TRANSCRIPTIONAL REGULATION OF THE HUMAN PROSTATIC ACID PHOSPHATASE GENE

Tissue-specific and androgen-dependent regulation of the promoter constructs in cell lines and transgenic mice

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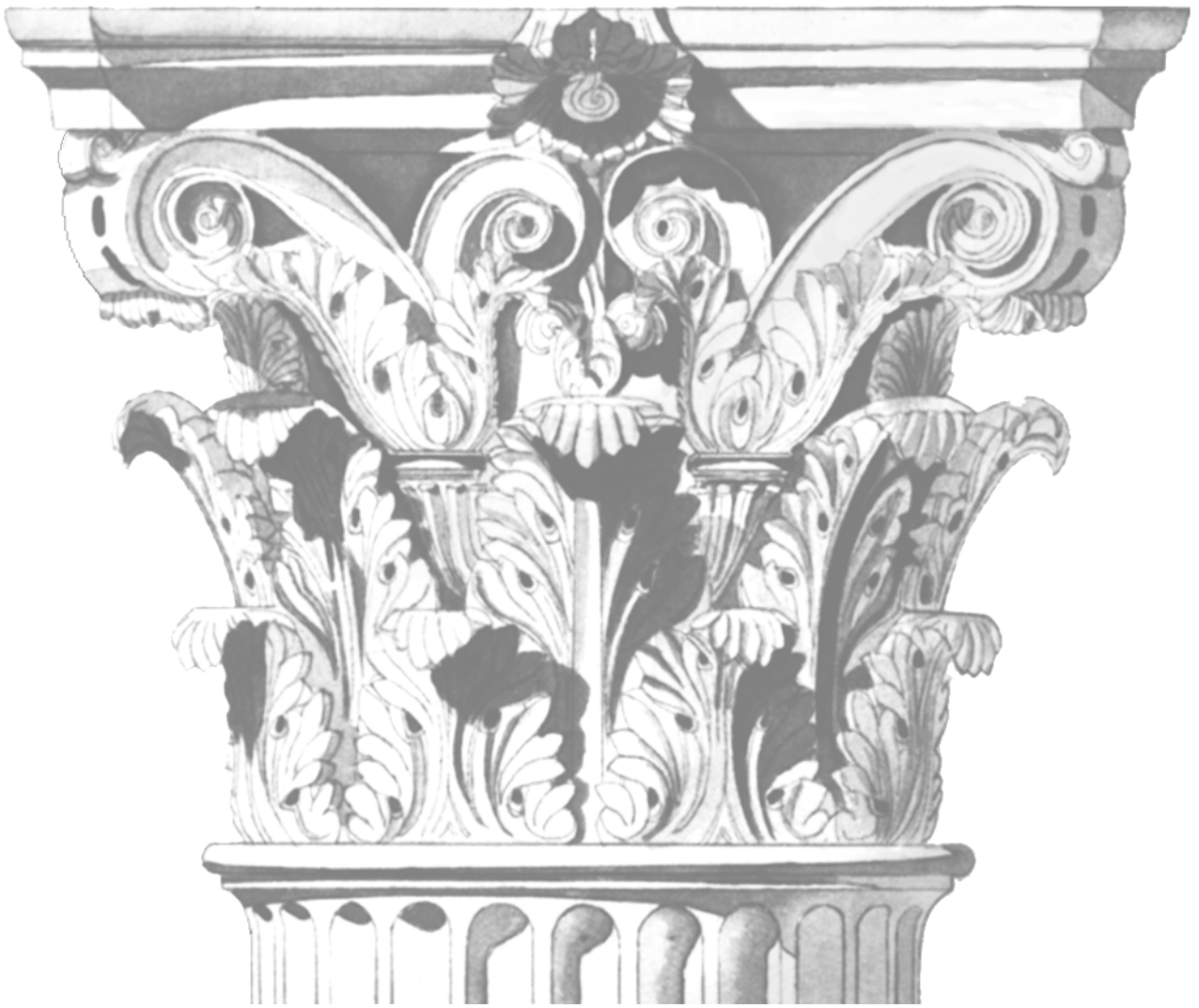
OULU 2002



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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in the Auditorium 9 of the University Hospital of Oulu, on August 9th, 2002, at 12 noon.

OULUN YLIOPISTO, OULU 2002

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ISBN 951-42-6762-1 (URL: http://herkules.oulu.fi/isbn9514267621/)

ALSO AVAILABLE IN PRINTED FORMAT
Acta Univ. Oul. D 687, 2002
ISBN 951-42-6761-3
ISSN 0355-3221 (URL: http://herkules.oulu.fi/issn03553221/)

OULU UNIVERSITY PRESS
OULU 2002

Shan, Jingdong, Transcriptional regulation of the human prostatic acid phosphatase gene Tissue-specific and androgen-dependent regulation of the promoter constructs in cell lines and transgenic mice

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2002

Abstract

Human prostatic acid phosphatase (hPAP) was the first laboratory parameter used for prostate cancer diagnosis, whereas the mechanisms behind the androgen regulation and tissue-specific expression of this prostate epithelium-specific differentiation antigen are not yet clear.

In this study, a transient transfection model and transgenic animal model have been set up for functional analysis of the promoter and first intron region of the hPAP gene. The promoter constructs covering the region-734/+467 of the gene were functional in both prostatic and nonprostatic cells. Although hPAP constructs included two putative AREs with *in vitro* AR-binding ability at -178 and +336, androgen treatment had little effect on the promoter activity of the gene in transiently transfected cells. The hPAP fragment -734/+467 could trigger the expression of the CAT reporter gene and restrict the expression mainly in the prostates of transgenic mice.

The DNA-binding site with the sequence GAAAATATGATA of a regulatory protein involved in prostate-specific and androgen receptor-dependent gene expression was identified from rPB promoter. The exact same 12 bp sequence was found in the first intron +1144/+1155 of the hPAP gene. Five homologous sequence, A, B, C, D and E, were located in the -734/+467 region of the hPAP gene, where site C and E could bind the regulatory protein in EMSA. Deletion of site C decreased the transcriptional activities significantly compared to those of corresponding wild-type constructs in LNCaP cells when androgens were present. Deletion of site E or both sites D and E increased the promoter activity in LNCaP when androgens were absent.

In conclusion, androgens could not directly regulate hPAP expression via receptor-binding to the AREs in LNCaP cells. The promoter and first intron fragment -734/+467 of the hPAP gene could direct and restrict the gene expression mainly in prostate epithelium. A prostatic regulatory protein binds to multiple sites with the GAAAATATGATA or homologous sequences along the regulatory areas of the hPAP gene with different affinities, modulating the prostate-specific expression of the gene in a bidirectional manner, depending on the hormone status.

Keywords: transgenic mice, transcription factors, human prostatic acid phosphatase gene, promoter, transcription, androgen receptor, DNA-binding sites, prostate, transfection

Acknowledgements

The present study was carried out at the Research Center for Molecular Endocrinology, the WHO Collaborating Center for Research on Productive Health, Faculty of Medicine, and Biocenter Oulu, University of Oulu, during the years 1995-2002.

I wish to express my deepest gratitude to my supervisor, Professor Pirkko Vihko, M.D., Ph.D., for giving me the opportunity to prepare my thesis as a member of her group. Her board knowledge of scientific issues, unique enthusiasm, excellent guidance and unending support has been of great value to me and essential for the completion of this work. I also wish to present my sincerest gratitude to the President of the Academy of Finland, Professor Reijo Vihko, M.D., Ph.D., for inviting me here, introducing me into the field of molecular endocrinology, and for his encouragement during these years. I owe my warmest gratitude to Dr. Katja Porvari, for her unreserved advice and unstinted help at all the stages of my thesis work.

I am very obligated to Docent Pekka Kallio, Ph.D., Sari Mäkelä, M.D., Ph.D., for their constructive criticism on the manuscript of this thesis. I wish to thank Sandra Barboczy for her careful revision of the language of the manuscript.

I am indebted to all my co-authors for their invaluable contribution to this work. I am also grateful to all my co-workers in the research center for sharing their knowledge and expertise, especially to Airi Vesala, Mirja Mäkeläinen, Marja-Riitta Hurnasti, Marja-Liisa Norrena, and Pirkko Ruokojärvi for their skillful technical assistance. I owe my special acknowledgements to Anja Raatikainen, Sirpa Annanperä for their valuable work. I would like to extend my warmly thanks to Docent Veli Isomaa, Ph.D., for his support during the years, to Juhani Heikkilä and Jaakko Heikkilä for their help in computer application, to Annakaisa Herrala, Riitta Kurkela, Aija Lindfors, Yan Li and Olayiwola Oduwole for their friendship.

Finally, I owe my sincerest gratitude to my wife Hongmin Tu, my son Yuanqi Shan, and my parents; without their empathy and support, this work would have never come to the end.

This study was mainly supported by Biocenter Oulu and the research council for Health, the Academy of Finland.

Oulu, June 2002

Jingdong Shan

Abbreviations

ACP	acid phosphatase
AF-1	activation function-1
AF-2	activation function-2
ALP	alkaline phosphatase
AP-1	activator protein-1
AR	androgen receptor
ARE	androgen response element
bp	base pair
BRCA1	breast cancer susceptibility gene-1
CAT	chloramphenicol acetyltransferase
CREB	cAMP response element binding protein
cDNA	complementary deoxyribonucleic acid
CMV	cytomegalovirus
DBD	DNA-binding domain
DHT	5α-dihydrotestosterone
E2	17β-estradiol
EGF	epidermal growth factor
EMSA	electrophoretic mobility shift assay
ER	estrogen receptor
FGF	fibroblast growth factor
GAD	glyceraldehyde-3-phosphatase dehydrogenase
GR	glucocorticoid receptor
GRIP 1	glucocorticoid receptor-interacting protein-1
hK2	prostate-specific human glandular kallikrein
hPAP	human prostatic acid phosphatase
HRE	hormone response element
ICAM-1	human intercellular adhesion molecule-1
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL	interleukin
kb	kilobases

kDa	kilodalton
LBD	ligand-binding domain
LBP	ligand-binding pocket
MMTV	mouse mammary tumor virus
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor-κB
NLS	nuclear localization signal
nt	nucleotide
PCR	polymerase chain reaction
PDEF	prostate-derived-Ets-factor
pI	isoelectric point
PKC	protein kinase C
pNPP	paranitrophenylphosphatase
PPARγ	peroxisome proliferators-activated receptor γ
PR	progesterone receptor
PSA	prostate-specific antigen
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
R1881	17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one
RAR	retinoic acid receptor
RSV	rous sarcoma virus
rPAP	rat prostatic acid phosphatase
rPB	rat probasin
RXR	retinoic-X receptor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SRE	steroid response element
STAT	signal transducers and activators of transcription
SV	simian virus
TAD	N-terminal transactivation domain
TGF	transforming growth factor
THR	thyroid hormone receptor
TIF2	human transcriptional intermediary factor 2
TK	tyrosine kinase
TNFα	tumor necrosis factor-α

List of original publications

This thesis is based on the following articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented

I Ruokonen M, Shan J, Hedberg P, Patrikainen L, Vihko P (1996) Transfecting well-differentiated prostatic cancer cell line LNCaP. Biochem Biophys Res Comm 218: 794-796.

II Shan J, Porvari K, Ruokonen M, Launonen V, Hedberg P, Karhu A, Oikarinen J, Vihko P (1997) Steroid-involved transcriptional regulation of human genes encoding prostatic acid phosphatase, prostate-specific antigen, and prostate-specific glandular kallikrein. Endocrinology 138: 3764-3770.

III Patrikainen L, Shan J, Porvari K, Vihko P (1999) Identification of the Deoxyribonucleic acid-binding site of a regulatory protein involved in Prostate-specific and androgen receptor-dependent gene expression. Endocrinology 140: 2063-2070.

IV Shan J, Porvari K, Kivinen A, Patrikainen L, Halmekytö M, Jänne J Vihko P (2002) Transcriptional regulation of the prostatic acid phosphatase gene – tissue-specific and androgen-dependent effects mediated by multiple binding sites of prostatic transcription factor. (Submitted).

Contents

1 Introduction

Human prostatic acid phosphatase (hPAP) is a prostate-specific differentiation antigen and the major phosphatase in differentiated prostate epithelial cells (Gyorkey 1973, Yam 1974). It is a secreted glycoprotein composed of two approximately 50-kDa subunits synthesized in the epithelial cells of the prostate (Derechin *et al.*1971, Vihko *et al*. 1978, 1978a). The enzyme dephosphorylates various phosphomonoesters with an acidic optimum *in vitro* (Yam 1974). It has been proposed that prostate cells, which express a low level of hPAP, may be at risk for malignant development (Reif *et al*. 1973), since the enzyme has a low expression in carcinoma cells (Hakalahti *et al*. 1993), although its secretory form in the circulation may be elevated (Reif *et al*. 1973, Foti *et al*. 1977, Loor *et al*. 1981, Vihko *et al*. 1980, 1981, 1985). hPAP has been used as a parameter for prostate cancer diagnosis in clinical laboratories for over half a century, owing to the property of its variable activities at different states of the disease in serum. Vihko *et al.* (1978b) developed the first radioimmunoassay to detect hPAP directly, instead of the activity measurement of hPAP applied in the clinic. Recently, another prostate-specific differentiation antigen, prostate-specific antigen (PSA), became the major clinical standard of prostate cancer detection because of its greater sensitivity in comparison to hPAP (Oesterling 1991). Even so, hPAP as a biomarker may still be useful if new assay technology is applied.

Because an extremely large amount of hPAP is present in spermatic fluid, it has been suggested to have a physiological role in fertility (Rönnberg *et al*. 1981, Coffey & Pienta 1987), but the enzyme's function in this respect remains largely unclear. Conversely, it seems that the cellular form of hPAP is involved in the growth regulation of prostate cells (Lin *et al*. 1992, Lin *et al*. 1994). This function of hPAP relies on the enzyme also exhibiting protein tyrosine phosphatase (PTP) activity (Li *et al*. 1984, Lin & Clinton 1986, Chevalier *et al*. 1988, Vihko *et al*. 1993), even though the structure of hPAP is quite different from typical PTPs. Recent studies suggest that c-ErbB-2 protein, a member of the erbB receptor tyrosine kinase (RTK) family and the gene that is frequently amplified in breast cancer patients, could be an *in vivo* substrate of the enzyme (Lin & Meng 1996, Meng & Lin 1998). It has been hypothesized that decreased cellular hPAP expression results in a highly tyrosine-phosphorylated ErbB-2, which subsequently initiates

constitutively active proliferation signals, leading to the androgen-independent phenotype of advanced prostate cancer (Meng *et al*. 2000).

The expression of hPAP is negligible in fetal tissue, but very high in normal, well-differentiated prostate cells in the post-pubertal and adult male. The effect of puberty on hPAP expression indicates that the prostate-specific enzyme is under androgen influence. However, androgen regulation of hPAP expression is as complicated as it is in the case of rat PAP (Porvari 1995). Different results of both down- and up-regulation of the hPAP gene by androgen were observed depending on models, methodologies, and even the experimental conditions (Henttu *et al*. 1992, Lin *et al*. 1993b). Two putative androgen response elements (AREs) at -178 and +336 have been shown to be able to bind androgen receptor (AR) efficiently *in vivo* (Virkkunen *et al.* 1994.), and it has be proposed that these two AREs might have a synergistic effect to mediate the androgen regulation of the gene. Functional analysis of the AREs is demanded to clarify the issue. On the other hand, hPAP expressed specifically from the prostate provides a suitable model to study tissue-specific gene regulation. Better understanding of the mechanism of prostate-specific gene regulation will facilitate the development of a new strategy of gene therapy for prostate cancer, the most commonly diagnosed cancer among men in Western industrialized countries.

In this work, a cell culture and transfection model has been set up for functional analysis of putative AREs in the promoter and first intron region of the hPAP gene. Hormonal regulation of the hPAP promoter has been compared with two other promoters of prostate-related genes PSA and prostatic-specific human glandular kallikrein (hK2), both in prostatic cell lines and non-prostatic cell lines. Prostate-specific gene regulation has also been investigated by using hPAP and rat Probasin (rPB) as models.

2 Review of the literature

2.1 Phosphatases

Hydrolysis of phosphate monoesters by phosphatases in biological systems is a crucially important process linked to energy metabolism, metabolic regulation, and a wide variety of cellular signal transduction pathways. Phosphatases are widely distributed in nature and differ greatly in their physical properties and tissue location. They can be classified according to several frameworks (Vincent *et al*. 1992). Based on their substrate type, they can be divided into non-specific phosphatases and protein phosphatases.

Non-specific phosphatases catalyze the hydrolysis of almost any phosphate ester, and they can be further divided into alkaline and acid phosphatase based on their optimal pH for catalysis. Alkaline phosphatases (ALP; EC 3.1.3.1.) work optimally near pH 8. Like other enzymes, ALP has many isoenzymes. In healthy adults, ALP is mainly derived from the liver, bones, and in less amounts from intestine, placenta, kidney, and leukocytes. Acid phosphatases (ACP; EC 3.1.3.2.) are a group of genetically distinct isoenzymes capable of hydrolyzing phosphate esters with an acid optimum to yield an inorganic phosphate group (Yam 1974). In addition, acid phosphatases can catalyze the phosphoryl transfer between a phosphoester and alcohols (Bodansky 1972, Vihko, 1978, van Etten 1982). Four forms of acid phosphatase isoenzymes exist at the structural level of genes (Moss *et al*. 1995). The erythrocytic and lysosomal forms are expressed in most cells, whereas, the prostatic and macrophagic forms have a more limited expression. Acid phosphatases can also be grouped by their molecular weight or their sensitivity to inhibition by tartrate. The erythrocytic and macrophagic forms are distinguished from the others in resisting inhibition by L-tartrate. The macrophagic forms have been linked with miscellaneous disorders, notably increased osteolysis, Gaucher's disease of spleen and hairy cell leukemia. The prostatic form has served as a marker for prostate cancer more than sixty years (Gutman & Gutman, 1938).

Protein phosphatases prefer phosphoproteins or phosphopeptides as substrates. The reversible phosphorylation of proteins, catalyzed by protein kinases and phosphatases, is a major mechanism for regulating many cellular processes, including intermediary metabolism, cell-cycle progression, DNA replication, transcription, protein translation,

and transport and secretory processes (Cohen 1989, Mumby & Walter 1993, Wera & Hemmings 1995). Phosphoprotein phosphatases are structurally and functionally diverse enzymes that are represented by three distinct gene families. Two of these, the PPP and PPM families, dephosphorylate phosphoserine and phosphothreonine residues, whereas the protein tyrosine phosphatases (PTPs) dephosphorylate phosphotyrosine amino acids (Barford 1996, Tonks & Neel 1996). The dual-specificity phosphatases, a subfamily of PTPs, dephosphorylate all three phosphoamino acids (Keyse 1995). Within each family, the catalytic domains are highly conserved, with functional diversity endowed by regulatory domains and subunits. The PPP and PPM families are metalloenzymes and dephosphorylate their substrates in a single reaction step using a metal-activated nucleophilic water molecule. In contrast, the PTPs catalyze dephosphorylation by using a cysteinyl-phosphate intermediate. Most of the structurally diverse PTPs contain one highly conserved 240-amino acid catalytic domain, with the exception of some members, such as low molecular weight PTPs (Dixon 1996, Tonks & Neel 1996, Zhang *et al*. 1998). These catalytic domains are defined by the presence of a PTP signature motif, (I/V)HCXAGXXR(S/T)G, which forms the 'catalytic pocket' of the enzyme (Ostman *et al*. 1994, Nell & Tonds 1997). The signature motif functions as a phosphate-binding cradle in which a cysteine residue is sterically positioned for a nucleophilic attack on the phosphorous atom of the substrate's phosphotyrosyl residue.

2.2 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 the prostatic fluid (Vihko 1978, Vihko *et al*. 1978a, 1978b, 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-Gryszkiewicz *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 it has an optimum pH of 4-6.

2.2.1 Gene structure of hPAP

The gene encoding hPAP is located at chromosome 3q21→qter (Winqvist *et al*. 1989). The cDNA encodes a 354-residue protein with a calculated molecular mass of 41126 Da (Vihko *et al*. 1988, Sharief & Li, 1992). In the 5'-end, the cDNA codes a signal peptide of 32 amino acids. Three putative asparagine-linked glycosylation sites were found in the PAP sequence: Asn-Glu-Ser (residues 62-64), Asn-Phe-Thr (residue 188-190), and Asn-Glu-Thr (residue 301-303). The hPAP gene contains 10 exons with the sizes of 170, 96, 87, 153, 99, 93, 133, 83, 104, and 2098 bp, respectively (Sharief & Li, 1992, Virkkunen *et al*. 1994).The signal sequence and the first eight amino acids of the protein were encoded by exon 1. The rest of the coding region and 3'-untranslated region are covered

by exons 2-10 and 10, respectively. One alu-type repetitive sequence was found upstream of the proximal promoter and also two copies in the 3'-untranslated region of the gene. The major transcription start site was located 50 nt upstream from the ATG codon of the gene, using human prostatic carcinoma or benign hyperplasia tissue as a source of RNA in primer extension analysis. When RNA from the prostatic carcinoma cell line LNCaP was used in S1 nuclease and primer extension mapping, two clear transcription initiation sites for hPAP were found around 56 and 91 nt upstream of the translation initiation cordon (Banas *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 the cDNA is explained by multiple polyadenylation signals (AATAAA) following two copies of alu-type repetitive sequences in the 3' non-coding region.

2.2.2 Protein structure of hPAP

Native hPAP exists as a dimer (Derechin *et al*. 1971, Luchter-Wasyl & Ostrowski 1974, Vihko *et al*. 1978) of two catalytically inactive subunits non-covalently bound together to form an active enzyme (Kuciel *et al*. 1990). These subunits are indistinguishable by gel filtration, SDS-PAGE, or tryptic mapping (Luchter-Wasyl & Ostrowski 1974). Each monomer of the enzyme was shown to contain three N-linked glycosylation sites (Risley & van Etten 1987, Morris et al. 1989), and these asparagine residues are occupied by two high mannose-type carbohydrate moities (Asn 62 and Asn 301) and one partially sialylated, fucosylated biantennary or triantennary complex (Asn 188), respectively (Risley & van Etten 1987, Jakob *et al* 2000). The hPAP monomer has been shown by spectrophotometric titration to contain three disulphide bonds between Cys129/Cys340, Cys183/Cys281, and Cys315/Cys319, and no free sulphydryl groups (van Etten *et al*. 1991), while the protein crystallographic study of homologous rat PAP, which has 75% identity in amino acid sequence with hPAP (Roiko *et al*. 1990), suggests that only two disulphide bonds existing (Schneider *et al*. 1993). The existence of two free sulfhydryl groups in hPAP has been demonstrated by biochemical studies (Ostanin *et al*. 1994). hPAP has a similar three-dimensional structure to rat PAP , especially in the active site region, where the amino acid sequences are entirely conserved (Jakob *et al*. 2000). Both hPAP and rPAP are homodimers in which subunits are related by a 2-fold axis (Schneider *et al*. 1993, Jakob *et al*. 2000). Each subunit is comprised of two domains. The larger domain is an α/β type 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 by long-chain excursions (residues 125-227 in hPAP) 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 enables the enzyme to accept a large variety of substrates (Heller 1987). hPAP has been classified as a histidine phosphatase (McTigue & van Etten, 1978a, Ostrowski 1978, van Etten 1982), since the crucial intermediate is a phosphoroamidate, namely, phosphohistidine.

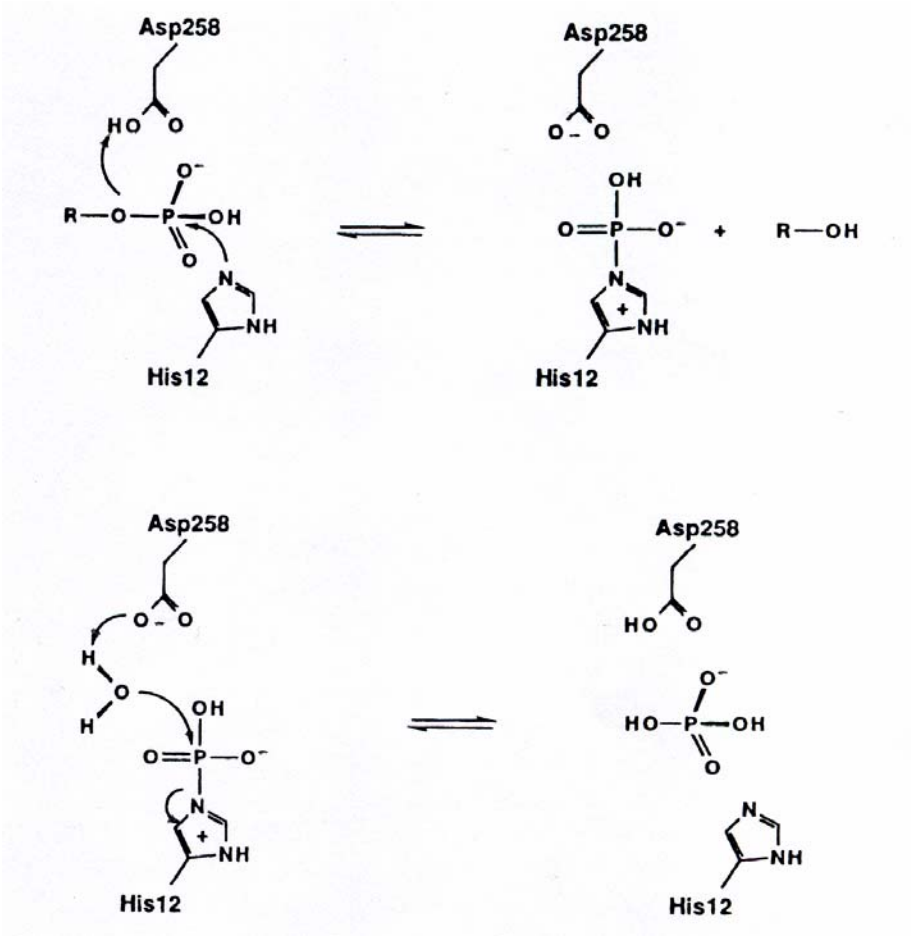

Fig. 1. Minimal reaction mechanism of rPAP. Modified from Lindqvist *et al*. 1994.

The rate-limiting step is the breakdown of the covalent phosphoenzyme intermediate by the attack of water on the phosphoroamidate, resulting in the formation of a noncovalent enzyme-inorganic phosphate complex (Fig. 1). The active site was shown to contain two arginines probably involved in the binding of the negatively charged phosphate group of the substrate (McTigue & van Etten 1978b). The presence of carboxylic acid residues, Asp or Glu, at the active site was indicated by Saini & van Etten (1979) and van Etten (1982). Site-directed mutagenesis of hPAP shows His 12 acts as an acceptor of the phospho group, Asp 258 is a proton donor for the substrate-leaving group, and His 257 may participate in substrate-binding or may facilitate the breakdown of the phosphoenzyme complex (Ostanin *et al*. 1994). These results are consistent with the crystal structure of rPAP (Schneider *et al*. 1993). Most of the active site residues of rPAP come from the loops after strands 1 and 4 of the large domain. Residues Arg11, His12 and Arg15 are part of a sequence motif RHGXRXP characteristic for acid phosphatase

(van Etten *et al*. 1991). These residues are part of a cluster of conserved amino acid residues in the center of the active site, consisting of residues of Arg11, His12, Arg15, Arg79, His257, and Asp258. The active site of hPAP and rPAP are very similar in three-dimensional structure, except for the conformation of the Arg15 side chain (Lindqvist *et al*. 1993, Jakob *et al*. 2000). A recent study (Zhang *et al*. 1997) shows that Trp174 was at the active site of the human enzyme, because it was protected by the competitive inhibitor tartrate in the DNPS-Cl modification studies. This is also consistent with the location of a homologous residue in the structure of the rat enzyme.

Denaturation-renaturation and subunit reassociation studies show that hPAP activity depends on dimer formation (Kuciel *et al*. 1990). Oligomerization of rPAP using site-directed mutagenesis found that mutants W106E and H112D, as well as the double mutant W106E/H112D, are monomers without catalytic activity or the ability to bind tartrate (Porvari *et al*. 1994). The His112 side-chain forms a hydrogen bond with the Asp76 side-chain between two subunits, and the side-chains of Trp106 are stacked on top of each other across the twofold axis relating the two subunits (Schneider *et al*. 1993). Since the PAP active-site is located far from the subunits' interface, these results indicate that the formation of dimers induces structural changes which propagate to the active site.

2.2.3 Heterogeneity of hPAP

Human PAP from native sources possesses molecular heterogeneity. Starch-gel electrophoresis gives 20 bands for hPAP from normal prostatic tissue (Smith & Whitby 1968), while isoeletric focusing gives up to 30 variants of hPAP from normal, malignant, or hyperplastic prostatic tissue, 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 accounts for part of the heterogeneity of the isoelectric point (pI) of hPAP (Smith & Whitby 1968). Post translational deamidation of glutamine and asparagine (van Etten et al. 1991) also contributes to the observed variation in the pI of hPAP (Chu *et al*. 1978). The carboxyterminal amino acid of hPAP can be threonine, glutamic acid, or aspartic acid, corresponding to residues 349-351 of the cDNA-derived hPAP sequence, indicating that the carboxy-terminus is susceptible to post-translational proteolysis (van Etten et al. 1991). The involvement of phosphorylated sugars and the structural differences at the amino-terminal end of the protein as the cause of the heterogeneity have been ruled out (McTigue & van Etten 1982, Taga *et al*. 1983).

Different hPAP isoenzymes from prostate tissue and semen have similar substrate specificities (Smith & Whitby 1968, Lin *et al*. 1983), while their susceptibility to inhibition by Fe^{3+}, Ca^{2+}, or La^{3+} and their pH optima differ (Rönnberg *et al*. 1981, Lin *et al*. 1983). The property of inhibition by L-tartrate has proved to be a good basis for purifying the enzyme to homogeneity by affinity chromatography. Two forms of hPAP isoenzyme were purified from prostate tissue (Smith & Whitby 1968, Vihko *et al*. 1978, McTigue &Van Etten 1982, Taga *et al*. 1983). The major form I, which has been reported to be the form present in seminal fluid (Lam *et al*. 1979), and the minor form II have a similar molecular mass of approximately 100 kDa, but different pI of 4.9 and 5.5,

respectively. These forms differ in the structure of the carbohydrate moieties. Lin et al. (1983) purified two forms of hPAP of 120 kDa, pI 4.7-4.9, and 100 kDa, pI 4.8-5.3, respectively, from seminal plasma. It was suggested that proteolytic processing of a high-molecular-weight precursor molecule is the cause of the two forms, which are thus products of the same gene (van Etten & Waheed 1985). Lee *et al*. (1991) purified two hPAP isoenzymes from seminal plasma possessing similar substrate and inhibitor specificity, but having partly different immunological properties. Heterogeneity of hPAP could also be caused by the existence of true isoenzymes consisting of different polypeptide chains.

2.2.4 Physiological function of hPAP

Human PAP is one of the prostate epithelium-specific differential antigens (Yam 1974, Lam *et al*. 1982, Kamoshida & Tsutsumi 1990). There are two forms of hPAP: one is intracellular and the other is secreted (Vihko 1979, Lad *et al*. 1984). hPAP is secreted in high amounts into seminal fluid (Rönnberg *et al*. 1981), and the serum activity of the enzyme is frequently elevated in patients with prostate carcinomas and is correlated with tumor progression (Gutman & Gutman 1938, Choe *et al*. 1980, Griffiths 1980, Vihko *et al*. 1980, 1981, and 1985). hPAP was a main diagnostic marker for prostate cancer until the arrival of the prostate-specific antigen. As a large quantity of hPAP is present in spermatic fluid, it has been suggested to have a physiological role in fertility (Coffey & Pienta 1987) and may affect the mobility of sperm (Lin & Clinton 1987). *In vitro*, hPAP hydrolyzes phosphorycholine, which is found in semen (Saini & van Etten 1981), as well as phosphocreatine, an intracellular high-energy compound present in seminal plasma (Lee *et al*. 1988).

Cellular hPAP has been implicated to be involved in the growth regulation of prostate cells (Lin *et al*. 1992, Lin *et al*. 1994). In prostate carcinomas, the activity of hPAP within the cells is lower than that in normal or benign hypertrophic prostates (Reif *et al.* 1973, Foti *et al*. 1977, Loor *et al*. 1981), although its activity may be elevated in the circulation (Yam 1974, Chu *et al*. 1979). Both the mRNA level and the protein level of hPAP in prostate carcinomas are decreased compared to those in normal and hyperplastic prostate (Hakalahti *et al*. 1993, Alaiya *et al*. 2001). Prostate cancer cells LNCaP that express cellular hPAP have a slow growth rate, compared with two other prostate cancer cells, PC-3 and DU-145, that lack the endogenous hPAP expression (Lin *et al*. 1992, Lin *et al.* 1994). Subclones of LNCaP cells, such as clone-51 and clone-81, grow faster than cells with a smaller passage number, for example clone-33, whereas clone-51 and clone-81 resemble advanced hormone-refractory prostate cancer and express less hPAP (Lin *et al.* 2000). Introduction of cellular hPAP into PC-3 cells results in a decreased cell growth rate. When cellular hPAP activity decreased upon passage, the cellular growth rate returned to normal. There is a strong inverse correlation between cellular hPAP activity and the cell growth of LNCaP cells that express endogenous hPAP. The growth rate regulation function of hPAP is due to the fact that the enzyme possesses intrisic protein tyrosine phosphatase (PTP) activity (Li *et al.* 1984, Boissonneault *et al.* 1995). In prostatic epithelial cells, there is an inverse correlation between protein tyrosin kinase

(PTK) and PAP activity, the proliferating cells showing high PTK and low PAP activity, while differentiated cells exhibit high PAP and low PTK activity (Lin & Meng 1987, Tessier *et al*. 1989). Most of known PTPs serve as negative regulators of critical signal transduction pathways (Dixon 1996, Streuli 1996, Tonks & Neel 1996, Chernoff 1999, Li & Dixon 2000) and are able to suppress the transformation phenotype induced by PTKs in cultured cells (Woodford-Thomas *et al*. 1992, Zheng *et al*. 1992). hPAP is copurified with PTP activity and exhibits a high specificity toward phosphotyrosine proteins (Li *et al*. 1984, Lin & Clinton 1986). It has been shown that hPAP dephosphorylates epidermal growth factor receptor preferentially at neutral pH, resulting in decreased PTK-specific activity of the receptor protein (Lin & Clinton 1988). Recent studies show that the phosphotyrosine level of a 185-kDa protein (pp185) is correlated with the cell growth rate, but inversely correlated with the cellular hPAP activity in prostate cancer cells (Lin & Meng 1996, Lin et al. 1998, Meng & Lin 1998). Subsequently, pp185 has been identified as c-ErbB-2 (Meng & Lin 1998), a member of the ErbB receptor tyrosine kinase family. Site-directed mutagenesis studies demonstrated that the p-Tyr level of ErbB-2 in cells expressing H12A or D258A mutant hPAP is similar to that in control cells without endogenous hPAP expression, where His-12 and Asp-258 are in the active site of the enzyme. These results confirm that c-ErbB-2 is indeed an *in vivo* substrate of the cellular form of hPAP (Zhang *et al*. 2001).

Several lines of evidence indicate that cellular hPAP functions as a neutral PTP. Nevertheless, hPAP shares little sequence homology with other typical PTPs. It does not contain a 240- residue conserved PTP domain or a PTP signature motif with a catalytic Cys residue located in, although an Arg-Asn-Arg-Tyr-Pro sequence has been found in hPAP (residues 54-58), lysosomal acid phosphatase (Pohlmann *et al*. 1988, Vihko *et al*. 1988, I, van Etten *et al*. 1991), and PTPs from rat brain and human placenta (Chernoff *et al*. 1990, Guan *et al*. 1990). There are two free cysteine residues, Cys-183 and Cys-281, in hPAP, and Cys-183 has been proposed to participate in substrate-binding or catalysis (Schneider *et al*. 1993) and functions as a phosphate acceptor (Ostanin *et al*. 1994). Mutation of either Cys-183 or Cys-281 has little effect on the acid phosphatase activity toward the substrate paranitrophenylphosphate (pNPP) or the PTP activity, as indicated by the *in vivo* phosphotyrosine level of c-ErbB-2 (Zhang *et al*. 2001). LNCaP cells expressing C183A, C281A, or wild-type hPAP had a decreased p-Tyr level of ErbB-2 compared to the control cells. Furthermore, transient expression of C183A, C281A, or wild-type enzyme, but not H12A or D258A, decreased the growth rate of C-81 LNCaP cells that lack endogenous hPAP expression. These results indicate that both the acid phosphatase and PTP activity of hPAP share the same active site and apparently use the same amino acid residues for catalysis.

Interaction between hPAP and ErbB-2 is involved in the androgen-promoted growth of human prostate cancer cells. The androgen-responsive phenotype of human prostate cancer cells is associated with a low phosphotyrosine (p-Tyr) level of ErbB-2, which is regulated by cellular hPAP. In prostate cancer cells, the p-Tyr level, but not the protein level, of ErbB-2 is inversely correlated with the androgen-responsiveness of cell proliferation. Androgen-stimulated cell growth concurs with a 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). The tyrosine phosphorylation of ErbB-2 regulated by cellular hPAP plays a key role in regulating androgen-mediated proliferation signalling (Fig.2).

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).

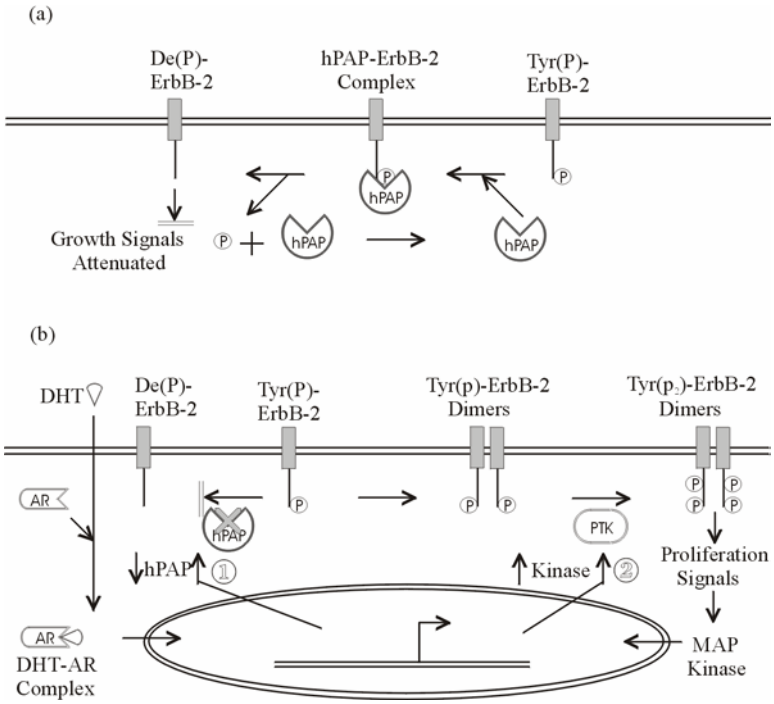

Fig. 2. Proposed model: Interaction of hPAP and ErbB-2 is involved in androgen-mediated cell proliferation. (a) In hPAP (PAcP)-expressed human prostate epithelia, ErbB-2 has a low level of tyrosine phosphorylation, due to dephosphorylation by cellular hPAP. Tyrosine-dephosphorylated ErbB-2 is inactive and cannot transduce cell growth signals. (b) Androgen induces a cascade of transcriptional signals which include one pathway to suppress the expression of cellular hPAP, and another pathway to activate ErbB-2-specific tyrosine kinases. Tyrosine-phosphorylated ErbB-2 proteins form homodimers and subsequently activate the downstream MAP kinase signalling pathway, leading to a rapid growth rate. Modified from Meng *et al*. 2000.

No mutation of the hPAP gene has been reported so far. The production of hPAP gene knock-out mice would be important for demonstration of the physiological role of the enzyme.

2.2.5 Tissue-specific expression of hPAP

Immunochemistry using specific monoclonal 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). Immunoreactive hPAP is present subcellularly within the cytoplasmic secretory vesicles in the apical areas of epithelial cells, and is also found in the microvilli of the apical lining membrane, and frequently in secreting blebs extruding into the glandular lumen (Mori & Wakasugi 1985). These findings indicate the secretory nature of hPAP, for after translation, it is transferred to the secretory vesicles via the rough endoplasmic reticulum and the Golgi apparatus and expelled into the glandular lumen by exocytosis (Lin *et al.* 1986a). *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). Northern blot analysis detected no specific hPAP mRNA in human liver, lung, pancreatic cancer tissue, placenta, breast cancer cells, mononuclear blood cells, or acute promyelocytic leukaemia cells (Solin *et al*. 1990). The mRNA was also not detected in spleen, thymus, testis, ovary, small intestine, colon or peripheral blood leukocyte (Zelivianski *et al*. 1998).

Immunoreactive hPAP has been found in some non-prostate cells and tissues, such as leukocytes, kidney, spleen, placenta, pancreas, liver, stomach, granulocytes, neutrophils (Li *et al.* 1980, Shaw *et al*. 1981, Yam *et al*. 1981, Aumüller & Seitz 1985, Waheed *et al.* 1985), anal gland and urethral gland of males (Kamoshida & Tsutsumi 1990), crypt epithelium of the duodenum (Drenckhahn *et al*. 1987), pancreatic islet cell carcinomas (Choe *et al*. 1978), and breast tumor cells (Li *et al*. 1980). It has been proposed that the acid phosphatase detected in many organs was not hPAP, but an immunologically related acid phosphatase (Höyhtyä *et al*. 1987, Lam *et al.* 1989, Solin *et al*. 1990, Lin *et al.* 1990). The extraprostatic observations of hPAP may partly be due to immunological cross-reactivity with lysosomal acid phosphatase (LAP), which is a transmembrane protein expressed in almost all tissues (Waheed *et al.* 1985). There is a 49% identity between the mature polypeptide chains of these two enzymes (Peters *et al*. 1989). Some LAP antibodies have been shown to recognize hPAP (Choe *et al*. 1982), and the enzyme has been found to possess similar antigenic determinants in both terminal regions (Lee *et al*. 1991b). Lin *et al*. (1990) showed that acid phosphatases from spleen and lung share at least one common antigenic epitope with hPAP. A novel testicular acid phosphatase gene has been cloned recently (Yousef *et al*. 2001). The enzyme has approximately 50% homology with both hPAP and LAP, and the positions of cysteine residues, the N-glycosylation sites, and the histidine in the catalytic site are conserved among the three proteins.

Little is known about the mechanism of the tissue-specific regulation of the hPAP gene at the molecular level. Zelivianski *et al*. (1998) cloned a 1.4-kb of DNA that flanks the 5' region (-1356/+87) of the hPAP gene and characterized the promoter activity in LNCaP

cells. This 1.4-kb flank region could drive low expression of the CAT reporter gene, but it has not been shown if this promoter activity is prostate-specific.

2.2.6 Androgen regulation of hPAP

The expression of hPAP is negligible before adolescence, but it has a high expression level in normal, well-differentiated prostate epithelial cells (Gyorkey 1973, Yam 1974). This effect of the puberty was the first indication that the expression of hPAP is under androgen influence. Although the up-regulation of rPAP by androgens appears to be clear (Porvari 1995), the situation in the case of hPAP is complicated. For example, significant levels of hPAP in circulation have been reported in the patients treated for advanced prostate cancer with androgen deprivation (Chu *et al*. 1979, Grayhack *et al*. 1987), suggesting that, at least in this disease case, hPAP expression may not require androgens.

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 the synthetic androgen, R1881, was present in charcoal-stripped serum (Henttu & Vihko 1992). Accordingly, 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 Horoszewicz *et al*. (1983) and Lin *et al*. (1993a). 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 (Langeler *et al*. 1993). Controversial results were obtained concerning androgen regulation of the hPAP mRNA level in LNCaP cells. Henttu *et al*. (1992) demonstrated the down-regulation of hPAP mRNA by androgen, while Lin *et al*. (1993) reported an up-regulatory effect of androgen. It has been suggested that cell density in the culture affects the expression of hPAP (Lin *et al*. 1994). Cell culture density does not only affect the level of hPAP mRNA, but also modulates androgen regulation of hPAP mRNA (Lin & Garcia-Arenas 1994). In high density cultured LNCaP cells, which mimic the differentiated state of cells, androgen suppresses the mRNA level of hPAP (Lin *et al*. 2000). Androgens have similar effects on hPAP expression in LNCaP cells of different passage numbers, such as clone-33, clone-51, and clone-81, resulting in an approximately 30% diminution of hPAP at the mRNA level in all three clones. In addition, the amounts of PAP in a well-differentiated human prostatic carcinoma tumor (PC-82), which was grown in nude mice, were significantly increased after castration (van Steenbrugge *et al*. 1983).

Dulinska *et al*. (1997) studied the effect of DHT on the level of hPAP mRNA using tissue slices from various benign prostatic hyperplastic glands. The absence of DHT in the incubation medium led to a gradual, significant decrease of the hPAP mRNA level. Addition of the hormone induced hPAP mRNA in a time- and dose-dependent manner. The results indicate that DHT is necessary to sustain the expression of hPAP in hyperplastic prostates. Two studies with contradictory results as regards to the hormonal regulation hPAP in normal men have been published, suggesting that the direction of androgen action is dose-dependent (Dondero *et al*. 1976, Carpino *et al*. 1994). Androgen

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

2.2.7 Other regulators of the hPAP gene

Henttu and Vihko (1993) studied changes occurring in the biosynthesis of hPAP and PSA in LNCaP cells treated with growth factors. Epidermal growth factor (EGF) was found to reduce the secretion of hPAP and PSA by the cell, as the result of a lowered steady-state level of the corresponding mRNA. The fibroblast growth factors (FGF), aFGF and bFGF, down-regulated the secretion of hPAP and PSA, but aFGF did not affect the levels of the mRNA and bFGF down-regulated only the level of the hPAP mRNA. Transforming growth factor-β1 (TGF-β1) significantly increased the steady-state level of hPAP mRNA, but did not affect the accumulation of hPAP or PSA in culture medium. Treatment of LNCaP with activin A, a member of the TGF-β family, resulted in growth and morphological changes that were accompanied by the up-regulation of hPAP and PSA (Zhang *et al*. 1997b).

2.3 Androgen receptor action

2.3.1 Androgen receptor

Androgens (testosterone [T] and 5α-dihydrotestosterone [DHT]) control the development, differentiation, and function of male reproductive and accessory sex tissues, such as the seminal vesicle, epididymis, and prostate. Other organs and tissues, such as skin, skeletal muscle, bone marrow, hair folices, and brain, are also under the influence of androgen. The principal action of androgen is to regulate gene expression through the androgen receptor (AR), which belongs to the superfamily of nuclear receptors. Nuclear receptors are ligand-inducible transcription factors that mediate the signals of a broad variety of fat-soluble hormones, including the steroid and vitamin D3 hormones, thyroid hormones retinoids (Evans 1988, Beato 1989, Truss and Beato 1993). Approximately 70 members of the nuclear receptor superfamily members have been identified (Moras & Gronemeyer 1998). Only some of them are ligand-binding receptors, while others belong to the subfamily of so-called orphan receptors for which specific ligands have not yet been identified or may not even exist (O'malley & Conneely 1992). AR can modulate gene expression directly by interacting with specific elements in the regulatory regions of target genes (Reigmen *et al*. 1991) or indirectly by activating various growth factor signalling pathways (Peterziel *et al*. 1999).

2.3.1.1 Structural features

Like other members of the nuclear receptor superfamily, AR has four major functional regions (Fig. 3): the N-terminal transactivation domain (TAD), a central DNA-binding domain (DBD), a C-terminal ligand-binding domain (LBD), and a hinge region connecting the DBD and LBD (Mangelsdorf *et al*. 1995). Two autonomous transactivation functions, a constitutively active activation function (AF-1) originating in the N-terminal and a ligand-dependent activation function (AF-2) arising in the LBD, are responsible for the transcriptional activity of nuclear receptors (Gronemeyer & Laudet 1995).

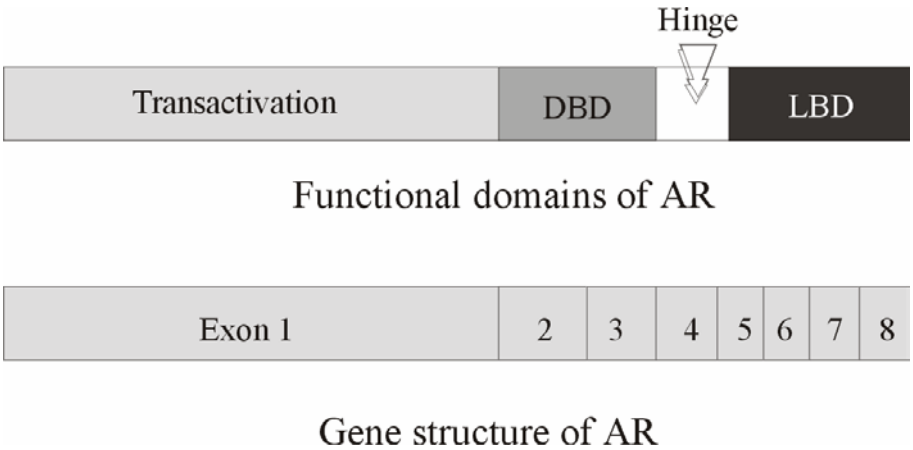

Fig. 3. Schematic structure of the androgen receptor. The androgen receptor gene was cloned in 1988 by Chang and co-workers (1988) and Lubahn *et al*. (1988 a, b) and soon after by several others (Tilley *et al*. 1989, Trapman *et al*. 1988, Brinkmann *et al*. 1989). There are eight exons encoding the receptor with a large exon 1 required for transactivation and exons 2-8 encoding a DNA-binding domain, hinge region, and hormone binding domain.

The DBD of AR exhibits a high degree of amino acid sequence identity to other members of the glucocorticoid receptor (GR) subfamily, the progesterone receptor (PR), and mineralocorticoid receptor (MR). Consequently, the four receptors recognize very similar, if not identical, hormone response elements (HREs). Two zinc fingers in the DBDs of the nuclear receptors differ both structurally and functionally (Härd *et al*. 1990, Schwabe *et al*. 1990, Luisi *et al*. 1991). The first zinc finger contains so-called P-box (Gly, Ser, and Val) that dictates the sequence specificity of binding to HRE (Berg 1989, Freedman 1992, Freedman & Luisi 1993). A five amino acid-residue long D-box of the GR superfamily is located in the N-terminal side of the second zinc finger. The D-box is important in specifying the half-site spacing requisite at the HRE (Dahlman-Wright *et al*. 1991). In addition, the D-box provides the entire dimerization interface for DBD-DBD interaction.

Conformation changes resulting from the binding of androgens to the LBD located at the C-terminal end of the molecule are responsible for activating the androgen response.

Despite the low sequence identity of as low as 20% between the LBDs of different nuclear receptor families, all NRs share a similar fold in this region. They are comprised of up to 12 helices and a small -sheet arranged in a so-called α-helical sandwich. In the agonist-bound conformation, H12 serves as a 'lid' to close the ligand-binding pocket (LBP), whereas in the antagonist-bound conformation, H12 is positioned in a different orientation, thus opening the entrance to the LBP. About 50% of mutations reported in the LBD of AR have been found to be associated with prostate cancer (PC) or androgen insensitivity syndrome (AIS). The replacement of threonine 877 with alanine in AR LBD is a frequent mutation in prostate cancer patients and corresponds to the mutation found in LNCaP cells (Veldsholte *et al*. 1990a, b, Taplin *et al*. 1995, Suzuki *et al*. 1996). The T887A substitution allows the AR to be activated by binding to cortisol or other steroids, and even to antiandrogens such as flutamide, thereby promoting prostate cancer cell growth (Taplin *et al*. 1999, Wang *et al*. 2000, Zhao *et al*. 2000). The structures of the complexes of wild-type and T877A-mutant AR LBD with DHT provide clear explanations for the differential affinities of these nuclear receptors for androgens as well as for the T877A mutant's ability to respond to other ligands (Matias *et al*. 2000, Sack *et al*. 2001). In addition, the R726L AR mutant is known to be activated by estradiol (Elo *et al*. 1995)

The transactivation functions of AF-1 and AF-2 are located in the TAD and the LBD, respectively, of nuclear receptors, and the activity of them is dependent on the recruitment of coactivator molecules to form active preinitiation sites for gene transcription (Onate *et al*. 1998, Bevan *et al*. 1999). Receptors with a deletion of their LBD are constitutively active, suggesting that the AF-1 is ligand-independent. Strong AF-2 was demonstrated in LBDs of retinoic acid receptor (RAR) (Durand *et al*. 1994), retinoic-X receptor (RXR) (vom Baur *et al*. 1998), vitamin D receptor (Jiménez *et al*. 1999), GR (Sheldon *et al*. 1999), PR (Onate *et al*. 1998), Peroxisome proliferator-activated receptor (PPARγ) (Nolte *et al*. 1998), estrogen receptor (ER) (Tora *et al*. 1989), and thyroid hormone receptor (THR) (Barettino *et al*. 1994), but not in AR (Berrevoets *et al*. 1998, Bevan *et al*. 1999). AF-2 in the LBD of nuclear receptors forms a hydrophobic cleft that binds the LXXLL motif of the p160 family of transcriptional coactivators (Onate *et al*. 1995, Voegel *et al*. 1998, Glass *et al*. 1997, McKenna *et al*. 1999), which are associated with histone acetyl transferase activity and can recruit CBP, pCAF, and other coactivitors required for chromatin modification (Chen *et al*. 1997). Although p160 coactivators can bind to the AR LBD in a hormone-dependent manner (Ghadessy *et al*. 1999, Lim *et al*. 2000), the AR LBD demonstrates very little activation function when fused to heterologous DBD. AR transactivation function is dependent on the strong AF-1 activity of TAD, as a result of ligand-activated interactions between the TAD and the LBD. A nonconservative asparagine to lysine substitution in AR residue 727, which is located in the LBD and did not alter the ligand-binding characteristics of the AR, but disrupted LBD interactions with AR TAD and with the TIF2. This substitution was encountered in a phenotypically normal man with subfertility and depressed spermatogenesis (Lim *et al*. 2000). A FXXLF motif in the AR N-terminal interacts with different regions of the AR AF-2/LBD to stabilize the hormone-receptor complex (He *et al*. 1999, 2000, 2001), and mediate androgen receptor-specific interactions with FXXLF-motif-containing coregulators, such as ARA 70/RFG, ARA 55/Hic-5, and ARA 54 (He *et al*, 2002).

The function of the AR hinge region, defined by residues 628-669, is not yet well understood. A sequence located between residues 628 and 657 within the hinge region contains a short stretch of basic amino acids that resemble the nuclear targeting signal of GR and has been described to form part of a bipartite nuclear localization signal (NLS) (Zhou *et al*. 1994). A recent study shows that the AF-2 of AR LBD is inhibited by the cognate hinge region (Wang *et al*. 2001). A mutant AR with a deletion of residues 628-646 in the hinge region exhibited transactivation activity that was more than double that of the wild-type AR. The negative modulatory activity of residues 628-646 were exerted via the coactivator (TIF2) pathway in a manner independent of c-Jun, c-Fos, c-Jun/c-Fos, or NcoR corepressors.

2.3.1.2 Androgen receptor coregulators

The transcriptional activity of AR is affected by coregulators that influence a number of functional properties of AR, including ligand selectivity and DNA binding capacity. AR coregulators participate in DNA modification of target genes, either directly through modification of histones or indirectly by the recruitment of chromatin-modifying complexes, as well as functioning in the recruitment of the basal transcriptional machinery (Heinlein & Chang 2002). Some of the better characterized coregulators are members of the p160 family, ARA70, ARA55, ARA54, ARA267-α, Smad-3, and AIB1 (Yeh *et al*. 1999a). ARA55 and ARA70 both allow the activation of AR by 17β-estradiol (E2), with ARA70 being the most effective coactivator for conferring androgenic activity to E2 (Miyamoto *et al*. 1998, Yeh *et al*. 1998, Fujimoto *et al*. 1999). Furthermore, both ARA55 and Smad-3 have been suggested to function as bridges for cross-talk between transforming growth factor-β signalling pathway and androgen/AR action (Fujimoto *et al*. 1999, Kang *et al*. 2001). Aberrant AR coregulator activity due to mutation or altered expression levels may be a contributing factor in the progression of diseases related to AR activity, such as prostate cancer (Heinlein & Chang 2002).

2.3.1.3 Mechanisms of action

Ligand-binding initiates a series of events leading to the regulation of target genes by the receptor. The occupied receptor undergoes an allosteric change in its LBD, and was dissociated from heat shock proteins, such as hsp90, hsp70, and hsp56 (Roy *et al*. 2001), dimerized, and translocated, if it is not already present into the nucleus. Upon binding to a hormone response element (HRE), the receptor dimer recruits coactivators to form an active pre-initiation complex and interacts with basal transcription machinery to trigger the transcription of the target genes (Fig. 4).

Once an antagonist-bound AR dimer binds to the ARE, it recruits corepressors, such as N-CoR and SMRT and, indirectly, histone deacetylases, such as RPD3, leading to transrepression, presumably due to chromatin compaction. In addition to the classical mode, AR can modulate the transcription by the direct protein-protein interaction with other factors in a ligand-dependent manner, for which DNA binding by AR is not

necessary (Palvimo *et al.* 1996, Aarnisalo *et al.* 1998, Fronsdal *et al.* 1998, Slagsvold *et al.* 2001).

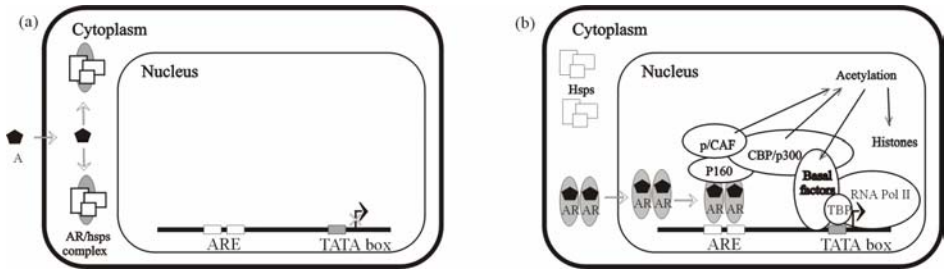

Fig. 4. Proposed models for AR transactivation. (a) Androgens diffuse into target cells and bind to androgen receptors which exist in the form of complexes with heat shock proteins (hsps, marked with blocks) in the cytoplasm. (b) Androgen receptors were dissociated from hsps, dimerized, translocated into the nucleus. Upon binding to an androgen response element (ARE), the receptor dimer recruits coactivators such as p160 family and CBP/p300 that possess histone acetyltransferase activity or recruits histone acetyltransferase p/CAF to form an active preinitiation complex and interacts with basal transcription machinery to trigger the transcription of the target genes.

2.3.1.4 Ligand-independent activation

Nuclear receptors may also be activated by signalling pathways that originated at the cell surface. NRs, along with other transcription factors, are regulated by reversible phosphorylation (Orti *et al.* 1992). Kinase-mediated signal transduction pathways could affect the activity of NRs (Burnstein & Cidlowski 1993). The consensus phosphorylation sites found in AR indicate that AR could be a substrate for the DNA-dependent protein kinase, protein kinase A, protein kinase C, mitogen-activited kinase, and casein kinase II (Blok *et al.* 1996). This hypothesis was supported by the observation that protein kinase A and protein kinase C could enhance AR transactivation (Ikonen *et al.* 1994, Nazareth *et al.* 1996). A recent study demonstrated that the HER2/Neu-mitogen-actived protein kinase pathway could phosphorylate AR, which might facilitate recruitment of AR coregulators to AR (Yeh *et al.* 1999b). Indeed, AR could be activated in an androgen-independent way by growth factor or cytokine signalling pathways, like those initiated by epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), keratinocyte growth factor and IL-6, which would elicit AR-mediated transcriptional activation (Culig *et al.* 1994, Reinikainen *et al.* 1996, Hobisch *et al.* 1998).

2.3.2 Selective DNA binding by androgen receptor

All of the class I receptors, GR, PR, MR, and AR, recognize response elements that are organized as inverted repeats of 5'-TGTTCT-3'-like sequences with a three nucleotide spacer (Beato 1989). Receptor distribution and hormone metabolism can explain part of the steroid-specificity *in vivo* (Funder 1993, Cleutjens *et al*. 1997). Other ways of imposing selectivity have been proposed at the chromatin level (Beota *et al*. 1995) and at the level of cooperativity of receptors with other transcription factors (Truss & Beota 1993). A recent study demonstrated that selective DNA binding by AR (Table 1) could be a mechanism for hormone-specific gene regulation (Schoenmakers *et al*. 2000).

Table 1. Classification of androgen response elements

AREs	Sequence	Reference
High affinity, non-specific		
GRE177	GTTACA AAC TGTTCT	Beato *et al*. 1989
C3(1) ARE	AGTACT TGA TGTTCT	Claessens *et al*. 1989
GRE2 TAT	TGTACA GGA TGTTCT	Beato *et al*. 1989
PSA ARE1	AGCACT TGC TGTTCT	Reigman *et al*. 1991
SLP-HRE-3	GAAACA GCC TGTTCT	Loreni *et al*. 1988
High affinity, AR-specific		
PB-ARE-2	GGTTCG TGG AGTACT	Rennie *et al*. 1993
SLP-HRE-2	TGGTCA GCC AGTTCT	Loreni *et al*. 1988
SC ARE 1, 2	GGCTCT TTC AGTTCT	Verrijdt *et al*. 1999
Low affinity, non-specific		
PB-ARE-1	ATAGCA TCT TGTTCT	Rennie *et al*. 1993
MVDP pARE	TGAAGT TCC TGTTCT	Darne *et al*. 1997
GPX5	ATCCTA TGT TGTTCT	Lareyre *et al*. 1997
CRP2	AGAAGA AAA TGTACA	Devos *et al*. 1997
Low affinity, AR-specific		
SC ARE	AGCAGG CTG TGTCCC	Haelans *et al*. 1999

Adapted from Claessens *et al*. 2001.

The androgen-specific regulation of the rat probasin (PB) gene can be explained by the presence of an androgen response element (ARE) in its promoter (PB-ARE-2), specifically recognized by AR but not by GR (Claessens *et al*. 1996, Kasper *et al*. 1999). The direct repeat nature of this ARE prohibited GR binding. The first zinc finger of the AR is not involved in the specific recognition of the PB-ARE-2, but rather the second zinc finger and part of the hinge region that contribute to this specificity (Schoenmakers

et al. 1999). An alternative dimerization mechanism might explain this specificity (Claessens *et al*. 2001). Since the spacer between the two hexamers in the PB-ARE-2 must be three nucleotides, the only alternative could be a head-to-tail dimerization of the DNA-bound AR-DBDs. This would be analogous to the DNA-binding by heterodimers of the RXR with many other NRs (Glass 1994). Point mutations in the right half of the perfect direct repeat can abolish dimeric binding, while the same mutations in the left half do not. Apparently, the first DBD must bind to the right half with high affinity in order to enable the cooperative binding the dimeric partner to the left half.

2.4 Prostate-specific gene expression

Prostate cancer is the most frequently diagnosed cancer among men in Western industrialized countries. The mechanisms behind the malignant transformation and prostate cancer development are only partially known (Pentyala *et al*. 2000). Prostate-specific gene regulation is under extensive study recently, because it provides further insight into the mechanism of the development of prostate cancer and furthermore offers new strategies for prostate cancer treatment.

2.4.1 Prostate-specific promoters

Gene therapy for prostate cancer has moved rapidly into a developmental phase in which practical considerations for safety and efficacy have dominated efforts. Because of their wide range of infectivity, high achievable titers, and relatively low risk of secondary mutagenesis, recombinant adenoviruses have been chosen as vectors for therapeutic genes, including tumor suppressor genes (Eastham *et al*. 1995, Gotoh *et al*. 1997, Steiner *et al*. 2000), 'suicide' genes (Eastham *et al*. 1996, Martiniello-Wilks *et al*. 1998, Blackburn *et al*. 1999), and immunomodulatory genes (Simons *et al*. 1999, Hull *et al*. 2000). Tissue-specific promoters are the most critical in this strategy. By placing transgenes under the transcriptional control of tissue-specific promoters, one should be able to improve safety and avoid autoimmunity or bystander toxicity. Therefore, several composite prostate-specific promoters/enhancers based on rPB, PSA, and human prostate-specific gladular kallikrein (hK2) have been developed for this purpose (Zhang *et al*. 2000, Latham *et al*. 2000, Xie *et al*. 2001), and rPB promoter is the most commonly used one.

Rat PB was initially identified as one of the several prostate-specific genes (Dodd *et al*. 1983). Although its function is still unclear, PB is a member of the lipocalin superfamily and is found both in the nucleus of prostate epithelial cells and in prostatic secretions (Spence *et al*. 1989). The PB promoter from –426 to +28 was sufficient to target expression of the bacterial chloramphenicol acetyltransferase (CAT) gene to the prostate in transgenic mice (Greenberg *et al*. 1994). Cooperative binding of AR to two distinct AREs (nucleotides –236 to –223 and –140 to –117) in the androgen response region (ARR) is required for maximum androgen induction of gene expression (Kasper *et al*. 1994, 1999). The presence of one copy of the ARR of PB, although sufficient for

developing animal models for 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 the PB ARR (-244 to –96) upstream of the minimal promoter, leading to very strong androgen induction (Zhang *et al*. 2000).

2.4.2 Prostate-specific enhancers

PSA is one member of the human kallikrein gene family. It is expressed primarily in normal, hyperplastic, and malignant prostatic epithelia (Aumüller *et al*. 1990, Young *et al*. 1991). Although the promoter plays an important role in PSA expression, the experiment in transgenic mice has shown that it is insufficient to confer strong androgen responsiveness and cell type-specificity *in vivo* (Cleutjens *et al*. 1997b). The expression of androgen-responsive PSA is regulated by an upstream enhancer (PSE) (Schuur *et al*. 1996). The enhancer was originally identified as a 1.6-kb fragment containing sites recognized by AR, AP-1, and CREB. Early studies showed that the expression of PSA was directly regulated by AR via three distinct AREs, in which AREI and AREII are located at the promoter region of the gene and AREIII is contained within a 440-bp strong enhancer element core located at -4.2kb of the transcription start site (Cleutjens *et al*. 1996, Schuur *et al*. 1996, Pang *et al*. 1997, Cleutjens *et al*. 1997a). This enhancer has been shown to be both necessary and sufficient for maximal and cell type-specific PSA expression (Cleutjen *et al*. 1997b, Brookes *et al*. 1998). Recently, five additional low-affinity AREs close to AREIII were identified within a 170-bp region of the PSE (Huang *et al*. 1999). In addition to PSA, other androgen-regulated genes, such as the prostatic binding protein C3 (1) gene and mouse vas deferens protein, are also regulated by enhancers separated from the promoter region (Claessens *et al*. 1989, Fabre *et al*. 1994). PSA enhancer has also been used in gene therapy trials of prostate cancer (Gotoh *et al*. 1998, Wu *et al*. 2001).

2.4.3 Androgen receptor and prostate-specific gene expression

Androgen receptor plays a central role in prostate-specific gene expression. Genes uniquely regulated by the AR typically contain multiple AREs that in isolation are of low DNA-binding affinity and transcriptional activity. However, specific combinations of AREs in their native promoter or enhancer result in highly cooperative DNA binding by AR and high levels of transcriptional activation.

Androgen-regulated promoters of PSA and PB contain two classes of AREs dictated by their primary nucleotide sequence that function to mediate cooperativity. Class I AREs display conventional guanine contacts. Class II AREs have distinctive atypical sequence features and, upon binding to AR, the DNA structure is dramatically altered through allosteric interaction with the receptor. Class II sites stabilized AR binding to adjacent class I sites and resulted in synergistic transcriptional activity and increased hormone sensitivity (Reid *et al*. 2001). Cooperative binding of AR to at least four tandem,

nonconsensus AREs in the PSE region of PSA stimulates transcription synergistically (Huang *et al*. 1999).

2.4.4 Prostate-specific regulatory proteins

AREs are found in the promoters of genes expressed in different tissues, including prostate, brain, kidney, liver, and testis (Chang *et al*. 1995). It is unlikely, therefore, that AR alone regulates tissue-specific expression of the prostate-related genes. Indeed, 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). The first well-characterized prostate-specific regulatory protein is Nkx3.1. Nkx3.1 is an androgen-regulated, prostate-specific homeobox gene (He *et al*. 1997, Prescott *et al*. 1998). Mice heterozygous for targeted disruption of Nkx3.1 have abnormal prostate morphology with overgrown and dysplastic epithelium, and the situation is more severe in Nkx3.1-null mice (Bhatia-Gaur *et al*. 1999). These results suggested that Nkx3.1 exerts a growth-suppression effect on prostate epithelial cells and controls differentiated glandular functions. Loss of Nkx3.1 expression in human prostate cancers correlates with tumor progression (Bowen *et al*. 2000). Recently, a novel prostate epithelium-specific Ets transcription factor was identified (Oettgn *et al*. 2000). This prostate-derived-Ets-factor (PDEF) acts as an androgen-independent transcriptional activator of the PSA promoter. PDEF also directly interacts with the DBD of AR and enhances androgen-mediated activation of the PSA promoter. Interestingly, the recognition sequence of PDEF is very similar to the protected sequence in the C3 (1) gene. Preliminary experiments indicated that PDEF seemed to bind functionally to the C3 (1) intronic enhancer (Claessens *et al*. 2001).

In addition, several cell type-specific protected regions have been identified in the upstream enhancer of PSA (Farmer *et al*. 2001). The nature of the factors binding to those elements is not yet known. The sequences of those elements clearly diverge from the PDEF binding site, indicating the existence of other prostate-specific regulatory proteins which might play important roles alone or cooperatively with AR and other transcription factors in prostate-specific gene expression.

3 Outlines of the present study

Human prostatic acid phosphatase is a prostate-specific differentiation antigen and the major acid phosphatase in differentiated prostate epithelial cells, and it has be suggested to be the main protein tyrosine phosphatase in prostate cancer cells. This study was designed to expand the knowledge of the transcriptional regulation, particularly hormonal and tissue-specific regulation of the genes expressed specifically in prostate, using hPAP as a model. Information gained from this study is expected to be valuable for the projects where prostate-specific gene expression is needed e.g. in prostate gene targeting or prostate cancer gene therapy. The special aims were:

- to set up a transfection method for prostatic cell line LNCaP, the only commercially available cell line that can produce endogenous hPAP, to be able to study the gene regulation of the hPAP at the transcription level in cell culture model.

- to analyze the functionality of the hPAP promoter constructs in prostatic and non-prostatic cell lines and in transgenic mice, to be able to identify regulatory regions important for prostate-specific gene expression.

- to identify prostate-specific transcription factor binding sites in prostate-related genes and to evaluate the functions of prostate-specific regulatory elements in the transcriptional regulation of the hPAP gene, in order to clarify the mechanisms for tissue-specific expression of the hPAP gene.

4 Materials and methods

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

4.1 Cell culture

The human prostate carcinoma cell lines LNCaP and PC-3, the monkey kidney cell lines CV-1 and COS-1, the human breast carcinoma cell lines MCF-7 and T47D, the human endometrial adenocarcinoma cell line HEC-1, and human liver carcinoma cell line HEP-1 were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured according to the instructions from the supplier.

4.2 Preparation of plasmid constructs for reporter gene analysis

Fragments of hPAP, hPSA, hK2, and rat probasin gene were generated by PCR. Oligonucleotides containing sites for restriction enzymes were used as primers. Fragments were first cloned into pCR$^{2.1}$TA-cloning vector (Invitrogen, San Diago, CA) and then subcloned into pCAT Basic expression vector (Promega, Madison, WI), or directly cloned into the pCAT basic expression vector and the pBLCAT4 promoter vector (Promega, Madison, WI), respectively. 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 using the T7 Sequencing Kit (Pharmacia Biotech, Uppsala, Sweden).

4.3 Transient transfection assays

The transfections were performed using the lipofection method (DOTAP or FUGENE 6, Boehringer Mannheim, Mannheim, Germany), as recommended by the manufacturer. Before transfection, 1 x 10^6 cells/100-mm dish (DOTAP) or 2 x 10^5 cells/35-mm dish (FUGENE 6) were plated 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 tumor virus (MMTV) reporter construct (GMCAT, American Type Culture Collection). After 20-24 h of transfection, the cell medium was replaced with medium containing charcoal-stripped FCS, and hormones (R1881, dexamethasone or ORG2058) 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 the transfection.

4.4 CAT, β-galactosidase, and protein assays

Cell lysates were prepared using a freezing-thawing procedure, and CAT activity measurements were performed using the fluor diffusion assay (Neumann *et al*. 1987, Eastman 1987). Transfection efficiency was estimated by determining the β-galactosidase activity of the samples according to the method of Rosenthal (1987). The protein contents were measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) and the results were presented as relative activity. Statistical analyses of the difference between the means were performed with ANOVA. The means were regarded as statistically significant when $p < 0.05$.

4.5 RNA extraction and slot blotting

Total RNA from tissue was isolated by the CsCl-Gradient method (Davis *et al*. 1986). Total RNA from cultured cells was isolated using TRIzol™ reagent (Life Technologies, Grand Island, NY) as recommended by the manufacturer. 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.

4.6 Nuclear extracts

Nuclear extracts were made from LNCaP, PC-3, CV-1, or COS-1 as described by Dignam et al. (1983). The nuclear extracts prepared from Hela cells were obtained from Promega.

4.7 EMSAs and competition assay

Double-stranded DNA fragments containing various putative regulatory elements from hPAP or rat probasin gene were used as probes in EMSA to test their interactions with nuclear factors. The probes were end-labelled with [α-^{32}P] dCTP (3000 Ci/mmole, Amersham Pharmacia, Little Chalfont, UK) using Klenow fragment of DNA polymerase I or with [γ-^{32}P] ATP (3000 Ci/mmole, Amersham Pharmacia, Little Chalfont, UK) using T4 polynucleotide kinase. To abolish non-specific binding of the nuclear proteins, poly(dI-dC)/poly(dI-dC) was used as a competitor. In some cases, a 50-200 excess of unlabelled oligoduplexes were used as competitors to confirm specific DNA-protein interactions. The DNA-protein complexes were separated from unbound probes using a 4% or 5% nondenaturing polyacrylamide gel. To test if the same factors could bind to different DNA sequences, different DNA fragments were used as competitors to perform EMSA in a competition assay.

4.8 UV-cross-linking analysis

Oligonucleotides of the hPAP gene and rat probasin gene with 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.

4.9 DNase I footprinting

In the case of the hPAP promoter study, a restriction enzyme digested fragment was labelled with 3'-end-filling using Klenow enzyme to generate the probe. In the case of the rat probasin promoter study, one of the 5'-end labelled primers was used to perform the PCR reaction to generate the 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 DNase I (Boeringer 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 probes were digested by the Maxam-Gillbert method. The resulting DNA fragments were electrophoresed on a 6% sequencing gel.

4.10 Generation and detection of transgenic mice

Two different hPAP promoter/reporter gene constructs were used in transgenic production: the regulatory regions of -734/+50 and -734/+467 were cloned in front of the chloramphenicol acetyltransferase (CAT) gene in pCAT basic vector as described before. The hPAP promoter-driven 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. The tail DNAs of some mice representing line -734/+467 were analyzed by Southern blot. Transgene copies were quantified by slot blot hybridization.

4.11 *In situ* hybridization

A PCR fragment of pCAT, corresponding to nucleotides 2347-2701, was cloned into pCRII-TOPO vector (Invitrogen). BamHI- and XhoI-linearized forms of this construct were used as templates for *in vitro* transcription. SP6 and T7 RNA polymerases were used to transcribe [α-^{35}S] CTP-(1250 Ci/mmole, Amersham Pharmacia, Little Chalfont, UK) labelled antisense and sense RNA probes, respectively. A control probe was synthesized similarly from pTRI-β-actin construct (Ambion, Austin, TX). *In situ* hybridization was carried out according to Mustonen *et al.* (1998) using 6 μm sections of formalin-fixed, paraffin-embedded tissue samples from adult transgenic and control mice. Hoechst 33258 (Sigma, St. Luois, MO) was used to stain nuclei.

4.12 Western blotting and ECL detection

PC-3 cells were transfected with pCAT-231/+467 or pCAT-734/+467 constructs as described. Equal amounts of cells were lysed with SDS loading buffer, and aliquots of the samples were run on a 12% SDS-polyacrylamide gel. The proteins were transferred onto PVDF membrane. CAT protein was detected using polyclonal CAT antibody (5’ prime–3’ prime, Boulder, CO), horseradish peroxidase-linked secondary antibody, and light-emitting substrate according to the ECL Western blotting protocol (Amersham Life Science).

5 RESULTS

5.1 Transfecting well-differentiated prostatic cancer cell line LNCaP

A lipofection method has been set up for the transient transfection of LNCaP cells (I). The LNCaP cells were originated from a lymph node metastatic lesion of human prostatic adenocarcinoma. The relatively high degree of differentiation makes LNCaP cells difficult to maintain in cell culture conditions. Their low ability to attach to the growth support, the slow growth rate, the demand for a high percentage of serum in the culture medium, the tendency of making colonies, and the modest uptake of DNA are features typical of such well-differentiated cells. Lipofection was chosen as a method because it is the gentlest way for transfection. The disadvantage of the DEAE dextran method is the multiple wash steps, and the cytotoxicity of DEAE destroying most of the LNCaP cells. The high induction of positive control pMMTV with 10 nM androgen showed that LNCaP could be transfected after careful optimization of the conditions. Among three tested control plasmid, the cytomegalovirus promoter containing plasmid (pCMVβ-gal) was the most efficient one, while the pSVβ-gal and the pRSVβ-gal were only modestly active in LNCaP cells.

After the production of more efficient transfection reagents, DOTAP was replaced with FUGENE6. The method was modified by plating 5 x 10^5 cells/35mm plate for LNCaP, while 2 x 10^5 cells/35mm plate were grown for other cells before transfection. The total amount of DNA per plate was reduced to 2 μg. The longer culture time before and after transfection compared to other cells is still needed for LNCaP to obtain comparable protein levels for the consequent CAT assay and protein assay. Since LNCaP is the only commercially available cell line that expresses endogenous hPAP, transfecting LNCaP cells makes it possible to study the promoter activity of the hPAP gene, as well as the hormonal regulation of the hPAP promoter.

5.2 Promoter activity of the hPAP gene and comparison with promoters of the PSA and hK2 genes

The activities of several promoter fragments of the hPAP gene that were linked to a pCAT reporter plasmid were determined in the androgen-dependent LNCaP, which is able to secrete proteins of hPAP, hPSA, and hK2. Some of the pCAT constructs covering the entire region -734/+467 of the hPAP gene and the androgen receptor expression vector were transiently transfected into an androgen-independent prostatic cell line PC-3 and a nonprostatic cell line CV-1 apart from the LNCaP cells. The activities of the hPSA (-620/+40) and hK2 (-493/+27) promoter constructs were also analyzed in these three cell lines to allow the comparison of androgen-involved transcriptional regulation of these three prostate-related genes. The promoter constructs of the hPAP gene were functional in both prostatic and nonprostatic cells (II). Although hPAP constructs covered two putative AREs with *in vitro* AR-binding ability at -178 and +336, androgen treatment had little effect on the activity of the proximal promoter of the hPAP gene. The lack of androgen response was also evident in the case of the hPAP -1652/+43 construct, which covered two putative AREs at -1576 and -178 with weak and strong *in vitro* AR-binding capacity, respectively. All the the hPAP promoter activities without androgen *vs*. the activities with androgen were not statistically different in any of the tested cell lines. On the other hand, hPSA and hK2 promoter constructs were also functional in all three cell lines, and androgens were able to stimulate the transcriptional activity of these promoters in PC-3 and CV-1 cells. However, androgen activation of hPSA and hK2 promoters could not be clearly demonstrated in LNCaP cells, even though the control promoter pMMTV showed a significant response to androgen treatment in this cell line.

Because synthetic androgen R1881 failed to regulate the hPAP promoter, the effects of glucocorticoid and progesterone on the transcriptional regulation of the hPAP proximal promoter were also tested in PC-3 cells co-transfected with respective steroid receptor expression vectors. Only minor changes in the CAT activities were observed, similar to the effects of androgen treatment. These results indicated that steroids could not directly regulate hPAP gene expression *via* receptor binding to putative AREs at -178 and +336. On the contrary, transcriptional activities of the promoters of the kallikrein family genes were induced significantly by glucocorticoid dexamethasone and progesterone ORG2058 in addition to androgen in PC-3 cells over expressing respective steroid receptors. In the case of the hPSA promoter, dexamethasone was the most powerful activator, while ORG2058 stimulated hPSA promoter activity to a similar extent as did androgen. In contrast, the induction of the hK2 promoter of glucocorticoid was the weakest among the tested steroids. These results indicate that the putative AREs in the proximal promoters of hPSA and hK2 are not androgen specific, offering a molecular basis for the expression of these genes outside the prostate in tissues containing steroid receptors.

Among the hPAP constructs, the region -734/+467 gave the lowest reporter activities in all three tested cell lines, but this construct seemed to be more active in prostatic cells. The first intron fragment decreased the activity of the hPAP promoter region -734/+50 especially in CV-1 cells (II).

To study the transcriptional regulation of hPAP promoter constructs in transgenic mice, two regulatory regions of -734/+50 and -734/+467 were cloned in front of the CAT

gene in pCAT basic vector. The hPAP promoter-driven reporter gene fragments were released and used to generate transgenic mice by the pronucleus microinjection technique (IV). Eight and twelve founder mice for the -734/+50 and -734/+467 constructs were obtained respectively, while two founders, -734/+50 VIII and -734/+467 III, were unable to transmit the transgene to their offspring due to infertility or possible defective incorporation of the transgene. In addition, four founders (-734/+50 V and VII; -734/+467 II and VIII) transmitted the transgenes to a low number of their offspring and were therefore excluded from the expression studies.

Expression of the transgene was analyzed by the slot blot technique, but was too low to be detected in lines -734/+50 III, IV and -734/+467 V. Extensive tissue distribution analysis was performed for lines -734/+50 I and -734/+467 IV, which represented the highest expression levels of the reporter gene directed by the respective regulatory region. Transgene expression was low and nearly constant in different tissues of line -734/+50 I mice, whereas different lobes of prostate were the main tissues for the reporter expression in -734/+467 IV mice. Expression patterns of the -734/+467 XI and XII were similar to that seen in line IV, indicating that the significant amount of marker mRNA in prostate was not due to the integrating site of the transgene.

In situ hybridization showed the presence of CAT transcripts in the prostate epithelium of -734/+467 IV mice, but not in -734/+50 I mice, supporting the detected difference between the expression levels of the reporter in these lines. CAT signals were absent from other tissues examined from both lines, although control probing with β-actin testified the integrity of mRNA in the samples (IV).

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

The 5’-flanking region (-426/+28) of the rat probasin gene, which has been shown to direct prostate-specific gene expression in transgenic mice, was used to identify the exact DNA-binding site of a putative prostate-specific transcription factor. Promoter activity analysis by CAT assay revealed that the construct pCAT PB -244/+52 was equally well induced by androgens both in prostatic LNCaP and nonprostatic COS-1, MCF-1, HEC-1, and HEP-1 cell lines (III). This indicated that although the probasin promoter region -244/+52 was important for androgen regulation, it did not act in a prostate-specific manner. Androgen induction of the promoter was decreased to 50% in LNCaP cells upon the 5’-deletion of the construct from -278 to -244. Since no effect was observed in COS-1 cells, the sequence around -278 to -244 was shown to be crucial for prostate-specific gene expression. DNA-protein interaction study within the region from -310 to -96 by DNase I footprinting exhibited a protected window between the nucleotides -251 and -240 when prostatic LNCaP and PC-3 nuclear extracts were used. The sequence of this area is 5’-GAAAATATGATA-3’. EMSA using rPB -257/-235 as a probe, which covering the 12 bp sequence, displayed a strong shift band also when LNCaP or PC-3 nuclear extracts were used. Mutation of two consecutive nucleotides in the 5’-end, 3’-end, or in middle of that

12 bp sequence abolished the formation of the specific DNA-protein complex. Deletion of the 12 bp from pCAT PB -426/+52 or mutation of those six nucleotides within the 12 bp sequence simultaneously in pCAT PB -426/+52 significantly decreased androgen induction in LNCaP cells compared to the wild-type 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. Glucocorticoid could not induce the promoter activity of PB -278/+52, indicating that the putative prostate-specific transcription factor might interact specifically with AR.

The GAAAATATGATA sequence was also found at the first intron (+1144/+1155) of the hPAP gene. EMSA using hPAP +1136/+1164 as a probe showed a strong specific shift band when LNCaP or PC-3 nuclear extracts were used, and an rPB -57/-235 fragment could compete off the shift band (III). These results suggested that the putative prostatic regulatory protein could also play a role in the transcriptional regulation of the hPAP gene.

5.4 The prostatic regulatory protein and tissue-specific regulation of hPAP promoter constructs

The hPAP promoter and the first intron fragment of hPAP (-734/+467) could trigger the expression of the reporter gene in transgenic mice and restrict the expression mainly in the prostate, whereas hPAP-734/+50 had lower promoter activity and the reporter gene was expressed nearly constantly in different tissues. In LNCaP cells, the transcriptional activity of the TK promoter in pBLCAT4 vector was increased fourfold when the hPAP +57/+467 intron fragment was included in the construct. A similar effect was seen in the breast cancer cell line T47D, but not in COS-1 kidney cells (IV). These results indicated the presence of regulatory elements involved in tissue-specific gene expression at +57/+467 of the hPAP gene.

Comparion analysis using computer program revealed that there are five sequences similar to the identified GAAAATATGATA binding site in the hPAP -734/+467 region. These sites are located at -580/-569, -257/-246, -151/-140, +218/+229, and +244/+255, named site A, B, C, D, and E, respectively. The prostate-specific binding site at +1144/+1155 of the hPAP gene, identical to the original one (named Pb) found in the promoter of the probasin gene, is marked by F. The site F is known to bind prostatic transcription factor most efficiently and shows 75% nucleotide identity with sites A, C, D, and E, and 58% identity with site B. A specific DNA-protein complex was detected in EMSA when hPAP +239/+269 was used as a probe, which contains the site E, when LNCaP or PC-3 nuclear extracts were used. No complex was detected, however, when COS-1 or Hela nuclear extracts were used. Perfect core sequences of transcription factor GATA and SOX-5 were found in hPAP +239/+269, but neither the GATA consensus sequence nor the SOX-5 consensus could compete off the shift band in competition assay. UV-cross-linking study using equally sized probes, which contained binding sites for E,

F, or Pb, indicated that nuclear proteins bound to respective oligos were at least the same in size. Nuclear extracts from LNCaP or PC-3 could also form a complex with site C, but not with site A, B or D. The competition assay using hPAP +239/+269 as a probe and different oligos as competitors showed that the capacity of the putative prostatic regulatory protein bound to different sites could be estimated roughly as +++ for site F, ++ for site Pb, and + for both sites E and C (Fig. 5).

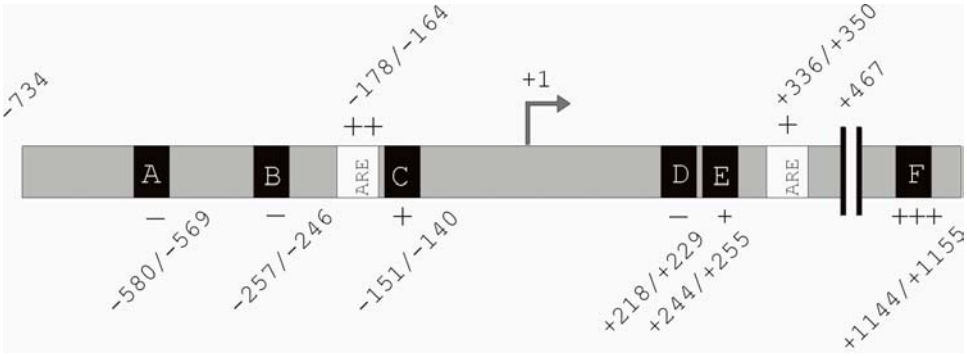

Fig. 5. Location of the prostate-specific DNA-binding site GAAAATATGATA related sequences and potential androgen response elements in the schematic representation of the regulatory region of the hPAP gene. Homologs of GAAAATATGATA (A-F) were found using FINDPATTERNS (in https://seqweb.csc.fi) and are shown by black boxes with the nucleotide location underneath. Binding capacities of the elements for the prostatic protein *in vitro* are also marked (-, +, +++). AREs are shown by white boxes with the sequence position above. *In vitro* androgen receptor binding capacities are indicated by + or ++ above AREs.

Deletion of site C from constructs hPAP -231/+50, hPAP -734/+50, and hPAP -734/+467 did not change the promoter activities significantly in prostatic LNCaP cells in the absence of androgens, although a slight decrease in the mean activities of the mutants took place compared to the respective wild types. In the presence of androgens, however, each of the constructs lacking site C showed a statistically significant decrease in transcriptional activities compared to that of corresponding wild-type constructs in LNCaP cells. Significant changes were not detected in reporter gene activity when the wild-type and deletion constructs were analyzed in the nonprostatic COS-1 cells.

Deletion of site E from the construct hPAP -734/+467 significantly increased transcriptional activity compared to the wild type in the absence of androgens in LNCaP cells, while no change was detected in the presence of androgens. Also, no change could be seen in COS-1 cells either in the absence or presence of androgens. When both sites C and E were deleted from the hPAP -734/+467 constructs, the results were similar to the case lacking only site E. The reporter gene activity of hPAP -734ΔC/+467ΔE was significantly higher than that of the wild-type construct in a hormone-depleted environment. The double mutation (ΔCE) showed diminished activity compared to the wild type and ΔE constructs in the presence of androgens. However, the decrease was not statistically significant in either case, suggesting that the deletion of site E probably has a

mild activating effect on the construct also in the androgen-supplemented environment, being able to overcome the inhibitory effect of the site C deletion. On the other hand, the function of ΔCE was similar to that of the wild type and ΔE regardless of the hormone status in the COS-1 cell.

The double deletion ΔDE had the highest mean value for transcriptional activity among the hPAP-734/+467 construct and its mutants, showing 4-fold activation compared to the wild type in LNCaP cells without androgen treatment. Furthermore, the increase in the reporter gene activity of hPAP -734/+467ΔDE compared to that of the respective ΔE mutant was statistically significant. These results suggest that site D could act as a binding site for the prostatic regulatory protein *in vivo*. Significant changes in the transcriptional activity between ΔDE and hPAP -734/+467 were not detected in COS-1 cells. The activity pattern of hPAP -734ΔC/+467ΔDE in LNCaP cells resembles that of ΔDE, with a masked •C effect in the presence of androgens. Also in this case, the observed functional activation of ΔCDE compared to the wild-type construct in hormone-depleted medium was statistically significant. Similar to the behaviour of the other constructs, functionality of the triple mutant and the wild type was comparable with or without androgen treatment in COS-1 cells.

Anti-androgen flutamide treatment resulted in decreased activity of the double mutant compared to that in the absence of androgens. The decrease was statistically significant and the remaining activity was similar to the corresponding value in the presence of androgens. These results suggest that ligand-independent action of the androgen receptor could be the mechanism behind the transcriptional activation of the constructs lacking site E or both sites D and E in LNCaP cells in the absence of androgens.

Taken together, deletion of the different binding sites of the prostatic transcription factor resulted in bidirectional effects depending on the location of the sites and the hormone status in LNCaP cells. Site C at the proximal promoter of hPAP acts as a positive regulatory element in prostatic cells in an androgen-dependent manner, while site E or sites D and E act as negative regulatory element(s) in the androgen-depleted environment. Flutamide treatment was able to block the detected transcriptional activation after D and E deletion, indicating the essential role of the androgen receptor in the event by a ligand-independent mechanism.

6 Discussion

6.1 Promoter activity and hormonal regulation of the hPAP gene

Promoter constructs of hPAP covering the entire gene region of -734/+467 were functional in both prostatic and nonprostatic cell lines, but these constructs could not be induced with androgen, glucocorticoid, or progesterone in transfection assays (II). There was no androgen response evident in the case of the hPAP -1652/+43 construct tested in LNCaP and PC-3 cells. Previously Virkkunen *et al*. (1994) reported that the SREs of the hPAP gene at -178 and +336 and the corresponding ones in rat PAP are able to bind AR in vitro. Furthermore, putative SREs of the human and rat PAP genes at -1576 and -1612, respectively, have weak *in vitro* AR-binding capacities. Therefore our transfection results indicate that androgens cannot directly regulate hPAP gene expression via receptor binding to those SREs. On the other hand, androgens down-regulate hPAP mRNA in LNCaP cells under similar culture conditions (Henttu *et al*. 1992, Lin *et al.* 1994). In nuclei run-on experiments, it seems that androgens stimulate the transcription of hPAP mRNA in low density cells, while, the transcription of hPAP mRNA is suppressed by androgen in high density cells (Zelivianski *et al*. 1998). These results suggest that androgens could regulate hPAP expression at the level of transcription. One possibility for the contradiction in phenomenon is that the distal parts of the hPAP gene contain additional SRE(s) that might cooperate with the SREs in the proximal areas and be essential for optimal androgen action. There are several examples of genes where the androgen effects cannot be demonstrated maximally without the interaction of several SREs, as in the case of the probasin gene (Rennie *et al*. 1993, Kasper *et al.* 1994). Another possibility is that the hPAP gene needs another transcription factor(s) along with AR to enhance transcription and define prostate-specific expression. The response element(s) for this factor(s) could be far upstream or somewhere else in the gene (II). Zelivianski *et al*. (2000) studied the cis-regulatory elements of the hPAP promoter covering the region from -2899 to +87. Two regions of transcriptional suppression were identified and located at -2899/-2583 and -2583/-1305, whereas the fragment of -1305/-779 had a transcriptional activation function. It has not been reported if these different 5'-deletion constructs could respond to androgen stimulation.

Contrary to hPAP promoter constructs, hPSA and hK2 promoters showed a weak androgen induction in LNCaP cells, but a strong induction in PC-3 or CV-1 cells. This indicates that the hPAP gene might be differently regulated by androgen compared to the hPSA or hK2 gene (II). In fact, androgen stimulation of the proliferation of prostate epithelial cells coincides with a decrease in hPAP and an increase in hPSA mRNA (Henttu *et al*. 1992, Lin *et al*. 2000). The difference in the effects of androgens on hPAP and hPSA expression could be due to the observation that the cellular form of hPAP functions as a negative regulator by dephosphorylating c-ErbB-2/neu oncoprotein, resulting in the down-regulation of cell proliferation (Lin & Meng 1996, Lin *et al*. 1998, Meng & Lin 1998), while PSA may activate an insulin-like growth factor (IGF) by hydrolyzing its binding protein (IGF-BP), leading to the stimulation of cell growth (Cohen *et al*. 1992, 1994). Androgen down-regulation of hPAP could, in part, be via the PKC signal transduction pathway (Henttu & Vihko 1996). TPA, a ligand of PKC, and DHT have similar effects on PKC activity as well as the hPAP expression in LNCaP cells (Lin *et al*. 2000).

Androgen up-regulation of the PSA gene could be due to a direct interaction of the androgen/AR complex with the functional ARE in the promoter of the gene (Cleutjens *et al*. 1996, 1997a). The different androgen response of the PSA or hK2 promoter in LNCaP and PC-3 might be due to the different AR coregulators in these two cell lines. Fujimoto *et al*. (2001) examined the expression level of some of the AR coactivators in LNCaP, PC-3, DU-145 cancer cell lines, benign prostatic tissue sample, and prostate cancer tissue specimens. In the cell lines, coactivator SRC1 was expressed ubiquitously at almost equal amounts, whereas other coactivators, such as ARA55, ARA54, TIF2, and RAC3, displayed cell line-specific expression.

The promoter activities of PSA and hK2 could also be induced by glucocorticoid and progesterone, indicating that the SREs in the promoters of the PSA and hK2 genes are not androgen-specific, as in the case of the rat probasin gene (Claessens *et al*. 1996). The androgen-specific regulation of the PB gene can be explained by the presence of an ARE in its promoter, specifically recognized by AR but not GR (Kasper *et al*. 1999).

6.2 The putative prostatic regulatory protein, DNA binding sites and prostate-specific expression of the hPAP gene

A 12-bp binding site located at -251/-240 in the rat Pb promoter was identified from the 5'-flanking area (-426/+52) of the gene, which has been shown to be sufficient for targeting gene expression to the prostatic epithelium (Greenberg *et al*. 1994, 1995). Deletion of this element from the PB promoter construct significantly decreased the androgen induction of the promoter in prostatic cells but not in nonprostatic cells. Although the -251/-240 was important for androgen induction in prostatic cells, at least 20 5'-flanking nucleotides were needed for maximal effect. However, these flanking nucleotides did not bind any sequence-specific proteins of their own. These nucleotides may help the putative prostatic regulatory protein to bind to its response element (III). Similar observation has been seen in the NF-κB response element of the human

intercellular adhesion molecule-1 (ICAM-1) gene (Paxton *et al*. 1997). ICAM-1 is induced when NF-κB binds to a modified NF-κB site after the stimulation of tumor necrosis factor-α (TNFα). In addition to this binding site, both specific 5'- and 3'-flanking sequences are necessary for TNFα induction. When either of the AREs at the rPB -286/+52-CAT construct are mutated, androgen induction is almost completely lost (Kasper *et al*. 1994), although the constructs contain the DNA-binding site of the putative prostatic regulatory protein. These results suggest that the regulatory protein is not obligatory for the function of AR in the rPB promoter. However, the presence of this factor can duplicate or even triplicate the effect of androgens, indicating that there is a synergistic action between the prostatic transcription factor and AR (III).

Relatively few prostate-specific transcription factors have been characterized so far: one is Nkx3.1, which is an androgen-regulated, prostate-specific homeobox protein (He *et al*. 1997, Prescott *et al*. 1998). Nkx3.1 preferentially binds to the TAAGTA sequence (Steadman *et al*. 2000); another is prostate-derived Ets-factor (PDEF, Oettgen *et al*. 2000). Among the Ets family, PDEF prefers binding to a GGAT rather than a GGAA core. PDEF could be a potential partner of Nkx3.1 (Chen *et al*. 2002). Coimmunoprecipitation analyses demonstrated that Nkx3.1 and PDEF are physically associated in prostate epithelial cells. Cotransfection analyses revealed that Nkx3.1 can abolish the transcriptional activation function of PDEF on the PSA promoter. The binding sites of Nkx3.1 and PDFE are clearly different from GAAAATATGATA, the binding site of the putative prostatic regulatory protein, suggesting that the prostatic regulatory protein could be a new prostate-specific transcription factor.

The exact same 12-bp sequence has also been found in the first intron of the hPAP gene. EMSA shows that the prostatic regulatory protein could also bind to this element (F: +1144/+1155), suggesting that the transcription factor could regulate hPAP expression (II). Five homolog sequences, sites A (-580/-569), B (-257/-246), C (-151/-140), D (+218/+229), and E (+244/+255), have been found in the hPAP -734/+467 region, which has been shown to trigger the reporter gene expression and restrict the expression mainly in prostate epithelium. EMSA showed that the putative prostatic regulatory protein could bind to sites C and E, but to not A, B, or D (Fig.5). The behavior of site C is similar to that of the prostatic binding site in the rPB promoter, since the transcription efficiency was decreased in the presence of androgen after the deletion of site C from the hPAP constructs. The location of site C at -151/-140 of the hPAP proximal promoter, 13 nucleotides downstream from an ARE, is similar to the position of the prostate-specific binding site at -251/-239 of the PB promoter, which is only three nucleotides away from one downstream ARE. However, it is currently uncertain whether androgen action and site C-mediated function are connected directly or indirectly. Sites A and B at the proximal promoter of hPAP are unable to bind transcription factors *in vitro*, which does not, however, completely disqualify the possibility of a regulatory role for the sites *in vivo*. Sites D and E are located in the first intron of the hPAP gene. Surprisingly, the deletion of site E from the hPAP -734/+467 construct caused an increase in transcriptional activity in the absence of androgens compared to the wild-type construct. Further deletion of the site D concomitant to site E was able to intensify the effect in hormone-depleted medium. This could mean that site E is a low-affinity binding site for the prostatic regulatory protein and possibly the close proximity of sites D and E improves the binding capacities of both sites *in vivo* (IV).

Site C is located in the promoter region, while sites D and E are located in the first intron of the hPAP gene. The orientation of site E is opposite to other homolog sites present in the hPAP gene. Furthermore, the distance between site E and the nearest ARE in the hPAP gene is about six times longer than in the case of site C. These could open the possibility of differential regulatory responses for the elements. Our recent studies have indicated that the closer the prostate-specific binding site of rPB is to an ARE, the more powerful the activating effect is in the presence of androgens (unpublished data). AR is a potential connecting molecule in the detected bidirectional gene regulation via C and D/E elements binding to the prostatic transcription factor. Anti-androgen flutamide could block the transcriptional activation of the hPAP -734/+467 construct after the deletion of E and D elements in hormone-depleted conditions, suggesting a mechanism of the ligand-independent action for AR (IV). Interestingly, a bidirectional regulation process has also been seen in the case of the glycoprotein hormone α-subunit gene. Pituitary cell type-dependent activation and repression is mediated though an upstream regulator of the gene. Differences in the regulatory mechanisms were observed, but elements mediating the activation or repression seemed to be closely juxtaposed or even overlapped (Wood *et al*. 1999).

A sequence sharing approximately 80% identity with the GAAAATATGATA sequence was located in the hPSA (-4196/-4185) enhancer area. The DNA fragment containing this GAAGATATTATC sequence could not form a specific DNA-protein complex when LNCaP or PC-3 nuclear extracts were used in EMSA (II). The prostatic regulatory protein binds to element F of the hPAP gene with high affinity, while it binds with moderate affinity to the binding site in rPB promoter and with low affinity to elements C and E of the hPAP gene (IV). Preliminary methylation interference experiments using the hPAP fragment containing element F as a probe suggested that the thirtieth nucleotide C is also involved in DNA-protein interaction (unpublished data). This could explain why element F has the highest affinity with the prostatic transcription factor. The existance of multiple binding sites for a regulatory protein with different affinities within one gene is quite common. At least eight high and low affinity AR binding sites have been identified in the promoter and enhancer regions and have been implicated in the androgen-mediated regulation of PSA. Inspection of the PSA promoter and enhancer sequence revealed the existence of at least 11 putative Ets-binding sites (Oettgen *et al*. 2000). Some of these sites are in close proximity to AR-binding sites, and EMSA demonstrated that PDEF could bind to some of these sites with different affinities.

The importance of the putative bidirectional regulation of the whole hPAP gene through the GAAAATATGATA and homologous sequence during different biological conditions is not yet clear. The first indication of the androgen-dependency of hPAP expression is the appearance of the protein following sexual maturation (Yam 1974). This could mean that in normal prostatic cells, the hPAP gene is activated when the androgen concentration in the circulation reaches the levels of adulthood. Therefore, a mechanism controlling the expression of the hPAP gene could exist where the multiple prostate-specific DNA-binding sites mediate either activation or repression, depending on the relevant physiological level of androgens in the serum.

6.3 LNCaP model and transgenic mice model

The LNCaP cells were originated from a lymph node metastatic lesion of human prostatic adenocarcinoma, and have been widely used in the study of prostate cancer (Vaalara *et al.* 1998, 2000). Unlike prostatic cell lines PC-3 and DU-145, the growth of LNCaP cells is androgen-dependent, and the cells could be transformed to androgen-independent clones (van Steenbrugge *et al.* 1991). The secretion of PSA and hPAP, two major prostate epithelium-specific differentiation antigens, serves conventionally as a marker of androgen action on prostate cells (Andrews *et al.* 1992, Henttu *et al.* 1992). The androgen-responsive feature of LNCaP also makes it a useful model in studying the transcriptional regulation of prostate-related genes, since the expression of many prostate-specific proteins requires functionally differentiated, androgen-responsive cells. LNCaP is even more valuable in examining the transcriptional regulation of the hPAP gene because it is the only commercially available human prostate cancer cell line which can express endogenous hPAP (Horoszewice *et al.* 1983, Andrews *et al.* 1992, Henttu *et al.* 1992). The androgen-independent prostatic cancer cell line PC-3 does not express detectable levels of AR mRNA (Brolin *et al.* 1992) or hPAP mRNA in Northern blot analysis (Solin *et al.* 1990), and PC-3 cells are unable to secrete hPSA or hK2 (Garcia-Arenas *et al.* 1995). In PC-3 cells, the hPAP gene seems to be normal according to Southern blot analysis (Garcia-Arenas *et al.* 1995) and partial sequencing (unpublished data). The forced expression of AR in PC-3 cells cannot trigger the transcription of the hPAP gene (Garcia-Arenas *et al.* 1995). Most likely, some transcription factors essential for hPAP expression in LNCaP cells are missing from PC-3 cells.

Preparing a method for the transfection of LNCaP cells offers a fascinating possibility for studying the promoter activity of the hPAP gene and the transcriptional regulation of the hPAP promoter in a cell culture model. The density of the cells at the moment of plating is important for successful transfection (I). Cell culture density can influence gene expression and protein levels of biologically important molecules, including growth factors and their receptors (Singh *et al.* 1996, Monget *et al.* 1998, Pfeiffer *et al.* 1998), in epithelial cells. In LNCaP, the cell culture density not only affects the level of hPAP mRNA (Lin *et al.* 1994), but also modulates the androgen regulation of hPAP mRNA (Lin & Garcia-Arenas 1994). The optimal cell density plated for transfection is 1 x 10^6cells/100mm plate (I). Under this growth condition, androgen suppresses hPAP mRNA and stimulates the secretion of hPAP (Henttu *et al.* 1992, Lin *et al.* 1993a, Lin *et al.* 1993b). LNCaP maintained at such a high density with a slow growth rate could mimic the differentiated state of prostate epithelial cells (Lin *et al.* 1994).

The puberty effect indicates that the expression of the hPAP gene is under androgen influence. In normal rat prostate, two of the transcripts encoding PAP were up-regulated by androgens, whereas one of the mRNA species was insensitive to the hormone status. The androgen-sensitive transcripts were detectable 4 days after castration (Porvari *et al.* 1995). These results indicate that PAP gene expression in a normal prostate is a complicated process and is not exclusively androgen-regulated. The mRNA studies using tissue slices from various benign prostatic hyperplastic glands suggested that DHT is necessary to sustain the expression of hPAP in hyperplastic prostates (Dulinska *et al.* 1997). Results from the transient transfection of LNCaP cells might reflect the case in the

cancer status. A number of changes might occur in LNCaP cancer cells compared to normal prostate epithelial cells. A T877A mutation at the LBD of AR in LNCaP makes the receptor responsive to other steroid hormones. AR coactivators could be also differently expressed in LNCaP compared to the normal prostate cells or other prostate cancer cell lines. Normal prostate cells express the receptor protein phosphatase PTP, whereas LNCaP cells do not. PTP regulates the PKC pathway to restore E-cadherin-dependent adhesion of the prostate epithelial cells via its interaction with RACK1 (Hellberg *et al*. 2002). It is possible that the control of hPAP expression is disturbed in prostate cancer cells and may have little linkage to the function of the AR (Garcia-Arenas *et al*. 1995).

The first intron fragment +57/+467 of the hPAP gene decreases promoter activity in transient transfections, while the reporter gene led by hPAP -734/+467 has a relatively high expression in the prostate of transgenic mice compared to that by hPAP -734/+50. This might be due to the difference between the normal prostate cells and cancer cells, but it could be also due to the difference between the cell culture and animal models. In the cell culture model, cells lack the interaction with other types of cells. The cells might then lose the *in vivo* autocrine or paracrine influences. Interleukin-6 (Il-6) is an autocrine factor in human prostate cancer (Giri *et al*. 2001). Il-6 protein concentrations are increased approximately 18-fold in clinical localized prostate cancers when compared to normal prostate tissue. Normal and neoplastic prostatic epithelial cells in culture, with the exception of LNCaP cells, secrete Il-6. The concentration of the Il-6 receptor is increased eightfold in prostate cancer tissues and increased in prostate cancer cell lines. Addition of exogenous Il-6 to primary epithelial cells in culture or to LNCaP cells leads to phosphorylation of signal transducers and activators of transcription 3 (STAT3) and increases in net cell proliferation. Furthermore, the Il-6 signal transduction pathway could cross-talk with AR signal transduction pathway (Ueda *et al*. 2002). Immunoprecipitation and transactivation studies showed a direct interaction between the amino acids 234-558 of the AR and STAT3 following Il-6 treatment of LNCaP cells. Inhibitors of MAPK and JAK decreased Il-6-induced phosphorylation of MAPK and activation of the AR N-terminal domain. Although native AR was always co-transfected in the cell culture model, it is quite possible that AR is not fully activated in LNCaP cells.

The transgenic animal model provides the *in vivo* information of gene regulation, but this model is expensive and time-consuming. The variation in copy number of the transgene and the integration site could sometimes cause problems.

6.4 Conclusion

This study focused on the promoter activities and hormonal regulation of the hPAP gene in transient transfections of cell lines, as well as the promoter activities in transgenic mice models. Androgen regulates the hPAP gene differently in LNCaP cells than it does the other prostate-related genes hPSA and hK2; androgens could not directly regulate hPAP expression via receptor binding to AREs located at -178 of the proximal promoter or at +336 of the first intron of the gene. The promoter and first intron fragment -734/+467 of the hPAP gene could direct and also restrict the gene expression mainly in prostate

epithelium. A prostatic regulatory protein could bind to multiple binding sites with the GAAAATATGATA or homologous sequence along the promoter and the first intron of the hPAP gene with different affinities, and hence could be involved in the regulation of the prostate-specific expression of the hPAP gene in a bidirectional manner, depending on the hormone status.

The hPAP gene is a good model in studying hormone regulation and tissue/cell-specific regulation of gene expression, although the real physiological role of hPAP is not yet clear. Since no mutation of the hPAP gene has been found in clinics so far, the generation of PAP gene-deficient knock-out mice becomes very important in demonstrating the exact function of PAP, and may also be helpful to clarify the hormonal regulation and prostate-specific regulation of the gene. Study of the hPAP promoter may have a significant medical impact. The further understanding of the molecular mechanisms behind the androgen regulation and prostate-specific regulation of hPAP promoter might lead to the improvement of the clinical practice for prostate cancer by using therapeutic genes under the tight control of a highly active and prostate-specific promoter.

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