA clones was used to analyze by Northern blotting PA mRNA expression in the PC 82 and PC EW human prostate tumors (Figure 4). At least seven different hybridizing transcripts were observed ranging in size from 0.6 to 5.6 kb. The 1.5 kb band represents the major mRNA species. To obtain additional information identical blots were hybridized with the

3'-BamHI - EcoRI fragment of PA 525 and the NcoI - PstI PA 525 fragment as probes (Figure 4b,c). The 0.6 kb, 0.9 kb and the abundant 1.5 kb mRNA transcripts could not be detected by the NcoI - PstI probe. The BamHI - EcoRI fragment specifically hybridized with the five larger mRNAs. These data can be explained by assuming that PA 75 corresponds to the major 1.5 kb mRNA species and PA 424 to the 0.9 kb mRNA. PA 525 represents the major part of the 1.9 kb transcript.

DISCUSSION

In this study the characterization of two novel PA cDNA clones, PA 525 and PA 424, is described and evidence is presented showing that these cDNAs correspond to a 1.9 and a 0.9 kb PA mRNA species, respectively. PA 525 results from alternative splicing, PA 424 from intron retention and alternative polyadenylation as compared to PA 75, which represents the major PA 1.5 kb transcript. The AAGAAA polyadenylation motif of PA 424 is remarkable. As compared to the AATAAA consensus sequence, the AAGAAA signal has been reported to be very weak (Birnsteil et al., 1985).

It is presumed that PA 525 and PA 424 encode variant forms of the major 35 kD PA protein. Even if the 145 bp intervening sequence of PA 424 is spliced out, the resulting protein will be different from PA 75. PA 424 contains the information for a protein of 156 amino acids, PA 525 will be able to encode a 218 amino acid protein. There are data available indicating that alternative forms of the PA protein exist. Purified PA protein preparations contain, in addition to the 35 kD protein, at least one or two 20 - 25 kD proteins, which specifically interact with monoclonal antibodies against PA (Gallee et al., 1986). These proteins are likely candidates as products of different PA mRNA species. Alternatively they can be the result of proteolytic degradation of the 35 kD protein species.

The histidine residue at position 41, the aspartic acid at 96 and the serine at position 189, which are important for the proteolytic activity of kallikreins (Carter & Wells, 1988), are also found in PA 75 (see Figure 3). Histidine 41 and aspartic acid 96 will be present in the protein products of PA 525 and 424. However, the C-terminal part will be completely different and serine 189 will be lacking. No doubt, this will strongly influence the enzymatic properties of these proteins (Carter & Wells, 1988). At present, it is not yet possible to predict a cellular function of the PA 525 and 424 proteins. Expression of PA 525 and 424 and characterization of purified PA 525 and 424 protein can establish whether these proteins show (modified) enzymatic activity.

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REFERENCES

Birnsteil,M.L., Busslinger,M. & Strub,K. (1985) Cell 41, 349–359.
Carter,P. & Wells,J.A. (1988) Nature 332, 564–568.
Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. & Rutter,W.J. (1979) Biochemistry 18, 5294–5299.
Chu,M.T. (1986) Urology 27, 487–491.
Feinberg,A.P. & Vogelstein,B. (1983) Anal. Biochem. 132, 6–13.
Fukushima,D., Kitamura,N. & Nakanishi,S. (1985) Biochemistry 24, 8037–8043.
Gallee,M.P.W., Vroonhoven,C.C.J.van, Korput,J.A.G.M.van der, Kwast,T.H.van der, Kate,F.J.W.ten, Romijn,J.C. & Trapman,J. (1986) Prostate 9, 33–45.
Hoehn,W.,Schroeder,F.H., Riemann,J.F., Joebsis,A.C. & Hermanek,P. (1980) Prostate 1, 95–104.
Hoehn,W., Wagner,M., Riemann,J.F., Hermanek,P., Williams,E., Walter,R. & Schrueffer,R. (1984) Prostate 5, 445–452.
Huyn,T.V., Young,R.A. & Davis,R.W. (1985) DNA cloning Vol I, pp.49–78. IRL press, Oxford.
Kuriyama,M., Wang,M.C., Lee,C.L., Killian,C.S., Papsidero,L.D., Inaji,H., Loor,R.M. Lin,M.F., Nishiura,T., Slack,N.H., Murphy,G.P. & Chu,T.M. (1982a) J. Natl. Cancer Inst. 68, 99–105.
Kuriyama,M., Wang,M.C., Lee,C.L., Killian,C.S., Papsidero,L.D., Inaji,H., Lin,M.F., Nishiura,T., Slack,N.H., Murphy,G.P. & Chu,T.M. (1982b) Cancer Res. 41, 3874–3876.
Lilja,H. (1985) J. Clin. Invest. 76, 1899–1903.
Lilja,H. & Laurell,C.B. (1984) Scand. J. Clin. Lab. Invest. 44, 447–452.
Lundwall,A. & Lilja,H. (1987) FEBS Letters 214, 317–322.
Maniatis,T., Fritsch E.F. & Sambrook,J. (1982) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratories, New York.
Sanger,F., Nicklen,S. & Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
Schedlich,L.J., Bennets,B.H. & Morris,B.J. (1987) DNA 6, 429-437.
Stamey,T.A., Yang,N., Hay,A.R., McNeal,J.E., Freiha,F.S. & Redwine,E.B.A. (1987) N. Engl.J. Med. 317, 909–916.
Steenbrugge,G.J.van, Groen,M., Romijn,J.C. & Schröder,F.H. (1984) J. Urol. 131, 812–817.
Stein,B.S., Vangore,S., Petersen,R.O. & Kendall,A.R. (1982) Am. J. Surg. Pathol. 6, 553–557.
Takeuchi,T., Kuriyama,M., Fujihiro,S., Fujimoto,Y., Okana,M. & Nishiura,T. (1983) J. Surg. Onc. 24, 157–160.
Wang,M.C., Valenzuela,L.A., Murphy,G.P. & Chu,T.M. (1979) Invest. Urol. 17, 159–163.
Watt,K.W.K., Lee,P.J., M'Timkulu,T., Chan,W.P. & Loor,R. (1986) Proc. Natl. Acad. Sci. USA 83, 3166–3170.

CHAPTER III

CHARACTERIZATION OF THE PROSTATE-SPECIFIC ANTIGEN GENE: A NOVEL HUMAN KALLIKREIN LIKE GENE.

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SUMMARY

Using Prostate-specific Antigen cDNA fragments as hybridization probes a clone containing the information for the gene encoding Prostate-specific Antigen was isolated from a human genomic DNA library. The complete gene (about 6 kb) was sequenced and shown to be composed of four introns and five exons. Two major transcription initiation sites were found. The sequence of the promoter region revealed the presence of various well known transcription regulatory elements including a TATA box. A high percentage of homology was found between the Prostate-specific Antigen gene and the hGK-1 gene (82%). This homology extended into the promoter region. Two previously described variant Prostate-specific Antigen cDNAs can now be explained by intron retention and alternative splicing of the primary transcript.

INTRODUCTION

Tissue kallikreins and kallikrein-like proteins are a subgroup of closely related serine proteases (see for recent reviews Drinkwater et al., 1988 and MacDonald et al., 1988). They are able to cleave specific precursor proteins at highly selective sites, generating in this way the mature, biologically active proteins. Kallikrein-like proteins are produced by many different tissues, including salivary glands, kidney, prostate and pancreas (Drinkwater et al., 1988; MacDonald et al., 1988). In rat and mouse kallikrein-like proteases are encoded by a multigene family (Mason et al., 1983; Ashley et al., 1985; Gerald et al., 1986; Evans et al., 1988; Chen et al., 1988). The total number of kallikrein-like genes in these species is between 15 and 25. In man the gene family seems to be much smaller (Baker & Shine, 1985; Fukushima 1985; Schedlich et al., 1987). So far, the existence of three different members has been described. One gene (hGK-1) was completely sequenced (Schedlich et al., 1987), of two others (Prostate-specific Antigen (PA) (Lundwall & Lilja, 1987; Riegman et al., 1988) and tissue (pancreas/kidney) kallikrein (Baker & Shine, 1985; Fukushima et al., 1985) the cDNA structure has been established.

PA is a 35 kD glycoprotein, that is exclusively synthesized by the epithelial cells of the prostate gland (Wang et al., 1979,1981; Watt et al., 1986). Its natural substrates probably are proteins, secreted by seminal vesicles, which cause the gel like structure of the semen (Lilja, 1985; McGee & Herr, 1988). Recently, PA has attracted much attention as a reliable marker for human prostate cancer (Kuriyama et al., 1980; Killian et al., 1985,1986; Stamey et al., 1987). Normally, PA is not detectable in the serum. In case of invasive growth and/or metastasis of prostate tumors significantly elevated PA levels can be measured. Because of its clinical importance, detailed knowledge of regulation of PA expression is of high interest.

Previously we reported the molecular cloning and characterization of three different PA cDNAs and the detection of seven different PA transcripts (Riegman et al., 1988). In this study the isolation and structure of the PA gene including part of the promoter region is described. In addition the mutual relationship between various variant PA cDNAs is clarified.

METHODS

A human genomic library (partially MboI digested DNA in lambda EMBL 3) was kindly provided by Dr. G. Grosveld (Rotterdam). The library was screened with a [^{32}P]-labelled (Feinberg & Vogelstein, 1983) 380 bp EcoRI - SacI fragment derived from the cDNA clone PA 525 (Riegman et al., 1988). Duplicate nitrocellulose filters were hybridized overnight at 65°C in 6x SSC containing 10x Denhardt, 0.1% SDS, and 100 μg/ml salmon sperm DNA. Filters were washed twice in 3x SSC for 20 minutes at 65°C and twice in 1x SSC for 20 minutes at 65°C and once at 0.3x SSC for 10 minutes at 65°C. Filters were exposed to X-ray films for 18 hours at -70°C using intensifier screens. Positive phages were purified by two isolation cycles.

Phages were propagated, DNA isolated and fragments were subcloned into pUC9 using standard procedures (Maniatis et al., 1982; Davis et al., 1986). Nucleotide sequences of appropriate fragments subcloned into M13mp18/19 were determined by the dideoxy chain termination method (Sanger et al., 1977) using sequenase (USB, Cleveland) and dGTP or dITP.

Primer extension was perfomed using a method essentially identical as described by Geliebter et al. (1986), but omitting the dideoxy nucleotides in the reverse transcriptase mix. As primer a 22mer oligonucleotide with the sequence 5'-TGCAGCACCAATCCACGTCACG-3' was used. This oligonucleotide is PA-specific (compare Figure 2 position 74 to 87 and 1323 to 1330 in hGK-1 (Schedlich et al., 1987). PolyA$^+$ RNA was isolated from the human prostate tumors PC EW and PC 82, using standard procedures. Annealing of primer (5 ng) with polyA$^+$ RNA (3 μg) was for 6 hours at 65°C.

RESULTS

Using PA cDNA fragments as hybridization probes a genomic DNA fragment (designated 5P1) containing the complete PA gene was isolated. The restriction map of clone 5P1 is depicted in Fig. 1a. A more detailed map of the region at which the PA gene is situated is presented in Fig. 1b. Fig. 2 shows the complete sequence of the PA gene including parts of the 5'- and 3'-flanking regions. Comparison of the genomic sequence with that of the most abundant PA cDNA (PA 75, corresponding to a 1.5 kb mRNA; Fig. 1c and Riegman et al., 1988) revealed that the PA gene is composed of five exons and four introns. The total size of the gene is approximately 6 kb. As determined by primer extention experiments, using polyA+ RNA from two different

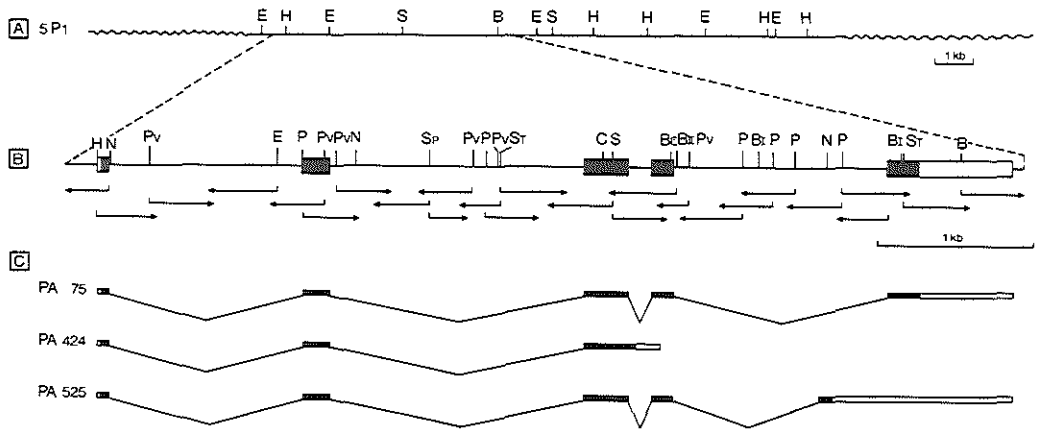


FIGURE 1: Genomic organization of the PA gene.

A. Partial restriction map of the genomic clone 5P1.

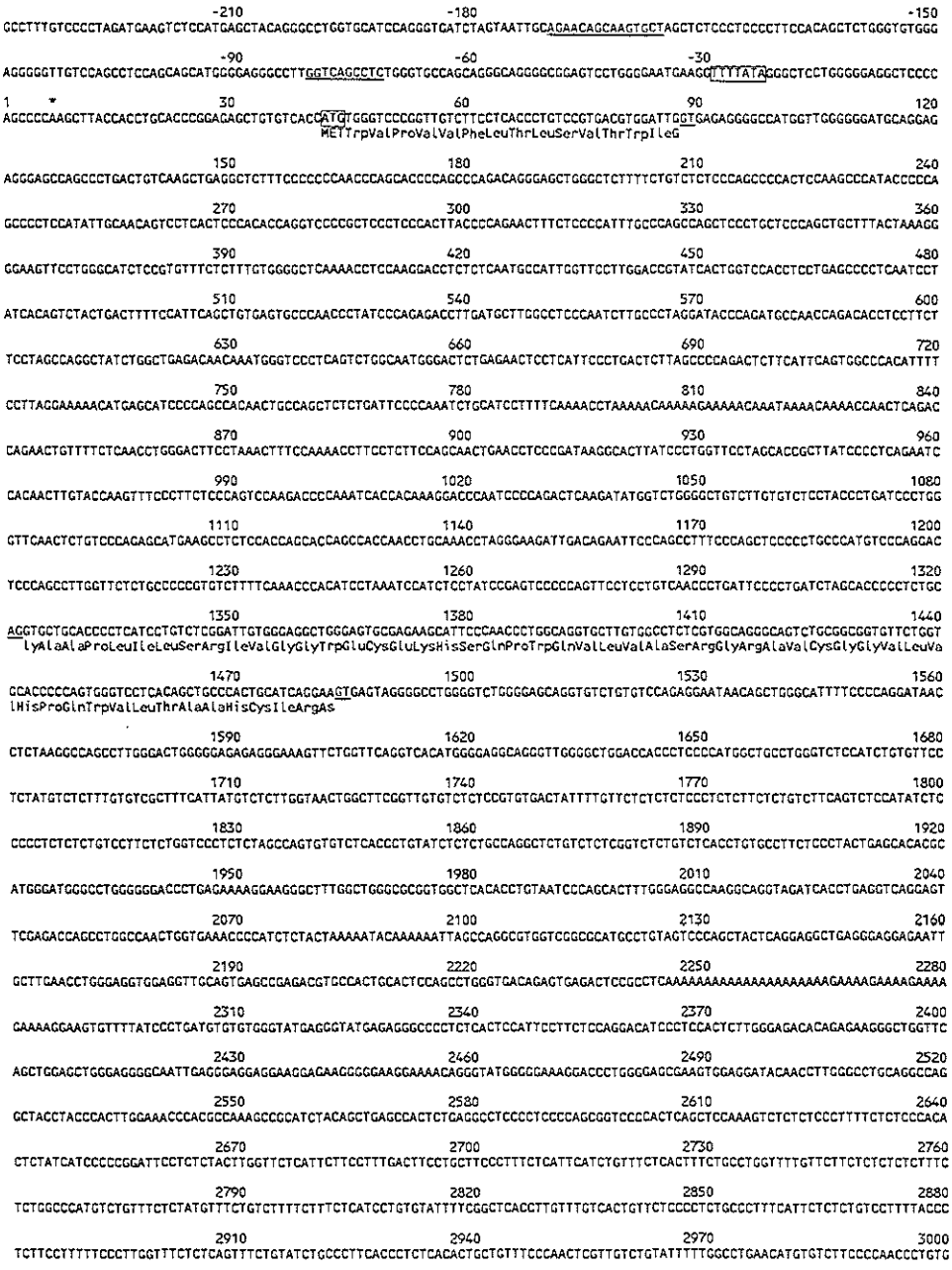
B. Restriction map of the genomic region containing the PA gene. Arrows indicate the sequence strategy.

C. Organization of the different PA cDNA's described so far (see Lundwall & Lilja, 1987; Riegman et al., 1988).

Closed boxes correspond to the open reading frame. Open boxes represent the remaining regions of the various cDNA's. The boxes in B. correspond to the major PA transcript (see also cDNA clone PA 75). B=BamHI, BI=BglI, BII=BglII, C=ClaI, E=EcoRI, H=HindIII, N=NcoI, P=PstI, Pv=PvuII, S=SacI, Sl=SalI, Sp=SphI and St=StuI.

human prostate tumors (PC 82, PC EW), two transcriptional initiation sites were found 42, respectively 35 bp upstream from the first ATG of the open reading frame (see Fig. 3). The exons have a size of 81 or 87 bp (exon I), 160 bp (exon II), 287 bp (exon III), 137 bp (exon IV) and 793 bp (exon V). The respective introns have a size of 1235, 1628, 145 and 1373 bp. Exon I contains in addition to the leader sequence the information for the major part of the signal peptide. Exon II encodes the last two amino acids of the signal peptide, the seven amino acids of the pro-fragment and the amino-terminal region of the mature protein. Exon V provides information for the carboxy-terminal part (51 amino acids) of the protein and the complete 3'-non-coding region.

FIGURE 2: Nucleotide sequence of the PA gene. Numbering starts at the first transcription start site; the second transcription initiation site is indicated by an asterix. Putative transcriptional regulatory elements, splice consensus sequenses and polyadenylation signals are underlined. The TATA box and the translational start codon are boxed. The stop codon is indicated by an asterix in the protein sequence.



-210 -180 -150
GCCTTTGTCCCCTAGATGAAGTCTCCATGAGCTACAGGGCCTGGTGCATCCAGGGTGATCTAGTAATTGCAGAACAGCAAGTGCTAGGCTCTCCCTCCCCTTCCACAGCTCTGGGTGTGGG

-90 -60 -30
AGGGGGTTGTCCAGCCTCCAGCAGCATGGGGAGGGCCTTGGTCAGCCTCTGGGTGCCAGCAGGGCAGGGGCGGAGTCCTGGGGAATGAAGCTTTTATAGGGCTCCTGGGGGAGGCTCCCC

1 * 30 60 90 120
AGCCCCAAGCTTACCACCTGCACCCGGAGAGCTGTGTCACCATGTGGGTCCCGGTTGTCTTCCTCACCCTGTCCGTGACGTGGATTGGTGAGAGGGGCCATGGTTGGGGGGATGCAGGAG
METTrpValProValValPheLeuThrLeuSerValThrTrpIleG

150 180 210 240
AGGGAGCCAGCCCTGACTGTCAAGCTGAGGCTCTTTCCCCCCAACCCAGCACCCCAGCCCAGACAGGGAGCTGGGCTCTTTTCTGTCTCTCCCAGCCCCACTCCAAGCCCATACCCCCA

270 300 330 360
GCCCCTCCATATTGCAACAGTCCTCACTCCCACACCAGGTCCCCGCTCCCTCCCACTTACCCCAGAACTTTCTCCCCATTTGCCCAGCCAGCTCCCTGCTCCCAGCTGCTTTACTAAAGG

390 420 450 480
GGAAGTTCCTGGGCATCTCCGTGTTTCTCTTTGTGGGGCTCAAAACCTCCAAGGACCTCTCTCAATGCCATTGGTTCCTTGGACCGTATCACTGGTCCACCTCCTGAGCCCCTCAATCCT

510 540 570 600
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630 660 690 720
TCCTAGCCAGGCTATCTGGCTGAGACAACAAATGGGTCCCTCAGTCTGGCAATGGGACTCTGAGAACTCCTCATTCCCTGACTCTTAGCCCCAGACTCTTCATTCAGTGGCCCACATTTT

750 780 810 840
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870 900 930 960
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990 1020 1050 1080
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1110 1140 1170 1200
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1230 1260 1290 1320
TCCCAGCCTTGGTTCTCTGCCCCCGTGTCTTTTCAAACCCACATCCTAAATCCATCTCCTATCCGAGTCCCCCAGTTCCTCCTGTCAACCCTGATTCCCCTGATCTAGCACCCCCTCTGC

1350 1380 1410 1440
AGGTGCTGCACCCCTCATCCTGTCTCGGATTGTGGCAGGCTGGGAGTGCGAGAAGCATTCCCAACCCTGGCAGGTGCTTGTGCCCTCTCGTGGCAGGGCAGTCTGCGGCGGTGTTCTGGT
lyAlaAlaProLeuIleLeuSerArgIleValGlyGlyTrpGluCysGluLysHisSerGlnProTrpGlnValLeuValAlaSerArgGlyArgAlaValCysGlyGlyValLeuVa

1470 1500 1530 1560
GCACCCCCAGTGGGTCCTCACAGCTGCCCACTGCATCAGGAAGTGAGTAGGGGCCTGGGGTCTGGGGAGCAGGTGTCTGTGTCCAGAGGAATAACAGCTGGGCATTTTCCCCAGGATAAC
lHisProGlnTrpValLeuThrAlaAlaHisCysIleArgAs

1590 1620 1650 1680
CTCTAAGGCCAGCCTTGGGACTGGGGGAGAGAGGGAAAGTTCTGGTTCAGGTCACATGGGGAGGCAGGGTTGGGGCTGGACCACCCTCCCCATGGCTGCCTGGGTCTCCATCTGTGTTCC

1710 1740 1770 1800
TCTATGTCTCTTTGTGTCGCTTTCATTATGTCTCTTGGTAACTGGCTTCGGTTGTGTCTCTCCGTGTGACTATTTTGTTCTCTCTGTCCCTCTCTTCTCTGTCTTCAGTCTCCATATCTC

1830 1860 1890 1920
CCCCTCTCTCTGTCCTTCTCTGGTCCCTCTCTAGCCAGTGTGTCTCACCCTGTATCTCTCTGCCAGGCTCTGTCTCTCGGTCTCTGTCTCACCTGTGCCTTCTCCCTACTGAGCACACGC

1950 1980 2010 2040
ATGGGATGGGCCTGGGGGGACCCTGAGAAAAGGAAGGGCTTTGGCTGGGCGCCGTGGCTCACACCTGTAATCCCAGCACTTTGGGAGGCCAAGGCAGGTAGATCACCTGAGGTCAGGAGT

2070 2100 2130 2160
TCGAGACCAGCCTGGCCAACTGGTGAAACCCCATCTCTACTAAAAATACAAAAAATTAGCCAGGCGTGGTCGGCGCCATGCCTGTAGTCCCAGCTACTCAGGAGGCTGAGGCAGGAGAATT

2190 2220 2250 2280
GCTTGAACCTGGGAGGTGGAGGTTGCAGTGAGCCGAGACGTGCCACTGCACTCCAGCCTGGGTGACAGAGTGAGACTCCGCCTCAAAAAAAAAAAAAAAAAAAAAGAAAAGAAAAGAAAA

2310 2340 2370 2400
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2430 2460 2490 2520
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2550 2580 2610 2640
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2670 2700 2730 2760
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2790 2820 2850 2880
TCTGGCCCATGTCTGTTTCTCTATGTTTCTGTCTTTTCTTTCTCATCCTGTGTATTTTCGGCTCACCTTGTTTGTCACTGTTCTCCCCTCTGCCCTTTCATTCTCTCTGTCCTTTTACCC

2910 2940 2970 3000
TCTTCCTTTTTCCCTTGGTTTCTCTCAGTTTCTGTATCTGCCCTTCACCCTCTCACACTGCTGTTTCCCAACTCGTTGTCTGTATTTTTGGCCTGAACATGTGTCTTCCCCAACCCTGTG

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                                3030                                3060                                3090                                3120
TTTTTCTCACTGTTTCTTTTTCTCTTTTGGAGCCTCCTCCTTGCTCCTCTGTCCCTTCTCTCTTTCCTTATCATCCTCGGCTCCTCATTCCTGCGTCTGCTTCCTCCCCAGCAAAAGCGTG
                                                                                                                                nLysSerVal

                                3150                                3180                                3210                                3240
ATCTTGCTGGGTCGGCACAGCCTGTTTCATCCTGAAGACACAGGCCAGGTATTTCAGGTCAGCCACAGCTTCCCACACCCGCTCTACGATATGAGCCTCCTGAAGAATCGATTCCTCAGG
IleLeuLeuGlyArgHisSerLeuPheHisProGluAspThrGlyGlnValPheGlnValSerHisSerPheProHisProLeuTyrAspMetSerLeuLeuLysAsnArgPheLeuArg

                                3270                                3300                                3330                                3360
CCAGGTGATGACTCCAGCCACGACCTCATGCTGCTCCGCCTGTCAGAGCCTGCCGAGCTCACGGATGCTGTGAAGGTCATGGACCTGCCCCACCCAGGAGCCAGCACTGGGGACCACCTGC
ProGlyAspAspSerSerHisAspLeuMetLeuLeuArgLeuSerGluProAlaGluLeuThrAspAlaValLysValMetAspLeuProThrGlnGluProAlaLeuGlyThrThrCys

                                3390                                3420                                3450                                3480
TACGCCTCAGGCTGGGGCAGCATTGAACCAGAGGAGTGTACGCCTGGGCCAGATGGTGCAGCCGGGAGCCCAGATGCCTGGGTCTGAGGGAGGAGGGGACAGGACTCCTAGGTCTGAGGG
TyrAlaSerGlyTrpGlySerIleGluProGluGluP

                                3510                                3540                                3570                                3600
AGGAGGGCCAAGGAACCAGGTGGGGTCCAGCCCACAACAGTGTTTTTTGCCTGGCCCGTAGTCTTGACCCCAAAGAAACTTCAGTGTGTGGACCTCCATGTTATTTCCAATGACGTGTGT
                                                                    heLeuThrProLysLysLeuGlnCysValAspLeuHisValIleSerAsnAspValCys

                                3630                                3660                                3690                                3720
GCGCAAGTTCACCCTCAGAAGGTGACCAAGTTCATGCTGTGTGCTGGACGCTGGACAGGGGCCAAAAGCACCTGCTCGGTGAGTCATCCCTACTCCCAAGATCTTGAGGGGAAAGGTGAG
AlaGlnValHisProGlnLysValThrLysPheMetLeuCysAlaGlyArgTrpThrGlyGlyLysSerThrCysSer

                                3750                                3780                                3810                                3840
TGGGGACCTTAATTCTGGGCTGGGGTCTAGAAGCCAACAAGCATCTGCCTCCCCTGCTCCCCAGCTGTAGCCATGCCACCTCCCCGTGTCTCATCTCATTCCCTCCTTCCCTCTTCTTTG

                                3870                                3900                                3930                                3960
ACTCCCTCAAGGCAATAGGTTATTCTTACAGCACAACTCATCTGTTCCTGCGTTCAGCACACGGTTACTAGGCACCTGCTATGCACCCAGCACTGCCCTAGAGCCTGGACATAGCAGTGA

                                3990                                4020                                4050                                4080
ACAGACAGAGAGCAGCCCCTCCCTTCTGTAGCCCCCAAGCCAGTGAGGGGCACAGGCAGGAACAGGGACCACAACACAGAAAAGCTGGAGGGTGTCAGGAGGTGATCAGGCTCTCGGGGA

                                4110                                4140                                4170                                4200
GGGAGAAGGGGTGGGGAGTGTGACTGGGAGGAGACATCCTGCAGAAGGCGGGAGTGAGCAAACACCTGCCCCCAGGGGAGGGGAGGGCCTGCGGCACCTGGGGGAGCAGAGGAACAGCAT

                                4230                                4260                                4290                                4320
CTGGCCAGGCCTGGGAGGAGGGGCCTAGAGGGCGTCAGGAGCAGAGAGGAGGTTGCCTGGCTGGAGTGAAGGATCGGGGCAGGGTGCGAGAGGGAAGAAGGACCCCTCCTGCAGGGCCTC

                                4350                                4380                                4410                                4440
ACCTGGGCCACAGGAGGACACTGCTTTTCCTCTGAGGAGTCAGGAACTGTGGATGGTGCTGGACAGAAGCAGGACAGGGCCTGGCTCAGGTGTCCAGAGGCTGCCGCTGGCCTCCCTATG

                                4470                                4500                                4530                                4560
GGATCAGACTGCAGGGAGGGAGGGCAGCAGGGATGTGGAGGGAGTGATGATGGGGCTGACCTGGGGGTGGCTCCAGGCATTGTCCCCACCTGGGCCCTTACCCAGCCTCCCTCACAGGCT

                                4590                                4620                                4650                                4680
CCTGGCCCTCAGTCTCTCCCCTCCACTCCATTCTCCACCTACCCACAGTGGGTCATTCTGATCACCGAACTGACCATGCCAGCCCTGCCGATGGTCCTCCATGGCTCCCTAGTGCCCTGG

                                4710                                4740                                4770                                4800
AGAGGAGGTGTCTAGTCAGAGAGTAGTCCTGGAAGGTGGCCTCTGTGAGGACCCACGGGGACAGCATCCTGCAGATGGTCCTGCCCCTTGTCCCCACCGACCTGTCTACAAGGACTGTCCT

                                4830                                4860                                4890                                4920
CGTGGACCCTCCCCTCTGCACAGGAGCTGGACCCTGAAGTCCCTTCCCCACCGGCCAGGACTGGAGCCCCTACCCCTCTGTTGGAATCCCTGCCCACCTTCTTCTGGAAGTCGGCTCTGG

                                4950                                4980                                5010                                5040
AGACATTTCTCTCTTCTTCCAAAGCTGGGAACTGCTATCTGTTATCTGCCTGTCCAGGTCTGAAAGATAGGATTGCCCAGGCAGAAACTGGGACTGACCTATCTCACTCTCTCCCTGCTT

                                5070                                5100                                5130                                5160
TTACCCTTAGGGGTGATTCTGGGGCCCCACTTGTCTGTAATGGTGTGCTTCAAGGTATCACGTCATGGGGCAGTGAACCATGTGCCCTGCCCGAAAGGCCTTCCCTGTACACCAAGGTGGT
          GlyAspSerGlyGlyProLeuValCysAsnGlyValLeuGlnGlyIleThrSerTrpGlySerGluProCysAlaLeuProGluArgProSerLeuTyrThrLysValVa

                                5190                                5220                                5250                                5280
GCATTACCGGAAGTGGATCAAGGACACCATCGTGGCCAACCCCTGAGCACCCCTATCAACTCCCTATTGTAGTAAACTTGGAACCTTGGAAATGACCAGGCCAAGACTCAAGCCTCCCCA
lHisTyrArgLysTrpIleLysAspThrIleValAlaAsnPro *

                                5310                                5340                                5370                                5400
GTTCTACTGACCTTTGTCCTTAGGTGTGAGGTCCAGGGTTGCTAGGAAAAGAAATCAGCAGACACAGGTGTAGACCAGAGTGTTTCTTAAATGGTGTAATTTTGTCCTCTCTGTGTCCTG

                                5430                                5460                                5490                                5520
GGGAATACTGGCCATGCCTGGAGACATATCACTCAATTTCTCTGAGGACACAGATAGGATGGGTTGTCTGTGTTATTTGTGGGGTACAGAGATGAAACAGGGGTGGGGATCCACACTGAG

                                5550                                5580                                5610                                5640
AGAGTGGAGAGTGACATGTGCTGGACACTGTCCATGAAGCACTGAGCAGAAGCTGGAGCGCACAACGCACCAGACACTCACAGCAAGGATGGAGCTGAAAACATAACCCACTCTGTCCTGG

                                5670                                5700                                5730                                5760
AGGCACTGGGAAGCCTAGAGAAGGCTGTGAGCCAAGGAGGGAGGGTCTTCCTTTGGCATGGGATGGGGATGAAGTAAGGAGAGGGACTGGACCCCCTGGAAGCTGATTCACTATGGGGGG

                                5790                                5820                                5850                                5880
AGGTGTATTGAAGTCCTCCAGACAACCCTCAGATTTGATGATTTCCTAGTAGAACTCACAGAAATAAAGAGCTGTTATACTGTGGTTTATTCTGGTTTGTTACATTGACAGGAGACACAC

                                5910
TGAAATCAGCAAAGGAAACAGGCATCTAAGTGG
```

Seven distinct PA transcripts have been detected (Riegman et al., 1988). In addition to cDNA corresponding to the 1.5 kb mRNA (PA 75), two cDNAs have been characterized in detail: PA 424 and PA 525. Both contain an extra internal fragment as compared to PA 75 cDNA (Fig. 1c and Riegman et al., 1988). In addition, PA 424 is truncated at the 3'-terminus because of the use of an alternative polyadenylation signal sequence (Riegman et al., 1988). From the organization of the PA gene it can now clearly be established that PA 424 contains as extra information the complete retained small intron III (Figure 1c). PA 525 results from the use of an alternative splice acceptor site upstream from position 4608 instead of the site at position 5051 used in PA 75 (see Figure 2).

In general, the most important regulatory elements are located within a region of 200 bp upstream from the transcription start site. This region in the PA gene is characterized by the presence of several well known regulatory elements. A variant TATA-box (TTTATA) is found at position -28 to -23, a GC-box at -53 to -48, a CACCC-box at -129 to -125. Interestingly, starting at position -170 the imperfect palindrome AGAACAGCAAGTGCT is found, which is closely related to reverse complement of the consensus sequence for steroid (glucocorticoid, progesterone, androgen (Ham et al., 1988)) hormone receptor binding (TGTACANNNTGTC/TCT). Another, to our knowledge not yet described, repeat is located in the region -123 to -72. Both the sequences GGGAGGG and CAGCCTC are present in duplicate at a mutual distance of 7 nucleotides.

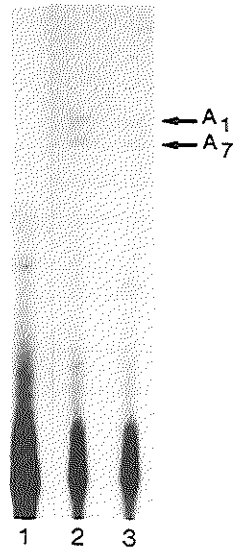

FIGURE 3: Primer extension analysis of PA mRNA. As size marker the sequence, performed with the 22-mer oligonucleotide as a primer, of the corresponding genomic region, as determined by the dideoxy chain termination method was used. Lane1: control tRNA; lane 2: PC 82 poly A^+ RNA lane 3: PC EW poly A^+ RNA. The two transcription initiation sites are indicated by arrows, the number indicates the corresponding position of the nucleotide in the genomic sequence (see Figure 2).

DISCUSSION

In this study we determined the structure of the PA gene. Comparison with other kallikrein-like genes (mouse, rat and the human hGK-1 gene) revealed a similar organization (Mason et al., 1983; Ashley et al., 1985; Gerald et al., 1986; Evans et al., 1988; Schedlich et al., 1987). Transcription in rat and mouse kallikrein-like genes initiates at positions comparable to the ones found here (Mason et al., 1983; Chen et al., 1988). All genes are composed of five exons and four introns; the splice junction sites are completely conserved. Rat and mouse kallikrein-like genes are somewhat smaller than PA and hGK-1, mainly because of the smaller size of the last intron. As expected, the highest percentage of homology is found with the human gene hGK-1 (see Fig. 4 and Schedlich et al., 1987). The various regions show a mutual homology ranging from 75 to 92 percent. The highest homology is found in the promoter region and in the protein coding region of exon V (over 90 %); the structural similarity is the smallest in the first exon, encoding the non-translated leader and the major part of the signal sequence, and in exon IV (75-77 %). Intron regions are as well conserved as the protein coding parts.

PA is expressed at high level in the prostate (Lundwall & Lilja, 1987; Riegman et al., 1988). Recently data have been published, indicating that hGK-1 is also expressed in the prostate gland, although at a lower level (Chapdelaine et al., 1988). The third known human kallikrein-like gene, tissue kallikrein, is expressed in pancreas and kidney (Fukushima et al., 1985). Comparison of the promoter regions of the three human

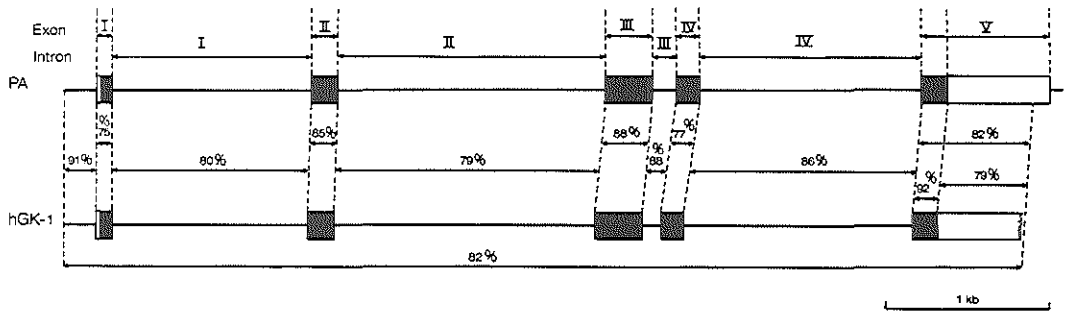

FIGURE 4: Comparison of the organization of the PA gene with that of the hGK-1 gene. The hGK-1 sequence data are from Schedlich et al. (1987).

```
PA      -190  CAGGGTGATCTAGTAATTGCAGAACAGCAAGTGCTAGCTCTCCCTCCCCTTCCA
hGK-1         ...AC.........TG..TG............G...............

PA      -136  CAGCTCTGGGTGTGGGAGGGGGTTGTCCAGCCTCCAGCAGCATGGGGAGGGCCT
hGK-1         .......______.....................................

PA       -82  TGGTCAGCCTCTGGGTGCCAGCAGGGCAGGGGCGGAGTCCTGGGGAATGAAGGT
hGK-1         ........A...A.......A.......A______G.......A.........C

PA       -28  TTTATAGGGCTCCTGGGGGAGGCTCCCC -1
hGK-1         ______........CA.......C....
```

Figure 5: Comparison of the sequence of the promoter region of the PA gene with that of the hGK-1 gene. Dots represent identical nucleotides. Putative transcriptional regulatory elements are underlined. The hGK-1 sequence data are from Schedlich et al. (1987).

kallikrein-like genes can, therefore, provide important information about elements involved in tissue specific gene expression. The promoter region of the hGK-1 gene has been described (Schedlich et al., 1987). So far, however, no data are available about the structure of the tissue kallikrein gene. As depicted in Fig. 5 the putative regulatory elements present in the PA promoter region are in general also present in the 5'-flanking region of hGK-1. A difference which can be of interest is the G at -170 in the HRE (Hormone Responsive Element) of hGK-1 instead of A, which makes that the putative hGK-1 HRE deviates from the consensus sequence. At present, experiments are in progress to find out whether or not PA/hGK-1 transcription is steroid dependent and which sequences are involved in prostate specific gene expression.

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REFERENCES

Ashley,P.L. & MacDonald,R.J. (1985) Biochem. 24, 4512-4520.
Baker,A.R. & Shine,J. (1985) DNA 4, 445-450.
Chapdelaine,P., Paradis,G., Tremblay,R.R. & Dube,J.Y. (1988) FEBS Lett. 236, 205-208.
Chen,Y-P. Chao,J. & Chao,L. (1988) Biochem. 27, 7189-7196.

Davis,L.G., Dibner,M.D. & Battey,J.F. (1986) Basic Methods in Molecular Biology, Elsevier, NY.
Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1988) Trends Biochem. Sci. 13, 169–1722.
Evans,B.A., Drinkwater,C.C. & Richards,R.I. (1988) J. Biol. Chem. 262, 8027–8034.
Feinberg,A.P. & Vogelstein,P. (1983) Anal. Biochem. 132, 6-13.
Fukushima,D., Kitamura,N. & Nakanishi,S. (1985) Biochem. 24, 8037-8043.
Gallee,M.P.W., Vroonhoven,C.C.J.van, Korput,J.A.G.M.van der, Kwast,T.H.van der, Kate,F.J.W.ten, Romijn,J.C. & Trapman,J. (1986) Prostate 9, 33–45.
Geliebter,J., Zeff,R.A., Melvold,R.W. & Nathenson,S.G. (1986) Proc. Natl. Acad. Sci. USA 83, 3371–3375.
Gerald,W.L., Chao,J. & Chao,L. (1986) Biochim. Biophys. Acta 866, 1–14.
Ham,J., Thomson,A., Needham,M., Webb,P. & Parker,M. (1988) Nucl. Acids Res. 16, 5263–5275.
Killian,C.S., Yang,N., Emrich,L.J., Vargas,F.P., Kuriyama,M., Wang,M.C., Slack,N.H., Papsidero,L.D., Murphy,G.P. & Chu,T.M. (1985) Cancer Res. 45, 886–891.
Killian,C.S., Emrich,L.J., Vargas,F.P., Yang,N., Wang,M.C., Priore,R.L., Murphy,G.P. & Chu,T.M. (1986) J. Natl. Cancer Inst. 76, 179–185.
Kuriyama,M., Wang,M.C., Papsidero,L.D., Kilian,C.S., Shimano,T., Valenzuela,L., Nishiura,T., Murphy,G.P. & Chu,T.M. (1980) Cancer Res. 40, 4658–4662.
Lilja,H. (1985) J. Clin. Invest. 76, 1899–1903.
Lundwall,A. & Lilja,H. (1987) FEBS Letters 214, 317–322.
Macdonald,R.J., Margolius,H.S. & Erdös,E.G. (1988) Biochem. J. 253, 313–321.
Maniatis,T., Fritsch,E.F. & Sambrook,J. (1982) Molecular cloning: A Laboratory Manual, Cold Spring Harbor, NY.
Mason,A.J., Evans,B.A., Cox,D.R., Shine,J. & Richards,R.I. (1983) Nature 303, 300–307.
McGee,R.S. & Herr,J.C. (1988) Biol. Reprod. 39, 499–510.
Riegman,P.H.J., Klaassen,P., Korput,J.A.G. van der, Romijn,J.C. & Trapman,J. (1988) Biochem Biophys. Res. Commun. 155, 181–188.
Sanger,F., Nicklen,S. & Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
Schedlich,L.J., Bennetts,B.H. & Morris,B.J. (1987) DNA 6, 429–437.
Stamey,T.A., Yang,N., Hay,A.R., McNeal,J.E., Freiha,F.S. & Redwine,E. (1987) New Engl. J. Med. 317, 909–916.
Wang,M.C., Valenzuela,L.A., Murphy,G.P. & Chu,T.M. (1979) Invest. Urol. 17, 159–163.
Wang,M.C., Papsidero,L.D., Kuriyama,M., Valenzuela,L.A., Murphy,G.P. & Chu,T.M. (1981) Prostate 2, 89–96.
Watt,K.W.K., Lee,PJ., M'Timkulu,T., Chan,WP. & Loor,R. (1986) Proc. Natl. Acad. Sci. USA 83, 3166–3170.

CHAPTER IV

THE PROSTATE-SPECIFIC ANTIGEN GENE AND THE HUMAN GLANDULAR KALLIKREIN-1 GENE ARE TANDEMLY LOCATED ON CHROMOSOME 19.

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FEBS letters (1989) 247, 123-126.

SUMMARY

Using a Prostate-specific Antigen cDNA as a hybridization probe, clones containing the kallikrein genes encoding Prostate-specific Antigen, human Glandular Kallikrein-1 and Tissue Kallikrein were isolated from a human genomic library. Clones containing the Prostate-specific Antigen gene and the human Glandular Kallikrein-1 gene overlap and span a region of about 36 kb. The two genes are aligned in a head to tail orientation at a mutual distance of 12 kb. Southern blot analysis of DNA from a panel of human-hamster hybrid cells with specific probes revealed the genes to be situated on chromosome 19. Assuming that the Tissue Kallikrein gene is located in the same cluster, the distance to the Prostate-specific Antigen gene and the human Glandular Kallikrein-1 gene must be at least 15 kb.

INTRODUCTION

Kallikreins and kallikrein-like proteins are a subgroup of the serine protease family which show high degree of substrate specificity (see for recent reviews Drinkwater et al., 1988 and MacDonald et al., 1988). In mouse and rat kallikreins are encoded by a large multigene family (Maso et al., 1983; Ashley et al., 1985; Gerald et al., 1986; Evans et al., 1987; Chen et al., 1988). In the mouse genome at least 24 kallikrein genes have been identified, which are clustered in groups of up to 11 genes on chromosome 7 (Mason et al., 1983). Ten of the mouse kallikrein genes appear to be pseudogenes (Evans et al., 1987). A family of 15 to 20 kallikrein genes has been found in the rat genome (Gerald et al., 1986). At least 4 of these genes are functional (Ashley et al., 1985). Three human kallikrein genes have been described: Prostate-specific Antigen (PA) (Lundwall & Lilja, 1987; Riegman et al., 1988,1989), human Glandular Kallikrein (hGK-1) (Schedlich et al., 1987) and (pancreas/kidney) tissue kallikrein (Baker & Shine, 1985; Fukushima et al., 1985; Evans et al., 1988). Both the PA and hGK-1 gene are completely sequenced, their mutual homology is 82% (Schedlich et al., 1987; Riegman et al., 1989). PA is exclusively synthesized by the epithelial cells of the prostate gland (Wang et al., 1979,1981; Watt et al., 1986; Gallee et al., 1986). Its presumed function is dissolving the seminal coagulum by digesting proteins secreted by the seminal vesicles, that cause the gel like structure of the semen (Lilja, 1985; McGee & Herr, 1988). An elevated level of PA in the serum is a reliable marker for prostate cancer (Kuriyama et al., 1980; Kilian et al., 1985; Stamey et al., 1987). Expression of the hGK-1 gene, like that of PA, seems also to be restricted to the prostate (Chapdelaine et al., 1988). The physiological function of hGK-1 has not yet been established. Tissue kallikrein is a protease that cleaves the precursor kininogen to

release small vasoactive peptides or kinins (Schachter, 1980). It has been suggested that the kallikrein-kinin system is involved in hypertension (Carretero & Scicli, 1980).

Recently, the tissue kallikrein gene has been mapped on chromosome 19 (Evans et al., 1988). In this study the chromosomal location of the PA and hGK-1 genes is described and the size and presumed clustering of the human kallikrein gene family are discussed.

METHODS

A human genomic library (partially Mbo I digested DNA in lambda EMBL 3) was kindly provided by Dr. G. Grosveld (Rotterdam). The library was screened with a [^{32}P]-labeled (Feinberg & Vogelstein, 1983) 380 bp EcoRI-SacI fragment derived from the PA cDNA clone PA 525 (Riegman et al., 1988). Duplicate nitrocellulose filters were hybridized overnight at 65°C in 6xSSC containing 10x Denhardt, 0.1% SDS, and 100 μg/ml salmon sperm DNA. Filters were washed twice in 3x SSC for 20 min at 65°C, twice in 1x SSC for 20 min at 65°C and once in 0.3x SSC for 10 min at 65°C. Filters were exposed to X-ray films for 18 h at -70°C using intensifying screens. Positive phages were purified by two isolation cycles. Phages were propagated, DNA isolated and a restriction map was made using standard procedures (Maniatis et al., 1982). Fragments were subcloned in a plasmid vector for detailed mapping. For sequencing fragments were subcloned in M13mp18/19. Sequencing was done by the dideoxy chain termination method (Sanger et al., 1977), using sequenase (USB, Cleveland).

Somatic cell hybrids were generated by fusion of human cells with the hamster A3 cell line as described (Geurts van Kessel et al., 1983). DNA of 15 of these hybrid cell lines was isolated and analyzed by Southern blotting using standard procedures. The probe used for hybridization was the 1600 bp cDNA clone PA 525 (Riegman et al., 1988). The labeling, hybridization and washing conditions were identical to those used for the screening of the genomic library as described above.

RESULTS AND DISCUSSION

A fragment of a PA cDNA clone was used to screen a genomic library (see method section). Four different clones (designated 4P1 (18.5 kb), 5P1 (15.5 kb), 7P1 (17 kb) and 9P1 (18 kb)) were isolated. Partial restriction maps of 4P1, 5P1 and 9P1 are shown in Figure 1. Sequence analysis of specific fragments proved that 7P1 contains the tissue kallikrein gene (Evans et al., 1988) and 9P1 the hGK-1 gene (Schedlich et al., 1987); 4P1 and 5P1 largely overlap and both contain the complete PA gene. The exon/intron organization and the sequence of the PA gene has been described in detail elsewhere (Riegman et al., 1989). The organization of the hGK-1 and tissue kallikrein gene is derived from Schedlich et al. (1987) and Evans et al. (1988). Hybridization experiments with terminal fragments revealed that 4P1/5P1 and 9P1 contain a small

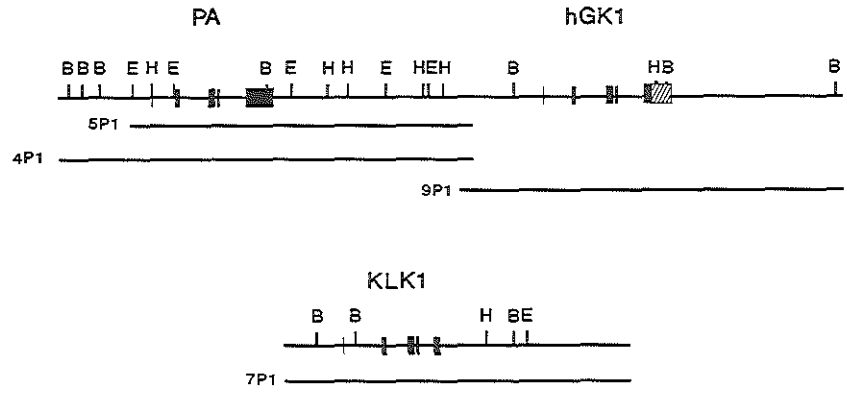

FIGURE 1: Partial restriction map of the genomic clones 4P1, 5P1 and 9P1 (A) and 7P1 (B). Organization of the PA, hGK-1 and tissue kallikrein gene is from (Schedlich et al., 1987; Evans et al., 1988; Riegman et al., 1989). The closed boxes represent exons. The hatched area in the hGK-1 gene indicates a putative 3'-nontranslated region of the cDNA. B=BamHI; E=EcoRI; H=HindIII.

common region. In agreement with this finding, a 3'-fragment of 4P1/5P1 and a 5'-fragment of 9P1 hybridized with the same band in BamHI, HindIII and BamHI + HindIII digests of genomic DNA (data not shown). Comparison of the restriction maps of 4P1/5P1 and 9P1 with that of the earlier described clone lambda HGK1 (Schedlich et al., 1987) confirmed the tandem arrangement on the PA and hGK-1 gene. The two genes are positioned in a head to tail orientation and are separated by a stretch of approximately 12 kb. Together the clones span a region of 36 kb.

In the mouse genome a large kallikrein gene family (about 25 genes) is found to be located in clusters on chromosome 7 (Evan et al., 1987). Similarly, the rat kallikrein gene family counts 15-20 members (Gerald et al., 1986). Southern blot analysis of genomic DNA with hGK-1 and tissue kallikrein probes indicated that the human kallikrein family is much smaller, and it has been suggested that it comprises only three genes (Schedlich et al., 191987; Baker & Shine, 1985). To substantiate these observations, similar experiments were carried out using a PA cDNA probe (Figure 2). In all digests only a limited number of bands could be visualized. Because the complete

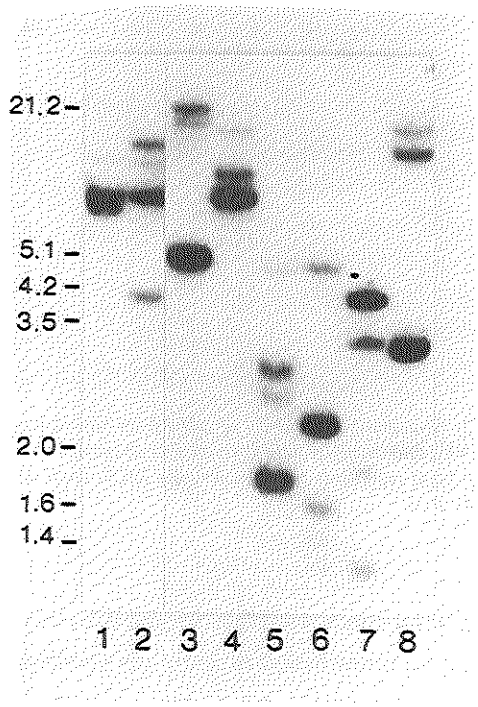

FIGURE 2: Southern blot analysis of human genomic DNA hybridized with PA cDNA clone 525 (Riegman et al., 1988,1989). DNA was digested with BamHI (lane 1); BglII (2); EcoRI (3); HindIII (4); NcoI (5); PstI (6); PvuII (7); SacI (8).

sequence of the PA gene (Riegman et al., 1989), the hGK-1 gene (Schedlich et al., 1987) and most of the tissue kallikrein gene has been published (Evans et al., 1988) and restriction maps of the corresponding genomic areas are available it could be deduced that the hybridizing genomic fragments represent the complete PA, hGK-1 and tissue kallikrein genes or parts of the three genes. Under the same conditions the PA probe detected a large series of kallikrein genes in mouse DNA (data not shown). These findings firmly established that the human kallikrein gene family indeed encompasses only three closely related genes: PA, hGK-1 and tissue kallikrein.

To determine the chromosomal location of the PA and hGK-1 gene, BamHI digested DNA from 15 different human-hamster cell hybrids containing characteristic sets of human chromosomes was analyzed by Southern blotting using a PA cDNA probe. Examples of the experiments are shown in Figure 3. In control human DNA three bands are detected (Figure 3B).Two of these bands (a weak 13 kb signal and a strong 8 kb signal) represent the PA gene; the 7.6 kb fragment contains the hGK-1 gene; the

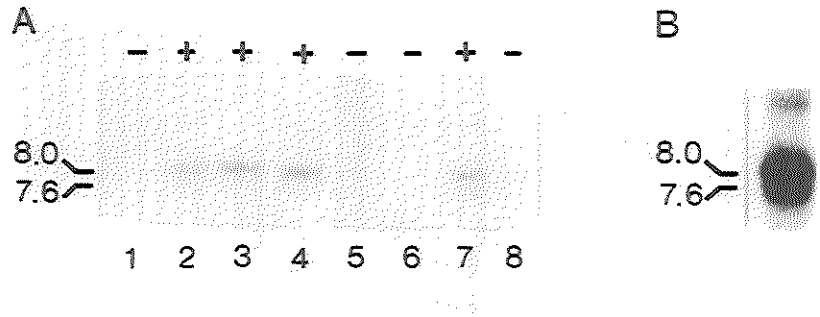

FIGURE 3: Southern blot analysis of DNA from human-hamster hybrid cells (lanes 1 - 8) (A) and human control DNA (B) hybridized with PA 525 (see Riegman et al., 1988,1989). DNA was digested with BamHI. The 8.0 kb band represents the PA gene, the 7.6 kb band the hGK-1 gene. A positive hybridization signal is indicated by a +.

predicted 8 kb tissue kallikrein fragment cannot be seen in this experiment. Hamster DNA does not cross-hybridize under the conditions used (data not shown). The results, as summarized in table I, clearly show that the PA and the hGK-1 gene are situated on chromosome 19. Recently, the tissue kallikrein gene was reported to be located on the same chromosome (Evans et al., 1988). Clustering of the human kallikrein genes would be in accordance with the close linkage of kallikrein genes found in the mouse genome (Evans et al., 1987). In this species the distance between the genes in the various clusters can be as small as 3-5 kb. It was already concluded that the region between the PA gene and the hGK-1 gene is considerably larger (about 12 kb, see Figure 1). It seems reasonable to assume that, if the kallikrein family is clustered, the tissue kallikrein gene is aligned in the same orientation as the PA and hGK-1 gene. In this case a minimal distance of 15 kb between tissue kallikrein and PA or hGK-1 can be predicted from the restriction maps of the clones presented in Figure 1 combined with those of two other published clones (Schedlich et al., 1987; Evans et al., 1988). Alternatively, although located on the same chromosome, the tissue kallikrein gene may not be closely linked to PA and hGK-1. Additional chromosome walking or pulse field electrophoresis experiments will have to settle this point.

TABLE I: CORRELATION BETWEEN THE PRESENCE OR ABSENCE OF PA AND hGK-1 AND HUMAN CHROMOSOMES IN THE HUMAN-HAMSTER HYBRID CELL LINES.

CHROMOSOME	CHROMOSOME / PA + hGK-1 (NO. OF HYBRIDS)				CONCORDANCE (%)
	+/+	-/-	+/-	-/+	
1	5	2	3	5	47
2	2	5	0	8	47
3	4	3	2	6	47
4	8	2	3	2	67
5	6	2	3	4	53
6	7	2	3	3	60
7	6	2	3	4	53
8	6	4	1	4	67
9	5	2	3	5	47
10	4	3	2	6	47
11	7	0	5	3	47
12	8	1	4	2	60
13	4	3	2	6	47
14	6	2	3	4	53
15	6	3	2	4	60
16	8	3	2	2	73
17	10	0	5	0	67
18	5	3	2	5	53
19	10	5	0	0	100
20	8	2	3	2	67
21	7	1	4	3	53
22	8	2	3	2	67
X	5	2	3	5	47
Y	0	4	1	10	26

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REFERENCES

Ashley,P.L. & MacDonald,R.J. (1985) Biochem. 24, 4512–4520.
Baker,A.R. & Shine,J. (1985) DNA 4, 445–450.
Carretero,O.A. & Scilci,A.G. (1980) Am. J. Physiol. 238, F247–F255.
Chapdelaine,P., Paradis,G., Tremblay,R.R. & Dube,J.Y. (1988) FEBS Lett. 236, 205–208.
Chen,Y.P., Chao,J. & Chao,L. (1988) Biochem. 27, 7189–7196.
Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1988) Trends Biochem. Sci. 13, 169–172.

Evans,B.A., Drinkwater,C.C. & Richards,R.I. (1987) J. Biol. Chem. 262, 8027-8034.
Evans,B.A., Yun,Z.X., Close,J.A., Tregear,G.W., Kitamura,N, Nakanishi,S., Callen,D.F., Baker,E., Hyland,V.J., Sutherland,G.R. & Richards,R.I. (1988) Biochem. 27, 3124–3129.
Feinberg,A.P. & Vogelstein,P. (1983) Anal. Biochem. 132, 6–13.
Fukushima,D., Kitamura,N. & Nakanishi,S. (1985) Biochem. 24, 80378043.
Gallee,M.P.W., Vroonhoven,C.C.J. van, Korput,J.A.G.M. van der, Kwast,T.H. van der, Kate,F.J.W. ten, Romijn,J.C. & Trapman,J. (1986) Prostate 9, 33–45.
Gerald,W.L., Chao,J. & Chao,L. (1986) Biochim. Biophys. Acta 866, 1-14.
Geurts van Kessel,A. ,Tetteroo,P.A.T., Borne,K. von dem, Hagemeijer, A. & Bootsma,D. (1983) Proc. Natl. Acad. Sci. USA 80, 3748–3752.
Killian,C.S., Yang,N., Emrich,L.J., Vargas,F.P., Kuriyama,M., Wang,M.C., Slack,N.H., Papsidero,L.D., Murphy,G.P. & Chu,T.M. (1985) Cancer Res. 45, 886-891.
Killian,C.S., Emrich,L.J., Vargas,F.P., Yang,N., Wang,M.C., Priore,R.L., Murphy,G.P. & Chu,T.M. (1986) J. Natl. Cancer Inst. 76, 179–185.
Kuriyama, M., Wang, M.C., Papsidero,L.D., Kilian,C.S., Shimano,T., Nishiura,T., Valenzuela,L., Murphy,G.P. & Chu,T.M. (1980) Cancer Res. 40, 4658–4662.
Lilja,H. (1985) J. Clin. Invest. 76, 1899–1903.
Lundwall, A. & Lilja, H. (1987) FEBS Lett. 214, 317–322.
Macdonald,R.J., Margolius,H.S. & Erdös,E.G. (1988) Biochem. J. 253, 313–321.
Maniatis,T., Fritsch,E.F. & Sambrook,J. (1982) Molecular cloning: A Laboratory Manual, Cold Spring Harbor, NY.
Mason,A.J., Evans,B.A., Cox,D.R., Shine,J. & Richards,R.I. (1983) Nature 303, 300–307.
McGee,R.S. & Herr,J.C. (1988) Biol. Reprod. 39, 499–510.
Riegman,P.H.J, Klaassen,P., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1988) Biochem. Biophys. Res. Commun. 155, 181-188.
Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1989) Biochem. Biophys. Res. Commun. (in press)
Sanger,F., Nicklen,S. & Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
Schachter,M. (1980) Pharmacol. Rev. 31, 1–17.
Schedlich,L.J., Bennetts,B.H. & Morris,B.J. (1987) DNA 6, 429-437.
Stamey,T.A., Yang,N., Hay,A.R., McNeal,J.E., Freiha,F.S. & Redwine,E. (1987) New Engl. J. Med. 317, 909–916.
Wang,M.C., Valenzuela,L.A., Murphy,G.P. & Chu,T.M. (1979) Invest. Urol. 17, 159–163.
Wang,M.C., Papsidero,L.D., Kuriyama,M., Valenzuela, L.A., Murphy,G.P. & Chu,T.M. (1981) Prostate 2, 89–96.
Watt,K.W.K., Lee,P.J., M'Timkulu,T., Chan,W.P. & Loor,R. (1986) Proc. Natl. Acad. Sci. USA 83, 3166–3170.

CHAPTER V:

CHARACTERIZATION OF THE HUMAN KALLIKREIN LOCUS.

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Genomics (accepted for publication).

SUMMARY

The human kallikrein gene family is composed of three members: Tissue Kallikrein (KLK1), Prostate-specific Antigen (PA or APS) and human Glandular Kallikrein-1 (hGK-1 or KLK2). The three genes have previously been isolated and mapped to chromosome 19q13.2–q13.4 (Schedlich et al., 1987; Evans et al., 1988; Riegman et al., 1989a; Ropers & Pericak-Vance, 1990). Further analysis of an area of 110 kb surrounding the kallikrein genes by CHEF electrophoresis and chromosome walking showed clustering of the three genes. The KLK1 gene is positioned in the opposite orientation of the APS and KLK2 genes in the order KLK1–APS–KLK2. The APS and KLK2 gene are separated by 12 kb (see also Riegman et al., 1989b); the distance between KLK1 and APS amounts 31 kb. A CpG island was detected in the region between KLK1 and APS. Preliminary data indicate that this CpG island is located directly adjacent to a gene, which is unrelated to the kallikreins, and which seems to be ubiquitously expressed.

INTRODUCTION

The kallikrein gene family represents a group of serine proteases, which share a high degree of mutual structural homology. The biochemical role of these kallikreins is the selective cleavage of specific polypeptide precursors (Drinkwater et al., 1988; MacDonald et al., 1988; Clements, 1989).

In mouse and rat, kallikreins are encoded by large multigene families. In the mouse genome at least 24 genes have been identified (Mason et al., 1983; Evans et al., 1987). Expression of 11 of these genes has been confirmed; 10 of the genes are presumed to be pseudogenes (Lundgren et al., 1984; Ullrich et al., 1984; Evans & Richards, 1985; van Leeuwen et al., 1986; Blaber et al., 1987; Drinkwater et al., 1987; Drinkwater et al., 1988). The mouse kallikrein genes are clustered in groups of up to 11 genes on chromosome 7 (Evans et al., 1987). The rat kallikrein gene family has been estimated to contain 20 members (Gerald et al., 1986; Evans, 1987; Pritchet & Roberts, 1987; Chen et al., 1988). So far, the structures of 11 of these genes have been identified (Chen et al., 1988; Wines et al., 1989).

The human kallikrein gene family is most probably composed of only 3 genes, Prostate-specific Antigen (APS or PA), human Glandular Kallikrein (KLK2 or hGK-1) and Tissue Kallikrein (KLK1) (Riegman et al., 1989b). APS and KLK2 are both exclusively expressed in the prostate (Wang et al., 1981; Papsidero et al., 1981; Gallee et al., 1986; Chapdelaine et al., 1988; Morris, 1989). The presumed physiological function of APS is the liquefaction of the seminal coagulum by digestion of seminal vesicle-derived proteins (Lilja, 1985; McGee & Herr, 1988). APS is a well-known prostate tumor marker (see for a recent review Gittes, 1991). KLK1 is expressed in kidney, pancreas and salivary glands (Baker & Shine, 1985; Fukushima et al., 1985; Angerman et al., 1989). It is considered to play an important role in the regulation of blood pressure,

local blood flow, and/or sodium balance, by cleavage of precursor kininogen, thereby releasing small vasoactive peptides or kinins (Schachter, 1979; Margolius, 1984; Sharma, 1988; Clements, 1989; Pravenec et al., 1991). The precise physiological function of KLK2 is unknown.

The APS gene has a length of 5.8 kb and its complete sequence has been published (Riegman et al., 1989a); the KLK2 gene has a size of 5.2 kb and its complete structure has also been elucidated (Schedlich et al., 1987; Schedlich & Morris, 1988). The APS and the KLK2 genes show an overall homology of 82% (Riegman et al., 1989a). The APS gene is located upstream from the KLK2 gene at a distance of 12 kb (Riegman et al., 1989b). The KLK1 gene has a size of approximately 4.5 kb and the exon sequences and exon/intron junctions of this gene have been determined (Evans et al., 1988). The organization of the kallikrein genes (5 exons, 4 introns) is conserved among the different species (reviewed by Clements, 1989). All three human kallikrein genes have been assigned to chromosome 19q13.2-q13.4 (Evans et al., 1988; Sutherland et al., 1988; Riegman et al., 1989b; Schonk et al., 1990; Ropers & Pericak-Vance, 1990).

We are interested in the regulation of transcription of the human kallikrein genes, in particular APS and KLK2. For this purpose a detailed physical map of the kallikrein gene locus was constructed. The data obtained showed that all three human kallikrein genes are clustered in a small area of 60 kb. In addition, a CpG island was found in the kallikrein locus, which most probably represents the promoter region of an unrelated gene.

METHODS

Chromosome walking A human genomic DNA library (partially MboI digested DNA in lambda EMBL 3) was kindly provided by Dr. G. Grosveld (Rotterdam). The library was screened with mixtures of α^{32}P-dATP labelled (Feinberg and Vogelstein, 1983) probes (see Table 1) derived from previously isolated genomic clones. Before use, the probes were checked on human genomic DNA Southern blots for their unique hybridization profile. Duplicate nitrocellulose filters were hybridized overnight at 65°C in 6xSSC containing 10x Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA. Filters were washed twice in 3xSSC for 20 min at 65°C, twice in 1x SSC for 20 min at 65°C, and once in 0.3xSSC for 10 min at 65°C. Subsequently, filters were exposed to a Kodak AR film at -70°C using intensifying screens. Positive phages were isolated and simultaneously selected by several purification cycles. Phages were propagated, DNA was isolated, fragments were subcloned into plasmids, and restriction maps were prepared using standard procedures (Sambrook et al., 1989).

Southern blotting Blots containing DNA digests of 31 unrelated Caucasians were kindly provided by Dr. R.H. Lekanne Deprez (Rotterdam). The labelling of probes, hybridization and washing conditions were identical to those used in screening the genomic library as described above.

Contour-clamped homogeneous electric field (CHEF) electrophoresis Agarose blocks (80 µl, 0.5%) containing immobilized DNA of $0.5x10^6$ HeLa or T24 cells were rinsed with H_2O, washed three times in 10-20 volumes TE (10mM Tris pH 7.5, 1mM EDTA) for 2 h at room temperature and prior to digestion equilibrated in restriction buffer overnight at 4°C under gentle rotation. Digestion was done in a total volume of 200 µl for 6 h with 20-40 U of the appropriate restriction enzyme. The enzyme was added in two equal portions at the beginning and after 3 h digestion. Subsequently, the blocks were directly layered on gel, 1/3 block per lane. Electrophoresis was carried out in 1% agarose and 0.5xTBE for 20 h at 15°C, 150 V, 165 mA and a switch time of 25 sec (Chu et al., 1986). After electrophoresis, gels were stained by gently rocking in 0.5xTBE containing 0.5 µg/ml ethidium bromide for 30 min at room temperature. Gels were destained in distilled water for 30 min at room temperature. Sizes were determined by comparison with marker bands of intact yeast (Saccharomyces cerevisiae) chromosomal DNA and ladders of phage lambda DNA multimers. DNA was transferred to Hybond N^+ filters (Amersham) by electroblotting in 0.5xTBE at 120 V, for 2 h in a cooled Bio-Rad transblot cell. Filters were wetted with 0.4N NaOH for 15 min, rinsed in 5xSSC and hybridized. Hybridization and washing conditions were identical to those described by den Dunnen et al. (1987). Labelling conditions were identical to those used for screening the genomic library as described above.

Northern blotting Isolation of total cellular RNA from the different cell lines was carried out by the guanidinium thiocyanate method (Chirgwin et al., 1979). Northern blot analysis was performed with glyoxal denatured RNA. RNA (20 µg/lane) was separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (GeneScreen, NEN). Probes were labelled as described above. Hybridization was in the presence of 50% formamide at 42°C. After washing twice with 2xSSC at 20°C, twice with 2xSSC, 0.1% SDS for 20 min at 65°C and once with 1x SSC at 20°C, filters were exposed to Kodak AR film at −70°C using intensifying screens.

RESULTS

Previously, three genomic clones containing the three human kallikrein genes have been characterized (Riegman et al., 1989a,b). Clones 4P1 and 9P1 overlap and thereby physically link the APS and KLK2 genes at a distance of 12 kb; clone 7P1 contains the KLK1 gene, but does not overlap with 4P1 or 9P1 (Riegman et al., 1989b). In order to isolate clones with additional, flanking DNA fragments, the genomic library was hybridized with a mixture of terminal fragments isolated from 4P1 (probe BB700), 9P1 (probe 21.1) and 7P1, (probes 6.25 and 11.1 flanking the KLK1 gene) (see Figure 1A,B, Table 1 and Methods). Four of the clones obtained in this screen (8P2, 3F1, 3P2 and 10P2), which contain genomic fragments with maximal extra information flanking the APS/KLK2 gene cluster and the KLKI gene, are depicted in Figure 1A. Together, clones 8P2, 7P1 and 3F1 represent a genomic segment of 30 kb containing the KLK1 gene, whereas clones 3P2, 4P1, 9P1 and 10P2 form a fragment of 63 kb enclosing the APS and KLK2 genes. Restriction mapping and cross-hybridization experiments showed that these two segments did not overlap.

Table 1. Hybridization probes for CHEF-electrophoresis and chromosome walking

Code	Length in kb	Restriction sites	Origin of genomic DNA clone
11.1	1.4	StuI-BamHI	7P1
16.1	0.3	EcoRI-BglI	8P2
6.25	0.5	NcoI-BsshII	7P1
14.1	0.5	SalI[1]-PvuII	3P2
BB700	0.7	BamHI-BamHI	4P1
21.1	1.1	ApaI-BglII	9P1

[1] *Restriction site originates from multiple cloning site.*

To investigate a presumed close linkage of the KLK1 and the APS/KLK2 genes, genomic DNA was subjected to CHEF-electrophoresis. Blots were hybridized with specific probes from the KLK1 and APS/KLK2 regions. The results of these experiments are shown in Figure 2. Probe 11.1 and probe 14.1, isolated from the KLKI and APS/KLK2 region, respectively, hybridized with MluI fragments of different size in HeLa cell DNA [a small fragment of approx. 40 kb and a 400 kb fragment, respectively (Figure 2A,B, lane 1)]. However, both in NruI- and in SalI-digested DNA the two probes hybridized with a fragment of an identical length [400 kb and 150 kb, respectively (see Figure 2A,B, lanes 2 and 3)]. Summarizing, these data suggest that the KLK1 gene is linked to the APS/KLK2 region in a segment of 150 kb (SalI fragment) or less.

Probes 11.1 and BB700 both hybridized with a 50 kb BsshII fragment from T24 DNA, whereas probe 21.1, which is derived from a region downstream of the KLK2 gene, recognized a 250 kb BsshII fragment (see Figure 2C). First of all, these findings mean that at least one of the two BssHII sites present in 9P1, and separating probes BB700 and 21.1, is digested (see Figure 2C). Secondly, it implies that KLK1 must be located upstream of APS, otherwise the hybridization data obtained with the BsshII and the SalI digests cannot be explained (see also Figure 1).

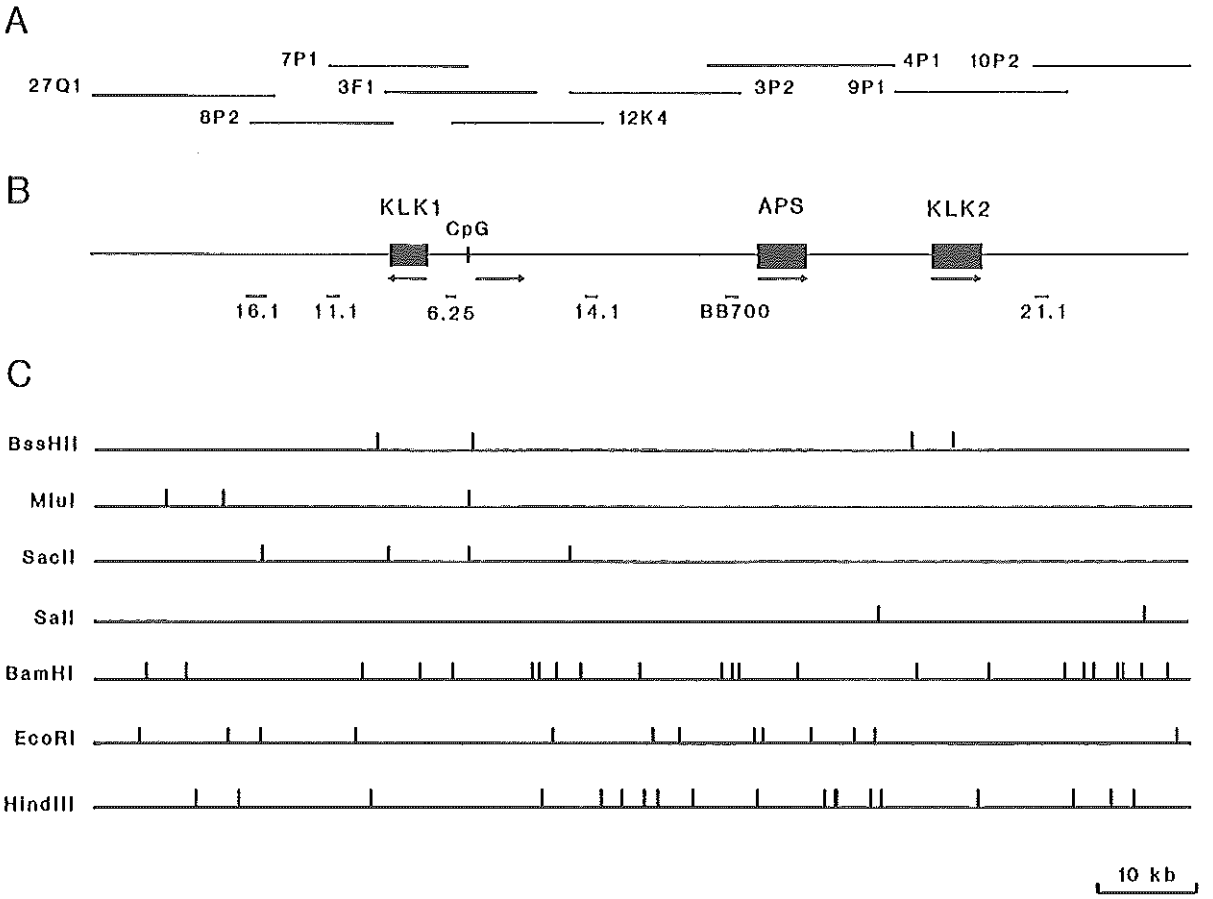

FIGURE 1: *Physical map of the human kallikrein locus.*

A. Position of overlapping genomic clones in the kallikrein locus.

B. Localization of the KLK1, APS(PA) and KLK2(hGK-1) gene in the kallikrein locus (closed boxes). The position of the CpG island is represented by an open box. The arrows show the direction of transcription of the kallikrein genes and the novel gene. Hybridization probes are given by horizontal bars.

C. Restriction map of the kallikrein locus. The restriction sites were determined on cloned genomic DNA fragments. No NruI sites were found in the clones.

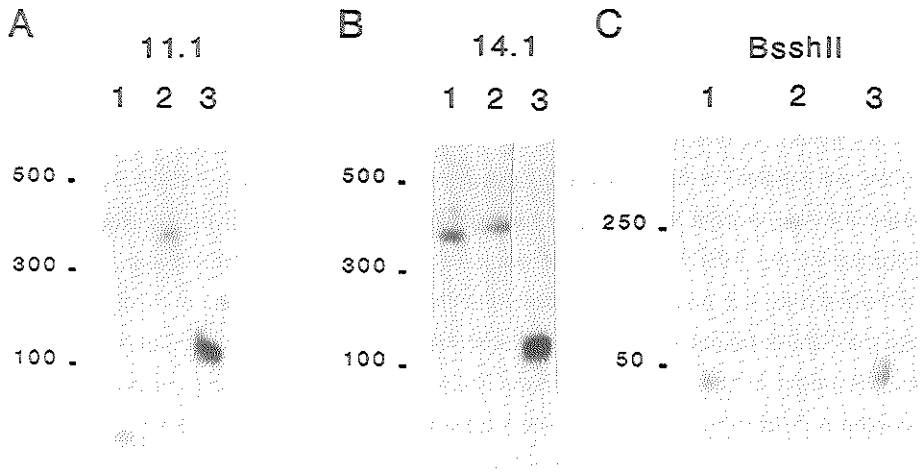

FIGURE 2: Analysis of the kallikrein locus by CHEF-electrophoresis, using APS/KLK2 and KLK1 region specific hybridization probes.
Panels A and B: HeLa cell DNA digested with MluI (lane 1) NruI (lane 2) and SalI (lane 3), hybridized with probe 11.1 (panel A) and 14.1 (panel B). Panel C: T24 DNA digested with BsshII and hybridized with 11.1 (lane 1), BB700 (lane 2) and 21.1 (lane 3). Size markers are given in kb.

The finding that probes 11.1 and BB700 both hybridized with a 50 kb BsshII fragment suggested that APS and KLK1 could be located in the same fragment, implying a distance of about 30 kb between KLK1 and APS and an identical orientation for KLK1, APS and KLK2. Alternatively, the two hybridizing 50 kb fragments, although of the same size, were not identical. To discriminate between these two possibilities, the genomic library was screened with probes 16.1, 6.25 and 14.1 (Table I). This resulted in the isolation of clones 27Q1 and 12K4 (see Figure 1A). Clone 12K4 hybridized with both 14.1 and 6.25 and restriction map analysis of 12K4 confirmed overlap with clones 3F1 and 3P2. This clone was probably underrepresented in the library, because it was not picked up in a previous screen with probe 6.25. Together the isolated clones represent a fragment of 113 kb, containing the KLK1, APS and KLK2 genes. Summarizing, the results obtained show that (1) the KLK1, APS and KLK2 genes are closely linked, (2) KLK1 is located upstream of APS, (3) the orientation of

KLK1 is opposite of that of APS and KLK2, (4) the distance between KLK1 and APS amounts 31 kb.

Previously, several different Restriction Fragment Length Polymorphisms (RFLPs) have been reported for the human kallikrein genes. EcoRI and HindIII RFLPs were detected with a KLK1 cDNA probe with a frequency of 8%, as studied in 12 individuals (Baker & Shine, 1985); a two allele 1.8/2.0 kb MspI RFLP was found, using a KLK2 cDNA probe, as studied in 60 unrelated Caucasians (Hermens et al., 1990). To extend the latter finding, another RFLP survey was performed with a KLK2 cDNA probe (pGK10A; Riegman et al., 1991), using DNA samples of 31 unrelated individuals. This analysis confirmed the 1.8 and 2.0 kb MspI RFLP in a 0.8 to 0.2 ratio. In 1 of the 31 MspI digested DNAs tested an additional 0.6 kb MspI fragment was detected (Figure 3A). So far, the origin of this unique fragment is unclear. In a TaqI digest, in one of the DNA samples a 5 kb fragment was found instead of the common 2.5 kb fragment (Figure 3B). No RFLPs were observed with the pGK10A probe in BglI, BglII, DraI and PstI digests.

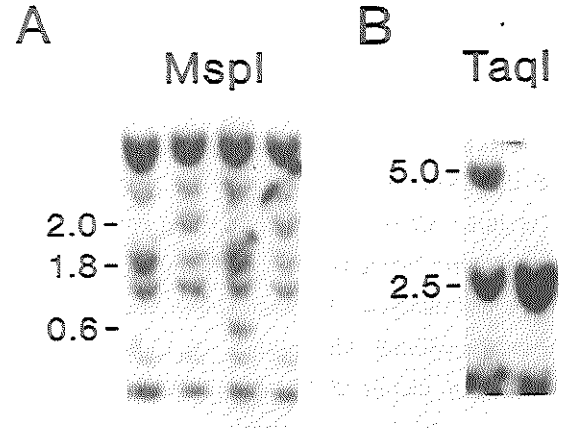

FIGURE 3: *Southern blot analysis of genomic DNA for detection of RFLPs in the kallikrein locus with the KLK2(hGK-1) cDNA probe pGK10A. Panel A: MspI digestion; Panel B: TaqI digestion. Sizes of fragments are presented in kb. The various lanes contain DNA from unrelated individuals.*

A restriction map of the kallikrein locus was constructed for the enzymes BamHI, EcoRI, HindIII and the rare cutting enzymes SalI, SacII, MluI and BssHII (see Figure 1C). About 28 kb upstream of APS and close to the KLK1 promoter region a BsshII, a MluI and a SacII site were found to be clustered in a NcoI-BssHII segment of only 0.4 kb (see Figures 1C and 4A). Digestion of this segment with MspI resulted in a series of small fragments ranging from 70 to 100 bp (data not shown). These data indicate the presence of a CpG island in this segment. Because CpG islands usually indicate the presence of a promoter region, probes A, B and C, which overlap and/or flank the CpG island (see Figure 4A), were used for hybridization of Northern blots with RNA from four different human cell lines (Figure 4B). In all cell lines a transcript of approx. 4.8 kb was detected, using probe C; no clear hybridizing mRNA was found with probes A and B. A high expression level was observed in HepG2 (liver), and LNCaP (prostate) cells; somewhat lower expression in HeLa (cervix) and T47D (breast) cells [in T24 (bladder) cells a very low level of the 4.8 kb mRNA species was found (data not shown)]. The results suggest that the orientation of the novel gene is identical to that of the APS and KLK2 genes. Preliminary sequence analysis of the SmaI-SacI fragment (Figure 4A) indicates that the gene has not been described earlier.

DISCUSSION

Previous studies have shown that the three human kallikrein genes [KLK1, APS and KLK2] are located within a relatively small segment of chromosome 19q13.2–q13.4 (Evans et al., 1988; Sutherland et al., 1988; Riegman et al., 1989b; Schonk et al., 1990; Ropers & Pericak-Vance, 1990). Here we have presented evidence of the clustering of the three genes in an area of 60 kb in the gene order [KLK1- -31kb- -PSA- -12kb- -KLK2]. The orientation of KLK1 is opposite to that of APS/KLK2.

Clustering of kallikrein genes has also been reported in the mouse and rat genomes (Evans et al., 1987; Wines et al.,1989). However, most of the 24 or more mouse kallikrein genes are much more closely linked than the human genes. The region between two mouse kallikrein genes can be as small as 3.3–7.0 kb (Evans et al., 1987; Wines et al., 1989). A strong conservation in gene order between the human chromosome 19q13.1-q13.4 region and 17 loci in a 20 cM proximal part of mouse chromosome 7, including the kallikrein locus, has been documented (Saunders & Seldin, 1990; Nadeau et al., 1991). Because of this conservation, the novel gene located between KLK1 and APS (as described in this study) might also be situated at a similar

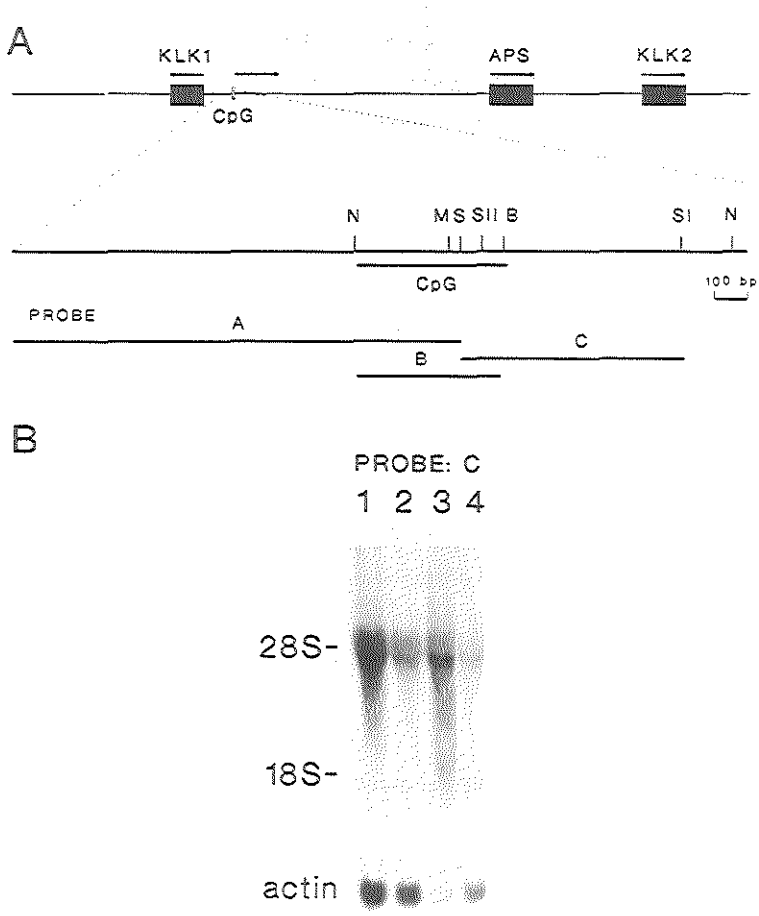

FIGURE 4: *Characterization of the novel gene.*

A. Restriction map of the DNA fragment containing the CpG island and the localization of the single-copy probes A, B and C generated from this area. B=BsshII, M=MluI, S=SmaI, SI=SacI and SII=SacII

B. Northern blots containing RNA isolated from the human cell lines HepG2 (1), HeLa (2), LNCaP (3), T47D (4), hybridized with probe C.

position in the mouse genome, flanked by (clusters of) kallikrein genes.

The human kallikrein locus is located in a myotonic dystrophy (DM) relevant segment, which has been assigned to 19q13.2-q13.3 (le Beau et al., 1989). By linkage analysis with variable simple sequence motifs the following order of genes closely surrounding the DM locus on 19q13.2–qter was determined: cen-CKM-ERCC1-DM-D19S50-

[RRAS,KLK]-D19S22-ter (Smeets et al., 1991). KLK represents the complete kallikrein locus, which is now known to enclose four genes in the order [KLK1–unknown gene-APS-KLK2]. The RRAS and the SNRP70 gene are closely linked to the kallikrein locus (Schonk et al., 1990). However, the novel gene between KLK1 and APS cannot be identical to RRAS or SNRP70, because the transcripts of these genes (1 kb; and a major 1.7 or a minor 3.9 kb, respectively) are different in size from the 4.8 kb transcript from this gene (Theissen et al., 1986; Lowe et al., 1987; Spritz et al., 1987). For further investigation of the novel gene, the characterization of the cDNA is essential. Experiments in this direction are in progress.

The human kallikrein family is extremely small as compared to the large kallikrein families in mice and rats. Among others, the mouse kallikrein family encompasses enzymes involved in the activation of the growth factors EGF and NGF (Lundgren et al., 1984; Ullrich et al., 1984; Evans & Richards, 1985; van Leeuwen et al., 1985; Blaber et al., 1987; Drinkwater et al., 1987; Reviewed by Clements, 1989). In the here characterized human kallikrein locus, genes encoding proteins with such an important function could not be identified. Therefore, most likely in humans the enzymes involved in maturation of growth factor precursors will be encoded by genes which are not or only to a limited extend homologous to the kallikrein genes.

Acknowledgments We are indebted to Dr. G. Grosveld for a genomic library, to Dr. R. Lekanne Deprez for DNA blots for RFLP analysis and Mrs. Paula Delfos and Mr. F.L. van der Panne for photography. This work was supported by a grant from the Netherlands Cancer Society (KWF).

REFERENCES

Angerman,A., Bergmann,C. & Appelhans,H. (1989) Biochem. J. 262, 787-793.
Baker,A.R. & Shine,J. (1985) DNA 4, 445–450.
Beau,M.M.le, Ryan,D. & Pericak-Vance,M.A. (1989) Cytogenet. Cell. Genet. 51, 338–357.
Blaber,M., Isackson,P.J. & Bradshaw,R.A. (1987) Biochemistry 26, 6742-6749.
Chapdelaine,P., Paradis,G., Tremblay,R.R. & Dube,J.Y. (1988) FEBS Lett. 236, 205–208.
Chen,Y.P., Chao,J. & Chao,L. (1988) Biochemistry 27, 7189-7196.
Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. & Rutter,W.J. (1979) Biochemistry 18, 5294–5299.
Chu,G., Volrath,D. & Davis,R.W. (1986) Science 234, 1582-1585.
Clements,J.A. (1989) Endocrine Rev. 10, 393–419.

Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1987) Biochemistry 26, 6750-6756.
Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1988a) J. Biol. Chem. 263, 8565-8568.
Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1988b) Trends Biochem. Sci. 13, 169-172.
Dunnen,J.T.den, Bakker,E., Klein-Breteler,E.G., Pearson,P.L. & Ommen,G.J.B.van (1987) Nature 329, 640-642.
Evans,B.A. & Richards,R.I. (1985) EMBO J. 4, 133-138.
Evans,B.A., Drinkwater,C.C. & Richards,R.I. (1987) J. Biol. Chem. 262, 8027-8034.
Evans,B.A., Yun,Z.X., Close,J.A., Tregear,G.W., Kitamura,N, Nakanishi,S., Callen,D.F., Baker,E., Hyland,V.J., Sutherland,G.R. & Richards,R.I. (1988) Biochemistry 27, 3124-3129.
Feinberg,A.P. & Vogelstein,B. (1983) Anal. Biochemistry 132, 6-13.
Fukushima,D., Kitamura,N. & Nakanishi,S. (1985) Biochemistry 24, 8037-8043.
Gallee,M.P.W., Vroonhoven,C.C.J.van, Korput,J.A.G.M.van der, Kwast,T.H.van der, Kate,F.J.W.ten, Romijn,J.C. & Trapman,J. (1986) Prostate 9, 33-45.
Gerald,W.L., Chao,J. & Chao,L. (1986) Biochim. Biophys. Acta 866, 1-14.
Gittes,R.F. (1991) New Eng. J. Med. 324, 236-245.
Hermens,R., Coerwinkel,M., Trapman,J., Riegman,P.H.J., Vlietstra,R.J., Smeets,H. & Wieringa,B (1990) Nucl. Acids Res. 18, 208.
Leeuwen,B.H.van, Evans,B.A., Tregear,G.W. & Richards,R.I. (1986) J. Biol. Chem. 261, 5529-5535.
Lilja,H. (1985) J. Clin. Invest., 76, 1899-1903.
Lowe,D.G., Capon,D.J., Delwart,E., Sakaguchi,A.Y. & Naylor,S.L. (1987) Cell 48, 137-146.
Lundgren,S., Ronne,H., Rask,L. & Peterson,P.A. (1984) J. Biol. Chem. 259, 7780-7784.
MacDdonald,R.J., Margolius,H.S. & Erdös,E.G. (1988) Biochem. J. 253, 313-321.
Margolius,H.S. (1984) Annu. Rev. Physiol. 46, 309-326.
Mason,A.J., Evans,B.A., Cox,D.R., Shine,J. & Richards,R.I. (1983) Nature 303, 300-307.
McGee,R.S. & Herr,J.C. (1988) Biol. Reprod. 39, 499-510.
Morris,B.J. (1989) Clin. Exp. Pharm. Phys. 16, 345-351.
Nadeau,J., Grant,P. & Kosowsky,M. (1991) Mouse Genome 89, 31-36.
Papsidero,L.D., Wang,M.C., Horoszewicz,J.S., Leong,S.S, Kuriyama,M., Valenzuela,L.M., Murphy,G.P. & Chu,T.M. (1981) In "The prostatic cell, structure and function" Part A, Alan R. Liss, Inc., New York, 435-443.
Pravenec,M., Kren,V., Kunes,J., Scicli,G., Carretero,O.A., Simonet,L. & Kurtz,T.W. (1991) Hypertension 17, 242-246.
Pritchet,D.B. & Roberts,J.L., (1987) Proc. Natl. Acad. Sci. USA 84, 5545-5549.
Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M.van der, Romijn,J.C. & Trapman,J. (1989a) Biochem. Biophys. Res. Commun. 159, 95-102.
Riegman,P.H.J., Vlietstra,R.J., Klaassen,P., Korput,J.A.G.M.van der, Romijn,J.C. & Trapman,J. (1989b) FEBS Lett. 247, 123-126.
Riegman,P.H.J., Vlietstra,R.J., Korput,H.A.G.M.van der, Romijn,J.C. & Trapman,J. (1991) Mol. Cell. Endocrinol. 76, 181-190.
Ropers,H.H. & Pericak-Vance,M.A. (1990) Cytogenet. Cell. Genet. 55, 218-228.
Sambrook,J., Fritsch,E.F. & Maniatis,T. (1989) Cold Spring Harbor Laboratory press NY.
Saunders,A.M. & Seldin,M.F. (1990) Genomics 8, 525-535.
Schachter,M. (1979) Pharmacol. Rev. 31, 1-17.
Schedlich,L.J., Bennetts,B.H. & Morris,B.J. (1987) DNA 6, 429-437.

Schedlich,L.J. & Morris,B.J. (1988) Clin. Exp. Pharm. & Phys. 15, 339-344.
Schonk,D., Coerwinkel-Driessen,M., Dalen,I.van, Oerlemans,F., Smeets,B., Schepens,F., Hulsebos,T., Cockburn,D., Boyd,Y., Davis,M., Rettig,W., Shaw,D., Roses,A., Ropers,H.H. & Wieringa,B. (1989) Genomics 4, 384–396.
Schonk,D., Dijk,P.van, Riegman,P., Trapman,J., Holm,C., Willcocks,T.C., Sillekens,P., Venrooij,W.van, Wimmer,E.M., Geurts van Kessel,A., Ropers,H.H. & Wieringa,B. (1990) Cytogenet. Cell. Genet. 54, 15–19.
Sharma,J.N. (1988) Gen. Pharmacol. 19, 177–187.
Smeets,H.J.M, Hermens,R., Brunner,H.G., Ropers,H–H. & Wieringa,B. (1991) Genomics 9, 257–263.
Spritz,R.A., Strunk,K., Surowy,C.S., Hoch,S.O., Barton,D.E. & Francke,U. (1987) Nucl. Acids Res. 15, 10373–10391.
Sutherland,G.R., Baker,E., Hyland,V.J., Callen,D.F., Close,J.A., Tregear,G.W., Evans,B.A. & Richards,R.I. (1988) Cytogenet. Cell. Genet. 48, 205–207.
Theissen,H., Etzerodt,M., Reuter,R., Schneider,C., Lottspeich,F., Argos,P., Luhrmann,R. & Phillipson,L. (1986) EMBO J. 5, 3209–3217.
Ullrich,A., Gray,A., Wood,W.I., Hayflick,J. & Seeburg,P.H. (1984) DNA 3, 387-392.
Wang,M.C., Valenzuela,L.A., Murphy,G.P. & Chu,T.M. (1979) Invest. Urol. 17, 159–163.
Wines,D.R., Brady,J.M., Pritchett,D.B., Roberts,J.L. & MacDonald,R.J. (1989) J. Biol. Chem. 264, 7653–7662.

CHAPTER VI

IDENTIFICATION AND ANDROGEN-REGULATED EXPRESSION OF TWO MAJOR HUMAN GLANDULAR KALLIKREIN-1 (hGK-1) mRNA SPECIES.

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SUMMARY

The screening of an oligo(dT)-primed prostate cDNA library with a human Glandular Kallikrein-I (hGK-1) genomic DNA fragment resulted in the isolation of two different hGK-1 cDNAs. An 1.2 kb cDNA (pGK-1) contains an open reading frame of 510 bp, encoding the major part of the previously predicted hGK-1 protein [Schedlich et al., DNA 6, 429-437 (1987)]. This cDNA contains a 3'-untranslated region of 677 nucleotides and terminates in a poly(A) stretch, preceded by the canonical AATAAA polyadenylation signal. A second cDNA (pGK-10A), with a size of 1.5 kb, contains an open reading frame of 669 nucleotides preceded by 16 nucleotides of the 5'-untranslated region. pGK-10A differs from pGK-1 by the presence of an additional 37 bp fragment, interrupting the protein coding region of hGK-1, which results from the use of an alternative splice donor site of intron IV of the hGK-1 gene. The mature protein (excluding presumed pre- and propeptides) as deduced from the pGK-10A cDNA sequence, has a size of 199 amino acids and differs at the COOH-terminus from the 237 amino acid hGK-1 protein. The alternatively spliced mRNA comprises approximately 20 % of the hGK-1 transcripts, as deduced from analysis of mRNA from prostate cells by PCR amplification of specific fragments.

The regulation of hGK-1 mRNA expression was studied in different human prostate tumors and cell lines by Northern blotting, using a hGK-1 specific oligonucleotide probe. A high level of hGK-1 expression was found in the androgen-dependent tumors PC 82 and PC EW. hGK-1 mRNA was also present in the androgen-sensitive LNCaP cell line, but undetectable in the androgen-insensitive prostate tumors PC 133, PC 135 and the PC 3 cell line. In LNCaP cells, the expression of hGK-1 mRNA was strongly induced by androgens. Regulation of expression of the closely related Prostate-specific Antigen (PA) gene showed a similar pattern.

INTRODUCTION

Human Glandular Kallikrein-I (hGK-1) belongs to the family of kallikrein-like serine proteases (MacDonald et al., 1988; Clements, 1989). The biochemical role of the kallikrein-like proteases is the selective cleavage of specific polypeptide precursors (MacDonald et al., 1988; Drinkwater et al., 1988; Clements, 1989).

In the mouse and in the rat, kallikrein-like proteins are encoded by a large multigene family of 15-25 members (Mason et al., 1983; Ashley et al., 1985; Gerald et al., 1986; Evans et al., 1987; Wines et al., 1989). The human kallikrein gene family is much smaller and encompasses only three genes, which encode human Glandular Kallikrein-I (hGK-1) (Schedlich et al., 1987) Prostate-specific Antigen (PA) (Riegman et al., 1989a), and tissue (pancreatic/renal/salivary gland) kallikrein (Evans et al., 1988). The three genes are clustered on chromosome 19 with the PA and hGK-1 genes aligned in a head to tail orientation at a distance of only 12 kb (Riegman et al., 1989b). There is a striking similarity in the organization of the kallikrein genes, which is also highly conserved among different species (Clements et al., 1989). All genes are composed of

5 exons and 4 introns and show a high degree of mutual homology, which amounts 82% for PA and hGK-1, (Schedlich et al., 1987; Riegman et al., 1989a). Expression of hGK-1 is, similar to PA, so far only detected in prostate tissue (Chapdelaine et al., 1988a; Morris, 1989). Expression of tissue kallikrein is found in kidney (Baker and Shine, 1985), pancreas (Fukushima et al., 1985), and salivary glands (Angerman et al., 1989), but not in the prostate gland (Morris, 1989).

Tissue kallikrein cleaves the precursor kininogen to release small vasoactive peptides or kinins (Schachter, 1980). The presumed function of PA, a well known prostate tumor marker (see for recent reviews Chu and Murphy, 1986 and Stamey et al., 1989), is the liquefaction of the seminal coagulum by digestion of seminal vesicle derived proteins, which cause the gel-like structure of the semen (Lilja, 1985; McGee and Herr, 1988). The precise physiological function of hGK-1 is unknown.

We previously reported the structure and expression of the PA gene (Riegman et al., 1988, 1989a, b). In this study the structure of two major hGK-1 mRNA species is presented, and the regulation of hGK-1 expression is described.

MATERIALS AND METHODS

Isolation of a hGK-1 genomic DNA clone: A human genomic DNA library (partially MboI digested DNA in lambda EMBL 3) was kindly provided by Dr. G. Grosveld (Rotterdam). The library was screened with an α^{32}P-dATP labelled (Feinberg and Vogelstein, 1983), 380 bp EcoRI - SacI fragment derived from the PA cDNA clone PA 525 (Riegman et al., 1988). Duplicate nitrocellulose filters were hybridized overnight at 65°C in 6x SSC containing 10x Denhardt's solution, 0.1% SDS, and 100 μg/ml salmon sperm DNA. Filters were washed twice in 3x SSC for 20 min at 65°C, twice in 1x SSC for 20 min at 65°C, and once at 0.3x SSC for 10 min at 65°C. Subsequently, filters were exposed to an X-ray film for 18 h at -70°C using intensifying screens. Positive phages were purified by two isolation cycles. Phages were propagated, DNA was isolated and fragments were subcloned into pUC9, and a restriction map was prepared using standard procedures (Maniatis et al., 1982). To identify the clones, fragments were sequenced by the dideoxy chain termination method (Sanger et al., 1977), using sequenase (USB, Cleveland).

Isolation of hGK-1 cDNA clones: A lambda gt10 prostate cDNA library (Clontech, Palo Alto) was screened with an α^{32}P-dATP labelled (Feinberg and Vogelstein, 1983), 1300 bp PstI - PstI fragment isolated from the hGK-1 genomic clone 9P1. This fragment contains exon III and IV, intron III, and parts of intron II and intron IV sequences (see figure 1B). Filters were treated, DNA was isolated, subcloned and the clones of interest sequenced as described above.

Oligonucleotides: Oligonucleotides were synthesized in an Applied Biosystems DNA synthesizer.

Primer A: hGK-1 forward PCR primer, also used for RNA hybridization [position 864-904, hGK-1 cDNA (figure 2)]:

5'-CATCCAGAAAGGCCAAGTGATGCCAGAACGTGAGGTGGACT-3'

Primer B: PA probe used for RNA hybridization [position 1262-1302, PA 75 cDNA, (Riegman et al., 1988)]:
5'-GGTCCAGTCCCTCTCCTTACATCATCCCCATCCCATGCCAA-3'
Primer C: hGK-1 PCR reverse primer [position 589-610, hGK-1 cDNA (figure 2)]:
5'-ATGTGTGCTAGAGCTTACTCTG-3'

RNA preparation: Isolation of total cellular RNA from the different cell lines was carried out by the guanidinium thiocyanate method (Chirgwin et al., 1979).

Amplification of cDNA: 100 ng PC 82 RNA was incubated in 15 μl of a 33 mM Tris.HCl pH 8.3 buffer, containing 250 mM KCl, 0.3 mM EDTA and 100 ng of primer A. The mixture was incubated for 3 min at 80°C and for 3 h at 60°C. Then 25 μl RT-mixture (24 mM Tris-HCl pH 8.3, 16 mM $MgCl_2$, 8 mM DTT, 4 mM dNTP's, 0.1 mg/ml actinomycin D) and 7 units AMV Reverse Transcriptase (Promega, Madison) were added. Incubation was continued for 2 h at 42°C. The reaction was terminated by the addition of 500 μl TE buffer (10 mM Tris.HCl, pH7.6 and 1 mM EDTA).

Amplification by the Polymerase Chain Reaction (PCR) was performed in the following mixture: 50 μl 2x Taq-buffer (50 mM KCl, 10 mM Tris.HCl pH 8.3 and 1.5 mM $MgCl_2$), 10 μl dNTP mix (2 mM each), 17 μl BSA (1 mg/ml), 6 μl primer A (0,1 μg/μl), 6 μl primer C (0,1 μg/μl), 10 μl first strand cDNA, 1 unit Taq polymerase (Amersham, UK). This mixture was overlayered with 70 μl paraffin oil. Twenty four amplification cycles (denaturation: 1 min at 92°C; annealing: 2 min at 55°C; extension: 5 min at 70°C) were carried out. The final extension reaction was for 10 min at 70°C. The PCR products were analyzed on a 2% agarose gel, blotted on a nitrocellulose filter and hybridized with the α^{32}P-dATP labelled (Feinberg and Vogelstein, 1983), 700 bp PvuII - HindIII fragment of pGK-10A (see figure 1). The PCR products were blunt ended with T4 polymerase and Klenow DNA polymerase and subsequently subcloned in M13 for sequencing using standard procedures (Maniatis et al., 1982).

Northern blot analysis: Northern blot analysis was performed with glyoxal denatured RNA. RNA (20 μg/lane) was separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (Genescreen, NEN, Boston). Oligonucleotides A and B were end labeled to a specific activity of $5x10^8$ dpm/μg with γ^{32}P-ATP and T4 polynucleotide kinase (Maniatis et al., 1982). Hybridization was at stringent conditions in the presence of 50% formamide for 16 h at 40°C. After washing twice with 2x SSC at 20°C, twice with 2x SSC, 0.1% SDS for 20 min at 55°C and once with 1x SSC at 20°C, filters were dried and exposed to X-ray film at -70°C using intensifying screens.

Prostate tumor lines and prostate tumor cell lines: PC 82, PC EW, PC 133 and PC 135 prostate tumors were propagated as transplants on male nude mice (Hoehn et al., 1980; van Steenbrugge et al., 1984; Hoehn et al., 1984), frozen in liquid nitrogen directly after removal and kept at -70°C until further use. The LNCaP clone 9 (Horoszewicz et al., 1980; van Steenbrugge et al., 1989), and PC 3 (Kaighn, 1979) in vitro growing cell lines were cultured in RPMI 1640 supplemented with 5% FCS and antibiotics, and harvested just prior to RNA isolation.

For examination of androgen-regulated gene expression, LNCaP cells were grown for a period of six days in medium containing steroid-depleted (dextran-charcoal treated) serum. Next, the synthetic androgen R1881 (NEN, Boston) was added to a final concentration of 0.1 nM. Incubation was continued for six days, cells were harvested and directly used for RNA isolation. A control culture was continuously grown in steroid-depleted medium during the same time period.

RESULTS:

A PA cDNA fragment was used for the isolation of the closely related hGK-1 gene from a human genomic DNA library (see method section). One of the clones identified, designated 9P1, was found to contain the complete hGK-1 gene. A partial restriction map of the 18 kb insert, in which the position and the proposed organization of the hGK-1 gene (5 exons, 4 introns; Schedlich et al., 1987) are indicated, is shown in figure 1A and 1B.

Previously, the sequence of the hGK-1 gene has been described (Schedlich et al., 1987). However, no data are available as yet on the structure and regulation of expression of hGK mRNA. Because it was recently reported, that hGK-1 is exclusively expressed in the prostate gland (Morris, 1989), a prostate library was screened with a hGK-1 genomic DNA fragment for the isolation of hGK-1 cDNA (see method section). The longest hGK-1 cDNA clone identified had a size of 1.5 kb. This clone, designated pGK-10A, was completely sequenced and its structure compared to the sequence of the hGK-1 gene (see figure 1D, figure 2 and Schedlich et al., 1987). pGK-10A terminates with a poly(A) tail, preceded by the canonical AATAAA polyadenylation signal. This sequence is at the 3'-end 145 nucleotides longer than the published genomic hGK-1 sequence. pGK-10A lacks the first 26 nucleotides of the 42 nucleotides long 5'-untranslated region (Chapdelaine et al., 1988a). A shorter hGK-1 cDNA clone of 1.2 kb, designated pGK-1, also contains a poly(A) tail and the complete 3'-untranslated region, but lacks the 5'-untranslated region and the first 273 nucleotides of the open reading frame. In addition, sequence alignment of pGK-1 and pGK-10A showed the presence of an additional, internal fragment of 37 nucleotides in pGK-10A (GTGAGTCATCCCTACTCCCAACATCTGGAGGGGAAAG). This sequence is located at the position where during processing of the hGK-1 transcript intron IV is spliced out (see figure 1B,C,D and figure 2). Comparison with the genomic sequence, (Schedlich et al., 1987), revealed that for processing of pGK-1 the predicted splice donor site of intron IV (GGG/GTGAGT) is used, whereas in the case of pGK-10A an alternative splice donor site (AAG/GTGAGT), which is located 37 nucleotides downstream, is used instead. This not only gives rise to a slightly longer cDNA, but also to a difference in the open reading frame. A protein product derived from pGK-10A will, after removal of pre- and propeptide, have a size of 199 amino acids (instead of 237) of which the COOH-terminal 13 amino acids differ from the postulated mature hGK-1 protein structure (figure 1D and Schedlich et al., 1987).

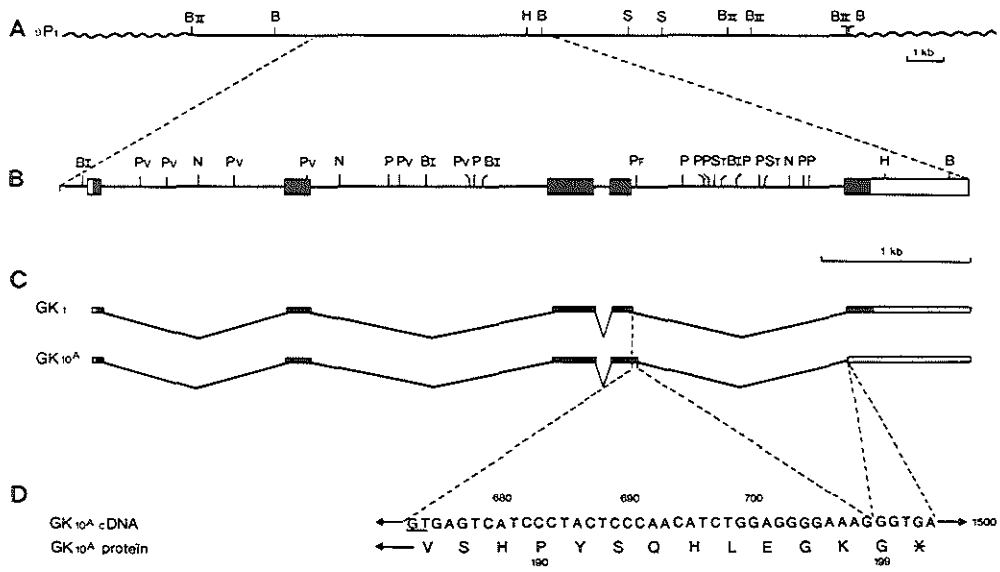

FIGURE 1: *Organization of the hGK-1 gene.*

A. Partial restriction map of the genomic DNA clone 9P1.

B. Fine map of the DNA fragment, on which the hGK-1 gene is situated. The boxes represent the predicted exons, as derived from Schedlich et al. (1987) and from this study. B=BamHI, BI=BglI, BII=BglII, C=ClaI, H=HindIII, N=NcoI, P=PstI, Pf=PflMI, Pv=PvuII, S=SacI, Sl=SalI, Sp=SphI and St=StuI.

C. Splice pattern of hGK-1 and hGK-10A mRNA. Closed boxes correspond to the open reading frame. Open boxes represent the 5'- and 3'- untranslated regions of the two mRNAs.

D. Nucleotide sequence of the fragment present in hGK-10A, and the corresponding amino acid sequence. Numbers are based upon those used in the hGK-1 sequence (figure 2). The hGK-1 splice donor site (GT) is underlined.

To investigate the frequency of alternative splicing at the AAG/GTGAGT sequence, a hGK-1 cDNA fragment was amplified by PCR, using RNA from the PC 82 prostate tumor and primers derived from hGK-1 exon IV and exon V sequences (see methods section and figures 2 and 3). Although in general it is difficult to use PCR for quantification of mRNA, in this specific situation: comparison of two mRNAs of almost identical size, using one set of primers, such a quantification is allowed. The hGK-1-

A

```
                    30                  ...                 60                  90                 120
                   GGAC ACCTGTGTCAGCATGTGGGACCTGGTTCTC TCCATCGCCTTGTCTGTGGGGTGCACTGGT GCCGTGCCCCTCATCCAGTCTCGGATTGTG

                   150                 180                 210                 240
GGAGGCTGGGAGTGTGAGAAGCATTCCCAA CCCTGGCAGGTGGCTGTGTACAGTCATGGA TGGGCACACTGTGGGGGTGTCCTGGTGCAC CCCCAGTGGGTGCTCACAGCTGCCCATTGC

                   270                 300                 330                 360
CTAAAGAAGAATAGCCAGGTCTGGCTGGGT CGGCACAACCTGTTTGAGCCTGAAGACACA GGCCAGAGGGTCCCTGTCAGCCACAGCTTC CCACACCCGCTCTACAATATGAGCCTTCTG

                   390                 420                 450                 480
AAGCATCAAAGCCTTAGACCAGATGAAGAC TCCAGCCATGACCTCATGCTGCTCCGCCTG TCAGAGCCTGCCAAGATCACAGATGTTGTG AAGGTCCTGGGCCTGCCCACCCAGGAGCCA

                   510                 540                 570                 600
GCACTGGGGACCACCTGCTACGCCTCAGGC TGGGGCAGCATCGAACCAGAGGAGTTCTTG CGCCCCAGGAGTCTTCAGTGTGTGAGCCTC CATCTCCTGTCCAATGACATGTGTGCTAGA

                   630                 660                 ...     690                 720
GCTTACTCTGAGAAGGTGACAGAGTTCATG TTGTGTGCTGGGCTCTGGACAGGTGGTAAA GACACTTGTGGGGGTGATTCTGGGGGTCCA CTTGTCTGTAATGGTGTGCTTCAAGGTATC
                                                                  \                 /
                                                                   TGTGAGTCATCCCTACTCCCAACATCTGGAGGGGAAAG
                   750                 780                 810                 ...     840
ACATCATGGGGCCCTGAGCCATGTGCCCTG CCTGAAAAGCCTGCTGTGTACACCAAGGTG GTGCATTACCGGAAGTGGATCAAGGACACC ATCGCAGCCAACCCCTGAGTGCCCCTGTCC

                   870                 900                 930                 960
CACCCCTACCTCTAGTAAATTTAAGTCCAC CTCACGTTCTGGCATCACTTGGCCTTTCTG GATGCTGGACACCTGAAGCTTGGAACTCAC CTGGCCGAAGCTCGAGCCTCCTGAGTCCTA

                   990                 1020                1050                1080
CTGACCTGTGCTTTCTGGTGTGGAGTCCAG GGCTGCTAGGAAAAGGAATGGGCAGACACA GGTGTATGCCAATGTTTCTGAAATGGGTAT AATTTCCTCCTCTCCTTCGGAACACTGGCT

                   1110                1140                1170                1200
GTCTCTGAAGACTTCTCGCTCAGTTTCAGT GAGGACACACACAAAGACGTGGGTGACCAT GTTGTTTGTGGGGTGCAGAGATGGGAGGGG TGGGGCCCACCCTGGAAGAGTGGACAGTGA

                   1230                1260                1290                1320
CACAAGGTGGACACTCTCTACAGATCACTG AGGATAAGCTGGAGCCACAATGCATGAGGC ACACACACAGAAGGATGACGCTGTAAACAT AGCCCACGCTGTCCTGGGGGCACTGGGAAG

                   1350                1380                1410                1440
CCTAGATAAGGCCGTGAGCAGAAAGAAGGG GAGGATCCTCCTATGTTGTTGAAGGAGGGA CTAGGGGGAGAAACTGAAAGCTGATTAATT ACAGGAGGTTTGTTCAGGTCCCCCAAACCA

                   1470                1500                1530
CCGTCAGATTTGATGATTTCCTAGCTGGAC TTACAGAAATAAAGAGCTATCATGCTGTGG TTAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

B

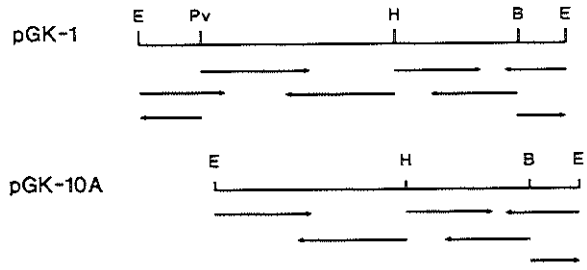

FIGURE 2: Nucleotide sequence of pGK-1 and pGK-10A.

A. The nucleotide sequence of the hGK-1 cDNA clones pGK-1 and pGK-10A. +1 indicates the presumed transcription initiation site (Schedlich et al., 1987). The positions of the oligonucleotides A and C used for PCR and Northern blotting (see Materials and Methods), and the polyadenylation signal are underlined. The translation start site and the hGK-1 and hGK-10A stop codons are indicated by dots. The position and the sequence of the hGK-10A specific fragment is indicated (see also figure 1D).

B. Strategy used for sequencing of hGK-1 and hGK-10A. E=EcoRI, Pv=PvuII, H=HindIII, B=BamHI.

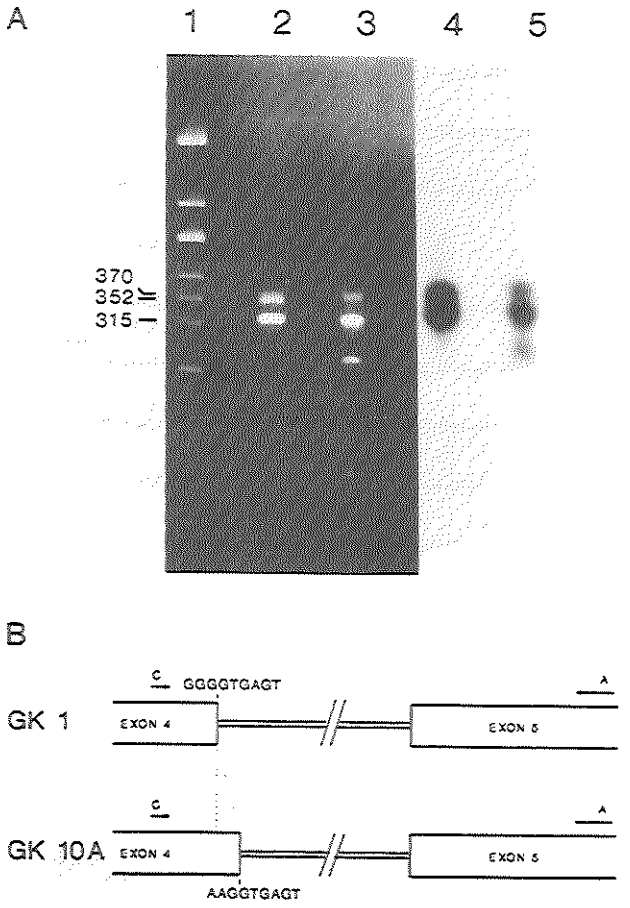

FIGURE 3: Size and structure of hGK mRNA-specific PCR products using exon 4 and exon 5 primers.
A. Agarose gel electrophoresis of the hGK-1 PCR products. Lane 1: Marker pBR322 x HinfI/EcoRI. Lane 2: hGK-1 PCR products. Lane 3: hGK-1 PCR products x PflMI. Lanes 4 and 5 are a Southern blot analysis of lanes 2 and 3, hybridized with a hGK-1 specific probe.
B. Schematical representation of the formation of the two hGK-1 mRNA PCR products as caused by alternative splicing of intron IV. A and C indicate the positions of the oligonucleotides which were used for amplification.

specific primers A and C were chosen at regions with minimal homology between hGK-1 and PA cDNA. Three fragments of different sizes were found after amplification, which all proved to be hGK-1 PCR products because of specific hybridization with a

hGK-1 probe (see figure 3 lanes 2 and 4). Controls without reverse transcriptase did not give rise to a PCR signal (data not shown). The expected sizes of amplified fragments derived from pGK-10A-and pGK-1-like transcripts are 352 and 315 bp, respectively. Two of the three amplified fragments correspond to these sizes, and their identity was confirmed by sequencing. The third hGK-1 PCR product, which migrated as a fragment of approximately 370 bp, turned out to be an artefact. It could not be digested with PflMI, that cleaves the 352 bp fragment, but not the 315 bp fragment (figure 3 lanes 3 and 5). We were also unable to subclone the 370 bp fragment. Subsequently, the 370 bp band was found to be an anneal product of complementary hGK-10A and hGK-1 strands, because under denaturing electrophoresis conditions only the 315 and 352 nucleotide strands were detected (data not shown).

As deduced from three independent PCR amplification reactions the ratio of 315 and 352 bp fragments was calculated to be 4 to 1, indicating that alternative splicing takes place in approximately 20 per cent of the hGK-1 transcripts. Primers A and C were used for screening by PCR of eleven additionally isolated hGK-1 cDNA clones for the presence of the 352 bp fragment. Two of the cDNA clones contained this fragment and in 9 clones the 315 bp fragment was detected. This also indicates that the majority of transcripts uses the GGG/GTGAGT splice donor site of intron IV. On the other hand it shows that a substantial percentage of the mature hGK-1 mRNA results from alternative splicing.

Comparison of the hGK-1 cDNA sequences with the hGK-1 genomic sequence (Schedlich et al., 1987), gave rise to three discrepancies. Two are located in the 3'-untranslated region: an extra C is present in the gene at position 1270 of the cDNA, and a C is lacking in the gene at position 1182 of the cDNA. In addition, in the open reading frame at position 705 a T is found in the cDNA sequence, where a G is present in the genomic sequence. However, this has no effect on the amino acid sequence.

To study hGK-1 expression, RNA of different prostate tumors and cell lines was isolated and analyzed by Northern blotting using the hGK-1 specific oligonucleotide A as a hybridization probe. This sequence is completely absent in the closely related PA mRNA (compare Riegman et al., 1989a). The results are shown in figure 4A. Expression of hGK-1 was high in the androgen-dependent prostate tumors PC EW and PC 82 and also clearly detectable, although at a lower level, in the androgen-sensitive prostate tumor cell line LNCaP. At least five hGK-1 transcripts were identified. The most prominent mRNA has a size of 1.5 kb, which must be composed of the two

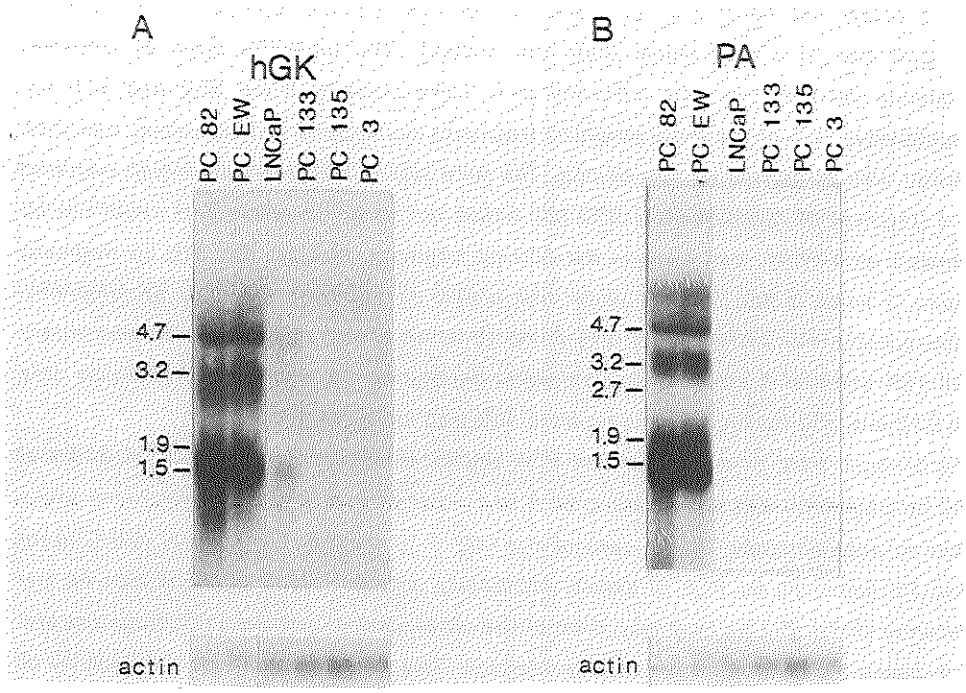

FIGURE 4: hGK-1 and PA mRNA expression in prostate tumors/cell lines.
Northern blots of RNA isolated from different prostate tumors/cell lines: PC EW, PC 82, LNCaP, PC 3, PC 133, PC 135. The blots were hybridized with (A) the hGK-1 or (B) the PA specific oligonucleotide. Hybridization with an actin probe was used as a control.

mRNA species described above. The other transcripts have sizes of 1.9, 2.7, 3.2 and 4.7 kb, respectively. The latters can be the results of alternative splicing. A second possibility is that these transcripts are precursors of the mature mRNAs. hGK-1 transcripts could not be detected in the androgen-independent prostate tumors PC 133 and PC 135 and the androgen-independent prostate cell line PC 3.

To compare the expression of hGK-1 with that of PA, a PA-specific oligonucleotide (primer B), was synthesized at a region with minimal homology between hGK-1 and PA cDNA (see method section). The specificity of this primer was checked by dot blot hybridization with the appropriate PA and hGK-1 cDNA fragments (data not shown). The results of the Northern blot hybridization are depicted in figure 4B. Similar to hGK-1, expression of PA was clearly detectable in the androgen-responsive prostate

tumors. Overexposure of the film reveals low PA mRNA levels in LNCaP cells (data not shown), but absence of PA transcripts in the androgen-independent prostate cells (figure 5B). The most prominent PA mRNA is 1.5 kb, the other PA transcripts have sizes of 1.9, 3.2, 4.7 kb and 5.6 kb (see also Riegman et al., 1988).

Comparison of the intensity of the PA band with that of hGK-1 in the various cell lines indicates some variation in expression levels. In the LNCaP cell line, expression of hGK-1 seems higher than that of PA, whereas in PC 82 and PC EW tumors PA expression is slightly higher. Whether this represents a difference in regulation of expression in the three tumor cell lines remains to be substantiated.

One of the main differences between the androgen-responsive and androgen-unresponsive prostate tumors/cell lines is the presence of the androgen receptor in the former (Trapman et al., in press). To test the effect of androgens on hGK-1 and PA mRNA expression, LNCaP cells were grown for six days in the presence and in the absence of the synthetic androgen R1881 (0.1 nM). Subsequently, RNA was isolated and used for Northern blotting. The hybridization probes, which were used for hGK-1 and PA mRNA detection were identical to the ones described above. The results of the experiments are illustrated in figure 5. It can clearly be seen that hGK-1 mRNA expression is strongly induced by addition of R1881 to the culture medium (figure 5A).

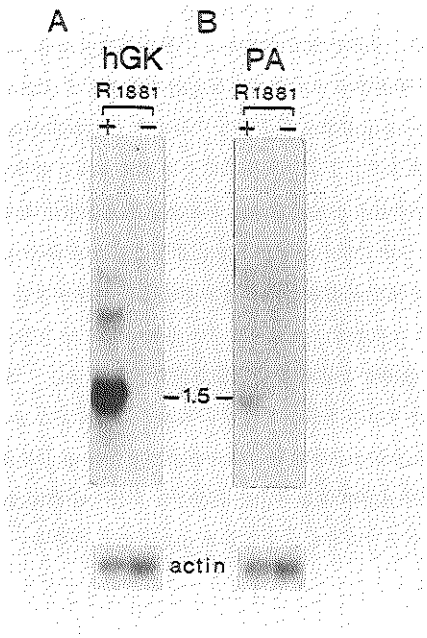

FIGURE 5: Androgen-responsive expression of hGK-1 and PA mRNA.
Northern blots of LNCaP RNA, which was isolated from cells grown in the absence or in the presence of the synthetic androgen R1881 (see Materials and Methods): The blots were hybridized with (A) the hGK-1 or (B) the PA specific oligo-nucleotide. Hybridization with an actin probe was used as a control.

Although, because of the lower expression level less clear, PA mRNA expression is, similar to hGK-1, androgen-dependent (figure 5B), indicating an identical mechanism of regulation of expression for both genes. Essentially identical results were obtained when higher R1881 concentrations (up to 100 nM) or shorter incubation periods (6 h) were used (Riegman, unpublished).

DISCUSSION

In this study the identification of two different cDNA clones (pGK-1 and pGK-10A) corresponding to two hGK-1 mRNAs is described. Both are derived from the hGK-1 gene, which is organized in 5 exons and 4 introns (Schedlich et al., 1987). The two transcripts result from differential splicing of intron IV sequences. This intron contains a cryptic splice donor site (AAG/GTGAGT) located 37 nucleotides downstream of the major splice donor site (GGG/GTGAGT). Interestingly, the sequence of the alternative splice site perfectly matches the splice donor consensus sequence ([A\C]AG/GT[G\A]AGT; Shapiro and Senapathy, 1987), which in general seems to be preferred. Similarly, the closely related PA gene contains in intron IV the same AAGGTGAGT sequence (see Riegman et al., 1989a and figure 6). In contrast to hGK-1, amplification of PA cDNA did not result in the synthesis of a specific fragment derived from an alternatively spliced mRNA (data not shown). We were also unable to isolate a PA cDNA clone containing an extended exon IV fragment as a result of the use of an alternative splice site. Why the sequence GGG/GTGAGT is preferred above the AAG/GTGAGT sequence in hGK-1, and why TCG/GTGAGT is the only splice donor site of PA intron IV used, is unknown. However, these data clearly illustrate that hGK-1 (and PA) RNA splicing not only depends on the sequences directly surrounding the splice donor site, but also on additional, so far unidentified structural elements.

The sequence of the postulated hGK-1 protein product is identical to the protein structure as predicted from the hGK-1 gene structure (Schedlich et al., 1987). A protein derived from hGK-10A will deviate from hGK-1 beginning at amino acid 187, because the insertion of 37 nucleotides causes a shift in the reading frame. In addition, the protein will be truncated at the COOH-terminus (the size is 199 amino acids as compared to 237 for hGK-1). As a result, the serine residue at position 189 in

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hGK-1  ACTTGTGGG/GTGAGTCATCCCTACTCCCAACATCTGGAGGGGAAAG/GTGAGTGAAGACCC
PA     ..C..CTC./......................G....T.........../.......GG....T
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FIGURE 6: Sequence comparison of the intron IV splice donor sites in the hGK-1 and the PA gene. Dots represent identical nucleotides. Predicted splice sites are indicated by a slash and the sequences correlated with the splice donor consensus sequences are underlined. Sequence data are from Riegman et al. (1989a) and Schedlich et al. (1987).

hGK-10A, which is essential for serine protease activity (Carter and Wells, 1988), is lacking in hGK-10A (see figure 1). As a consequence the protein product of hGK-10A mRNA will be devoid of serine protease activity, unless the essential function of serine-189 can be taken over by a different amino acid. The serine residues located at positions 188 and 192 are possible candidates for such a role. An effect of further differences in size and amino acid composition between the hGK-1 and hGK-10A COOH-terminal region on serine protease activity and specificity is hard to predict. Conclusive answers will only be obtained after characterization of the purified hGK-10A and hGK-1 protein.

Expression of hGK-1 and PA mRNA is strongly androgen-responsive. Therefore, it is no surprise that hGK-1 and PA transcripts were not detected in the androgen-independent prostate tumors/cell lines PC 133, PC 135 and PC 3, which contain no or only very small amounts of androgen receptor (Trapman et al., in press). It is at present unknown at which level the regulation of hGK-1 and PA mRNA expression by androgens takes place. Because at least one putative androgen responsive element ([G\A]GAACAGCAAGTGCT) is identified in the 5'-flanking region of the hGK-1 and PA gene, part of the regulation could be directly at the level of transcription (Riegman et al., 1989a; Ham et al., 1988).

Expression of the kallikrein gene family has extensively been studied in the mouse and rat. In both species the kallikrein gene family is much larger than in man, which complicates experiments focused on expression of individual members (Mason et al., 1983). Two kallikrein-like genes (S3 and P1) were found to be expressed in the rat ventral prostate (Brady et al., 1989). Expression of both genes is androgen-responsive (Clements et al., 1988; Winderickx et al., 1989). Similarly, in the dog prostate an

androgen-responsive serine protease has been identified (Chapdelaine et al., 1988b). In mice as well as in rats many of the kallikrein-like proteins are expressed in a steroid-responsive manner in the salivary (submaxillary) glands. In general, the effects of testosterone supplementation to castrated animals seem to be rather slow (van Leeuwen et al., 1987; Clements et al., 1988; Chapdelaine et al., 1988b; Winderickx et al., 1989). This could mean that the effect of testosterone is not directly on the level of gene transcription. On the other hand, there is evidence that an androgen-dependent process of cellular differentiation has to take place, before an effect on specific kallikrein gene expression can become manifest. In the latter case the possibility remains that (part of) the testosterone effect is directly on gene transcription (van Leeuwen et al., 1987). In one study (Clements et al., 1988), a rat kallikrein gene (K1) was found to be expressed androgen-dependent in submaxillary glands and androgen-independent in kidney tissue. The expression of true kallikrein (PS or rGK-1 in rat, and mGK-6 in mouse) is independent of male sex hormones (van Leeuwen et al., 1987; Clements et al., 1988). It will be clear from these observations that the regulation of expression of the kallikrein-like genes can be very complex and includes tissue specific processes. It is tempting to speculate that tissue specific transcription factors play a role in this regard.

The hGK-1 protein contains, like the PA protein, a signal peptide, indicating that it will be secreted. If this is true, hGK-1 may be present in the serum in case of prostate cancer, as has been demonstrated for PA (Chu and Murphy, 1986; Stamey et al., 1989). This indicates that (like PA) hGK-1 could serve as a prostate tumor marker. In fact, some of the antibodies used for measuring of PA serum levels might cross-react with hGK-1. It is unclear whether the until recently unknown expression of a second, closely related protein will influence the accuracy of PA serum assays. This will depend on the properties of the antibodies applied, the concentration of hGK-1 in the serum and the regulation of hGK-1 and PA expression. Although so far the hGK-1 and PA expression patterns were found to be very similar, some differences were noticed when the ratios of PA and hGK-1 expression in LNCaP cells were compared with those in PC EW and PC 82 tumors. However, the PA/hGK-1 ratios might also be different in various LNCaP sublines (Riegman, unpublished). Fluctuations in PA and hGK-1 mRNA levels were recently also reported in primary prostate tumors (Henttu et al., 1990). Further experiments are needed to substantiate the clinical relevance of these observations.

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REFERENCES

Angerman,A., Bergmann,C. & Appelhans,H. (1989) Biochem. J. 262, 787-793.
Ashley,P.L. & MacDonald,R.J. (1985) Biochem. 24, 4512–4520.
Baker,A.R. & Shine,J. (1985) DNA 4, 445–450.
Brady,J.M., Wines,D.R. & MacDonald,R.J. (1989) Biochem. 28, 5203-5210.
Carter,P. & Wells,J.A. (1988) Nature 332, 564–568.
Chapdelaine,P., Paradis,G., Tremblay,R.R. & Dubé,J.Y. (1988a) FEBS Lett. 236, 205–208.
Chapdelaine,P., Potvin,C., Ho–Kim,M–A., Larouche,L., Bellemare,G., Tremblay,R.T. & Dubé,J.Y. (1988b) Mol. Cell. Endocrinol. 56, 63–70.
Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. & Rutter,W.J. (1979) Biochem. 18, 5294–5299.
Chu,T.M. & Murphy,G.P. (1986) Urology 27, 487–491.
Clements,J.A., Matheson,B.A., Wines,D.R., Brady,J.M., MacDonald,R.J. & Funder,J.W. (1988) J.Biol.Chem. 263, 16132-16137.
Clements,J.A. (1989) Endocrine Rev. 10, 393–419.
Drinkwater,C.C., Evans,B.A. & Richards,R.I. (1988) Trends Biochem. Sci. 13, 169–172.
Evans,B.A., Drinkwater,C.C. & Richards,R.I. (1987) J. Biol. Chem. 262, 8027-8034.
Evans,B.A., Yun,Z.X., Close,J.A., Tregear,G.W., Kitamura,N., Nakanishi,S., Callen,D.F., Baker,E., Hyland,V.J., Sutherland,G.R. & Richards,R.I. (1988) Biochem. 27, 3124-3129.
Feinberg,A.P. & Vogelstein,P. (1983) Anal. Biochem. 132, 6-13.
Fukushima,D., Kitamura,N. & Nakanishi,S. (1985) Biochem. 24, 8037-8043.
Gerald,W.L., Chao,J. & Chao,L. (1986) Biochim. Biophys. Acta 866, 1-14.
Ham,J., Thomson,A., Needham,M., Webb,P. & Parker,M. (1988) Nucl. Acids Res. 16, 5263–5275.
Henttu,P., Lukkarinen,O. & Vihko,P. (1990) Int. J. Cancer 45, 654–660.
Hoehn,W., Wagner,M., Riemann,J.F., Hermanek,P., Williams,E., Walther,R. & Schrueffer,R. (1984) Prostate 5, 445–452.
Hoehn,W., Schroeder,F.H., Riemann,J.F., Joebsis,A.C. & Hermanek,P. (1980) Prostate 1, 95–104.
Horoszewicz,J., Leong,S., Chu,T., Wasjman,Z., Friedman,M., Papsidero,L., Kim,U., Chiu,L., Kakati,S., Arya,S. & Sandberg,A. (1980) Prog. Clin. Biol. Res. 37, 115–132.
Kaighn,M.E., Narayan,K.S., Ohnuki,Y., Lechner,J.F. & Jones,L.W. (1979) Invest.Urol. 17, 16-23.
Leeuwen,B.H. van, Penschow,J.D., Coghlan,J.P. & Richards,R.I. (1987) EMBO J. 6, 1705–1713.
Lilja,H. (1985) J. Clin. Invest. 76, 1899–1903.
MacDonald,R.J., Margolius,H.S. & Erdös,E.G. (1988) Biochem. J. 253, 313-321.
Maniatis,T., Fritsch,E.F. & Sambrook,J. (1982) Molecular cloning: A Laboratory Manual, Cold Spring Harbor, NY.

Mason,A.J., Evans,B.A., Cox,D.R., Shine,J. & Richards,R.I. (1983) Nature 303, 300–307.
McGee,R.S. & Herr,J.C. (1988) Biol. Reprod. 39, 499–510.
Morris,B.J. (1989) Clin. Exp. Pharm. Phys. 16, 345–351.
Riegman,P.H.J, Klaassen,P., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1988) Biochem. Biophys. Res. Commun. 155, 181-188.
Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1989a) Biochem. Biophys. Res. Commun. 159, 95–102.
Riegman,P.H.J., Vlietstra,R.J., Klaassen,P., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1989b) FEBS Lett. 247, 123–126.
Sanger,F., Nicklen,S. & Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
Schachter,M. (1980) Pharmacol. Rev. 31, 1–17.
Schedlich,L.J., Bennetts,B.H. & Morris,B.J. (1987) DNA 6, 429-437.
Shapiro,M.B. & Senapathy,P. (1987) Nucl. Acids Res. 15, 7155–7174.
Stamey,T.A., Kabalin,J.N., McNeal,J.E., Johnstone,I.M., Freiha,F., Redwine,E.A., Yang,N. & Ferrari,M. (1989) J. Urol. 141, 1070–1090.
Steenbrugge,G.J.van, Groen,M., Romijn,J.C. & Schröder,F.H. (1984) J. Urol. 131, 812–817.
Steenbrugge,G.J.van, Groen,M., Dongen,J.W.van, Bolt,J., Korput,H.van der, Trapman,J., Hasenson,M. & Horoszewicz,J. (1989) Urol. Res. 17, 71-77.
Trapman,J., Ris–Stalpers,C., Korput,J.A.G.M.van der, Kuiper,G.G.J.M., Faber,P.W., Romijn,J.C., Mulder,E. & Brinkmann,A.O. J. Steroid Biochem. In Press.
Winderickx,J., Swinnen,K., Dijck,P.van, Verhoeven,G. & Heyns,W. (1989) Mol. Cell. Endocrinol. 62, 217–226.
Wines,D.R., Brady,J.M., Pritchett,D.B., Roberts,J.L. & MacDonald,R.J. (1989) J. Biol. Chem. 264, 7653–7662.

CHAPTER VII

THE PROMOTER OF THE PROSTATE-SPECIFIC ANTIGEN GENE CONTAINS A FUNCTIONAL ANDROGEN RESPONSIVE ELEMENT

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Molecular Endocrinology (1991) 5, 1921–1930.

SUMMARY

Expression of Prostate-specific Antigen (PA) mRNA was tested at various time periods after incubation of the human prostate tumor cell line LNCaP with the synthetic androgen R1881. Androgen-stimulated expression was observed within 6 hours after addition of R1881 to the cells. Run-on experiments with nuclei isolated from LNCaP cells showed that expression of the PA gene could be regulated by R1881 on the level of transcription. DNase I footprints of the promoter region of the PA gene (−320 to +12) with nuclear protein extracts from LNCaP cells showed at least four protected regions. The protected areas include the TATA-box, a GC-box sequence and a sequence AGAACAgcaAGTGCT at position −170 to -156, which closely resembles the reverse complement of the consensus sequence GGTACAnnnTGTTCT for binding of the glucocorticoid receptor and the progesterone receptor. Fragments of the PA promoter region were cloned in front of the CAT reporter gene and cotransfected with an androgen receptor expression plasmid into COS cells in a transient expression assay. CAT activity of COS cells grown in the presence of 1 nM R1881 was compared to untreated controls. A 110-fold induction of CAT activity was found if a −1600 to +12 PA promoter fragment was used in the construct. By further deletion mapping of the PA promoter a minimal region (−320 to −155) was identified as being essential for androgen-regulated gene expression. Mutation of the sequence AGAACAgcaAGTGCT (at −170 to −156) to AAAAAAgcaAGTGCT almost completely abolished androgen inducibility of the reporter gene constructs. One or more copies of the sequence AGAACAgcaAGTGCT cloned in front of a TK (thymidine kinase) promoter-CAT reporter gene confers androgen regulation to the reporter gene. These findings provide strong evidence for transcription regulation of the PA gene by androgens via the sequence AGAACAgcaAGTGCT. Interestingly, in addition to the AGAACAgcaAGTGCT element, an upstream region (−539 to −320) is needed for optimal androgen inducibility of the PA promoter.

INTRODUCTION

Androgen-regulated gene expression, which plays an important role in male sexual development, is mediated through the androgen receptor (AR). Recently, the cDNA of the human AR has been isolated and the structure of the protein was determined (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988; Faber et al.,1989). In addition, the protein was functionally characterized (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988; Faber et al.,1989; Rundlet et al., 1990; Simental et al., 1991; Jentser et al., in press).

The AR is a member of the superfamily of ligand-responsive transcription factors, which includes steroid, thyroid hormone and retinoic acid receptors (Evans, 1988; Green & Chambon, 1988; Beato, 1989; Carson-Jurica et al., 1990). These ligand-responsive transcription factors can bind to specific DNA sequences and regulate the transcriptional activity of their target genes (Beato, 1989; Carson-Jurica et al., 1990; Yamamoto, 1985). All receptors share a similar functional domain structure. The

N-terminal region is essential for the transactivating function, however, it is not very well conserved among the various members of the receptor family. The DNA-binding domain of about 70 amino acids is highly conserved. It contains eight conserved cysteine residues, which interact with two zinc ions, and is arginine- and lysine-rich. The ligand-binding domain has a size of approximately 250 amino acids and is located in the C-terminal region of the protein. The ligand binding domain contains also the information for receptor dimerization and, at least in part of the receptors, a second transactivating domain. DNA-binding and transactivation are controlled by ligand-binding.

The DNA structures to which steroid receptors bind are imperfect palindromic sequences. The consensus sequence of the responsive element for the glucocorticoid receptor (GR) and progesterone receptors (PR), which is also referred to as PRE/GRE, is GGTACAnnnTGTTCT, the sequence of the ERE (estrogen responsive element) is GGTCAnnnTGACC (Beato, 1989). Steroid receptors bind to this motif as homodimers (Tsai et al., 1988; Kumar & Chambon, 1988; Eriksson & Wrange, 1990; Fawell et al., 1990; Dahlman-Wright et al., 1991). Studies concerning the DNA-binding domain structure of the GR and the estrogen receptor (ER), which appeared to be strikingly similar, have shown that this domain consists of two perpendicularly orientated alpha helices (Härd et al., 1990; Schwabe et al., 1990). Specific amino acids in the N-terminal helix of this zinc twist (Vallee et al., 1991) are supposed to interact with the major groove in one half site of the HRE. This helix was also found to be involved in the recognition of the cognate DNA element (Green et al., 1988; Mader et al., 1989; Umesuno et al., 1989; Zilliacus et al., 1991). Because the DNA-binding domain is a well conserved region among the ligand-responsive transcription factors, and the structures of the ER and GR DNA-binding domain appeared to be similar, this model presumably represents a common form of DNA interaction for this family of transcription factors, including the AR.

Although the expression of many genes is known to be androgen-dependent, so far, the number of genes of which the transcription is directly regulated by androgens is limited. The best known example is the mouse mammary tumour virus (MMTV) promoter, which is regulated by glucocorticoids and progestins, but is also able to confer androgen responsiveness to a reporter gene, although less efficiently (Cato et al., 1987, 1988; Ham et al., 1988; Otten et al., 1988). Mutation of the GRE/PREs in this promoter also abolish regulation by androgens. These data indicate that a motif,

similar or identical to the GRE/PRE consensus sequence is essential for recognition by the AR. Cellular genes, which are at least partially regulated on the level of transcription by androgens include the rat C3(1) gene (Page & Parker,1982), the mouse RP2 gene (Rheaume et al., 1989) and the rat SVS IV and V genes (Higgins & Hemmingway, 1991). Out of these the C3(1) is studied in most detail. In the promoter region and in the first intron of the C3(1) gene, restriction fragments, which are able to bind the AR have been identified (Rushmere et al., 1987; Claessens et al., 1990). In the first intron a sequence is present, which closely resembles the PRE/GRE consensus sequence. This sequence conferred androgen responsiveness to the TK promoter (Claessens et al., 1989). In addition, the DNA-binding domain of the AR, expressed in E. coli, was found to be able to interact with this sequence (de Vos et al., 1991). However, the function of this GRE/PRE-like sequence has not been tested in the context of the C3(1) promoter.

As part of our studies on prostate-specific and androgen-regulated gene expression we molecularly cloned the gene encoding Prostate-specific Antigen (PA) (Riegman et al., 1989a). The organization of the PA gene was determined and the gene was completely sequenced. The PA gene, which contains five exons over a length of approximately 6 kb, is located on the long arm of chromosome 19, in the region q13.3-qter (Riegman et al., 1989b; Schonk et al., 1990). Adjacent to the PA gene, the closely related human Glandular Kallikrein-1 (hGK-1) gene is situated (Riegman et al., 1989b; Schedlich et al., 1987). Expression of PA is only detected in human prostate tissue (Wang et al., 1979, 1981; Gallee et al., 1986). In a previous study we have shown that PA (and hGK-1) mRNA expression is androgen-responsive (Riegman et al., 1991).

The promoter of the PA gene contains a variant TATA-box (TTTATA); two transcription initiation sites (positions +1 and +7) were identified. The open reading frame starts at position +42 in the first exon. In the promoter region of the PA gene a motif was found at position -170 to -156, which closely resembles the PRE/GRE consensus sequence (Riegman et al., 1989a). In this study we provide evidence that this sequence is essential for androgen-regulated transcription of the PA gene. In additon, it was found that a more upstream region acts synergistically with this element for optimal androgen inducibility.

MATERIALS AND METHODS

Cell culture: For preparation of nuclear protein extracts, LNCaP cell were cultured in RPMI 1640 supplemented with 5% fetal calf serum and antibiotics. For examination of androgen-regulated gene expression, LNCaP cells were grown for a period of six days in RPMI 1640 containing 5% steroid-depleted (dextran-charcoal treated) serum. Next, the synthetic androgen R1881 (NEN, Boston) was added to a final concentration of 1 nM. Cells were harvested at different time points after R1881 treatment and directly used for RNA isolation. Control cultures were continuously grown in steroid-depleted medium during the same time periods.
For transfection, cells were grown in Dulbecco's Modification of Eagle's Medium (D-MEM) supplemented with 5% steroid-depleted (dextran-charcoal treated) serum.
RNA preparation: Isolation of total cellular RNA from the different cell lines was carried out with the guanidinium thiocyanate method (Chirgwin et al., 1979).
Northern blot analysis: Northern blot analysis with PA and hGK-1 specific oligonucleotides as probes was performed as described (Riegman et al., 1991). The PA probe 5'-GGTCCAGTCCCTCTCCTTACATCATCCCCATCCCATGCCAA-3', used for RNA hybridization, is located at position 1262-1302 in PA 75 cDNA (Riegman et al., 1988). The specific hGK-1 probe used for RNA hybridization 5'-CATCCAGAAAGGCCAAGTGATGCCAGAACGTGAGGTGGACT-3' is located at position 864-904 in hGK-1 cDNA (Riegman et al., 1991). Oligonucleotides were synthesized in an Applied Biosystems DNA synthesizer.
Run-on experiments: Nuclear run-on assays were carried out as described (Linial et al., 1985), with some minor modifications. LNCaP cells were grown for 3 days in RPMI 1640 supplemented with 5% steroid-depleted (dextran-charcoal treated) foetal calf serum and antibiotics. Next, to 4 x 10^7 cells R1881 was added to an end concentration of 1 nM and incubation was continued for 24 hours; control cells were kept on steroid-depleted medium. For the isolation of nuclei, cells were washed twice in PBS and subsequently homogenized by 20 strokes with a B pestle in a glass dounce homogenizer in 8 ml "homogenizer buffer" (10 mM Tris pH 7.5, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5% NP-40 and protease inhibitors 0.1mM PMSF, 1 mM leupeptin and 1% aprotinin) and centrifuged for 5 minutes at 1000xg and 0°C. About 2x10^7 nuclei were frozen in 210 µl "freezing buffer" (40% glycerol, 50 mM Tris pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, 3 mM $CaCl_2$ supplemented with protease inhibitors, 0.1mM PMSF, 1 mM leupeptin and 1% aprotinin). The preparations were stored under liquid nitrogen until use. For transcription elongation, 210 µl nuclei suspension was thawed and added to 25 µl [α^{32}P]UTP (250 µCi, >3000Ci/mmol) and 60 µl of 5x "run-on buffer": 25 mM Tris pH 8.0, 12.5 mM $MgCl_2$, 750 mM KCl, 1.25 mM ATP, 1.25 mM CTP, 1.25 mM GTP and protease inhibitors 0.1mM PMSF, 1 mM leupeptin and 1% aprotinin, and incubated for 30 minutes at 30°C. The reaction was terminated by addition of 10 U RQ DNase (Promega, Madison) and incubation for 15 minutes at 30°C. Proteins were degraded by the addition of 75 µl 5x "SET": 5% SDS, 25 mM EDTA, 10 mM Tris pH 7.5 and 100 µg/ml Proteinase K. This mixture was incubated for 90 minutes at 42°C. The RNA was extracted with an equal volume of phenol/chloroform (1:1), and precipitated by addition of 0.3 volumes of 10M NH_4OAc and an equal volume of isopropanol. After 15 minutes at -70°C the RNA was pelleted in a microfuge for 10 minutes. The pellet was dissolved in 50 µl TE and centrifugated over a G-50 column. The RNA was overnight precipitated at -20°C after addition of 1/9 volumes of 3M NaOAc and 2.5 volumes of 100% ethanol, pelleted and dissolved in 100 µl H_2O. The slotblot was prepared by application of 5 µg denatured plasmid per slot to a nitrocellulose filter with a Schleicher and Schull slot blot apparatus. The

plasmids used were pA 75 (Riegman et al., 1988), Hamster actin (1.2 kb PstI–PstI fragment in pSP 65) and pUC 9. Pre-hybridiszation in 4 ml: 10 mM TES pH 7.4, 0,2% SDS, 10 mM EDTA, 0,3 M NaCl, 1x Denhardt's and 250 μg/ml yeast tRNA for 24 hours at 65°C. For hybridization the denatured RNA was added (2–4x10^7 cpm) and incubation was continued for 48 hours at 65°C. Filters were washed 4 times with 2xSSC for 30 minutes at 65°C. Filters were exposed to X-ray film at –70°C using intensifying screens and films were analyzed on a BioRad videodensitometer model 620.

Construction of plasmids: All plasmid constructs were prepared using standard methods (Sambrook et al., 1989). The human AR expression plasmid pAR_0 was described previously (Brinkman et al., 1989). The promoter-less basis plasmid pRV_4CAT, which was used for cloning promoter region fragments from the PA and the hGK-1 genes in front of the CAT reporter-gene, was derived from pSV_2CAT (Gorman, 1985) by exchange of the 505 bp AccI-HindIII fragment, containing the SV 40 early promoter, with the 141 bp PvuII-HindIII fragment from pUC19 containing the multiple cloning site. PA-CAT constructs pPA1–CAT (SmaI–HindIII; –1600/+12), pPA2–CAT (EcoRI–HindIII; –630/+12), pPA3–CAT (BglII–HindIII; –539/+12), pPA4–CAT (XhoII–HindIII; –320/+12) and pPA5–CAT (NheI–HindIII; –155/+12) were generated by ligation of the appropriate fragments in the multiple cloning site of pRV_4CAT. One (pARE1–TK–CAT), two (pARE2–TK–CAT) and three (pARE3–TK–CAT) AREs were cloned in front of a TK promoter CAT construct (pBL-CAT2) (Luckow & Schütz, 1987). Oligonucleotides 5'–GATCCTTGCAGAACAGCAAGTGCTAGCTG–3' (positioned –174/–152 in the PA gene) and 5'-GATCCAGCTAGCACTTGCTGTTCTGCAAG–3' were annealed and subsequently cloned in the BamHI site of pBL-CAT2. Clones were checked by sequencing using the dideoxy chain termination method (Sanger et al., 1977).

PCR mediated mutation of the ARE: For mutation of the ARE (-170/–156) in the PA promoter region the fragment (–545 to –148) of pPA_2CAT was amplified by the Polymerase Chain Reaction (PCR). The forward PCR primer (–545/–521) positioned over the BglI site (underlined) 5'–CCACAAGATCTTTTTATGATGACAG–3' and the reverse PCR primer (–185/–148) positioned over the NheI site (underlined) 5'-GGAGAGCTAGCACTTGCTTTTTTGCAATTACTAGATCA–3' and the ARE, carrying two mutations (underlined). Amplification by PCR was performed as described (Riegman et al., 1991), except that 18 amplification cycles (denaturation: 1 minute at 92°C; annealing: 3 minutes at 55°C; extension: 4 minutes at 72°C) were carried out and the final extension reaction was for 4 minutes at 72°C. The PCR products were cut with BglII and NheI and isolated from a 2% agarose gel. The isolated fragment was religated in BglII–NheI digested plasmids pPA1–CAT and pPA2–CAT. The new plasmids pPA1–mICAT and pPA2–mICAT were checked by sequencing.

Transfections: COS cells were transfected by the calcium phosphate precipitation method (Chen & Okyama, 1987), using 5 x 10^5 cells and 5 μg PA-CAT constructs and 5 μg pAR_0.

Cat assay: CAT activity was determined by the butyryl CoA phase extraction assay (Seed & Sheen, 1988). All experiments were carried out at least four times in duplicate with two separate plasmid isolations.

DNase I footprints: The XhoII–HindIII (–320/+12) PA promoter fragment was blunt-ended and ligated in the SmaI site of pUC9. The PstI–EcoRI fragment of pAF 1.1, which contains the XhoII–HindIII fragment in the proper orientation was labeled by filling in the EcoRI site with the Klenow fragment of DNA polymerase and [α^{32}P]–dATP (Sambrook et al., 1989). Nuclear protein extracts were prepared from LNCaP cells, cultured in RPMI 1640 supplemented with 5% fetal calf serum and antibiotics, as

described (Gorski et al., 1987) and stored under liquid nitrogen until use. For DNase I footprints the "Hotfoot" footprint kit (Stratagene, La Jolla) was used. The assay conditions were as described (Harrington et al., 1988) except that 10 ng labeled DNA (0.8–1 x 10^4 cpm), 20 μg nuclear protein extracts and 0.2–0.5 U DNaseI (Stratagene) were used in the assay mixture. Assay mixtures without nuclear protein extracts were treated with 0.02 U DNaseI. G/A sequence reactions of the same fragment were run alongside the footprint as a marker (Maxam & Gilbert, 1980).

RESULTS

Regulation of PA transcription

Recently, PA and hGK-1 mRNA expression was shown to be upregulated in the human prostate tumor cell line LNCaP by long term androgen treatment (Riegman et al., 1991). To determine more precisely the kinetics of the androgen response, Northern blots containing RNA from different time points after R1881 stimulation were hybridized with a PA-specific oligonucleotide (Figure 1A). The most prominent PA mRNA has a size of 1.5 kb (Riegman et al., 1991). As can be seen, the PA mRNA expression is increased within 6 hours after addition of androgens and reaches its

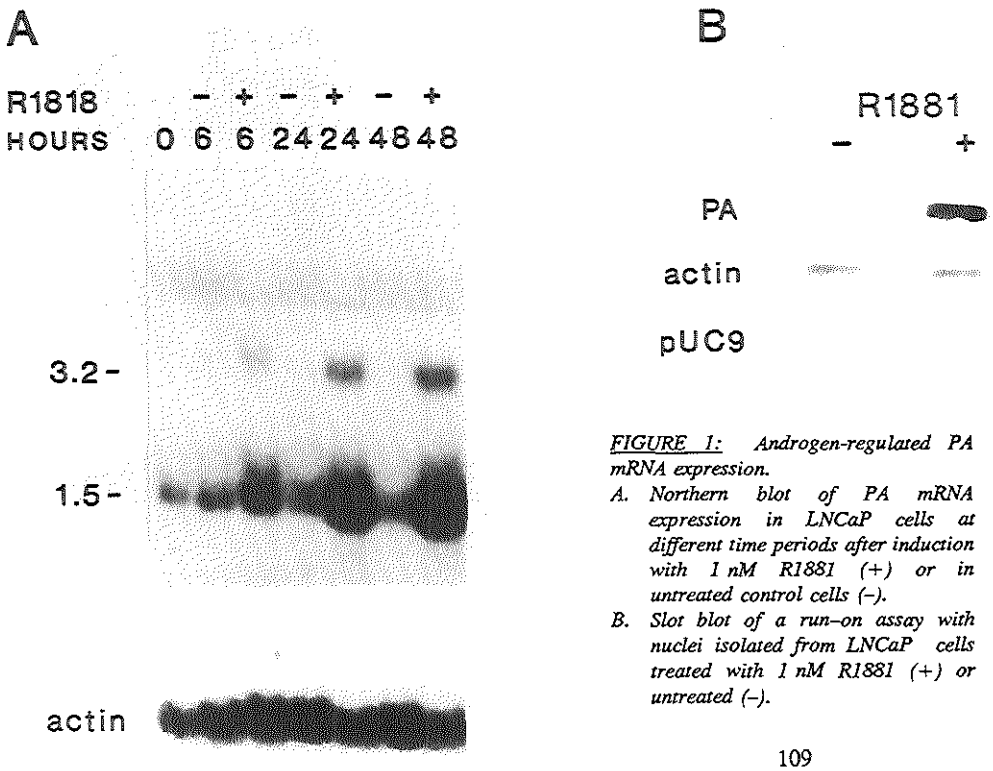
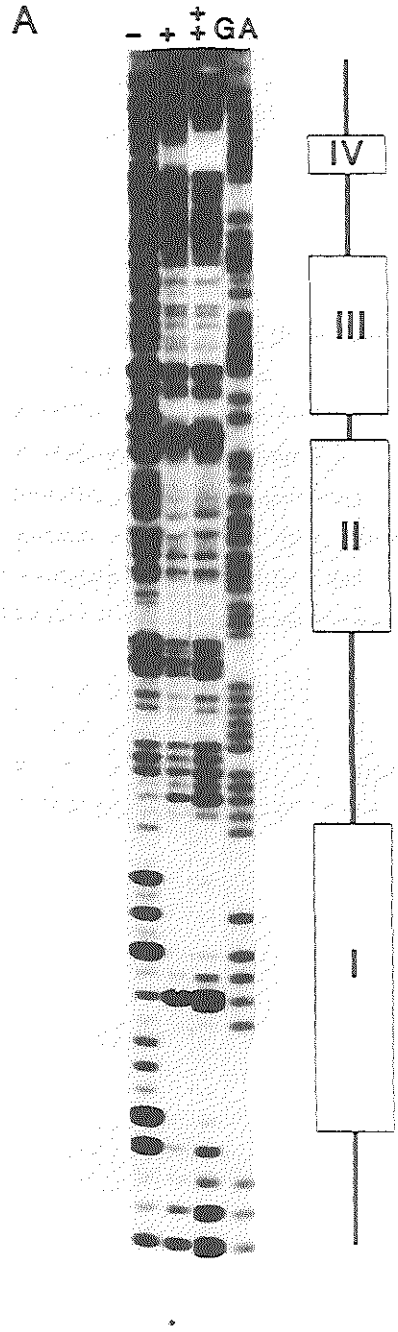


FIGURE 1: Androgen-regulated PA mRNA expression.
A. Northern blot of PA mRNA expression in LNCaP cells at different time periods after induction with 1 nM R1881 (+) or in untreated control cells (–).
B. Slot blot of a run–on assay with nuclei isolated from LNCaP cells treated with 1 nM R1881 (+) or untreated (–).

maximum at about 24 hours. Similar results were obtained for hGK-1 mRNA expression (data not shown).

In order to investigate whether (part of) androgen regulated PA mRNA expression could be on the level of transcription, run-on experiments were performed. Nuclei with intact transcription units were isolated from LNCaP cells, which were cultured for 24 hours in medium supplemented with steroid-depleted serum either in the absence or presence of 1 nM R1881. Transcription was continued in vitro in the presence of ^{32}P-labeled UTP. The radioactive RNA was hybridized in a slot blot containing PA cDNA plasmid and the appropriate controls. The results, as presented in Figure 1B, clearly show that the transcription of the PA gene is strongly increased by R1881. Densitometric scanning of the film indicate an induction of at least 20-fold by androgen treatment.



Footprint analysis of the PA promoter

A restriction fragment containing the promoter of the PA gene (XhoII-HindIII fragment, from -320 to +12) was incubated with nuclear extract from LNCaP cells in a DNAseI protection assay. The footprint over this fragment shows at least four protected regions (Figure 2A). Previously, four elements

B

FIGURE 2: Site specific binding of LNCaP Nuclear Proteins to the PA promoter.

A. DNaseI footprint analysis of the fragment -320 to +12 of the PA promoter. lane (G/A) represents the G/A Maxam and Gilbert sequence of the fragment; lane (-) represents the fragment treated with DNaseI, lane (+) is the fragment incubated with LNCaP nuclear protein extract and treated with 0.2 U DNaseI; lane (++) is with fragment and LNCaP nuclear protein extract treated with 0.5 U DNaseI. Protections are represented by boxes.

B. Partial sequence of the PA promoter region. Protections found in the DNaseI footprint are boxed. The two transcription start sites are indicated by arrows.

strongly resembling the consensus sequences of cis-acting regulatory elements, including a TATA-box, GC-box, CACCC-box and GRE/PRE, were recognized in the promoter region of the PA gene (Riegman et al., 1989a). The protections found in the DNaseI footprints partly coincide with these elements. The first protection (I) from -16 to -30, which is composed of two small, separate protected fragments, includes the TATA box (TTTATA, at -28 to -23) (see Figure 2A and 2B). The GC-box (-54 to -45), is part of protected region (II), which is positioned from -70 to -45. Region (III) is composed of a small footprint (-82 to -76) and a large protected region (-118 to -88). The former mostly protects a sequence (TT)GGTCAG, which is closely related to the TRE-consensus sequence [TPA-Responsive Element: T(T/G)AGTCAG]. An obvious binding sequence for a well characterized transcription factor is absent in the region -118 to -88. The putative CACCC-box (GGGTGTG; -129 to -123) is not protected by LNCaP nuclear proteins under the experimental conditions applied. Interestingly, the sequence at position -170 to -156 (AGAACAgcaAGTGCT), which resembles the PRE/GRE consensus sequence was found to be included in the large footprint (IV), positioned from -203 to -156.

Analysis of the PA promoter by deletion mapping

A series of PA promoter fragments was cloned in front of the CAT (Chloramphenicol Acetyltransferase) reporter gene, resulting in pPA1–CAT to pPA5–CAT (see Methods and Figure 3). pPA–CAT constructs were transfected to LNCaP cells in order to study the transcription regulation of the PA promoter, however, no significant CAT activity was detected. Cotransfection of LNCaP cells with pPA1–CAT and the AR expression plasmid (pAR_0) (Brinkman et al., 1988) also gave negative results. The low CAT activity in LNCaP cells can be explained by the extremely low transfection efficiency of the cells, however, the absence of a strong tissue-specific enhancer element in our constructs, which hampers high expression, cannot be ruled out. Unfortunately, other prostate cell lines, which express PA mRNA and which can be used for transfection studies are not available.

In order to be able to study androgen-regulation of the PA promoter, COS cells were cotransfected with each of the pPA–CAT constructs and pAR_0 in the absence and the presence of 1 nM R1881. Progesterone receptors and glucocorticoid receptors were previously shown to be absent in the transfected COS cells (Trapman et al., 1988). Omission of pAR_0 from the transfection mix resulted in very low CAT activity in the cell lysates (data not shown). Cotransfection of pPA1–CAT (–1600 to +12) with pAR_0 in the presence of 1 nM R1881 resulted in a high CAT activity. The relative induction value as compared to CAT activity measured in the absence of R1881 is 110 (Figure 3). Similar results were obtained with the constructs pPA2–CAT and pPA3–CAT, which start at –630 and –539, respectively.

Further deletion of the PA promoter enabled the identification of a region involved in the androgen-regulated gene expression. A profound reduction of the relative induction by a factor 13, accompanied by a drop in the absolute CAT activity in the presence of R1881, was found after removal of the fragment -539 (pPA3–CAT) to -320 (pPA4–CAT). However, expression of pPA4–CAT still is 5–fold induced in the presence of R1881. After removal of the fragment -320 (pPA4–CAT) to -155 (pPA5–CAT) the PA promoter activity is low and can no longer be upregulated by R1881. These results indicate that within the –320 to –155 fragment a DNA motif must be located, which is essential for androgen-regulated transcription of the PA promoter. Importantly, this region contains at position –170 to –156 the sequence AGAACAgcaAGTGCT, which resembles the PRE/GRE consensus sequence (Riegman et al., 1989a). Similar results as described above were obtained if HeLa cells were cotransfected with pAR_0 and with all

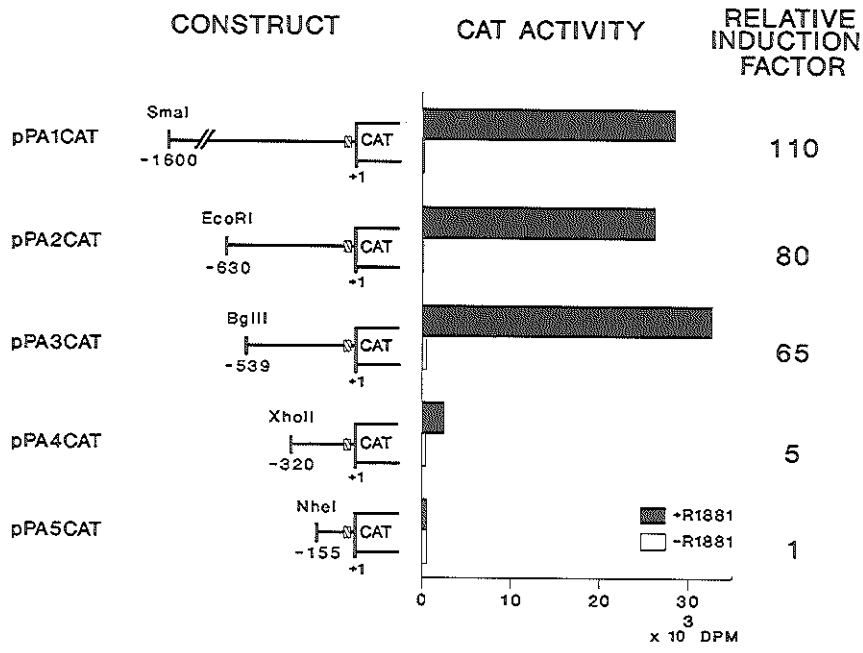


FIGURE 3: Cotransfection of pPA-CAT constructs and pAR_0 in COS cells in the presence and in the absence of 1 nM R1881. Experimental details are as described in the Method section. The TATA-box is represented by a hatched square.

the PA-promoter constructs. However, the absolute values obtained were considerably lower (data not shown).

Definition of the Androgen Responsive Element

PRE/GREs have been reported to be able to confer androgen responsiveness to a reporter gene (Cato et al., 1987, 1988; Ham et al., 1988; Otten et al., 1988). To determine the role of the AGAACAgcaAGTGCT motif in the androgen-regulated expression of the PA promoter, the sequence was mutated to AAAAAAgcaAGTGCT in pPA1-CAT and in pPA2-CAT, resulting in pPA1-mICAT and pPA2-mICAT, respectively (see Methods). The results of the pAR_0 cotransfection experiments with these mutated constructs are illustrated in Figure 4. In both constructs, the mutation resulted in an almost complete abolishment of the transcription activation by R1881.

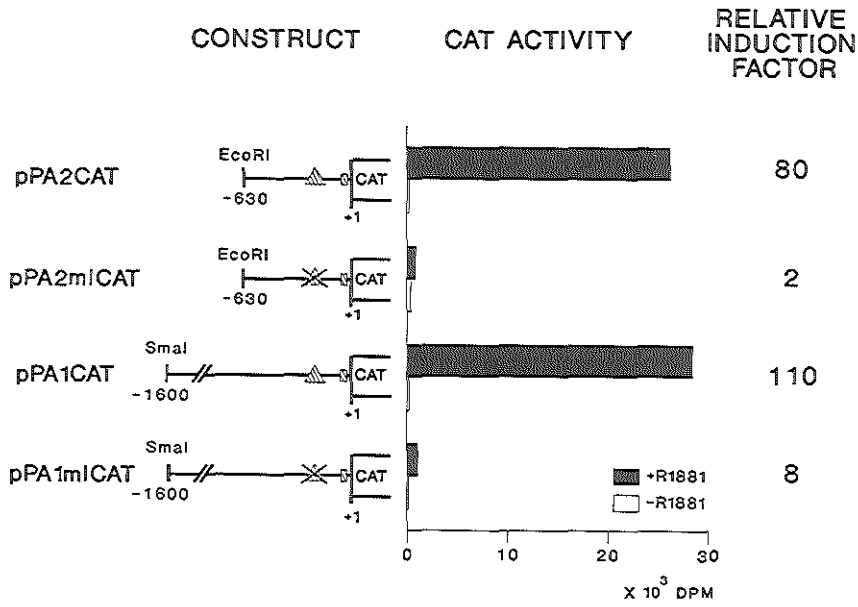


FIGURE 4: Cotransfection of mutated pPA-CAT constructs and pAR_0 in COS cells in the presence and in the absence of 1 nM R1881. In pPA1-mICAT and pPA2-mICAT the ARE sequence (-170/-156) AGAACAgcaAGTGCT was mutated to AAAAAAgcaAGTGCT. Experiments were carried out as described in the Methods section. The TATA-box is represented by a hatched square; the ARE by a hatched triangle.

Especially with the shortest, mutated construct (pPA2-mICAT) almost no residual CAT activity was detected. These data strongly suggest the importance of the AGAACAgcaAGTGCT motif in androgen-regulated gene expression. Because androgen-inducibility is almost completely lost in pPA2-mICAT, the results further indicate that one or more transcription factors, which bind to the -539 to -320 fragment and which are involved in androgen-regulated expression of the PA promoter (see above), do not act separately, but in concert with the androgen receptor, binding to the AGAACAgcaAGTGCT motif.

To obtain additional evidence that the sequence AGAACAgcaAGTGCT can act as an Androgen Responsive Element (ARE), it was taken out of the context of the PA promoter and 1,2 or 3 copies were linked to a TK-promoter CAT reporter gene (pBL-CAT2) construct. The results of the cotransfections with pAR_0 are depicted in Figure 5. A relative induction factor of 2 with one ARE, 8 with two AREs and 16 with three AREs was measured. Expression of the pBL-CAT2 control construct without ARE was not inducible by R1881.

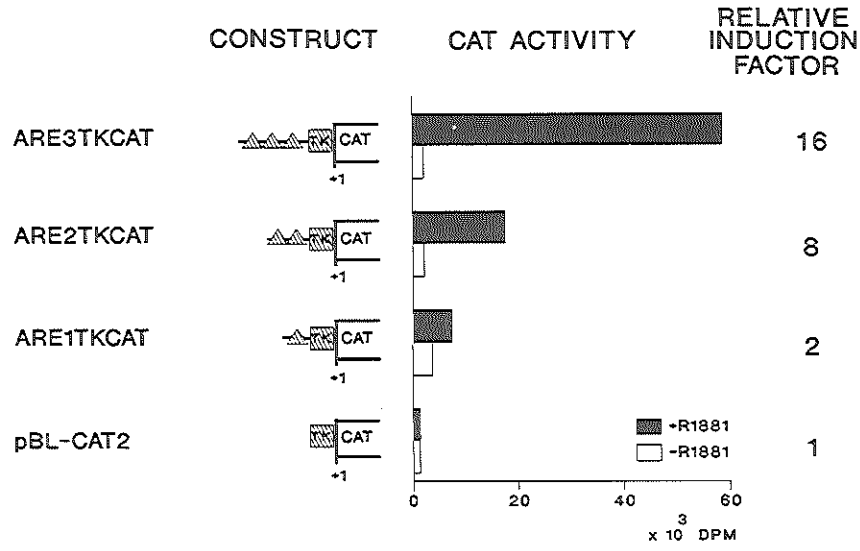


FIGURE 5: Cotransfection of ARE-TK-CAT constructs and pAR_0 in COS cells in the absence and in the presence of R1881. 1,2 or 3 copies of the ARE (-174/-152) in the PA promoter were cloned in front of a TK-promoter CAT reporter gene construct (pBL-CAT2). Experimental details are described in Methods. The ARE is represented by a hatched triangle.

DISCUSSION

Androgen-responsive mRNA expression can be regulated on the level of transcription (directly and/or indirectly), RNA processing and RNA stability. Various genes have been described to be regulated by androgens, both in a negative and in a positive fashion. Well known examples of upregulating are the rat C3(1) gene (Page & Parker, 1982), the mouse RP2 gene (Rheaume et al., 1989) and the rat SVS IV and V genes (Higgins & Hemmingway, 1991). Downregulated by androgens in the rat prostate are TRPM-2 (Montpetit et al., 1986; Leger et al., 1987) and TGF-beta (Kyprianou & Isaacs, 1989). In LNCaP cells AR mRNA expression is downregulated by androgen treatment (Quarmby et al., 1990; Trapman et al., 1990). It is unknown of most of these genes whether a direct effect of androgens on transcription of the gene, by activation of the AR, is an important part of the regulatory mechanism.

PA (Prostate-specific Antigen) is among the genes which are regulated by androgens (Riegman et al., 1991). In this study we have shown that mRNA expression of the PA gene can be upregulated on the level of transcription by the synthetic androgen R1881. This will represent at least part of the total of androgen-responsive regulation

mechanisms, which determine the final PA mRNA level. Using a series of different experimental approaches, we have provided evidence that the promoter region of the PA gene contains a functional ARE at position −170 to −156 (AGAACAgcaAGTGCT). This element can function both in the context of the PA promoter and as a separate element, if cloned in front of the TK promoter.

It has been established for several steroid receptors that they bind to their responsive element in the form of a homodimer, one component of the dimer interacting with one half-site of the palindromic sequence (Tsai et al., 1988; Kumar & Chambon, 1988; Eriksson & Wrange, 1990; Fawell et al., 1990; Dahlman-Wright et al., 1991). Critical bases in the GRE were identified by determination of the effect of point mutations in the PRE/GRE consensus sequence on the trans-activating capacity of the GR (Nordeen et al., 1990). The sequence of the functional ARE in the PA promoter is closely related to the reverse complement consensus sequence AGAACAnnnTGTACC for binding of the GR and PR (Carson-Jurica, 1990). Comparison of the ARE sequence at position −170 to −156 AGAACAnnnAGTGCT (differences with the reverse complement of the consensus PRE/GRE are underlined) with the GRE mutation data showed that the T at position -156 would cause some (about 70%) reduction in GR-mediated transcription activation (Nordeen et al., 1990). No data were available on the two other differences, the A at position −161 instead of T and the G at position −158 instead of A. So, as far as studied up to now the −170 to −156 PA-ARE will be able to act as an effective GRE/PRE. It remains to be investigated, however, whether the other two changes within the PA-ARE sequence as compared to the GRE/PRE consensus sequence or differences in the flanking sides can differentially affect regulation by GR, PR or AR.

In synthetic promoter constructs, synergistic activity has been described for the GR and PR with factors binding to the SP-1, Oct-1, NF1 or CACCC-box consensus sequence; two GRE/PREs can also act synergistically (Schüle et al., 1988a,b; Strahle et al., 1988; Allan et al., 1991). Binding of PR or GR to the PRE/GRE facilitates binding of Oct-1 to the octamer-binding motif in the MMTV promoter (Brüggemeier et al., 1991). Similarly, in other GR, PR or ER-regulated natural promoters evidence has been collected for cooperation between a steroid receptor and other transcription factors (Cato et al., 1988; Day et al., 1990; Martinez et al., 1991). No such data have been available so far for the AR. The region −539 to −320 in the PA promoter has a marked enhancing effect on the R1881-induced upregulation of the promoter, without affecting basal activity (see Figure 3). Mutation of the ARE in pPA2-CAT resulted in an almost

complete loss of the transcription activation by R1881 (see Figure 4). These findings imply that the −539 to −320 region acts synergistically with the ARE. So far we have not been able to identify an obvious homology between sequences in this fragment with a consensus sequence of a well described regulatory element. Further fine mapping of the −539 to −320 fragment is needed to establish its exact role in transcription activation of the PA promoter. Furthermore, it remains to be established whether this region has a similar function in prostate cells. The CACCC motif in the PA promoter (position −129 to −125), which is located close to the ARE, has no effect on R1881-induced expression, because deletion of this element did not result in a significant change in CAT activity in cells treated with R1881 or in untreated cells (data not shown).

Expression of hGK-1 mRNA can also be regulated by R1881 (Riegman et al., 1991). The homology between the promoter regions of the PA and hGK-1 genes is very high (91% from −190 to +1) (Schedlich et al., 1987; Riegman et al., 1989a). Preliminary data indicate that the R1881-regulated mRNA expression of this gene is mediated by the GGAACAnnnAGTGCT motif, which is located at the same position as the ARE in the promoter region of the PA gene (Riegman et al., 1989). The hGK-1 motif has one difference with the PA–ARE, a G instead of an A residue at −170 (GGAACAgcaAGTGCT). It has been shown that such a change, if introduced in a GRE, has no marked consequences for the transcriptional activity of the GR (Nordeen et al., 1990), so it will most probably also not affect AR activity. A striking difference between the PA and the hGK–1 promoter regions is the presence of an Alu repeat in the hGK-1 promoter at position −322 to −627. This repeat is situated between the AR-binding motif and the region, which shows synergism with the ARE in the PA promoter. So far, however, no experimental data have been collected to attribute a function to the repeat.

PA belongs to the kallikrein gene family (Clements, 1989). The promoter regions of these closely related genes are conserved in different species. Like PA and hGK–1, at least part of the mouse (mGK–3, mGK–4 and mGK–6) and rat (rGK–2, RSGK–2 and rGK–8) kallikrein genes show regulation of mRNA expression by androgens (van Leeuwen et al., 1987; Clements et al., 1988; Clements, 1989; Winderickx et al., 1989). At a position identical to that of the PA–ARE the alignment of the various promoters indicates in general the presence of an element with less homology to the PRE/GRE consensus. This finding suggests that the PA–ARE is probably not conserved

as a functional ARE in promoter regions of most of the androgen-regulated rat and mouse kallikrein genes.

In studies concerning the androgen-regulated expression of the rat prostate C3(1) gene, a strong androgen-binding region has been located in the first intron (Rushmere et al., 1987; Claessens et al., 1990). This fragment also confers androgen responsiveness to the TK promoter (Claessens et al., 1989). It contains the sequence AGTACGtgaTGTTCT, resembling the PRE/GRE consensus. Mutation of this sequence abolished androgen responsiveness of the TK–CAT construct described above. The PA–ARE and the C3(1)–ARE have an opposite orientation and differ in only 3 nucleotides (two of which are in the GRE/PRE consensus) AGAACAgcaAGTGCT (differences are underlined).

Construction of transgenic mice indicate that all information needed for prostate-specific and androgen-regulated expression of the C3(1) gene resides in a 9.5 kb fragment containing the complete C3(1) gene with additional 4 kb upstream and 2.5 kb downstream (Allison et al., 1989). No induction was seen if fragments of the C3(1) gene were cloned directly in front of the CAT gene or in front of a TK–CAT construct and transfected to prostate cells (Parker et al., 1988). Similarly, we were unsuccessful in expressing pPA–CAT constructs in LNCaP cells. This implies that in both cases (tissue-specific) element(s) could be missing from the context of the total transcription machinary. Another explanation can be a low transfection efficiency of the various prostate cell lines. The identification of new (tissue-specific) regulatory elements from either the PA or the C3(1) gene will be of extremely importance for further analysis of androgen-regulated gene expression in the prostate. Experiments in this direction are in progress.

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REFERENCES

Allan,G.F., Ing,N.H., Tsai,S.Y., Srinivasan,G., Weigel,N.L., Thompson,E.B., Tsai,M–J. & O'Malley,B.W. (1991) J. Biol. Chem. 266, 5905-5910.

Allison,J., Zhang,Y–L. & Parker,M.G. (1989) Mol. Cell. Biol. 9, 2254-2257.

Day,R.N., Koike,S., Sakai,M., Muramatsu,M. & Maurer,R.A. (1990) Mol. Endocrinol. 4, 1964–1971.

Beato,M. (1989) Cell 56, 335–344.

Brinkmann,A.O., Faber,P.W., Rooij,H.C.J. van, Kuiper,G.G.J.M., Ris,C., Klaassen,P.,

Korput,J.A.G.M. van der, Voorhorst,M.M., Laar,J.H. van, Mulder,E, & Trapman,J. (1989) J. Steroid Biochem. 34, 307–310.
Brüggemeier,U., Kalff,M., Franke,S., Scheideret,C. & Beato,M. (1991) Cell 64, 565-572.
Carson–Jurica,M.A., Schrader,W.T. & O'Malley,B.W. (1990) Endocrine Rev. 11, 201–220.
Cato,A.C.B., Henderson,D & Ponta,H. (1987) EMBO J. 6, 363-368.
Cato,A.C.B., Heitlinger,E., Ponta,H., Klein-Hitpass,L., Ryffel,G.U., Bailly,A., Rauch,C. & Milgrom,E. (1988) Mol. Cell. Biol. 8, 5323–5330.
Cato,A.C.B., Skroch,P., Weinmann,J., Butkeraitis,P. & Ponta,P. (1988) EMBO J. 7, 1403-1410.
Chang C., Kokontis.J. & Liao,S. (1988) Proc. Natl. Acad. Sci. USA 85, 7211–7215.
Chen,C. & Okyama,H. (1987) Mol. Cell. Biol. 7, 2745–2752.
Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. & Rutter,W.J. (1979) Biochemistry 18, 5294–5299.
Claessens,F., Rushmere,N.K., Davies,P., Celis,L., Peeters,B. & Rombauts,W.A. (1990) Mol. Cell. Endocrinol. 74, 203–212.
Claessens,F., Celis,L., Peeters,B., Heyns,W. & Verhoeven,G. (1989) Biochem. Biophys. Res. Comm. 164, 833-840.
Clements,J.A., Matheson,B.A., Wines,D.R., Brady,J.M., MacDonald,R.J. & Funder,J.W. (1988) J. Biol. Chem. 263, 16132-16137.
Clements,J.A. (1989) Endocrine Rev. 10, 393–419.
Dahlman–Wright,K., Wright,A., Gustafsson,J–A. & Carlstedt-Duke,J. (1991) J. Biol. Chem. 266, 3107-3112.
Eriksson,P. & Wrange,O. (1990) J. Biol. Chem. 265, 3535–3542.
Evans,R.M. (1988) Science 240, 889–895.
Faber,P.W., Kuiper,G.G.J.M., Rooij,H.C.J. van, Korput,J.A.G.M. van der, Brinkmann,A.O. & Trapman,J. (1989) Mol. Cell. Endocrinol. 61, 257–262.
Gorski,K., Carniero,M. & Schibler,U. (1987) Cell 47, 767–776.
Fawell,S.E., Lees,J.A., White,R. & Parker,M.G. (1990) Cell 60, 953–962.
Gallee,M.P.W., Vroonhoven,C.C.J van, Korput,J.A.G.M. van der, Kwast,T.H. van der, Kate,F.J.W. ten, Romijn,J.C. & Trapman,J. (1986) Prostate 9, 33–45.
Gorman,C. (1985) DNA cloning a practical approach vol III. Glover,D.M. (ed), IRL press Oxford pp 143–190.
Green,S. & Chambon,P. (1988) Trends Gen. 4, 309–314.
Green,S., Kumar,V., Theulaz,I., Wahli,W. & Chambon,P. (1988) EMBO J. 7, 3037-3044.
Ham,J., Thomson,A., Needham,M., Webb,P. & Parker,M (1988) Nucl. Acids Res. 16, 5263–5277.
Härd,T., Kellenbach,E., Boelens,R., Maler,B.A., Dahlman,K., Freedman,L.P., Carlstedt-Duke,J., Yamamoto,K.R., Gustafsson,J.A. & Kaptein,R. (1990) Science 249, 157–160.
Harrington,M.A., Jones,P.A., Imagawa,M. & Karin,M. (1988) Proc. Natl. Acad. Sci. USA 85, 2066-2070.
Higgins,S.J. & Hemingway,A.L. (1991) Mol. Cell. Endocrinol. 76, 55–61.
Jenster,G., Korput,H.A.G.M. van der, Vroonhoven,C. van, Kwast,Th.H. van der, Trapman,J. & Brinkmann,A.O. (1991) Mol. Endocrinol. (In Press).
Kumar,V. & Chambon,P. (1988) Cell 55, 145–156.
Kyprianou,N. & Isaacs,J (1989) Mol. Endocrinol. 3, 1515–1522.
Leeuwen,B.H. van, Penschow,J.D., Coghlan,J.P. & Richards,R.I. (1987) EMBO J. 6, 1705-1713.

Leger,J.G., Montpetit,M.L. & Tenniswood,M.P. (1987) Biochem. Biophys. Res. Commun. 147, 196–203.
Linial,M., Ginderson,N. & Groudine,M. (1985) Science 230, 1126–1132.
Lubahn,D.B., Joseph,D.R., Sar,M., Tan,J., Higgs,H.N., Larson R.E., French,F.S. & Wilson,E.M. (1988) Mol. Endocrinol. 2, 1265–1275.
Luckow,B. & Schütz,G. (1987) Nucl. Acids Res. 15, 5490.
Mader,S., Kumar,V., Verneuil,H. de & Chambon,P. (1989) Nature 338, 271-274.
Martinez,E., Dusserre,Y., Wahli,W, & Mermod,N. (1991) Mol. Cell. Biol. 11, 2937–2945.
Maxam,A.M. & Gilbert,W. (1980) Methods Enzymol. 65, 499–560.
Montpetit,M.L., Lawlep,K.R. & Tenniswood,M.P. (1986) Prostate 8, 25–36.
Nordeen,S.K., Suh,B.J., Kühnel,B. & Hutchinson III,C.A. (1990) Mol. Endocrinol. 4, 1866-1873.
Otten,A.D., Sanders,M.M. & McKnight,G.S. (1988) Mol. Endocrinol. 2, 143–147.
Page,M.J. & Parker,M.G. (1982) Mol. Cell. Endocrinol. 27, 343-355.
Parker,M.G., Webb,P., Mills,J.S., Needham,M. & Tilly,R. (1988) J. Steroid Biochem. 30, 47-51.
Quarmby,V.E., Yarbrough,W.G., Lubahn,D.B., French,F.S. & Wilson,E.M. (1990) Mol. Endocrinol. 4, 22–28.
Rheaume,C., Barbour,K.W., Tseng-Crank,J. & Berger,F.G. (1989) Mol. Cell. Biol. 9, 477–483.
Riegman,P.H.J., Klaassen,P., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1988) Biochem. Biophys. Res. Commun. 155, 181–188.
Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1989a) Biochem. Biophys. Res. Commun. 159, 95-102.
Riegman,P.H.J., Vlietstra,R.J., Klaassen,P., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1989b) FEBS Lett. 247, 123–126.
Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1991) Mol. Cell. Endocrinol. 76, 181-190.
Rundlett,S.E., Wu,X–P. & Miesfeld,R.L. (1990) Mol. Endocrinol. 4, 708–714.
Rushmere,N.K., Parker,M.G. & Davies,P. (1987) Mol. Cell. Endocrinol. 51, 259-265.
Sambrook,J., Fritsch,E.F. & Maniatis,T. (1989) Molecular cloning: A Laboratory Manual, Cold Spring Harbor Laboratory press NY
Sanger,F., Nicklen,S. & Coulson,A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463–5467.
Schedlich,L.J., Bennetts,B.H. & Morris,B.J. (1987) DNA 6, 429–437.
Schonk,D., Dijk,P. van, Riegman,P., Trapman,J., Holm,C., Willcocks,T.C., Sillekens,P., Venrooij,W. van, Wimmer,E.M., Geurts van Kessel,A., Ropers,H.H. & Wieringa,B. (1990) Cytogenet. Cell. Genet. 54, 15–19.
Schüle,R., Muller,M., Otsuka-Murakami,H. & Renkawitz,R. (1988) Nature 332, 87-90.
Schüle,R., Muller,M., Kaltschmidt,C. & Renkawitz,R. (1988) Science 242, 1418-1420.
Schwabe,J.W.R., Neuhaus,D. & Rhodes,D. (1990) Nature 348, 458-461.
Seed,B. & Sheen,J–Y. (1988) Gene 67, 271–277.
Simental,J.A., Sar,M., Lane,MV., French,FS. & Wilson,EM. (1991) J. Biol. Chem. 266, 510-518.
Strahle,U., Schmid,W. & Schutz,G. (1988) EMBO J. 7, 3389–3395.
Trapman,J., Klaassen,P., Kuiper,G.G.J.M., Korput,J.A.G.M. van der, Faber,P.W., Rooij,H.C.J. van, Geurts van Kessel,A., Voorhorst,M.M., Mulder,E. & Brinkmann,A.O. (1988) Biochem. Biophys. Res. Commun. 153, 241-248.
Trapman,J., Ris-Stalpers,C., Korput,J.A.G.M. van der, Kuiper,G.G.J.M., Faber,P.W., Romijn,J.C., Mulder,E. & Brinkmann,A.O. (1990) J. Steroid Biochem. 37,

837–842.
Tsai,S.Y., Carlstedt–Duke,J., Weigel,N.L., Dahlman,K., Gustafsson,J.A., Tsai,M.J. & O'Malley,B.W. (1988) Cell 55, 361–369.
Umesono,K. & Evans,R.M. (1989) Cell 57, 1139–1146.
Vallee,B.L., Coleman,J.E. & Auld,D.S. (1991) Proc. Natl. Acad. Sci. USA 88, 999-1003.
Vos,P. de, Claessens,F., Winderickx,J., Dijck,P. van, Celis,L., Peeters,B., Rombauts,W., Heyns,W. & Verhoeven,G. (1991) J. Biol. Chem. 266, 3439-3443.
Wang,M.C., Valenzuela,L.A., Murphy,G.P. & Chu,T.M. (1979) Invest. Urol. 17, 159–163.
Wang,M.C., Papsidero,L.D., Kuriyama,M., Valenzuela,L.M., Murphy,G.P. & Chu,T.M. (1981) Prostate 2, 89–96.
Winderickx,J., Swinnen,K., Dijck,P. van, Verhoeven,G. & Heyns,W. (1989) Mol. Cell. Endocrinol. 62, 217–226.
Yamamoto K.R. (1985) Annu. Rev. Genet. 19 209–252.
Zilliacus,J., Dahlman-Wright,K., Wright,A., Gustafsson,J–A. & Carlstedt-Duke,J. (1991) J. Biol. Chem. 266, 3101-3106.

CHAPTER VIII

GENERAL DISCUSSION

The experimental work described in this thesis was initiated in order to gain insight in the process of gene expression in the normal prostate and in prostate carcinoma. The development of the prostate is initially controlled by epithelial-stromal cell interactions. Normal growth and development of the prostate is androgen-dependent. Absence of androgens or functional androgen receptors (ARs) strongly inhibits the development of the prostate and maintenance of morphology and functional activity of the adult prostate, including expression of PA and hGK-1.

Kallikrein genes:

We have characterized the human kallikrein locus. The human kallikrein gene family consists of three genes: PA, HGK-1 and tissue-kallikrein (KLK-1). These genes share a high percentage of mutual structural homology. The molecular cloning of these genes allows the analysis of PA, HGK-1 and KLK-1 gene expression, including detailed analysis of the promoter regions of the respective genes.

Comparison of the human kallikrein genes with kallikrein genes of other species showed considerable structural conservation (discussed extensively in Chapter I 4.3). The expression of the individual kallikreins is differentially regulated by steroid hormones (see Chapter I 4.2). Differential regulation was also found for the tissue-specific expression of the individual kallikreins. Since the homology between the different kallikrein genes extends in the promoter regions (see Figure 4.2.1), promoter sequences can be compared to certain aspects of transcription regulation of the various genes. Therefore, the kallikrein genes form a unique model system for hormone-responsive and tissue-specific transcription regulation. In this study a beginning has been made with the unraveling of the mechanism of kallikrein expression in the human prostate.

PA transcription regulation as a model for androgen-responsive and prostate-specific gene expression:

A functional ARE was identified at position -170 to -155 in the promoter region of the PA gene. It could be determined that the androgen-responsive PA mRNA expression is largely determined by the presence of this ARE and a more upstream region (-539 to -320). Synergism was demonstrated between the ARE and the upstream region (Riegman et al., 1991, Chapter VII). This model will allow the determination of AR regions involved in synergistic co-operation between the AR and other transcription

factors by analysis of the activity of mutated ARs in cotransfection experiments. Identification of the response element(s) involved in the synergistic interaction, and subsequent characterization of the transcription factor(s) binding to this element will contribute to a better understanding of the process of androgen-responsive transcription regulation.

Although it is clear that PA expression can function as a good model system for basal androgen-responsive transcription regulation, so far, one of the major limitations of this model is that analysis of PA promoter activity in prostate cells, through transfection with PA promoter–CAT reporter gene constructs, gave negative results. For this reason we were unable to collect data on androgen-responsive and tissue-specific regulation of the PA gene in prostate cells. On the other hand, it is remarkable that PA promoter activity could be observed in non-prostate cell lines like COS and HeLa in the presence of the AR (although HeLa cell values were very low). The differences observed could be explained in several ways:

(I) Low transfection efficiency of the only human prostate cell line (LNCaP) available for such studies. Many different transfection methods have been tried, but none gave rise to a measurable PA promoter. Presently, experiments are in progress to test the activity of a more sensitive reporter gene (luciferase) in LNCaP cells, when hooked to the PA promoter. Preliminary experiments indicate that this reporter gene can indeed be used for measuring of PA promoter activity.

(II) The absence of (tissue-specific) element(s), which are essential for high PA expression, in the PA promoter constructs used. If present, these elements might be located far outside the proximal promoter region, as described for several other genes like albumin and the globin genes (Pinkert et al., 1987; Herbst et al., 1989; Orkin, 1990). Identification of prostate-specific enhancer elements and incorporation of these elements in PA promoter-CAT constructs could make it possible to study androgen-regulated transcription in prostate cells.

(III) Absence of the proper extra-cellular signals triggering for high PA expression in LNCaP cells. It is not unlikely that part of the high level of PA expression in the intact prostate is caused by factors produced by stromal cells, which interact with the epithelial cells. Stromal cells or conditioned medium from stromal cells are absent in the transfection system used, and have not been tested as yet.

AR, PR and GR target gene specificity:

The ARE in the promoter region of the PA gene and the ARE in the C3(1) gene are highly homologous to the PRE/GRE consensus sequence. No differences were found in the protein contact sites within the PRE/GRE between PR and GR (Cairns et al., 1991). Comparison of AR binding to PRE/GRE sequences by methylation interference showed no difference in PR, GR and AR binding to the different responsive elements (Marschke unpublished results). So, it seems that the responsive element by itself does not determine whether a gene containing a PRE/GRE/ARE is a specific target for one special steroid receptor. The presence of the responsive element in the regulatory region is, however, a prerequisite for a gene to be a target for the PR, GR or AR. Synergism, cooperativity between different transcription factors, and expression levels of PR, GR or AR may be the most important determinants in the target gene specificity. This hypothesis is consistent with the data obtained by comparing the promoter regions of the kallikrein genes and the regulation their mRNA expression in different cell types (see Chapter I 4.3). That cooperativity between other (tissue-specific) transcription factors and the AR can be of importance for androgen-responsive transcription regulation has recently been shown in the case of mouse ß–glucuronidase expression. The ß–glucuronidase gene is constitutively expressed in all cells, but ß–glucuronidase mRNA expression is exclusively in epithelial kidney cells responsive to androgens. This response requires an intact GRE(ARE) and cooperative binding of an androgen-inducible kidney-specific factor in a complex regulatory region present in intron 9 of the gene (Ganschow et al., 1991). The AR and the androgen-inducible kidney-specific factor act synergistically in this system. A similar mechanism was found in the regulation of the RP–2 gene (Rhee et al., 1991). There is no indication for a cell-specific component in the synergism between the ARE and the upstream fragment, because both COS and HeLa cells seem to contain factors which can cause the effect. Whether in prostate cells different, prostate-specific, transcription factors are important for cooperation with the AR remains to be established.

REFERENCES

Cairns,C., Gustafson,J-A. & Carstedt-Duke,J. (1991) Mol. Endocrinol. 5, 598-604.

Ganschow,R.E., Wang,B., Lund,S.D. & Porter,S.C. (1991) 73rd meeting of the Endocrine Society, Abstract 1470. Mol. Cell. Biol.CB?

Herbst,R.S., Friedman,N., Darnell,J.E. & Babiss,L.E. (1989) Proc. Natl. Acad. Sci. USA 86, 1553-1557.

Pinkert,C.A., Ornitz,D.M., Brinster,R.L. & Palmiter,R.D. (1987) Genes Dev. 1, 268-276.

Renkawitz,R. (1990) Trends Gen. 6, 192-197.

Rhee,M Dimaculangan,D. & Berger,F.G. (1991) Mol. Endocrinol. 5, 564-572.

Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M. van der & Trapman,J. (1991) Mol. Endocrinol. 5, 1921-9130.

SUMMARY:

In our aim to generate a model to study gene expression in the human prostate, PA cDNAs and the corresponding gene were isolated. PA was chosen, because it is exclusively synthesized by the epithelial cells of the prostate. In Chapter II the isolation of three different PA cDNAs from a PC 82 prostate cDNA library is described. Oligonucleotides deduced from the PA amino acid sequence were used as probes. Seven different PA transcripts were detected in RNA isolated from the prostate tumors PC 82 and PC EW, ranging in size from 0.5 to 5.6 kb. PA 75 cDNA represents the major 1.5 kb mRNA. PA 424 correlates with a 0.9 kb transcript and PA 525 with a 1.9 kb mRNA species. PA 75 contains the almost complete open reading frame encoding the PA preproprotein. PA 525 contains an additional 0.4 kb fragment, as a result of alternative splicing. PA 424 contains a 0.15 kb fragment, as a result of intron retention, and a poly(A) tail preceded by an AAGAAA motif. The predicted protein products of PA 525 and PA 424 will be different from PA 75 at the C-terminal end and will probably be devoid of serine protease activity.

The isolation and characterization of the PA gene is described in Chapter III. The PA gene was isolated from a human genomic library, using PA cDNA fragments as a probe. The complete gene, about 6 kb, was sequenced and shown to be composed of four introns and five exons. Two transcription start sites located 7 bp apart were identified. The PA gene was shown to share 82% homology with the hGK-1 gene. The homology extends into the promoter region of both genes, which includes various well known transcription regulatory elements.

In addition to the PA gene, the two other human kallikrein genes, hGK-1 and KLK1, were molecularly cloned. The hGK-1 gene shares the highest homology with PA and is, like PA, exclusively expressed in prostate tissue. The third gene KLK1 is expressed in kidney, pancreas and salivary glands. In Chapter IV the chromosomal localization and the chromosomal organization of the PA and hGK-1 genes is described. Because the genomic clones containing the PA and hGK-1 genes overlap, it could be deduced that the PA and hGK-1 genes have identical orientations and are separated by 12 kb. Both genes were assigned to chromosome 19, by Southern blot analysis of DNA from a panel of human-hamster hybrid cells with specific probes. Further mapping of the surroundings of the human kallikrein genes is presented in Chapter V. These human kallikrein genes showed clustering on a segment of 60 kb. The KLK1 gene is positioned

in the opposite orientation of the APS and KLK2 genes in the order KLK1–APS–KLK2. The distance between KLK–1 and PA was estimated to be 31 kb. An unknown gene is located between the KLK1 and PA genes within the human kallikrein locus. This gene has the identical orientation as the PA and hGK–1 genes and expresses a 4.8 kb transcript in all cell lines tested so far.

In order to be able to distinguish and compare PA and hGK-1 mRNA expression, hGK–1 cDNAs were cloned. The molecular cloning of the hGK–1 cDNAs is described in Chapter VI. Two different hGK–1 cDNA clones were isolated from a prostate cDNA library with a genomic DNA fragment as probe. pGK–1 represents the major 1.5 kb mRNA. pGK–1 contains the almost complete open reading frame encoding a predicted hGK–1 preprotein. pGK–10A contains an additional 37 bp fragment, as a result of alternative splicing. The mature protein (excluding presumed pre- and propeptides) as deduced from the pGK-10A cDNA sequence, has a size of 199 amino acids and differs at the COOH–terminus from the 237 amino acid hGK-1 protein. The alternatively spliced mRNA comprises approximately 20 % of the hGK-1 transcripts.

The cDNA sequences enabled the design of PA and hGK–1 specific oligonucleotides. The regulation of PA and hGK-1 mRNA expression was studied in different human prostate tumors and cell lines by Northern blotting, using the PA and hGK-1 specific oligonucleotides as probes. A high level of PA and hGK-1 expression was found in the androgen-dependent tumors PC 82 and PC EW. PA and hGK-1 mRNA was also present in the androgen-sensitive LNCaP cell line, but undetectable in the androgen-insensitive prostate tumors PC 133, PC 135 and the PC 3 cell line. In LNCaP cells, the expression of PA and hGK-1 mRNA was strongly induced by androgens.

The identification of a functional androgen response element in the promoter region of the PA gene is described in Chapter VII. Run-on experiments with nuclei isolated from LNCaP cells showed that at least part of the androgen-responsive regulation of PA mRNA expression is on the level of gene transcription. DNase I footprints of the promoter region of the PA gene with nuclear extracts from LNCaP cells showed protections, which include the TATA–box, an Sp1–binding site, and a sequence AGAACAgcaAGTGCT, at position –170 to -156, resembling the PRE/GRE consesus sequence. Fragments of the PA promoter region were cloned in front of the CAT reporter gene and cotransfected with an androgen receptor expression plasmid into COS cells in a transient expression assay. CAT activity of COS cells grown in the presence of androgens was compared to untreated controls. A 110–fold induction of

CAT activity was found if a −1600 to +12 PA promoter fragment was used in the construct. By further deletion mapping of the PA promoter a minimal region (−320 to −155) was identified as being essential for androgen-regulated gene expression. Mutation of the sequence AGAACAgcaAGTGCT (−170 to −156) almost completely abolished androgen inducibility of PA promoter-reporter gene constructs. One or more copies of the sequence AGAACAgcaAGTGCT could confer androgen-responsiveness to a heterologous promoter. Synergism was demonstrated between the androgen responsive element and an upstream region (−539 to −320).

SAMENVATTING:

Met als doel een model te genereren om gen expressie in prostaatcellen te bestuderen, werden PA cDNAs en het corresponderende gen gekloneerd. Gekozen werd voor het PA gen, omdat PA alleen tot expressie komt in de epitheliale cellen van de prostaat. In hoofdstuk II wordt de isolatie van drie verschillende PA cDNAs uit een PC 82 cDNA bank beschreven. Als probes werden oligonucleotiden gebruikt waarvan de sequentie was afgeleid van de PA aminozuur volgorde. In RNA geisoleerd uit de prostaat tumoren PC 82 en PC EW, konden zeven verschillende transcripten worden gedetecteerd, die varieerden in grootte van 0,5 tot 5.6 kb. Het cDNA PA 75 vertegenwoordigt het meest abundante PA transcript met een lengte van 1,5 kb. PA 424 komt overeen met een PA transcript van 0,9 kb en PA 525 met één van 1,9 kb. PA 75 bevat bijna het gehele "open lees frame", dat codeert voor het PA preproeiwit. Door "alternative splicing" bevat PA 525 een extra 0,4 kb fragment. PA 424 bevat een extra 0,15 kb fragment, omdat een intron in zijn geheel niet "gespliced" wordt, en een poly(A) staart voorafgegaan door een AAGAAA sequentie. Alleen aan de C-terminus van de voorspelde eiwit produkten van PA 424 en PA 525 zullen verschillen zijn te vinden met het eiwit produkt van PA 75 en waarschijnlijk bezitten deze eiwitten geen serine protease activiteit.

De isolatie en karakterisatie van het PA gen wordt beschreven in hoofdstuk III. Van het complete gen, dat een lengte heeft van ongeveer 6 kb, werd de sequentie in zijn geheel bepaald. Het gen bleek te bestaan uit vier intronen en vijf exonen. Twee transcriptie startplaatsen werden geidentificeerd, die op een afstand van 7 baseparen van elkaar liggen. Het PA gen vertoont 82% homologie met het hGK-1 gen. Deze homologie loopt door tot in de promotoren van beide genen en bevat bindingsplaatsen voor bekende transcriptiefaktoren.

Naast het PA gen werden twee andere humane kallikreine genen gecloneerd, hGK-1 en KLK-1. hGK-1 is het meest homoloog aan het PA gen en expressie van dit gen vindt, net als de expressie van het PA gen, alleen plaats in prostaat weefsel. Het derde gen (KLK-1) komt to expressie in de nier, pancreas en speeksel klieren. In hoofdstuk V wordt de chromosomale organisatie en localisatie van de PA en hGK-1 genen beschreven. Uit het feit dat de geisoleerde genomische cloons waarop PA en hGK-1 gelegen zijn met elkaar overlappen, kon worden afgeleid, dat PA en hGK-1 dezelfde orientatie hebben en dat de genen 12 kb uit elkaar liggen. Analyse van "Southern blots"

met DNA afkomstig uit een serie mens-hamster hybride cellen met specifieke probes toonde aan, dat beide genen op chromosoom 19 liggen. In hoofdstuk V wordt beschreven hoe de omgeving van de humane kallikreine genen verder in kaart werd gebracht. Het bleek dat de humane kallikreine genen geclusterd liggen op een gebied van 60 kb. Het KLK1 gen heeft een tegenovergestelde orientatie ten opzichte van PA en hGK-1 (KLK2) in de volgorde KLK1-APS-KLK2. De afstand tussen de PA en KLK-1 genen kon worden bepaald op 31 kb. Een ander, nog onbekend gen ligt tussen het KLK-1 gen en het PA gen in, in het humane kallikreine locus dat is gelocaliseerd op chromosoom 19q13.2-13.4. Dit gen heeft dezelfde orientatie als PA en hGK-1 en brengt een 4,8 kb RNA transcript tot expressie in de geteste humane cellijnen.

Om onderscheid te kunnen maken tussen PA en hGK-1 mRNA expressie werden hGK-1 cDNAs gecloneerd. Deze cDNAs worden gepresenteerd in hoofdstuk VI. Twee verschillende hGK-1 cDNAs werden geisoleerd uit een prostaat cDNA bank met als probe een genomisch fragment. Het cDNA pGK-1 vertegenwoordigt het meest voorkomende hGK-1 transcript met een lengte van 1,5 kb. pGK-1 bevat bijna het gehele "open lees frame", dat codeert voor het voorspelde hGK-1 preproeiwit. Door "alternative splicing" bevat pGK-10A een extra fragment van 37 bp. Het eiwit (zonder de veronderstelde pre- en propeptides) afgeleid van pGK-10A bestaat uit 199 aminozuren en verschilt aan de C-terminus van het 237 amino zuren tellende hGK-1 eiwit. De "alternative splice", zoals die is gevonden in pGK-10A, omvat 20% van de hGK-1 transcripten. De sequentie van de cDNAs maakte het mogelijk om PA en hGK-1 specifieke oligonucleotiden af te leiden. Met behulp van "Northern Blots" en de specifieke oligonucleotiden als probes werd de regulatie van expressie van PA en hGK-1 bestudeerd in humane prostaat tumoren en cellijnen. PA en hGK-1 komen hoog tot expressie in de androgeen afhankelijk groeiende prostaat tumoren PC 82 en PC EW. PA en hGK-1 transcripten waren ook detecteerbaar in de androgeen gevoelige cellijn LNCaP, maar niet in de androgeen ongevoelige prostaat tumoren PC 133 en PC 135 en de ongevoelige cellijn PC 3. The expressie van PA en hGK-1 kon sterk geinduceerd worden in LNCaP cellen gegroeid in de aanwezigheid van androgenen.

In hoofdstuk VII wordt de identificatie van een functioneel androgeen responsief element in de promoter van het PA gen beschreven. Met behulp van "run-on" experimenten uitgevoerd met kernen, geisoleerd uit LNCaP cellen, kon worden vastgesteld, dat tenminste een gedeelte van de androgeen responsieve regulatie van PA mRNA plaatsvindt op het niveau van transcriptie. "DNase I footprints" met het

fragment −320 tot +12 van de promoter van het PA gen, uitgevoerd met nucleaire eiwit extracten van LNCaP cellen, lieten bescherming zien van de "TATA-box", een Sp1-bindingsplaats en de sequentie AGAACAgcaAGTGCT, die sterke gelijkenis vertoond met de PRE/GRE consensus sequentie, op positie −170 to −156. Fragmenten van de PA promoter werden gecloneerd voor het CAT reporter gen en vervolgens gecotransfecteerd met een androgeen receptor expressieplasmide naar COS cellen in een "transient expression assay". De CAT activiteit van deze COS cellen, die werden behandeld met androgenen, werd vergeleken met onbehandelde, getransfecteerde COS cellen. Een 110-voudige inductie van CAT activiteit werd gevonden met het CAT reporter gen construct, dat het fragment van −1600 tot +12 van de PA promoter bevatte. Met behulp van "deletion mapping" van dit gedeelte van de PA promoter kon een minimaal fragment (−320 tot −155) worden geidentificeerd, dat essentieel bleek te zijn voor androgeen gereguleerde gen expressie. Mutatie van de sequentie AGAACAgcaAGTGCT (−170 tot −156) maakte de PA-promoter reporter gen constructen vrijwel ongevoelig voor androgenen. Eén of meerdere kopieen van deze sequentie AGAACAgcaAGTGCT kunnen ervoor zorgen, dat een heterologe promoter gereguleerd kan worden door androgenen. Tussen dit androgeen responsieve element en een fragment (−539 tot −320) werd synergisme aangetoond.

CURRICULUM VITAE

4 mei 1959	Geboren te Rotterdam.
1977	Eindexamen HAVO aan de Scholengemeenschap Van Oldenbarnevelt te Rotterdam.
1977 - 1982	Leraren Opleiding Zuid West Nederland te Delft. Biologie 2° graads lesbevoegdheid. Natuurkunde 3° graads lesbevoegdheid.
1982 - 1986	Rijks Universiteit Leiden, Subfaculteit Biologie. Hoofdvak: Moleculaire Genetica, afd. Pathologie, Erasmus Universiteit Rotterdam. Dr. J. Trapman, Prof. Dr. D. Bootsma (afd. Genetica). Bijvak: Celbiologie, afd. Celbiologie Dr. W. de Priester, Prof. Dr. T.M. Konijn. Onderwijsbevoegdheid: 1° graads lesbevoegdheid Biologie.
1986-1991	Promotie onderzoek op de afdeling Pathologie van de Erasmus Universiteit Rotterdam. Promotor: Prof. Dr. D. Bootsma (afd. Genetica). Co-promotor: Dr. J. Trapman.
1991-heden	Aanstelling als tijdelijk wetenschappelijk onderzoeker werkzaam aan de genomische karakterisatie van het meningioma tumor suppressor gen op de afdeling Pathologie van de Erasmus Universiteit Rotterdam. Onder leiding van: Dr. E.C. Zwarthoff.

PUBLICATIONS:

- Molecular cloning and characterization of novel prostate antigen cDNAs.
  Riegman,P.H.J, Klaassen,P., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1988) Biochem. Biophys. Res. Commun. 155, 181-188.

- Expression of (proto)oncogenes and genes encoding growth factors and growth factor receptors and prostate antigen in human prostate carcinoma cells.
  Trapman,J., Jenster,G., Riegman,P.H.J., Klaassen,P., Korput,J.A.G.M. van der, Steenbrugge,G.J. van and Romijn,J.C. (1988) Prog. Cancer Res. Ther. 35, 102–109.

- Characterization of the prostate-specific antigen gene : a novel human kallikrein-like gene.
  Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1989) Biochem. Biophys. Res. Commun. 159, 95-102.

- The prostate-specific antigen gene and the human glandular kallikrein 1 gene are tandemly located on chromosome 19.
  Riegman,P.H.J., Vlietstra,R.J., Klaassen,P., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1989) FEBS Lett. 247, 123–126.

- An Msp1 RFLP detected by the human glandular kallikrein gene (hGK) on chromosome 19q.
  Hermens,R., M.Coerwinkel, Trapman,J., Riegman,P.H.J., R.J.Vlietstra, Smeets,H. and Wieringa,B (1990) Nucl. Acids Res. 18, 208.

- Assignment of seven genes to distinct intervals on the midportion of human chromosome 19q around the myotonic dystrophy gene region. Schonk,D.P. van Dijk,P., Riegman,P.H.J., Trapman,J., Holm,C., Craig,I., Sillekens,P., van Venrooy,W., Wimmer,E., Geurts van Kessel,A., Ropers,H.H. and Wieringa,B. (1990) Cytogenet. Cell. Genet. 54, 15–19.

- Identification and androgen-regulated expression of two major human glandular kallikrein-1 (hGK–1) mRNA species.
  Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M. van der, Romijn,J.C. & Trapman,J. (1991) Mol. Cell. Endocrinol. 76, 181–190.

- The promoter of the Prostate-specific Antigen gene contains a functional androgen responsive element.
  Riegman,P.H.J., Vlietstra,R.J., Korput,J.A.G.M. van der & Trapman,J. (1991) Mol. Endocrinol. 5, 1921-9130.

- Characterization of the human kallikrein locus.
  Riegman,P.H.J., Vlietstra,R.J., Suurmeijer,L., Cleutjens,C.B.J.M & Trapman,J. Genomics (in press).

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