PROSTATIC GENE EXPRESSION: PROBASIN, HUMAN PROSTATIC ACID PHOSPHATASE AND MACROPHAGE INHIBITORY CYTOKINE-1 AS MODEL GENES

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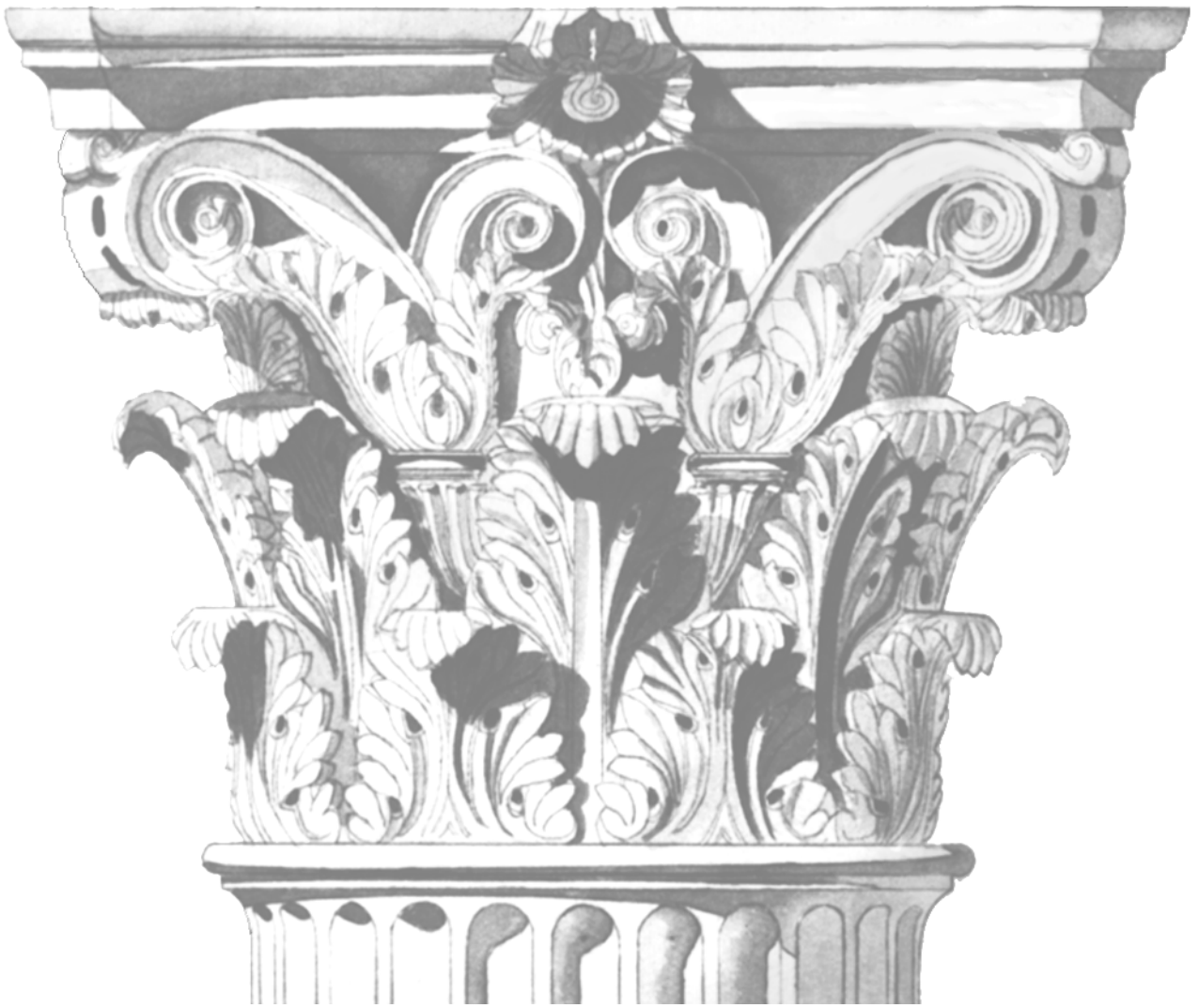

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Abstract

Gene products that are only expressed in one tissue or cell type are useful models for investigating the biochemical and molecular mechanisms of tissue/cell-specific gene regulation. The regulatory regions of such genes are also practical tools in gene therapy.

In this work, prostate-specific and androgen-dependent gene regulation was investigated by using human prostatic acid phosphatase (hPAP) and rat probasin (rPB) as models. In DNase I footprinting, a protected 12 bp region was found in the PB gene between the nucleotides -251 and -240 only with nuclear extracts of prostatic origin. The sequence of this area was GAAAATATGATA. Weak interaction could be detected between the DNA-binding domain of AR and the prostatic transcription factor. The results also suggested that the prostatic regulatory protein makes AR binding to its response element more effective and concomitantly magnifies the effect of androgen.

A hPAP construct containing the sequence between the nucleotides -734 and +467 in front of the CAT reporter gene was highly expressed in the prostate of transgenic mice. Five homologues (A-E) for our previously identified prostate-specific GAAAATATGATA DNA-binding site were found in the area where the sites C and E could bind the regulatory protein in EMSA.

The prostatic transcription factor complex bound to the GAAAATATGATA site was purified and characterized from a suspension-adapted mass culture of PC-3 prostate cancer cells by using sequence-specific DNA affinity chromatography, mass spectrometry and supershifts. Several potential transcription factors were identified, but only USF2 was confirmed to be part of the transcription factor complex.

Two PC-3 cell line variants (anchorage-dependent and suspension-adapted, anchorage-independent variants) were used as a model for advanced, androgen-independent prostate cancer. Genes that were overexpressed in a suspension-adapted PC-3 cell line were further investigated, since they can be considered as putative markers of metastatic activity. The macrophage inhibitory cytokine-1 (MIC-1) gene, which was overexpressed in the suspension-adapted PC-3 cell line, was further investigated in order to clarify the mechanism behind aggressive cell growth and androgen-independent gene regulation. In patient specimens, MIC-1 had no or low expression in benign prostatic hyperplasia and normal prostate but high in prostatic cancer and therefore it could be a useful marker for aggressive prostate cancer. Indomethacin increased the expression of MIC-1 in PC-3 cells, and apoptosis was also induced in this cell line but not in saPC-3 cell line suggesting a block in MIC-1 inducible apoptosis pathway.

Keywords: androgen receptor, DNA-binding sites, gene expression, promoter, prostate, transcription factors

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Oulu, February 2004

Lila Patrikainen

Abbreviations

AIbZIP	Androgen-induced bZIP
APHR	Aphrodisin
AR	Androgen receptor
ARBS	Androgen receptor-binding site
ARE	Andogen response element
Bp	base pair
BPH	Benign prostatic hyperplasia
COX	cyclooxygenase
DHT	5α-dihydrotestosterone
EMSA	Electromobility shift assay
FGF	Fibroblastic growth factor
GST	Glutathione-S-transferase
GR	Glucocorticoid receptor
hPAP	Human prostatic acid phosphatase
kb	kilobase
kDa	kilodalton
MIC-1	Macrophage inhibitory cytokine-1
MMTV	Mouse mammary tumour virus
mRNA	messanger ribonucleic acid
MUP	Rat α2-urinary globulin
NF-I	Nuclear factor-I
NSAID	Non-steroidal anti-inflammatory drug
nt	nucleotide
OBP	Rat odorant-binding protein
PDEF	Prostate epithelium-specific Ets transcription factor
PKC	Protein kinase C
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PSA	Prostate-specific antigen
PTP	Protein tyrosine phosphatase
RARE	Retinoid acid receptor element

rPB	Rat probasin
TARP	T cell receptor γ-chain reading frame protein
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TPA	12-o-tetradecanoyl phorbol-13-acetate
uPA	Urokinase-type plasminogen activator
USF-2	Upstream factor-2
Wt	wild type

List of original articles

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Patrikainen L, Shan J, Porvari K and Vihko P. (1999). Identification of the DNA-binding site of a regulatory protein involved in prostate-specific and androgen receptor dependent gene expression. Endocrinology, 140:2063-70

II Shan J, Porvari K, Kivinen A, Patrikainen L, Halmetkytö M, Jänne J & Vihko P (2003) Tissue-specific expression of the prostatic acid phosphatase promoter constructs. Biochem. Biophys. Res Comm. 311:864-869

III Kivinen A, Patrikainen L, Kurkela R, Porvari K & Vihko P. USF2 is connected to GAAAATATGATA element and associates with androgen receptor dependent transcriptional regulation in prostate. Prostate. In press

IV Patrikainen L, Kurkela R, Hirvikoski P, Porvari K, Soini Y & Vihko P. Macrophage inhibitory cytokine 1 highly expressed in prostatic adenocarcinoma but does not induce apoptosis in an aggressive prostate cancer cell model. Manuscript

Contents

1 Introduction

Gene products that are only expressed in one tissue or cell type are useful models for investigating the biochemical and molecular mechanisms of tissue/cell-specific gene regulation. The regulatory regions of such genes are also practical tools in gene therapy. Genes that are expressed in a prostate-specific manner, e.g. rat probasin (rPB) (Greenberg *et al.* 1994) and prostatic acid phosphatase (PAP) (Aumüller & Seitz 1985, Mori & Wakasugi 1985, Solin *et al*. 1990), provide useful insight into prostate-specific gene expression.

As the basic molecules involved in the regulation of gene expression are present in many tissue types, the molecular basis for the diversity of tissue-, cell- and gene-specific transcription must be determined by additional factors. Androgen receptor (AR) mediates a wide variety of hormonal effects by direct modulation of gene expression. For example, PB is up-regulated by androgens (Dodd *et al.* 1983, Matusik *et al.* 1986, 1991), while both up- and down-regulation by androgen have been reported for hPAP (Horozewicz *et al.* 1983, Henttu & Vihko 1992, Lin *et al.* 1993). Selective regulation of target genes by AR is achieved by, for example, tissue-specific distribution of ligands and tissue-restricted interaction of transcription factors and cofactors. The diversity of AR actions is also due to the nature of the bound ligands (reviewed by Heinlein & Chang 2002).

The development of prostate cancer and the mechanisms underlying the malignant transformation in prostate cancer are only partially known. However, it is common that prostate cancer transforms to an androgen-independent disease over time. Prostate cancer is a common disease, and better understanding of the molecular mechanisms is essential in order to develop new diagnostic and therapeutic strategies. It is essential to generate new tests for the diagnosis of prostate cancer and for potential discrimination between the androgen-dependent and androgen-independent varieties of prostate cancer. Consequent to the discovery of metastasis-related genes, serum markers for cancer metastasis may become available in the near future.

In this work, prostate-specific and androgen-dependent gene regulation was investigated by using hPAP and rat rPB as models. A transcription factor complex binding to a sequence involved in prostate-specific gene regulation was purified and characterized. Two PC-3 cell line variants (anchorage-dependent and suspension-adapted, anchorage-independent variants) were used as a model for advanced, androgen-

independent prostate cancer. The genes that were overexpressed in a suspension-adapted PC-3 cell line were further investigated, since they can be considered as putative markers of metastatic activity. The macrophage inhibitory cytokine-1 (MIC-1) gene, which was overexpressed in the suspension-adapted PC-3 cell line, was further investigated in patients and cell models in order to clarify the mechanism underlying aggressive cell growth and androgen-independent gene regulation.

2 Review of the literature

2.1 Genes expressed specifically in the human prostate

A number of human genes have been identified that are specifically expressed in the human prostate, including prostate-specific antigen (PSA), prostatic acid phosphatase, human kallikrein 2, prostate-specific membrane antigen, prostate-specific transglutaminase and prostate stem cell antigen (Aumüller & Seitz 1985, Aumüller *et al.* 1990, Clements 1983, Israeli *et al*. 1997, Dubbink *et al*. 1996, Reiter *et al*. 1998). The promoter sequences responsible for the prostate-specific expression of these genes have been cloned, and the unravelling of their transcriptional regulation is ongoing and will provide prostate-specific promoter fragments that can activate therapeutic agents selectively in prostate cells. The PSA gene promoter has been most extensively studied and revealed the existence of a proximal prostate-specific promoter with an upstream prostate-specific enhancer that are both required for high, androgen-regulated activation of PSA expression (Cleutjens *et al*. 1997, Schuur *et al*. 1996). In addition, the the rat probasin gene area responsible for tissue-specific gene expression is well characterized (Greenberg *et al*. 1994). Other human genes reported are noncoding riboregulators, DD3 and PCGEM1 (Bussemakers *et al*. 1999, Srikantan *et al*. 2000), serine protease, TMPRSS (Paoloni-Giacobino *et al*. 1997), small nuclear proteins, T-cell receptor γ-chain alternate reading frame protein, and prostate/rectum/colon gene (Wolfgang *et al*. 2000, Liu *et al*. 2001), tumour suppressor genes, NKX3.1 and prostate androgen-regulated transcript-1 (He *et al*. 1997, Lin *et al*. 2000), coregulator of the androgen receptor, PDEF and AIbZIP (Oettgen *et al*. 2000, Qi *et al*. 2002), androgen–regulated gene, PMEPA-1 (Xu *et al*. 2000), the human prostate-specific gene, HPG-1 (Herness & Rajesh, 2003) and the calcium channel protein, transient receptor potential-8 (Tsavaler *et al*. 2001). Most of the genes have not been extensively examined for the prostate-specificity and/or for their role in carcinogenesis. Thus, their application in early diagnosis or immunotherapy of prostate cancer and benign prostatic hyperplasia (BPH) remains unclear.

2.2 Probasin

Probasin is an abundant, 20-22 kDa, heparin-binding protein present in rat prostate extracts (Matuo et al. 1985, 1989). The rPB gene encodes an androgen- and zinc-regulated protein specific to the dorsolateral epithelium of prostate. In vivo, probasin appears both in the secretions and in the nucleus of prostatic epithelial cells (Spence *et al.* 1989).

Probasin is structurally related to the lipocalin superfamily, showing sequence similarity to other members, such as rat odorant-binding protein (OBP), aphrodisin (APHR), rat α2-urinary globulin (MUP), retinol-binding protein and bovine β-lactoglobulin (Newcomer *et al.* 1984, Laurent *et al.* 1985, Ali and Clark 1988, Pevsner *et al.* 1988, Magert *et al.* 1995, 1999, Lehman McKeeman *et al.* 1992, Clark *et al.* 1984).

2.2.1 Structure of probasin

The PB gene is located at chromosome Xq22, and it consists of seven exons and six introns, which span a 14.9 kb area. Exon size varies from 29 to 209 bp. Exon 1 contains the 5´-untranslated region, the signal peptide and the NH-terminal of the mature protein. Exon 6 contains the COOH-terminal, and exon 7 contains only the 3´-untranslated region with the 3´-polyadenylation signal (AATAA) starting 20 bp upstream of the 3´-end (Kasper *et al.* 2000). Rat PB exon 1 contains the signal peptide and the first ten amino acids of the mature protein. Two AUG codons follow the signal peptide sequence. The initiation of transcription at the first AUG codon in mRNA results in a secreted protein, while initiation at the second AUG sequence results in in a nuclear protein (Spence *et al.* 1989).

Probasin is considered to be a member of the outlier lipocalins that have more divergent sequences compared to those of the kernel lipocalins. According to a computer search, probasin shares 28% of identical residues with OBP and APHR, 18% of identical residues with rat α_2-urinary globulin and major urinary protein and 15% with bovine β-lactoglobulin and retinol-binding protein (Kasper *et al.* 2000). Two of the outlier lipocalins, odorant-binding protein and aphrodisin, share several common features with probasin. These include the distinctive C-X-X-X-C motif that is absent in most lipocalins. The several highly conserved motifs that appear to be a hallmark of the lipocalins include the G-X-W motif in exon 2 and the PB cysteine residues at positions 79 (exon 3) and 171 (exon 6) (Flower *et al.* 1996). These motifs are present in the PB gene, suggesting that it shares a common tertiary structure with the other lipocalins despite a difference of about 80% in the primary structure of these proteins. The secondary structure of probasin resembles that of retinol-binding protein in the number and position of the eight β-strands and the α-helix, thus predicting that probasin is a member of these ligand-carrier proteins (Flower *et al.* 1996, Sansom *et al.* 1994).

2.2.2 Function of probasin

Probasin is a 20-22 kDa heparin-binding protein with near identical chromatographic properties, but only 0.2%-1% of the mitogenic activity, of bovine brain heparin-binding growth factor-1 (Matuo *et al.* 1989).

PB mRNA is bifunctional, resulting in the synthesis of the same protein either with a signal peptide that is cleaved to give the mature secreted protein or without a signal peptide that is translocated to the nucleus (Spence *et al.* 1989).

The putative structure of PB parallels that of the pheromone carriers APHR, rat α2 urinary globulin and MUP and the olfactory carrier OBP, suggesting that probasin may be a pheromone carrier as well. However, PB is synthesized in the rodent prostate and secreted into seminal fluid, indicating that PB secretion appears as a result of copulation. Thus, it seems unlikely that PB would function as a pheromone prior to copulation, but it might mark the female to ward off other males. It is possible that PB serves as a modulator of transcription (Kasper *et al.* 2000). The specific ligand for probasin has not been identified, and the roles of the nuclear and secreted forms of probasin remain unclear. Functionally, PB gene expression is specific to the prostate (Matuo *et al.* 1986, Matusik *et al.* 1986), and probasin levels rise rapidly during sexual maturation and differentiation of the prostate (Matuo *et al.* 1985, Matusik *et al.* 1986). Moreover, both androgens and zinc serve as regulators of PB gene expression (Dodd *et al.* 1983, Matusik *et al.* 1986).

2.2.3 Prostate-specific expression of probasin

Probasin is detected in all lobes of the rat prostate and the seminal vesicles, with maximum PB expression in the lateral lobe (100%) followed by the dorsal (33%), anterior (14%) and ventral (4%) lobes and the seminal vesicles (2%) (Spence *et al.* 1989, Dodd *et al.* 1983, Matusik *et al.* 1986). The prostate-specific expression of probasin has been confirmed in both *in vitro* and *in vivo* studies. Greenberg *et al.* (1994) reported that the –426/+28 bp PB promoter linked to a reporter gene was specifically expressed in prostatic epithelium in transgenic mice. During sexual maturation, at 2-7 weeks of age, CAT activity increased markedly, whereas it dramatically decreased after castration of adult male transgenic mice. Testosterone, but not dexamethasone, was able to increase CAT activity up to the pre-castration levels. A transgenic animal model for prostate cancer was also developed by using this regulatory region of the rPB gene (Greenberg *et al.* 1995). In addition, a large fragment of the rPB (LPB) promoter (-11500 to +28) can greatly elevate the levels of transgenic expression induced by androgen and zinc (Yan *et al.* 1997).

Yeung *et al*. (2003) recently identified and characterized a prostate-specific, androgen-independent, protein-binding site in the probasin promoter region –93/–67. Identification of the proteins binding to this region revealed the participation of nuclear factor I (NF-I) or a closely related protein, although other unknown proteins are also involved. Site-directed mutagenesis of the protected nucleotides within the region –93/-67 resulted in a significant reduction in the expression of the PB promoter.

2.2.4 Androgen regulation of probasin

Initially, the PB gene was shown to be androgen-regulated, since its mRNA concentrations dropped to nearly undetectable levels following castration (Dodd *et al.* 1983, Matusik *et al.* 1986, 1991). A PB gene –426/+28 fragment linked to the CAT reporter gene reacted to androgen receptor and androgen treatment, but not to glucocorticoid or dexamethasone treatment (Greenberg *et al.* 1994, Rennie *et al.* 1993). Furthermore, cyclin D, a potent inhibitor of AR activity (Knudsen *et al.* 1999, Petre *et al.* 2002), is able to mute the gene expression of PB (Petre-Draviam *et al.* 2003). Dnase I protection assays identified two androgen response elements (AREs), which were later renamed as androgen receptor binding sites (ARBS-1 between the positions –236 and –223 and ARBS-2 between the positions –140 and –117), since both binding sites were essential for biological activity. Although ARBS-2 bound the androgen receptor with higher affinity than ARBS-1, Dnase I protection assays showed both sites to be always occupied, even at the lowest concentration of AR tested. A single point mutation at either site greatly reduces androgen receptor binding to both sides and effectively decreases androgen-induced CAT gene activity by more than 95% (Kasper *et al.* 1994, 1999). When both AR-binding sites are in the wt configuration, purified AR binds selectively to both ARBS-1 and ARBs-2, whereas glucocorticoid receptor (GR) only occupies both sites at the highest concentration of purified receptor (800 ng) tested (Kasper *et al.* 1999). Claessens *et al.* (1996) and Schoenmakers *et al.* (1999) confirmed that PB ARBS-2 selectively binds AR. They concluded that androgen-specific recognition of PB ARBS-2 required AR residues in the second Zn finger, the C-terminal extension of the DNA-binding domain of AR, in addition to 12 C-terminal residues, demonstrating that the selectivity by which AR binds to PB ARBS-2 was different from that used by GR to bind to the GRE consensus motif.

Recently, another androgen-responsive region has been described between the nucleotides –705/-426. This region is an enhancer containing two androgen receptor-binding sites (ARBS-3 and ARBS-4), and these two new ARBSs interact in a cooperative manner with the previously described androgen response region –244/-96) (Zhang *et al.* 2003).

2.3 Human prostatic acid phosphatase

Human prostatic acid phosphatase (hPAP) is a glycoprotein synthesized in the epithelial cells of the prostate gland (Derechin *et al.* 1971, Ostrowski *et al.* 1976, Risley & van Etten 1987, Hakalahti *et al.*1993), from which it is secreted into prostatic fluid (Vihko 1978, Vihko *et al.*1978, Rönnberg *et al.* 1981). This enzyme hydrolyzes a wide range of alkyl and aryl orthophosphate monoesters, including phosphotyrosine (Apostol *et al.*1985, Vihko *et al.* 1993) and nucleotides (Dziembor-Gryzkiewicz *et al.* 1979) and has also been found to dephosphorylate macromolecules, such as phosphopeptides and phosphoproteins (Wasylewska *et al.* 1983, Li *et al.* 1984). hPAP is categorized as an acid phosphatase, since in has an optimum pH of 4-6.

2.3.1 Structure of hPAP

The gene encoding hPAP is located at chromosome 3q21-qter (Winqvist *et al.* 1989). cDNA encodes a 354-residue protein with a calculated molecular mass of 41126 Da. The hPAP gene contains 10 exons of 170, 96, 87, 153, 99, 93, 133, 83 104 and 2098 bp, respectively. A 32 amino acid signal sequence and the first eight amino acids of the protein are encoded by exon 1, and the rest of the coding region and the 3´-untranslated region are covered by the exons 2-10 and 10, respectively (Virkkunen *et al*. 1994). When prostatic carcinoma or benign hyperplasia tissue is used as a source of RNA, a major transcription start site can be found 50 nt upstream of the ATG codon of the gene (Sharief & Li, 1992, Virkkunen *et al*. 1994). The length of the 3´ non-coding region in hPAP cDNA varies between 646 and 1913 bp, although, in Northern blot analysis, a 3.3 kb mRNA species is usually observed (Vihko *et al.* 1988, Sharief *et al.* 1989, Solin *et al.* 1990). The heterogeneity in the length of cDNA is explained by the multiple polyadenylation signals (AAATAA) following two copies of alu-type repetitive sequences in the 3´non-coding region.

The hPAP protein is a homodimer with subunits related by a 2-fold axis (Schneider *et al.*1993, Jakob *et al.* 2000). Each subunit comprises of two domains. The larger domain of α/β type is composed of a central seven-stranded mixed β-sheet with helices on both sides, while the smaller α domain contains six α-helices and is formed mostly of a long-chain excursion from the first domain. The hPAP active site, which contains an essential histidine (His12) residue (Davidson 1990), is located in a large open cleft between the two domains. This allows the enzyme to accept a large variety of substrates (Heller 1987).

Human PAP from native sources possesses molecular heterogeneity. Isoelectric focusing gives up to 30 variants of hPAP from normal, malignant or hyperplastic prostatic tissue and sera from patients with prostate cancer and seminal fluid (Chu *et al.* 1978, Vihko *et al.* 1978a, 1978b, Taga *et al.* 1983, Hibbard *et al.* 1983, Seitz & Aumüller 1985, Mack *et al.* 1987). Variation in the amount of sialic acid and other sugar residues and post-translational deamination of glutamine and asparagine account for part of the heterogeneity of hPAP (Smith & Whitby 1968, van Etten *et al.* 1991). Furthermore, the carboxyterminal amino acid of hPAP can be threonine, glutamic acid or aspartic acid (van Etten *et al.* 1991). The heterogeneity of hPAP could also be due to the existence of true isoenzymes consisting of different polypeptide chains.

2.3.2 Function of hPAP

Human PAP is one of the prostate epithelium-specific differential antigens (Yam 1974, Lam *et al.*1982, Kamoshida & Tsusumi 1990). There are two forms of hPAP: one is intracellular and the other secreted (Vihko 1979, Lad *et al.* 1984). The physiological substrate of hPAP is unknown. However, hPAP is secreted in large amounts into seminal fluid (Rönnberg *et al.*1981), suggesting a physiological role in fertility (Coffey & Pienta 1987). The serum activity of the enzyme is frequently elevated in patients with prostate carcinoma and correlates with tumour progression (Gutman & Gutman 1938, Choe *et al.*

1980, Griffith 1980, Vihko *et al.* 1980, 1981 and 1985). *In vitro*, hPAP hydrolyzes phosphorycholine, which is found in semen (Saini & van Etten 1979), and phosphocreatine, an intracellular high-energy compound present in seminal plasma (Lee *et al.* 1988).

hPAP possesses intrinsic protein tyrosine phosphatase (PTP) activity (Li *et al.* 1984, Porvari *et al*. 1994, Boissonneault *et al.* 1995), and several lines of evidence indicate that cellular hPAP functions as a neutral PTP, although it shares little sequence homology with other typical PTPs (Guan *et al*. 1990, Chernoff *et al*. 1990, van Etten *et al*.1991). hPAP also has amidolytic activity on a seminal vesicle protein semenogelin I. The enzyme is able to cleave both peptide substrates derived from the semenogelin sequence and native semenogelin I. The main cleavage sites are at Tyr 292 and Ser 170 (Brillard-Bourdet *et al.* 2002). In addition, hPAP interacts with ErbB-2, a member of the erbB receptor tyrosine kinase family, in the androgen-promoted growth of human prostate cancer cells. Androgen-stimulated cell growth concurs with down-regulation of cellular hPAP, an elevated p-Tyr level of ErbB-2 and the activation of mitogen-activated protein kinases (Meng *et al.* 2000). Cellular hPAP can down-regulate prostate cancer cell growth, at least partially, by dephosphorylating c-ErbB-2. Therefore, decreased cellular hPAP expression in cancer cells may be involved in prostate cancer progression (Lin *et al.* 2001).

2.3.3 Expression of hPAP in prostate

Immunohistochemistry using specific antibodies against PAP has demonstrated that the enzyme is located in the columnar, secretory epithelial cells of the prostate (Aumüller & Seitz 1985, Mori & Wakasugi 1985, Lilja & Abarahamsson 1988, Sinha *et al.* 1988, Lam *et al.* 1989, Hakalahti *et al.* 1993). *In situ* hybridization analysis has shown that hPAP mRNA is confined to the glandular and ductal epithelial cells of the prostate, and that stromal cells are devoid of this mRNA (Hakalahti *et al.* 1993). No hPAP mRNA has been detected in human liver, lung, pancreatic cancer tissue, placenta, breast cancer cells, mononuclear blood cells or acute promyelocytic leukaemia cells (Solin *et al.* 1990), nor in spleen, thymus, testis, ovary, small intestine, colon or peripheral blood leukocytes (Zelivianski *et al.* 1998).

Little is known about the mechanism of tissue-specific regulation of the hPAP gene at the molecular level. Zeliavinski *et al.* (2002) have shown that, in addition to the basic promoter, the region between –1258/-779 is able to enhance the PAP promoter activity in PC-3 and DU-145 human prostate cancer cells, but not in non-prostate cancer cells, indicating that this region is involved in governing the cell type-specific expression of the hPAP gene.

2.3.4 Androgen regulation of hPAP

In cell culture models, both up- and down-regulation by androgens have been reported for hPAP. The amount of hPAP released into LNCaP cell culture medium was decreased to 26% of the control level in 7 days when a synthetic androgen, R1881, was present in

charcoal-stripped serum (Henttu & Vihko 1992). Accordingly, 5α-dihydrotestosterone (DHT) treatment was found to decrease the activity of hPAP in these cells. A stimulatory effect of androgen on hPAP secretion has been confirmed by Horozewicz *et al.* (1983) and Lin *et al.* (1993). hPAP mRNA levels are also increased when tissue slides derived from benign prostatic hyperplasia are treated with DHT and fibroblastic growth factor (bFGF) (Dulinska *et al.* 2002). A biphasic pattern of the effect of androgen on LNCaP cells has been reported: stimulation of growth and inhibition of hPAP secretion was detected at less than 1nM concentrations of androgen, while an opposite effect was observed at higher concentrations (Henttu *et al.* 1992, Langler *et al.* 1993). Shan *et al.* (1997) showed that reporter constructs of hPAP promoter covering the region –734/+467 were functional in both prostatic and nonprostatic cell lines in transient transfections. This region contained two putative AREs, which have been shown to have androgen receptor-binding ability *in vitro*, but the promoter could not be induced with androgen, glucocorticoid or progesterone, indicating that steroids cannot directly regulate hPAP gene expression via receptor binding to these AREs.

Androgen may regulate hPAP expression differently in diverse physiological or pathological conditions.

Both up- and down-regulation of hPAP by 12-o-tetradecanoyl phorbol-13-acetate (TPA), a protein kinase C (PKC) activator, has been reported (Henttu & Vihko 1996, Lin *et al*. 2001). Lin *et al*. (2001) reported that TPA is able to increase the secretion of hPAP in a dose- and time-dependent fashion in the androgen-responsive LNCaP cell line. This TPA stimulation of hPAP secretion was more potent than the conventional stimulating agent DHT at the same concentration. Furthermore, the action of TPA and DHT on hPAP secretion was blocked by five different PKC inhibitors. DHT, as well as TPA, could rapidly modulate PKC activity. Therefore, PKC can regulate hPAP secretion and may also be involved in the DHT action on hPAP secretion.

2.4 Prostatic gene expression

Prostate cancer is the most frequently diagnosed cancer among men in Western industrialized countries. The identification of prostate-specific gene products and prostate-specific gene promoters is important for the development of gene therapy of prostate cancer. Promoters that are uniquely active in prostate tissues can be used to restrict the expression of therapeutic genes to prostate cancer cells.

2.4.1 Prostatic promoters and enhancers

Various gene therapy strategies for prostate cancer have been examined. Eastham et al. (1996) reported that direct intratumoral injection of an adenovirus vector encoding the herpes simplex virus thymidine kinase (HSV-tk) under the regulation of the Rous sarcoma virus long terminal repeat regressed nude mouse subcutaneous tumours of prostate cancer cells. Hall *et al*. demonstrated the synergistic therapeutic effects of androgen ablation and HSV-tk gene transfer plus GCV treatment (1999). Blackburn et al.

(1998) developed a heat-inducible fusion gene comprising cytosine deaminase and HSV-tk genes and described a unique application of hyperthermia in prostate cancer therapy. The use of prostate-specific promoters may allow substantial, but safe, escalation of the vector dosage and may enable effective delivery of toxin or apoptosis-inducing genes to prostate and cancer cells, which may be disseminated or metastasized in the body. Several composite prostate-specific promoters/enhancers based on rPB, PSA, T cell receptor γ-chain reading frame protein (TARP) and human prostate-specific glandular kallikrein (hK2) have been developed for this purpose (Zhang et al. 2000, Latham et al. 2000, Xie et al. 2001, Cheng et al. 2003, Furuhata et al. 2003) (Fig 1).

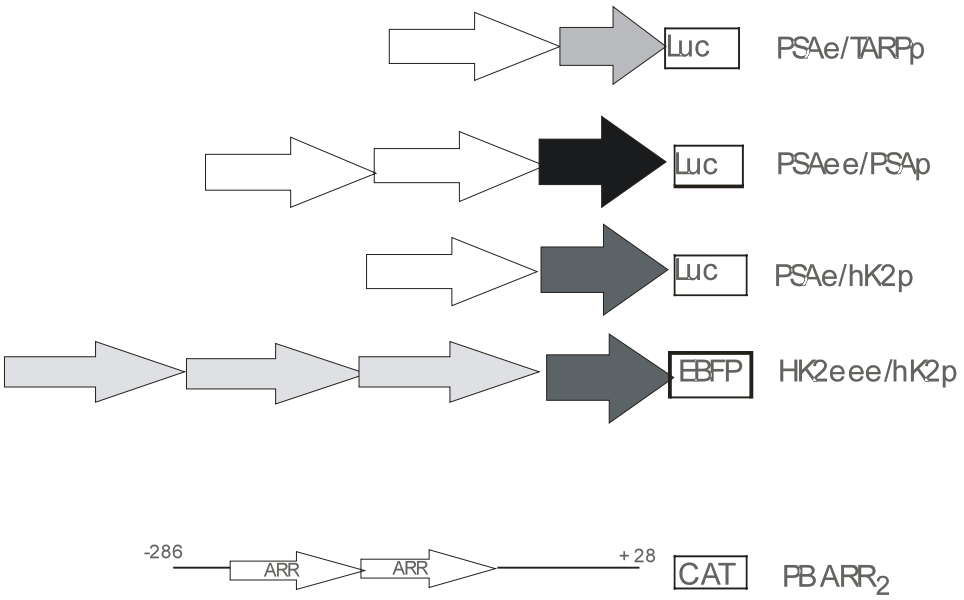

Fig. 1. Examples of prostate-specific promoters/enhancer constructs used to express the luciferase (Luc), enhanced green fluorescent protein (EGFP) and chloramphenicol acetylase transferase (CAT) reporter genes. PSAe, indicated as an open arrow, is a 1.4 kb enhancer of the PSA gene. In these examples PSAe has been inserted as 1-2 copies in front of the minimal promoter of TARP (TARPp, gray arrow), PSA (PSAp black arrow) and hK2 (hK2p dark gray arrow). HK2e is a 1.2 kb enhancer of the hK2 gene. A triplicate copy of this enhancer has been inserted in front of hK2p. PB ARR2 is a construct containing two copies of the rPB gene androgen response region –244/-96 (Modified from Zhang *et al*. 2000, Latham *et al*. 2000, Xie *et al*. 2001, Cheng *et al*. 2003).

The best-characterized androgen-responsive gene in the human prostate gland is KLK-3, the gene coding for prostate-specific antigen (PSA). KLK-3 transcription is regulated through at least two AREs, one in the proximal promoter and one in an upstream enhancer sequence (Cleutjens et al. 1997a). An experiment with transgenic mice showed that the promoter is insufficient to confer strong androgen responsiveness and cell type specificity in vivo (Cleutjens et al. 1997b). However, a chimeric sequence consisting of the prostate-specific TARP promoter and the PSA enhancer is expressed specifically in

TARP-positive prostate cancer cell lines. In addition, in the LNCaP cell line, the transcriptional activity of this chimera is 20-fold compared to the activity of a regulatory sequence consisting of the PSA promoter and the PSA enhancer (Cheng et al. 2003).

The two androgen receptor-binding sites (ARBS-1 between the positions –236 and –223 and ARBS-2 between the positions –140 and –117) in the PB gene androgen response region (ARR) –244/-96 are essential for the biological activity of the gene (Kasper et al. 1994, 1999). The presence of one copy of the ARR of PB, although sufficient for developing animal models of prostate cancer (Greenberg et al. 1995), may be insufficient for clinical gene therapy. This concern led to the development of a small 500-bp composite promoter, ARR_2PB, which contains two copies of PB ARR upstream of the minimal promoter, leading to very strong androgen induction (Zhang et al. 2000). Fuhurata et al. (2003) also designed a modified PB promoter that is activated by the retinoids-retinoid receptor complex instead of the androgen-AR complex, to target transgene expression in androgen-independent prostate cancer. In this modified PB promoter area (–426 to +25), AREs were substituted by three of the six copies of synthetic RARE. Furthermore, the combination of retinoid treatment and adenovirus-mediated gene transfer of the modified PB-driven HSV-tk gene resulted in significant growth suppression of the androgen-independent prostate cancer cells in the presence of the prodrug ganciclovir.

2.4.2 Prostatic regulatory proteins

A binding site for prostate-specific factor has been found in the first intron of the C3 (1) gene within the enhancer (Celis *et al.* 1993). The binding site for the tissue-specific regulatory protein, together with NF-1 and Octamer Transcription factor (OTF), is located in an 80-bp region upstream of a functional ARE (Claessens *et al.* 1990). Only a few genes encoding transcription factors with intense expression in prostate, but scant or no expression in several other tissues, have been characterized. One of these genes is an androgen-regulated NKX3.1 homeobox gene that maps to 8p21, a region deleted in prostate cancer (He *et al.* 1997). PDEF is a prostate epithelium-specific Ets transcription factor, which interacts with the androgen receptor and activates prostate-specific antigen gene expression (Oettgen *et al.* 2000). Androgen-induced bZIP (AIbZIP) is a 395 amino acid protein with homology to cyclic, AMP-responsive, element-binding protein/activating transcription factors. The gene expression of AIbZIP is up-regulated by androgens, it has a highly tissue-specific expression profile, and it is expressed at higher levels in cancerous prostate cells compared with noncancerous prostate cells (Qi *et al.* 2002).

2.5 Molecular changes associated with prostate cancer

At the cellular and molecular levels, genetic aberrations drive the formation and aggressiveness of prostate cancer. Every carcinoma focus is presumed to arise from a single cell that accumulates genomic changes affecting the regulatory genes, resulting in

a growth or survival advantage. Additional changes lead to local invasion and metastasis. Mutations in classic oncogenes or tumour suppressor genes are uncommon in primary prostate cancer, and no mutations specific to prostate cancer have been identified (Bookstein *et al.* 2001). However, several molecular or genetic changes have been found. The molecular basis of prostate cancer includes inheritable and somatic genetic changes (tumour suppressor genes, loss of heterozygosity, gene targets and regions of chromosomal gain, CpG island promoter methylation, invasion and metastasis suppressor genes, telomere shortening and genetic instability). Changed gene expression of proliferation-related genes and changes in the androgen receptor, apoptosis and stress-response genes have some potential as biomarkers and therapeutic targets in prostate cancer (reviewed by DeMarzo *et al.* 2003) (Table 1).

Table 1. Selected genes proposed to be involved in prostate cancer initiation and progression or in modifying the risk of prostate cancer development (modified from DeMarzo et al. 2003)

Gene	Proposed function
	1. Germline alterations
	Mutations causing decreased activity of the gene
Macrophage scavenger receptor 1 (MSR1)	Anti-infectious, scavenger receptor
ELAC2	Metal dependent hydrolase
Ribonuclease L (RNASEL)	Anti-infectious, apoptosis
	Polymorphisms affecting prostate cancer risks
Androgen receptor	Cell proliferation, survival, and differentiation
Cytochrome P450c17 alpha (CYP17)	Androgen metabolism
5α-reductase (SRD5A2)	Androgen metabolism
	2. Somatic alterations
	Promoter hypermethylation resulting in gene silencing
Pi class of glutathione-S-transferase (GSTP1)	Carcinogen detoxification
	Loss of heterozygosity and point mutation
Phosphatase and tensin homolog (PTEN)	Cell survival and proliferation
Tumour protein 53 (TP53, also P53)	Cell survival and proliferation, genome stability
	Loss of heterozygosity and haploinsufficiency
NKX3-1	Cell differentiation and proliferation
Cyclin-dependent kinase inhibitor IB (CDKN1B, P27KIP1)	Cell proliferation
	Point mutations
Core promoter element binding protein (COPEB, also KLF6)	Transcription regulator
Androgen receptor (AR)	Cell proliferation, survival and differentiation
	Amplification
Androgen receptor	Cell proliferation, survival and differentiation
	Overexpressed at mRNA and protein level
Telomerase reverse transcriptase (HTERT)	Cell immortality
Hepsin (HPN)	Transmembrane protease
Fatty acid synthase (FASN)	Fatty-acid synthesis
α-methylacyl-CoA racemase (AMACR)	Fatty-acid metabolism, branched chain
Enhancer of zeste homolog 2 (EZH2)	Transcription repressor, cell proliferation
Myelocytomatosis oncogene (MYC)	Cell proliferation
B-cell leukemia/lymphoma 2 (BCL2)	Cell survival

RNASEL and MSR1 are candidate susceptiblity genes for prostate cancer, and they are involved in the pathogenesis of prostate cancer. Both genes take part in the host response to infectious agents, and mutations might hence reduce the ability to eradicate certain infectious agents within the prostate, resulting in a chronic inflammatory reaction (Carpten *et al*. 2002, Xu *et al*. 2002). The gene encoding RNASEL has been mapped to

the critical region of 1q24-25, thus implicating this gene as a candidate for hereditary prostate cancer 1 locus (HPC1). (Carpten *et al*. 2002). ELAC2 is a candidate for the hereditary prostate cancer 2 locus (Tavtigian *et al*. 2001). Genes with common sequence variants may also have a role in prostate carcinogenesis. Examples of polymorphic genes are SRD5A2, which encodes the predominant isozyme of 5α-reductase in the prostate (reviewed by DeMarzo *et al*. 2003), and the enzyme P450c17α encoded by the CYP 17 gene, which catalyzes the conversion of progesterone and pregnenolone into precursors of potent androgens. However, a recent study failed to reveal any statistically significant overall associations of CYP17 genotypes with the prostate cancer risk (Madigan *et al*. 2003). The AR gene located on the X chromosome constains a variable number of CAG repeats ranging from 8 to 31. Decreased transactivation activity and binding affinity for androgens is associated with an increased number trinucleotide repeats and it may confer a protective effect in terms of prostate cancer risk (Ekman *et al*. 1999)

Classic tumour suppressors generally show biallelic inactivation, usually by a point mutation in one allele coupled with a deletion or rearrangement of the other, although other changes may also be involved (reviewed by DeMarzo *et al*. 2003). NKX3-1, which is expressed in normal prostate epithelium and is decreased in prostate tumour cells is an example of such genes. . In mice, loss of even one Nkx3.1 allele causes prostatic epithelial hyperplasia and eventual prostatic intraepithelial neoplasia (PIN) formation (Bhatia-Gaur *et al.* 1999). Genetic inactivation of TP53 is also frequently seen in metastatic and hormone-refractory lesions of prostate cancer (reviewed by DeMarzo *et al*. 2001). PTEN, which is responsible for dephosphorylation and inactivation of phosphatidylinositol-3,4,5-trisphosphate is mutated in up to a third of hormone-refractory prostate cancers (Wang *et al*. 1998). Loss of PTEN in primary prostate cancer correlates with high Gleason scores and advanced stage (McMenamin *et al.* 1999). The CDKN1B protein is expressed at high concentrations in healthy prostate epithelium, and loss of CDKN1B also correlates with poor prognosis in prostate cancer (Guo *et al*. 1997). Loss of heterozygosity COBEP has been found in a majority of primary prostate tumours (Narla *et al*. 2001). In high-grade proste cancer, genetic alterations of COBEP are only rarely detected (Chen *et al*. 2003). Elo *et al*. (1997) also reported that a loss of heterozygosity of chromosome 16q23, which contains the 17HSD 2 gene, is significantly associated with metastatic and aggressive behaviour of prostate cancer. Silencing of the gene by hypermethylation may also be linked to prostate carcinogenesis. The promoter region of GSTP1 is silenced in this way in a majority of cancer lesions (Nelson *et al*. 2001).

Amplification of the region on chromosome 8q correlates with the aggressiveness of the tumour. One candidate for amplification on 8q is the MYC gene, since it is amplified in several cases, correlating with poorer prognosis in prostate cancer (Sato *et al*. 1999). The androgen receptor gene is also commonly amplified at a relatively late stage of prostate cancer (Visakorpi *et al*. 1995). Linja *et al.* (2002) analyzed BPH, untreated and hormone-refractory locally recurrent carcinomas, as well as prostate cancer xenografts, and showed that in hormone-refractory tumors AR has on avarage 6-fold higher expression than in androgen-dependent tumors or BPH. Androgen-independent tumors with gene amplification expressed, on avarage, showed 2-fold higher level of AR than the refractory tumors without the gene amplification. The findings demonstrate that AR

signaling pathway is important in the progression of prostate cancer during endocrine treatment.

The genes overexpressed in prostate cancer include BCL2, an antiapoptotic factor, and AMACR, which has a key role in β-oxidation of dietary branched-chain fatty acids (reviewed by DeMarzo *et al*. 2003). EZH2, is a developmental regulatory gene that is a transcriptional repressor and is found in higher concentrations in metastatic prostate cancers than in primary tumours. In addition, clinically localized prostate cancers that express higher concetrations on EZH2 show a poorer prognosis and therefore it could be used as a marker that distinguishes indolent prostate cancer from those at risk of lethal progression (Varambally *et al*. 2002).

2.5.1 Hormone- refractory prostate cancer

Most prostate cancers express androgen receptors and regress on withdrawal of androgens. Progression of prostate cancer is characterized by a transition from an androgen-dependent to an androgen-independent phenotype, which determines the patient's clinical outcome. Therefore, much research has focused on androgen signalling in prostate cancer and how cells that are initially hormone-dependent become hormone-independent in view of growth. By using different LNCaP cell line variants, some *in vitro* cell models have been developed to investigate the molecular mechanisms of androgen-independent growth of prostate cancer cells (Vaarala *et al.* 2000, Karan *et al.* 2002, Härkönen *et al*. 2003). The following genes have been found to be overexpressed in an androgen-independent cell type LNCaP-C81: c-MYC, c-MYC purine-binding transcription factor (PuF), macrophage migration inhibitory factor (MIF), macrophage inhibitory cytokine-1 (MIC), lactate dehydrogenase-A (LDH-A), guanine nucleotide-binding protein Gi, α-1 subunit (NBP), cyclin-dependent kinase-2 (CDK-2), prostate-specific membrane antigen (PSM), cyclin H (CCNH), 60S ribosomal protein L10 (RPL10), 60S ribosomal protein L32 (RPL32) and 40S ribosomal protein S16 (RPS16) (Karan *et al.* 2002). Vaarala *et al.* (2000) also reported that tissue-type plasminogen activator, interferon-inducible protein p78 (MxB), follistatin, fatty acid-binding protein 5, annexin I, the interferon-inducible gene 1-8U and phospholipase D1 were highly overexpressed in an androgen-independent LNCaP cell line. However, these genes were not expressed in another androgen-independent cell line, PC-3, which has the functional and morphologic characteristics of poorly-differentiated prostatic adenocarcinoma (Kaight *et al.* 1979). These results suggested that this cell line has a different strategy for androgen-independent growth.

2.5.2 Macrophage inhibitory cytokine 1

The gene-encoding macrophage inhibitory cytokine 1 (MIC-1), which is also called PDF (AC: AF003934, PLAB (AC: U88323), NAG-1 (AC: AF173860), PTGFB (AC: AF008303) and HP00296 (AB000584), was initially cloned from activated macrophages, and it encodes a protein that bears the structural characteristics of a transforming growth

factor β (TGF-β) superfamily cytokine. MIC-1 is able to inhibit lipopolysaccharide-induced macrophage TNF-α production, suggesting that MIC-1 acts in macrophages as an autocrine regulatory molecule (Bootcov *et al.* 1997). TNF-a is a widely studied and important macrophage proinflammatory cytokine that causes a rage of local and systemic biological effects (Maini *et al*. 1995). MIC-1 mRNA is present in small amounts in kidney, pancreas and prostate and in large amounts in placenta (Paralkar *et al.* 1998, Moore *et al.* 2000, Fairlie *et al.* 1999). Breast, colon and prostate cancer tissues express large amounts of MIC-1 (Brown *et al.* 2001). It has also been suggested that MIC-1 acts as an antitumorigenic and proapoptotic protein (Baek *et al.* 2001).

2.5.2.1 Structure of MIC-1

The gene encoding MIC-1 is located at chromosome 19p13.1-13.2. The gene is composed of two exons and contains one single intron that interrupts the coding sequence within the prepro-domain of the corresponding protein (Böttner *et al.* 1999). As with all of the TGF-β superfamily members, MIC-1 is synthesized as a 308-amino acid polypeptide that encompasses a 29-amino acid signal peptide, a 167-amino acid propeptide and a 112-amino acid mature region. The mature protein is secreted as a disulfide-linked homodimer comprising two 112 amino acid mature regions, which is released from the propeptide after intracellular cleavage at a typical RXXR furin-like cleavage site (Bootcov *et al.* 1997). Unlike the other TGF-superfamily members studied, the MIC-1 mature peptide can be correctly folded and secreted without a propeptide (Bauskin *et al.* 2000, Fairlie *et al.* 2000). As with many cytokines, polymorphisms of MIC-1 have also been documented. The MIC-1 gene has at least two major allelic variants, a single nucleotide change leads to the replacement of the histidine with an aspartic acid. The markedly differing properties of these two amino acids suggest that this substitution may have functional consequences (Fairlie *et al.* 2001).

2.5.2.2 Function of MIC-1

MIC-1 is able to inhibit tumour necrosis factor production from lipopolysaccharide-stimulated macrophages (Bootcov *et al.* 1997), to induce cartilage formation and the early stages of endochonadal bone formation (Paralkar *et al.* 1998) and to inhibit the proliferation of primitive hemopoietic progenitors (Hromas *et al.* 1997).

Several findings suggest that MIC-1 has a role in the regulation of tumour cell growth. In some cases, MIC-1 is involved in tumour cell growth suppression and in other cases in tumour cell growth progression. MIC-1 has antitumorigenic capacity in colon carcinoma and glioblastoma cells in mice, and it has been suggested that its antitumorigenic activity may involve both autocrine and paracrine mechanisms. The latter may involve antiangiogenic effects of MIC-1 (Baek *et al.* 2001, Albertoni *et al.* 2002). The MIC-1 promoter region is a target for the p53 tumour suppressor gene product, and it can also suppress tumour cell growth *in vitro* through the TGF-β-signalling pathway, although very high concentrations of MIC-1 are required to elicit this effect (Tan *et al.* 2000). It

has been suggested that MIC-1 has no effect on cell proliferation in prostate cancer, but reduces cell adhesion and, consequently, induces cell detachment (Bauskin *et al.* 2003). The very high level of MIC-1 mRNA in the human placenta suggests that MIC-1 may be important for placental function and/or fetal development (Moore *et al.* 2000).

MIC-1 is also highly and specifically expressed in prostate (Welsh *et al.* 2001, Karan *et al.* 2002) and colorectal cancer tissues (Buckhaults *et al.* 2001), suggesting a role in tumour development or progression. In prostatic DU 145 cells, MIC-1 was able to down-regulate the expression of the antiapoptotic gene metallothionein 1E and the cell adhesion genes RhoE and catenin δ1, leading to reduced cell adhesion and induced cell detachment (Liu *et al.* 2003). MIC-1 can also induce the invasiveness of gastric cancer cells by up-regulating the urokinase type plasminogen activator system via the extracellular signal-regulated kinase-1/2-dependent pathway (Lee *et al.* 2003).

2.5.2.3 Differential expressions of MIC-1 gene in cancer and hyperplasia

Direct evidence for links between MIC-1 and cancer have been obtained from serial analyses of gene expression, which have indicated that MIC-1 is one of a very limited set of genes for secreted proteins in which expression is up-regulated in both adenomatous polyps and colorectal carcinoma (Buckhaults *et al.* 2001). Similar findings, obtained with microarray technology, have also been reported in prostate cancer (Welsh *et al.* 2001). Brown *et al.* (2001) documented an association between elevated serum MIC-1 and metastatic colorectal, prostate and breast cancer.

2.5.2.4 Regulation of MIC-1

MIC-1 was first cloned on the basis of its increased mRNA expression associated with macrophage activation. Although MIC-1 is not expressed in resting macrophages, stimulation of these macrophages by several biological mediators, including tumour necrosis factor α, interleukin 1 and macrophage-colony stimulating factor, induce its expression. Retinoid acid (RA) and phorbol 12 myristate 13-acetate (PMA) also enhance the expression of MIC-1, although its induction is dependent on the level of cell differentiation. MIC-1 is more responsive to RA/PMA in monocytes than more immature precursors (Bootcov *et al.* 1997).

Although originally identified in activated macrophages, MIC-1 can be expressed in several tissues. In prostate, MIC-1 expression is regulated by androgens, since orchiectomy of rats resulted in a dramatic decrease of MIC-1 expression. 5αDHT treatment resulted in a time-dependent increase in MIC-1 expression (Paralkar *et al.* 1998). In human lung cells MIC-1 expression is induced by p53 (Kannan *et al.* 2000), but in glioblastoma cells anoxia induces MIC-1 expression independently of p53 (Albertoni *et al.* 2002). Furthermore, retinoic acid and retinoid acid receptor effectively induce MIC-1 expression in normal lung cells but not in lung adenocarcinoma cells. *In vivo*, MIC-1 expression is not observed in normal tracheobronchial epithelium, whereas no expression was found in sections of human lung tumours, either. These results suggest that the

induction of MIC-1 expression by retinoids in normal and carcinoma cells is regulated by distinct mechanisms and associated with different biological process. (Newman *et al.* 2003).

In human colorectal cancer cells, MIC-1 expression is increased and apoptosis is induced by treatment with some nonsteroidal anti-inflammatory drugs (NSAIDs), which are cyclooxygenase (COX) inhibitors (Baek *et al.* 2001). Pnn, a nuclear and cell adhesion-related protein, may also have some role in the regulation of MIC-1 gene expression (Shi *et al.* 2001). The promoter region –133/+41 of MIC-1 contains three Sp1 transcription factor-binding sites, and the corresponding transcription factors bind to these sites and transactivate MIC-1 expression. In addition, the chicken ovalbumin upstream promotertranscription factor 1 can interact with the C-terminal region of Sp1 and Sp3 proteins and induce MIC-1 promoter activity through the Sp1 and Sp3 transcription factors (Baek *et al.* 2001) (Fig 2).

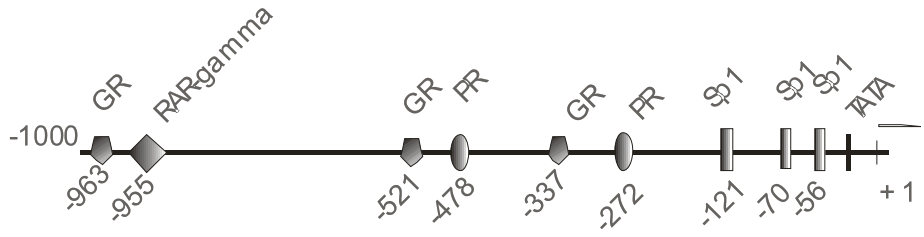

Fig. 2. Location of Sp1-transcription factors and TATA-box and pontential binding sites of steroid hormone receptors in the MIC-1 gene –1000/+1 region according to Baek *et al.* 2001.

3 Outlines of the present study

This study was designed to enhance our knowledge of transcriptional regulation, particularly hormonal and tissue-specific regulation of the genes expressed in prostate or prostate cancer, using rPB, hPAP and MIC-1 as models. The information gained from this study is expected to be valuable for projects where prostate-specific gene expression is needed, e.g. prostate gene targeting or prostate cancer gene therapy. The special aims were:

1. to identify prostate-specific transcription factor binding sites in prostate-related, androgen-regulated genes and to evaluate the functions of prostate-specific regulatory elements in the transcriptional regulation of the hPB and hPAP gene, in order to clarify the mechanisms behind tissue-specific expression of these genes
2. to purify the transcription factor(s) binding to the prostate-specific regulatory elements
3. to identify and characterize nonspecific genes that are overexpressed in androgen-independent, poorly differentiated prostatic adenocarcinoma and cells
4. to study gene expression of MIC-1 in prostate cancer

4 Materials and methods

Detailed descriptions of the materials and methods are presented in the original articles I-IV.

4.1 Cell culture

Prostate carcinoma cell lines LNCaP and PC-3, green monkey kidney COS-1, breast carcinoma cell lines BT-20, T-47D, MCF-7 and MDA-MB-361 (brain metastasis), placental choriocarcinoma cell lines JEG-3 and JAR, endometrial carcinoma cell lines RL95-2, HEC-1-A and KLE, kidney clear-cell carcinoma cell line CAKI-2, transformed embryonic kidney cell line 293, liver carcinoma cell lines HepG2 and SK-HEP-1, colorectal carcinoma cell lines HT-29 and HCT-8 and epidermoid carcinoma cell line A-431 were obtained from American Type Culture Collection (Manassas, VA). Prostate epithelial cells (PrEC) were obtained from Clonetics (San Diego, CA). The cells were maintained as recommended by the supplier. Part of the cells were treated with 20 μM indomethacin for 24 h. Human skin primary fibroblasts were cultured in DMEM containing 10% FCS, 2.5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin.

4.2 Adaptation of PC-3 cells to grow in suspension

Anchorage-dependent PC-3 cells were maintained in T-flasks in DMEM/Nutrient Mix F-12 (Ham) containing 10% FCS, 2.5 mM L-glutamine and 2.4 g/l $NaHCO_3$. The medium was supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin. For large-scale cell production, PC-3 cells were first trypsinated into suspension cultures in spinner flasks. After an adaptation period, the PC-3 cells were transferred into a 2-litre bioreactor. When cell density reached 3.5×10^6 cells/ml, the culture was scaled up into a 30-litre bioreactor. When the suspension culture was initiated in spinner flasks and further scaled up to bioreactors, the concentration of $NaHCO_3$ was decreased to 1.2 g/l and 0.1 %.

Synperonic F68 was added into the culture medium of the PC-3 cells to minimize shear force effects. Aeration of the bioreactors (2-litre Biostat MD and 30-litre Biostat UD, B.Braun Biotech International) was provided by an automatic gas mixing system, and gas was passed into the culture medium through silicon tubing in a manner that resulted in bubble-free diffusion. pO_2 was controlled with air/oxygen and nitrogen and pH with CO_2. The culturing conditions in the bioreactors were: O_2 50 %, temperature 37ºC, mixing 60 rpm, and pH 7.1. An addition of $NaHCO_3$ was needed to keep the medium pH constant during the culture. PC-3 cells were cultured in Biostat UD 30 continuously in a repeated fed-batch mode. The viability of PC-3 cells was monitored with Trypan blue staining.

4.3 Nuclear extracts

Nuclear extracts were prepared as described by Dignam *et al.* (1983). The nuclear extracts prepared from Hela cells were obtained from Promega.

4.4 GEX constructs

Fragments of the androgen receptor were amplified by PCR and cloned to the pGEX - 2TK vector ructs and preparation of GST fusion proteins (Pharmacia Biotech, Uppsala, Sweden) downstream of the glutathionine-S-transferase (GST) sequence. Constructs were sequenced to confirm that no mutations were introduced during the PCR. The plasmids were transformed into Escherichia *coli* invα cells. The GST fusion proteins were prepared as described by Smith and Johnson, 1988.

Complementary oligonucleotides from the rPB gene promoter area (200-400 pmol) were annealed and labelled. GST fusion protein beads and PC-3 nuclear extracts were incubated overnight at 4°C. After incubation, the interacting proteins were removed by centrifugation. The labelled oligonucleotide was then incubated with the remaining supernatant, and EMSA was performed.

4.5 Preparation of plasmid constructs for reporter gene analysis

Fragments of the hPAP and rPB genes were generated by PCR. The fragments were first cloned into a pCR$^{2.1}$TA-cloning vector (Promega, Madison, WI) or directly cloned into the pCAT basic expression vector and the pBLCAT4 promoter vector (Promega, Madison, WI), respectively. Oligonucleotides containing sites for restriction enzymes were used as primers. Primers containing 3 copies of the pros regulatory element were annealed, and the insert was ligated to the pBLCAT 2 vector containing two binding sites of ARE (gift from Dr. Jorma Palvimo). Deletion constructs and mutation constructs were made using the Quickchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All the constructs were confirmed by sequencing with the T7 Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden).

4.6 Transient transfection assays

Transient transfections of the cell lines with plasmid DNA were performed using the lipofection reagents DOTAP or Fugene 6, (Roche, Indianapolis, IN) as recommended by the manufacturer (Roche). Before transfection, $1x10^6$ cells/100-mm dish (DOTAP) or $2x10^5$ cells/35-mm dish (Fugene 6) were cultured for 72 h (LNCaP) or 24 h (other cells). Steroid receptor expression vectors and control plasmid (pCMVβ, Clontech, Palo Alto, CA) were co-transfected in particular experiments. The steroid response was verified by transfecting the mouse mammary tumour virus (MMTV) reporter construct (GMCAT, American Type Culture Collection). After 20-24 h of transfection, the cell medium was replaced by medium containing charcoal-stripped FCS, and hormones (R1881 or dexamethasone) were added as indicated in the experiments. The cells were collected and lysed after 72 h (LNCaP) or 48 h (other cells) of incubation from the beginning of transfection.

4.7 CAT, β-galactosidase and protein assays

CAT measurements were performed using the fluor diffusion assay method (Neumann *et al.* 1987, Eastman 1987). Transfection efficiency was estimated by determining the β-galactosidase activity of the samples with the method of Rosenthal (1987). Protein contents were measured by the Bio-Rad protein assay. The CAT activities were divided by the β-galactosidase activities and the protein contents of the samples, to normalize the results. When Fugene 6 was used for transfection, CAT activities were divided only by the protein contents of the samples to normalize the results.

4.8 EMSAs, supershifts and competition assay

Probes for EMSA were prepared by labelling double-stranded oligonucleotides containing various putative regulatory elements from hPAP or rPB gene with Klenow enzyme and 5'-[α^{32}P]deoxycytidine triphosphate (3000 Ci/mmole, Amersham Pharmacia, Little Chalfont, UK). The probe was incubated with nuclear extracts or purified GAAATATGATA-binding proteins. Varying amounts of ds poly(dI-dC) (Amersham Biotech, Piscataway, New Jersey) were added, depending on the stage of purification. In competition experiments, 100- or 500-fold molar excess of unlabelled specific or nonspecific ds oligonucleotide was added to the reaction mixture. The DNA-protein complexes were separated from unbound DNA using 4% nondenaturing polyacrylamide gel. Supershift experiments were carried out by incubating the nuclear extract with 2-10 μg of antibody for 1 hour at 4 °C either before or after incubation with the probe. The polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, California).

4.9 Dnase I footprint analysis

5´-end labelled primers were used to perform the PCR reaction to generate the rat probasin promoter probe. The DNA-protein binding reaction was carried out by incubating DNA probes with the nuclear extracts in binding buffer (20 mM HEPES, 75 mM NaCl, 2 mM dithiothreitol, 20% glycerol and 5 µg poly(dI-dC)) for 30 min at room temperature. The concentration of $MgCl_2$ was adjusted to 10 mM in the reaction. The samples were incubated for 1 min with freshly diluted DnaseI (Boehringer Mannheim), and the reactions were stopped by adding 140 µl of stop solution (192 mM sodium acetate, 32 mM EDTA and 0.14% SDS). The samples were digested with proteinase K, further extracted with phenol-chloroform and ethanol-precipitated. The probe was digested by the Maxam-Gillbert method. The resulting DNA fragments were electrophoresed on a 6% sequencing gel.

4.10 Generation and identification of transgenic mice

The hPAP promoter/reporter gene constructs were cloned in front of the chloramphenicol acetyltransferase (CAT) gene in the pCAT basic vector as described before. The hPAP promoter-driver reporter gene fragments were released from the vector sequence by restriction digestion and separated by gel electrophoresis. The appropriate DNA fragments were used to generate transgenic mice by the pronucleus microinjection technique. The presence of the transgene in DNAs from tail biopsies was analyzed by PCR or Southern blot. Transgene copies were quantified by slot blot hybridization.

4.11 RNA extraction, Northern blotting and slot blotting

Total RNA from tissue was isolated by the CsCl-gradient method (Davis *et al.* 1986). Total RNA was isolated from cultured cells, which were grown on dishes using either TRIzol (Life Technologies, Gaithesburg, Maryland) or EUROzol (EuroClone, Wetherby, UK) reagent. Poly-A RNA was isolated using oligo(dT)-cellulose (Amersham Pharmacia, Little Chalfont, UK). Northern blots were carried out as recommended by the membrane manufacturer (Boehringer Mannheim, Mannheim, Germany). Slot blot of RNA samples was prepared as described by Solin *et al.* (1990) and hybridized with ^{32}P-labelled pCAT DNA. Control probing of the blots was performed with nick-translated glyceraldehyde-3-phosphate dehydrogenase cDNA (GAD, AC: X02231). Densitometric analysis was used to quantify the mRNA signals of the samples.

4.12 Patient specimen and in situ hybridization

The MIC-1 and GAPD cDNAs were amplified by PCR using oligonucleotides with T7 binding sites. The antisense and sense RNA probes were transcribed from PCR products by using T7 RNA polymerases (Promega, Madison, WI). A PCR fragment of pCAT was cloned into the pCRII-TOPO vector (Invitrogen, San Diego, CA). BamHI- and Xho-I-linearized forms of this construct were used as templates for *in vitro* transcription. A control probe was synthesized similarly from the pTRI-β-actin construct (Ambion, Austin, TX). *In vitro* transcription reactions with the polymerases were performed according to the manufacturer´s specifications in the presence of ^{35}S-CTP.

The *in situ* hybridization reactions were performed as described previously by Chotteau-Lelievre *et al.* (1994) and Mustonen *et al.* (1998), using 6 μm sections of the formalin-fixed, paraffin-embedded tissue samples from 28 patients with BPH and/or prostate cancer or adult transgenic and control mice. Hematoxylin-eosin or Hoechst 33258 (Sigma, St. Louis, MO) was used to stain nuclei. Tissue specimens were collected from patients undergoing radical prostatectomy, biopsy or transurethral resection of the prostate. The hematoxylin stained specimens were examined by a pathologist to ensure that they contained both benign and malignant tissue, or malignant tissue only. The patients with prostate cancer were classified according to the Gleason classification system (Gleason *et al*. 1974).

4.13 UV cross-linking

Oligonucleotides of the hPAP gene and the rPB gene of the same length were used as probes to investigate if the same nuclear protein(s) binds to different elements. A DNA-protein binding reaction was performed as in EMSA. After 20 minutes' incubation at room temperature, the reaction mixtures were exposed to UV light in a Spectrolinker XL-1000 for 10 min. Proteins were separated on a 12% SDS-polyacrylamide gel. The gel was subjected to electrophoresis for 1-2 h at 75 V, dried and exposed to Kodak X-AR films.

4.14 Purification and identification of the complex binding to the GAAAATATGATA site

Suspension-adapted PC-3 cells were used in the purification of GAAAATATGATA-binding proteins. The maximum cell density was 3×10^6 cells/ml, and 85×10^9 cells were harvested weekly. For a purification run, a total of 2.6×10^{11} cells in 85 litres of cell culture media were prepared to yield 3 g of crude nuclear extract. The GAAATATGATA-binding protein complex was purified by a combination of ammonium sulfate precipitation, two sequential anion exchange columns and a sequence-specific DNA affinity column. Fractions from the different purification steps were assayed by EMSA for DNA-binding activity, using ds rPB -257/-235 as a probe. The fractions were

analyzed by SDS-PAGE and silver staining. Several protein bands appeared concurrently at a 0.6 M NaCl concentration.

To identify the purified proteins, the protein bands were excised from the gel and digested with trypsin, and the peptides were examined with MALDI-TOF MS. The masses of tryptic peptides were compared against the SWISS-PROT and NCBI human protein database using the Mascot search algorithm (Perkins *et al.* 1999).

4.15 GEM clones

mRNA from PC-3 and suspension-adapted PC-3 cells was used for Human UniGEM V v2.0 Custom Screening (Incyte Genomics, Inc., St Louis, Missouri). The mRNAs were labelled as described in http://www.incyte.com. Relative expression values for 9182 cDNAs were obtained for both PC-3 cell lines. Clones selected according to screening were purchased from Incyte Genomics (St. Louis, Missouri) and further sequenced by using an ABI377 automatic sequencer (Applied Biosystems, Branchburg, New Jersey) to confirm their identity.

4.16 Measurement of cell viability and apoptosis

5 x 10^5 cells (PC-3) were attached to 6-well plates or 2 x 10^6 transferred to a 50 ml spinner (suspension adapted PC-3). Part of the cells were treated with 20 μM indomethacin for 48 h.

The cells were lyzed and assays for the measurement of double-stranded DNA were performed by the FluoReporter Blue Fluorometric dsDNA Quantification Kit (Molecular Probes, Eugene, Oregon) as recommended by the manufacturer. Fluorescence measurements were carried out using excitation at 360 nm and detection at 460 nm.

For measurement of apoptosis, 1-2 x 10^4 cells were cultured in a 96-well plate for 48 h. Part of the cells were treated with 20 μM indomethacin. Apo-ONE™ Homogeneous Caspase-3/7 Assay (Promega, Madison, Wisconsin) was used for the detection of caspase-3/7 activity in cell culture as recommended by the manufacturer. Normalized caspase activity was obtained by dividing the results from the caspase activity assay by the DNA contents.

5 Results

5.1 Identification of the DNA-binding site of a regulatory protein involved in prostate-specific and androgen receptor-dependent gene expression

A 5´flanking region (-426/+28) of the rat prostatic probasin (rPB) gene shown to be sufficient to direct prostate-specific expression in transgenic mice was used to identify the exact DNA-binding site of a putative prostate-specific transcription factor. CAT reporter gene analyses revealed that the construct pCAT PB -244/+52 was equally well induced by androgens in both prostatic LNCaP and nonprostatic COS-1, MCF-7, HEC-1 and HEP-1 cell lines, indicating that although the probasin gene region -244/+52 was important for androgen regulation, it was not regulated in a prostate-specific manner. Further studies suggested that the region -278/-240 was most crucial for prostate-specific expression. The sequence -426/-279 could be considered a silencer area, especially in nonprostatic cells. In DNase I footprinting, a protected 12 bp region was found between the nucleotides -251 and -240 only with nuclear extracts of prostatic origin. The sequence of this area was GAAAATATGATA (Fig. 3).

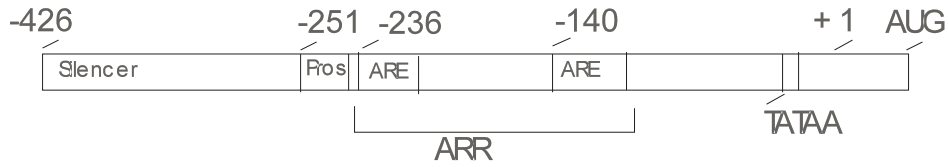

Fig. 3. Location of the prostate-specific DNA-binding site GAAAATATGATA (pros) in the schematic representation of the rPB –426/+52 region.

Deletion of this area decreased androgen induction significantly in transient transfections of prostatic cells compared to the wild-type reporter construct. The pCAT PB –257/+52 construct, which contained the binding site of the putative prostatic regulatory protein

and six additional 5´-nucleotides, did not show nearly so good androgen induction as pCAT PB –278/+52. This suggested that at least 20 additional 5´-nucleotides of the binding site were needed for the regulatory protein to confer optimal androgen induction of the probasin promoter. Glucocorticoids were incapable of increasing the induction of the pCAT PB -278/+52 reporter construct compared to that of pCAT PB -244/+52 in the prostatic cell line LNCaP, suggesting that the putative prostate-specific protein acts as an inducer only when androgen and androgen receptor are present.

The GAAAATATGATA sequence was also found at the first intron (+1144/+1155) of the hPAP gene. EMSA, with hPAP +1136/+1164 as a probe, showed a strong specific shift band when LNCaP or PC-3 nuclear extracts were used, and an rPB –257/-235 fragment could compete off the shift band. These results suggested that the putative prostatic regulatory protein could also play a role in the transcriptional regulation of the hPAP gene.

5.2 Prostatic regulatory proteins and tissue-specific regulation of hPAP promoter constructs

A hPAP construct containing the sequence between the nucleotides -734 and +467 in front of the CAT reporter gene was highly expressed in the prostate of transgenic mice, while the proximal promoter -734/+50 alone achieved low levels of CAT mRNA in all tissues analyzed. Five homologues (A-E) for our previously identified prostate-specific GAAAATATGATA DNA-binding site were found in the area (Figure 4).

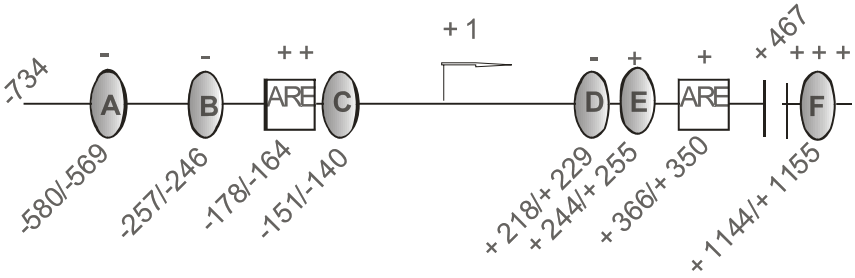

Fig. 4. Location of the prostate-specific DNA-binding site GAAAATATGATA related sequences and potential androgen response elements in the schematic represantation of the regulatory region of the hPAP gene. Binding capacities of the elements for the prostatic protein *in vitro* are also marked (-, +, +++). *In vitro* androgen receptor binding capacities are indicated by + or ++ above AREs.

The competitive reactions in electrophoretic mobility shift assays and UV cross-linking studies suggested that the same nuclear factor binds to the GAAAATATGATA and the sites C and E. The deletion of the element C from the reporter constructs decreased their transcriptional activity in the presence of androgen, while increased activity in the absence of androgens, maximized by concomitant deletion of site D and blocked by antiandrogen flutamide, was detected after removal of element E. These events took place

in LNCaP, but not in COS-1 cells. In summary, the region -734/+467 of hPAP contains regulatory elements involved in prostate-specific and androgen-dependent gene expression. The binding sites of the prostatic transcription factor in the constructs mediated bidirectional regulatory events. Transcriptional activation or repression, depending on the hormonal environment and the location of the site, was detected. The androgen receptor is probably linked in both processes in a ligand-dependent or -independent way.

5.3 Purification of the complex binding to the GAAAATATGATA site

The prostatic transcription factor complex bound to the previously identified GAAAATATGATA site was purified and characterized from a suspension-adapted mass culture of PC-3 prostate cancer cells by using sequence-specific DNA affinity chromatography, mass spectrometry and supershifts. Several potential transcription factors were identified, but only USF2 was confirmed to be part of the transcription factor complex. However, USF2 did not bind directly to GAAAATATGATA.

The prostate specificity of the GAAAATATGATA-binding protein was also further analysed by EMSA. Nuclear extracts from breast, liver, epidermoid and endometrial cell lines did form complexes with the probe containing GAAAATATGATA, but these complexes were weak compared to those of prostatic origin. The interaction between the androgen receptor (AR) and the prostatic regulatory protein was studied by using glutathione-S-transferase pull-downs and transient transfections. Some weak interaction could be detected between the DNA-binding domain of AR and the prostatic transcription factor.

The results on transient transfections showed that a TK-CAT construct containing 3 copies of a GAAAATATGATA sequence 25 nucleotides apart from ARE was more efficiently induced by androgens than a construct where 60 nucleotides separated the GAAAATATGATA and ARE. This suggests that physical proximity between the androgen response element and GAAAATATGATA is important for their co-operation. When PB-CAT constructs with or without GAAAATATGATA, i.e. (pCAT PB –278/+52) and (pCAT PB –244/+52), were transfected to PC-3 cells in the presence of variable amounts of AR expression vector together with androgen, strong induction was already achieved by using 20-40 ng of AR expression vector with the construct containing GAAAATATGATA, but the activity of the construct lacking this sequence increased gradually along with the rising AR levels. The result suggests that the prostatic protein complex makes AR binding to its response element more effective and concomitantly magnifies the effect of androgen.

5.4 Differential gene and MIC-1 expression in prostate cancer and benign hyperplasia

Differentially expressed genes in two PC-3 cell type variants (anchorage-dependent PC-3 cells and suspension-adapted saPC-3 cells) were identified by using cDNA array technology. Genes activated during suspension culturing can be considered putative markers of metastatic activity, since similar events probably take place during cancer cell spread from tissues. In PC-3 cells, the overexpressed genes were mostly large, structural proteins participating in cell interactions. Human collagen type VI (Chu et al 1990, Zeichen et al, 2000), human basement membrane heparane sulfate proteoglycan (Kallunki and Tryggvason 1992) and fibronectin 1 (Donald et al 1998) are examples of such genes. Interferon-inducible proteins were also overexpressed in this cell line. In saPC-3 cells, growth factors and enzymes were overexpressed. Genes involved in regulation of apoptosis, such as translationally controlled tumor protein 1 (Li *et al*. 2001) were also upregulated in saPC-3 cells. Five genes (asparagine synthetase, human serine protease 1, human stanniocalcin homologue, NAD-dependent methylene tetrahydrate dehydrogenase cyclohydrolase (NMDMC) and macrophage inhibitory cytokine 1 (MIC-1)), which had prominent expression in the suspension-adapted cell line, were further analyzed by Northern blot hybridization in prostatic cell lines, benign prostatic hyperplasia (BPH) (3 patients), normal prostate (1 patient) and prostate cancer tissues (5 patients). This study suggested that MIC-1 was expressed in cancer but not in BPH.

In addition, MIC-1 gene expression was studied in specimens of 28 patients with prostate cancer and BPH by *in situ* hybridization. In our results, MIC-1 had no or low expression in BPH and normal prostate, but high expression in prostatic cancer, and it could therefore be a useful marker for prostate cancer. In our study, MIC-1 expression was not overexpressed in tumours with high Gleason scores (Gleason score >4+4, 10 patients) compared to moderately or well differentiated carcinoma (Gleason score < 4+4).

Several transcription factors are known or suggested to regulate MIC-1 gene expression (Paralkar et al. 1997, Bootcov et al. 1997, Kannan et al. 2000, Baek et al. 2001). To clarify the possible regulatory pathway of MIC-1 in prostate cancer progression, these factors and their differential gene expression in PC-3 versus saPC-3 cell lines were analyzed by using microarray data. Genes encoding P53, interleukin 2, TNF-α and TGF-β3 were expressed at equal levels in both cell lines . The MIC-1 promoter area –1521/-1 was analyzed by the TESS transcription factor site search program (http://www.cbil.upenn.edu/cgi-bin/tess/tess/tess33_if=1&RQ=WELCOME). In this search, potential binding sites for estrogen receptor, glucocorticoid receptor and progesterone receptors were found. However, according to the microarray, the genes encoding these receptors are not expressed in the PC-3 or saPC-3 cell lines. Binding sites for retinoid acid receptors were also found, and the gene expression of RXRγ was approximately 2-fold in the saPC-3 cell line compared to the PC-3 cell line, suggesting its possible role in the regulation of the MIC-1 gene. Interleukin 6 is also differentially expressed in the two PC-3 cell lines, and potential response elements for this factor are also found in the MIC-1 promoter. When PC-3 cells were treated with a NSAID compound, cyclooxygenase (COX) inhibitor indomethacin, MIC-1 expression was induced. Apoptosis is also induced by indomethacin in this cell line. In saPC-3 cell line

MIC-1 expression is already high and indomethacin is able to increase MIC-1 expression only in a small extent. Indomethacin induces apoptosis in this cells line but in a lesser extent than in PC-3 cells. The level of apoptosis in saPC-3 cells – either treated or not-treated – is very low compared to PC-3 cells. Similarly to MIC-1, peroxisome proliferator-activated receptor α (PPARα) is known to be activated by COX inhibitors (Lehmann et al. 1997), and a potential response element was also found in the promoter area of MIC-1. In the microarray, the gene encoding PPARα was not expressed. On the other hand, COX inhibitors can also activate the PPARγ pathway (Yamazaki et al. 2002). In the microarray, PPARγ, which can form a heterodimer with RXRγ, is equally expressed in both cell lines, suggesting that the indomethacin induction of MIC-1 could be regulated by the PPARγ pathway.

Recent data also suggest that MIC-1 increases cell invasiveness, the activity of the urokinase-type plasminogene activator (uPA) and the expression of the urokinase-type plasminogen activator receptor (uPAR) in gastric cancer cells (Lee *et al.* 2003). In the microarray, the signal activity of uPA in PC-3 cells was 1.4-fold compared to saPC-3 cells, and the signal activity of uPAR was 1.4-fold in saPC-3 cells compared to PC-3 cells. Although some data indicate that uPA gene is amplified in PC-3 cells (Helenius *et al*. 2001), the results suggest that MIC-1 may have a similar function in prostate cancer cells as in gastric cancer cells.

6 Discussion

6.1 Identification and androgen regulation of the binding site of a prostatic protein

A rPB gene 5´flanking area (-426/+52), which has been shown to be sufficient in targeting gene expression to the prostatic epithelium (Greenberg *et al.* 1994,1995), was used as a tool in the identification of the DNA-binding site of a putative prostate-specific regulatory protein. A protected 12 bp region was found between the nucleotides -251 and -240 only with a nuclear extract of prostatic origin. The presence of this binding site increased the expression of the probasin promoter together with androgen and AR in prostatic cancer cell lines, but not in nonprostatic cell lines. GR was shown to be incapable of cooperating with this regulatory protein, indicating that the function of this protein is AR-dependent. It was also interesting to see that although the rPB -251/-240 area was shown to be important for androgen induction of the rPB gene in prostatic cells, at least 20 5´flanking nucleotides were needed for maximal effect. However, these 5´flanking nucleotides did not bind any sequence-specific proteins of their own. These nucleotides can, for example, help the prostatic protein to bind to its response element. Similar observations have been made with the NF-κB response element of the human intercellular adhesion molecule-1 (ICAM-1) gene. This gene is induced when the tumour necrosis factor α (TNFα) binds to the modified NFκB site. In addition to this binding site, however, both of the specific 5´- and 3´-flanking sequences are necessary for TNF-α induction (Paxton *et al.* 1997).

When either of the AREs at the rPB –286/+52-CAT construct is mutated, androgen induction is almost completely lost (Kasper *et al.*1994), although the construct contains the DNA-binding site of the putative prostatic regulatory protein. This result suggests that the prostatic protein is not obligatory for the function of ARs in the rPB gene 5´flanking area. However, the presence of this factor can duplicate or even triplicate the effect of androgens, suggesting that there is a synergistic action between the putative prostate-specific transcription factor and ARs. The fact that this factor was unable to cooperate with the GR and the physical closeness of the androgen response elements and the DNA-

binding site of the prostatic protein further support this idea. These results suggest that the prostatic protein does not act independently, but enhances the AR-mediated induction if GAAAATATGATA is physically close to ARE and enhances the transactivation function of AR. GST-AR fusion proteins in conjunction with a sensitive absorption assay indicated that there was a specific interaction between the protein complex and the DBD of AR. AR co-operates in a similar manner with the DNA-binding protein complexes in human prostate-specific antigen and human kallikrein 2, which are also prostatic and androgen-responsive genes (Sun *et al.* 1997, Zhang *et al.* 1997). In the case of human kallikrein 2, c-Fos protein was shown to take part in the protein complex that interacts with AR and was also required for complex formation (Sun *et al.* 1997). β-catenin can also interact with liganded AR, and DBD of the receptor is necessary for this interaction (Pawloski *et al*. 2002, Chesire *et al*. 2002). We suggest that GAAAATATGATA elements located close to AREs can potentiate AR binding and thereby make an impact on the low-affinity AREs present in prostate-specifically expressed genes. Low-affinity AREs have been found in, for example, the PSA enhancer (Huang *et al.* 1999), which also contains GAAAATATGATA-like sequences close to these AREs. The biochemical definition of how specific combinations of AR-interacting proteins generate cell-type and tissue-specific effects represents a major challenge for the future.

AR is known to associate with a variety of transcription factors, such as general transcription factors (GTFs), the steroid receptor coactivator (SRC) family, the PIAS [protein inhibitor of activated signal transducer and activator of transcription (STAT)] family, filamentous actin (f-actin)-binding proteins and coactivators that influence the nuclear-cytoplasmic trafficking of AR (reviewed by Heinlein *et al.* 2002). The prostate-specific transcription factors identified so far also interact with AR. One of these is PDEF, a prostate-derived member of the Ets transcription factor family (Oettgen *et al.* 2000, Yamada *et al.* 2000). PDEF interacts with the androgen receptor and cooperatively enhances androgen-mediated activation (Oettgen *et al.* 2000). In addition, the homeodomain transcription factor NKX3.1 is expressed predominantly in prostate, with low levels also detectable in testis (He *et al.* 1997, Prescott *et al.* 1998). Androgen-responsively expressed NKX3.1 is believed to play an important role in prostate cell proliferation and differentiation (Bhatia-Gaur *et al.* 1999). A recent study demonstrated that NKX3.1 is capable of interacting physically with PDEF (Chen *et al.* 2002). Furthermore, the recently identified Androgen-Induced bZIP (AIbZIP) protein is suggested to be a putative androgen-regulated transcription factor exclusively in prostate as well as in breast and prostate cancer cell lines. AIbZIP appeared to be expressed at higher levels in prostate cancer cells compared to non-cancerous prostate cells (Qi *et al.* 2002).

The DNA-binding site was observed not only in the rPB 5´flanking area, but also in the hPAP gene, which is known to be expressed in a prostate-specific manner (Solin *et al*. 1990). Five homologous sequences for our previously identified prostatic GAAAATATGATA DNA-binding site were found from the hPAP proximal promoter –734/+50. A CAT-reporter gene construct containing this area was significantly expressed in the prostate of transgenic mice, while the proximal promoter alone achieved low levels of CAT mRNA in all tissues analyzed. It is possible that the prostatic factor is conserved between species. The intronic area +57/+467 was shown to be important for the androgen-activated expression of the hPAP gene. The GAAAATATGATA-like elements

found in the regulatory areas of hPAP might have an impact on the tissue-specific expression of the gene, as shown in the case of probasin.

6.2 Purification of the GAAAATATGATA-binding protein

We purified the potential factors interacting with the GAAAATATGATA sequence and characterized the co-operation between AR and the putative regulatory factor(s). The results strongly suggested that the prostatic factor is a complex. USF2 could be confirmed to be part of the complex. We also showed that the prostatic complex bound to GAAAATATGATA behaves like an AR coactivator.

Though, in EMSA, the rPB -257/-235 fragment formed clear, specific complexes with nuclear extracts of prostatic origin, weak but specific complexes were also formed with nuclear extracts from breast, liver, epidermoid and endometrial cell lines. However, previous findings (I, Zhang *et al.* 2000) have shown that, in transient transfections, the probasin-CAT construct that contains the GAAAATATGATA site gives higher activity in transient transfections, compared to a construct without that element, only in prostatic cell lines. These results suggest that the complexes formed in EMSA with non-prostatic nuclear extracts may lack some interactions crucial for the activation of the gene.

The majority of the potential GAAAATATGATA-associated factors identified in this study are widely expressed. Thus, none of them alone are sufficient to account for cell type-dependent activities, and additional factors are therefore needed. In several studies, ubiquitous factors have been shown to interact with a tissue-specific factor to promote tissue-specific gene expression. Alternatively, non-tissue-specific factors may be incorporated into a complex, and the specific combination of the complex may mediate tissue-specific gene expression. AR is known to interact with a variety of nuclear regulatory proteins (Heinlein *et al.* 2002). Interaction with different regulatory factors modulates its interaction with DNA and the basal transcription machinery and thereby contributes to cell type specificities in androgen-mediated gene expression.

The upstream stimulatory factors USF1 and USF2 are members of the evolutionarily conserved E-box (CANNTG) binding the basic-helix-loop-helix-leucine-zipper (bHLH-zip) family of transcription factors (Sirito *et al.* 1992). We confirmed the presence of USF2 by supershift experiments. We were not able to confirm the presence of USF1 using antibody directed against an epitope at the carboxy terminus of USF1. However, it is known that USF2 preferentially heterodimerizes with USF1, and it is thus plausible that both USF factors are present when the complex forms. USF factors are ubiquitously expressed, but they are known to interact with tissue-specific factors to promote cell-specific regulation (Quang *et al.* 1999). They also play important roles in the estrogen-inducible transcriptional regulation of breast cancer cells (Xing & Archer 1998). USF factors have been suggested to participate in combinatorial interactions between multiple non-tissue-specific factors and thereby to contribute to tissue-specific expression (Johnson & Owens 1999). Domains important for the transcriptional activity of the USF proteins have been localized to their amino-terminal region. The amino-terminal mutant USF2 efficiently binds DNA, but is unable to transactivate due to the absence of transactivation domains (Heckert *et al.* 2000). The carboxy terminus includes the region

responsible for the DNA-binding properties of the protein (Qyang *et al.* 1999). In this study, the GAAAATATGATA-associated complex formation was totally prevented by the anti-USF2 amino-terminal antibody but not by the carboxy-terminal antibody. This suggests that USF2 contributes to the GAAAATATGATA complex formation rather by protein-protein interaction than by direct binding to the element. Thus, the GAAAATATGATA element is preferably associated with a complex of regulatory factors than a single transcription factor.

In addition to USF proteins, a group of other transcription factors was obtained by purification. Some of them have been reported to act as activators, while some others seem more commonly to play an inhibitory role. This conforms well to our unpublished results, suggesting that the GAAAATATGATA-associated complex could either repress or activate transcription in prostate cells, depending on the absence or presence of androgen, respectively. One of the potential repressors is hnRNPA1, which belongs to the heterogeneous nuclear ribonuclear protein (hnRNP) superfamily. hnRNPs were originally identified based on their ability to bind to single-stranded pre-mRNA and to control its processing and nuclear export. However, an increasing number of hnRNPs appear to be multifunctional, participating both in transcriptional and in post-transcriptional events. Several proteins in this family have been shown to act as double- or single-stranded DNA-binding transcription factors. Recently, particular members of the hnRNP family were reported to be capable of modifying steroid hormone-directed gene regulation. One of them is the estrogen response element-binding protein ERE-BP, which competes with the estrogen receptor for binding to its response element (Chen *et al.* 1998). Equally, the vitamin D response element-binding protein VDRE-BP, which belongs to the hnRNPA family, is capable of squelching vitamin D receptor (VDR)-mediated transactivation (Chen *et al.* 2000). Another factor identified in this study was the nuclear receptor coactivator 62000 Da (NCoA-62), which is known as a VDR interacting protein (Baudino *et al.* 1998). NCoA-62 interacts with members of the nuclear receptor superfamily and enhances the transcriptional activities of liganded VDR, retinoic acid, estrogen, and glucocorticoid receptor (Zhang *et al.* 2001).

Participation in the Notch-signaling pathway is the best-characterized function of the J kappa recombination signal-binding protein RBP-Jκ, while it may also function as a transcriptional repressor by interacting with coactivators of the basal transcription machinery (Olave *et al.* 1998). Transcription factor NRF has been reported to inhibit transcription factor NF-κB activity by direct protein-protein interactions with NF-κB (Nourkbakhsh *et al.* 2001). Similarly to NRF, RBP-Jκ and hnRNPA1 have also been reported to interfere with the control of NF-κB-dependent transcription (Oswald *et al.* 1998, Hay *et al.* 2001). NF-κB is a key transcription factor involved in the regulation of many cellular processes, including normal and malignant cellular growth and differentiation. Certain nuclear hormone receptors have been shown to interact physically with NF-κB (McKay *et al.* 1999). Previous studies have shown NF-κB and AR to be mutual antagonists (Palvimo *et al.* 1996). NF-κB has been shown to be constitutively activated in several types of cancer cells, including the androgen-independent prostate cancer cells PC-3 and DU-145. Recent studies support a role for NF-κB in prostate cancer progression (Chen *et al.* 2002).

Damage-specific DNA binding protein 1 (DDB1) was initially identified as a subunit of DDB, which is a ubiquitous and highly conserved nuclear factor that functions in the

DNA repair system. However, DDB1 has been shown to interact with trans-activators of several genes. It may function as part of a signal transduction process that regulates gene transcription in response to DNA damage (Watanabe *et al.* 1999). Furthermore, it has been reported to be closely related to the regulatory factor BRF-2, which contributes to liver-specific gene expression (Krishamoorthy *et al.* 1997).

6.3 Gene expression in androgen-independent, poorly-differentiated prostatic adenocarcinoma

We studied the differential expression of genes in two PC-3 cell lines by cDNA microarray. The differential expression pattern of the genes is assumed to reflect differences in prostate cancer progression. Althought the anchorage-dependent PC-3 cell line already have metastatic features, the saPC-3 cell line may be a useful model for very aggressive prostate cancer. 21 genes were selected from the microarray for closer investigation, and the differential expression of five of these genes was analysed by Northern blotting in different prostate cancer cell lines and in patient material. Since one of the genes, MIC-1, was expressed in prostate cancer but not in prostatic hyperplasia, its expression was studied in more detail. *In situ* hybridisation with 28 patients with prostatic carcinoma confirmed that MIC-1 mRNA is abundant in cancer but not in hyperplasia. Large amounts of MIC-1 have been previously detected by immunohistochemistry in biopsy specimens of breast, colon and prostate cancer, but not within normal epithelial cells of these organs (Brown *et al.* 2001). Baek *et al.* 2001 showed that MIC-1 is an antitumorigenic and proapoptotic protein, and that its expression is increased by COX inhibitors, such as indomethacin and sulindac sulfide, in human colorectal cells. However, COX-2-specific inhibitors were not able to increase MIC-1 expression. In our results, MIC-1 expression is induced by indomethacin in PC-3 cells. Apoptosis is also induced by indomethacin in this cell line, but to a lesser extent in the saPC-3 cell line, suggesting that the apoptotic pathways in the latter cell line are blocked. A recent study showed MIC-1 to be overexpressed in an androgen-independent LNCaP prostate cancer cell line compared to an androgen-sensitive LNCaP cell line (Baek *et al.* 2002). In our study, this gene was also expressed in an androgen-independent cell line, indicating reactivation of the gene by a so far unknown mechanism typical of some cancer cells. Similar reactivation has been shown to take place in the case of the gene encoding serine protease TMPRSS2 in prostate cancer tissue specimens (Vaarala *et al.* 2001). It is obvious that MIC-1 has a different function in prostate cancer than in macrophages or colorectal cells. In prostatic DU 145 cells, MIC-1 is able to down-regulate the expression of the antiapoptotic gene metallothionein 1E and the cell adhesion genes RhoE and catenin δ1, leading to reduced cell adhesion and induced cell detachment (Liu *et al.* 2003). MIC-1 can also induce the invasiveness of gastric cancer cells by up-regulating the urokinase-type plasminogen activator system via an extracellular signal-regulated kinase-1/2-dependent pathway (Lee *et al.* 2003). In the microarray, the signal activity of uPA in PC-3 cells was about 1.5-fold compared to saPC-3 cells, suggesting that MIC-1 may have a similar function in prostate cancer cells as in gastric cancer cells.

The genes that have previously been shown to increase cell attachment were fibronectin 1 (Sites *et al.* 1996), integrin alpha 1 (Briesewitz *et al.* 1993), human collagen type VI (Zeichen *et al.* 2000) and human basement membrane heparane sulfate proteoglycan (Kallunki and Tryggvason 1992). All these genes were overexpressed in the anchorage-dependent PC-3 cell line, suggesting that saPC-3 cells have lost their adhesive potential. Donald *et al.* (1998) also showed that highly metastatic prostate cancer cells (ML, MLL and AT-3) possess less adhesive potential relative to extracellular matrix components, such as fibronectin 1, than nonmetastatic cells. It is possible that, in our material, MIC-1 had a role in the reduced cell adhesion.

Information given by cDNA array is a valuable tool in cancer research, because genes with prominent or very low expression can be compared with expression data available on other disease states. For example, the discovery of a gene with high expression in PC-3 and saPC-3 cells, but with no or low expression in BPH or normal prostate, would reveal a potential new prostate cancer marker. In our data, MIC-1 could be such a marker.

6.4 Conclusion

This study focused on the tissue-specific promoter activities and androgen regulation of rPB and hPAP genes. The gene expression profile in androgen-independent prostate cancer was also studied. The GAAAATATGATA element in the probasin promoter –251/-240 region was found to be involved in the prostate-specific and androgen-dependent regulation of this gene. Precisely the same 12 bp sequence was found in the first intron +1144/+1155 of the hPAP promoter. Five homologous sequences along the promoter and the first intron of the hPAP gene could bind the prostatic regulatory protein with different affinities and could hence be involved in the regulation of the prostate-specific expression of the hPAP gene in a bidirectional manner, depending on the hormone status. The promoter and first intron fragment –734/+467 of the hPAP gene could direct and restrict the gene expression mainly in prostatic epithelium. We were not able to purify any single prostate-specific transcription factor, but a complex, and one part of this complex is the transcription factor USF-2. This complex was shown to have weak interactions with the DBD of AR.

The further understanding of the molecular mechanisms behind the androgen regulation and the prostate-specific regulation of rPB and hPAP promoter might lead to improvement of the clinical practice for prostate cancer by using therapeutic genes under the strict control of a highly active and prostate-specific promoter.

The MIC-1 gene had high expression in saPC-3 cells, which served as a model for the androgen-independent, metastatic phase of prostate cancer. Indomethacin induced MIC-1 expression in PC-3 and saPC-3 cells, and apoptosis was also induced in PC-3 cells, but not in saPC-3 cells. MIC-1 has previously been described as a proapoptotic gene (Baek *et al*. 2001), but it is obvious that, in saPC-3 cells, this apoptotic activity is blocked. *In situ* hybridization showed that the MIC-1 gene was expressed in prostate cancer tissue with no or low expression in BPH or normal prostate, suggesting that this gene could be a potential new marker for prostate cancer.

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