REGULATION OF PROSTATE-SPECIFIC ANTIGEN EXPRESSION

REGULERING VAN PROSTAAT-SPECIFIEK ANTIGEEN EXPRESSIE

PROEFSCHRIFT

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Voor mijn ouders
Voor Jos

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Abreviations

List of abbreviations

aa	amino acid
AF	transactivation function
AIS	androgen insensitivity syndrome
AP	anterior prostate
AR	androgen receptor
ARE	androgen response element
bp	base pairs
BPH	benign prostate hyperplasia
cAMP	adenosine cyclic-3':5'-monophosphate
cDNA	complementary deoxyribonucleic acid
COUP-TF	chicken ovalbumin upstream promoter-transcription factor
CTD	C-terminal repeat domain
DBD	DNA binding domain
Dex	dexamethasone
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
DP	dorsal prostate
EcR	ecdysone receptor
EGF	epidermal growth factor
ER	estrogen receptor
ERE	estrogen response element
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GTF	general transcription factor
hGK-1	human glandular kallikrein-1
HRE	hormone response element
hsp	heat shock protein
IGF-I	insulin-like growth factor I
Kb	kilo base pairs
kDa	kilo Dalton
KGF	keratinocyte growth factor
KLK1	human tissue kallikrein
KLK2	human glandular kallikrein-1
LBD	ligand binding domain
LNCaP	lymph node carcinoma of the prostate (cell line)
LP	lateral prostate
LTR	long terminal repeat
mGK	mouse glandular kallikrein
MMTV	mouse mammary tumor virus
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NLS	nuclear localization signal
NMR	nuclear magnetic resonance

PCR	polymerase chain reaction
PG	parotid salivary gland
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
PRE	progesterone response element
PSA	Prostate specific antigen
R1881	17α-methyl-17β-hydroxyestra-4,9,11-trien-3-one
R5020	17α,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione
RAR	retinoic acid receptor
RLU	relative light units
RNA pol II	ribonucleic acid polymerase II
RU486	RU 38486 (mifepristone)
RT	room temperature
RXR	retinoic X receptor
SLG	sublingual salivary gland
SMG	submandibular salivary gland
T	testosterone
TAF	TBP associated factor
TAT	tyrosine amino transferase
TBP	TATA binding protein
TF	transcription factor
TFIIIA	transcription factor III A (Xenopus laevis)
TR	thyroid hormone receptor
TIC	transcription initiation complex
TIF	transcription intermediary factor
TK	thymidine kinase
TSS	transcription start site
UTR	untranslated region
VDR	vitamin D3 receptor
VP	ventral prostate
X-Gal	5-bromo-4-chloro-3-indoyl βD-galactosidase

Chapter I

INTRODUCTION

Chapter I

I.1. THE STEROID HORMONE RECEPTOR FAMILY

Steroid hormones are widely distributed, cholesterol-derived, small hydrophobic molecules. They mediate a variety of biological functions, including tissue development, differentiation and homeostasis. Mammalian steroid hormones (androgens, glucocorticoids, mineralocorticoids, estrogens and progestins) exert their function by binding to the corresponding intracellular steroid hormone receptor. This binding triggers a complex set of molecular events, including protein-protein and protein-DNA interactions.

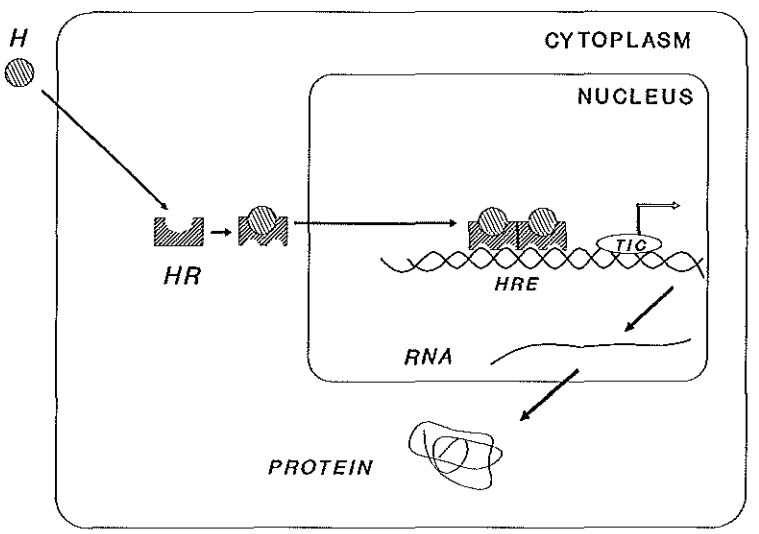

Figure I.1. A basic model for activation of gene expression by steroid hormones. H: steroid hormone, HR: steroid hormone receptor, HRE: hormone response element, TIC: transcription initiation complex.

As early as 29 years ago, Jensen et al. (1968) proposed a model for the action of steroid hormones, that is basically still valid today. According to this model (see Figure I.1), circulating steroids enter target cells by diffusion through the cell membrane. In the cell they interact with their cognate receptors. Upon ligand binding the receptor is activated and converted into a tight nuclear binding state. In the nucleus it regulates transcription by binding to the hormone response element (HRE) in the regulatory region of target genes, thereby activating RNA polymerase II in the transcription initiation complex (TIC). According to more recent studies the original model has been refined and in some cases modified, as will be outlined below.

Cellular concentrations of steroid hormone receptors are very low, and therefore they are not easy to study. The molecular cloning of cDNAs encoding the steroid hormone receptors was an essential contribution to the current knowledge on transcriptional regulation by steroid hormones.

I.1.1. NUCLEAR RECEPTORS

The steroid receptors are members of the family of nuclear receptors, that comprises over 60 different proteins in vertebrates and insects. Elucidation of the primary structure of the nuclear receptors provides understanding of their modular structure and functional domains. All receptors are characterized by a central DNA binding domain (DBD), that targets the receptor to specific DNA sequences (HREs). The DBD is composed of two highly conserved zinc coordinating domains. The N-terminal modulating domain is highly variable in size and in amino acid composition by comparing the various receptors, and contains transactivation functions. The C-terminal part of the receptor encompasses the ligand-binding domain (LBD), that is partially conserved between the various family members.

Phylogenetic studies indicate a common ancestor of nuclear receptors (Amero et al. 1992, Laudet et al. 1992, Detera-Wadleigh & Flemming 1994, Mangelsdorf et al. 1995). In this view, the individual receptors originate from a single precursor gene by processes of gene duplication, rearrangement, mutation, exon shuffling and transposition (O'Malley 1989, Dorit et al. 1990, Amero et al. 1992, Keese & Gibbs 1992, Laudet et al. 1992). Based upon phylogenetic analysis, the nuclear receptor family can be divided into three major subfamilies (Gronemeyer & Laudet, 1995). Subfamily I contains as best defined members the receptors for thyroid hormone (TRα and β), vitamin D (VDR), retinoic acid (RARα, β and γ), the retinoic X receptors (RXRα and β), the peroxisome proliferator activated receptors (PPARα, β and γ), and the Drosophila ecdysone receptor (EcR). Subfamily II contains the majority of the so called orphan receptors. For orphan receptors, the ligands have not been identified as yet, or they are active without specific ligand binding. Subfamily III encompasses the steroid hormone receptors except for EcR.

Starting with the human glucocorticoid receptor (GR) (Hollenberg et al. 1985, Miesfeld et al. 1986) and estrogen receptor (ER) (Green et al. 1986, Greene et al. 1986) the cDNAs of all human steroid hormone receptors were cloned [progesterone receptor (PR) (Mishari et al. 1987); mineralocorticoid receptor (MR) (Arriza et al. 1987) and the androgen receptor (AR) (Chang et al. 1988, Lubahn et al. 1988a, Trapman et al. 1988)]. Surprisingly, ten years after the cloning of the ER (now renamed ERα), the isolation of a second ER cDNA, ERβ was reported (Kuiper et al. 1996, Mosselman et al. 1996). The schematical organization and the structural homology between the various domains of the different steroid receptors are depicted in Figure I.2. With the exception of ER, the DBDs of the steroid receptors show more than 75 percent homology, whereas the homology between the LBDs is approximately 50 percent. At the C-terminal end the ERs are slightly longer.

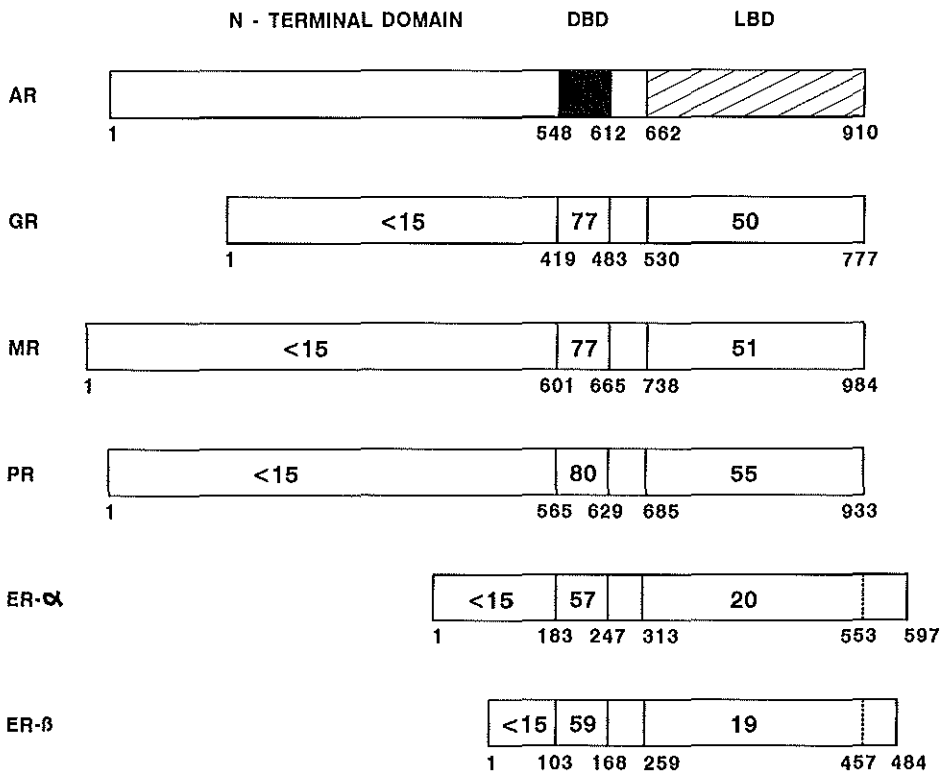

Figure I.2. Structural homology of steroid hormone receptors. The structural homology in the N-terminal domain, DNA-binding domain (DBD; black box) and ligand binding domain (LBD; hatched box) of the human androgen receptor, glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, estrogen receptor α and estrogen receptor β. Numbers in the open bars represent the percentage of homology.

I.1.2. STRUCTURAL ANALYSIS OF STEROID RECEPTORS

The separate domains of steroid hormone receptors contain one or more functional units involved in their specific function (see Figure I.3 for a schematical representation). As pointed out above, they include domains for DNA binding and ligand binding. Additionally, they include regions essential for transcription regulation, dimerization and for effective transport to the nucleus.

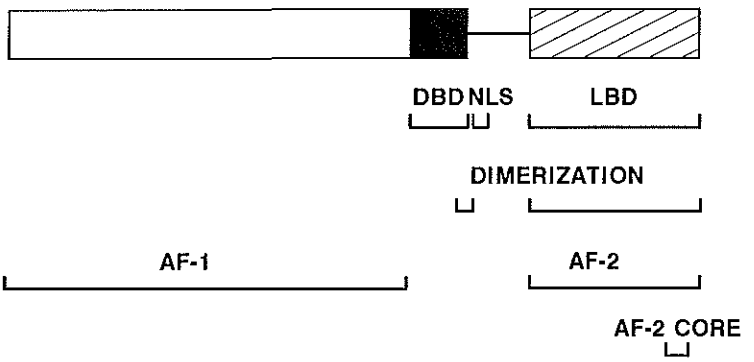

Figure I.3. Schematic illustration of the structural/functional organization of steroid hormone receptors. The N-terminal domain is shown as an open box, the DBD and LBD as black and hatched boxes, respectively. Domain functions are depicted under the schematic receptor representation. NLS: nuclear localization signal, AF: transactivation function.

The N-terminal domain

The N-terminal domain of most steroid receptors is very long (601, 565, 419 and 548 amino acid residues for MR, PR, GR and AR respectively). In contrast, both ERα and ERβ contain much shorter N-terminal domains (183 and 103 amino acids, respectively). The N-terminal domains of all steroid hormone receptors including the ERs, contain at least one region with a hormone dependent transactivation function (AF-1), which is essential for the maximal transcriptional response of hormone-inducible promoters. AF-1 is constitutively active in truncated receptors, that lack the LBD. Furthermore, AF-1 activity is cell and promoter dependent (Bocquel et al. 1989, Tasset et al. 1990, Muller et al. 1991, Dieken & Miesfeld 1992, McEwan et al. 1993, 1994, Tzuckerman et al. 1994, reviewed in Evans 1988, Green & Chambon 1988, Carson-Jurica et al. 1990, Gronemeyer & Laudet 1995). Deletion mapping of the N-terminal domain established that the AF-1 regions of the different steroid receptors are structurally distinct, and are located at different positions (see Figure I.4) (Hollenberg et al. 1987, Hollenberg & Evans 1988, Tora et al. 1989, Simental et al. 1991, Meyer et al. 1992, Pakdel et al. 1993, Metzger et al. 1995a, Dahlman-Wright et al. 1994 and 1995, Jenster et al. 1995, Chamberlain et al. 1996). The AF-1 region in the N-terminal domain of the GR was mapped to an acidic 185 amino acid segment close to the N-terminus of the receptor (Hollenberg et al. 1987). Internal deletions in this region revealed the presence of a 41 amino acid core region, crucial for activity (Dahlman-Wright et al. 1994 and 1995). The AF-1 region of the PR is located in a proline-rich 91 amino acid sequence, adjacent to the DBD (Meyer et al. 1992). Two PR isoforms are known, designated PR-A and PR-B, respectively (see Figure I.4).

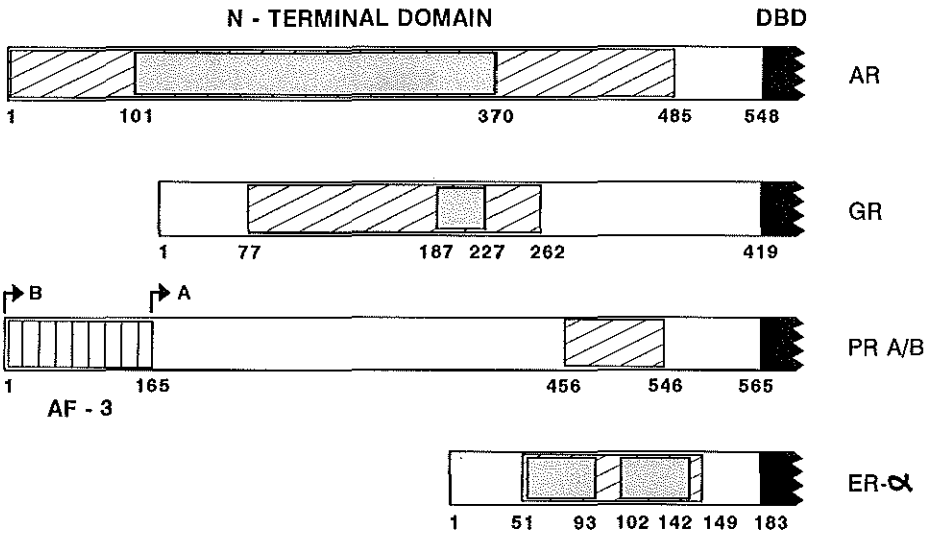

Figure I.4. Location of N-terminal transactivation units in the individual steroid hormone receptors. The N-terminal domain is shown as an open box, the DBD as black box. The AF-1 regions are depicted by hatched boxes; core regions are represented by grey boxes.

In PR-B, besides the AF-1 region, an additional activation function (AF-3) has been defined (Sartorius et al. 1994). This AF-3 region is located in the most N-terminal 164 amino acids specific for PR-B, and enhances the activity of AF-1. The AF-1 function of ER-α was assigned to a 99 amino acid hydrophobic, proline-rich region, which differs from the proline-rich AF-1 region in the PR (Metzger et al. 1995). Two AF-1 subfragments were found to synergize independently with the AF-2 function in the receptor LBD (see below). Almost the entire N-terminal domain was necessary for maximal AR activity (Simental et al. 1991, Jenster et al. 1995). A 260 amino acid core region starting at position 101 contains approximately 50 percent of the maximal activity (Jenster et al. 1995). This core region contains a relatively high number of acidic amino acids. Chamberlain et al. (1996) recently identified two small subfragments within the core region involved in the transactivation function of AF-1.

The DNA binding domain

The DBD mediates the sequence specific interaction of the receptor with DNA (Schwabe et al. 1990, Freedman 1992), and contains sequences involved in dimerization of receptor molecules (Umesono & Evans 1989, Luisi et al. 1991). The DBD contains nine conserved cysteine residues (see for a review Laudet et al. 1992). Originally, the DBDs of steroid hormone receptors were proposed to form two zinc finger motifs, identical to those observed in the Xenopus laevis transcription factor IIIA (TFIIIA) (Miller et al. 1985, Evans & Hollenberg 1988).

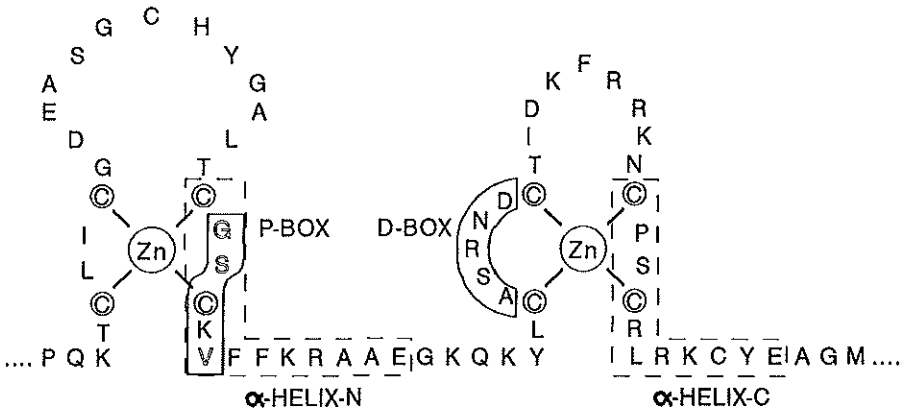

Figure I.5. Functional motifs in the androgen receptor DNA binding domain. The sequence shows the two Cysteine-Zinc coordinations and the N- and C-terminal α-helices (boxed in dashed lines). The P-box and D-box, involved in DNA recognition and dimerization, respectively, are shown as open boxes. The amino acids in the P-box essential in ARE/GRE/PRE versus ERE recognition are in open characters.

However, mutational and NMR/crystallographic analysis of GR and ER DBD demonstrated that the steroid hormone receptor DBD is folded differently, into so-called zinc-domain-helix-extended regions. The two zinc domains contain the zinc-ions, that are each coordinated by four cysteine residues (Figure I.5). The two α-helices that follow the zinc domains (indicated by a dashed box in Figure I.5) are packed perpendicularly to each other, and hydrophobic side-chains form an extensive hydrophobic core between the two helices (Freedman et al 1988, Hard et al. 1990, Schwabe et al. 1990, 1993a and 1993b, Luisi et al. 1991, Freedman 1992).

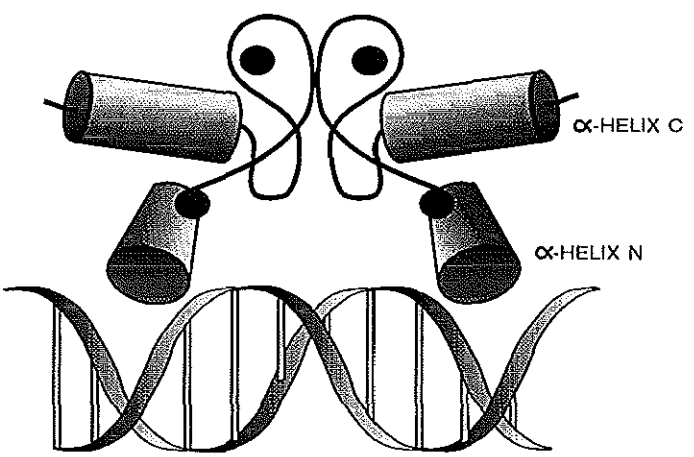

Figure I.6. Model of a steroid hormone receptor DBD dimer, bound to DNA. Black dots are zinc ions, the N- and C-terminal α-helices are represented by cylinders.

Figure I.6 shows a schematical presentation of two steroid receptor DBDs bound to DNA. They bind to adjacent major grooves from one side of the DNA double helix. The molecules make extensive contacts to the phosphate backbone at one side, orienting the DBDs such that the recognition helices enter the major groove. This allows surface side-chains to make sequence-specific contacts to the DNA (Luisi et al. 1991, Schwabe et al. 1990, Suzuki & Yagi 1994).

Steroid hormone receptor homodimers predominantly recognize imperfect palindromic sequences, with a 3 bp spacer between the 6 bp half-sites. The consensus high affinity binding site for steroid receptor homodimers except for ERs is GGT/AACAnnnTGTTCT (GRE/PRE/ARE) (Nordeen et al. 1990, Roche et al. 1992, Lieberman et al. 1993, Lombès et al. 1993); the consensus sequence for ER binding is AGGTCAnnnTGACCT (ERE) (Martinez et al. 1987, Beato et al. 1989). Recent studies on synthetic promoters have indicated that steroid receptors might also interact with direct repeats with different spacings between half-sites (Kato et al. 1995a; Aumais et al. 1996). However, the transactivation efficiency mediated by these direct repeats was considerably less than the activity directed by classical palindromic sequences. In natural promoters, functional activity of a region containing several HRE half-sites has been postulated (Kato et al. 1992, Ho et al. 1993). In these promoters, additional regulatory elements contribute to maximal hormone stimulated activity.

The determinants for HRE specificity have been studied in detail for GR and ER. Mutational analyses and domain swapping of GR and ER have resulted in the identification of the P-box, at the base of the N-terminal zinc coordinating domain, involved in the recognition of the HRE sequence (Green et al. 1988). Three amino acids essential for receptor interaction with its cognate response element were identified (open characters in Figure I.5) (Umesono et al. 1989). Exchange of these three amino acids of the ER P-box (EGxxA) to the corresponding amino acid residues in the GR (GSxxV), changed the specificity, and resulted in high transcriptional activity of a GRE directed reporter gene (Mader et al. 1989). Mutations of three P-box amino acid residues in the GR DBD (GSxxV) to those in the ER (EGxxA), resulted in a mutated protein that bound with high affinity to the ERE, but retained some affinity to a GRE (Zilliacus et al. 1991). The second zinc containing domain encompasses a sequence, named D-box (indicated in Figure I.5), that facilitates cooperative DNA binding of two receptors by protein-protein interactions, and thereby determines half-site spacing (see also Figure I.6) (Umesono et al. 1991, Luisi et al. 1991, Dahlman-Wright et al. 1991).

In contrast to steroid receptors, most other nuclear receptors form predominantly heterodimers, and recognize direct repeats with various spacings between the two half-sites (Green 1993, Mangelsdorf & Evans 1995). RXR is a key factor in this respect, because it is the heterodimerization partner of RAR, TR, VDR, PPAR and some orphan receptors (Gronemeyer & Laudet 1995, Mangelsdorf & Evans 1995). The direct repeat half-sites closely resemble the consensus half site for ER binding (TGACCT), and are in general separated by a 1 bp spacer in case of RXR-PPAR dimers, 3 bp for RXR-VDR, 4 bp for RXR-TR, and 1, 2 or 5 bp in case of RXR-RAR heterodimers. Crystallographic and extensive mutational analyses revealed the formation of distinct, asymmetric DBD interfaces in the individual heterodimers, discriminating between direct repeats with different half-site

spacing (Zechel et al. 1994, Rastinejad et al. 1995). So, steric hindrance between receptor pairs is involved in spacer recognition, it also determines the polarity of the receptor pair on the response element.

In steroid receptors the DBD is linked to the LBD by the so-called hinge region, which contains the bipartite nuclear localization signal (NLS) necessary for translocation to the nucleus.

The ligand binding domain

The C-terminal region of steroid receptors of approximately 250 amino acids contains the ligand binding function (Kumar et al. 1986, Giguère et al. 1986, Rusconi & Yamamoto 1987, Gronemeyer et al. 1987, Jenster et al. 1991). The integrity of the complete domain is important for steroid binding, because all deletions and the majority of point mutations in this region abolish ligand binding. LBD sequences also play a role in receptor dimerization (Fawell et al. 1990, Nemoto et al. 1994). Furthermore, most steroid receptors (ER, PR and GR) have been shown to contain transactivating activity in this region (Hollenberg & Evans 1988, Bocquel et al. 1989, Gronemeyer 1991, Danielian et al. 1992, Tzuckerman et al. 1994). This transactivating function (AF-2) depends on hormone binding for activity. The AF-2 domains of the various receptors contain at their C-terminus an autonomous, constitutively active, but weak activation function, the AF-2 core region (see Figure I.3) (Danielian et al. 1992, Meyer et al. 1992, Tzuckerman et al. 1994). The integrity of the core domain is crucial for AF-2 function. To date, no apparent AF-2 activity has been identified in the AR LBD, despite the high degree of homology between AR and the other steroid hormone receptors in the AF-2 core region (Pierrat et al. 1994).

In contrast to other nuclear receptors, unliganded steroid receptor LBDs are associated with a large multiprotein complex composed of heat shock proteins (hsp90, hsp70 and hsp56), that maintains the receptor in a transcriptionally inactive form (Smith & Toft 1993, Pratt 1993, Bohen et al. 1995). Heat shock proteins are thought to be involved in proper folding of the LBD (Pratt 1993, Bohen et al. 1995, Fang et al. 1996). It has also been suggested that hsp-receptor association is implicated in intracellular trafficking and nuclear import (Smith & Toft 1993, Prat 1993, Pratt et al. 1993).

The subcellular localization of the individual steroid hormone receptors is still a matter of dispute. Experimental evidence supports a model of constant bi-directional shuttling of the receptor/chaperone complex between the nucleus and the cytoplasm (Guiochon-Mantel et al. 1991, Chandran & DeFranco 1992, Dauvouis et al. 1993, Madan & DeFranco 1993). Probably, the unliganded ER and PR are predominantly nuclear (Yilkomi et al. 1992), and the unliganded GR and MR cytoplasmic (Guiochon-Mantel et al. 1991, Madan & DeFranco 1993). Immunohistochemical studies of major androgen target tissues demonstrated that the AR was predominantly present in the nucleus in either the absence or presence of hormone (Husman et al. 1990, Sar et al. 1990, van der Kwast et al. 1991). In transiently transfected cell lines, unliganded AR has been detected both in the nucleus and in the cytoplasm, depending on the cell line tested (Jenster et al. 1993, Zhou et al. 1993).

Hormone binding results in the dissociation of receptor associated chaperone proteins (Smith & Toft 1993), and initiates conformational changes necessary for dimerization, DNA binding and interaction with transcriptional mediators (Carson-Jurica et al 1990, Gronemeyer 1992, Truss & Beato 1993, Beekman et al. 1993).

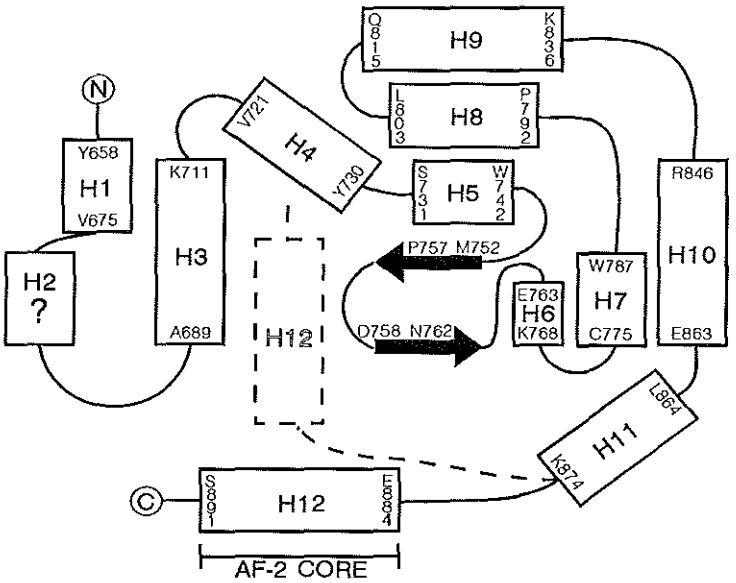

Figure I.7. Predicted model of the androgen receptor ligand binding domain. The model is adapted from Renaud et al. 1995, and represents an antiparallel α-helical sandwich, containing 12 α-helices (H1 to H12) and 2 short β-strands (S1 and S2). The α-helices H1 to H11 are organized in a three layer structure, in which H4, H5, H6, H8 and H9 are sandwiched between H1 to H3 and H7, H10 and H11. In the unliganded state, H12 protrudes from the LBD, whereas H12 folds towards the LBD in the liganded state (shown by the dashed box) (Renaud et al. 1995, Wurtz et al. 1996). In the model, the postulated positions of α-helices 1 and 3 to 12, and β-strands 1 and 2 of the AR LBD are indicated; helix 2 might be absent in AR.

Recently, the three dimensional structures of RXRα (Bourguet et al. 1995), RARγ (Renaud et al. 1995) and TRα (Wagner et al. 1995) LBDs have been elucidated. They were shown to fold in an anti-parallel α-helical sandwich, containing 11 to 12 α-helices and 2 short β-strands. The overall homology of the various LBDs might be sufficient to predict the folding of the steroid receptor LBDs (see Figure I.7 for AR; Wurtz et al. 1996). Homology modeling of the unliganded RXRα and GR LBDs according to the structure of the ligand bound RARγ LBD predicted a common ligand-binding pocket, involving mainly hydrophobic residues. Mutant studies support this common three dimensional structure. Alignment of all nuclear receptors to this model has resulted in the proposal of a common, so-called 'mouse trap-like' mechanism of ligand binding (Wurtz et al. 1996). By folding back to the LBD core, α-helix 12, containing the AF-2 core sequence, comes in close contact with the ligand and α-helix 4, and seals the ligand binding pocket like a lid (see Figure I.7). The conformational change induced by hormone binding creates an interaction surface which presumably allows binding of transcriptional co-activators, or interaction (direct or indirect) with the N-terminal domain, exposure of the nuclear localization signal, and allows receptor dimerization (Picard & Yamamoto 1987, Picard et al. 1988, Yiikomi et al. 1992, Wong et al. 1993, Langley et al. 1995, Kraus et al. 1995, Beato et al. 1995,1996b, Wurtz et al. 1996, Doesburg et al. 1997).

Previously, so-called heptad repeat residues were proposed to trigger dimerization of ligand bound LBD (Lees et al. 1990, Forman & Samuels 1990). However, the crystal structure of the ligand bound RARγ LBD dimer showed that these heptad repeat residues contribute to the overall stability of the LBD fold and their mutation can only indirectly affect the dimer interface (Bourguet et al. 1995). In RXRα, the dimerization interface is supposed to be formed by helix 10 (Bourguet et al. 1995, Wurtz et al. 1996).

Antagonists inhibit the biological effects of steroid hormones by competing for binding to the receptor (Wakeling 1992). They are frequently applied in the treatment of hormone dependent disorders, including hormone dependent malignancies. Most studied in this respect are the anti-estrogen Tamoxifen, the anti-progestin RU486, and the anti-androgen cyproterone acetate. In general, steroidal antagonists are thought to induce a different conformational change upon binding to the LBD. Several studies have indeed shown that this indeed the case (Moudgil et al. 1989, Weigel et al. 1992, Allan et al. 1992a and 1992b, Beekman et al. 1993, Kallio et al. 1994, Kuil & Mulder 1994, Kuil et al. 1995). The conformational state induced by antagonists results in a receptor without, or with reduced transcriptional activity. Different antagonists may affect different aspects of receptor function, such as hsp dissociation, dimerization, binding to DNA, and interaction with general or specific transcription factors and co-activators, and with the N-terminal domain of the receptor (Meyer et al. 1990, Baulieu et al. 1990, Gronemeyer et al. 1992, Agarwal 1994, Metzger et al. 1995b, Langley et al. 1995, Kraus et al. 1995, Doesburg et al. 1997).

Steroid receptor specificity

Despite the shared consensus DNA-binding site, AR, GR, MR and PR mediate diverse cellular responses. Several, mutually not exclusive mechanisms to achieve specificity have been proposed (Katzenellenbogen et al. 1996, Beato et al. 1996b). One of the molecular mechanisms, that can account for specificity includes sequence differences in natural HRE sequences, which can deviate considerably from the consensus, high affinity binding sequence (Claessens et al. 1996). Other mechanisms can include differences in the DNA context of the HRE (Bear & Yamamoto 1994, Scheller et al. 1996), differential affinities to general or sequence specific transcription factors (Adler et al. 1992 and 1993, Ho et al. 1993, Pearse & Yamamoto 1993), different capacities to modulate chromatin structures (Archer et al. 1994a,b and 1995, Mymrik et al. 1995, Truss et al. 1995, Beato et al. 1996a), and ligand availability (Funder et al. 1993, Russell & Wilson 1994, Kralli et al. 1995).

I.1.3. INTERACTION WITH GENERAL AND SEQUENCE SPECIFIC TRANSCRIPTION FACTORS, AND CO-ACTIVATORS

Hormonal activation and nuclear import are prerequisites for DNA bound steroid receptors before performing their transcription regulation function. By binding to the HRE in promoter or enhancer regions of target genes, steroid hormone receptors initiate the assembly, or increase the stability of the transcription initiation complex, resulting in modulation of target gene expression. The transcription initiation complex is composed of RNA polymerase II and basal or general transcription factors (GTFs). GTFs interact with the core promoter elements, and are sufficient to direct RNA polymerase specificity and low levels of transcription.

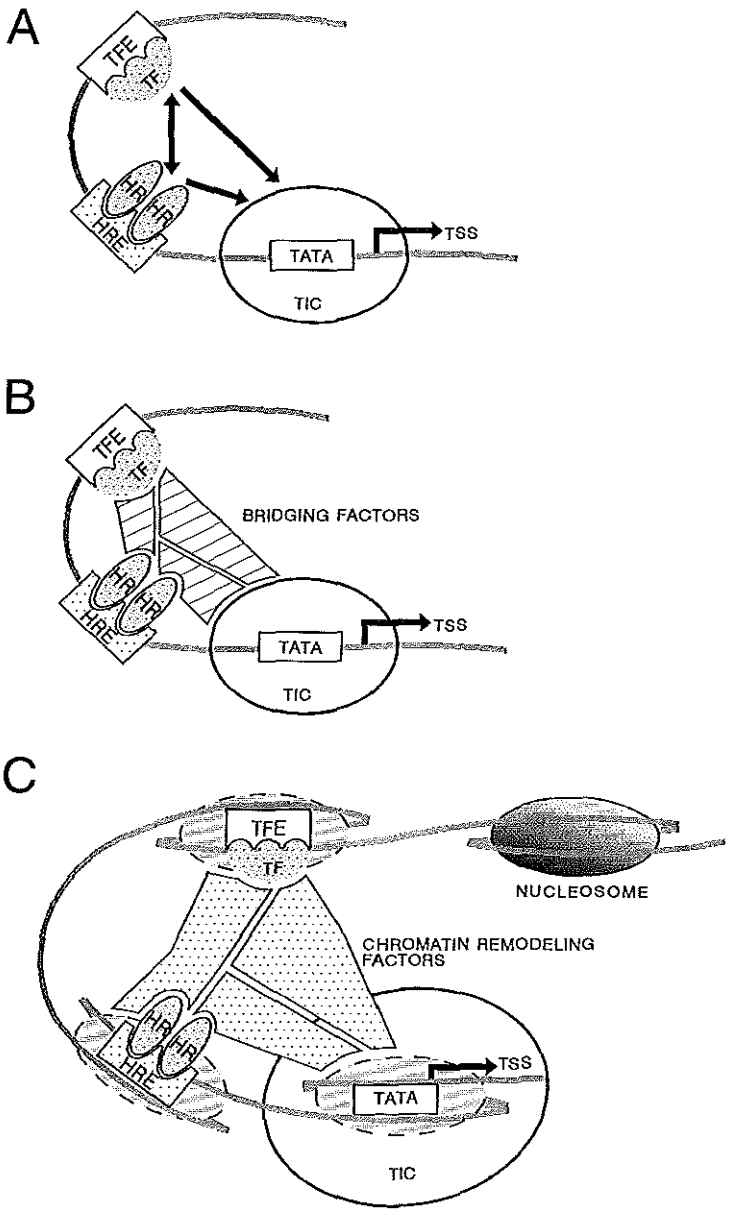

Figure I.8. Schematic representation of interactions between steroid hormone receptors and other nuclear proteins involved in transcriptional activation. These interactions can be either direct (A) or indirect via bridging factors (B) or chromatin remodeling factors (C). HR: Steroid hormone receptor, TF: transcription factor, HRE: hormone response element, TFE: TF binding element, TIC: transcription initiation complex, TSS: transcription start site.

Figure I.8A schematically represents the simplified building of the activated transcription initiation complex. In this model, direct interactions are proposed between a steroid receptor homodimer and the transcription initiation complex. Additionally, direct interactions are proposed between the steroid receptor dimer and a second specific transcription factor bound to its cognate DNA element, resulting in synergistic cooperativity. However, accumulating evidence indicates that interactions cannot only be direct, but also indirect, via transcription intermediary factors (TIFs) or co-activators. These co-activators can function as bridging factors between the various components of the transcription machinery (see Figure I.8B as an example). Furthermore, specific transcription factors and co-activators can play a role in chromatin remodeling, thereby enhancing the activity of the transcription initiation complex (Figure I.8C). The study of protein-protein interactions in the formation of specific, stable transcription initiation complexes is a rapidly developing research field, and currently investigated in many different laboratories.

Interaction with general transcription factors

RNA polymerase II is the central player in transcription initiation. It interacts with several GTFs, in particular through the C-terminal repeat domain (CTD) of its largest subunit. Two different mechanisms for the assembly of the transcription initiation complex have been proposed. For many years it was believed, that the initiation complex is assembled in an ordered step-wise fashion (reviewed by Buratowski 1994, Zawel & Reinberg 1995, Orphanides et al. 1996). Starting with recognition of the TATA-box by TFIID, followed by recognition of this complex by TFIIB, recruitment of the TFIIF/RNA polymerase II complex, and finally binding of TFIIE and TFIIH. TFIIA can bind to the initiation complex at any stage after TFIID binding, and stabilizes the complex. More recently, the concept of a preformed complex, containing RNA polymerase II and many GTFs has been postulated (Koleske & Young 1994 and 1995, Halle & Meisterernst 1996, Orphanides et al. 1996). Such a holoenzyme could be directly recruited to the promoter by sequence specific transcription factors.

All GTFs are composed of two or more subunits, and cDNAs encoding most subunits have been molecularly cloned (reviewed by Burley & Roeder 1996, Orphanides et al. 1996). The TFIID complex is composed of the TATA-box binding component TBP and a variety of other subunits, named TBP-associated factors (TAFs). The TFIID complex seems to be the central part in the communication between RNA polymerase II and the sequence specific transcription factors, such as steroid receptors, either direct or via co-activators (see Figure I.8).

Steroid receptors are found to interact with several GTFs. Transactivation by ER is enhanced by overexpression of TBP, and direct *in vitro* interaction between the two proteins has been demonstrated (Sadovsky et al. 1995). Also, a direct interaction between steroid hormone receptors and several TAF_{II}s has been detected. Transactivation by ER requires $TAF_{II}30$ (Jacq et al. 1994), and *Drosophila* $TAF_{II}110$ interacts with the DBD of the PR (Schwerk et al. 1995). Furthermore, interactions of ER and PR and TFIIB have been reported (Ing et al. 1992). The *in vivo* significance of these *in vitro* observed interactions, and effects on transcriptional activation remain to be established.

Interaction with sequence-specific transcription factors

Steroid hormone receptors not only interact, directly or indirectly, with GTFs but also with sequence-specific transcription factors. On artificial promoters, containing properly spaced binding sites, many sequence-specific transcription factors cooperate synergistically with steroid hormone receptors (Schüle et al. 1988, Strähle et al. 1988). In several natural steroid responsive genes, synergism between two or more HREs has also been described. One of the first examples of synergistic activity of HREs was found in the promoter of the chicken vitellogenin II gene. Synergistical activity of both ER and GR, and ER and PR has been demonstrated (Ankebauer et al. 1988, Cato et al. 1988a). Also, cooperativity between two EREs has been described (Burch et al. 1988). Although the mechanism of this synergistical activation is not clear, it has been suggested that it is based upon cooperative binding of receptors to multiple HRE sequences (Ankebauer et al. 1988, Tsai et al. 1989).

The best studied natural promoters showing synergistical effects of steroid hormone receptors and other sequence specific transcription factors are the MMTV-LTR (mouse mammary tumor virus long terminal repeat) promoter, the TAT (tyrosine aminotransferase) promoter and the ovalbumin promoter. The MMTV promoter contains two separated regions interacting with GR, PR and AR (Scheidereit et al. 1983, von der Ahe et al. 1985, Cato et al. 1988b), a distal region, with high homology to the HRE consensus sequence, and a proximal region that consists of a cluster of three perfect HRE half-sites. The individual half-sites do not show receptor binding. The notion that different receptor binding sequences act synergistically, is based upon the observation that mutation of any of the HRE motifs strongly reduced hormone inducibility (Cato et al. 1988b). The MMTV promoter contains also NF-1 and Oct binding sites, and both are required for optimal induction by steroid hormone receptors. However, in *in vitro* transcription assays, on naked DNA templates, the steroid receptors do not synergize, but rather compete with NF-1 for binding to the promoter (Kalff et al. 1990, Brüggemeier et al. 1990, 1991). Functional studies indicated that the observed synergism between steroid receptors and NF-1 might be mediated by the organization of the DNA in chromatin (Brüggemeier et al 1991, Beato et al. 1996a). Hormone induction is believed to cause a displacement or disruption of the nucleosome positioned over the hormone responsive region, which enables free access of NF-1 to its binding site, resulting in optimal transcriptional activity (reviewed by Beato & Sánchez-Pacheco 1996).

Evidence about the role of the chromatin structure in the liver-specific and glucocorticoid regulated activity of the rat TAT gene has also been collected. In the TAT enhancer region, 2.5 Kb upstream of the transcription start-site, three candidate GRE sequences have been identified (Jantzen et al. 1987, Grange et al. 1989). Functional synergism between the GR and the liver enriched transcription factors HNF-3 (previously named HNF-5) and HNF-4 has been described (Rigaud et al. 1991, Nitsch et al. 1993, Roux et al. 1995). Results obtained by *in vivo* footprinting suggest that the GR may function, at least in part, by allowing access of HNF-3 to its DNA binding site through an alteration of the chromatin structure. In addition to the synergistic action of proteins binding to the -2.5 Kb enhancer region itself, this enhancer region also acts synergistically with a region 5.4 Kb upstream of the transcription start site. The latter region is almost inactive in itself, but it cooperatively enhances the glucocorticoid induced activity of the -2.5 Kb enhancer region (Grange et al. 1989).

Synergistical cooperativity between steroid hormone receptors and another sequence specific transcription factor was also observed in the ovalbumin gene. Synthesis of ovalbumin mRNA in chicken oviducts is controlled by estrogens and progestins, primarily at the level of transcription (O'Malley et al. 1979). Both a functional ER and PR binding site are found in the proximal promoter region, and in a far upstream enhancer several synergistically acting ERE half-sites have been identified (Compton et al. 1986, Tora et al. 1988, Kato et al. 1992). Besides the steroid hormone receptors, a chicken ovalbumin upstream promoter (COUP) element was found to be essential for efficient transcription of the gene (Sagami et al. 1986). The transcription factor binding to this element, COUP-TF is a well known orphan receptor (Wang et al. 1989).

Besides for the extensively studied promoters discussed above, synergistical effects of sequence specific transcription factors and steroid hormone receptors were also suggested for several other natural occurring promoters. A subset of these will be discussed in section I.2.4.

Interaction with co-activators

Transcriptional interference or squelching between steroid hormone receptors has suggested the existence of common co-activators or TIFs (Meyer et al. 1989, Bastian & Nordeen 1991). By protein-protein interaction-screening assays [the yeast two hybrid system (Chien et al. 1991), or others] several proteins interacting with steroid hormone receptors have been identified (see Table I.1 for an overview of candidate co-activators). At the moment the description co-activator is loosely defined by the various authors (Horwitz et al. 1996, and references therein). In this section steroid hormone receptor co-activators are defined as proteins that interact with DNA bound steroid hormone receptors, and positively influence receptor mediated transcriptional activity either by bridging between the steroid receptor and the basal transcription machinery, by chromatin remodeling, or by both. For most candidate co-activators, an interacting partner of the basal transcription machinery is yet unknown. The *in vivo* relevance of many of the co-activators discovered in mammalian and yeast protein-protein interaction systems needs to be further determined. Most candidate co-activators are identified by their ability to interact with the ligand bound receptor LBD. Some of the best studied examples will be briefly discussed below.

SUG1/TRIP1, a component of the RNA polymerase II holoenzyme, was shown to interact with the AF-2 domain of the ER LBD (vomBaur et al. 1996). Furthermore, SUG1 was demonstrated to interact with TBP (Swaffield et al. 1995) and $TAF_{II}30$ (vomBaur et al. 1996). It was postulated that steroid hormone receptors can use SUG1/TRIP1 to recruit the complete basal transcription machinery in one single step (Beato et al. 1996b). Recent data indicate that SUG1 is a subunit of the 26S proteosome complex. It therefore may affect transcriptional activation by steroid receptors indirectly, by controlling the turnover rates of other regulatory factors (Rubin et al. 1996).

TIF2/GRIP1 functionally interacts with the LBD of ER, GR, PR and AR in a ligand-dependent manner (Voegel et al. 1996, Hong et al. 1996). TIF2/GRIP1 is thought to bridge the AF-2 core region and one of the GTFs. TIF2/GRIP1 is expressed in many tissues, and enhances transcriptional activity of AF-2 of the various receptors in mammalian cells and in yeast.

SRC-1 was isolated as a protein interacting with ligand bound PR LBD (Oñate et al. 1995). SRC-1 is ubiquitously expressed and enhances transcription activation by PR, GR and ER. Recently, SRC-1 and ERAP160/p160 (Halachmi et al. 1994) were shown to be

structurally closely related (Kamei et al. 1996). McInerney et al. (1996) suggested that SRC-1 might act as an adaptor protein that facilitates the productive association of N-terminal domain and the LBD of the ER.

ARA_{70} was isolated as a protein binding to AR LBD (Yeh & Chang 1996). The protein exclusively interacts with the agonist bound and not with the antagonist bound AR. In cotransfection experiments in ARA70 negative DU145 prostate cells, ARA_{70} enhanced AR-dependent transcription 10-fold, but had only a minimal effect on ER, GR and PR.

TABLE I.1. Candidate transcriptional intermediary factors or co-activators for steroid hormone receptors

CO-ACTIVATOR	STEROID RECEPTOR		TARGET	REFERENCES
	partner	domain		
SUG1/TRIP1	ER	AF-2	TBP/TAF_{II}30	Swaffield et al. 1995 vomBauer et al. 1996
ARA 70	AR	LBD	?	Yeh et al. 1996
GRIP1/TIF2	ER/GR/PR/AR	AF-2/LBD	?	Voegel et al. 1996 Hong et al. 1996
SRC-1/p160/ERAP 160	ER/GR/PR	LBD	CBP/p300	Oñate et al. 1995 Halachmi et al. 1994 Kamei et al. 1996
RIP 140	ER/GR	AF-2	?	Cavaillès et al. 1994,1995
RAP 46	ER/GR/PR	LBD	?	Zeiner et al. 1995
GRIP 170	GR	DBD	?	Eggert et al. 1995
hRPF 1	GR/PR	LBD	TAF_{II}18 ?	Huibregtse et al. 1995 Imhof et al. 1996
PML	PR	AF-1/2	?	Guiochon-Mantel et al. 1995
TIF1 (TIF1α)	ER/GR	AF-2	chromatin	Le Douarin et al. 1995,1996 vomBauer et al. 1996
BRG1/hBRM	ER/GR	?	chromatin	Muchardt et al. 1993 Chiba et al. 1994
HMG-1	PR/ER	?	chromatin ?	Oñate et al. 1994 Verrier et al. 1995
CBP/p300	GR	LBD	nucleosomal core histones	Kamei et al. 1996 Yao et al. 1996

Candidate co-activators described so far were shown or presumed to interact with the basal transcription machinery. Co-activators can also interact with components of the chromatin, resulting in nucleosome remodeling. TIF1 interacts with the AF-2 domain of ligand bound ER and GR *in vitro* and in yeast (Le Douarin et al., 1995, vomBauer et al. 1996). Recently, it was shown that TIF1 also interacts with two mouse heterochromatin proteins, mHP1α and mMOD1, likely involved in chromatin structure and function (LeDouarin et al. 1996). A yeast two hybrid screen to identify proteins interacting with HP1α, identified a protein closely related to TIF1. This newly identified protein was named TIF1β (TIF is now TIF1α). So far it is not known whether TIF1β interacts with steroid hormone receptors.

Hbrm and BRG1, two human homologs of the yeast proteins SWI2/SNF2, are believed to play a role in the remodeling of nucleosomes by the displacement of histone H2A/H2B dimers. This function might involve the proposed helicase activity of hbrm/BRG1 (Winston et al. 1992, Côte et al. 1994, Beato & Sánchez-Pacheco 1996). It has been shown that hbrm and BRG1 can cooperate with the ER and GR (Muchardt et al. 1993, Chiba et al. 1994).

CBP and the related p300 protein were shown to interact with the LBD of the GR, and with SRC-1/ERAP160/p160 co-activators (Ogryzko et al. 1996). CBP and p300 were found to be histone acetyltransferases of all four core histones in nucleosomes. Acetylation of histone tails presumably destabilizes the nucleosome and thereby facilitates access by regulatory proteins. A direct link between histone acetylation and transcriptional activation was recently shown for yeast GCN5 and its human homolog (Wang et al. 1997). Because CBP and p300 can interact with a wide array of different transcription factors, they were postulated to be involved in the cross-talk between multiple signal transduction pathways (Kamei et al. 1996, Yao et al. 1996).

The DNA bending protein HMG-1 has been reported to stimulate the PR and ER DNA binding (Oñate et al. 1994, Verrier et al. 1995). Co-immunoprecipitation experiments showed that both HMG-1 and PR can be found in a complex with the PRE although it appeared that HMG-1 enhanced PR binding without stably participating as a component of the final PRE/PR complex (Prendergast et al. 1994). The physiological relevance of enhanced steroid receptor/HRE binding by HMG-1 needs to be determined.

Besides co-activators, also co-repressors have been described. For the non-steroid nuclear receptors TR, RAR and RXR, two candidate co-repressor proteins have been identified, a 270 kDa protein N-CoR (Hörlein et al. 1995, Kurokawa et al. 1995) and the related 168 kDa protein SMRT (Chen & Evans 1995). Members of the TR/RAR/RXR family of nuclear receptors can bind to DNA in the absence of ligand and actively repress transcription. N-CoR and SMRT are thought to exert their function by their interaction with DNA bound, unliganded TR/RXR and RAR/RXR heterodimers. Upon ligand binding, the co-repressor dissociates and thereby allows interaction with co-activators, and subsequent transcriptional activation by the ligand bound receptor dimer. So far nuclear co-repressors for steroid hormone receptors have not been reported. It might be argued that co-repressors for steroid receptors do not exist, because the unliganded steroid receptor is unable to bind to DNA.

I.1.4. CROSS-TALK OF STEROID HORMONE RECEPTORS

Alternative pathways of receptor activation

For many years, ligand binding was assumed to be a prerequisite for activation of steroid hormone receptors. With the observation that modulation of kinase activity can cause activation of steroid hormone receptors in the absence of hormone (reviewed by Weigel 1996), it became apparent that there are alternative, steroid-independent, pathways.

In addition to the nuclear receptor signal transduction pathway, there are three general mechanisms of signal transduction by extracellular components that involve protein phosphorylation (reviewed by Hill & Treisman 1995, Karin & Hunter 1995). Figure I.9 represents a schematical and simplified overview of these general mechanisms.

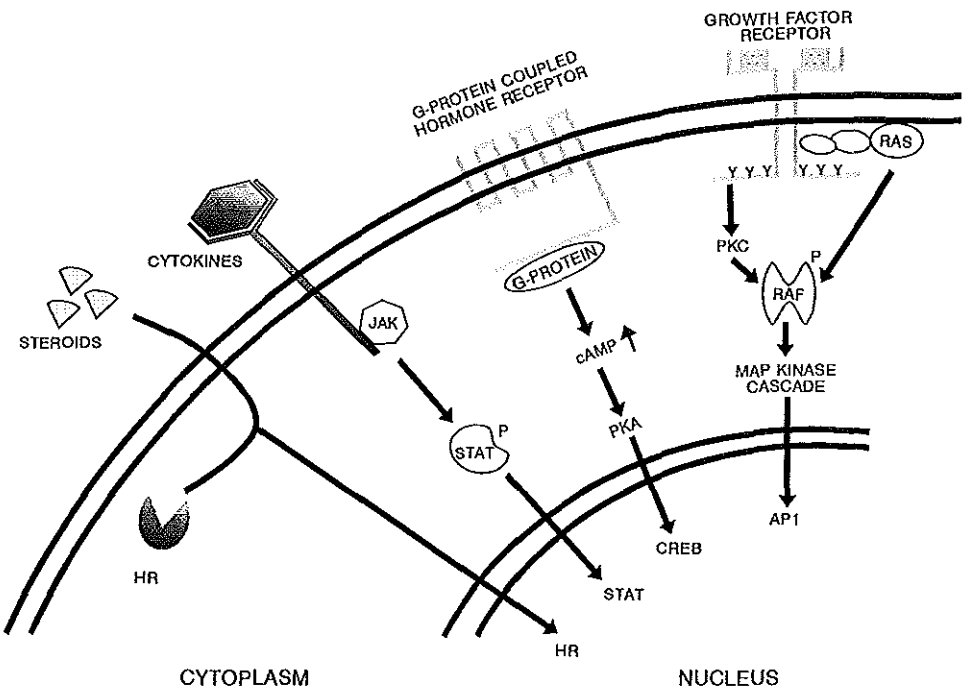

Figure I.9. General mechanisms of signal transduction. HR: steroid hormone receptors. Y indicates tyrosine residues.

One pathway involves the mitogen-activated protein (MAP) kinase cascade. Growth factor or peptide hormone binding results in transmembrane receptor dimerization, that triggers auto-phosphorylation of tyrosine residues, followed by activation of intrinsic protein tyrosine kinase activity. This in turn leads to the activation of the MAP kinase family of serine/threonine protein kinases. This signal transduction pathway also involves the PKC family of protein kinases. Ultimately the MAP kinase pathway leads to activation of AP1

family of transcription factors in the nucleus. A second signal transduction pathway involves the intracellular Janus protein tyrosine kinases (JAKs). Upon polypeptide (for example cytokines and interferon) binding to a specific transmembrane receptor, JAKs phosphorylate members of the Stat family of transcription factors. Following dimerization, phosphorylated Stat protein is able to bind DNA at the Stat-response element and regulate expression of target genes. The third mechanism is formed by the G protein coupled "seven transmembrane" receptors. In this pathway, binding of the ligand, for example dopamine, ultimately results in rise of the cAMP concentration in the cell. cAMP then activates the cAMP dependent protein kinase (PKA), which translocates to the nucleus, where it can phosphorylate its specific target proteins, including the CREB transcription factor.

Ligand independent activation of steroid hormone receptors was initially discovered for the chicken PR, that can be transcriptionally activated by both a protein kinase A activator and a phosphatase 1 and 2A inhibitor (Denner et al. 1990). Subsequent studies showed membrane receptor mediated signal transduction pathway coupled activation of PR by dopamine (Power et al. 1991). At present, a considerable amount of evidence for ligand-independent activation of mammalian PR in transiently transfected mammalian cells and in animal models is available (reviewed by Power et al. 1992, and O'Malley et al. 1995).

Steroid-independent receptor activation in many cases involves growth factor/receptor tyrosine kinase pathways. Recently, it has been shown that a serine residue (Ser 118), essential for full transcriptional activation of the ER, can be phosphorylated by mitogen-activated protein (MAP) kinase *in vitro* (Kato et al. 1995b) and by EGF and IGF-1 activation, and activated K-ras *in vivo* (Kato et al. 1995b, Bunone et al. 1996). Curtis et al. (1996) demonstrated that ER-α knockout mice lack the estrogen-like response mediated by EGF in ER positive mice, clearly indicating the coupling of EGF and ER signaling pathways *in vivo*. At present, a wide array of growth factors, mitogens and other compounds affecting phosphorylation by PKA or PKC activation have been shown to be able to activate the unliganded steroid hormone receptors or enhance steroid-dependent transcriptional activity (reviewed by Weigel 1996).

The outcome of the effect of growth factors and mitogens on steroid-independent receptor activation can be influenced remarkably by the cell type, and culture condition used (Nordeen et al. 1995, Reinikainen et al. 1996, Nazareth & Weigel 1996). Similarly, the effect of ligand independent activation of steroid receptors is promoter dependent. Therefore, it is important to study the mechanism of ligand independent activation in more detail, and establish its *in vivo* relevance. Steroid-independent receptor activation might contribute to the failure of endocrine therapy in several human tumors.

Cross-talk of steroid hormone receptors with other specific transcription factors

The cross-talk between steroid hormone receptors and other signal transduction pathways not only leads to alternative activation of the steroid hormone receptor, but the cross-talk can also work in the opposite direction. Ligand bound GR can act as a transcriptional activator of Stat5 and enhances Stat5-dependent transcription (Stöcklin et al. 1996). Phosphorylated Stat5 and the liganded GR are able to form a complex, and cooperate in transcriptional activity of a Stat5 inducible promoter. DNA binding by the GR is not necessary for the transcriptional activity of the Stat5/GR complex since a GR mutant, lacking DNA binding,

is still functional. It is suggested that the strong transactivation domain of the GR enhances Stat5 action. As a result of Stat5/GR dimerization, glucocorticoid induction of a GR target gene is strongly repressed.

Several groups have reported interaction between steroid hormone receptors and AP-1 components (fos and jun) (reviewed by Herrlich & Ponta 1994). Depending on the promoter, the cell line and the specific receptor, both negative and positive regulatory interactions have been described (Shemshedini et al. 1991). The mechanism by which this cross-talk acts is still elusive. Evidence leading early on to the idea that AP-1 and steroid hormone receptors form complexes which abort DNA binding, might represent an *in vitro* artefact. A recently postulated alternative hypothesis is that AP-1 and steroid hormone receptors do not interact directly, but via transcriptional mediators (Pfahl 1993, Beato et al. 1995, Gronemeyer & Laudet 1995, Kallio et al. 1995). This hypothesis is supported by the recent discovery that activation of nuclear receptors requirs CBP or p300 (Kamei et al. 1996, Yao et al. 1996). As discussed above, CBP (Kwok et al. 1994) and p300 (Eckner et al. 1994) directly interact with the LBD of several steroid hormone receptors. Previous studies already revealed that CBP/p300 is required for AP-1 activation (Arias et al. 1994, Arany et al. 1995, Lundblad et al. 1995). Combination of these results led to the proposition that inhibition of AP-1 by nuclear receptors and vice versa might be the result of competition for limiting amounts of CBP/p300 present in target cells (Kamei et al. 1996).

Negative cross-talk has also been observed between NF-κB and members of the steroid hormone receptor family (Stein & Yang 1995, Marx 1995). Best studied in this respect, is the mediation of immunosuppression by glucocorticoids. RelA, the p65 subunit of the composite transcription factor NF-κB, is not only inhibited by GR in stimulating NF$_\kappa$B-responsive genes but also antagonizes GR action on GRE-containing promoters (Ray & Prefontaine 1994, Caldenhoven et al. 1995, Scheinman et al, 1995a). Additional research showed that direct binding of the GR to NF-κB perhaps comprises only part of the molecular mechanism of glucocorticoid suppressed immunity. Expression of IκBα, the NF-κB inhibitory protein that keeps NF-κB trapped in the cytoplasm, is increased by glucocorticoids. As a result, NF-κB might be retained in its inactive form in the cytoplasm, even under conditions where it would normally be released to migrate to the nucleus, and act as transcriptional regulator (Auphan et al. 1995, Scheinman et al. 1995b).

Cross-talk has also been observed between AR and GR, and calreticulin. Calreticulin, a ubiquitously expressed intracellular Ca^{2+}-binding protein has been shown to bind to integrins as well as to nuclear receptors. Calreticulin binds to a six amino acid motif, KxFFK/RR, found in the DBD of all nuclear receptors (Burns et al. 1994, Dedhar et al. 1994). These amino acids are crucial for DNA binding, and calreticulin is believed to block the ability of steroid receptors to bind to their response elements, and thereby inhibit transcriptional activity. Calreticulin may represent a link between cellular phenotype, via the extracellular matrix, and specific gene expression (Dedhar 1994).

I.2. ANDROGEN REGULATED AND PROSTATE SPECIFIC GENE EXPRESSION

The male sex hormones or androgens [testosterone (T) and its metabolite 5α-dihydrotestosterone (DHT)] play a central role in development of the male phenotype during fetal life and puberty, and control male fertility and sexual behavior in adults. T is produced by the Leydig cells in the testis. In target tissues, T can be converted into the more active DHT by the enzymes 5α-reductase 1 and 2 (Andersson et al. 1989, 1991). 5α-Reductase 1 is predominant in nongenital skin, whereas 5α-reductase 2 is mainly expressed in the male urogenital tract (Thigpen et al. 1993). Both T and DHT exert their biological functions by activation of the AR. High AR expression is mainly restricted to the male urogenital system, including the prostate.

I.2.1. ANDROGEN REGULATED PROSTATE DEVELOPMENT

The presence of a functional AR and the appropriate androgenic hormone are essential for the development and maintenance of the prostate (Cunha et al. 1987, Cunha 1994). The prostate is an exocrine male reproductive organ, that is only present in mammals. The prostate gland is composed of ductal-acinar structures with a complex architecture. The acini and ducts are lined by a double layer of epithelial cells, the basal and luminal cells. Along the secretory epithelial cells, a third epithelial cell type, the neuroendocrine cells, which form a small proportion of the epithelial compartment, can be found. A role in prostate growth and differentiation, and in homeostasis has been proposed (reviewed by Noordzij et al. 1995). The acini and ducts are surrounded by stromal tissue, that is mainly composed of smooth muscle cells, connective tissue and lymphatic cells. The function of the basal ductal cells is essentially unknown, although it has been suggested that prostate stem cells are located within this cell layer (Bonkhoff & Remberger, 1996). The luminal epithelial cells are regarded as highly differentiated, and produce many components of the seminal fluid. The best known of these is prostate specific antigen (PSA), but large amounts of prostate acid phosphatase (PAP) and Prostate-Specific Protein (PSP_{94}) are also secreted.

The prostate is situated immediately below the bladder and surrounds the urethra. Prostate morphology is different in various species. In the human prostate, regions with different anatomy are fused together to form a compact organ with a chestnut-like shape. In contrast, the prostate of rodents is organized in dorsal, lateral, ventral and anterior lobes, each of which has a distinct function, in keeping with its unique branching pattern and the production of specific proteins (Cunha et al. 1987).

In humans, prostate development initiates around the 10th week of gestation from solid epithelial outgrowths (prostatic buds) of the urogenital sinus. Embryonic prostate organogenesis is strictly androgen dependent. During the initial steps of prostate development the AR is expressed in the mesenchymal compartment, but undetectable in the epithelial cells. However, it has been well established that epithelial cell differentiation is regulated by androgens (Cunha et al. 1987, Cunha 1994). It is assumed that androgen regulated growth factors and extracellular matrix components produced by the mesenchyme drive the initial androgen regulated differentiation of the prostate epithelium (see Figure I.10). Keratinocyte growth factor (KGF), an androgen regulated member of the fibroblast growth factor family

that is expressed in stromal cells, is suggested to be involved in androgen regulated prostate development (Yan et al. 1992, Sugimura et al. 1993, Rubin et al. 1995, Cunha et al. 1996).

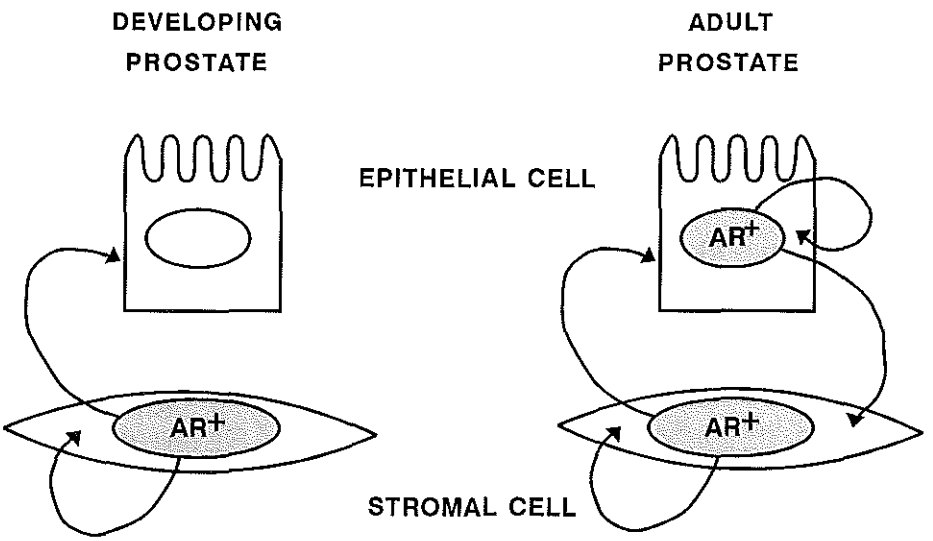

Figure I.10. Schematic representation of androgen driven interactions between epithelial and mesenchymal cells during prostate development and between epithelial cells and stromal cells in the mature prostate. AR+ indicates the presence of functional androgen receptors.

During puberty a second, androgen-dependent, step in prostate growth and development takes place, ultimately resulting in the formation of the adult prostate and the production of prostate specific proteins (Cooke et al. 1991, Donjacour & Cunha 1993). In these later stages of development, AR expressed in the epithelial cells is also an essential factor. Androgens are indispensable for the maintenance of prostate structure and function, because orchiectomy or inhibition of AR activity by anti-androgens leads to prostate involution, concomitant with a rapid loss of luminal epithelial cells. DHT is the androgenic hormone responsible for prostate development, since patients with 5α-reductase deficiency show rudimentary or absent prostate glands, despite the presence of functional AR and T (Wilson et al. 1993).

Besides their role in normal prostate development and differentiation, androgens are implicated in prostate carcinogenesis (see for reviews Henderson et al. 1991, Karp et al. 1996) and benign prostatic hyperplasia (BPH). For example, prostate cancer develops only rarely in men, who have been castrated before puberty or in early adulthood (Pienta & Esper 1993). The role of the AR in prostate cancer will be discussed in section I.2.7.

I.2.2. THE ANDROGEN RECEPTOR

cDNAs encoding the full length human AR have been cloned by several groups (Chang et al. 1988a,b, Lubahn et al. 1988a,b, Trapman et al. 1988, Faber et al. 1989, Tilley et al. 1989). The open reading frame of the AR cDNA (approximately 2.7 Kb) is flanked by very long 5' and 3' untranslated regions (UTRs) (1.1 Kb and 6.8 Kb, respectively). The AR gene is located on the X chromosome at q11.2-q12, and spans at least 90 Kb (Faber et al. 1989, Kuiper et al. 1989, Lubahn et al. 1989). The gene is composed of 8 exons, the N-terminal domain is encoded by part of exon 1, the DBD is encoded by exons 2 and 3, and exons 4 to 7 and part of exon 8 encode the LBD.

The N-terminal domain of the AR contains several homopolymeric amino acid stretches, including long, glutamine (encoded by CAG) and glycine (encoded by GGN) stretches (Chang et al. 1988b, Faber et al. 1989). Especially the glutamine stretch is highly polymorphic (Caskey et al. 1992, Edwards et al. 1992, Sleddens et al. 1992). Cloned AR cDNAs encode proteins of 910 and 919 amino acid residues with an apparent molecular mass of 110-114 kDa (Faber et al. 1989, Quarmby et al. 1990, Wilson et al. 1992, Jenster et al. 1991, 1994). The variability in the lenght of the CAG and GGN repeats has resulted in confusion concerning the exact size of the AR, and the numbering of the individual amino acids. In this chapter, the numbering of the amino acid numbers correspond to an AR composed of 910 amino acid residues (20 Gln and 16 Gly). A variant (87 kDa) form of the AR has also been described (Wilson & McPhaul 1994, 1996). This truncated receptor is believed to originate from translation initiation at the first internal translation start codon (methionine, position 188). It represents about 10% of the total AR level in fetal tissues and adult genital skin fibroblasts. Its *in vivo* relevance is unknown.

Northern blot analysis revealed the presence of two hAR mRNA species of approximately 11 and 8.5 Kb, the latter as a result of alternative splicing in the 3' UTR (Trapman et al. 1988, Faber et al. 1991, Wolf et al. 1993). In many cell lines and tissues, androgens decrease the level of AR mRNA, however, in some tissues and possibly also at certain developmental stages, AR mRNA is upregulated by androgens (Quarmby et al. 1990, Burnstein et al 1995, Dai et al. 1996, Dai & Burnstein 1996). The physiological role of this differential auto-regulation is unknown as yet. In LNCaP prostate cells, androgen induced decrease of the AR mRNA level is found to be the result of a decrease in transcription rate (Blok et al. 1992, Wolf et al. 1993). The observed upregulation of AR protein in LNCaP cells by androgens (Kongrad et al. 1991, Wolf et al. 1993) might be the result of stabilization of the ligand (and DNA) bound AR during the protein extraction procedures (Kemppainen et al. 1992, Wolf et al. 1993, Zhou et al. 1995a).

Immunohistochemical staining of human tissues with specific AR directed antibodies revealed a high level of AR expression in tissues of the male urogenital system. Low levels of expression were found in several other tissues including female mammary gland, kidney, liver, brain, genital skin fibroblasts and keratinocytes, hair follicles and cardiac muscle (Ruizeveld de Winter et al. 1991, Kimura et al. 1993). At variance with AR expression in humans, rodents show high AR expression in salivary glands.

Starting from the intact AR, deletion mapping of the N-terminal domain has been performed to identify the regions essential for AF-1 transactivating activity. A large part (amino acid residues 1 to 485) was found to be necessary for maximal transcriptional activity of the AR (Simental et al. 1991, Jenster et al. 1991, 1995). A core segment, covering more than 50 percent activity has been defined between amino acids 101 and 370 (Jenster et al.

1995). Recently, Chamberlain et al. (1996) postulated the presence of two noncontiguous transactivation units in the AF-1 core region of the rat AR. The first region (AF-1a) consists of a 16 amino acid segment, of which the inner 14 amino acids (position 154 to 167) are predicted to form a β-turn followed by an amphipatic α-helix. An identical region is present in the human AR (positions 172 to 185). An acidic activator (AF-1b) has been postulated between amino acids 295 to 359. The comparable region (amino acids 296 to 360) in the human AR is also acidic, which is not unexpected because the N-terminal domain is highly conserved between species. Deletion of the individual AF-1 subfragments resulted in a small reduction of transcriptional activity of the mutant receptors. However, an AF-1a and AF-1b double mutant receptor showed less than 10 percent transcriptional activity compared to wild type AR. Interestingly, in addition to the AF-1 core region active in the full length receptor, amino acids 360 to 485 of the human AR N-terminal domain function as a strong transactivation domain (tau-5) in a constitutively active AR mutant lacking the entire LBD (Jenster at al. 1995). This observation suggests interactions between the N-terminal domain and LBD. Recent in vivo protein-protein interaction studies with separate AR LBD and N-terminal domains demonstrated that such a functional interaction is possible (Langley et al. 1995, Doesburg et al. 1997). This interaction is ligand dependent, and might be direct or indirect. However, it is unknown whether interaction between the LBD and N-terminal domain really occurs in the full length AR.

For all steroid hormone receptors except for AR a transactivation function in the LBD (AF-2) has been demonstrated. Although the AF-2 core sequence is present in the AR LBD, AR mutants composed of DBD and LBD do not display a clear transactivation function (Simental et al. 1991, Jenster et al. 1991, Wurtz et al. 1996).

Recently, it was shown that the co-activator TIF2(GRIP1) can functionally interact with GAL4(DBD)AR(LBD) fusion proteins (Voegel et al. 1996,Hong et al. 1996). As already discussed, this interaction is not AR specific. A more specfic interaction can be found between AR LBD and ARA70 (Yeh & Chang 1996). Co-transfection of ARA70 and AR expression plasmids to ARA70 minus prostate cells leads to upregulation of reporter gene expression. These effects were much less pronounced for GR and PR.

AR, like the other steroid hormone receptors, shows a basal phosphorylation level, and hormone binding initiates additional phosphorylation events. Most of the hormone induced phosphorylation sites are located in the N-terminal domain (Kempainen et al. 1992, Kuiper et al. 1993), with phosphorylation occurring predominantly at serine and threonine residues. Recently, three phosphorylation sites in the human AR have been identified, two sites, Ser80 and Ser93, in the N-terminal domain, and Ser641 in the hinge region (Zhou et al. 1995b). The effect of loss of each individual phosphorylation site on transcriptional activity was studied by mutation of the individual serine residues to alanine. Mutation of Ser81 and Ser94 had little effect, whereas mutation of Ser641 resulted in a small reduction of transcriptional activity.

I.2.3. ANDROGEN RECEPTOR MUTATIONS IN HUMAN DISEASE

Mutations in the AR gene have been demonstrated in the androgen insensitivity syndrome (AIS), Kennedy's disease, and in prostate cancer (Gottlieb et al. 1997). Best documented are the mutations in AIS. The initial recognition of AIS dates 60 years back, when individuals

with a female phenotype, but with undescended testes instead of ovaria were reported (Petterson & Bonnier, 1937). AIS constitutes a wide clinical spectrum of phenotypes. The complete form of AIS is characterized by an external female phenotype in combination with a 46, XY karyotype (reviewed by Quigley et al. 1995). Patients with complete AIS show intra-abdominal testes, a blind ending vagina, absence of the uterus and ovaries, and gynaecomastia. Besides the complete form, mutations in the AR gene can result in partial impairment of AR function leading to partial AIS (pAIS). Clinical indications for pAIS can be abnormal sexual development with a predominant male phenotype (micropenis, hypospadias), or clitoromegaly in individuals with a predominantly female phenotype. A variety of different mutations in the AR causing AIS have been reported (reviewed by Pinsky et al. 1992, Brinkmann & Trapman 1992, McPhaul et al. 1993, Quigley et al. 1995, Brown 1995, Gottlieb et al. 1997). They include large deletions, frameshifts and point mutations. Especially the mutated receptors showing a single amino acid substitution have revealed important information concerning the structure and function of the AR. Most point mutations resulted in inhibition of ligand or DNA binding. Only a few mutations have been detected in the N-terminal domain, all leading to the synthesis of a truncated protein.

A disease related to AIS is 5α-reductase deficiency, an androgen (DHT) biosynthesis defect. 5α-Reductase deficiency caused by mutations in the 5α-reductase type 2 gene leads to male pseudohermaphroditism with rudimentary or absent prostate glands (Wilson et al. 1993). The occurrence of this disorder indicates the physiological importance of DHT, and proposes a different role for T and DHT in AR mediated activity.

Kennedy's disease (a slowly progressing spinal and bulbar muscular atrophy) is linked to an expansion of the glutamine (CAG) stretch in the N-terminal domain of the AR to over 40 residues (La Spada et al. 1991). In the normal population, this stretch ranges from 11-35 elements with an average of 21 (Caskey et al. 1992, Edwards et al. 1992, Sleddens et al. 1992). Depending on the cell and promoter tested, the expanded glutamine stretch has been shown to cause a minor decrease, or no effect at all on transactivational activity (Jenster et al. 1994, Kazemi-Esfarjani et al. 1995). Choong et al. (1996) recently reported that the expanded CAG repeat reduces mRNA and protein expression, and does not alter AR functional activity. The possible role of AR mutations in prostate cancer will be discussed in section I.2.6.

I.2.4. FUNCTIONAL AREs IN ANDROGEN RECEPTOR TARGET GENES

Androgen responsive gene expression can be regulated at the level of transcription, RNA processing, RNA stability and translation. Regulation of transcription can be direct or indirect. Direct regulation is the result of direct interaction of the activated AR with one or more androgen response elements (AREs) in the regulatory regions of the gene. In secondary response genes, androgen regulation of transcription is indirect and needs protein synthesis. In this case, androgen regulation of one gene can trigger regulation of the secondary response gene.

Although many androgen-regulated genes have been identified, for most of them only limited information on the mechanism of androgen regulation is available. A serious drawback in the study of directly androgen regulated genes is the lack of cell lines expressing both the gene of interest and the AR. Therefore, functional analysis of potential androgen response sequences is mostly studied in heterologous cells, and in cells cotransfected with

an AR expression vector. An exception forms the analysis of the androgen regulation of the PSA promoter in LNCaP human prostate cancer cells (Horoszewicz et al. 1983), which showed endogenous expression of both AR and PSA. Many genes known to be regulated by androgens also display a cell or tissue specific expression pattern. Especially in this situation, structural analysis of complex regulatory elements involved in tissue-specific and androgen regulated gene expression will depend on the availability of cell lines expressing the target gene, which implicates the expression of all necessary transcription factors, (including AR) and co-activators.

Table I.2. Functional AREs in human and rodent androgen-regulated genes.

GENE	ARE-SEQUENCE	POSITION	REFERENCES
PSA (ARE-I) (h)	AGAACA gca AGTGCT	- 170	Riegman et al. 1991b
PSA (ARE-II) (h)	GGATCA ggg AGTCTC	- 394	Cleutjens et al. 1996
PSA (ARE-III) (h)	GGAACA tat TGTATC	~- 4200	Cleutjens et al. 1997
Factor IX (h)	AGCTCA gct TGTACT	- 36	Crossley et al. 1992
hGK-1 (= hKLK2) (h)	GGAACA gca AGTGCT	- 170	Murtha et al. 1993
C3(1) core II (r)	AGTACG tga TGTTCT	+ 1359	Claessens et al. 1989
Probasin (ARE-I) (r)	ATAGCA tct TGTTCT	- 241	Rennie et al. 1993
Probasin (ARE-II) (r)	AGTACT cca AGAACC	- 140	Rennie et al. 1993 Claessens et al. 1996
Slp (HRE 3) (m)	AGAACA gcc TGTTTC	- 1922	Adler et al. 1991
Gus (m)	AGTACT tgt TGTTCT	+ 7833	Lund et al. 1991
ODC (m)	AGAACA agt GGGACT	- 924	Crozat et al. 1992
arMEP24 (m)	TGTTGA gag AGAACA	- 896	Ghyselinck et al.1993
MVDP (m)	TGAAGT tcc TGTTCT	- 111	Fabre et al. 1994
ARE CONSENSUS	**GG$^{T}_{A}$ACA nnn TGTTCT**		Roche et al. 1992

(h): human, (r): rat, (m): mouse.

At present, only a limited number of functional AREs have been characterized and studied in detail. Table I.2 summarizes the functional AREs identified in human and rodent androgen-regulated genes. None of the functional AREs in natural genes is completely identical to the ARE consensus sequence GGT/AACAnnnTGTTCT. However, in most AREs one half site is almost or completely identical to the consensus sequence. Exceptions are the rat probasin ARE-2 and the ARE in the mouse arMEP24 promoter. In most functional AREs at least 5 of the 6 nucleotides (positions 2,3 and 5 in each half-site, underlined in Table I.2) most critical for high affinity AR binding and functional activity (Nordeen et al. 1990, Roche et al. 1992) are present.

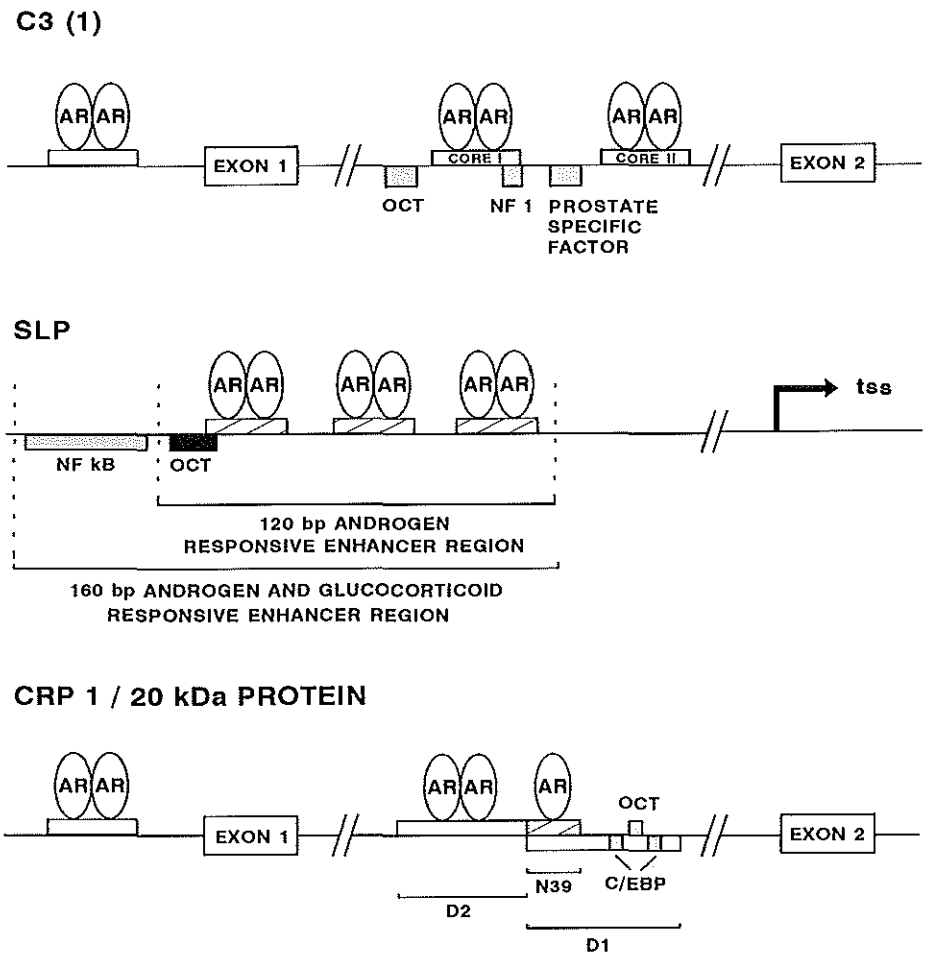

Figure I.11. Schematic representation of (putative) regulatory elements involved in androgen-regulated expression of the C3(1), Slp, and crp1/20 kDa protein. Tss represents the transcription start site. Core I and II in the C3(1) gene are AR binding sites. N39, D1 and D2 in the CRP1/20 kDa protein represent candidate complex enhancer regions.

Best documented examples of androgen regulated genes are the C3(1) (prostatic binding protein) gene (expressed in rat ventral prostate), the probasin gene (expressed in rat dorsolateral prostate), the mouse Slp genes (sex limiting protein) and GUS (β-glucuronidase) both expressed in kidney, and the human PSA gene.

The C3(1) gene

The androgen regulated C3(1) gene encodes the C3 glycoprotein component of rat prostatic binding protein (Heyns et al. 1978). Androgen regulation occurs at least in part at the transcriptional level (Page & Parker 1982). For many years C3(1) expression was thought to be confined to the ventral prostate, but recently C3(1) expression was also detected in the lacrimal gland (Allison et al. 1989, Buttyan & Slawin 1993, Vanaken et al. 1996). A schematical representation of the (putative) regulatory regions of the C3(1) gene is shown in Figure I.11. Three candidate AREs have been found in the C3(1) gene, one in the proximal promoter (-150/-136, AGAACActgGTTTCA), and two in the first intron: AGAACAactTGGCTA [Core I (+1307/+1321)] and AGTACGtgaTGTTCT [Core II (+1359/+1375)] (Claessens et al. 1989, 1993, De Vos et al. 1991, Tan et al. 1992). Although *in vitro* binding of AR to the promoter ARE and both Core I and II has been observed, only Core II is active in transfection experiments. Activity of Core II is strongly enhanced by surrounding sequences including Core I, candidate OCT and NF-I binding sites, and a binding site for an unidentified prostate-specific factor (Celis et al. 1993). Zhang et al. (1990) showed tissue-specific differences in proteins binding to the CCAAT box at position -63 to -55, and postulated the involvement of the C/EBP transcription factor family in C3 promoter activity. Additionally, they observed binding of an androgen regulated protein to the proximal promoter (position -149 to -119) region (Zhang et al. 1993). It is at present unknown whether these two proximal promoter regions contribute to maximal androgen regulated gene expression in the ventral prostate. The functional analysis of putative regulatory sequences was hindered by lack of appropriate cell cultures expressing C3(1). This problem might have been overcome recently by the discovery of C3(1) expression in primary cultures from lacrimal gland (Vanaken et al. 1996).

The mouse sex-limited protein (*Slp*) gene

Slp is a duplicated complement *C4* gene, whose expression is androgen-dependent in several tissues including liver and kidney due to an inserted provirus, that functions as a hormone-dependent enhancer. A schematical drawing of the candidate regulatory elements in the enhancer fragment is presented in Figure I.11. A 160 bp fragment directs both androgen and glucocorticoid response, whereas a 120 bp subfragment is activated by AR in CV-1 cells, and by both AR and GR in T47D cells. In the 120 bp androgen responsive enhancer, three tandemly repeated HRE-like sequences are present (Adler et al. 1992). The most proximal HRE3 [AGAACAggcTGTTTC (−1922/ −1908)] is sufficient to confer both androgen and glucocorticoid induction if linked to a TK promoter. Mutational analysis and protein binding assays demonstrated that in addition to the AR binding HRE3, other sequences including two HRE-like structures GTAATTatcTGTTCT (-1954/-1940) and TGGTCAggcAGTTCT (−1938/−1924), and several non-receptor binding elements, including an OCT binding site, contribute to the characteristic androgen response of this complex enhancer (Adler et al. 1993). The observation that the OCT binding site was occupied in males, but not in females, and only in tissues expressing *slp*, in *in vivo* footprinting experiments, provided evidence that it is involved in tissue-specific androgen regulation (Scarlett & Robins 1995). Scheller et al. recently reported that a NF-κB binding site present in the 160 bp, but not in the 120 bp

enhancer fragment might be important for GR induced activity as observed in CV-1 cells (Scheller et al. 1996). In CV-1 cells, one or more proteins binding to the NF-κB binding site, allow GR mediated activity of the enhancer. In T47D cells these sequences seem not essential for glucocorticoid induced activity.

The cystatin related protein (*crp*) 1 / 20 kDa protein gene

Expression of the *crp*1 gene, also referred to as 20 kDa protein gene in the ventral prostate and lacrimal gland of the male rat strongly depends on androgens (Ho et al. 1989, Winderickx et al. 1990, 1994). DNaseI footprinting revealed the presence of a region in the proximal promoter interacting with the AR DBD. This putative ARE (GGGAACaagTGTACT, -139 to -125) differed strongly from the ARE consensus sequence and binds AR with low affinity in band shift experiments. Mutation of the ARE strongly affected the androgen response of a proximal promoter fragment (-271 to +11) (Devos 1995). A second candidate region for androgen regulation was found in the first intron (see Figure 1.11). It contains a cluster of three ARE-like half sites (underlined) spanning the 39 bp fragment N39 (CAGAGCAGATCATGTACTGGCAATGGTTCTTACCTGTCCT: +2684/+2722), which shows AR binding and confers weak androgen response to a heterologous promoter. Additional sequences surrounding this cluster of ARE-like sequences are needed for full activity (Ho et al. 1993). These include a flanking upstream region [D2], that showed high AR binding affinity and androgen, but not glucocorticoid induced enhancer activity (see Figure I.11). In D2, initially no candidate ARE sequences were identified. However, as a result of the establishment of the functional activity of AREs that deviate considerably from the consensus sequence (Table I.2), two candidate AREs can be predicted, TGATCAtttGGTGAT (+2559 to +2573) and GGATCAttcAATTCA (+2637 to +2651). Like in the Slp and C3 promoters, other transcription factors, including OCT and C/EBP (see D1 in Figure I.11), are supposed to be involved in the full AR specific response of the 20 kDa Protein/*crp*1 gene (Ho et al. 1993).

The probasin gene

The rat probasin gene codes for a nuclear and secreted protein of the dorsolateral prostate. Its expression is *in vivo* regulated by androgens (Dodd et al. 1983), and to a lesser extent by glucocorticoids (Matusik et al. 1991). In the probasin promoter, two fragments [ARBS-1: ATAGCATCTTGTTCTTTAGT (-241/-223) and ARBS-2: GTAAAGTACTCCAAGAACCTATTT (-140/-117), putative AREs are underlined], that are important for androgen-regulation of the promoter and that bind AR, can be found (Rennie et al. 1993, Kasper et al. 1994). Although both sequences were able to interact with AR outside the probasin promoter context, initially both AR binding sites were found to be individually functional inactive (Kasper et al. 1994). Even three copies of the two separate AR binding sites failed to give rise to androgen-induced reporter gene activity. The putative ARE in the ARBS-2 region (PB-ARE2: AGTACTccaAGAACC, -136 to -123) represents the first AR binding site which selectively interacts with the AR-DBD and not with the GR-DBD (Claessens et al. 1996). From studies with swapped half-sites, it was deduced that the right hand half-site, 5'-AGAACC-3' excludes GR binding. Reporter constructs, containing three copies of PB-ARE-2 linked to a minimal TK promoter, showed in transfections induction upon androgen treatment but not after glucocorticoid treatment, indicating that PB-ARE-2 represents a functionally active, AR specific ARE. The functional activity of PB-ARE-2 was unexpected, because of the previous negative results of Kasper et al. (1994). The observed discrepancy might be due to differences in cell lines and culture conditions used.

The β-glucuronidase (*gus*) gene

The mouse *gus* gene is constitutively expressed in almost all tissues, but its expression is androgen regulated in kidney cells (Lund et al. 1988). The identification of the regulatory elements involved in this androgen regulated expression benefitted from the occurrence of three natural genetic variants displaying different phenotypes of androgen regulated *gus* expression (Lund et al. 1991). The presence of an androgen-inducible DNAseI hypersensitive site in intron 9 coincided with the androgen responsive phenotype. Careful analysis of this region revealed the presence of a functional ARE (AGTACTtgtTGTTCT, +7833 to +7847) which cooperates with a 57 bp segment at position +7990 to +8046 to generate maximal androgen response. Mice showing a complete absence of the androgen response were found to have a mutation in the ARE sequence, whereas mice with a reduced androgen response showed a deletion of the +8005 to +8026 segment. This region is recognized by an androgen inducible and kidney specific transcription factor (Lund et al. 1991).

The human coagulation factor IX gene

The presence of a functionally active ARE in the factor IX gene has been suggested by natural occurring mutations. A mutation at position -26, within the putative ARE sequence (AGCTCAgctTGTACT; -36 to -22) in the promoter of this gene caused a variant form of Hemophilia B Leyden, a bleeding disorder due to a very low level of factor IX (Crossley et al. 1992). Unlike the classical cases, the patient carrying this -26 mutation did not recover during puberty, when circulating T levels start to rise. Despite the mutation in the ARE sequence, the mechanism of failure of androgen response is not clear. Although AR can bind to this ARE, AR is only a weak activator of factor IX promoter activity in transient transfection experiments. The putative ARE sequence overlaps with a HNF-4 binding site (TTGTACTTTGGT; positions -28 to -17). The -26 mutation has been shown to completely block HNF-4 binding, and it has been suggested that Hemophilia throughout life is a result of the complete loss of HNF-4 binding (Reijnen et al. 1990). CCAAT-box binding proteins, binding to a region (positions +1 to +18) just downstream of the first major transcription start site (Reijnen et al. 1992), have been suggested to be involved in the phenotypical recovery of Hemophilia B Leyden around puberty in most patients (Pickets et al. 1993).

The human glandular kallikrein-1 (hGK-1) gene

hGK-1 (also known as KLK2 and hK2) is a member of the human kallikrein-like serine protease family. This family consists of three members, hGK-1, tissue kallikrein KLK1, and the extensively studied PSA gene (see sections I.2.5., I.2.6 and Chapters III, V and VI). Both PSA and hGK-1 expression are regulated by androgens at the level of transcription (Riegman et al. 1991b, Wolf et al. 1992). The proximal promoter regions of both genes show 80 percent homology. Expression of the hGK-1 gene is restricted to the prostate. Transcriptional activity of hGK-1 promoter fragments is induced upon androgen treatment (Murtha et al. 1993). In the proximal promoter a putative ARE (GGAACAgcaAGTGCT; −170 to -156) was identified, this sequence differs at only one position from the functionally active ARE at the same position in the PSA gene (Riegman et al. 1991b, Chapter III). Deletion of one half-site of this putative ARE completely abolished androgen response of hGK-1 promoter fragments, and two copies of the ARE sequence conferred hormone response to a minimal TK promoter (Murtha et al. 1993). These results clearly indicate that the sequence at position -170 to -156 in the hGK-1 gene represents a functionally active ARE.

Other androgen regulated genes

Transcription of the prostate arginine esterase gene, a dog kallikrein-like gene related to the human PSA and hGK-1 genes, is regulated by androgens (Gauthier et al. 1993). Two putative ARE sequences could be identified (Arg-est-ARE2: AGGGCTataGGTCCT at positions -230 to -216, and Arg-est-ARE1: AGGACAacaGGTGTT at positions -165 to -151) (Dubé et al. 1995). Although Arg-est-ARE1 was shown to interact with the AR, no significant effect on androgen regulation of a heterologous promoter was observed in transfection experiments. Arg-est-ARE2 was not tested in this respect. It might be postulated that cooperative action of both AREs is needed to obtain measurable androgen-induced activity.

Mouse vas deferens protein (MVDP) is exclusively expressed in the epithelial cells of the vas deferens. In the MVDP gene promoter two ARE sequences, one in the proximal promoter: TGAAGTtccTGTTCT (−111/-97) and one more upstream: AGAACAtgcTGCTCT (-1186 /−1172), are present (Fabre et al. 1994). Only the proximal ARE is functionally active when fused to a heterologous promoter. Although a minimal MVDP promoter fragment (-121 to +41) is sufficient for androgen regulation, the region -510 to + 41 gave maximal androgen induction. Putative binding sequences for several sequence specific transcription factors, including Sp1, NF-1, OCT and CCAAT box binding factors were found to be located in this region.

Ornithine decarboxylase (ODC) is an ubiquitously expressed enzyme involved in polyamine biosynthesis. Androgen regulated expression is found in ventral prostate, seminal vesicle and kidney of rodents. The mouse ODC promoter region contains an ARE sequence (AGAACAagtGGGACT) at position -924 to -910. This sequence binds AR *in vitro* and an ODC promoter fragment containing this sequence confers a low level of androgen response to a reporter gene (Eisenberg & Jänne 1989, Crozat et al. 1992).

The androgen-regulated murine epididymal protein (arMEP24) is an androgen-dependent glutathione peroxidase-like protein synthesized and secreted by the mouse epididymis (Ghyselinck et al. 1993). An ARE sequence has been found in the upstream promoter region (TGTTGAgagAGAACA; -896 to -882). Two or three copies of this sequence are able to add limited androgen regulation to a TK promoter.

Functional transcriptional analysis of a 481 bp region of the mitochondrial aspartate aminotransferase (mAAT) gene showed that this region, containing two putative ARE sequences (GGAAAAgacTGTTCT at position -1439 to -1424 and TCTCCAtctTGTTCT at -1465 to -1450) is sufficient for androgen regulated expression of the gene (Juang et al. 1995).

From the combined data presented above several general conclusions can be drawn:
(i) Functional AREs can deviate considerably from the consensus high affinity binding site.
(ii) Androgen regulated genes in general contain two or more AREs, which act synergistically.
(iii) In addition to AREs, binding sites for ubiquitous transcription factors, and for tissue specific transcription factors are essential for high, androgen regulated promoter activity.
(iv) Different mechanisms of AR specificity can occur, including ARE sequence, binding sites for other transcription factors and cell specific factors.

I.2.5. REGULATION OF PSA EXPRESSION

PSA is a single chain glycoprotein with a molecular weight of approximately 33 kDa, first isolated by Wang et al. (1979). The protein was independently isolated by others, and therefore is known under different names, as γ seminoprotein (Schaller et al. 1987) and P-30 (Graves et al. 1990). PSA is a member of the family of human kallikrein-like serine proteases. As mentioned before, other members of this family are hGK-1 and tissue kallikrein KLK1. PSA is synthesized in, and secreted by the luminal epithelial cells of the human prostate. Mature PSA is composed of 237 amino acids, its precursor contains a 17 amino acid signal peptide. For full proteolytic activity, posttranslational processing is needed (reviewed by Clements 1994, and Peehl 1995). *In vivo,* PSA may function to liquify the seminal coagulum by proteolysis of seminogelin and fibronectin (Lilja et al. 1985). Laminin, IGF-BP3, TGF-β and cell surface receptors have also been suggested as substrates for PSA (Lee et al. 1989, Cohen et al. 1992, Killian et al. 1993, Webber et al. 1995).

PSA cDNA (Lundwall & Lilja 1987, Riegman et al. 1988, Henntu & Vihko 1989) and the PSA gene (Riegman et al. 1989a, Lundwall et al. 1989) have been isolated and completely sequenced. The gene consists of five exons, and spans a region of approximately 5 Kb. The PSA gene is clustered with the hGK-1 and KLK-1 genes in the order (KLK1)-(PSA)-(hGK-1), in an area of 60 Kb on human chromosome 19q13.2-13.4 (Evans et al. 1988, Riegman et al. 1989b, Schonk et al. 1990, Riegman et al. 1992). PSA, and also hGK-1, is almost exclusively expressed in the human prostate and prostate derived tumors and tumor cell lines (Chapdelaine et al. 1988, Trapman et al. 1988, Riegman et al. 1988, 1991a, Henntu et al. 1990 and 1992, Henntu & Vihko 1992, Young et al. 1991, 1992, Nevalainen et al. 1993). PSA mRNA expression does not only show cell specificity, but is also tightly regulated by androgens (Riegman et al. 1991a,b, Henntu et al. 1992, Young et al. 1991, 1995). Androgen-stimulated expression is at least partially regulated at the level of transcription (Riegman et al. 1991b, Wolf et al. 1992).

At present, three functional AREs have been identified in the PSA regulatory regions. Two functionally active AR binding sites were identified in the proximal PSA promoter, at positions -170 (ARE-I; AGAACAgcaAGTGCT) and −394 (ARE-II; GGATCAgggAGTCTC), respectively (Riegman et al. 1991b, Cleutjens et al. 1996; Chapter III). In transient transfection experiments in LNCaP prostate tumor cells, the high affinity AR binding site, ARE-1 (-170) by itself gave rise to weak (2-fold) stimulation in the presence of androgens. ARE-1 (-170) had to cooperate with the low affinity AR binding site, ARE-II at position -394 to obtain maximal (~6-fold) androgen induction of the proximal PSA promoter. A 6 Kb PSA promoter construct showed much higher (~3000-fold) androgen-induced activity when transfected to LNCaP cells. A high affinity AR-binding site (ARE-III; GGAACAtatTGTATC), was identified in this long PSA promoter fragment, ~4200 bp upstream of the transcription start site (Cleutjens et al. 1997; Chapter V). ARE-III, like ARE-I, closely resembles the ARE consensus sequence; whereas ARE-II, which binds AR with lower affinity, deviates more. Although all three AREs contributed to maximal androgen inducibility, the presence of ARE-III in the upstream enhancer region was absolutely essential for high androgen regulated activity of the 6 Kb PSA promoter. ARE-III (~-4200) turned out to be part of a complex, very strong androgen regulated enhancer region (Schuur et al. 1996, Cleutjens et al. 1997).

The upstream enhancer region showed synergistic cooperation with the proximal PSA promoter, and was found to be composed of at least three separate, but cooperating regulatory regions. Although the presence of ARE-III was a prerequisite, it was not sufficient for strong androgen regulated activity of the enhancer region. The importance of the upstream enhancer for androgen regulated PSA expression was confirmed by the presence of a strong, androgen regulated DNase I hypersensitive site at this position in LNCaP cell chromatin.

Transiently transfected PSA promoter constructs were active in cells with endogenous PSA expression (LNCaP prostate cells), but inactive in cells without endogenous PSA, with the exception of T47D cells (Schuur et al. 1996, Cleutjens et al. 1997, Chapter V). The cell specificity was retained within a 440 bp core enhancer region, encompassing the upstream ARE-III. (Cleutjens et al. 1997, Chapter V). Interestingly, the 6 Kb PSA promoter did not only show largely prostate cell specific activity in transient transfection experiments, but was also capable to direct prostate specific transcription in transgenic mice (Chapter VI).

PSA promoter activity is not AR specific. In T47D mammary tumor cells, PR stimulated expression of a PSA promoter driven reporter gene could be demonstrated. Similarly, in LNCaP cells stably transfected with a GR expression vector, activated GR was able to stimulate endogenous PSA expression, as well as the activity of a transfected, PSA promoter driven reporter gene (Cleutjens et al. 1997, Chapters IV and V).

I.2.6. PSA EXPRESSION IN PROSTATE CANCER

In Western countries, adenocarcinoma of the prostate is the most frequently diagnosed tumor in men, and the second leading cause of male cancer death (Parker et al. 1996). Therapeutic approaches for prostate cancer depend on the stage of the malignancy. In clinically localized prostate cancer, surgical curability is feasible through complete excision of the prostate (radical prostatectomy). First line therapy of locally invasive or metastatic disease is generally based upon androgen ablation, or blockade of AR function by anti-androgens. Unfortunately, the effects of endocrine therapy are only temporary and after a variable period of time, a finally hormone refractory tumor continues to grow.

PSA is expressed in the vast majority of prostate cancers, although its expression level is decreased and more heterogeneous in undifferentiated tumors (Keillor & Aterman 1987, Gallee et al. 1990, Partin et al. 1993, Ruizeveld de Winter et al. 1994). Incidentally, prostate cancers can even be PSA negative (Mai et al. 1996). Although PSA is considered to be the best tissue specific marker available, low PSA expression has been demonstrated in some male salivary gland derived tumors (vanKrieken 1993), and in mammary tumors, lactating breast tissue and in endometrial tissue in women (Diamandis et al. 1994, Yu & Diamandis 1994, Clements & Muktar 1994). Also in the non-prostatic tissues, expression of PSA was suggested to be steroid hormone-dependent (Yu & Diamandis 1994, Graves 1995).

PSA as serum marker of prostate cancer

Although not per se a tumor marker, increased serum PSA is a strong indication of prostate cancer (Catalona et al 1991, Oesterling 1991). In spite of its limitations, the serum PSA test is now a widely accepted method to screen for prostate cancer. Because occasionally inflammation of the prostate and BPH can also give rise to positive serum PSA values, additional tests, including a needle biopsy are needed for final proof.

Increase of serum PSA during endocrine or other therapy is generally considered as evidence for recurrence or progression of the tumor. What determines the increase in serum PSA during the development of an apparently hormone refractory tumor? Several alternative mechanisms can be proposed. One factor can be that, although PSA expression in individual tumor cells is decreased, the increase in tumor load leads to a net increase in serum PSA. Other hypotheses include hormone independent activation of the AR (Culig et al. 1994), or GR or PR induced PSA expression (this thesis). However, whether prostate tumors contain functionally active GR or PR is presently unknown. An alternative explanation could involve the induction of hormone independent PSA expression using an unidentified mechanism.

The currently widespread application of serum PSA-based prostate cancer screening has led to a considerable increase in the number of prostate cancer diagnoses, and a concomitant decrease in tumor stage at time of diagnosis (Rosen 1995, Bangma et al. 1995). Early detection might provide the opportunity to detect a larger number of prostate confined tumors, which might still be curable. On the other hand, it is a real possibility that PSA-based screening leads to overdiagnosis and subsequent overtreatment.

Because, like PSA, hGK-1 expression is prostate specific, it is a second candidate for application in prostate cancer diagnosis. The hGK-1 mRNA level has been estimated to amount 10 to 50 percent of PSA mRNA in prostate (Chapdelaine et al. 1988, Henntu et al. 1990, Young et al. 1992, Young 1996). In serum, the proportion of hGK-1 relative to PSA seems to be low (Piironen et al. 1996). Therefore, the clinical significance of serum hGK-1 tests remains elusive.

PSA mRNA as marker of circulating tumor cells

In addition to its role in the early detection of prostate cancer, and as a marker for monitoring therapy, PSA expression might have additional clinical relevance. Approximately 50 percent of patients with clinically localized prostate cancer show extra-prostatic disease upon histopathological screening of the prostate and regional lymphnodes (Epstein et al. 1993, Rosen et al. 1992). A substantial proportion of these patients will, despite radical prostatectomy, relapse with locally recurrent or metastatic disease. In patients with clinically localized prostate cancer, the detection of prostate cells in the circulation might be an indication of the presence of micrometastases, and thus might be a means to discriminate between the presence of locally confined tumors and tumors with extra-prostatic spreading. Establishment of otherwise undetectable micrometastases might lead to a different therapeutical approach.

Circulating prostate tumor cells can theoretically be detected by means of RT-PCR for mRNAs, specifically expressed by prostate (tumor) cells and not by other cells in the blood. Because PSA is almost exclusively expressed by prostate epithelial cells, it is a good candidate. Several reports, applying PSA mRNA based RT-PCR for detection of circulating tumor cells, have now been published (Hambdy et al. 1992, Moreno et al. 1992, Israeli et al. 1994, Katz et al. 1995, Cama et al. 1995, Jaakola et al. 1995). There is substantial variation in the percentage of patients with proven metastases, in which PSA mRNA is detectable in the blood. Because of the very low level of PSA mRNA in the blood, the method is prone to false negative and false positive (contamination) values. Therefore, the clinical relevance of the assay remains to be established.

Besides PSA-based RT-PCR, a second related analysis, RT-PCR based upon Prostate specific membrane antigen (PSM or PSMA) mRNA for detection of circulating prostate tumor cells is currently under investigation. Cloning of the cDNA encoding PSM (Israeli et

al. 1993) showed that it is a transmembrane glycoprotein. A high level of expression was observed in normal prostate and both primary and metastasized prostate tumors (Israeli et al. 1994). Recently, the prostate specificity of PSM expression has become a matter of debate. Examination of a set of normal tissues revealed the presence of significant PSM expression in duodenal mucosa, a subset of renal tubules and certain neurocrine cells in the colon crypts (Silver et al. 1997). Leek et al. (1995) suggested the existence of a second, related gene, which might further complicate the specificity of PSM.

I.2.7. THE ANDROGEN RECEPTOR IN PROSTATE CANCER

One of the most important questions in prostate cancer biology concerns the role of androgens. Initially, the growth of the majority of prostate tumors depends, like normal prostate development, on androgens. Therefore, as discussed above, therapy of metastasized tumors is generally based upon androgen ablation or blockade of AR function. After onset of endocrine therapy, most prostate tumors show regression. However, essentially all originally hormone responsive tumors become apparently hormone independent during time. From a clinical point of view it is very important to elucidate the mechanisms involved in androgen dependent and androgen refractory prostate tumor growth.

An important first question concerns the role of the AR. The majority of locally progressive, hormone refractory tumors show high AR expression, although more heterogeneous than in the normal prostate (van der Kwast et al. 1991, Sadi et al. 1991, Sadi & Barreck 1993, Chodak et al. 1992, Ruizeveld de Winter et al. 1994, Tilley et al. 1994, reviewed by Trapman & Brinkmann 1996). Interestingly, distant prostatic carcinoma metastases in bone express AR (Hobish et al. 1995) and the expression level seems even higher and more homogeneous than in locally recurrent tumor (Kleinerman, unpublished). An increased level of AR expression could allow cancer cells to continue androgen-dependent growth in the presence of a low level of serum androgen. AR overexpression could be the result of amplification of the AR gene, as recently observed in a proportion of hormone refractory prostate tumors (Visakorpi et al. 1995, Koivisto et al. 1997). Importantly, AR gene amplification has never been detected in tumors prior to endocrine therapy.

Additionally, alternative AR activation could play a role in apparently androgen-independent prostate cancer. In prostate cancer cell lines, growth factors (for example IGF-I and KGF) have been found to induce AR mediated, promoter specific transcriptional activation (Culig et al. 1994). Furthermore, cross-talk between AR and both the PKA or PKC-signalling pathways has been reported (Ikonen et al. 1994, deRuiter et al. 1995, Nazareth & Weigel 1996).

Also, structural changes in the AR could account for androgen-independent activation of the receptor. A classical example is the point mutation in the AR gene in the LNCaP prostate cancer cell line (alanine to threonine substitution at position 868 in the LBD), which renders the receptor responsive to anti-androgens, estrogens and progestins (Veldscholte et al. 1990). Mutations in the AR gene have been detected in both primary prostate cancer and in metastases. Their prevalence in locally progressive, hormone dependent or refractory prostate tumors is low according to most studies (Newark et al. 1992, Culig et al. 1993, Suzuki et al. 1993, Gaddipati et al. 1994, Peterziel et al. 1995, Quigly et al. 1995, Koivisto et al. 1997). The proportion of mutations seems highest in late stage tumors (Taplin et al. 1995). For a limited number of tumors, it has been shown that the mutation can lead to altered

ligand specificity (Culig et al. 1993, Ris-Stalpers et al. 1993, Pieterziel et al. 1995, Taplin et al. 1995). At present the prevailing explanation for AR mutations in prostate cancer is, that they represent a mixture of functional and random mutations resulting from genetic instability (Trapman & Brinkmann, 1996).

In AR negative prostate tumors, none of the previously described mechanisms can account for the androgen-independent growth of the tumor. In these tumors, alternative tumor specific pathways must have bypassed the AR dependent process of growth stimulation. These pathways may implicate inactivation of tumorsuppressor genes and activation of oncogenes (Thompson 1990). However, the genes involved in the majority of prostate cancers have not been identified as yet.

Although in general AR stimulates prostate cancer growth, AR mediated tumor cell growth repression was recently also shown (Zhau et al. 1996). Whether the androgen-repressed phenotype is an incidental occurrence or may represent a larger subpopulation of hormone refractory prostate tumors remains to be established.

In conclusion, several not mutually exclusive mechanisms for the growth of hormone-refractory prostate tumors have been proposed. Elucidation of the mechanisms involved in hormone-refractory prostate tumor growth can ultimately lead to the improvement of the currently available therapeutic regimens.

I.2.8. PROSTATE SPECIFIC PROMOTERS IN PROSTATE CANCER MODELS

Progress towards a better understanding of the biology of prostate cancer, including its escape from regulation by androgens would benefit enormously from animal models displaying the full range of clinical stages of this disease. These models will also be indispensable for the development of new therapeutic approaches. Prostate cancer is essentially unique to humans. Generally spoken two different prostate cancer model systems can be envisaged: serial transplantable human xenografts and transgenic animal models. Both model systems display their own specific favorable and unfavorable features. Serially transplantable prostate cancer xenografts, although in an athymic nude mouse host, are of human origin (Nagabhushan et al. 1996, van Weerden et al. 1996). A major disadvantage of xenograft models is the variation of tumor take and growth rate and metastatic capacity, dependent on the site of transplantation. Furthermore, the effect of the immune system on tumor progression cannot be studied. Essentially, transgenic animals constitute a useful model for the study of multistep tumor progression and may serve as experimental system to develop and evaluate new therapeutic approaches. They provide a source of reproducible tumors with known genetic make-up. Major drawbacks of these models are the limitation to tumors of rodent origin (e.g. mouse), and the lack of knowledge of the most important genes involved in human prostate cancer, which can be applied to build the model.

A prerequisite of the establishment of a transgenic prostate cancer model is a promoter to target a heterologous gene with oncogenic properties to the appropriate prostate epithelial cells. Several attempts to generate inbred mouse prostate cancer models have been made. Schaffner et al. (1995) explored the capacity of the proximal 632 bp PSA promoter to induce prostate cancer in transgenic mice. However, mice carrying a Ha-*ras*T24 oncogene driven by the proximal PSA promoter, developed salivary gland and gastrointestinal tract tumors. Mutant Ha-*ras* expression was confirmed in salivary gland tumors, but not in gastrointestinal

tumors. Tumors arose in animals over 44 weeks of age. The late onset of tumorigenesis could indicate that the proximal PSA promoter driven oncogene expression was a secondary event.

A C3(1) promoter fragment was successfully applied to direct prostate tumor development in mice. Transgenic mice carrying a 5.7 Kb C3(1) promoter fragment linked to the SV40 large T antigen (Tag) developed prostate adenoma or adenocarcinoma in almost all males surviving to the age of 7 months (Maroulakou et al. 1994). All female mice carrying this transgene acquired mammary adenocarcinomas. Besides prostate and mammary adenocarcinomas, the mice developed other phenotypic changes including several proliferative lesions and malignancies leading to premature death. So this model seems not the most promising to generate a prostate cancer model.

Transgenic mice carrying the 426 bp probasin promoter driven SV40 large T antigen oncoprotein (TRAMP mice), develop progressive forms of prostatic cancer (Greenberg et al. 1995, Gingrich et al. 1996). Tumors have been detected specifically in the prostate as early as 10 weeks of age, and distant metastases can be found at 12 weeks (Gingrich et al. 1996). It is important to note that expression of Tag precedes the histological appearance of carcinomas. Furthermore, not all Tag expressing cells became hyperplastic or cancer cells (Gingrich et al. 1996). The same observation was made in the transgenic mouse model using the SV40 large T antigen oncogen, driven by the C3(1) promoter (Maroulakou et al. 1994). Cells expressing the transgene seem to be initially in a preneoplastic state and additional events are probably needed to confer the cells to the full malignant state. In all presently available transgenic prostate cancer models, strong viral oncogenes are targeted to the prostate. In the future, additional transgenic animal models, targeting gene products implicated in clinically important human prostate cancer might reveal extra information about the mechanisms underlying the different stages of prostate cancer initiation and progression.

I.3. SCOPE OF THIS THESIS

The goal of the present study was the identification and characterization of prostate specific transcription factors involved in growth and differentiation of the prostate. It was hypothesized that these factors could also play a role in the initial development and progressive growth of prostate cancer. To reach the target, two complementary experimental approaches were employed. The first approach included a search for novel, prostate specific members of known families of transcription factors, based upon the homology in the DNA binding domains. Using PCR techniques, expression in the prostate of the C/EBP transcription factor family was studied. The C/EBP family was selected as a potentially interesting candidate, because of literature data, suggesting the involvement of C/EBP in the prostate specific activity of the rat C3(1) promoter. As a result of this attempt, the C/EBPδ gene was cloned. C/EBPδ found to be the major C/EBP expressed in LNCaP cells (Chapter II), however, C/EBPδ expression appeared not to be prostate specific.

The second approach focussed on the mechanism of PSA expression. PSA is one of the main products of luminal prostate epithelial cells, the cell type which prostate cancer cells resemble most. Furthermore, high levels of PSA expression are only observed in prostate and prostate derived tumors. At the time of initiation of the work described in this thesis, one of the factors involved in the regulation of PSA expression was already identified, the AR. In the prostate, the AR is not only involved in the regulation of PSA expression, but is also essential for its development and maintenance of function. Although the AR is not prostate specific, AR expression is also high in other cells of the male urogenital tract, it was considered as an important candidate for further study. So, knowledge on molecular mechanisms involved in PSA gene expression would provide information on mechanisms of androgen regulated, and prostate-specific gene expression. As additional spin off, the knowledge on regulatory elements involved in prostate specific PSA expression might be used to direct high, prostate specific expression of a therapeutic gene to the prostate, and to develop animal prostate cancer models.

The analysis of the regulatory regions involved in androgen regulated and prostate specific expression of the PSA gene was initiated by transient transfection experiments in PSA and AR positive LNCaP prostate cells. The studies resulted in the identification of three functionally active androgen response elements in the PSA promoter, two (ARE-I and ARE-II) in the proximal promoter (Chapter III), and one (ARE-III) in a far upstream enhancer region (Chapter V). Because the sequence for high affinity DNA binding of the AR and several other steroid hormone receptors including the GR is identical, the mechanism underlying the apparent androgen specific induction of PSA promoter activity in LNCaP prostate cells was investigated (Chapter IV). The far upstream enhancer region, encompassing ARE-III, showed mainly LNCaP prostate cell activity in transient transfection experiments (Chapter V). In order to test whether a 6 Kb PSA promoter fragment containing both the upstream enhancer region and the proximal promoter, was able to direct prostate specific and androgen regulated activity *in vivo*, transgenic mice with the 6 Kb fragment and the proximal PSA promoter fragment linked to the LacZ reporter gene were generated. The results presented in Chapter VI clearly indicate that the 6 Kb promoter fragment is indeed capable to direct prostate specificity. Chapter VII summarizes our findings, reviews them in light of recent literature data and indicates future directions for research.

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Chapter II

THE HUMAN C/EBPδ (CRP3/CELF) GENE: STRUCTURE AND CHROMOSOMAL LOCALIZATION

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Chapter II

ABSTRACT

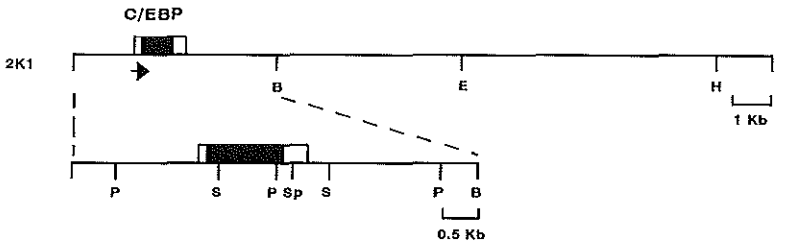
In an attempt to identify C/EBP-like transcription factors expressed in the prostate, a cDNA homologous to the mouse C/EBPδ (CRP3) and the rat CELF gene was isolated. A genomic clone containing the entire C/EBPδ gene was isolated using a cDNA fragment as a probe. The gene was characterized by restriction mapping and sequence analysis. By fluorescent in situ hybridization, using the biotinylated genomic clone as a probe, the C/EBPδ gene was assigned to the pericentromeric region of human chromosome 8, most probably to 8q11. This chromosomal localization was confirmed by analysis of a panel of human x hamster somatic cell hybrid DNA samples with a C/EBPδ specific STS. As a result, the C/EBPδ gene could be positioned between the PLAT and MOS locus.

INTRODUCTION

The CCAAT/enhancer-binding protein (C/EBP) family of transcription factors is important for regulation of the expression of many different genes (2). C/EBP-like proteins are members of the family of leucine zipper (bZIP) transcription factors, characterized by a basic DNA binding domain linked to heptad leucine repeats, which mediate dimerization. The DNA binding domain is well conserved between the different C/EBP-like proteins, but further structural homology is limited (14).

Expression of C/EBP-like proteins can be cell type dependent, and can vary during differentiation. C/EBP (13), hereafter called C/EBPα, is primarily expressed in liver and fat tissue, and at lower levels in intestine and lung (2,22). C/EBPα expression in these tissues is restricted to terminally differentiated cells. C/EBPβ (6) [also named CRP2 (24), AGP/EBP (4), IL-6DBP (16), NF-IL6 (1) and LAP (9)], C/EBPγ (6) [Ig/EBP (18)], and C/EBPδ (6) [CRP3 (24) or CELF (11)] are almost ubiquitously expressed in different tissues, but at varying levels. C/EBPδ expression is most abundant in kidney and testis.

To identify C/EBP-like transcription factors which are expressed in prostate, first strand cDNA of poly(A)$^+$ RNA from LNCaP cells, a human prostate carcinoma cell line, was prepared with a T12SITE primer (3' TTTTTTTTTTTTCCTAGGCTTAAGCGTACG 5'), using standard conditions. The first strand cDNA was then amplified by PCR using the SITE primer (3' CCTAGGCTTAAGCGTACG 5') and a primer specific to the DNA binding domain of C/EBP-like transcription factors (5' CAACATCGCGGTGCGCAAGAGC 3'). Both primers contain a restriction site (underlined; EcoRI and FspI, respectively), which were used to insert the PCR products in the pTZ19 vector (Pharmacia, Uppsala, Sweden). Twenty seven independent clones were isolated. Subsequent sequence analysis identified four clones containing an insert encoding a heptad leucine repeat. The four clones showed the same sequence, which turned out to be closely related to the 3' region of the mouse C/EBPδ (CRP3) gene (24) and the rat CELF gene (11), indicating the isolation of the human C/EBPδ (CELF) homoloque. The selective isolation of the C/EBPδ cDNA, and no other known or unknown C/EBP-like cDNAs, could implicate that the C/EBPδ mRNA is the most abundant C/EBP-like transcript in human prostate. Alternatively, the PCR conditions might have been in favour of this specific cDNA.

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                                                                  CCCGGGGCG
-120 CCCCCGCGGTGCCGGAGTCGGGGCGGGGCGTGCACGTCAGCCGGGGCTAGAAAAGGCGGC
-60  GGGGCTGGGCCCAGCGAGGTGACAGCCTCGCTTGGACGCAGAGCCCGGCCCGACGCCGCC
       M  S  A  A  L  F  S  L  D  G  P  A  R  G  A  P  W  P  A  E
1    ATGAGCGCCGCGCTCTTCAGCCTGGACGGCCCGGCGCGCGGCGCGCCCTGGCCTGCGGAG
       P  A  P  F  Y  E  P  G  R  A  G  K  P  G  R  G  A  E  P  G
61   CCTGCGCCCTTCTACGAACCGGGCCGGGCGGGCAAGCCGGGCCGCGGGGCCGAGCCAGGG
       A  L  G  E  P  G  A  A  A  P  A  M  Y  D  D  E  S  A  I  D
121  GCCCTAGGCGAGCCAGGCGCCGCCGCCCCCGCCATGTACGACGACGAGAGCGCCATCGAC
       F  S  A  Y  I  D  S  M  A  A  V  P  T  L  E  L  C  H  D  E
181  TTCAGCGCCTACATCGACTCCATGGCCGCCGTGCCCACCCTGGAGCTGTGCCACGACGAG
       L  F  A  D  L  F  N  S  N  H  K  A  G  G  A  G  P  L  E  L
241  CTCTTCGCCGACCTCTTCAACAGCAATCACAAGGCGGGCGGCGCGGGGCCCCTGGAGCTT
       L  P  G  G  P  A  R  P  L  G  P  G  P  A  A  P  R  L  L  K
301  CTTCCCGGCGGCCCCGCGCGCCCCTTGGGCCCGGGCCCTGCCGCTCCCCGCCTGCTCAAG
       R  E  P  D  W  G  D  G  D  A  P  G  S  L  L  P  A  Q  V  G
361  CGCGAGCCCGACTGGGGCGACGGCGACGCGCCCGGCTCGCTGTTGCCCGCGCAGGTGGGC
       P  C  A  Q  T  V  V  S  L  A  A  A  G  Q  P  T  P  P  T  S
421  CCGTGCGCACAGACCGTGGTGAGCTTGGCGGCCGCAGGGCAGCCCACCCCGCCCACGTCG
       P  E  P  P  R  S  S  P  R  Q  T  P  A  P  G  P  A  R  E  K
481  CCGGAGCCGCCCGCGCAGCAGCCCCAGGCAGACCCCCGCGCCCGGCCCCGCCCGGGAGAAG
       S  A  G  K  R  G  P  D  R  G  S  P  E  Y  R  Q  R  R  E  R
541  AGCGCCGGCAAGAGGGGCCCGGACCGCGGCAGCCCCGAGTACCGGCAGCGGCGCGAGCGC
       N  N  I  A  V  R  K  S  R  D  K  A  K  R  R  N  Q  E  M  Q
601  AACAACATCGCCGTGCGCAAGAGCCGCGACAAGGCCAAGCGGCGCAACCAGGAGATGCAG
       Q  K  L  V  E  L  S  A  E  N  E  K  L  H  Q  R  V  E  Q  L
661  CAGAAGTTGGTGGAGCTGTCGGCTGAGAACGAGAAGCTGCACCAGCGCGTGGAGCAGCTC
       T  R  D  L  A  G  L  R  Q  F  F  K  Q  L  P  S  P  P  F  L
721  ACGCGGGACCTGGCCGGCCTCCGGCAGTTCTTCAAGCAGCTGCCCAGCCCGCCCTTCCTG
       P  A  A  G  T  A  D  C  R
781  CCGGCCGCCGGGACAGCAGACTGCCGGTAACGCGCGGCCGGGGCGGGAGAGACTCAGCAA
841  CGACCCATACCTCAGACCCGACGGCCCGGAGCGGAGCGCGCCCTGCCCTGGCGCAGCCAG
901  AGCCGCCGGGTGCCCGCTGCAGTTTCTTGGGACATAGGAGCGCAAAGAAGCTACAGCCTG
961  GACTTACCACCACTAAACTGCGAGAGAAGCTAAACGTGTTTATTTTCCCTTAAATTATTT
1021 TTGTAATGGTAGCTTTTTCTACATCTTACTCCTGTTGATGCAGCTAAGGTACATTTGTAA
1081 AAAGAAAAAAAACCAGACTTTTCAGACAAACCCTTTGTATTGTAGATAAGAGGAAAAGAC
1141 TGAGCATGCTCACTTTTTTATATTAATTTTTACAGTATTTGTAAGAATAAAGCAGCATTT
1201 GAAATCGCCCCTGCTTCCTATATTCGCAGTGACTCCCGCCCGCCCGCCGCCGCCGGTCGG
1261 AGGACCCGGCTCGGAAGGGCGTTCCGGACCGCAGCCAGCCAGCACCTAGGGAGCCCGGGC
1321 GCCAGGTGTGTGTGTGGGGGGGGCGGGGGGATGGGCGCAGCGGCGAGCTACTCAGGAGAG
1381 AGGGTCTGTCGCTTTTAAAACGCATTAAAGGCTCTCTCCTGGCCTTATTTAACTTGCCTA
1441 AGCTAGGTGGAGCACGGCTGAGCTC
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Figure II.1. (A) Restriction map of the genomic clone (λ2K1) containing the C/EBPδ sequence. A more detailed restriction map of a region of 5.5 kb encompassing the entire gene is shown below. The open reading frame is shown as a dark box. The 5'- and 3'-untranslated regions are represented by open boxes. The horizontal arrow indicates the direction of transcription. B=BamH1; E=EcoR1; H=HindIII; S=Sst1; Sp=Sph1; P=Pst1. (B) Nucleotide and deduced amino acid sequence of the human C/EBPδ gene. The presumed TATAAA box and the polyadenylation signal are underlined. The position of the transcriptional start site, as indicated by an arrow, is drawn on basis of similarity to the mouse C/EBPδ gene. The poly(A) attachment site is indicated by an asterisk. The primer in the DNA binding domain, used for amplification of first strand cDNA is double underlined. The latter oligo and the reverse of the 962 to 983 sequence were used as STS for chromosomal mapping.

A human genomic library (Sau 3A partial digest in EMBL3) was screened with the C/EBPδ cDNA fragment as a probe. This resulted in the isolation of one clone (λ2K1), which contains the complete C/EBPδ gene (Fig.1A). The gene was characterized by restriction mapping and sequence analysis (Fig.1A,B). The human C/EBPδ turned out to be a one exon gene with a large open reading frame encoding a protein of 269 amino acids. Southern blot hybridization of genomic DNA with a human C/EBPδ probe showed the human C/EBPδ gene to be single copy (data not shown). The homology with the mouse C/EBPδ (CRP3) gene is 80% on nucleotide level and 85% on amino acid level. The homology with the rat CELF gene is 75% and 86%, respectively. When this work was in progress Kinoshita et al. reported the isolation of the human NF-IL6β gene (12). Although there is a difference of two amino acids (amino acid residues 2 and 13, respectively) and a homology of only 95% in the 3' untranslated region, the NF-IL6β and the human C/EBPδ gene most probably are two different allelic forms of the same gene.

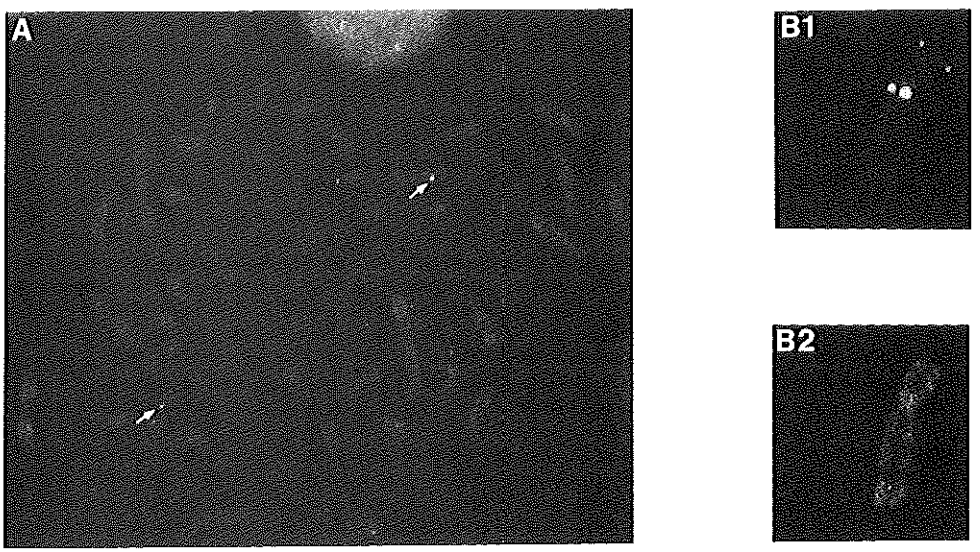

Figure II.2. (A) Competitive In Situ hybridization of human metaphase chromosome spreads with the genomic C/EBPδ clone λ2K1. The position of the fluorescently labeled C/EBPδ gene at chromosome 8 is indicated by an arrow. (B) Comparison of chromosome 8 In Situ hybridization with probe λ2K1 (B1) with R-banding (B2).

To determine the chromosomal localization of the C/EBPδ gene, simultaneous fluorescent in situ hybridization (FISH) and R-banding on metaphase chromosome spreads were performed (7,8). The biotinylated genomic clone λ2K1 was used as a probe. By comparison of the FISH results with the R-banding, the human C/EBPδ gene could be assigned to chromosome 8 (Fig.2). The results are very suggestive for an 8q11 localization, very close to the centromere (Fig.2B), however, mapping to the short arm at 8p11 cannot completely be ruled out.

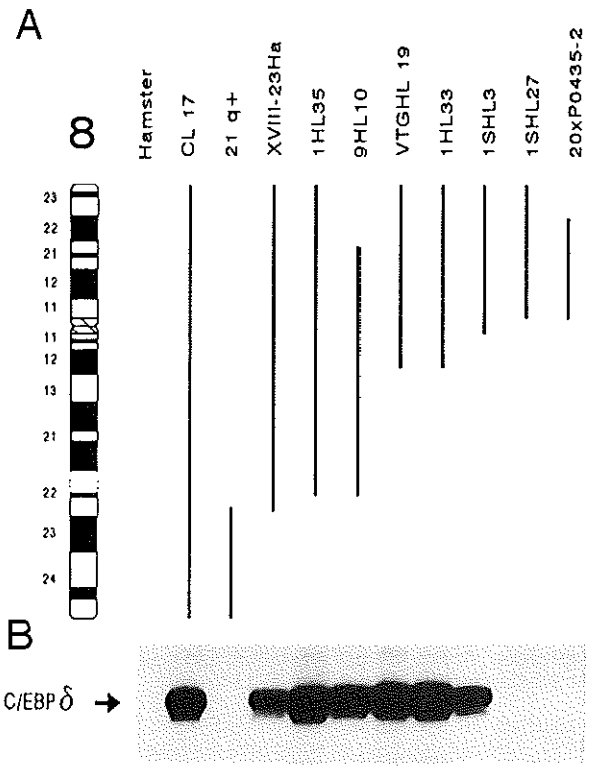

Figure II.3. Regional assignment of the C/EBPδ gene, using a human x hamster somatic hybrid cell panel. (A) Schematical representation of the chromosome 8 fragments present in the mapping panel (see also ref. 23). (B) Mapping of the C/EBPδ genomic fragment on the chromosome 8 panel. Radioactive PCR and PAGE analysis of the amplified C/EBPδ fragment were essentially as described (19).

To confirm the chromosomal localization, and to determine in more detail the regional position of the human C/EBPδ gene at the chromosomal map, a panel of human x hamster somatic cell hybrids, containing chromosome 8 as the single human chromosome (CL17), or chromosome 8 fragments (see Fig.3 and ref.23) was analyzed with a C/EBPδ specific STS (see legend to Fig.1). The results obtained are summarized in Figure 3. As expected, CL17 gave a positive signal of the correct size. All data obtained with hybrids containing chromosome 8 fragments are in agreement with the assignment of the C/EBPδ gene to the pericentromeric region of chromosome 8. Most importantly, the amplified C/EBPδ fragment was present in clone 1SHL3 and absent in 1SHL27 and 20xPO435-2 (Fig.3B). This positions the C/EBPδ gene between MOS at 8q11 and PLAT, which has been mapped to 8p12-q11.2 (23, and refs therein). The pericentromeric localization of the C/EBPδ gene links it to the autosomal dominant retinitis pigmentosa locus (RP1) (3).

Two other C/EBP genes, C/EBPα and DBP (15) are located at human chromosome 19 (10,20). The C/EBPβ gene is assigned to human chromosome 20 (10,21). So, with possibly one exception, the C/EBP gene family, as mapped so far, is scattered over the human genome. The murine C/EBPδ gene has recently been linked to chromosome 16 (cited in 24). The mouse MOS locus is at chromosome 4 (17); PLAT at chromosome 8 (5). This shows that the pericentromeric region of human chromosome 8 (8p11-q11) is not conserved in one specific mouse chromosomal segment.

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Chapter III

TWO ANDROGEN RESPONSE REGIONS COOPERATE IN STEROID HORMONE REGULATED ACTIVITY OF THE PROSTATE-SPECIFIC ANTIGEN PROMOTER

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Chapter III

SUMMARY

Transcription of the Prostate-specific Antigen (PSA) gene is androgen regulated. The PSA promoter contains at position −170 the sequence AGAACAgcaAGTGCT, which is closely related to the ARE (androgen response element) consensus sequence GGTACAnnnTGTTCT. This sequence is a high affinity androgen receptor (AR) binding site and acts as a functional ARE in transfected LNCaP cells. A 35 bp segment starting at -400 (ARR: Androgen Response Region; GTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTG) cooperates with the ARE in androgen induction of the PSA promoter. A construct with three ARR copies linked to a minimal PSA promoter showed a strong (104-fold), androgen induced activity. The ARR was also able to confer androgen responsiveness to a minimal thymidine kinase (TK) promoter. Both AR binding and transcriptional activity resided in a 20 bp ARR subfragment: CAGGGATCAGGGAGTCTCAC (2S). Mutational analysis indicated that the sequence GGATCAgggAGTCTC in the 2S fragment is a functionally active, low affinity AR binding site. Like AR, the glucocorticoid receptor (GR) was able to stimulate PSA promoter activity. Both the ARE and ARR are involved in dexamethasone regulatin of the PSA promoter. Both the AR and GR promoter constructs were 20- to 100- fold more active on ARR-PSA and ARR-TK promoter constructs than in other cell types (COS, HeLa, Hep3B and T47D cells), indicating (prostate) cell specificity.

INTRODUCTION

Androgens exert their function via the intracellular androgen receptor (AR), which is a member of the family of the steroid hormone receptors (see for reviews 1,2). Upon ligand binding, steroid receptors interact with specific DNA sequences and regulate the transcriptional activity of target genes (1-3). The DNA structures to which steroid receptors bind with high affinity are imperfect palindromic sequences, separated by a 3 bp spacer. The consensus sequence of the response element for the glucocorticoid receptor (GR), progesterone receptor (PR) and AR is identical: GGTACAnnnTGTTCT (HRE: hormone response element) (1-6). However, in natural target genes, the binding site can deviate considerably from the consensus sequence. Analysis of natural and synthetic promoters has shown that steroid receptors are able to act synergistically with a variety of other transcription factors (2,7-9). Synergistical interaction of two HREs has also been observed (7,8). At present it is unclear how specificity of the AR/GR/PR response operates. Several mutually not exclusive mechanisms have been proposed, including subtle differences in sequences flanking the HRE, differences in affinity to general or specific transcription factors, interaction with specific accessory proteins, or differences in cellular concentration of the specific receptors and ligands.

We use the Prostate-specific Antigen (PSA) gene as a model for androgen regulated gene expression. PSA is expressed at high level in the luminal epithelial cells of the prostate, and is absent or expressed at very low levels in other tissues. PSA is a member of the human kallikrein gene family, and is well known as a prostate-specific tumor marker (10,11). Further members of the kallikrein gene family are the hGK-1 gene and the tissue kallikrein gene (KLK-1) (12-15). The three genes are clustered in an area of 60 kb on chromosome 19q13.2-13.4 (15-18). In previous studies we and others have shown that PSA mRNA

expression is androgen-regulated (19-22). Androgen-stimulated expression of PSA is at least partially regulated at the level of transcription (22,23). A functional ARE (androgen responsive element) was defined at −170 which closely resembles the ARE consensus sequence (23).

In the present study we address the following questions: (i) which PSA promoter elements, additionally to the ARE (−170) are the major contributors to its androgen regulation, (ii) is PSA promoter regulation AR specific, and (iii) is steroid hormone regulation of the PSA promoter cell type specific.

MATERIALS AND METHODS

Cell culture

LNCaP cells were cultured in RPMI 1640, supplemented with 5 % fetal calf serum (Boehringer, Mannheim, Germany) and antibiotics. For transfection, cells were grown in Dulbecco's Modification of Eagle's Medium supplemented with 5 % steroid-depleted (dextran-charcoal treated) fetal calf serum. For examination of androgen-driven promoter activation by transfection, the synthetic androgen R1881 (New England Nuclear, Boston, MA) was added to a final concentration of 1 nM. For examination of glucocorticoid-regulated activation, a final concentration of 10 nM of the synthetic glucocorticoid dexamethasone (Sigma, St. Louis, MO) was used.

Construction of plasmids

All plasmid constructs were prepared using standard methods (24). The human AR expression plasmid pAR0, the rat GR expression plasmid PSTC GR 3-795 and the plasmid pRIT2TAR to produce AR(DBD) were described previously (25-27). The promoter-less basic plasmid pLUC, which was used for cloning of PSA promoter fragments in front of the LUC reporter gene, was derived from pSLA3 (28) by insertion of an oligonucleotide containing a multiple cloning site (MCS) (HindIII, PvuII, NheI, BglII and NcoI) in the HindIII and NcoI sites of PSLA3. PSA-LUC constructs, PSA-4-LUC (EcoRI/HindIII; -632/+12), PSA-5-LUC (BglII/HindIII, -539/+12), PSA-6-LUC (XhoII/HindIII; −324/+12) and PSA-7-LUC (NheI/HindIII; -157/+12) were generated by ligation of the appropriate fragments in the MCS of pLUC.

Constructs PSA-8 to PSA-11-LUC were obtained by exonuclease III digestion of PSA-2-CAT (-632/ +12)(23) from the SalI site. After exonuclease III incubation according to the 'Erase a base' protocol (Promega, Madison, WI), the plasmid was digested with HindIII and the derived promoter fragments were ligated in the MCS of pLUC. This resulted in the constructs PSA-8-LUC, starting at -488, PSA-9-LUC, starting at -456, PSA-10-LUC starting at -395 and PSA-11-LUC, starting at -376. Construct PSA-12-LUC was prepared by introduction of a PstI site at position -174 by PCR. The PCR product was digested with PstI and HindIII (+12) and isolated from a 1.5 % agarose gel. The isolated fragment was ligated in the PstI and HindIII sites of the pLUC MCS. One and three copies of the -400 to −366 oligomer were cloned in front of the PSA-12-LUC and the TK-LUC construct. Double-stranded oligonucleotides spanning ARR (-400 to -366) 5'GATCCGTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTG 3' were inserted in the BamHI site of PSA-12-LUC and TK-LUC. Double stranded oligonucleotides spanning three copies of the ARR-1S; GTGGTGCAGGGATCAGGGAG, ARR-2S;

CAGGGATCAGGGAGTCTCAC, ARR-3S region; GAGTCTACAATCTCCTG and the ARR-2S mutants (mutations are underlined) ARR-2S-1; CAGGGGATGAGGGAGTCTCAC, ARR-2S-2; CAGGGATCAGGGACTCTCAC and ARR-2S-3; CAGGGATCAGCGAGTCTCAC, containing SalI compatible ends were inserted in the SalI site of PSA-12-LUC. All constructs were verified by sequencing.

Transfections

LNCaP cells were transfected according to the calcium phosphate precipitation method essentially as described (29), using 1 x 10^6 cells per 25cm^2 flask, 5 μg of the appropriate PSA-LUC construct, and where indicated 2.5 μg pAR0 or PSTC GR 3-795 (GR expression vector). After overnight incubation with the precipitate, the culture medium was removed and replaced by phosphate buffered saline (PBS), containing 15% glycerol (incubation for 90 sec at room temperature). Subsequently, transfected cells were incubated in culture medium in the absence or presence of the appropriate hormone (R1881 or dexamethasone) for at least 24 h. Transfections were performed at least three times in duplicate, using at least two independent plasmid isolates.

Luciferase activities were corrected for variations in protein concentrations within the 100 μl cell extracts. Luciferase activities and relative induction factors are expressed as mean and standard error of the mean (SEM).

Luciferase assay

Cells were washed once with PBS and lysed in 300 μl lysis buffer (25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1 % Triton X-100, 15 % glycerol). Next, 100 μl Luciferin (0.25 μM) (Sigma)/ 0.25 μM ATP was added to 100 μl of each extract, and luciferase activity was measured in a LUMAC 2500 M Biocounter (LUMAC, Landgraaf, The Netherlands). After a delay of 2 sec (according to supplier), the light emission during 5 sec was recorded.

Gel retardation analysis

Nuclear extracts were prepared as described by Dignam et al. (30). Coupled transcription/translation of human AR cDNA cloned in BluescriptII-KS (31) was carried out according to the protocol of the manufacturer of the system (Promega). AR synthesis was in the presence of 10 μM $ZnCl_2$. Production in E. coli, and purification of AR(DBD) was done as described previously (27).

Double stranded oligonucleotide probes used in gel retardations:

```
ARE:      5' GATCCTTGCAGAACAGCAAGTGCTAGCTG     3'
               GAACGTCTTGTCGTTCACGATCGACCTAG

ARR:      5' GATCCGTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTG     3'
               GCACCACGTCCCTAGTCCCTCAGAGTGTTAGAGGACCTAG

ARR-1S:   5' GATCCGTGGTGCAGGGATCAGGGAG     3'
               GCACCACGTCCCTAGTCCCTCCTAG

ARR-2S:   5' GATCCAGGGATCAGGGAGTCTCACG     3'
               GTCCCTAGTCCCTCAGAGTGCCTAG

ARR-3S:   5' GATCCGAGTCTCACAATCTCCTGAG     3'
               GCTCAGAGTGTTAGAGGACTCCTAG
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ARR-2S-1:   5' GATCCAGGGATGAGGGAGTCTCACG      3'
                   GTCCCTACTCCCTCAGAGTGCCTAG

ARR-2S-2:   5' GATCCAGGGATCAGGGACTCTCACG      3'
                   GTCCCTAGTCCCTGAGAGTGCCTAG

ARR-2S-3:   5' GATCCAGGGATCAGCGAGTCTCACG      3'
                   GTCCCTAGTCGCTCAGAGTGCCTAG

ARR-2S-4:   5' GATCCAGGGATCAGGGAGTTCCACG      3'
                   GTCCCTAGTCCCTCAAGGTGCCTAG

ARR-2S-5:   5' GATCCAGGGAACAGGGTGTTCCACG      3'
                   GTCCCTTGTCCCACAAGGTGCCTAG
```

Probes were filled in with MMLV-reverse transcriptase in the presence of α-^{32}P-dATP, and subsequently isolated from non-denaturing polyacrylamide gel. For gel retardation assays, 20-50x10^3 cpm of each probe was added to 20 μl reaction mixture, containing 2 μg poly dIdC, 2 μg BSA, 10 μM $ZnCl_2$, 1 mM DTT and 2 μl 10x binding buffer (100 mM Hepes pH 7.6, 300 mM KCl, 62.5 mM $MgCl_2$ and 30 % glycerol), and in indicated cases 10 μg LNCaP nuclear protein, in vitro translated AR (7-10 fmol) or AR(DBD) (5 pmol). In experiments using the AR antibody Sp197 (epitope amino acid residues 1-20), 0.1 μl portions of antiserum were added to the reaction mixture (32). Incubation was for 30 min at RT. In addition to oligonucleotides described above, (100-fold excess of) double-stranded oligonucleotides containing a C/EBP binding site, 5'-GACCTTACCACTTTCACAATCTGCTAG-3' (33) and a GRE 5'-TCGACTGTACAGGATGTTCTAGCTACT-3' (Promega) were used in competition experiments. Samples were loaded on a 4 % polyacrylamide (19:1) gel and electrophoresed in a 50 mM Tris.HCl, 41.5 mM Boric acid, 0.5 mM EDTA buffer for 2 h at 150 V and RT. Subsequently, gels were fixed, dried and exposed to X-ray film.

RESULTS

Deletion mapping of the PSA promoter; effect of androgen receptor overexpression

In a previous study we analyzed androgen regulation of the PSA promoter in COS cells which were co-transfected with several different PSA promoter-chloramphenicol acetyltransferase (CAT) reporter gene constructs and the AR expression vector pAR0 (23). This resulted in the functional characterization of an ARE (AGAACAgcaAGTGCT), which is closely related to the consensus sequence, at position -170, and the identification of a second region, from -539 to -324, important for PSA promoter activity.

Essentially the same data were obtained in LNCaP cells which endogenously express the AR and PSA gene (Figure 1A). Transfection of PSA-4-LUC (−632/+12) or PSA-5-LUC (-539/+12) resulted in an approx. 7-fold higher LUC activity in the presence of R1881 than in its absence. Similar results were found with constructs containing longer promoter fragments [up to approx. 4 kb (data not shown)]. Removal of the -539 to −324 fragment (PSA-6-LUC) caused a 3.5-fold drop in relative induction. Subsequent removal of the -324 to -157 region (containing the ARE sequence at position -170), resulted in the complete abolishment of androgen induction (construct PSA-7-LUC).

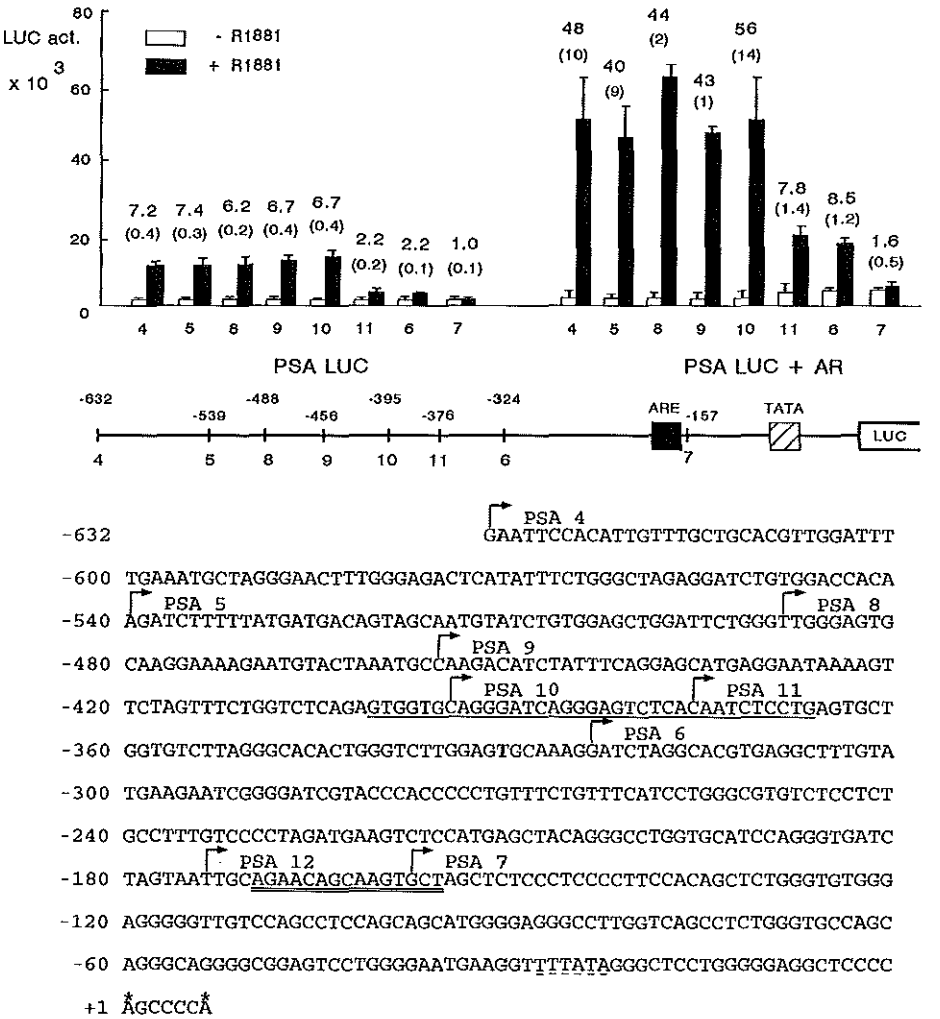


Figure III.1. Androgen regulation of the PSA promoter in LNCaP cells. (A) LNCaP cells were transfected with PSA-LUC constructs (left panel) and with PSA-LUC constructs plus the AR expression plasmid pAR0 (right panel) as described in Materials and Methods. Start positions of PSA promoter constructs are indicated below the figure and in Figure 1B. After overnight incubation with the precipitate, transfected cells were further incubated in the presence or absence of 1 nM R1881 for 24 h. The absolute activity and relative induction factor were calculated as the mean of five or more independent transfection experiments, which were all done in duplicate. Closed bar: Activity in the presence of R1881; open bar: activity in the absence of R1881. Hormone induction is given at the top of the bars. SEM of absolute activity is represented by a vertical stripe; SEM of induction is given in parentheses. The TATA box is represented by a hatched box; the ARE by a black box. (B) Sequence of the proximal PSA promoter. Transcription start sites are indicated by asterisks. Arrows indicate starting points of constructs used in transfections. The ARE sequence starting at position -170 is double underlined, the ARR(-400) is single underlined and the TATA box is indicated by stripes.

To investigate in more detail sequences in the -539 to −324 region important for PSA promoter activity, a series of exonuclease III deletions was generated (see Figure 1B for PSA promoter fragments in different constructs). Transfection of LNCaP cells with constructs PSA-8-LUC, PSA-9-LUC and PSA-10-LUC resulted in a high (6- to 7-fold), androgen-regulated PSA promoter activity, which is comparable to that of the PSA-5 construct. Importantly, construct PSA-11-LUC, starting at −376, showed a low (2.2-fold), androgen-induced activity, which is identical to that of PSA-6 (starting at −324). These results indicated sequences in the region -395 to -376 to be essential for high androgen-induced PSA promoter activity.

Co-transfection with the AR expression plasmid pAR0 resulted in considerable higher PSA promoter activity, both in absolute values as well as in relative induction levels without affecting the significance of the -324 to -157 region containing the ARE at position -170, and the -395 to −376 region (Figure 1A). The co-transfection experiments showed again that truncation of the promoter from -395 to -376 gives rise to a markedly lower androgen-induced activity (compare PSA-10 and PSA-11); deletion of the -324 to -157 region again resulted in a complete loss of androgen inducibility of the PSA promoter (compare PSA-6 and PSA-7).

Mutations in the ARE at -170 in construct PSA-4-LUC resulted in an almost complete inhibition of androgen activation of the PSA promoter (23, and data not shown), indicating cooperativity between the -395 to -376 region and the ARE sequence at position -170.

Androgen receptor binding to the ARE(-170) motif

To confirm that the sequence AGAACAgcaAGTGCT (-170) was able to interact directly with the AR, gel retardation experiments were done with LNCaP nuclear extracts, in vitro synthesized AR, and AR DNA-binding domain [AR(DBD)] produced in E. coli (Figure 2A-C). Using LNCaP nuclear extract, addition of an AR-specific antibody (Sp197) to the incubation mixture resulted in the visualization of a stable, specifically supershifted AR-ARE(-170) complex (compare Figure 2A, lanes 1-4). The retarded complexes visible in the absence of antibody Sp197 (lane 1) are considered to be non-specific because a 100-fold molar excess of unlabelled ARE(-170) did not inhibit the formation of these complexes (lane 3). Additionally, none of these bands was supershifted after adding the specific AR antibody (lane 2).

A specific AR-ARE(-170) retarded complex could be observed, if in vitro synthesized AR was used, addition of the Sp197 antibody again resulted in the formation of a much more stable supershifted complex (Figure 2B). Application of the AR(DBD) in the gel retardation assay revealed a clear AR(DBD)-ARE(−170) complex in the absence of antibody (Figure 2C).

Effect of the - 400 to -366 fragment (ARR) on basal PSA and TK promoter activity

To further elucidate the properties of the -395 to -375 region, one and three copies of an oligonucleotide, spanning this region [-400 to -366; GTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTG (denoted ARR, androgen response region) underlined in Figure 1B] were inserted in front of a "minimal" PSA promoter, starting at position -174. The "minimal" PSA promoter (PSA-12-LUC), which contains the TATA-box and ARE(-170), was approx. 6 times more active in R1881-treated, pARO co-transfected LNCaP cells than in the absence of hormone (Figure 3A). Constructs PSA-12.1s-LUC and PSA-12.1as-LUC, containing one ARR copy in sense and anti-sense orientation,

respectively, gave rise to an increased R1881-induced activity of the promoter (22- to 23-fold induction). PSA-12.3-LUC, with three ARR copies, showed a 185-fold higher promoter activity in the presence of R1881, clearly indicating cooperativity between the ARR motifs. Importantly, even three ARR copies did not affect basal PSA promoter activity in the absence of hormone. This suggested that ARR activity could be directly androgen regulated.

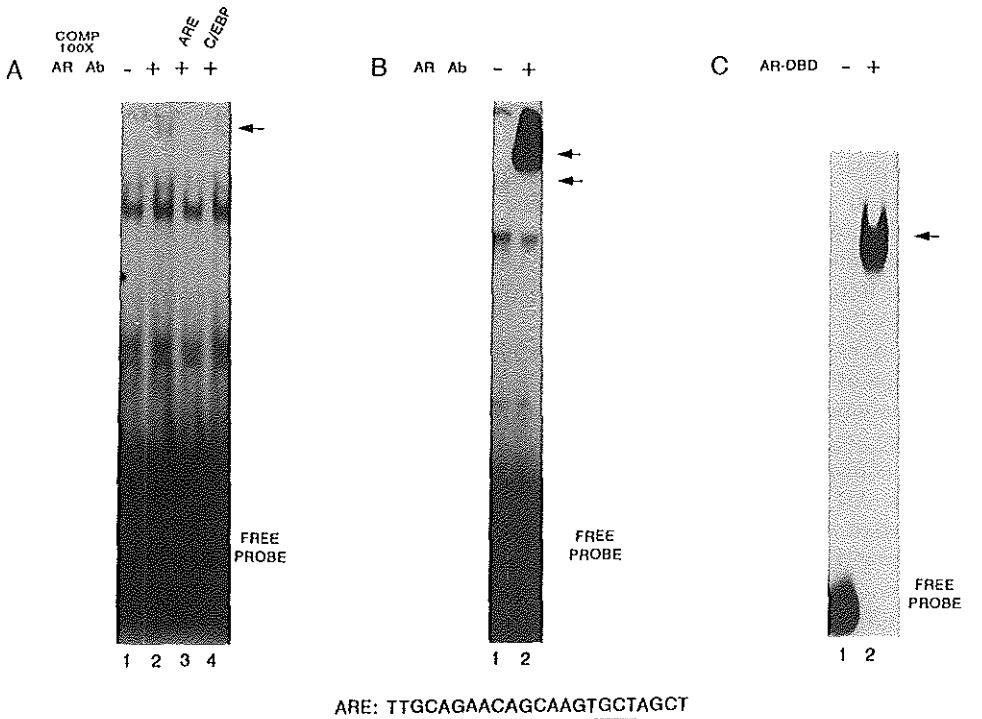


Figure III.2. Gel retardation analysis of AR/ARE(-170) complexes. (A) 20x10³ cpm ARE(-170) was incubated with LNCaP nuclear extract, and complexes were analyzed by PAGE as described in Materials and Methods. Incubations were in the absence (lane 1) or in the presence of antibody Sp197 (lanes 2-4). Incubation of ARE with Sp197, without nuclear extract did not give rise to a complex (data not shown). Specific, supershifted AR/ARE complexes are indicated by an arrow (lanes 2 and 4). Sequences of the oligonucleotides containing the ARE(-170) and the non-specific C/EBP competitor (lane 4) are given in Materials and Methods (see for the -170 ARE also the underlined sequence below the figure). (B) 40x10³ cpm ARE(-170) was incubated with in vitro synthesized full length AR, and AR/ARE(-170) complexes were analyzed by PAGE as described in Materials and Methods. Lane 1 and 2: ARE with reticulocyte lysate, containing in vitro synthesized AR. Incubations were in the absence (lane 1) and in the presence of antibody Sp197 (lane 2). Arrows indicate the position of the AR/ARE complex (lane 1) and supershifted AR/ARE complex (lane 2). (C) Gel retardation analysis of AR(DBD)/ARE(-170) complexes. Lane 1: Free ARE(-170) probe (50x10³ cpm). The arrow indicates the position of the AR(DBD)/ARE complex. Lane 2: 50x10³ cpm ARE(-170) was incubated with AR(DBD) expressed in E. coli, and purified as described in Materials and Methods. Subsequently, the reaction mixture was analyzed by PAGE.

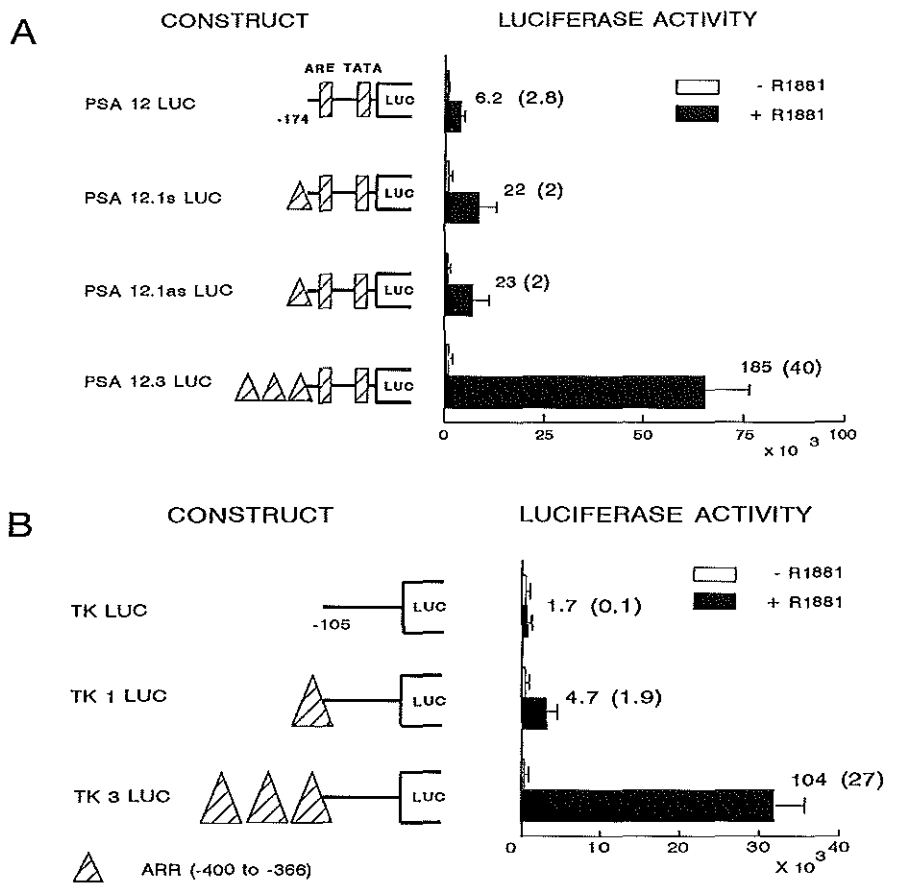


Figure III.3. (A) Effect of the -400 to -366 region (ARR) on basal PSA promoter activity in LNCaP cells overexpressing the AR. The ARR is represented by a hatched triangle, the ARE by a black box, and the TATA element by a hatched box. (B) Effect of the −400 to -366 region of the PSA promoter (ARR) on TK promoter activity in LNCaP cells overexpressing AR. The ARR is represented by a hatched triangle. The mean of luciferase activity and relative induction levels are from four independent, duplicate experiments. Experimental details are identical to those described in Figure 1A. The ARR sequence is single underlined in Figure 1B.

To investigate this further, one or three ARR copies were inserted in front of a minimal Thymidine Kinase (TK) promoter linked to the LUC gene and co-transfected with pAR0 to LNCaP cells. Basal TK promoter activity was hardly inducible by androgen (Figure 3B). The construct containing one ARR (TK-1-LUC) showed a 4.7-fold higher LUC activity in the presence of R1881 than in the absence of hormone. TK-3-LUC (with three ARR copies) showed a 104-fold induction. In the absence of hormone, the ARR-TK-LUC reporters had the same activity as the TK-LUC basal construct.

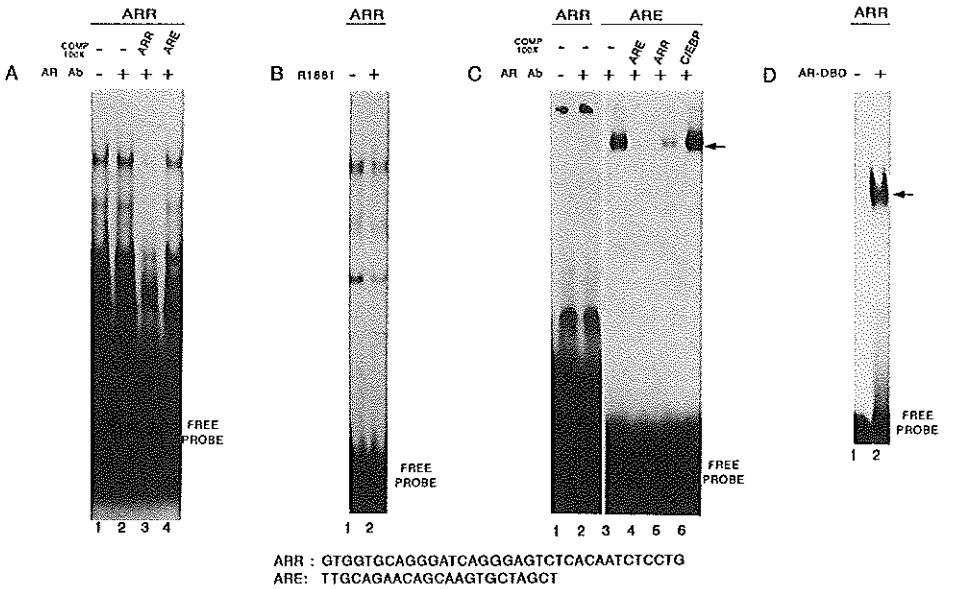


Figure III.4. Gel retardation analysis of AR/ARR complexes. (A) 40x10³ cpm ARR was incubated with LNCaP nuclear extract, and complexes were analyzed as described in Materials and Methods. Complex formation was in the absence (lane 1) or in the presence of antibody Sp197 (lanes 2-4). Incubation of ARR with Sp197, without nuclear extract did not give rise to a retarded band (data not shown). Competition experiments were in the presence of a 100-fold excess ARR(-400) (lane 3) and ARE(-170) (lane 4). ARR and ARE sequences are presented below the figure. (B) 40x10³ cpm ARR was incubated with nuclear extract of LNCaP cells grown in steroid depleted medium for 6 days and an additional 24 h in the presence (lane 2) or absence (lane 1) of 1 nM R1881. (C) 20x10³ cpm ARR was incubated with in vitro synthesized full length AR, in the absence (lane 1) and in the presence of antibody Sp197 (lane 2), and subsequently analyzed by PAGE as described in Materials and Methods. Lanes 3 to 6: 40x10³ cpm ARE incubated with in vitro synthesized AR. Complexes were analyzed by gel retardation assay as described in Materials and Methods. Incubations were in the presence of antibody Sp197. Competition was with 100-fold excess ARE(-170) (lane 4), ARR (lane 5), C/EBP (lane 6). The arrow indicates the position of supershifted AR/ARR and AR/ARE complexes. (D) Gel retardation analysis of AR(DBD)/ARR complexes. Lane 1: Free ARR probe. Lane 2: 50x10³ cpm ARR was incubated with AR(DBD) expressed in E.coli and purified as described in Materials and Methods and subsequently analyzed by PAGE. The arrow indicates the position of the AR(DBD)/ARR complex.

Gel retardation analysis of the androgen response region (ARR)

To find out whether the AR could directly interact with the ARR(-400 to -366), a series of in vitro protein-DNA interaction experiments was carried out. First of all, nuclear extract of LNCaP cells grown in the presence of R1881 was used to study proteins interacting with the ARR. Gel retardation analysis with this extract revealed the presence of at least three

specific complexes (Figure 4A, lanes 1-3). Incubation with antibody Sp197, did not result in a visible supershift [Figure 4A, lane 2; compare Figure 2A for ARE(-170) shifts], nor could one of the complexes be competed with an excess of unlabeled ARE(-170) (Figure 4A, lane 4). Identical complexes were formed with extracts from cells grown in the absence or in the presence of hormone, indicating that the expression or activity of none of the proteins visualized in Figure 4B is androgen regulated.

If in vitro synthesized AR was used in AR-ARR gel retardation assays, in the presence of Sp197 a very weak retarded complex could be detected (Figure 4C), suggesting the presence of a low affinity AR binding site in the ARR. In agreement with this observation, a 100-fold excess ARR could partially compete the formation of an AR-ARE(-170) complex (Figure 4C, lanes 3-6). A similar excess of unlabeled ARE completely inhibited the formation of the AR-ARE complex. The unrelated C/EBP oligo had no effect on AR-ARE complex formation. The most sensitive assay, gel retardation with AR(DBD) produced in E. coli clearly revealed the formation of an AR(DBD)-ARR complex (Figure 4D).

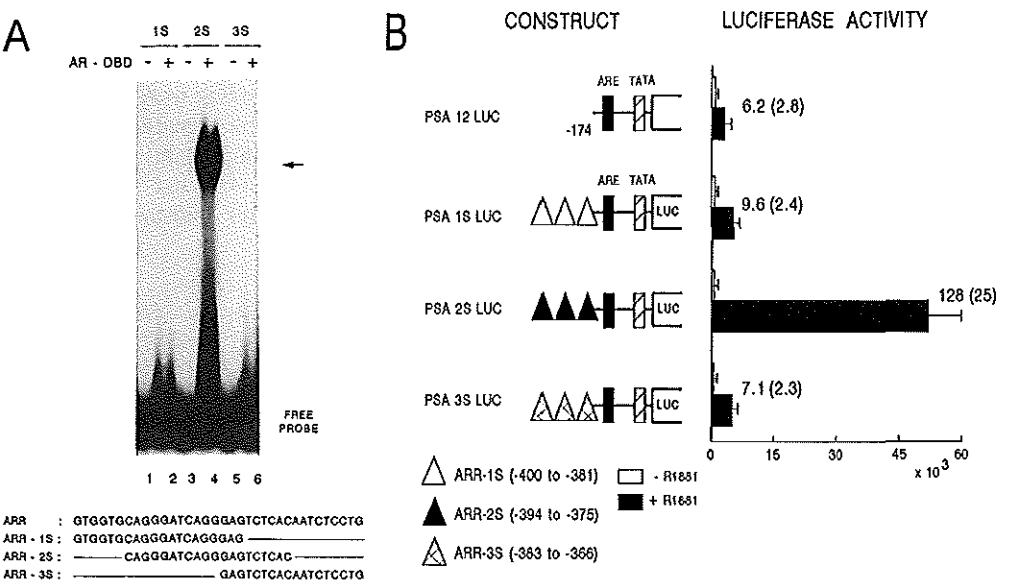
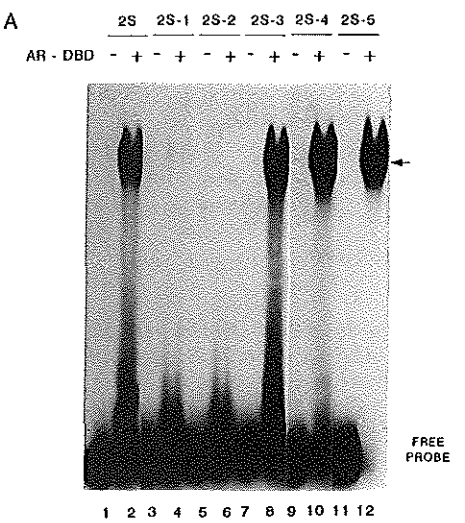


Figure III.5. Analysis of the AR binding segment in ARR. (A) Gel retardation analysis of AR(DBD)/ARR subfragment complexes. 50x10^3 cpm ARR-1S (lanes 1,2), -2S (lanes 3,4) and -3S (lanes 5,6) were incubated with AR(DBD) and analyzed by gel retardation assay as described in Materials and Methods. 1S, 2S and 3S sequences are shown below the figure. The arrow indicates the position of the AR(DBD)/ARR-2S complex. (B) Effect of the ARR-1S (-400 to -381), -2S (-394 to -375) and -3S (-383 to -366) on PSA basal promoter activity in LNCaP cells overexpressing the AR. The ARR-subfragments are represented by triangles, the ARE(-170) by a black box and the TATA box by a hatched box. Mean values and SEM are from three independent, duplicate experiments. Further experimental details are identical to those described in Figure 1A.

Next, three copies of 1S, 2S and 3S were inserted in front of the minimal PSA promoter construct PSA-12-LUC and co-transfected with pAR0 to LNCaP cells. The construct containing three copies of the 1S region (PSA-1S-LUC) gave rise to a 9.6-fold higher activity in the presence of R1881 than in the absence (Figure 5B). Construct PSA-2S-LUC, containing three copies of 2S gave rise to a 128-fold higher activity upon R1881 treatment. The construct with three copies of 3S (PSA-3S-LUC) produced a 7.1-fold higher activity in the presence of R1881. So, there is complete concordance between the presence of AR binding and functional, hormone dependent enhancer activity in 2S, and the absence of these activities in 1S and 3S.

Analysis of the AR binding site in ARR-2S

Sequence alignment showed that in 2S the highest percentage of homology to the ARE consensus sequence is in the sequence GGATCAgggAGTCTC. This sequence deviates in 2 out of 6 most essential positions, (positions 2,3 and 5 in each half-site, underlined) and overall in 6 out of 12 positions from the ARE consensus GGTACAnnnTGTTCT. To test whether this sequence could indeed be responsible for low affinity AR binding, gel retardation analyses were performed with five ARR-2S mutants (mutations are underlined). Two mutants expected to decrease AR affinity to the putative AR binding site ARR-2S-1 (GGATGAgggAGTCTC) and ARR-2S-2 (GGATCAgggACTCTC) and one presumed silent mutant ARR-2S-3 (GGATCAgcgAGTCTC) were tested for their AR binding capacity (Figure 6A). Gel retardation experiments confirmed our hypothesis: AR binding to ARR-2S-1 and ARR-2S-2 was almost completely abolished; ARR-2S-3 did not show a marked difference in AR affinity as compared to ARR-2S. AR(DBD) gel retardation with mutants with a higher homology to the consensus ARE sequence (ARR-2S-4 and ARR-2S-5: GGATCAgggAGTTCC and GGAACAgggTGTTCC, respectively) substantiated these findings.



ARR - 2S : CAGGGATCAGGGAGTCTCAC
ARR - 2S-1 : G
ARR - 2S-2 : C
ARR - 2S-3 : C
ARR - 2S-4 : TC
ARR - 2S-5 : A T TC

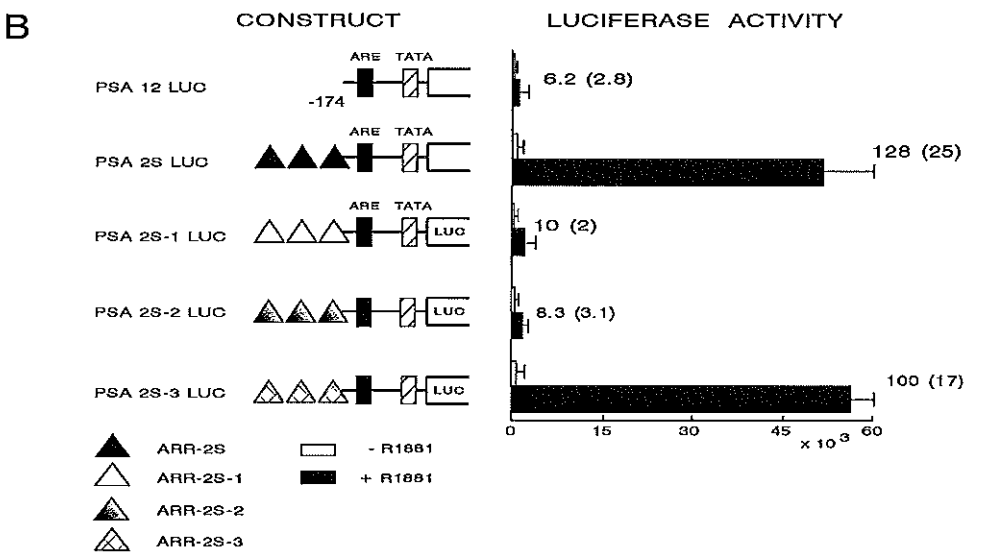


Figure III.6. Mutation analysis of the ARR-2S segment. (A) Gel retardation analysis of ARR-2S and ARR-2S mutants with AR(DBD). 50x10³ cpm ARR-2S (lanes 1,2) ARR-2S-1 (lanes 3,4), −2S-2 (lanes 5,6) -2S-3 (lanes 7,8), -2S-4 (lanes 9,10) and −2S-5 (lanes 11 and 12) were incubated with AR(DBD) and analyzed by gel retardation assay as described in Materials and Methods. Sequences of ARR-2S and ARR-2S mutants are shown below the figure. The arrow indicates the position of the AR(DBD)/probe complex. (B) Effect of the ARR-2S-1, -2S-2 and −2S-3 sequences on PSA basal promoter activity in LNCaP cells overexpressing the AR. ARR-2S and mutant ARR-2S oligo's are represented by triangles, the ARE(-170) by a black box and the TATA box by a hatched box. Mean values of luciferase activity and induction levels and their respective SEM are from three independent, duplicate experiments. Experimental details are as described in Figure 1A. Closed bar: Activity in the presence of R1881 (1 nM); Open bar: activity in the absence of hormone.

Next, mutated 2S-elements were tested in LNCaP cells for enhancer activity. To this end, three of the mutant oligonucleotides, 2S-1, 2S-2 (both abolishing AR-binding) and 2S-3 (spacer mutation), were cloned in front of the minimal PSA promoter construct PSA-12-LUC. PSA-2S-1-LUC and PSA-2S-2-LUC were hardly more active than PSA-12-LUC upon R1881 induction in AR co-transfected LNCaP cells (Figure 6B). Construct PSA-2S-3-LUC showed a 100-fold higher activity in the presence than in the absence of R1881. These data strongly suggest the importance of the GGATCAgggAGTCTC motif in androgen-regulated activity of the ARR in the PSA promoter.

Effect of glucocorticoid receptor overexpression on PSA promoter activity

Because on the one hand the DNA motif for high affinity AR and GR binding is identical and on the other hand AR and GR might show specificity on individual promoters, we investigated whether GR was able to mediate PSA promoter activity. LNCaP cells were co-transfected with the GR expression plasmid PSTC-GR and selected PSA-LUC constructs. Without GR co-transfection, no response of the different PSA promoters to dexamethasone was observed (data not shown). As depicted in Figure 7A, the pattern of PSA promoter activity induced by dexamethasone in the presence of GR turned out to be identical to AR

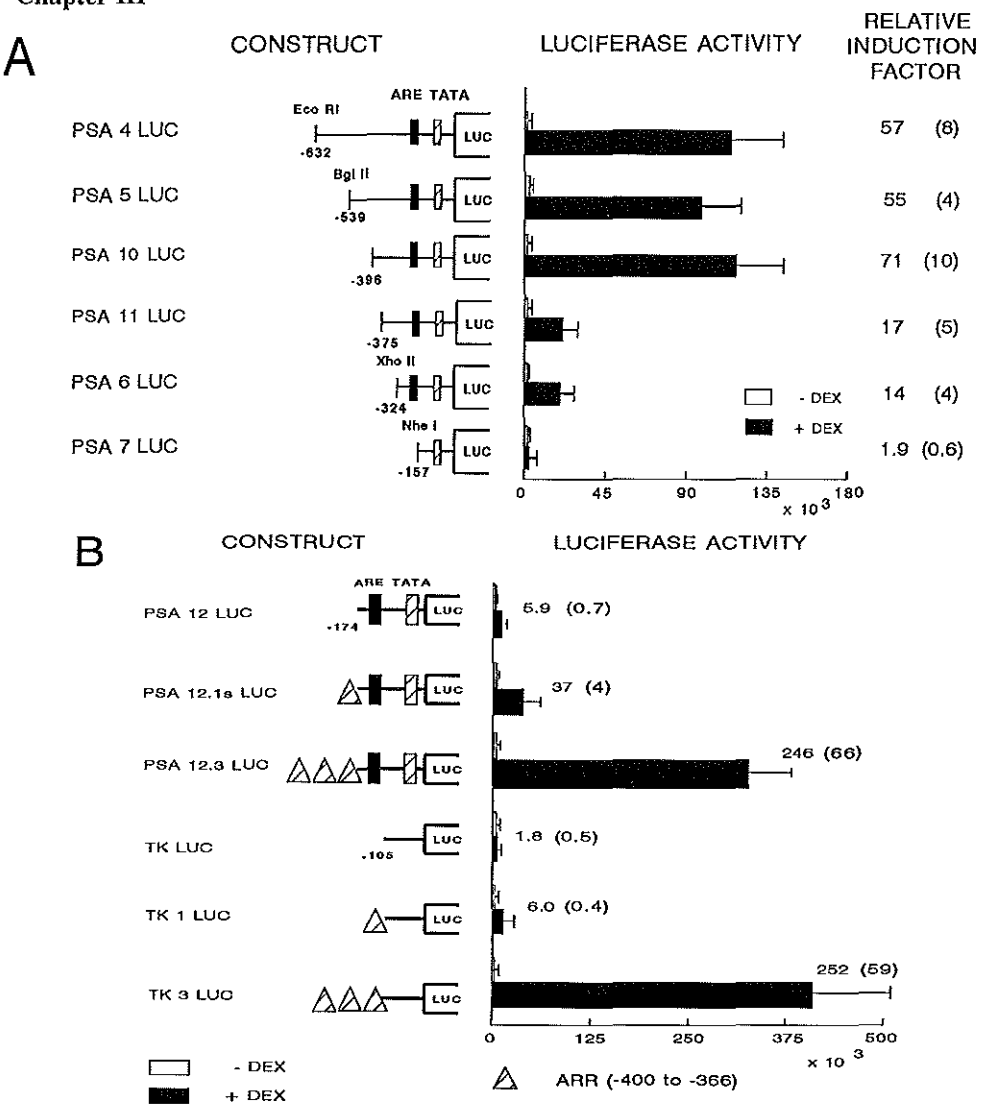


Figure III.7. Effect of glucocorticoid receptor overexpression on PSA promoter activity in LNCaP cells. (A) LNCaP cells were transfected with PSA promoter constructs in the presence of the GR expression plasmid PSTC-GR (see Materials and Methods for experimental details). After transfection, cells were cultured in the presence or absence of 10 nM dexamethasone. Closed bar: activity in the presence of dexamethasone; open bar: activity in the absence of hormone. Induction value of the various promoter constructs is indicated to the right of each black bar. Absolute luciferase activity and induction values are the means of at least four independent experiments performed in duplicate. SEM of luciferase activity is given by the horizontal bar; SEM of induction factor is given in parentheses. (B) Effect of the -400 to -366 region (ARR) on minimal PSA and TK promoter activity in LNCaP cells overexpressing the GR. The ARR is represented by a hatched triangle, the ARE by a black box and the TATA box by a hatched box. Further experimental details are as described in Figure 7A.

mediated activity (compare with Figure 1A): the ARE at position -170 could function as a GRE (glucocorticoid response element) and the ARR(-400 to -366) was needed for maximal dexamethasone inducibility.

Co-transfection of LNCaP cells with PSTC-GR and the constructs containing one and three copies of the ARR(-400) linked to the minimal PSA promoter or TK promoter showed a very strong synergistic, dexamethasone induced activity (Figure 7B). So, the GR seems to act exactly identical to the AR in activation of the PSA promoter. In fact, under the conditions used, the GR is an even more potent stimulator of PSA promoter activity than the AR.

Cell specificity of ARR activity

To investigate whether or not steroid hormone induced activity of ARR(-400) was LNCaP specific, PSA-12-LUC and TK-LUC constructs (see Figures 3 and 7B) and pAR0 or PSTC-GR were co-transfected to several non-prostate cell lines: COS, HeLa, Hep3B and T47D. In the non-prostate cell lines, the minimal PSA promoter construct PSA-12-LUC containing the ARE(-170), is at least 2.5-fold less induced by R1881 and dexamethasone as compared to LNCaP cells. Essentially identical results were obtained in comparising AR and GR induction of ARR-PSA and ARR-TK constructs. Both activated receptors were able to induce these promoters far better in LNCaP cells than in other cell types (AR/PSA-12.3-LUC: 42- to 97- fold; GR/PSA-12.3-LUC: 26- to 48-fold; AR/TK-3-LUC: 18- to 54- fold; GR/TK-3-LUC: 27- to 78-fold). Our data indicate that LNCaP cells contain one or more factors, which affect PSA minimal promoter activity and ARR(−400) activity. It is tempting to speculate that this factor(s) directly or indirectly interacts with the steroid receptors bound to its response elements.

Table III.1. Effect of R1881 (A) and dexamethasone (B) on ARR(-400) PSA promoter and ARR(-400) TK promoter activity in COS, HeLa, Hep3B, T47D and LNCaP cells co-transfected with (A) the androgen receptor expression plasmid pAR0 and (B) glucocorticoid receptor expression plasmid PSTC-GR.

CONSTRUCT	RELATIVE INDUCTION (+/- hormone)*				
	LNCaP	COS	HeLa	Hep3B	T47D
PSA-12-LUC**	4.4 ± 1.7	1.1 ± 0.1	1.1 ± 0.4	1.8 ± 0.4	1.1 ± 0.2
PSA-12.3-LUC	127 ± 28	2.0 ± 0.3	3.1 ± 0.8	3.1 ± 0.8	1.3 ± 0.2
TK-LUC	1.5 ± 0.2	1.1 ± 0.1	1.1 ± 0.3	1.1 ± 0.3	0.8 ± 0.1
TK-3-LUC	81 ± 22	2.1 ± 0.4	2.0 ± 0.9	4.4 ± 2.0	1.5 ± 0.3
PSA-12-LUC	8.9 ± 2.3	1.3 ± 0.3	3.5 ± 0.8	3.6 ± 1.2	1.8 ± 0.4
PSA-12.3-LUC	361 ± 83	9.7 ± 1.0	7.5 ± 1.3	14 ± 3.0	13 ± 4.7
TK-LUC	1.7 ± 0.4	0.8 ± 0.2	1.7 ± 0.7	1.0 ± 0.1	1.1 ± 0.1
TK-3-LUC	468 ± 101	13 ± 1.7	6.3 ± 1.9	17 ± 3.4	8.2 ± 2.6

*) Induction factor is the mean of three to seven independent, duplicate experiments ± SEM.
**) Constructs are as described in Materials and Methods.

Chapter III

DISCUSSION

In the present study, analysis of prostate-specificity and androgen-regulation of the PSA promoter is performed in LNCaP cells, the only available prostate derived cell line, that endogenously expresses the PSA gene in an androgen-dependent fashion (34). Two regions in the PSA promoter were identified, which are essential for androgen stimulation in LNCaP cells.

The first region (ARE) encompassed the imperfect palindromic sequence AGAACAgcaAGTGCT at -170 to -156 (see Figures 1B and 2). Comparison of this sequence with a GGTACAnnnTGTTCT ARE/GRE consensus sequence (4-6) revealed four deviations. However, none of these was at one of the six positions (positions 2,3 and 5 in each half-site), most critical for high affinity AR binding and/or functional activity (4,6). The ARE(-170) by itself gave rise to a weak activation of the PSA promoter (approx. 2-fold without pAR0 co-transfection). It had to cooperate with the second androgen response region: ARR (-400 GTGGTGCAGGGATCAGGGAGTCTCACAATCTCCTG -366) for high, androgen induced activity. This ARR contains a low affinity AR binding site. Results of both gel retardation and transfection experiments indicate an important role of the sequence GGATCAgggAGTCTC, which is a degenerated palindromic sequence (6 out of 12 positions are identical to the consensus ARE: GGTACAnnnTGTTCT). Low AR affinity can probably be explained by deviation from the consensus sequence at position 3 in the 5'-part (T instead of A) and at position 5 (T instead of C) in the 3'-part of the palindromic sequence.

Although several androgen-regulated genes have been described, only few functional AREs have been studied in detail. The mechanism of PSA promoter regulation by androgens seems different from other genes studied in this respect. In the MVDP gene promoter, two ARE sequences are present (35). Only the proximal ARE is functionally active, and no synergism between the two AREs was detected. In the probasin promoter, also two fragments, which are important for androgen-regulation of the promoter and which bind AR, can be found (36,37). Although both sequences are able to interact with AR outside the probasin promoter context, both AR binding sites are individually functionally inactive, even three copies of the two separated AR binding sites fail to give rise to androgen-induced reporter gene activity. This is in contrast to the AR binding sites in the PSA promoter, ARE(-170) and ARR-2S, which are clearly independently active, and when multimerized act synergistically (23, and this study).

Three candidate AREs have been found in the C3 gene; only one of them, Core II, C is functionally active in transfection experiments (38,40). Activity of Core II is strongly enhanced by surrounding sequences including candidate OCT-1 and NF−I binding sites (41).

Functional synergism between multiple ARE-like sequences and binding sites for other transcription factors has been found in the complex enhancer elements of the 20 kDa protein and Slp genes. In the androgen responsive enhancer in the promoter of the Slp gene, three tandemly repeated HRE-like sequences are present (42). Additionally, several non-receptor binding elements contribute to the characteristic androgen response of this complex enhancer (43). The first intron of the 20 kDa protein gene contains a cluster of three ARE-like half sites spanning a 39 bp fragment (N39) which shows AR binding and confers weak androgen responsivity to a heterologous promoter. Additional sequences surrounding this cluster of ARE-like sequences are needed for full activity of this enhancer (44). These additional

sequences include a region (D2) that shows high AR binding affinity and androgen induced transcriptional activity, but no candidate ARE sequences were identified. Taking into consideration the results obtained in our present study, which show that multiple weak AR binding sites can give rise to strong androgen inducibility, weak AREs might be postulated to be present in the D2 region. Like the Slp and C3 promoter, non-steroid receptor factors, including OCT-1 are supposed to be involved in establishment of the full AR specific response of the 20 kDa Protein promoter.

The complex promoters of the Slp and 20 kDa protein genes show an AR, but no GR response in transfection assays, although both AR and GR are able to bind and induce activity of smaller enhancer fragments (42,44). Both AR and GR are able to stimulate probasin promoter activity however AR is markedly more potent than GR in this respect (36). This in contrast to results on the PSA promoter presented in this study. Co-transfection of LNCaP cells with PSA-LUC constructs and a GR expression plasmid showed that GR can replace AR in high, steroid hormone regulated PSA promoter activation. This is not only related to the ARE(-170), but is also true for the ARR at −400. Preliminary evidence indicates that GR is also able to activate the promoter of the endogenous PSA gene in LNCaP cells (Cleutjens, unpublished). From these findings we conclude that the apparent AR specificity of the PSA promoter in LNCaP cells is due to the absence of other members of the steroid receptor family in this particular cell line. The absence of PSA expression in GR or AR positive, non-prostate cells must be explained by additional, inhibitory mechanisms in these cells or absence of other essential regulatory proteins involved in PSA expression.

Transfection experiments further indicated that ARR(-400) and minimal PSA promoter activity [including ARE(-170)] are cell dependent. We studied this aspect in more detail for ARR (−400). Even in the presence of high levels of AR and GR, ARR(-400) activity in LNCaP cells is much higher than in the non-prostate cell lines tested (Table I). This indicates that in addition to AR or GR, other factors are involved in steroid receptor regulated PSA promoter activation. These factors might be present in a higher concentration in LNCaP cells than in the non-prostate cell lines tested. Candidates regarding ARR(-400) could be the proteins detected in gel retardation experiments with the ARR(-400) and LNCaP nuclear extract (Figure 4A). However, these factors are not androgen regulated (Figure 4B) and in gel retardation experiments using nuclear extracts from the non-prostate cell lines COS, T47D, HeLa and Hep3B essentially the same protein complexes could be found (data not shown). The absence of LNCaP specific ARR(-400)/ protein complexes may be due to a relative weak or unstable interaction of these factors with the ARR(-400). Alternatively, specific factors affect AR and GR activity by protein-protein interaction.

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Chapter IV

BOTH ANDROGEN AND GLUCOCORTICOID RECEPTOR ARE ABLE TO INDUCE PROSTATE-SPECIFIC ANTIGEN EXPRESSION, BUT DIFFER IN THEIR GROWTH STIMULATING PROPERTIES OF LNCaP CELLS

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Chapter IV

SUMMARY

Androgen receptor (AR) positive LNCaP cells were stably transfected with a rat glucocorticoid receptor (GR) expression plasmid. Ligand binding studies in the generated cell lines revealed high affinity binding of the cognate ligands to their receptors. Transfection experiments with the newly derived cell lines showed that, like AR, GR can induce activity of a PSA promoter fragment linked to the luciferase gene. Similarly, dexamethasone can stimulate expression of endogenous PSA mRNA. Cell proliferation could be induced by R1881. In contrast, dexamethasone treatment of the GR positive sub-lines had no stimulatory effect on cell growth. In conclusion, the newly generated cell lines form together with the parental LNCaP cell line an attractive system to study the mechanism of specificity of steroid hormone regulation of gene expression. In addition, these cells can be applied to identify novel, steroid hormone-specific regulated gene(s).

INTRODUCTION

Steroid hormones affect many biological activities of the cell by modulating gene activity via interaction with specific nuclear receptors (1-3). Upon ligand binding, steroid receptors interact with specific DNA sequences and regulate the transcriptional activity of target genes (1-4). The glucocorticoid receptor (GR), mineralocorticoid receptor (MR), progesterone receptor (PR) and androgen receptor (AR) bind with high affinity to a DNA element composed of an inverted repeat, separated by a 3 bp spacer sequence. The consensus high affinity binding site for GR, MR, PR, and AR (HRE: hormone response element) is identical (5-8). Although there are genes which can be regulated by more than one specific steroid hormone receptor, the biological function of the different receptors is quite distinct. This presents the problem of specific gene activation in case multiple receptors, which recognize the same DNA binding site, are present in one and the same cell. At present it is essentially unknown how this mechanism operates. Several mutually not exclusive mechanisms, including subtle differences in receptor-DNA interaction, specificity of the interaction of the receptor with other proteins, receptor levels and ligand availability have been proposed (1-3).

LNCaP is an androgen-sensitive human prostatic carcinoma cell line (9), which expresses AR, but lacks GR and PR (10,11). It was previously shown that growth of LNCaP cells, maintained in steroid-depleted culture medium, is stimulated by the addition of androgens (11). The synthetic androgen R1881 at a concentration of 10^{-10} M increases the growth rate; a higher R1881 concentration is suboptimal to cell proliferation, and might even have no stimulatory effect at all. Androgen treatment of LNCaP cells increases the mRNA level, production and secretion of prostate specific antigen (PSA) (12-14).

In the present study we describe the generation and initial characterization of LNCaP sublines, in which the GR was stably expressed. In this way a system was established for comparison of AR and GR molecular and biological functions.

MATERIALS AND METHODS

Cell culture

LNCaP prostate cells were cultured in RPMI 1640 supplemented with 5 % fetal calf serum (Boehringer, Mannheim, Germany) and antibiotics. For transfection, cells were grown in Dulbecco's Modification of Eagle's Medium supplemented with 5 % fetal calf serum.

Plasmids and probes

The rat GR expression plasmid PSTC-GR(3-795) and the selection plasmid pSV_2Neo were described previously (15,16). PSA-61-LUC was generated by integration of a 6 Kb HindIII-HindIII (-6000/+12) fragment of the PSA promoter in the multiple cloning site of pLUC (17). Southern and Northern blots were hybridized with a rGR cDNA fragment (nucleotide 2256-2543) obtained by PCR amplification with primers: 5'−GAGTCTCACAAGACACTTCG−3' and 5'−GAAACATCCATGAGTACTG−3' and plasmid PSTC-GR(3-795) as template using standard methods. Northern blots were hybridized with the 320 bp EcoRI-ClaI fragment of PSA75 cDNA (18), and a 1.2 Kb PstI-PstI hamster actin cDNA fragment as a control.

Transfections

Stable transfection

LNCaP cells were transfected according to the calcium phosphate precipitation method essentially as described (19), using 3 x 10^6 cells per 10 cm dish, 10 μg PSTC-GR(3-795) and 2 μg pSV_2Neo. After overnight incubation with the precipitate, the culture medium was replaced by phosphate buffered saline (PBS), containing 15% glycerol (incubation for 90 sec at room temperature). Subsequently, transfected cells were incubated in culture medium for 24 h. Next, culture medium was replaced by maintenance medium supplemented with G418 (Gibco BRL, Grand Island, NY) at a concentration of 1.2 mg/ml. The resulting G418-resistant clones were seeded into 96-well plates, and selected for GR expression by immunohistochemistry (see below).

Transient transfection

The GR positive clones LNCaP-1B7 and LNCaP-1F5, and the parental LNCaP cells were transiently transfected according to the calcium phosphate method using 1 x 10^6 cells per 25cm^2 flask and 5 μg PSA-61-LUC. After overnight incubation with the precipitate, the culture medium was removed and cells were shocked in phosphate buffered saline (PBS), containing 15% glycerol (90 sec at room temperature). Subsequently, transfected cells were incubated in culture medium in the absence or presence of 10 nM of the synthetic glucocorticoid dexamethasone (Dex) (Sigma, St. Louis, MO) or 1 nM R1881 (DuPont NEN, Boston, MA) for 24 h. Transfections were performed three times in duplicate, using two independent plasmid isolates. Luciferase activities were corrected for variations in protein concentrations in 100 μl cell extract samples.

Luciferase assay

Cells were washed once in PBS, and subsequently lysed in 300 μl lysis buffer (25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1mM DTT, 1 % Triton X-100, 15 % glycerol). Next, 100 μl 0.1 μM luciferin (Sigma)/ 0.25 μM ATP was added to 100 μl extract, and luciferase activity was measured in a LUMAC 2500 M Biocounter (LUMAC, Landgraaf, The Netherlands). After a delay of 2 sec (according to the supplier), the light emission during 5 sec was recorded.

Immunohistochemistry

Immunostaining for GR was performed with the monoclonal anti-GR (rat) antibody Mab 7 (20). Cells were seeded at a density of 3 x 10^5 cells per well on sterile micro slides in four-well tissue culture plates (Heraeus Instruments, Hanau, Germany) in maintenance medium supplemented with G418, and cultured until 50-60% confluence. Next, 10 nM Dex was added, and the incubation was continued for 24 h. Cells were washed in PBS, and fixed for 10 min in 10% phosphate-buffered formalin (pH 7.4). Subsequently, the slides were rinsed in PBS (pH 7.4) and attached cells were made permeable in methanol (-20°C, 5 min), and acetone (-20°C, 2 min). After rehydration in PBS, the slides were incubated in 5% non-immune rabbit serum in PBS followed by overnight incubation in 1:1000 diluted monoclonal antibody Mab7 at 4°C. Excess antibody was removed and rGR immunoreactivity was visualized using rabbit anti-mouse immunoglobulin (DAKO, Glostrup, Denmark) as secondary antibody, and mouse monoclonal PAP complexes (DAKO) as third-step reagent. After three PBS washes, the slides were incubated with diaminobenzidine. The reaction was stopped in water. Cells were counterstained with Mayers hematoxylin.

Southern and Northern blot analysis

Total cellular DNA of LNCaP and GR positive sublines LNCaP-1F5 and LNCaP-1B7 was isolated using standard procedures (21). 10 μg DNA was digested with EcoRI for 16 h, electrophoresed on 0.8 % agarose gel and transferred to a Hybond N^+ membrane (Amersham, Cardiff, UK). Filters were hybridized at high stringency with random primed ^{32}P-labeled probes. Both DNA transfer and filter hybridization were carried out according to the protocol of the manufacturer.

Isolation of total cellular RNA from the different cell lines was carried out by the guanidinium thiocyanate method (22). Glyoxal denatured RNA (10 μg/lane) was separated by electrophoresis on a 1 % agarose gel and transferred to a nylon membrane (Gene Screen, DuPont NEN, Boston, MA). The blot was hybridized with random primed ^{32}P-labeled rGR, PSA or actin cDNA probes in 50 % formamide at 42°C, using standard conditions.

Radio ligand binding assay

Cells were cultured in maintenance medium until 50-60% confluence. To deplete for steroids, cell culturing was continued in RPMI 1640 supplemented with dextran-charcoal-stripped (dcc) serum for 48 h.

[^{3}H]-R1881 (87 Ci/mmol) and unlabeled R1881 (methyl-trienolone) were purchased from DuPont NEN. [^{3}H]-Dex (94 Ci/mmol) was obtained from Amersham. For radio ligand binding analysis, cells were rinsed in PBS and harvested as a single-cell suspension by trypsinization. Cells were washed four times, counted and resuspended in ice cold PBS. In order to measure the cellular AR and GR content and ligand affinity of both receptors, cells were incubated with serial [^{3}H]-R1881 dilutions (0.125-16 nM, in the absence and presence of a 100-fold molar excess of unlabeled R1881) or [^{3}H]-Dex dilutions (0.5-32 nM, in the absence and presence of a 100-fold molar excess of unlabeled Dex) for 90 min at room temperature. Free steroid was removed by extensive washing of the cells in ice cold PBS. Radioactivity was measured in a scintillation counter. Specific binding of [^{3}H]-R1881 and [^{3}H]-Dex was calculated by subtraction of non-specifically bound radioactivity from total bound radioactivity, and used for Scatchard analysis with the radioligand binding analysis program 'Ebda/Ligand' by GA Pherson from Elsevier-BIOSOFT. All assays were performed in triplicate.

Cell growth studies

Cells were trypsinized, seeded in RPMI medium containing 5% fetal calf serum in 25 cm^2 tissue culture flasks at a density of 5 x 10^4 cells, and cultured for three days. Subsequently, medium was replaced by medium containing 5% dcc serum, and the incubation was continued for an additional 3 day period. At this time point (t=0), medium was replaced by RPMI medium containing 5 % dcc serum and indicated hormones at different concentrations. R1881 was added to final concentrations of 10^{-11}, 10^{-10} and 10^{-9} M; Dex was added to final concentrations of 10^{-10}, 10^{-9} and 10^{-8} M, respectively. Control cultures without steroids were supplemented with 0.1 % (v/v) ethanol. At day 4, medium was renewed. At day 8, cells were washed in PBS, trypsinized and the cell number in each tissue culture flask was determined using a Bürker's cell counting chamber. Experiments were performed in triplicate.

RESULTS

Generation of GR expressing LNCaP transfectants

LNCaP cells were transfected with the GR expression vector PSTC-GR and the pSV_2Neo selection plasmid. Cells were grown in medium supplemented with G418 to select for transfected cells. After 3 weeks, G418 resistant clones were picked. Clones were immunohistochemically stained with the GR antibody MAb 7. Out of sixty G418 resistant clones, five showed strong reactivity with the antibody, indicating high GR expression. Thirty-two clones showed low or heterogeneous GR expression, whereas in the remaining clones, no GR immunoreactivity was observed. Staining of two clones with high levels of GR expression (LNCaP-1B7 and LNCaP-1F5), is shown in Figure 1A,B; staining of the parental LNCaP cells for GR was negative (Figure 1C). Note that the morphology of GR+ sublines is different from that of the parental cells, indicating a so far unexplained specific effect of the activated GR on cell physiology. LNCaP-1B7 and LNCaP-1F5 were selected for more detailed studies.

Characterization of LNCaP-1B7 and LNCaP-1F5

Southern blot analysis of EcoRI digested genomic DNA isolated from the LNCaP-1B7 and LNCaP-1F5 transfectants, demonstrated the presence of one or more copies of the complete CMV-GR cDNA fragment in both clones (lanes 2 and 3, Figure 2A; indicated by an arrow). The rat GR probe showed cross-hybridization to two fragments corresponding to the endogenous human GR gene (bands a and b; see also lane 1, containing parental LNCaP DNA). Both LNCaP-1B7 and LNCaP-1F5 DNA contained also at least one incomplete PSTC-GR fragment (bands c and d). Densitometric scanning of the blot revealed the presence of 6 to 7, and 2 complete copies of CMV-GR cDNA in LNCaP-1B7 and LNCaP-1F5, respectively.

Northern blot analysis of LNCaP-1B7 and LNCaP-1F5 RNA showed expression of GR mRNA of the expected size (2.7 Kb) in both clones, no hybridization signal was observed in the parental LNCaP cell line (Figure 2B).

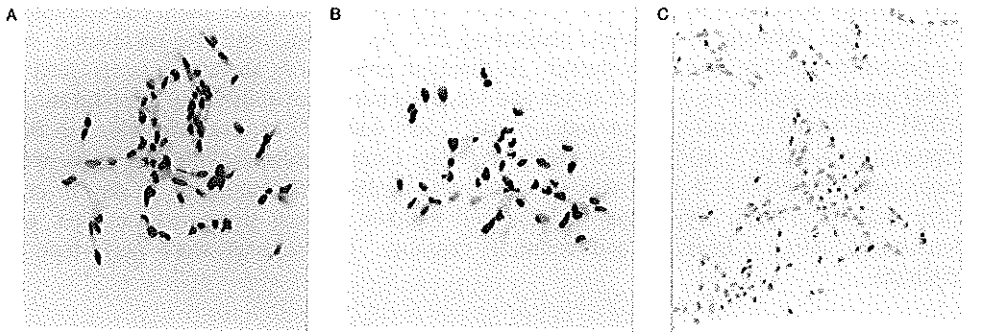

Figure IV.1. Immunohistochemical staining of GR positive LNCaP sub-lines LNCaP-1B7 (A), LNCaP-1F5 (B) and the parental LNCaP cells (C) with the anti rat-GR monoclonal antibody Mab7, utilizing the indirect PAP technique. Cells were counterstained with Mayer's hematoxylin. Prior to immunohistochemical analysis, cells were cultured for 24 h in the presence of Dex.

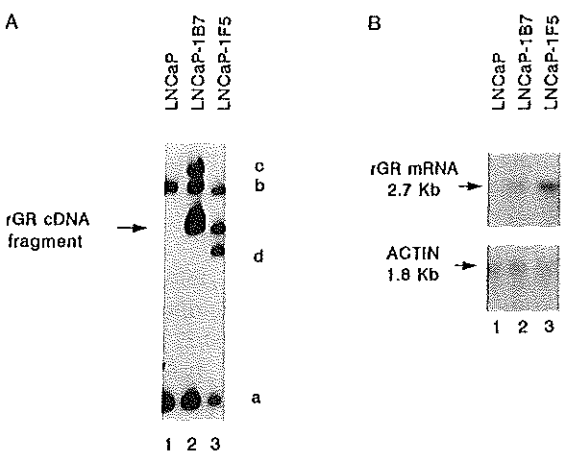

Figure IV.2. Southern and Northern blot analysis of the integrated GR cDNA (A), and GR mRNA expression (B) in GR+ LNCaP sublines and parental LNCaP cells. (A) Southern blot analysis of EcoRI digested genomic DNA from LNCaP (lane 1), LNCaP-1B7 (lane 2), and LNCaP-1F5 (lane 3). DNA (10 μg/lane) was hybridized with a rat GR cDNA probe, homologous to human GR cDNA. Bands a and b: endogenous human GR gene; bands c and d: integrated, partial rGR cDNA fragments. The arrow indicates the position of integrated, complete CMV-GR cDNA copies. (B) Northern blot analysis of 10 μg total RNA from LNCaP (lane 1), LNCaP-1B7 (lane 2), and LNCaP-1F5 (lane 3) hybridized with a rat GR cDNA probe. The lower panel shows β-actin mRNA expression.

Radio-ligand binding assays on LNCaP, LNCaP-1B7 and LNCaP-1F5 cells were performed to establish the number of GR and AR molecules per cell, and the respective dissociation constants for both receptors (Figure 3). The parental LNCaP cell line showed for R1881 a B_{max} of 75 pM, which is equivalent to approximately 15,000 AR molecules per cell (see legend to Figure 3). LNCaP-1B7 and LNCaP-1F5 contained approximately 30,000 and 32,000 AR molecules per cell, respectively, which is comparable to parental LNCaP cells (see above). All three cell lines showed an identical binding affinity for R1881 (K_d 1.1 nM).

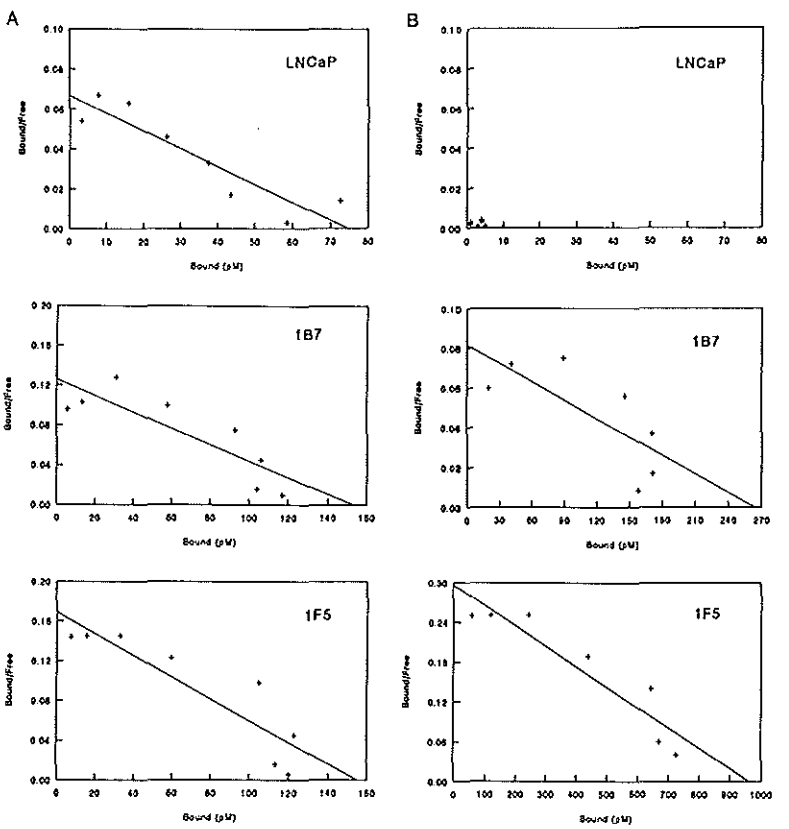

Figure IV.3. Analysis of glucocorticoid and androgen binding activity in parental LNCaP and the GR transfectant 1B7 and 1F5 cells. Scatchard representation of R1881 binding activity (panel A) and Dex binding activity (panel B) of LNCaP, LNCaP-1B7 and LNCaP-1F5 cells. Cellular AR and GR concentrations were deduced from (B_{max} (M) x N_{av})/number of cells per liter ($3x10^9$ in panel A, and $5x\ 10^9$ in panel B). Receptor bound [^{3}H]-steroid was measured by a whole cell assay in the presence and absence of a 100-fold molar excess of unlabeled hormone. The values shown are the mean of a triplicate experiment, and represent specific binding after subtraction of nonspecific binding.

As expected, in the parental LNCaP cell line, binding of Dex could not be detected. From the B_{max} values it could be deduced that LNCaP-1B7 contains approximately 31,000 GR copies per cell, and LNCaP-1F5 115,000 copies. The K_d for Dex binding was 3.1 nM in both LNCaP sublines.

GR activity in GR positive LNCaP sublines

To investigate whether the GR present in LNCaP-1B7 and LNCaP-1F5 cells was functionally active, the cells were transiently transfected with PSA-61-LUC, which contains a 6 Kb PSA promoter fragment. This promoter contains a strong androgen dependent enhancer region, approximately 4.2 Kb upstream of the transcription start site of the PSA gene (17,23). As expected, PSA-61-LUC showed clear, R1881 induced luciferase activity in transfected parental LNCaP cells. No luciferase activity was detected upon incubation of the PSA-61-LUC transfected parental LNCaP cells with Dex (Figure 4). Transient transfection of LNCaP-1B7 and LNCaP-1F5 cells with the PSA-61-LUC construct resulted in a comparable R1881 induced luciferase activity, both in absolute luciferase activity and in induction level (1310- and 1940-fold, respectively). Dex induced a slightly higher PSA-61-LUC activity, clearly indicating the transactivating capacity of the GR encoded by the stably integrated rGR expression vector (Figure 4).

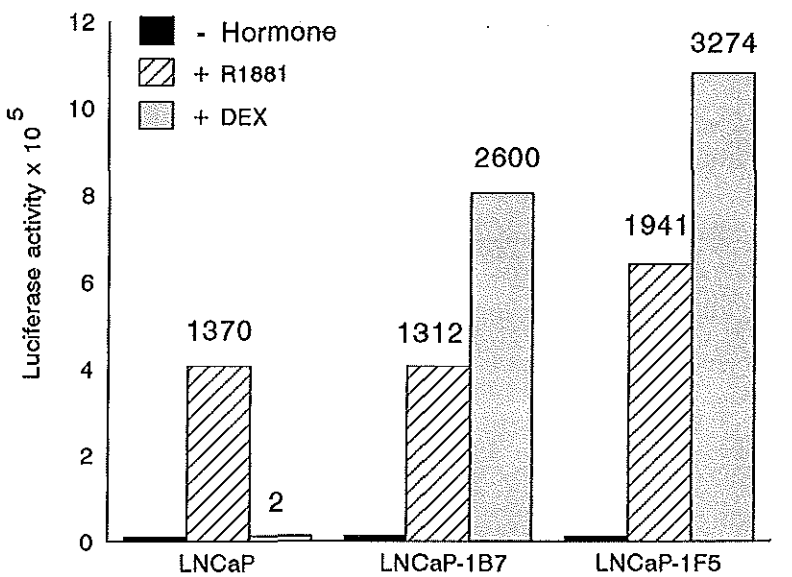

Figure IV.4. R1881 and Dex regulation of the PSA promoter activity in LNCaP and GR positive LNCaP sublines. LNCaP, LNCaP-1B7 and LNCaP-1F5 cells were transfected with the PSA-61-LUC reporter gene construct as described in Material and Methods. After overnight incubation with the precipitate, cells were incubated for 24 h either in the presence or absence of 1 nM R1881 or 10 nM Dex. Activity in the absence of hormone is indicated by a solid bar, activity in the presence of R1881 with a hatched bar and in the presence of Dex by a grey bar. Fold- induction is displayed on top of the bars.

Regulation of endogenous PSA mRNA expression

In previous studies we and others (12-14) have shown that PSA mRNA expression is induced upon androgen incubation of LNCaP cells. To investigate whether GR can replace AR in induction of the endogenous PSA gene, we performed Northern blot analysis with RNAs isolated from the parental LNCaP cell line, and from LNCaP-1B7 and LNCaP-1F5 cells, grown in the absence and in the presence of hormone (Dex or R1881). Hybridization with a PSA specific cDNA probe demonstrated that GR can replace AR in high, steroid hormone induced expression of the PSA gene (Figure 5). In the parental LNCaP cell line, PSA mRNA expression was induced by R1881 but not by Dex. In contrast, in the two GR positive clones, Dex treatment clearly resulted in stimulation of PSA mRNA expression, although to a slightly lower level (approximately 3-fold) than R1881 upregulated PSA mRNA.

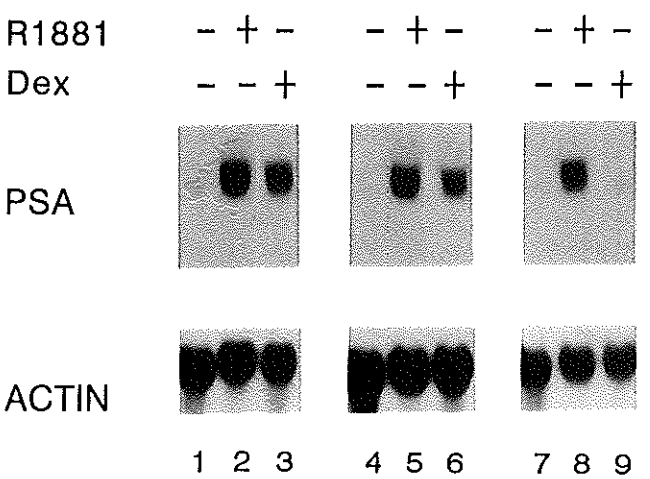

Figure IV.5. Expression of the rat GR renders the endogenous androgen-regulated PSA gene inducible by Dex in the LNCaP sublines 1B7 and 1F5. Northern blot analysis of 10 μg total RNA of LNCaP-1B7, LNCaP-1F5 and LNCaP cells hybridized with a PSA cDNA probe. Cells were treated for 24 h with Dex, R1881 or were grown in the absence of hormone. The lower panel shows hybridization of the β-actin cDNA probe as a control.

Regulation of cell proliferation

Growth of LNCaP cells depends on androgens in a concentration dependent fashion. Previous studies indicated a bell shaped dose-response curve for the stimulatory effect of androgens (11). Maximal growth stimulation of R1881 was observed at a concentration of 0.1 nM. To find out whether GR was able to replace AR in growth regulation of LNCaP cells, we compared the effects of different R1881 and Dex concentrations on growth of LNCaP cells, and of the two GR positive LNCaP sublines. At day 8 after addition of

hormone, all three cell lines showed a clear growth stimulation upon treatment with 0.1 nM R1881, and to a somewhat lower extent at 1 nM R1881. However, at none of the tested Dex concentrations a growth stimulatory effect was observed (Figure 6). Similar negative results were obtained with hydrocortisone (data not shown). These results clearly indicated that GR was unable to replace AR in growth stimulation of LNCaP cells.

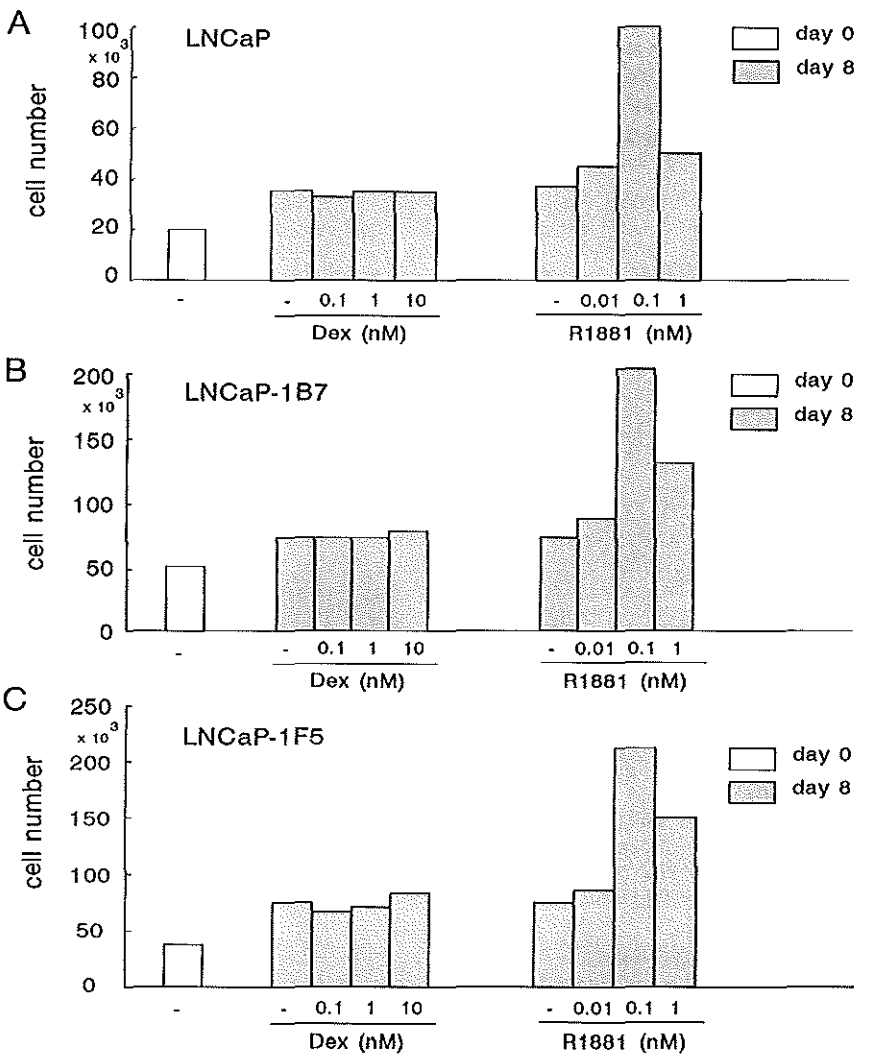

Figure IV.6. Effects of Dex and R1881 on cell growth of parental LNCaP, and the GR+ 1B7 and 1F5 cells. At day 0, and at day 8 of incubation in the absence or presence of hormone, cells were washed, trypsinized and counted. The values shown are the mean of experiments in triplicate.

DISCUSSION

In this paper we describe the generation and properties of LNCaP sublines, that express not only AR but also GR. These novel cell lines facilitate the direct comparison of GR and AR effects on cellular functioning. Furthermore, they can be employed for investigation of interference between GR and AR activated molecular and biological processes. We demonstrated that AR and GR positive cells behave identically in up-regulation of the expression of a transfected PSA promoter driven reporter gene, and the expression of the endogenous PSA gene. Interestingly, they were found to differ in hormone induced effects on cell proliferation.

At least part of the effects of steroid hormone receptors on gene expression is on transcription initiation. Upon ligand binding, steroid receptors interact with specific DNA sequences (HREs), and regulate the transcription of target genes. The GGT/AACAnnnTGTTCT consensus sequence for high affinity DNA binding of GR, MR, PR and AR is identical (5-8). Despite this common DNA binding site, the different receptors mediate distinct cellular responses. Many independent mechanisms to achieve specificity of the steroid hormone response have been proposed (see for reviews 3 and 24). These include differential affinities to natural receptor binding sites, or to binding sites in their natural DNA context (25-27), differential affinity to general and specific transcription factors (28-31), differences in interaction with receptor specific accessory proteins, or coactivators (32-34), differential modification of specific chromatin structures (35,36), differences in cellular concentration of the specific receptors (37), and variations in ligand availability (38,39).

The observation that PSA mRNA was Dex inducible in the GR expressing LNCaP sublines showed that the steroid receptor content determines at least in part the specific activation of the PSA gene in wild type LNCaP cells. Transient transfection of the 6 Kb PSA promoter to the LNCaP sublines resulted also in activation by both AR and GR. In contrast, in case of the MMTV promoter, differences have been reported on the effect of GR and PR on transiently transfected reporter gene constructs and stably integrated plasmids (35,36). It appears that the native chromatin structure can prevent PR activation, and permits GR stimulation of the stably integrated target gene.

AR activation of the PSA promoter involves at least three AREs, two in the 600 bp proximal promoter region, and one in a far upstream enhancer region (17,40). Although ultimate PSA promoter induction is comparable for AR and GR, it cannot be excluded as yet, that AR and GR affect the individual regulatory regions differentially. Such an observation has been made for GR and PR activation of the MMTV promoter, which contains four HREs. Differences might be accounted for by distinct chromatin structures over the individual HREs, and the ability of the different receptors to affect these structures, and/or the interaction with other specific transcription factors (31).

As shown above, GR expression cannot be detected in wild type LNCaP cells, this in contrast to normal human prostate tissue (41). However, in normal prostate highest GR expression is in the stromal compartment. In epithelial cells expression of AR appears much higher than GR expression. In prostate cancer, GR expression seems even to decrease. Therefore, GR might not be a major factor in PSA expression. No data are available about PR expression in prostate tissue, another candidate for regulation of PSA expression (17).

The stimulatory effect of androgens on LNCaP cell proliferation shows a bell-shaped dose-response curve (11,42). At low androgen concentrations (up to 10^{-10} M R1881), LNCaP cells proliferate in a dose dependent manner; at higher hormone concentration, the proliferation rate is less. The molecular mechanism of growth stimulation by androgens, including the remarkable dose response is not fully understood, although it has been proposed that TGF-β1 mediates at least part of the growth arrest observed at high androgen concentration (43). The cell growth studies clearly demonstrated that Dex was unable to induce proliferation of LNCaP-1B7 and LNCaP-1F5 cells. The molecular mechanism responsible for the differential effects of glucocorticoids and androgens on growth remains to be investigated. In a probably oversimplified view it can be hypothesized that a limited number differentially expressed genes are involved. Differential AR and GR regulated TGFβ and other growth factors or inhibitors, and their corresponding receptors should be considered in this regard.

Using a differential display PCR approach, we initiated a search for differentially expressed genes in the AR+GR+ LNCaP sublines. Although many genes appeared to be regulated by both GR and AR, so far one novel mRNA was detected, that could be up-regulated by AR, but not by GR (44). This observation demonstrates the validity of the model here presented. We expect that further analysis of the regulation of expression of this gene will provide information about the mechanism of AR specificity.

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Chapter V

AN ANDROGEN RESPONSE ELEMENT IN A FAR UPSTREAM ENHANCER REGION IS ESSENTIAL FOR HIGH, ANDROGEN-REGULATED ACTIVITY OF THE PROSTATE-SPECIFIC ANTIGEN PROMOTER

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Chapter V

ABSTRACT

Prostate-specific antigen is expressed at a high level in the luminal epithelial cells of the prostate, and is absent or expressed at very low levels in other tissues. PSA expression can be regulated by androgens. Previously, two functional androgen response elements were identified in the proximal promoter of the PSA gene. In order to detect additional, more distal control elements, DNaseI hypersensitive sites (DHSs) upstream of the PSA gene were mapped in chromatin from the prostate derived cell line LNCaP grown in the presence and absence of the synthetic androgen R1881. In a region 4.8 to 3.8 kb upstream of the transcription start site of the PSA gene, a cluster of three DHSs was detected. The middle DNAseI hypersensitive site (DHSII, at approximately − 4.2 kb) showed strong androgen responsiveness in LNCaP cells, and was absent in chromatin from HeLa cells. Further analysis of the region encompassing DHSII provided evidence for the presence of a complex, androgen responsive and cell specific enhancer. In transient transfected LNCaP cells, PSA promoter constructs containing this upstream enhancer region showed approximately 3000-fold higher activity in the presence than in the absence of R1881. The core region of the enhancer could be mapped within a 440 bp fragment. The enhancer showed synergistic cooperation with the proximal PSA promoter, and was found to be composed of at least three separate regulatory regions. In the center, a functionally active, high affinity androgen receptor binding site (GGAACATATTGTATC) could be identified. Mutation of this element almost completely abolished PSA promoter activity. Transfection experiments in prostate and non-prostate cell lines showed largely LNCaP cell specificity of the upstream enhancer region, although some activity was found in the T47D mammary tumor cell line.

INTRODUCTION

Prostate-specific antigen (PSA) is a kallikrein-like serine protease, that is almost exclusively synthesized by the luminal epithelial cells of the human prostate. It is well known as a prostate tumor marker (1,2). The PSA gene is a member of the human kallikrein gene family. Further members of the kallikrein gene family are the hGK-1 gene, which is also expressed in the prostate, and the tissue kallikrein gene (KLK1), which is expressed in the pancreas and kidney (3-6). The three genes are clustered in the order [KLK-1]-[PSA]-[hGK-1], in an area of 60 kb on human chromosome 19q13.2-13.4 (7). The PSA and hGK-1 genes are separated by 12 kb, the distance between KLK1 and PSA, which are transcribed from opposite strands is approximately 31 kb (7). PSA expression does not only show cell specificity, but is also tightly regulated by androgens, as mediated by the androgen receptor (AR) (8-12). The strong tissue specificity makes the PSA promoter a good candidate through which to deliver therapeutic genes in prostate cancer.

Two functionally active AR binding sites (androgen response elements, or AREs) were identified in the proximal PSA promoter, at positions -170 (ARE-I) and −394 (ARE-II), respectively (11,13). Although the proximal PSA promoter, including ARE-I and ARE-II, is more active in LNCaP prostate cells than in non-prostate cells, its activity is relatively low. This low level of activity suggested that the proximal PSA promoter is not sufficient to account completely for androgen regulation of the endogenous PSA gene, as observed in

LNCaP cells (11). This indicated to us that additional *cis*-acting control elements residing outside the proximal promoter might contribute to androgen regulated PSA gene expression. For several strong, tissue specific promoters, like those of the beta-globin and tyrosine amino transferase (TAT) genes, it has been well established that important control elements are located in regions far upstream of the proximal promoter (14-16). These distal enhancers cooperate with the proximal promoter for high expression of the specific gene.

To identify putative regulatory elements upstream of the PSA gene, DNaseI hypersensitive sites (DHSs) were mapped in chromatin from LNCaP prostate cells. Functional analysis of a DNaseI hypersensitive region far upstream of the PSA gene showed the presence of a complex, androgen regulated enhancer. In this study we present a detailed analysis of this strong enhancer, which contains a functionally active, high affinity AR binding site (ARE-III). Furthermore, we compare the AR binding affinity and the functionality of this novel ARE with that of the previously identified AREs -I and -II (11,13). An abstract describing parts of this work has been published previously (17).

While this work was in progress, Schuur et al. (18) reported the identification of a 1.6 Kb upstream enhancer fragment (-3.7 to -5.3). This fragment encompasses the 440 bp core enhancer region, which is the basis of the present study.

RESULTS

Mapping of DNaseI hypersensitive sites in the PSA upstream region

In a previous study we identified two regions in the PSA proximal promoter which are involved in androgen regulation (13). A functional active, high affinity, AR binding site, ARE-I (AGAACAgcaAGTGCT), was found to be present at position -170. ARE-I by itself gave rise to a weak (2-fold) stimulation of the PSA promoter activity in the presence of R1881. ARE-I had to cooperate with a second, low affinity AR binding site, ARE-II (GGATCAgggAGTCTC) at position -394 for maximal (approximately 6-fold) androgen induction of proximal PSA promoter activity in transfected LNCaP cells.

To identify additional regulatory elements, we mapped DHSs in the 31 kb region between the PSA and KLKI genes in chromatin from the prostate derived cell line LNCaP, grown in the presence and absence of androgens, and in HeLa cell chromatin. DNA from DNaseI treated nuclei was digested with EcoRI and evaluated for location of DHSs by Southern blot analysis with the appropriate hybridization probes. With two different probes, DHSs could be found (Figure 1). No other DHSs were detected over the 31 kb region with any of the probes tested (data not shown). Hybridization of EcoRI digested DNA from LNCaP cells with a 1.1 kb HindIII-EcoRI fragment, spanning exon 1 and intron 1 of the PSA gene, showed one DHS (DHSIV), which was most prominent in the presence of R1881 (Figure 1C). This DHS mapped to the proximal promoter region. Hybridization of genomic DNA from R1881 treated LNCaP cells with a 0.5 kb EcoRI-HindIII probe (-6 kb) revealed the presence of a cluster of three DHSs, approximately 4 kb upstream of the PSA gene (Figure 1A). The position of this cluster of DHSs could be confirmed by hybridization with a more downstream located probe (data not shown). Analysis of the same region in chromatin from LNCaP cells grown in the absence of hormone showed that DHSII at -4.2 kb is clearly androgen regulated. Intensity of DHSI, at approximately -4.8 kb, is also influenced by the presence of R1881 during LNCaP culturing. The weak DHSIII (at − 3.8 kb) could be found

both in the absence and presence of R1881. Although weak, DHSI and DHSIII might also be present in chromatin from HeLa cells, which do not express PSA (Figure 1B). In contrast, DHSII was clearly absent in HeLa cell chromatin, indicating cell specificity.

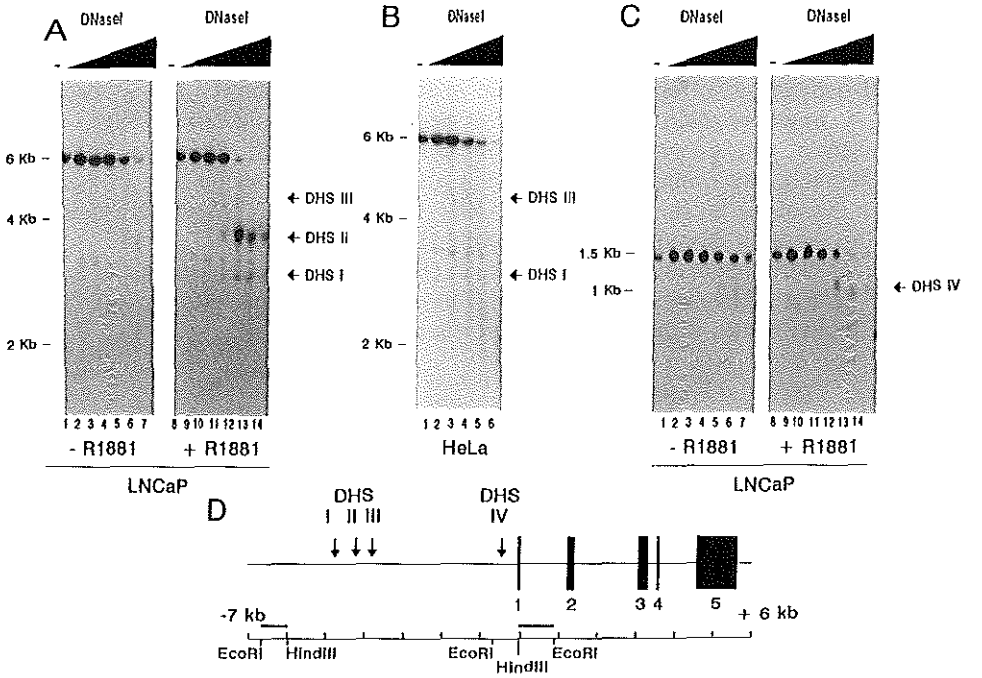


Figure V.1. DNAseI hypersensitive sites in chromatin upstream of the PSA gene. *Southern blot analysis of genomic DNA from nuclei of LNCaP (A, C) and HeLa (B) cells incubated with increasing amounts of DNaseI (lanes 2-7, and 9-14 in A; 2-6 in B; and 2-7, and 9-14 in C; Lanes 1 and 8 in A; 1 in B; 1 and 8 in C are controls without DNAseI treatment), and digested with EcoRI. Hybridization was with an EcoRI-HindIII probe at -6 kb (A,B) or a HindIII-EcoRI probe at +1 kb (C). Nuclei were isolated from LNCaP cells grown in the absence (A, lanes 1-7; C, lanes 1-7) or presence of R1881 (A, lanes 8-14; C, lanes 8-14), or (B) from HeLa cells grown in 5% complete foetal calf serum. (D) Schematical representation of the PSA gene. Black boxes represent the five exons of the PSA gene. Hybridization probes are indicated by horizontal bars in the partial restriction map. Positions of DHSs are indicated by arrows (DHSI-IV).*

Functional analysis of the DnaseI hypersensitive region upstream of the PSA gene

To identify the function of the DNA segment containing the upstream DHSs, a 6 kb PSA promoter fragment was inserted upstream of the luciferase reporter gene (PSA-61-LUC), and the activity of this fragment was compared with that of 2.2 kb (PSA-1-LUC) and 632 bp (PSA-4-LUC) PSA promoter fragments. Transient transfection to LNCaP cells showed for PSA-61-LUC a much higher (3000-fold) activity in the presence than in the absence of

R1881 (Figure 2A). PSA-1-LUC and PSA-4-LUC gave rise to a 6-fold and 4-fold induction, respectively, upon hormone treatment. These experiments clearly indicated the presence of a very potent enhancer between -6 and -2.2 kb.

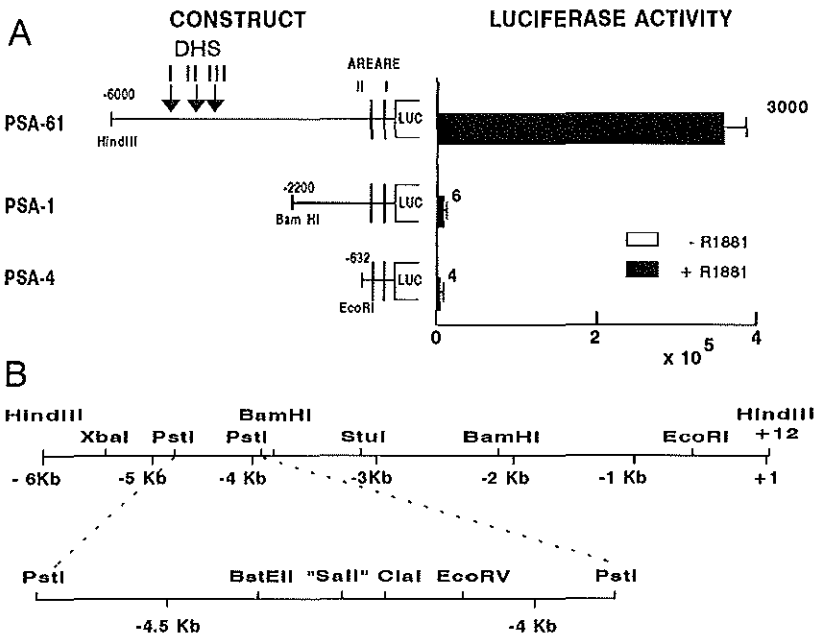


Figure V.2. Androgen regulation of the PSA promoter in LNCaP cells. (A) LNCaP cells were transiently transfected with PSA-LUC constructs as described in Materials and Methods. Following 4 h incubation with the plasmid precipitate, transfected cells were cultured for 24 h in the absence or in the presence of R1881 (1 nM). The absolute activity and relative induction factor were calculated as the mean of four or more independent experiments, which were done in duplicate. Closed bar: Activity in the presence of R1881; open bar: activity in the absence of R1881. The SEM of the absolute activity is represented by a horizontal stripe. The induction level is indicated at the right side of the bars. Positions of ARE-I and ARE-II in PSA promoter constructs are represented by black boxes. Positions of DHS I, II and III are indicated by arrows. (B) Partial restriction map of the PSA promoter. "SalI" represents the position of an artificial SalI site derived from the border of a human genomic DNA fragment in lambda EMBL3.

To map this upstream region in more detail, a 2.2 kb XbaI-StuI fragment (see Figure 2B for a partial restriction map of the PSA promoter), encompassing the three upstream DHSs, was inserted in front of the proximal PSA promoter in PSA-4-LUC, which starts at the EcoRI site at -632, and which contains ARE-I (-170) and ARE-II (-394) (13) (Figure 3A), giving rise to construct PSA-64-s-LUC. Transfection of PSA-64-s-LUC to LNCaP cells resulted in an even higher (over 6000-fold) induction of promoter activity upon R1881 activation (Figure 3A), indicating that the upstream enhancer activity resides within this 2.2 kb fragment. The 2.2 kb XbaI-StuI fragment in the opposite orientation gave rise to a similar high activity (PSA-64-as-LUC in Figure 3A).

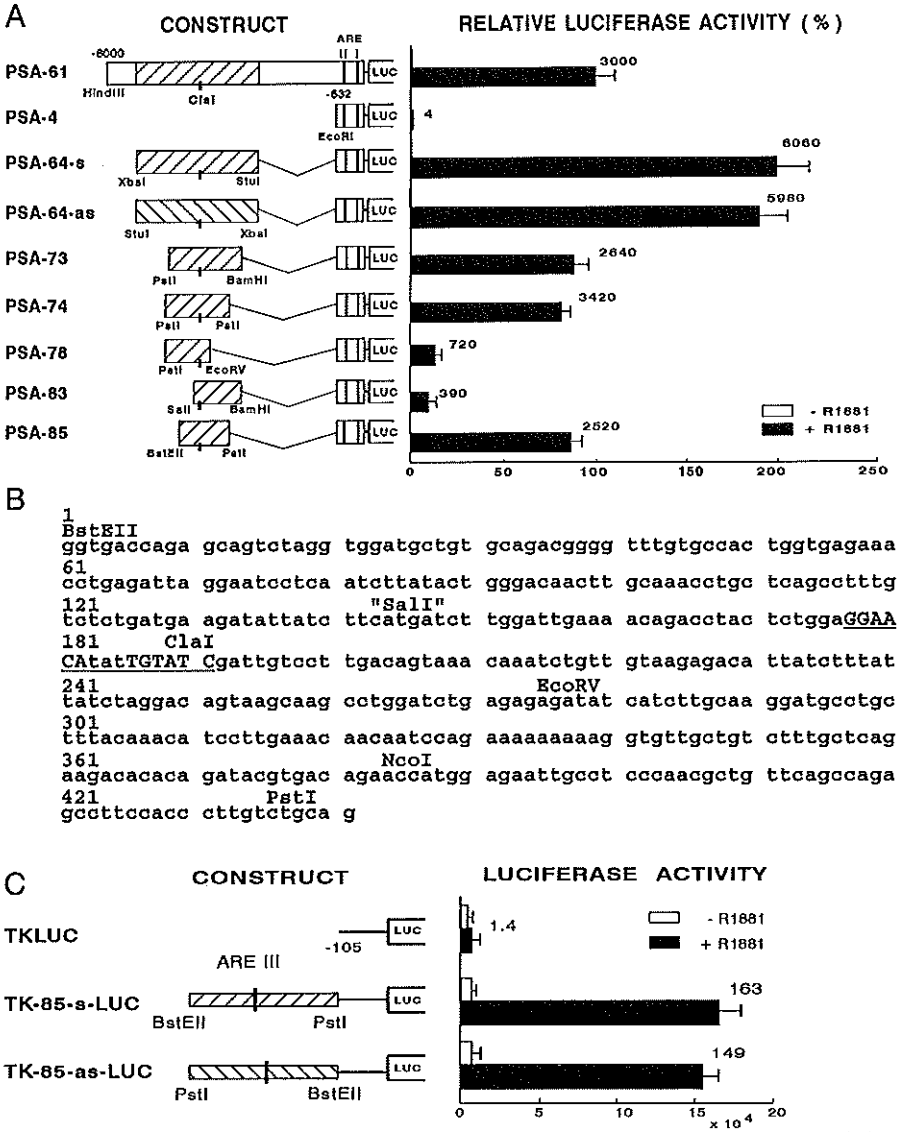


Figure V.3. Identification of the androgen regulated core enhancer region, upstream of the PSA gene. (A) Detailed deletion mapping of the upstream region of the PSA gene in transfected LNCaP cells. Experimental details of the transfections are described in Materials and Methods, and in the legend to Figure 2A. The activity of the PSA-61-LUC construct in the presence of R1881 is set at 100 %. The mean of the luciferase activity and the relative induction levels are from at least four independent experiments. (B) Sequence of the 440 bp BstEII-PstI upstream core enhancer fragment. Important restriction sites are indicated above the sequence. The ARE-III sequence is underlined, with the two half sites in capitals. (C) Effect of the core enhancer region on TK promoter activity in LNCaP cells. The hatched box represents the 440 bp core enhancer. Experimental details are as in Figure 2A.

To determine the borders of the upstream enhancer region, a series of truncated fragments was linked to the proximal PSA promoter in LUC reporter gene constructs. Both PSA-73-LUC (1 kb PstI-BamHI) and PSA-74-LUC (0.9 kb PstI-PstI) showed an induction and absolute activity, which was approximately 50% of PSA-64 activity. A further 200 bp 3' truncation in construct PSA-78-LUC (PstI-EcoRV) resulted in a 4-fold drop of activity upon R1881 treatment. Similarly, deletion of the distal end (PSA-83-LUC), resulted in an 8-fold reduction in activity. The 440 bp BstEII-PstI fragment (PSA-85-LUC) turned out to be the smallest fragment with strong enhancer activity (Figure 3A). We defined the BstEII-PstI fragment as the core enhancer. The sequence of this core enhancer is shown in Figure 3B. Because further 5'- or 3'- deletion resulted in a partial decrease of core enhancer activity, two or more separate enhancer elements must be present in this fragment. Most accurate calculations of the positions of DHSs -I, -II, and -III showed that DHSII is located within the core enhancer fragment, however, this is not the case for DHSI and DHSIII. Most likely, DHSI is situated close to the PstI site at -4.8 kb, and DHSIII close to the BamHI site at -3.8 kb (see Figure 2B).

To find out whether core enhancer activity was directly androgen regulated, the BstEII-PstI fragment was linked in both orientations to the TK promoter (TK-85-s-LUC and TK-85-as-LUC, respectively), and LNCaP cells were transfected with these constructs (Figure 3C). The result clearly showed that this 440 bp fragment contained orientation independent, intrinsic androgen responsive enhancer activity. This observation correlated with the strong androgen regulation of DHSII, linking results of the transient transfection studies with the activity of the promoter of the endogenous PSA gene. Additionally, the results indicated synergistic cooperation between the upstream enhancer and the proximal PSA promoter, because PSA-61-LUC and PSA-85-LUC were considerably more active than TK-85-LUC (Figures 2A, 3A and 3C, and data not shown).

Identification of an androgen response element in the core enhancer region

To identify candidate AR binding sites, DNaseI footprints were determined over four overlapping core enhancer segments, utilizing the purified AR DNA binding domain (AR-DBD). The only clear protection that was observed, was located in the middle part of the fragment, over the sequence 5'-ACTCTGGAGGAACATATTGTATCGATT-3', directly upstream of the ClaI site (Figure 4A). The protected area contained the sequence GGAACAtatTGTATC, which shows high homology (overall 9 out of 12 bp), with the consensus sequence GGT/AACAnnnTGTTCT for high affinity AR binding (19). Competition was found with a 100-fold excess ARE consensus oligo, but not with an excess of an NF-1 consensus oligo (Figure 4A, lanes 4 and 5), indicating specificity of the interaction. Although both the BstEII-SalI and the EcoRV-PstI subfragments contributed to maximal activity of the core enhancer (Figure 3A), AR binding was not observed in one of these fragments (data not shown). Gel retardation analysis of a double stranded oligonucleotide encompassing the upstream AR binding site (ARE-III: ggaGGAACAtatTGTATCgat) with AR-DBD confirmed that this fragment contains a specific, high affinity AR binding site (Figure 4B).

To test whether ARE-III was functionally active, the sequence was mutated to GCATAAtatTTCAAC in TK-85-s-LUC, resulting in construct TK-85-I-LUC. In transfection experiments, the mutated enhancer was no longer R1881 inducible (Figure 4C). This not only indicated that ARE-III was functionally active, but also provided evidence for a pivotal role of ARE-III in androgen regulation by the core enhancer region.

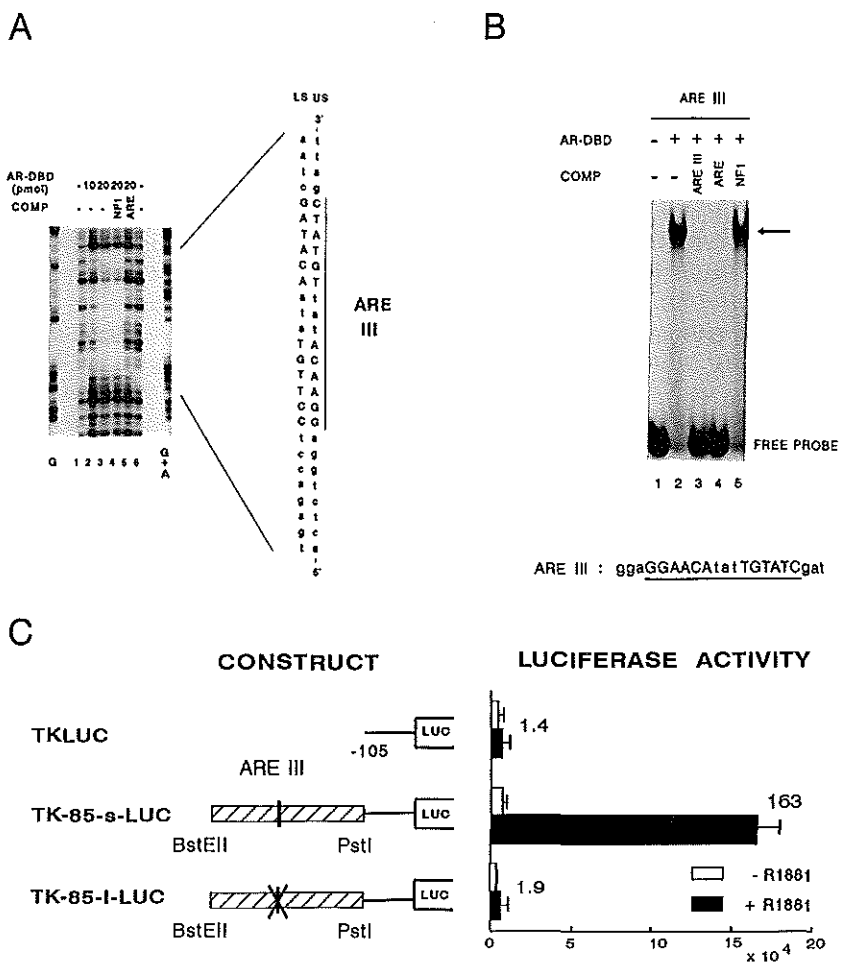
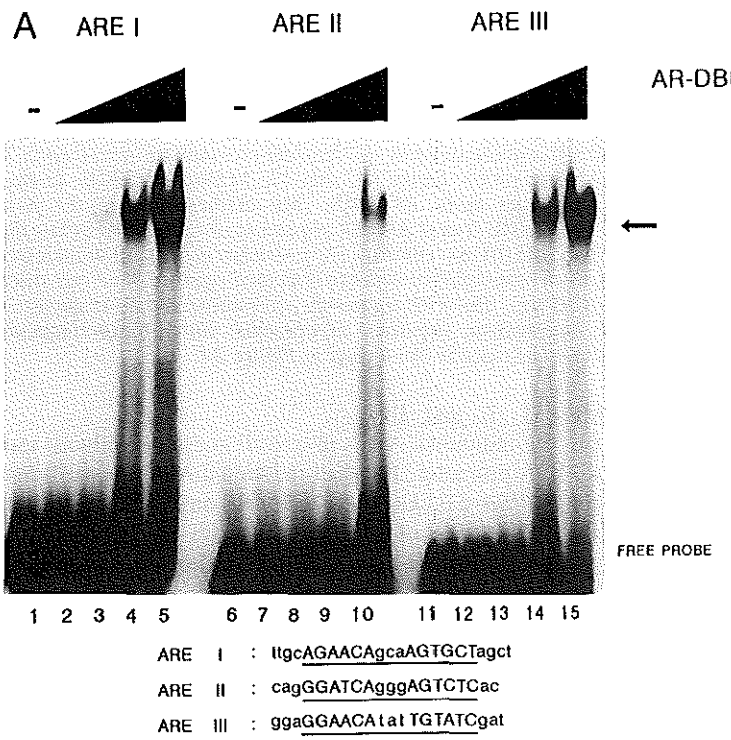


*Figure V.4. Identification of a functionally active AR binding site in the core enhancer region. (A) DNAseI footprint analysis over ARE-III. The lower strand of pHS2 ("SalI"-EcoRV fragment in the core enhancer) was 32P-end labelled, digested with DNaseI in the absence (lanes 1 and 6) and presence of 10 (lane 2) and 20 pmol (lanes 3-5) AR(DBD) fusion protein, and subjected to gel electrophoresis (see Materials and Methods). Lanes 4 and 5: competition with a 100-fold excess double stranded non specific (NF-1 consensus oligo, lane 4), and specific oligonucleotide (ARE consensus, lane 5). Maxam and Gilbert sequence reactions are run alongside the footprint (G and G+A). The sequence of the protected area is depicted at the right. The two ARE-III half sites are in capitals. LS: lower strand; US: upper strand. (B) Gel retardation analysis of the ARE-III*AR(DBD) complex. Experimental details are as described in Materials and Methods. Lane 1: free ARE-III probe; lanes 2-5: ARE-III probe incubated with AR(DBD). Lanes 3-5: competition with a 100-fold excess ARE-III oligo (lane 3), ARE consensus oligo (lane 4) and NF-1 consensus oligo (lane 5). The arrow indicates the position of the AR-III*AR(DBD) complex. The ARE-III sequence is presented below the figure. (C) The effect of ARE-III inactivation on the androgen regulated core enhancer activity in transiently transfected LNCaP cells. Experimental details are as described in Materials and Methods, and in the legend to Figure 2A. Closed bar: Activity in the presence of R1881 (1 nM); Open bar: activity in the absence of hormone. Mean values of luciferase activities and induction levels, and the SEM (horizontal stripe) are from three independent, duplicate experiments. The hatched bar in the constructs represents the core enhancer. ARE-III is given as a black box. The inactivated ARE-III is represented by a cross.*

Comparison of ARE-I, ARE-II, and ARE-III

The presence of (at least) three AREs [ARE-I(-170): AGAACAgcaAGTGCT; ARE-II(-394): GGATCAgggAGTCTC, and ARE-III (−4200): GGAACAtatTGTATC] in the PSA promoter raised the question of relative AR binding affinities of the individual AREs, and their separate contribution to overall androgen regulation of PSA promoter activity. To compare AR binding to these AREs, gel retardation analyses were performed with serial dilutions of purified AR-DBD (Figure 5A). ARE-I and ARE-III turned out to be high affinity AR-DBD binding sites, with comparable AR binding affinity. The *in vitro* interaction of AR-DBD with ARE-II was much weaker.

Next, three copies of ARE-III were inserted in front of the minimal TK promoter in TK-LUC, and the activity was compared with similar ARE-I and ARE-II TK-LUC constructs. Transient transfection experiments in LNCaP cells showed ARE-III to be functional active, albeit less than ARE-I: 10-fold, and 34-fold induction upon R1881 stimulation, respectively (see Figure 5B). However, both ARE-I and ARE-III were more active than ARE-II.



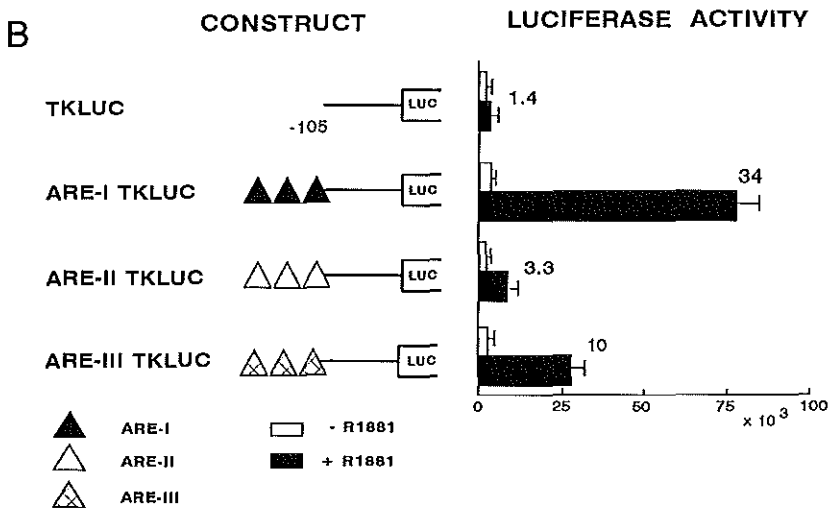


*Figure V.5. Comparison of ARE-I, ARE-II and ARE-III in vitro AR(DBD) binding and functional activity. (A) Gel retardation analysis of ARE-I, ARE-II and ARE-III complexed with AR(DBD). ARE-I (lanes 1-5), ARE-II (lanes 6-10), and ARE-III (lanes 11-15) ($50x10^3$ cpm) are incubated with an increasing amount of AR(DBD) and analyzed by gel retardation assay as described in Material and Methods. Lanes 1, 6 and 11: free probe. Lanes 2, 7 and 12: 30 fmol AR(DBD). In each panel, the following lane contains four times more AR(DBD). The arrow indicates the position of the ARE*AR(DBD) complex. ARE-I, ARE-II and ARE-III sequences are presented below the figure. (B) Effect of ARE-I, ARE-II and ARE-III on minimal TK promoter activity in transiently transfected LNCaP cells. LNCaP cells were cotransfected with the indicated reporter gene construct and the AR expression vector pSVARo (2.5 μg). The ARE-fragments are represented by triangles, mean values and SEM are from three independent, duplicate experiments. Further experimental details are described in Materials and Methods and in the legend to Figure 2A.*

Mutational analysis of AREs -I, -II, and -III

To investigate the role of the individual AREs in overall androgen induced transcriptional responsiveness of the 6 kb PSA promoter, for each individual ARE two different knock out mutations were introduced in PSA-61-LUC (see Materials and Methods for sequences of mutated AREs). Transient transfection of LNCaP cells with the resulting mutated PSA promoter-LUC constructs showed that all three AREs contributed to androgen regulation. ARE-I(-170) mutations resulted in an 80% reduction of promoter activity (Figure 6). Both mutations in ARE-II(-394) had a limited effect (50% or less reduction). Mutations in ARE-III had by far the most dramatic effect. As compared to wild type PSA-61-LUC, less than 1% of activity was retained in the mutated promoter. This finding indicated that ARE-III is not only a key element in the 440 bp upstream core enhancer, as shown in Figure 4C, but also in the context of the 6 kb PSA promoter.

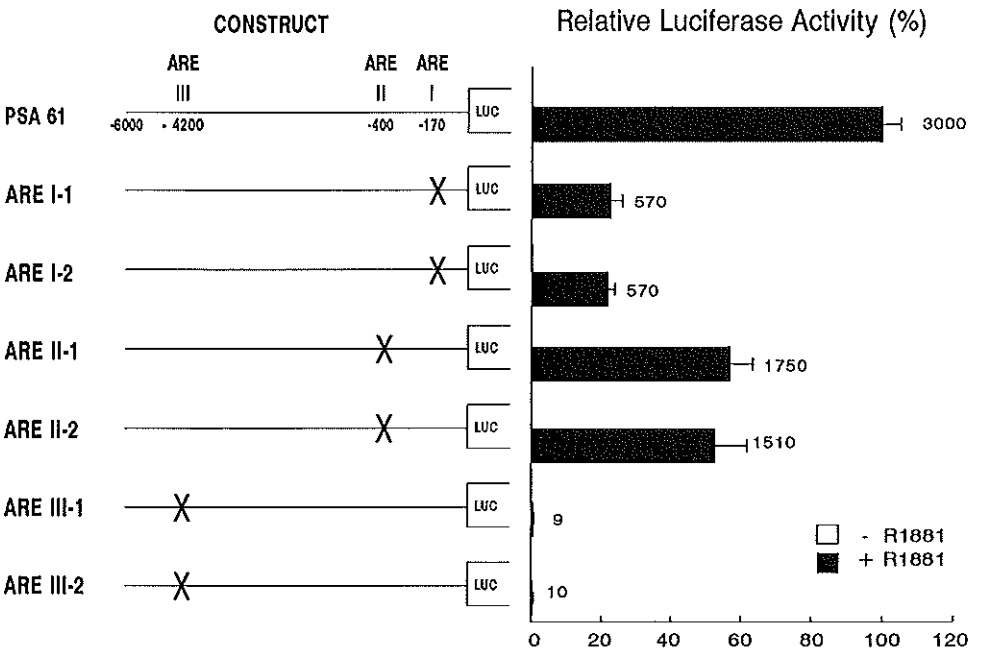


Figure V.6. The effect of the inactivation of ARE-I, ARE-II and ARE-III on androgen regulation of the PSA promoter. Experimental details are as described in Materials and Methods, and in the legend to in the legend to Fig 2A. PSA-61-LUC activity in LNCaP cells cultured in the presence of R1881 is set at 100%. The ARE mutations in the 6 Kb PSA-61-LUC construct are indicated by crosses. Mean values and SEM are from four independent, duplicate experiments. Hormone induction values are given at the right side of the bars.

Tissue specificity of the PSA promoter

Previous work in our laboratory showed that the proximal PSA promoter is more active in LNCaP prostate cells than in non-prostate cells (13). To study whether the androgen induced activity of the upstream core enhancer also showed cell specificity, reporter constructs PSA-61-LUC (Figure 2), TK-85-s-LUC and the TK-LUC control (Figure 3C) were cotransfected with the AR expression plasmid pSVARo to a series of AR negative prostate and non-prostate cell lines. In contrast to the high activity in LNCaP cells, TK-85-s-LUC showed hardly any activity over basal TK promoter activity in the androgen independent prostate cell lines PC-3 and DU145, and in COS (monkey kidney) and Hep3B (human hepatoma) cells (Table I). Furthermore, the 6 kb PSA promoter was hardly active in these cells. For comparison, MMTV-LUC was transfected to the same set of cell lines. In PC3 and DU145 cells, a low R1881 induced activity was detected. In COS, Hep3B and LNCaP cells, the MMTV promoter was strongly induced. Interestingly, in COS and HeLa cells, induction of MMTV promoter activity was 40-60 times higher than that of PSA-61. In contrast, in LNCaP cells, PSA-61-LUC was 6 times more active than MMTV-LUC. These findings clearly indicate cell specificity of the PSA promoter.

Table V.I. Effect of R1881 on PSA-61-LUC, TK-85-LUC and MMTV-LUC activity in PC3, DU145, COS, Hep3B cells, co-transfected with the AR expression plasmid pSVARo, as compared to LNCaP cells.

CONSTRUCT	RELATIVE INDUCTION (+/- R1881)*				
	PC3	DU145	COS	Hep3B	LNCaP
TK-85-s-LUC**	1.5 ± 0.2	1.2 ± 0.1	1.8 ± 0.3	1.3 ± 0.4	163 ± 17
TK-LUC	1.5 ± 0.2	1.1 ± 0.3	1.3 ± 0.4	1.1 ± 0.3	1.4 ± 0.2
PSA-61-LUC	1.7 ± 0.5	1.2 ± 0.1	1.4 ± 0.3	2.1 ± 0.8	3000 ± 224
MMTV-LUC	2.8 ± 1.5	2.6 ± 0.9	58 ± 20	115 ± 36	476 ± 64

*) Induction is the mean of three independent, duplicate experiments ± SEM.
**) Constructs are described in Materials and Methods.

The only tested cells, besides LNCaP cells, in which the PSA promoter was active was the human mammary carcinoma cell line T47D. Transfection of TK-85-s-LUC to T47D cells resulted in a 25-fold induction of luciferase activity upon R1881 stimulation, even in the absence of pSVARo cotransfection (Table IIA). PSA-61-LUC could be stimulated approximately 100-fold by R1881. Because T47D cells are known to contain the progesterone receptor (PR), and a low level of AR, and because R1881 can activate both AR and PR, experiments were repeated with the pure androgen dihydrotestosterone (DHT) and the pure progestin R5020. DHT stimulation of T47D cells transfected with TK-85-s-LUC and PSA-61-LUC resulted in a 4-fold and 15-fold induction of LUC activity, respectively. R5020 stimulation of T47D cells transfected with TK-85-s-LUC and PSA-61-LUC gave rise to induction levels, comparable to R1881 stimulation. Cotransfection of T47D cells with pSVARo slightly increased DHT induced PSA promoter activity (Table IIB). These results indicate that the PSA promoter is not completely cell specific, and also, that PSA promoter activity is not completely AR specific. However, comparison of PSA (PSA-61 and TK-85) and MMTV promoter activity in T47D cells with that in LNCaP cells (Tables I and II) still indicates a strong preference of the PSA promoter for LNCaP cells.

Table V.II. Effect of R1881, DHT and R5020 on PSA-61-LUC, TK-85-LUC and MMTV-LUC activity in T47D cells, without (A) and with co-transfection of the AR expression plasmid pSVARo (B).

CONSTRUCT	RELATIVE INDUCTION (+/- hormone)*			
	A R1881	DHT	R5020	B DHT
TK-85-s-LUC**	25 ± 4	4.3 ± 0.4	26 ± 3	8.2 ± 1.7
TK-LUC	1.3 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1.3 ± 0.3
PSA-61-LUC	97 ± 21	17 ± 4	157 ± 29	25 ± 7
MMTV-LUC	504 ± 50	228 ± 21	512 ± 60	478 ± 45

*) Induction is the mean of three independent, duplicate experiments ± SEM.
**) Constructs are described in Materials and Methods.

DISCUSSION

It is well established that PSA expression is directly androgen regulated, and cell specific. Previously, in transient transfection experiments with PSA promoter constructs, combined with in vitro protein-DNA interaction assays, two AREs were identified in the proximal PSA promoter: ARE-I at position -170, and ARE-II at -394 (11,13). Activity of a 632 bp proximal promoter was approximately 6-fold induced by R1881 in LNCaP cells. Cotransfection with an AR or glucocorticoid receptor (GR) expression plasmid resulted in an increase of absolute activity and induction level of the proximal promoter upon R1881 treatment. The low level of induction obtained with the endogenous AR suggested that the proximal PSA promoter was not sufficient to account completely for the androgen induction as observed for the endogenous PSA gene (11). In the present study we characterized a strong upstream enhancer, which is required for high, androgen regulated and cell specific expression of the PSA gene. The results described extend our own previous work, and the recent work of Schuur et al. (18). As compared to the latter study, two major differences seem obvious: (i) In the experiments described in the present study, approximately 50% of the activity of the upstream enhancer region is retained within the 440 bp BstEII-PstI (-4.3 to -3.9) fragment (see Figure 3A), whereas in the work of Schuur et al. essentially all activity is lost by deletion of sequences downstream of the XbaI site at -5.3 Kb. At the 3'-border of the enhancer region the differences are less dramatic. Deletion from the PstI site to the EcoRV site in constructs PSA-73 and PSA-74 results in an approximately 5-fold loss of activity (see Figure 3A); the comparable constructs in ref. 18 (CN70 and CN71) show a 2-fold drop in activity. Summarizing, we found the minimal region with high enhancer activity to be approximately 1 Kb smaller at the 5'-border. Although it can always be argued that different LNCaP sublines and culture conditions have been used, there is no obvious explanation for the differences observed. (ii) A second difference between the data in ref. 18 and our findings concerns the induction level of the 6 Kb PSA promoter (3000 as compared to 38). Part of the difference in induction might be accounted for by the reporter genes used (LUC and CAT, respectively). The higher sensitivity of the LUC assay enabled us to compare the properties of ARE-III in the upstream enhancer with those of ARE-I and ARE-II in the proximal promoter.

An important goal of the present study was the analysis of the chromatin structure in a 31 kb region upstream of the PSA gene by the identification of DHSs. Although other explanations are possible, DHSs in chromatin, which reflect structural alterations, are a strong indication for the interruption of the nucleosome structure due to binding of transcription factors to the DNA. In chromatin from LNCaP cells, three DHSs were found clustered in the area from 3.8 to 4.8 kb upstream of the PSA gene. DHSIII (at -3.8 kb) is weak and also present in chromatin from HeLa cells, which do not express PSA. DHSI (at -4.8 kb) is clearly androgen induced in LNCaP cells. DHSII (at -4.2 kb) is by far the most prominent: it is strongly androgen induced in LNCaP cells and absent in HeLa cells. The differences in structure between chromatin from LNCaP cells, grown in the presence and absence of R1881, and from HeLa cells indicated to us a functional role of the DHS cluster in androgen regulated and cell specific expression of the endogenous PSA gene.

In transient transfection experiments, the -4.8 to -3.8 region showed strong, androgen regulated enhancer activity (PSA-64-s-LUC and PSA-64-as-LUC in Figure 3A). It is not

clear whether sequences corresponding to DHSI and DHSIII are present in the shorter active enhancer construct PSA-73-LUC. Most likely, DHSI maps very close to the PstI site, which is at the 5' border of PSA-73-LUC; DHSIII maps close to the BamHI site, which determines the 3' border of PSA-73-LUC. An even shorter fragment, PSA-85-LUC, lacks both DHSI and DHSIII, but contains DHSII, which maps close to the ClaI site. The finding that PSA-85-LUC still shows approximately 50% of the enhancer activity suggests that, in transient transfections, DHSI and DHSIII sequences play no or only a minor part in PSA promoter activity. Whether they are required for proper expression of the endogenous PSA gene remains to be determined. The finding that ARE-III at -4.2 kb corresponds to DHSII in the chromatin suggests that ARE-III is not only essential in the PSA promoter in transient transfections, but also in androgen regulated expression of the endogenous PSA gene.

The upstream core enhancer most probably has a complex structure. We were unable to narrow down the size of the core enhancer to less than 440 bp without loosing substantial activity. Combined with the essential role of ARE-III in the enhancer, at least three separate active regions can be identified in the core enhancer: ARE-III, and the 5'- and 3'- end fragments (see Figure 3). In each of the two end fragments, one or more binding sites of ubiquitous or prostate specific transcription factors might be located. The possibility that these fragments contain one or more weak, so far not identified, AR binding sites cannot be excluded. In cooperation with ARE-III, and additional *cis*-acting sequences within the 130 bp SalI-EcoRV fragment, a complex enhancer might be formed with cooperative interactions between the different components. Further experiments are obviously required to elucidate the detailed composition of this core enhancer.

A functional ARE-III is a prerequisite for high activity of the upstream enhancer. Inactivation of ARE-III almost completely abolished core enhancer activity. In contrast, truncation of the 5'- and 3'-fragments resulted only in a partial reduction of enhancer activity. However, there is little doubt about a synergistic cooperation of ARE-III with other *cis*-acting elements in the core enhancer: when hooked to the TK promoter, the core enhancer, containing one ARE-III, is superior to even three copies of ARE-III coupled to the TK promoter. A mechanism explaining the central role of ARE-III could be AR induced DNA bending, enabling the direct or indirect interaction between other transcription factors in the core enhancer. Alternatively, ARE-III bound AR might be a key element in the interaction of the upstream enhancer with the proximal promoter region, or recruitment of the RNA polymerase II holoenzyme to the PSA promoter (20,21). In this respect the PSA upstream enhancer might function as a classical complex, steroid hormone regulated control region. Similar upstream glucocorticoid regulated enhancers, composed of GR binding sites, binding sites for the liver enriched transcription factors HNF-3 and HNF-4, and binding sites for more ubiquitous transcription factors have been identified for the TAT gene, which is highly expressed in liver parenchymal cells (15,16). The enhancer motifs restrict the hormonal activation of the TAT gene to liver cells, not only in cultured cells, but also in transgenic mice (22). The PSA gene is the first example of an androgen-regulated, prostate specific gene for which such a potent upstream enhancer is documented. Further study of this enhancer can be of great help for the identification of prostate specific transcription factors, and for the elucidation of the mechanism of cooperative interaction between the AR and other transcription factors.

The identification of three AREs in the PSA promoter in the present, and in our previous studies (11,13) readily raised the question of relative AR binding affinities and functional activities. ARE-I and ARE-III were found to be of similar potency, whereas ARE-II was less active. Mutational analysis indicated a clear synergistic cooperativity between ARE-I and ARE-III, and to a lesser extent ARE-II. However, inactivation of ARE-III had a far more impressive effect on the 6 kb PSA promoter than mutation of ARE-I. From these findings it can be concluded that the context in which the ARE is present has a pivotal effect on its functional activity. As indicated above, this might involve interactions with other transcription factors, including the spacing between specific *cis*-acting elements.

Although the PSA gene is the first example of a gene containing a very potent far upstream ARE, clear synergistical interaction between multiple ARE sequences has also be found in the proximal (600 bp) PSA promoter (see ref. 13, as discussed above) and in the proximal (426 bp), prostate specific rat probasin (PB) promoter (23). Both the proximal PSA and the PB promoter contain one high affinity and one low affinity, functionally active AR binding site (13,23). Although much more active in their natural setting, multimers of the different, separate AREs from the PSA promoter are functionally active when fused to a heterologous promoter (Figure 5B, and ref. 13). In contrast, cooperative binding of the AR to both AREs in the PB promoter is required for androgen induction (24).

To investigate cell specificity of PSA upstream core enhancer, we compared its activity in several prostate and non-prostate cell lines. Transient transfection experiments in (PSA negative) PC3, DU145, Hep3B and COS cells did not reveal any activity, although two of the cell lines (PC3, DU145) originate from a prostate background. The MMTV promoter showed a very limited activity in AR cotransfected PC3 and DU145 cells, but was clearly active in COS and HeLa cells. Together these findings indicate the absence of one or more transcription factor(s) or coactivator(s) essential for PSA promoter activity in these cells. Alternatively, a specific inhibitor of PSA promoter activity is present. The specificity of DHSII provided additional evidence for cell specific activity of the PSA promoter.

TK-85-LUC and PSA-61-LUC were both found to be active in T47D cells. In T47D cells, PSA promoter activity was not only mediated through the AR but also via the PR (Table II). These results indicate that activity of the 6 kb PSA promoter is not entirely prostate and androgen specific. However, PSA promoter activity in LNCaP cells is superior to T47D cell activity. The issue of receptor specificity should be investigated in more detail in cell lines containing comparable amounts of PR and AR, either endogenously or after cotransfection with the respective steroid receptor expression plasmid. In a similar type of experimental setup, we generated LNCaP sublines containing a stable transfected GR expression plasmid. This resulted in dexamethasone induction of the endogenous PSA gene, and GR regulated activity of the 6 kb PSA promoter in transient transfections (K.B.J.M. Cleutjens, in preparation). In summary, steroid hormone regulated expression of the PSA promoter depends on the properties of the cell line: the presence of AR, PR or GR is an essential, but not the only factor. Absence of specific inhibitors or presence of additional transcription factors and/or coactivators will also be essential.

Although PSA expression was originally thought to be strictly restricted to prostate epithelial cells, low PSA expression in mammary tumor cells has been recently published (25,26). The activity of the PSA promoter in transfected T47D cells, which are negative on Northern blots for endogenous PSA expression, could be in accordance with these findings.

It would be of interest to find out whether in mammary tumor tissue PSA expression is progesterone regulated, and could be used as a reliable marker for PR positive tumors. To obtain more definite information on tissue specificity of the 6 kb PSA promoter in both normal and tumor cells, animal studies with PSA promoter constructs are required. If the upstream enhancer is able to confer preferential expression of a target gene to prostate cells, *in vivo* applications in humans, including gene therapy for delivery of pharmaceutical reagents to the prostate, can be further explored.

MATERIALS AND METHODS

Cell culture

LNCaP cells (FGC), originally obtained from Dr. Horoszewicz (27), were cultured in RPMI 1640 and supplemented with 5% FCS and antibiotics. For transfection, cells were grown in DMEM supplemented with 5% steroid-depleted (dextran-charcoal treated) FCS. For examination of androgen induced DHSs, and androgen driven promoter activity in transfection experiments, the synthetic androgen R1881 (DuPont NEN, Boston, MA) was added to a final concentration of 1 nM. In indicated cases, DHT (Steraloids, Wilton, NH) and R5020 (DuPont NEN) were added to final concentrations of 100 nM and 10 nM, respectively.

HeLa, T47D and COS cells were grown in DMEM, Hep3B cells were grown in MEM-alpha, supplemented with 5% FCS and antibiotics. PC3 and DU145 cells were grown in RPMI 1640, supplemented with 7.5% FCS and antibiotics. For transfection, PC3 and DU145 cells were grown in DMEM.

Mapping of DHSs

Cultured cells (LNCaP cells, grown in the presence and absence of 1 nM R1881, and HeLa cells) were washed with ice cold phosphate buffered saline (PBS). Cells were suspended in 3 ml ice cold HS-buffer (15 mM Tris-HCl pH 7.4, 60 mM KCl, 15 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA and 5% glycerol, supplemented with 1 mM dithiothreitol, 0.15 mM spermine and 0.5 mM spermidine, directly prior to use). The cells were disrupted by passing 5 to 10 times through a 0.5 x 16 mm (25G) needle. Disruption was monitored by light microscopic examination. Nuclei were collected by centrifugation for 5 min at 2500 rpm, and resuspended in HS buffer to a final concentration of 5 X 10^7 nuclei/ml. Limited DNaseI digestion was carried out in a final volume of 0.5 ml HS-buffer containing 5 x 10^6 nuclei, 5 mM $MgCl_2$ and DNaseI (0-800 U; Boehringer Mannheim, Germany). The mixture was incubated for 30 min on ice, and the reaction was stopped by addition of 10 μl 0.5 M EDTA, 12.5 μl 20% SDS and 50 μl Proteinase K (10 mg/ml). Next, the sample was incubated overnight at 37 °C. Subsequent to phenol/chloroform extraction, the DNA was collected by isopropanol precipitation. The DNA was dissolved in 100 μl Tris-EDTA buffer and digested with EcoRI. Restriction fragments were separated by electrophoresis in a 1% agarose gel and transferred to a nylon membrane (Hybond N^+, Amersham, Cardiff, UK). Filters were hybridized at high stringency with random primed ^{32}P-labeled probes (as indicated in Figure 1) using standard procedures (28).

Construction of plasmids

All plasmid constructs were prepared using standard methods (28). The promoterless basic plasmid pLUC, PSA-4-LUC, TKLUC, the human AR expression plasmid pSVARo, the AR(DBD) expression plasmid pRIT2TAR and pMMTV-LUC were described previously (13,29-31). PSA-61-LUC was generated by insertion of the Hind III/HindIII (-6 kb/+12) fragment of the PSA promoter in the MCS (multiple cloning site) of pLUC. PSA-1-LUC was generated by ligation of the BamHI/HindIII (-2.2 kb/+12) fragment in the MCS of pLUC. PSA-64-s-LUC and PSA-64-as-LUC (XbaI-StuI, -5.4/-3.2 kb), PSA-73-LUC (PstI-PstI, -4.7/-3.9 plus PstI-BamHI -3.9/-3.8 kb), PSA-74-LUC (PstI-PstI, -4.7/-3.9 kb), PSA-78-LUC (PstI-EcoRV, -4.8/-4.1 kb), PSA-83-LUC (SalI-BamHI, -4.25/-3.8 kb) and PSA-85-LUC (BstEII-PstI, -4.35/-3.9 kb) were generated by insertion of the appropriate fragments in front of the proximal PSA promoter (−632/+12) in construct PSA-4-LUC. The artificial SalI site (−4.25 kb) was derived from the 5'-end of a human genomic DNA phage insert (4P1, see ref 7).

Constructs TK-85-s-LUC and TK-85-as-LUC were generated by insertion of the 440 bp BstEII-PstI fragment into the MCS of TKLUC. Constructs ARE-I-TKLUC and ARE-III-TKLUC were generated by cloning three copies of ARE-I, and ARE-III oligonucleotides in TKLUC, respectively (sequences of oligonucleotides are shown below). ARE-II-TKLUC was generated by ligation of the double stranded 3ARE-II oligonucleotide in the SalI site of TKLUC.

```
ARE-I    : 5' GATCCTTGCAGAACAGCAAGTGCTAGCTG      3'
           3'      GAACGTCTTGTCGTTCACGATCGACCTAG 5'

3ARE-II  : 5' TCGACAGGGATCAGGGAGTCTCACCAGGGATCA-
           3'      GTCCCTAGTCCCTCAGAGTGGTCCCTAGT-

               GGGAGTCTCACCAGGGATCAGGGAGTCTCACG      3'
               CCCTCAGAGTGGTCCCTAGTCCCTCAGAGTGCAGCT 5'

ARE-III  : 5' TCGACGAGGAACATATTGTATCGAG      3'
           3'      GCTCCTTGTATAACATAGCTCAGCT 5'
```

pHS1, pHS2, pHS3 and pHS4, which were the starting material for footprint experiments, were obtained by insertion of the blunt ended BstEII/ClaI, SalI/EcoRV, EcoRV/PstI and ClaI/NcoI fragments, respectively, into the SmaI site of pTZ19 (Pharmacia, Uppsala, Sweden).

Generation of ARE mutations

Mutations were introduced in ARE-I (-170), ARE-II (-394) and ARE-III (-4200) essentially according to the PCR method of Higuchi et al. (32). Standard amplification conditions were 30 cycles of denaturation for 1 min at 95°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C. The oligonucleotides which were used for the generation of the different mutations are listed below. Two different sets of outer primers were used, one set for ARE-I and -II mutations, and a separate set for ARE-III mutations. Substitutions in complementary

sets of inner primers ARE-I-1, -2; ARE-II-1 and -2; ARE-III-1 and -2 are underlined (see below). PSA-61-LUC was used as the template for the first PCR step. In the second PCR step, appropriate samples of the purified products of the first amplification reactions were mixed at a 1:1 ratio. The resulting PCR fragments were cloned and after sequencing, exchanged with the corresponding fragment of PSA-61-LUC.

```
ARE-I and -II outer primers:
forward primer : 5' CCACAAGATCTTTTTATGATGACAG 3'
reverse primer : 5' GCTCTCCAGCGGTTCCATCCTCTAG 3'

ARE-III outer primers :
forward primer : 5' CTTCTAGGGTGACCAGAGCAG  3'
reverse primer : 5' GCAGGCATCCTTGCAAGATG 3'

Inner primers:

ARE-I-1:  5' GTAATTGCACATTAGCAATGGGTAACTCTCCC 3'
          3' CATTAACGTGTAATCGTTACCCATTGAGAGGG 5'

ARE-I-2:  5' GTAATTGCATAGTAGCAAAAGGTAACTCTCCC 3'
          3' CATTAACGTATCATCGTTTTCCATTGAGAGGG 5'

ARE-II-1: 5' GGTGCAGGCATAAGGGATGCTCACAATCT 3'
          3' CCACGTCCGTATTCCCTACGAGTGTTAGA 5'

ARE-II-2: 5' GGTGCAGGCATTAGGCAACCTGACAATCT 3'
          3' CCACGTCCGTAATCCGTTGGACTGTTAGA 5'

ARE-III-1:5' CTCTGGAGCATAATATTTCAACGATTGTC 3'
          3' GAGACCTCGTATTATAAAGTTGCTAACAG 5'

ARE-III-2:5' CTCTGGAGTAGTATATTACAGCGATTGTC 3'
          3' GAGACCTCATCATATAATGTCGCTAACAG 5'
```

Transfections

Cells were transfected according to the calcium phosphate precipitation method essentially as described (33), using 1 x 10^6 cells per 25cm^2 flask and 5 μg of the appropriate PSA-LUC construct. Following 4 h incubation with the precipitate, the culture medium was replaced by PBS, containing 15% glycerol (incubation for 90 sec at room temperature). Subsequently, transfected cells were incubated in culture medium in the absence or presence of the appropriate hormone for 24 h. Transfections were performed in duplicate. Experiments were repeated at least three times using two independent plasmid isolates.

Luciferase assay

Cells were washed in PBS and lysed in 300 μl lysis buffer (25 mM Tris-phosphate pH 7.8, 8 mM $MgCl_2$, 1 mM DTT, 1 % Triton X-100, 15 % glycerol). Next, 0.1 ml Luciferin (0.25 μM) (Sigma)/ 0.25 μM ATP was added to 0.1 ml extract, and luciferase activity was measured in a LUMAC 2500 M Biocounter (LUMAC, Landgraaf, The Netherlands). After a delay of 2 sec (according to supplier), the light emission was recorded during 5 sec. Luciferase activities were corrected for variations in protein concentrations of the cell extracts. Luciferase activities and relative induction factors are expressed as mean and standard error of the mean (SEM) of at least three independent experiments.

DNAseI footprint analysis

Production and purification of AR(DBD) was done as described previously (13,30). Fragments for footprinting were generated by digestion of pHS1, 2, 3 and pHS4 with XbaI and SacI, or with SphI and EcoRI, to be able to identify protected windows on both the upper and lower strand. Subsequently, fragments were filled in with MMLV-reverse transcriptase (Boehringer) in the presence of α-^{32}P-dATP, and isolated from non-denaturing polyacrylamide gel. The DNaseI footprinting experiments were performed essentially according to Lemaigre et al. (34). Labelled probe (50,000 cpm) was incubated with 10-20 pmol AR(DBD) fusion protein for 30 min at 0 °C, in the presence of 10 μM $ZnCl_2$. In indicated cases, a 100-fold excess competitor oligo's (consensus ARE or consensus NF-1; 5'-GATCCAGGGAACAGGGTGTTCTACG-3', and 5'-ATTTTGGCTTGAAGCCAATATG-3', respectively)) was added. Digestion with 0.04 U DNaseI (Boehringer) was for 60 sec at 20 °C in a final volume of 50 μl. In the absence of AR(DBD), 0.025 U DNAseI was used. Following phenol/chloroform extraction and ethanol precipitation, DNA was dissolved in 5 μl formamide-dye-mix (98% formamide, 10 mM EDTA, 0.2% bromophenol blue and 0.2% xylene cyanol). After heating to 95 °C for 2 min and rapid cooling on ice, the DNA was separated on a denaturing (7M urea) 6 % polyacrylamide gel. G and (G+A) sequence reactions according to Maxam and Gilbert (35) of the same fragment were run as markers alongside each footprint. After electrophoresis, gels were fixed, dried and exposed to X-ray film.

Gel retardation analysis

The gel retardation experiments were performed as described previously (13). Double stranded oligonucleotides used in gel retardations:

```
ARE-I     : 5' GATCCTTGCAGAACAGCAAGTGCTAGCTG     3'
            3'     GAACGTCTTGTCGTTCACGATCGACCTAG 5'

ARE-II    : 5' GATCCAGGGATCAGGGAGTCTCAG    3'
            3'     GTCCCTAGTCCCTCAGAGTCCTAG 5'

ARE-III   : 5' TCGACGAGGAACATATTGTATCGAG    3'
            3'     GCTCCTTGTATAACATAGCTCAGCT 5'
```

Shortly, probes were filled in with MMLV-reverse transcriptase in the presence of α-^{32}P-dATP, and subsequently isolated from non-denaturing polyacrylamide gel. Labeled probe, 50,000 cpm, was incubated with AR(DBD) (30 fmol to 2 pmol). In indicated cases 100-fold excess ARE or NF-1 competitor oligonucleotides was added. Following 20 min incubation, samples were run on a 4 % non-denaturing polyacrylamide gel. Subsequently, gels were fixed, dried and exposed to X-ray film.

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Chapter VI

A 6 KB PROMOTER FRAGMENT MIMICS IN TRANSGENIC MICE THE PROSTATE-SPECIFIC AND ANDROGEN-REGULATED EXPRESSION OF THE ENDOGENOUS PROSTATE SPECIFIC ANTIGEN GENE IN HUMANS

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Chapter VI

ABSTRACT

Prostate-specific antigen (PSA) is a kallikrein-like serine protease, that is almost exclusively synthesized in the luminal epithelial cells of the human prostate. PSA expression is androgen regulated. Previously, we characterized *in vitro* the proximal promoter, and a strong enhancer region, approximately 4 Kb upstream of the PSA gene. Both regions are needed for high, androgen regulated activity of the PSA promoter in LNCaP cells. The present study aims at the *in vivo* characterization of the PSA promoter. Three transgenic mouse lines carrying the E.coli LacZ gene driven by the 632 bp proximal PSA promoter, and three lines with LacZ driven by the 6 Kb PSA promoter were generated. Expression of the LacZ reporter gene was analyzed in a large series of tissues. Transgene expression could not be demonstrated in any of the transgenic animals carrying the proximal PSA promoter. All three lines carrying the 6 Kb PSA promoter showed lateral prostate specific β-galactosidase activity. Transgene expression was undetectable until 8 weeks after birth. Upon castration, β-galactosidase activity rapidly declined. It could be restored by subsequent androgen administration. A search for mouse PSA-related kallikrein genes expressed in the prostate led to the identification of mGK22, which was previously demonstrated to be expressed in the submandibular salivary gland. Therefore, the 6 Kb PSA-LacZ transgene followed the expression pattern of the PSA gene in humans, which is almost completely prostate-specific, rather than that of mGK22 in mice. In conclusion, the 6 Kb promoter fragment appears to contain most, if not all, information for androgen-regulation and prostate-specificity of the PSA gene.

INTRODUCTION

Prostate specific antigen (PSA) is a 30-33 kDa glycoprotein, that is almost exclusively produced by the luminal epithelial cells of the human prostate. It is one of the predominant proteins secreted into the prostatic fluid. Serum PSA is a well known marker for diagnosis and monitoring of prostate cancer (1,2). The PSA gene (or KLK3) is a member of the human kallikrein gene family. Other members of the kallikrein gene family are the hGK-1(KLK2) gene, which is also expressed in the prostate, and the tissue kallikrein gene (KLK1), which is mainly expressed in the pancreas and kidney (3-6). The three genes are clustered within the 60 Kb kallikrein locus on chromosome 19 (7,8). PSA expression can be regulated by androgens (9-11). Previously, we and others characterized *in vitro* the 632 bp proximal promoter (11,12), and a strong, 440 bp enhancer region, approximately 4 Kb upstream of the transcription start site of the PSA gene (13,14). Both regions are needed for high, androgen regulated activity of the PSA promoter in LNCaP cells. Two functionally active androgen receptor binding sites (androgen response elements, or AREs) were identified in the proximal PSA promoter, at positions -170 (ARE-I) and −394 (ARE-II), respectively (11,12). The upstream enhancer showed synergistic cooperation with the proximal PSA promoter, and was found to be composed of at least three separate, but cooperating, regulatory regions. At -4.2 Kb, the presence of a functionally active, high affinity androgen receptor binding site (ARE-III) was established (14). Transient transfection of a 6 Kb PSA promoter fragment, containing both the proximal promoter and the upstream enhancer, linked to the luciferase reporter gene, to prostate and non-prostate cell lines showed largely LNCaP

prostate cell specific activity (13,14). The strong tissue specificity of the endogenous PSA gene *in vivo* and the 6 kb PSA promoter fragment in transient transfection experiments makes the PSA promoter a candidate to deliver therapeutic genes to prostate cancer cells. To explore this view, the present study aimed at the *in vivo* characterization of the PSA promoter in transgenic mice.

In mice, the kallikrein gene family is composed of 24 members, half of which are probably pseudogenes (15). Although structurally related to the PSA gene, none of the mouse kallikreins can be considered as the mouse homolog of human PSA, because of the different tissue distribution (16). All functional mouse kallikrein genes are expressed in the submandibular salivary gland (SMG). Individual genes show additional expression in pancreas, kidney, spleen and/or testis. So far, mouse kallikrein expression in the prostate has not been demonstrated. Two members of the closely related rat kallikrein gene family have been found to be expressed in both prostate and SMG (17). In order to compare PSA promoter specificity in transgenic mice with the promoter specificity of endogenous mouse kallikreins we determined which, if any, of the mouse kallikrein genes was expressed in prostate.

RESULTS

Activity of the PSA promoter LacZ fusion constructs in LNCaP cells

Previously, we characterized in transfection experiments the proximal promoter, and a strong 440 bp core enhancer region, approximately 4 Kb upstream of the transcription start site of the PSA gene (11,12,14). Two functionally active AREs were identified in the proximal PSA promoter, at positions -170 (ARE-I) and -394 (ARE-II), respectively (11,12). In the center of the 440 bp upstream enhancer region a third functionally active ARE, ARE-III (-4200) could be demonstrated (14). Although both the proximal promoter and the upstream region contributed to maximal androgen regulated and cell specific activity of the PSA promoter, the upstream enhancer was found to be essential for high activity (12,14). To investigate the regulatory regions of the PSA promoter in transgenic mice, two LacZ reporter gene constructs were designed (Figure 1A). In these constructs, the LacZ gene is driven by the 632 bp proximal PSA promoter (PSA-4-LACH), or by the 6 Kb PSA promoter fragment (PSA-61-LACH). The hormone induced activity of the constructs was tested in transiently transfected LNCaP cells. The PSA-4-LACH construct, co-transfected with the human androgen receptor expression plasmid pSVARo, was 7-fold more active in the presence than in the absence of the synthetic androgen R1881 (Figure 1A). In the absence of pSVARo, PSA-4-LACH showed a limited androgen inducibility (1.8 fold). Under these conditions PSA-61-LACH activity was induced 600-fold by R1881. These results are essentially identical to those obtained with comparable luciferase reporter gene constructs (12,14).

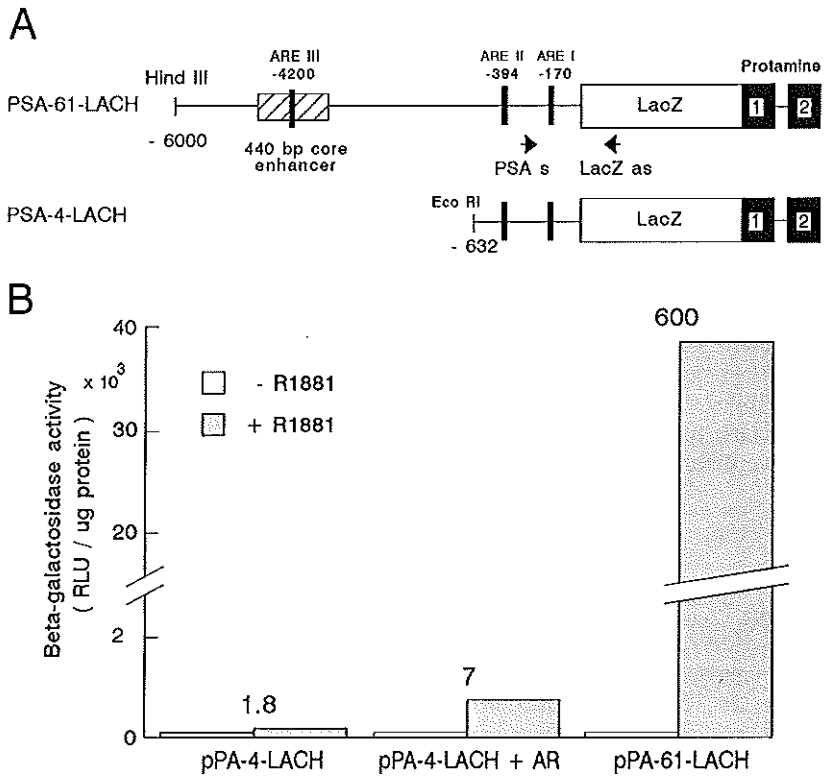


Figure VI.1. Structure and activity of the PSA-LACH constructs introduced in transfected LNCaP cells. (A) Schematical representation of constructs PSA-61-LACH and PSA-4-LACH. The 440 bp core enhancer region is represented by a hatched box, ARE sequences are indicated by black bars. The open box represents the LacZ open reading frame; numbered black boxes indicates exons 1 and 2 of the mouse protamine gene. Positions of primers used to identify transgenic animals are indicated below PSA-61-LACH. (B) LNCaP cells were transiently transfected with the PSA-4-LACH and PSA-61-LACH constructs, or with PSA-4-LACH plus the androgen receptor expression plasmid as described in Materials and Methods and ref. 14. Incubation with the plasmid precipitate was for 4 h. Induction values are given at the top of the bars.

Identification of transgenic mice

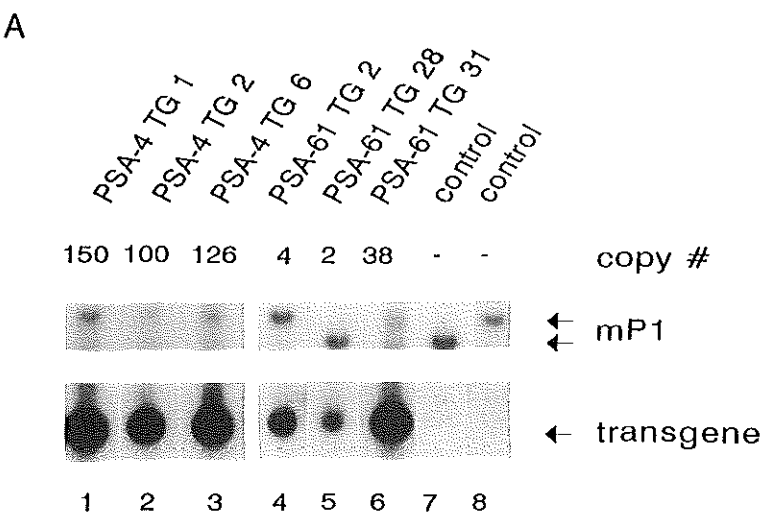
Both PSA-4-LACH and PSA-61-LACH were used to generate transgenic mice. Three PSA-4-LACH and five PSA-61-LACH founder animals were identified by PCR of tail DNA with primers PSA-s and LacZ-as (data not shown). Transmission of the transgene to their offspring was demonstrated for three PSA-61-LACH and all three PSA-4-LACH transgenic lines. One PSA-61-LACH male founder did not transmit the transgene; another PSA-61-LACH male founder was infertile. Comparison of the hybridization signals of the transgene and the endogenous mouse protamine-1 gene on Southern blots of genomic DNA revealed the presence of 4, 2 and 38 copies of the transgene in lines PSA-61 TG2, TG28 and TG31,

respectively. PSA-4 TG1, PSA-4 TG2 and PSA-4 TG6 carried approximately 150, 100 and 126 copies of the transgene (Figure 2A, lanes 4-6, and 1-3, respectively). Note that the endogenous mouse protamine-1 gene showed a restriction fragment length polymorphism, resulting in 6 and/or 8 Kb hybridizing fragments (Figure 2A lanes 7,8).

The 6 Kb, but not the 632 bp PSA promoter directs lateral prostate specific transgene expression

To determine the expression pattern of the transgene, male mice were sacrificed at 8 to 16 weeks of age, and β-galactosidase activity was measured in twenty-six different tissue lysates (see Materials and Methods). Thorough analysis of all three PSA-61-LACH transgenic mouse lines showed exclusive β-galactosidase activity in extracts from lateral prostate. In all other tissues, including the dorsal, ventral and anterior prostate lobes, LacZ expression was undetectable (as shown in Figure 2B for PSA-61 TG28). β-Galactosidase activity could also not be detected in extracts from tissues of virgin or lactating female transgenic mice (data not shown). In none of the tested tissues of PSA-4-LACH mice, β-galactosidase expression could be found (data not shown, and Figure 2C). For PSA-61-LACH, transgene activity was independent of the number of integrations, since β-galactosidase activity in the lateral prostate was comparable, despite the difference in copy numbers (4, 2 and 38, respectively). The level and specificity of transgene expression appeared independent of the integration site.

To screen for the presence of low levels of transgene expression in the different prostate lobes and Submandibular salivary gland (SMG), a known expression site of mouse kallikreins, we performed RT-PCR with transgene cDNA specific primers, and GAPDH as a control (See Materials and Methods). Again, transgene expression could only be detected in the lateral prostate (lane 2, Figure 2D).



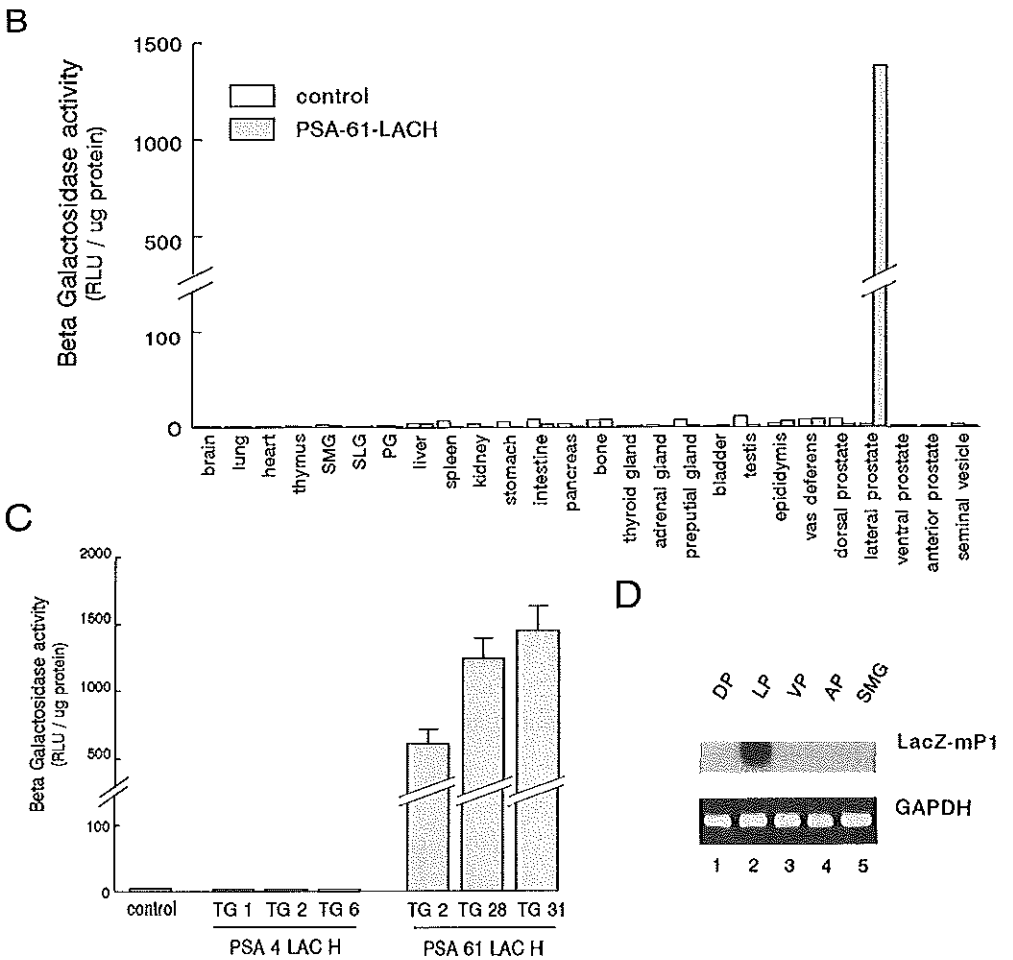


Figure VI.2. Characterization of transgenic mouse lines. (A) Southern blot analysis of the KpnI-SacI digested genomic DNA of PSA-4-LACH (lanes 1-3) and PSA-61-LACH (lanes 4-6) transgenic (TG) lines. Lane 7 contains DNA of a control mouse. DNA (10 µg/lane) was hybridized with a 175 bp mouse protamine cDNA probe (see Materials and Methods). By comparison of the intensity of the endogenous (see arrow heads) and transgene bands, the number of transgene copies present in the individual transgenic lines was determined (numbers on top of each lane). For PSA-4-LACH transgenic animals, two different exposure times of the same Southern blot are shown (A 4 h exposure of the transgene hybridizing fragment and a 40 h exposure of the endogenous mouse protamine gene). Note that the endogenous protamine fragment is polymorphic. (B) Liquid β-galactosidase assay of tissue extracts of 10-week old PSA-61-LACH TG28 male mice. (C) β-Galactosidase activity in lateral prostate lysates of PSA-4-LACH TG 1, 2 and 6 and PSA-61-LACH TG 2, 28 and 31 animals as compared to activity in control mice. (D) RT-PCR analysis of LacZ/Protamine transgene mRNA in RNA obtained from dorsal, lateral, ventral and anterior prostate and submandibular salivary gland of PSA-61-LACH TG 28 male mice. Experimental details are described in Materials and methods. The lower panel shows the result of RT-PCR analysis of ubiquitously expressed GAPDH mRNA. PCR products were separated over a 2 % agarose gel.

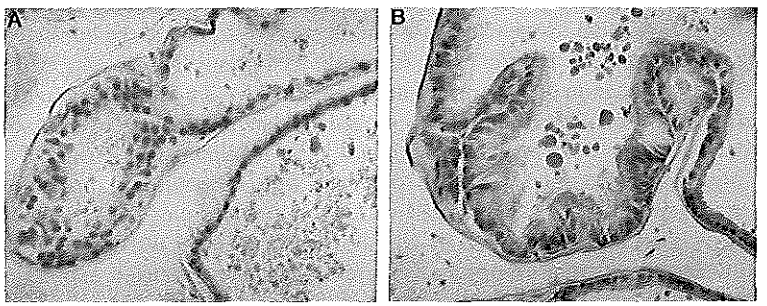


Figure VI.3. Transgene expression in the lateral prostate. (A,B) Whole mount X-gal staining, followed by neutral red counterstaining of 5 μm paraffine embedded sections of (B) lateral prostate of a 10 week PSA-61-LACH TG 28 male and (A) lateral prostate of a non-transgenic littermate (Magnification 400 x). Blue X-gal staining is shown as blue spots in the cytoplasm of luminal epithelial cells.

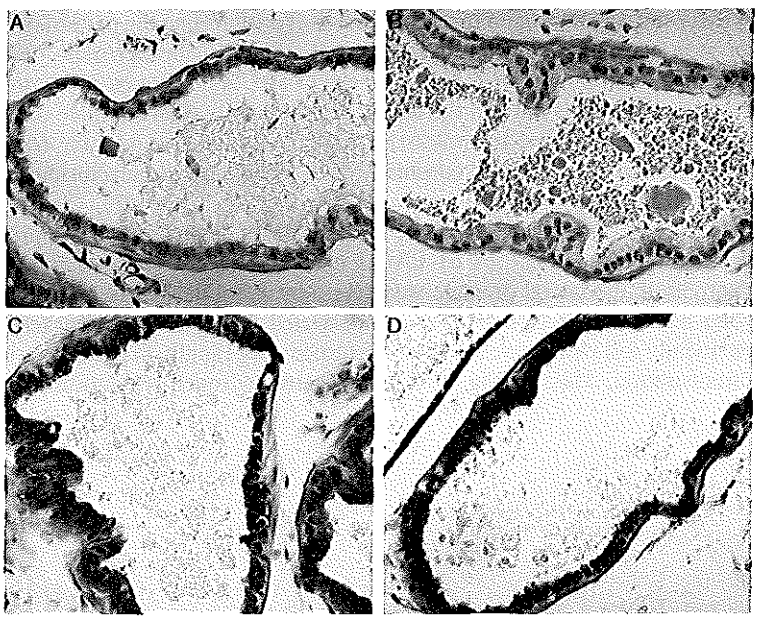


Figure VI.4. RNA in situ hybridization analysis of lateral prostate tissue sections of a 10 week old PSA-61-LACH TG 28 male transgenic mouse. Five μm sections of paraffine embedded tissue were incubated with a DIG-labeled protamine RNA probe. Hybridization was visualized with alkaline-phosphatase-conjugated antiDIG antibody (see Materials and Methods). (C,D) Tissue sections of a 14 week old PSA-61-LACH male transgenic mouse, (B) Non-transgenic littermate (antisense probe; 400 x magnification) (A) Incubation of a transgenic mouse prostate with a sense protamine riboprobe.

PSA-61-LACH expression is restricted to the luminal epithelial cells of the lateral prostate

Whole mount β-galactosidase staining, followed by sectioning of the paraffine embedded tissue was performed to investigate the cell type in the prostate expressing the LacZ gene. As demonstrated in Figure 3B, β-galactosidase staining was restricted to the luminal epithelial cells. Staining was concentrated at the basal site of the cytoplasm. No staining was found in the lateral prostate from age-matched control mice (Figure 3A). To further evaluate PSA-61-LACH expression, sections of the paraffine embedded lateral prostate of PSA-61-LACH positive and control mice, were analyzed by *in situ* hybridization using sense and antisense DIG-labeled protamine riboprobes. Results obtained with the antisense protamine probe revealed that transgene mRNA was localized within the cytoplasm of the luminal epithelial cells of the lateral prostate (Figure 4C,D). No hybridization signal was detected in control mice, or with a sense protamine riboprobe (Figure 4A,B). The restricted expression of the transgene to the luminal epithelial cells is consistent with endogenous PSA expression in the human prostate (18).

Developmental and hormonal regulation of PSA-61-LACH expression

PSA gene expression has been shown to be developmentally regulated and to follow plasma testosterone levels (19). In *in vitro* studies, expression of PSA mRNA and protein, and PSA promoter activity is strongly androgen regulated (9-14). To determine the pattern of the PSA-61-LACH transgene expression during development, lysates of lateral prostate tissues were prepared from line 28 males between 2 and 52 weeks of age. As indicated in Figure 5A, the dorsolateral prostate of 2 weeks old and the lateral prostate of 4 weeks old mice did not show significant β-galactosidase activity. In contrast, sexually mature males, ranging from 8 to 52 weeks of age, showed an almost constant, high level of β-galactosidase activity (approximately 1500 RLU/ug protein).

To obtain additional information on androgen inducibility of the 6 Kb PSA promoter in transgenic mice, sexually mature PSA-61-LACH males of line 28 were castrated, and β-galactosidase activity in the lateral prostate was determined at 4 days after castration, and at 2 days following hormone replacement. As demonstrated in Figure 5B, transgene activity decreased dramatically following castration, and returned very rapidly to pre-castration levels after DHT administration. This finding strongly indicates androgen regulation of transgene expression.

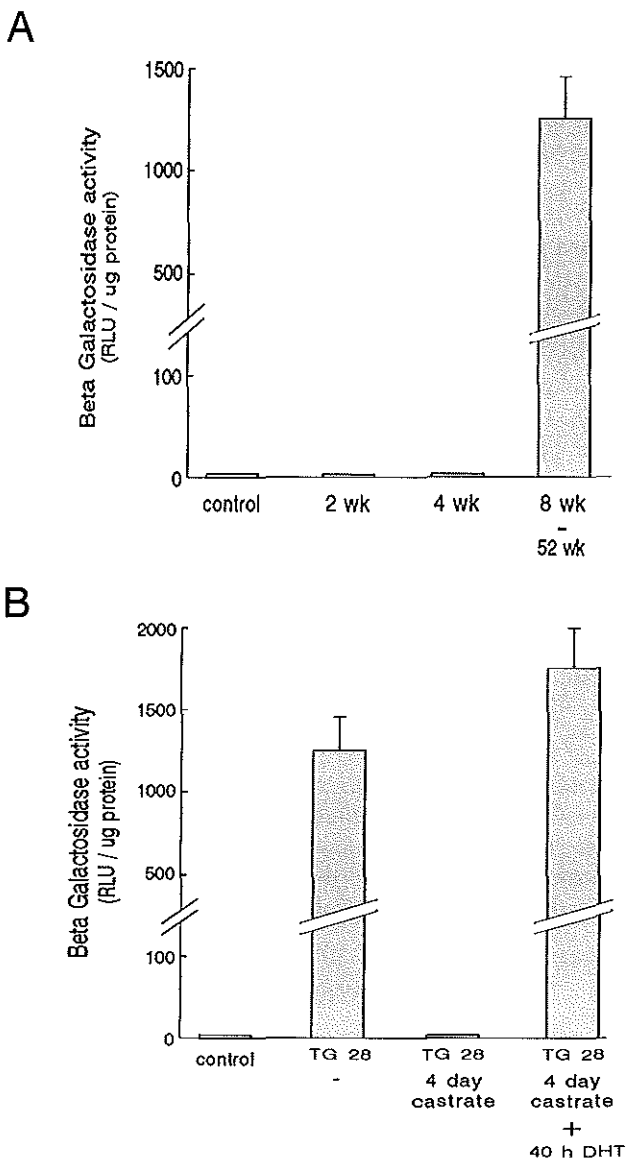


Figure VI.5. Developmental (A) and androgen (B) regulation of PSA-61-LACH expression. (A) β-Galactosidase activity in extracts of dorso-lateral prostate of 2 week, and lateral prostate of 4 week and 8 to 52 week old PSA-61-LACH TG 28 mice. (B) Androgen regulation of β-galactosidase activity in lateral prostate of PSA-61-LACH TG 28 mice. Mice were castrated at 10 weeks of age. After 4 days, part of the mice were supplemented with DHT or vehicle as described in Material and Methods. Lateral prostate of 10 week old PSA-61-LACH mice served as control.

Mouse kallikrein expression

In order to investigate mouse kallikrein gene expression in the prostate, RNA was isolated and RT-PCR was performed with primers overlapping highly conserved regions in exon 3 (KALK-3-s) and exon 4 (KALK-4-as) of all known mouse kallikrein genes (see GenBank data for mouse kallikrein sequences). Thirty-four cloned, 146 bp PCR fragments were sequenced. Thirty-two clones turned out to contain a mGK22 fragment (20), the 2 additional cDNA fragments were 94% identical, and both contained novel kallikrein sequences, with highest homology to mGK16 (91 and 92%, respectively) (21). Previously, mGK22 was found to be expressed in both male and female salivary glands, but absent in all other tissues tested (22). RT-PCR with mGK22 specific primers confirmed the presence of mGK22 mRNA in lateral prostate and SMG. mGK22 was absent in dorsal, ventral and anterior prostate (Figure 6). The expression level in SMG was much higher than in lateral prostate.

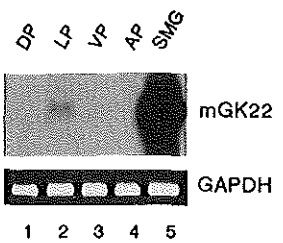


Figure VI.6. RT-PCR analysis of mouse Glandular Kallikrein 22 expression in the various lobes of the mouse prostate and male submandibular salivary gland. The RT-PCR products were blotted to Hybond N^+ membrane and hybridized with a random primed ^{32}P-labeled probe specific for the expected 634 bp cDNA fragment. RT-PCR of GAPDH mRNA in the RNA preparations of the different tissues is shown in the lower part.

DISCUSSION

Previously, we investigated the properties of the 632 bp proximal promoter, and a strong far upstream (-4 Kb) 440 bp enhancer region of the PSA gene in transfected LNCaP cells (11,12,14). Although both regions contributed to androgen regulated activity of the promoter, the presence of the 440 bp core enhancer was a prerequisite for high activity. A 6 Kb PSA promoter fragment, which contains both the proximal promoter and the upstream enhancer region, was mainly active in LNCaP prostate cells. However, PSA promoter activity was also observed in T47D mammary tumor cells (14).

In this study we showed that the 6 Kb and not the 632 bp PSA promoter is capable to direct reporter gene activity in transgenic mice. In three independent transgenic lines, carrying a LacZ reporter gene under control of the 6 Kb PSA promoter, hormonally and

developmentally regulated expression of the transgene was exclusively targeted to the luminal epithelial cells of the lateral prostate, which mimics the expression pattern of the endogenous PSA gene in the human prostate. This strongly suggests that the 6 Kb PSA promoter contains most, if not all, information for prostate specific activity. The specific expression of the transgene in the mouse lateral prostate is in agreement with the structural homology between the human prostate and the mouse lateral prostate, and the mouse kallikrein expression in the lateral prostate. The variable level of PSA expression in human breast cancer (23), and the activity of the 6 Kb PSA promoter in transiently transfected T47D human mammary tumor cells (14) could not be confirmed in normal breast tissue of female PSA-61-LACH transgenic mice (data not shown).

Transgene expression was assessed in a liquid β-galactosidase assay, by RT-PCR and by RNA *in situ* hybridization. Additionally, X-gal staining of the different tissues was performed. X-Gal staining of adult mouse tissues is complicated due to high endogenous β-galactosidase activity present in many tissues, including testis, epididymis, vas deferens, liver, intestine and prostate. This problem was overcome by modification of the standard protocols (24,25). Incubation at elevated temperature prior to staining (1 h at 50° C), and a raised pH (8.6) during the various incubation steps (see Materials and Methods) suppressed endogenous β-galactosidase activity, without noticeable loss of activity of the E.coli derived transgene. Only in epididymis, vas deferens and anterior prostate, endogenous β-galactosidase activity could be found at a long (over 16 h) staining period, which precludes detection of a low level of transgene expression in the X-gal assay (data not shown).

β-Galactosidase expression was undetectable in the PSA-4-LACH mice, despite the presence of 100 or more copies of the transgene in all three transgenic lines. Transient transfection of LNCaP cells with PSA-4-LACH (Figure 1) and also PSA-4-LUC constructs (12,14) showed a low activity of this 632 bp promoter fragment, especially in the absence of a co-transfected androgen receptor expression plasmid. The observation by Schaffner et al. (see ref. 26), that transgenic mice carrying a Ha-*ras*T24 oncogene, driven by the 632 bp proximal PSA promoter developed salivary gland and gastrointestinal tract tumors seems to be in contrast to our findings for this promoter. However, mutant Ha-*ras* expression was only confirmed in salivary gland tumors, and not in gastrointestinal tumors. Furthermore, the late onset of tumorigenesis could indicate that Ha-ras expression was a secondary event. This might be related to PSA expression in a subset of salivary gland tumors in humans (27). An alternative hypothesis is that Ha-*ras* intron or exon sequences affect the selectivity and level of expression of the oncogenic transgene.

The three PSA-61-LACH transgenic lines show a comparable level of lateral prostate specific, but copy number independent β-galactosidase expression. This could indicate that the PSA-61-LACH transgene cassette lacks elements, like matrix attachment regions or locus control regions, which might determine boundaries in chromatin structure, leading to copy number dependent and position independent activity of transgenes (see 28, and references therein). The 6 Kb PSA promoter fragment contains all DNAseI hypersenstive sites (which indicate important regulatory regions) in the 31 Kb region upstream of the PSA gene (see ref. 14). However, it might lack putative, so far unidentified regulatory sequences within the PSA gene, or in the flanking region downstream of the PSA gene, or even downstream of the hGK-1(KLK-2) gene, which is also prostate specifically expressed, and which is at a distance of 12 Kb in the human genome (7,8). Alternative explanations for copy number independent

activity are also possible. Although difficult to compare, the RT-PCR, and X-gal staining experiments suggest that the expression level of the transgene in PSA-61-LACH mice is not as high as that of the endogenous PSA gene in the human prostate. Although this might be due to the integration site and the properties of the LacZ and protamine part of the transgene cassette, it is a real possibility that one or more trans-acting factors, that direct high level PSA expression in the human prostate, are absent, or present in a much lower concentration in the mouse prostate. If this is indeed the case, these factors could be limiting factors in the expression of the transgene, which corresponds to a comparable activity of the 6 Kb PSA promoter in the three independent transgenic lines. This might also explain the low expression level of the mouse kallikrein mGK22 in the prostate. On the other hand, the latter might be caused by differences in promoter make up. Further analysis of mGK22 mouse kallikrein promoter activity in human prostate cell lines should provide additional information. In this regard, it is also interesting, that the 6kb PSA promoter driven transgene expression pattern was different from that of mGK22, which is expressed at a high level in SMGs. The PSA-61-LACH transgene follows the expression pattern of the endogenous PSA gene in humans, and not that of mouse kallikreins.

The 6 Kb PSA promoter is the first human promoter, that directs prostate specific expression in transgenic mice. Previously three rat promoters have been studied with respect to prostate specificity and applicability in the development of rodent prostate cancer models, rKLK8, C3(1) and probasin (29-35). Transgenic rats carrying a 2.5 Kb rKLK8 rat kallikrein promoter fragment did not show tissue specificity. Expression of the transgene was demonstrated in almost all tissues tested, including prostate, but was absent at the major sites of endogenous gene expression, the submandibular and sublingual salivary glands (29). Transgenic mice carrying a 6 Kb 5'-flanking region of the rat C3(1) gene linked to the β-galactosidase reporter gene (30) or a 9.5 Kb fragment carrying the C3(1) gene with 4 Kb upstream and 2 Kb downstream flanking sequences (31) did not direct transgene activity strictly to the prostate. Depending on integration site, expression was also detected in testis, heart, lung and skeletal muscle. Transgenic mice bearing a 5.7 Kb C3(1) promoter linked to the SV40 large T antigen region developed at 7 months a prostate adenoma or adenocarcinoma (32). Female mice carrying this transgene acquired mammary adenocarcinomas. The mice developed also other phenotypic changes including several proliferative lesions and malignancies leading to premature death. Greenberg et al. (33) reported on a 426 bp promoter fragment of the rat probasin gene directing CAT reporter gene expression to the prostate of transgenic mice. These transgenic mice showed CAT expression in dorsal, lateral and ventral prostate. Low levels of transgene expression were observed in the anterior prostate and in the seminal vesicles. Although prostate specific, the expression level of the transgene was dependent on the integration site, and did not strictly follow the expression pattern of the endogenous rat probasin gene, which is selectively expressed in the dorsolateral prostate. Co-integration of chicken lysozyme matrix attachment regions resulted in transgene expression in dorsolateral prostate of adult mice. Co-integration of matrix attachment sites was insufficient to facilitate high level and copy number dependent expression. Transgenic mice carrying the 426 bp Probasin promoter driven SV40 large T antigen oncoprotein, developed progressive forms of prostatic cancer (34,35).

Progress towards the understanding of the biology of prostate cancer benefits enormously from the availability of proper animal models displaying the whole range of clinical stages.

The present study provides a baseline for the generation of such models, utilizing the 6 kb PSA promoter hooked to the appropriate oncogenes. Because of its tissue specificity, and integration site independent, constant activity it might even be preferred above the probasin and C3(1) promoter driven prostate cancer models.

The observations as presented in this study, are not only relevant to the generation of mouse prostate cancer models, but also to gene therapy programs of human prostate cancer. The PSA gene is not only expressed in the luminal epithelial cells of the normal human prostate, but also in almost all prostate cancers. Therefore, the regulatory elements, that determine PSA expression in prostate cancer are of potential interest for building a promoter to drive expression of therapeutic genes in prostate cancer cells. The strict prostate specificity of the 6 Kb PSA promoter fragment strongly supports the applicability of this large promoter fragment, or derivatives, in gene therapy of human prostate cancer. Preliminary experiments, indicating prostate specificity of the 6 kb promoter driven TK gene in an adenovirus construct are in accordance with this view (Gotoh et al., unpublished results).

MATERIALS AND METHODS

Cell culture

LNCaP prostate cells were cultured as described (36). For examination of androgen driven promoter activity, the synthetic androgen, R1881 (DuPont NEN, Boston, MA), was added to steroid depleted medium to a final concentration of 1 nM.

Construction of plasmids

All plasmid constructs were prepared according to standard procedures (37). The human androgen receptor expression plasmid pSVARo and the LacZ containing reporter plasmid pLACH were described previously (38,39). A mouse protamine gene fragment (mP1, +95 to +625, see ref. 24) provides the LacZ cassette with an intron and the 3' untranslated region, including the polyadenylation signal. PSA-61-LACH was generated by integration of the blunt ended HindIII/HindIII (-6 kb/+12) fragment of the PSA promoter into the SmaI site of the pLACH multiple cloning site (MCS). PSA-4-LACH was generated by integration of the EcoRI/HindIII (-632 /+12 bp) PSA promoter fragment into pLACH.

Transient transfections

Cells were transfected according to the calcium phosphate precipitation method, essentially as described (14).

Generation and identification of transgenic mice

The 632 bp and 6 Kb PSA promoter driven LacZ genes were released from vector sequences by restriction digestion, purified by gel electrophoresis and prepared for injection according to standard methods (40). The appropriate fragments were microinjected into the male pronuclei of fertilized eggs of C57BL6xDBA2C (F1) mice. The presence of the transgene was established by PCR amplification on DNA from tail biopsies (40), using oligonucleotide primers PSA-s: 5'-TTGTCCCCTAGATGAAGTCTCCATGA-3' and LacZ-as: 5'-CGCCAGGGTTTTCCCAGTCACGAC-3' (indicated in Figure 1).

Transgene copy numbers were quantitated by phospho image analyses of Southern blots of tail DNA. To this purpose, 10 μg DNA was digested with KpnI and SacI, electrophoresed on 0.8 % agarose gel and transferred to Hybond N^+ membrane (Amersham, Cardiff, UK). Filters were hybridized at high stringency with a random primed ^{32}P-labeled protamine probe (see RNA *in situ* hybridization section). DNA transfer and filter hybridization were carried out according to the protocol of the manufacturer.

In indicated cases, mice were supplemented once a day with 5 mg Dihydrotestosterone (DHT) / Kg body weight. DHT in 100% ethanol was mixed with 9 volumes sesame oil, and injected subcutaneously.

Liquid β-galactosidase assay

β-Galactosidase activity was measured in lysates of LNCaP cells and mouse tissues using the Galacto-Light Plus™ chemiluminescent reporter assay (Tropix Inc., Bedford, MA). Two to 5 mg of mouse tissue was incubated in 100 μl lysis solution, transfected LNCaP cells were collected in 350 μl lysis solution. β-Galactosidase activity in 10 μl extract was corrected for variations in protein concentrations (protein microassay, Bio-Rad, München, Germany).

Whole mount β-galactosidase staining

Immediately after sacrificing, mouse tissues were fixed by perfusion fixation in 2% paraformaldehyde in a 0.1 M PIPES buffer (pH 6.9), containing 2 mM $MgCl_2$ and 1.25 mM EGTA. Tissues were dissected and fixed for an additional 60-90 min at RT. To inactivate endogenous β-galactosidase activity, tissues were washed three times for 30 min in PBS (pH 8.6). Subsequently, tissues were incubated in PBS for 60 min at 50°C. After cooling to RT, tissues were incubated in pre-staining solution (containing 2mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$ and 5 mM EGTA in PBS) for 60 min. After transfer to staining solution (pre-staining solution supplemented with 0.5 mg/ml X-Gal), incubation was continued for 6-24 h at RT. The reaction was stopped by extensive washing in PBS, and tissues were postfixed in 4% paraformaldehyde in PBS prior to paraffine embedding. Five μm sections were counterstained with neutral red.

RT-PCR

Isolation of total cellular RNA was carried out according to the guanidinium isothiocyanate method (41). Reverse transcription and PCR amplification of LACZ-protamine (primers LACZ-s and PRO1/2-as), mouse kallikreins (primers KALK- 3-s and KALK-4-as), mGK22 (mGK22-1/2-s and mGK22-4/5-as) and GAPDH (GAPDH-s and GAPDH-as) were performed on 1 μg total RNA in the single tube Access RT-PCR* system (Promega, Madison, WI), according to the protocol of the manufacturer. Annealing steps were at 58° C, except for the kallikrein cDNAs expressed in mouse prostate (primers KALK-3-s and KALK-4-as), which was at 50° C.

RT-PCR primers:

LACZ-s	: 5' AGCCATCGCCATCTG 3'
PRO1/2-as	: 5' GACGGCAGCATCTTCGCCTC 3'
KALK-3-s	: 5' TGCGGATCCTCAGGCTGGGGCAGCA 3'

KALK-4-as	: 5' TGTCAGATCTCCTGCACACAA/GCAT 3'
mGK22-1/2-s	: 5' CTAGGAGGGATTGATGCTGC 3'
mKG22-4/5-as	: 5' CCTCCTGAGTCTCCCTTACA 3'
GAPDH-s	: 5' GGTCTACATGTTCCAGTATGACTCC 3'
GAPDH-as	: 5' GAGACAACCTGGTCCTCAGTGTAGC 3'

The resulting PCR products were separated over a 2% agarose gel, and in indicated cases transferred to Hybond N^+ membrane. Filters were hybridized at high stringency with random primed ^{32}P-labeled probes specific for the expected cDNA fragment. The PCR product obtained with primers KALK-3-s and KALK-4-as was cloned in PCR-II (Invitrogen, Leek, The Netherlands), and resulting clones were sequenced.

RNA *in situ* hybridization

Sense and antisense digoxygenin (DIG) labeled protamine RNA probes were generated on a 175 bp protamine cDNA fragment, obtained by RT-PCR on mouse testis RNA with primers PRO-s (5' GAAGATGTCGCAGACGGAGG 3') and PRO-as (5' GATGTGGCGAGATGCTCTTG 3'). The PCR fragment was first cloned in pCR-II. After sequencing, the EcoRI-EcoRI cDNA fragment was re-cloned in pTZ19 (Pharmacia, Uppsala, Sweden). After linearization with HindIII, DIG labeled RNA was transcribed from the T7 promoter. Hybridization of 5 μm paraffine embedded sections, and visualization with alkaline phosphatase coupled anti-DIG antibodies and indoxil-nitroblue tetrazolium (NBT/BCIP) substrate were done essentially as described (42). Sections were counterstained with neutral red.

Experimental animals

In accordance with the NIH Guidelines for Care and Use of Laboratory Animals, all experiments were conducted using the highest standard for humane care.

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Chapter VI

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Chapter VII

GENERAL DISCUSSION AND FUTURE DIRECTIONS

Chapter VII

In an attempt to identify a candidate prostate-specific transcription factor, a novel member of the C/EBP family of transcription factors was cloned, C/EBPδ. As a model system, the molecular mechanism involved in androgen regulated and prostate specific expression of the PSA gene was extensively studied. During the course of the work described in this thesis three functionally active androgen response elements were identified in a 6 Kb PSA promoter fragment, two in the proximal promoter (ARE-I and ARE-II), and one in the far upstream enhancer region (ARE-III). The upstream enhancer region is probably complex and shows largely prostate specific activity in transfection experiments. Because the recognition sequence for the AR and several other steroid hormone receptors including the GR is identical, the mechanism underlying the apparent androgen specific induction of PSA promoter activity in LNCaP prostate cells was investigated. Finally, the capacity of two PSA promoter fragments in directing androgen regulated and prostate-specific activity of a reporter gene *in vivo* was studied.

<u>Cloning of a novel (candidate) prostate specific transcription factor of a known family</u>

For the isolation of a novel, prostate specific member of a known family of transcription factors, we focused on the C/EBP family. Comparison of band shifts of a CCAAT box (TTGGGCAAT) in the rat C3(1) promoter with liver, ventral prostate and testis nuclear extracts indicated prostate specificity of a member of the C/EBP family, which could be involved in the prostate specific expression of the C3(1) gene (Zhang et al. 1990). At the time our search for a C/EBP gene expressed in (human) LNCaP prostate cells was initiated, only one human member of the C/EBP family, C/EBPβ, had been identified. In rodents four different C/EBPs, including C/EBPβ had been cloned. C/EBPα had been implicated in liver and adipocyte specific gene expression. The C/EBP family members showed over 75 percent homology in the DNA binding domains, but the homology in the remaining parts of the proteins was limited. At present six different rodent C/EBPs have been identified, four of which have also been detected in humans (Akira et al. 1990, Kinoshita et al. 1992, Cleutjens et al. 1993, Antonson & Xanthopoulos, 1995, Chumakov et al. 1997). In the course of our study, we cloned the human C/EBPδ gene. Independently, others reported the isolation of the human C/EBPδ gene by screening of a genomic library with a probe encoding the C/EBPβ DNA binding domain (Kinoshita et al. 1992). C/EBPδ expression was detected in a variety of tissues, with the highest level of expression in lung, adipocytes and kidney. The expression of C/EBPδ in lung and kidney is strongly enhanced by inflammatory cytokines, whereas the expression in adipocytes is strongly increased by adipogenic hormones, including insulin and dexamethasone (Cao et al. 1991). In prostate, C/EBPδ expression is low, and upregulated by androgens (Cleutjens et al. unpublished results). Until now no evidence for a role of C/EBPδ in prostate specific gene expression has been obtained.

Although the cloning of C/EBPδ did not result in the identification of a prostate specific transcription factor, the approach to identify prostate transcription factors by means of cloning a novel member of a known transcription factor family is still valid. As explored for C/EBP, a prostate specific protein, binding to the consensus sequence for a specific transcription factor family in a band shift assay can be a first tool. Ideally, this binding site is present in a region known to be important for prostate-specific gene expression but this is not a prerequisite. Essentially, the approach of cloning a novel member of a known family of proteins can also be applied for the identification of a putative prostate specific co-activator.

Is the androgen receptor a key player in prostate specific gene expression ?

Possibly, prostate specific gene expression is not dependent upon one single strictly prostate specific transcription factor, but will be attained by a cooperative interaction of a set of transcription factors and co-activators, all showing partial tissue specificity. As a result, the target gene(s) will only be expressed at a high level in those cells which contain all essential factors. In this respect the AR might play a major role. AR is mainly expressed in cells of the male urogenital tract. Importantly, all genes showing (almost complete) prostate cell specific expression (including PSA, hGK-1, C3(1), probasin and prostate specific transglutaminase), were shown to be regulated by androgens (Heyns et al. 1978, Dodd et al. 1983, Riegman et al. 1991a,b, Young et al. 1991, 1992, 1995, Henntu et al. 1992, Ho et al. 1992, Wolf et al. 1992, Dubbink et al. 1996). Obviously, AR is not sufficient to direct prostate specific expression of these genes, because they are not expressed in other tissues of the urogenital tract. Both from endocrine manipulation of rats, and from the AR mutations in AIS, it is well established that AR is not only involved in tissue specific gene expression, but also essential for prostate development, and maintenance of its structure (Cunha et al. 1987, Cunha 1994, Quigly et al. 1995, Gottlieb et al. 1997).

Evidence for cooperative interaction of the AR with other factors was obtained by studying the cell specificity of the PSA promoter in transient transfection experiments. Reporter genes driven by the PSA promoter show largely LNCaP prostate cell specificity as compared to several other prostate and non-prostate derived cell lines, despite co-transfection of a human AR expression vector in these cells (Chapter V). This correlates with the expression of the endogenous PSA gene (with the exception of T47D cells). AR might not be able to stimulate PSA promoter activity in PSA negative cells due to the absence of essential cooperating transcription factors or co-activators. One AR specific co-activator has been claimed, ARA70. ARA70 was shown to interact with the AR ligand binding domain, and selectively enhance AR-dependent transcription (Yeh & Chang 1996). Although ARA70 is expressed in prostate, it is not prostate specific. Expression was demonstrated in almost all tissues tested, including AR negative tissues.

An alternative mechanism of absence of PSA expression in AR positive cells can be the presence of specific silencers in these cells, that block AR mediated PSA promoter activity. An indication for such a factor might be obtained by deletion mapping experiments in non-prostate cells. Deletion of the sequence to which the silencing factor binds would result in ubiquitous activity of the PSA promoter. Results obtained so far with the different PSA promoter constructs demonstrate that if a silencer protein is active, it must act on the 440 bp upstream enhancer region, because this fragment still shows prostate specificity (Chapter V)

Cooperative interaction of multiple AREs

Synergistic activity of multiple hormone response elements has been identified in many genes, including androgen responsive genes. The proximal promoter region of the probasin gene shows cooperative interaction of one high affinity and one low affinity AR binding site (Rennie et al. 1993, Kasper et al. 1994, Claessens et al. 1996). Clear cooperative activity between a high and a low affinity AR-binding sequence was also observed in the proximal PSA promoter (Chapter III). In case of the 6 Kb PSA promoter, the situation is even more complex, cooperative activity of three ARE sequences has been observed (Cleutjens et al. 1997, Chapter V). Figure VII.1. shows a schematical representation of the currently identified regulatory elements in the PSA promoter.

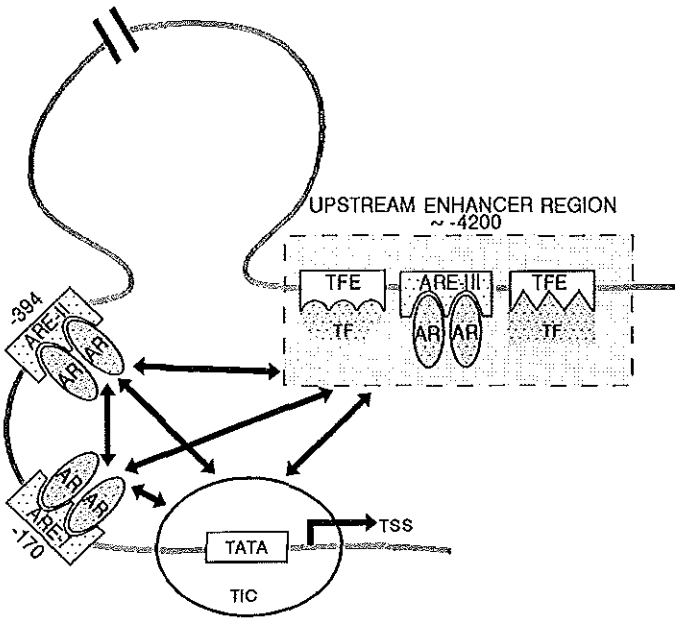

Figure VII.1 Schematical representation of regulatory elements in the promoter of the PSA gene. AR: androgen receptor, TF: transcription factor, TFE: TF binding element, TIC: transcription initiation complex, TSS: transcription start site.

In this model, interactions between the various AR homodimers and other transcription factors in the upstream enhancer region, and presumably in the proximal promoter region might be either direct or mediated by co-activators. Looping out of the intervening DNA sequences might contribute to the observed communication between the distal enhancer, and the transcription initiation complex and regulatory elements in the proximal promoter (Gothard et al. 1996, and references therein).

The observed cooperative activity of the low affinity AR binding sites ARE-1 (ATAGCAtctTGTTCT) in the probasin promoter and ARE-II (GGATCAgggAGTCTC) present in the proximal PSA promoter revealed the functional relevance of degenerate palindromic sequences with limited homology to the consensus ARE sequence and low AR binding affinity. Footprint or bandshift analysis with recombinant AR-DBD or the full length AR is an attractive experimental approach to identify candidate AREs, with either high or low AR binding affinity in the promoter or enhancer region of interest. However, functional activity of the candidate ARE can only be demonstrated by experiments in which multiple copies of the candidate ARE are shown to be sufficient to confer androgen regulated activity to a heterologous promoter and by mutational analysis of the candidate ARE.

To make the situation even more complex, in addition to palindromic repeats with a 3 bp spacer, directly repeated half-sites have been shown to act as response element for steroid hormone receptors, although with several fold lower binding affinity and transactivation efficiency (Kato et al. 1992 and 1995, Aumais et al. 1996). In natural androgen-regulated genes, a functional role of directly repeated half-sites has been implicated in the androgen-regulation of the crp1/20 kDa protein gene (Ho et al. 1993). A 39 bp fragment in the first

intron (N39) contains three ARE-like half-sites separated by 6 and 4 bp respectively. This fragment shows low AR-binding affinity and confers weak androgen responsivity to a heterologous promoter. In the upstream PSA enhancer region several ARE-like half-sites can be identified (see Chapter V), cooperativity of these half-sites with ARE-III cannot be excluded. However, until now no information about their functional relevance is available.

A complex upstream enhancer region

Inactivation of ARE-III, present in the centre of the 440 bp core of the upstream enhancer region, almost completely abolished enhancer activity, indicating its central role. Truncation of the 440 bp core enhancer region at either the 5' and 3' end resulted in partial reduction of androgen induced activity. The reduction in activity might be the result of deletion of binding sites for additional transcription factors. Additionally, the spacing between the upstream enhancer region and the proximal promoter might be a factor. The nature of the transcription factors, acting cooperatively with the AR in maximal androgen induced activity of the PSA core enhancer region has not been identified as yet. Although these factors might include ubiquitously expressed transcription factors, one or more should contribute to prostate specificity of the PSA promoter. Alternatively, a ubiquitously expressed transcription factor interacts with a prostate specific co-activator.

The central role of ARE-III in activity of the upstream enhancer region raises the possibility that binding of AR to ARE-III changes the chromatin structure in this upstream region, thereby allowing additional transcription factors to bind to the DNA, possibly followed by removal of the AR. This mechanism of cooperativity has previously been proposed for GR activation of the proximal MMTV-LTR promoter, and in an upstream enhancer region of the tyrosine aminotransferase (TAT) gene. The proximal MMTV promoter contains a cluster of three degenerated HREs, and NF-1 and Oct binding sites. All elements are required for optimal induction by steroid hormone receptors, as shown by mutational analysis (Cato et al. 1988). Functional studies indicated that the observed synergism between steroid hormone receptors and NF-1 might be mediated by the organization of the DNA in chromatin (Brüggemeier et al. 1991). Binding of the activated steroid hormone receptor is supposed to cause a displacement or disruption of the nucleosome positioned over the proximal promoter region, thereby enabling free access of NF-1 to its recognition sequence (reviewed by Beato & Sánchez-Pacheco 1996). In a complex liver specific and glucocorticoid regulated enhancer region, 2.5 Kb upstream of the TAT gene, functional cooperativity between multiple GREs and the liver enriched transcription factor HNF-3 has been observed (Rigaud et al. 1991, Nitsch et al. 1992, Roux et al. 1995). Results obtained by *in vivo* footprinting experiments over this enhancer region suggested a hit-and-run mechanism of transcriptional activation by GR (Rigaud et al. 1991). Activated GR binds to the GRE, which partially overlaps with the binding site for HNF-3, and modifies the local chromatin structure. Subsequently, GR leaves the region accessible to HNF-3. Binding of both HNF-3 and GR to this site is mutually exclusive. At present it is unknown if such a mechanism is also responsible for the activation of the upstream PSA enhancer region. However, the presence of a strong androgen regulated DNaseI hypersensitive site in the centre of the upstream enhancer region is a strong indication for hormone-induced nucleosome removal or disruption (Elgin 1988). Kinetics of protein interactions, as determined by *in vivo* footprinting is needed to determine whether or not AR binding to ARE-III induces chromatin remodelling, followed by binding of additional factors, and if so, whether AR is permanently or temporarily associated with the enhancer region.

Besides by remodelling of the chromatin structure by one factor, and subsequent binding of additional factors, synergistic activation between multiple transcription factors can be obtained by cooperative binding of transcription factors to adjacent DNA-binding sites (Ptashne et al. 1988, Tsai et al. 1989, Baniahmad et al. 1991, Brüggemeier et al. 1991). Cooperative interaction of independently DNA binding transcription factors with a common co-activator should also be considered as a mechanism for synergistic activity (Ptashne 1988, Bradshaw et al. 1991, Martinez et al. 1991).

As a first start to identify regulatory sequences, that in addition to ARE-III contribute to maximal androgen regulated and prostate-specific activity of this complex enhancer, *in vitro* footprinting experiments can be done using nuclear extracts from LNCaP and T47D cells, cultured in presence and absence of androgens and from a cell line in which the PSA promoter is inactive. Appropriate small fragments shown to be protected in footprints can be used in bandshifts to study in somewhat more detail the proteins binding to this region. Subsequently, these fragments can be deleted from the 440 bp enhancer region in reporter gene constructs, and activity of the resulting fragment can be compared with activity directed by the original fragment. Once regulatory elements involved in maximal enhancer activity are identified, the cloning and characterization of the transcription factor(s) binding to such an element can be initiated.

Especially when the observed synergistic activation in the upstream core enhancer is attained by AR induced chromatin remodelling, it will not be easy to determine isolated regulatory elements, since every small change in distance between AR and the additional elements, or minor changes of sequences surrounding these regulatory elements can have an effect on enhancer activity. So, results obtained by rather radical deletion mapping need to be substantiated by experiments in which the region of interest is replaced by an inert unrelated sequence of comparable length and by subtle mutational analysis.

<u>AR target gene specificity</u>

The consensus sequence for high affinity binding of all steroid hormone receptors, excluding ER is identical, GGT/AACAnnnTGTTCT. Despite the shared DNA-binding site, these receptors mediate diverse cellular responses. The common high affinity DNA-binding site raises the question of target gene specificity, if more than one receptor is expressed in one and the same cell. At present the picture emerges that several distinct molecular mechanisms may account for target gene specificity, including receptor expression level, receptor-DNA interaction, interaction of the receptor with other proteins, and ligand availability.

AR specific regulation of PSA expression in LNCaP cells is caused by the absence of GR in these cells (Chapter IV). No indication of differential activity of AR and GR on the individual regulatory elements in the PSA promoter has been found. Transient co-transfection of proximal PSA promoter constructs and AR or GR expression plasmids revealed an approximately 2-fold higher Dex induced promoter activity (Chapter III). The 6 Kb promoter was also slightly less activated by the AR. AR seemed slightly more active on the endogenous PSA gene. A lack of receptor specificity in mediating PSA promoter activity was also observed for AR and PR in T47D cells. These data suggest that an AR specific co-activator, or other AR specific protein-protein interactions are not essential for PSA promoter activity.

In contrast to the stimulation of PSA expression by both AR and GR, cell proliferation could only be induced by R1881. This suggests that genes involved in cell proliferation are differentially regulated. In a Differential Display search with a limited number of primers one

novel mRNA was detected, that could be up-regulated by AR, but not by GR (Steketee, unpublished results). In case the observed difference on the mRNA level is a reflection of a difference of transcription initiation, further analysis of the regulation of expression of this gene might provide information about the mechanism of AR specificity.

Recently, Claessens et al. (1996) reported on a selective ARE sequence. AR and GR DBDs bind to most functional AREs in natural androgen regulated genes, including core II of the C3(1) gene, HRE-3 of the Slp gene, PSA ARE-I and the AREs present in the hGK-1 and factor IX genes, with comparable affinity (Rundlett & Miesfeld 1995, Claessens et al. 1996). Furthermore both ARE-II and ARE-III of the PSA gene were found to interact with both AR and GR DBD (Cleutjens et al., unpublished results). However, ARE-2 (AGTACTccaAGAACC) in the probasin promoter region selectively interacts with the AR-DBD (Claessens et al. 1996). A situation that was reflected in transient transfection experiments with an ARE-II driven reporter gene. The functional C3(1) ARE is not specific. Studies with probasin/C3(1) swapped half-sites indicated that the right half site (5'-AGAACC-3' of probasin ARE-2 prevents GR-DBD binding. Comparison of the probasin ARE-2 sequence with all other known functional ARE sequences revealed that the AR specific binding affinity of probasin ARE-II can not be attributed to a single nucleotide. More detailed mutational experiments are needed to identify the mechanism determining the observed specificity of AR-DBD binding.

LNCaP prostate cells

The research on regulatory elements involved in androgen-regulated and prostate-specific expression of the PSA gene has benefitted from the availability of the LNCaP prostate cancer cell line, that endogenously expresses both AR and PSA (Horoszewicz et al. 1983). Initially this cell line was not easy to transfect and it was a challenge to find the proper culture and transfection conditions. Once the initial problems were overcome, the LNCaP cell line made a very suitable model system. The LNCaP cell line harbors one unfavorable property, the AR gene in the LNCaP cell line contains a point mutation in the LBD which renders this receptor responsive not only to androgens but also to estrogens, progestins and several anti-androgens (Veldscholte et al. 1990). For comparison it is very important to have additional human prostate cells available, expressing both AR and PSA. Hopefully, the recently developed cell line PC346C can fulfil this requirement (Romijn, personal communication, Dubbink et al. 1996).

In vivo application of PSA regulatory regions

The 6 Kb PSA promoter is the first human promoter, shown to direct prostate specific gene expression in transgenic mice. Out of the two rat promoters tested in transgenic mice for prostate specificity [C3(1), and probasin] (Allison et al. 1989, Buttyan & Slawin 1993, Greenberg et al. 1994), only the 426 bp rat probasin promoter fragment directed transgene expression almost exclusively to the prostate (Greenberg et al. 1994). Transgene expression (ventral and dorsolateral prostate) did not strictly follow the expression pattern of the endogenous rat probasin gene, which is selectively expressed in dorsolateral prostate. This indicates that additional regulatory regions surrounding or present in the probasin gene are needed for the tissue specific expression as observed for the endogenous gene.

Because of its strict prostate specific activity in transgenic mice, it can be argued that the 6 kb promoter contains most, if not all elements important for regulation of expression of the endogenous PSA gene in human tissues. These results strongly support the application of the PSA promoter in gene therapy of prostate cancer. Because the endogenous PSA gene is

expressed in the vast majority of prostate cancers, it is likely that all transcription factors and co-activators needed to drive a transgene PSA promoter are present. However, although difficult to judge, expression of the 6 Kb PSA promoter driven transgene in the mouse prostate did not look very high. Therefore, it might be beneficial to attempt to develop modified derivatives of the PSA promoter directing higher expression levels. To this end, the most important components directing prostate specific activity of the PSA promoter need to be determined. The 440 bp core enhancer seems a very attractive start point to identify such elements. Furthermore, since neither of the three ARE sequences in the PSA promoter is completely identical to the consensus sequence, substitution of these AREs by the consensus ARE might result in an even higher, prostate specific activity of the PSA promoter or PSA promoter derivatives.

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SUMMARY

In Western countries, adenocarcinoma of the prostate is the most frequently diagnosed tumor in men, and the second leading cause of male cancer death. Although early diagnosis, which can lead to successful surgical interference, is improving, a large proportion of patients presents with disseminated disease. Furthermore, a considerable number of surgically treated patients returns with metastases. First line therapy of locally invasive or metastatic disease is generally endocrine therapy. Unfortunately, the effects of this type of therapy are only temporary, and after a variable period of time, a then hormone refractory tumor continues to grow.

In the present study transcription factors involved in the development, growth and differentiation of the prostate were studied. These factors might also play a role in the initiation and progression of prostate cancer. The main focus is on androgen regulated gene expression. It was previously shown that the presence of a functional androgen receptor (AR), a member of the steroid hormone receptor family, and the appropriate androgenic hormones are essential for the development and maintenance of the prostate. Chapter I gives an overview of the current knowledge of the steroid hormone receptor family. Furthermore, androgen regulated and prostate specific gene expression are discussed.

To identify and characterize prostate specific transcription factors involved in growth and differentiation of the prostate, two complementary experimental approaches were followed. The first approach, a search for novel members of known families of transcription factors, resulted in the identification of the human C/EBPδ gene. The C/EBPδ gene was assigned to the pericentromeric region of chromosome 8. C/EBPδ was found to be the major C/EBP family member expressed in LNCaP prostate cells, however, C/EBPδ expression appeared not to be prostate specific (Chapter II).

The second experimental approach focussed on the regulation of prostate specific antigen (PSA) expression, the main subject of this thesis. PSA is a member of the human kallikrein gene family, further members of this family are the human glandular kallikrein (hGK-1) gene and the tissue kallikrein gene, KLK-1. The three genes are clustered in an area of 60 Kb on chromosome 19q13.2-13.4. PSA is expressed at high level in the luminal epithelial cells of the prostate, and at very low levels in other tissues. Expression of PSA is androgen regulated, this regulation is at least partially at the level of transcription initiation.

Chapter III reports the identification of two AR binding sites in the proximal PSA promoter, identified by means of bandshift analysis with recombinant AR protein. At position -170 a high affinity AR binding site, ARE-I (AGAACAgcaAGTGCT) was found to be present; ARE-II (GGATCAgggAGTCTC) at position -394, is a low affinity AR binding site. Functional activity of these candidate androgen response elements or AREs was shown by transient transfection experiments in PSA and AR positive LNCaP prostate tumor cells. The proximal PSA promoter, including ARE-I and ARE-II, is more active in LNCaP cells than in the PSA negative prostate cell lines DU145 and PC3 cells and in several non prostate cell lines. In LNCaP cells cotransfected with a glucocorticoid receptor (GR) expression plasmid, the pattern of PSA promoter activity induced by dexamethasone turned out to be identical to AR mediated activity.

Summary

In order to directly compare the functional activity of the AR and the closely related GR in prostate cells, a GR expression vector was stably integrated in AR positive LNCaP cells (Chapter IV). Interestingly, both AR and GR were found to upregulate expression of the endogenous PSA gene, but they were different in their growth stimulating properties of LNCaP sublines. These cell lines provide a novel system for comparison of AR and GR molecular and biological functions.

Its relatively low activity suggested that the proximal PSA promoter is not sufficient to account completely for androgen regulation of the endogenous PSA gene. In order to detect additional, more distal control elements, DNaseI hypersensitive sites (DHSs) upstream of the PSA gene were mapped in chromatin from the prostate derived cell line LNCaP, grown in the presence and absence of the synthetic androgen R1881. In a region 3.8 to 4.8 Kb upstream of the transcription start site of the PSA gene, a cluster of three DHSs was detected. The middle DHS (DHSII, at approximately -4.2 Kb) showed strong androgen responsiveness in LNCaP chromatin, and was absent in chromatin from HeLa cells. Further analysis of the region encompassing DHSII provided evidence for the presence of a complex, androgen responsive and cell specific enhancer. In transiently transfected LNCaP cells, PSA promoter constructs containing this upstream enhancer region showed approximately 3000-fold higher activity in the presence than in the absence of R1881. The core region of the enhancer could be mapped within a 440 bp fragment. The enhancer showed synergistic cooperation with the proximal PSA promoter, and was found to be composed of at least three separate regulatory regions. In the center, a functionally active, high affinity AR binding site was identified (ARE-III: GGAACAtatTGTATC at position -4.2 Kb). Mutation of this element almost completely abolished PSA promoter activity. To study whether the activity of the upstream core enhancer showed cell specificity, luciferase (LUC) reporter gene constructs were co-transfected with an AR expression plasmid to a series of AR negative prostate and non-prostate cell lines. Transient transfection experiments in (PSA negative) PC3, DU145, Hep3B and COS cells did not reveal any activity of the PSA upstream core enhancer, although PC3 and DU145 cell lines originate from the prostate. In addition to LNCaP cells, the PSA promoter is only active in T47D mammary tumor cells. However, the androgen induced activity of the PSA promoter in T47D cell is much lower than the activity in LNCaP cells. In T47D cells, the PSA-LUC reporter gene activity could also be induced by the activated progesterone receptor, again indicating that the PSA promoter is not completely AR specific (Chapter V).

Chapter VI describes the *in vivo* characterization of the PSA promoter. A 6 Kb PSA promoter fragment, but not a proximal 632 bp PSA promoter fragment, was sufficient to direct prostate specific expression of the bacterial LacZ reporter gene in transgenic mice. For both constructs three transgenic strains were generated. Integration was assayed by PCR analysis on tail DNA, and confirmed and quantitated by Southern blotting. LacZ expression was tested by RT-PCR, *in situ* hybridization, and β-galactosidase assay on whole tissues and tissue sections. First of all, lysates were prepared from 26 different tissues, and tested for β-galactosidase activity in a solution assay. In none of the mouse strains with the proximal (632 bp) PSA construct activity of the reporter gene could be detected. In contrast, all three strains with integration of the 6 Kb PSA promoter-LacZ

fragment showed expression of the transgene, which was completely prostate specific. The level of expression was comparable in all three transgenic strains, and independent of the number of integrated copies. Expression was absent in salivary glands, where many members of the mouse kallikreins are expressed at a high level, and in male and female mammary cells. Out of the different prostate lobes, reporter gene expression was exclusively found in the lateral prostate. Whole mount X-gal assay, followed by examination of staining in tissue sections, and *in situ* hybridization with a specific probe showed exclusive expression of the reporter gene in the luminal epithelial cells, like the endogenous PSA gene in the human prostate. The β-galactosidase assays could be confirmed by RT-PCR with reporter gene specific primers. Transgene expression was undetectable until 8 weeks after birth. Castration of transgenic mice rapidly led to down-regulation of β-galactosidase expression, which could be restored within 40 h by dihydrotestosterone supplementation. Both the developmental expression pattern and the hormone manipulation experiment indicated androgen regulation of the transgene, as found in human LNCaP cells. The results obtained strongly indicate prostate specificity of the 6 Kb PSA promoter in normal cells, and suggest that the 6 Kb promoter contains most, if not all elements important for regulation of expression of the endogenous PSA gene in human tissues. The strict prostate specificity of the 6 Kb PSA promoter fragment strongly supports its applicability in gene therapy of human prostate cancer. Furthermore, this fragment can be employed to direct oncogene expression to the prostate, to generate new mouse prostate cancer models.

The results as described in the previous chapters are reviewed in the light of recent literature data in Chapter VII. Additionally, future directions for research are proposed in this chapter.

SAMENVATTING

Adenocarcinoom van de prostaat is de meest voorkomende tumor bij mannen in westerse landen, en de op een na meest frequente oorzaak van kankersterfte. Ondanks de mogelijkheden tot vroege diagnose, met meer kans op succesvol chirurgisch ingrijpen, zijn er veel patiënten waarbij de ziekte reeds is uitgezaaid op het moment van diagnose. Daarnaast worden bij een substantieel deel van de patiënten die chirurgisch behandeld werden, na verloop van tijd toch metastasen waargenomen. Op dit moment is endocriene therapie de meest toegepaste behandeling bij gemetastaseerd prostaat-carcinoom. Helaas is het effect van deze vorm van therapie slechts tijdelijk, en na verloop van tijd groeit er een dan hormoon onafhankelijke tumor verder.

Het in dit proefschrift beschreven onderzoek richt zich op de identificatie van transcriptiefactoren die betrokken zijn bij de ontwikkeling, groei en differentiatie van de prostaat. Deze factoren spelen mogelijk ook een rol bij het ontstaan en de progressieve groei van prostaattumoren. Uit eerder onderzoek is reeds bekend dat de androgeenreceptor (AR), een lid van de familie van steroidhormoonreceptoren en bepaalde androgenen onmisbaar zijn voor de ontwikkeling en het instandhouden van de prostaat. In Hoofdstuk I wordt een overzicht gegeven van de huidige kennis van de familie van steroidhormoonreceptoren en met name van de structuur, de interactie met andere transcriptiefactoren en co-activatoren, en alternatieve mogelijkheden van activatie van de receptor. Daarnaast worden een aantal aspecten van androgeen gereguleerde en prostaatspecifieke genexpressie behandeld.

Twee complementaire strategieën werden gevolgd in de poging om prostaatspecifieke transcriptiefactoren te identificeren. De eerste aanpak, die onderzoek naar een nieuw, prostaat specifiek lid van een bekende familie van transcriptiefactoren behelst, resulteerde in de identificatie en karakterisering van het humane C/EBPδ gen. Het gen werd gelokaliseerd nabij het centromeer van chromosoom 8. C/EBPδ is het lid van de familie van C/EBP genen dat het hoogst tot expressie komt in LNCaP prostaatcellen. Helaas bleek C/EBPδ expressie niet prostaat specifiek (Hoofdstuk II).

De tweede experimentele benadering richtte zich op onderzoek naar de regulatie van de expressie van prostaatspecifiek antigeen (PSA), het hoofdthema van dit proefschrift. PSA is een lid van de familie van humane kallikreine genen. Andere leden van deze familie zijn het hGK-1 gen en het KLK-1 gen. Deze drie humane kallikreine genen liggen geclusterd in een gebied van 60 Kb op chromosoom 19q13.2-13.4. PSA komt hoog tot expressie in de luminale epitheliale cellen van de prostaat. De expressie is laag of afwezig in andere weefsels. Het expressieniveau van PSA wordt gereguleerd door androgenen, en deze regulatie vind op zijn minst gedeeltelijk plaats op het niveau van transcriptie.

Hoofdstuk III beschrijft de identificatie van twee AR bindingsplaatsen in de proximale promotor van het PSA gen mbv bandshift analyse met recombinant AR eiwit. Op positie -170 bevindt zich een bindingsplaats met hoge affiniteit voor de AR, ARE-I (AGAACAgcaAGTGCT); ARE-II (GGATCAgggAGTCTC) op positie -394, is een bindingsplaats met een lage affiniteit. De functionele activiteit van deze kandidaat androgeen-responsieve elementen of AREs werd bewezen mbv transiënte transfectie-experimenten in PSA- en AR-positieve LNCaP prostaat tumorcellen. Het proximale PSA promotor gebied,

inclusief ARE-I en ARE-II, is actiever in LNCaP cellen dan in de PSA negatieve prostaat cellijnen DU145 en PC3 en in een aantal niet-prostaat cellijnen. In LNCaP cellen transiënt gecotransfecteerd met een glucocorticoid receptor (GR) expressieplasmide, is het patroon van dexamethason geïnduceerde activiteit van de PSA promotor gelijk aan de activiteit tot stand gebracht door de geactiveerde AR.

Om de eigenschappen van de AR en de GR meer direct met elkaar te kunnen vergelijken werden, dmv stabiele integratie van een GR expressie plasmide, LNCaP sublijnen gegenereerd, die niet alleen de AR maar ook GR constitutief tot expressie brengen (Hoofdstuk IV). Zowel de AR als de GR bleek in staat de expressie van het endogene PSA gen te induceren, maar er bleek een verschil in hun groeistimulerende effect op LNCaP sublijnen. Deze cellijnen vormen een nieuw bruikbaar systeem voor de vergelijking van de moleculaire en biologische functies van de AR en de GR.

De relatief lage activiteit van de proximale PSA promotor suggereerde dat niet alle informatie die nodig is voor de androgeen gereguleerde expressie van het endogene PSA gen hierin aanwezig is. Om additionele, meer distaal gelegen regulerende gebieden te identificeren is gezocht naar DNase I hypergevoelige gebieden (DHS) in de chromatinestructuur voor het endogene PSA gen in de LNCaP cellijn, gekweekt met en zonder het synthetische androgeen R1881. In een gebied 3,8 tot 4,8 Kb voor de transcriptiestartplaats van het PSA gen, werd een cluster van drie DHS gevonden. De middelste DHS (DHSII, op ongeveer - 4,2 Kb) laat sterke gevoeligheid voor androgenen zien in LNCaP cellen, terwijl deze DHS niet aantoonbaar is in chromatine van HeLa cellen. Verdere analyse van het gebied rond DHSII gaf aanwijzingen voor de aanwezigheid van een complexe, androgeengevoelige en celspecifieke "enhancer". In transiënt getransfecteerde LNCaP cellen zijn PSA promotorconstructen die dit gebied omvatten ongeveer 3000 maal meer actief in de aanwezigheid dan in de afwezigheid van R1881. De kern van dit "enhancer" gebied wordt gevormd door een 440 bp fragment. De "kern-enhancer" werkt synergistisch samen met de proximale PSA promotor en bestaat uit tenminste drie afzonderlijke regulerende elementen. In het centrum werd een functioneel actieve bindingsplaats met sterke affiniteit voor de AR gevonden (ARE-III: GGAACAtatTGTATC op positie -4.2 Kb). Mutatie van dit element verhinderde bijna volledig de activiteit van de PSA promotor. Om mogelijk prostaatspecifieke activiteit van de nieuwe enhancer te onderzoeken werden een aantal AR negatieve prostaat en niet-prostaat afgeleide cellijnen gecotransfecteerd met reportergen constructen en een AR expressieplasmide. Transiënte transfectie in (PSA negatieve) PC3, DU145, Hep3B en COS cellen vertoonde geen activiteit van de PSA "kern-enhancer". De PSA promotor bleek naast in LNCaP cellen alleen in T47D borst tumorcellen actief te zijn. De door androgenen geïnduceerde activiteit van de PSA promotor in T47D cellen is echter veel lager dan in LNCaP cellen. In T47D cellen kan de activiteit van het PSA-LUC reportergen ook geïnduceerd worden door de geactiveerde progesteronreceptor, een nieuwe indicatie dat de PSA promotor niet geheel AR specifiek is.

Hoofdstuk VI beschrijft de *in vivo* analyse van de PSA promotor. Een 6 Kb, maar niet het proximale 632 bp fragment van de PSA promotor bleek prostaat specifieke expressie van het bacteriële LacZ reportergen in transgene muizen te kunnen induceren. Voor beide constructen werden drie onafhankelijke transgene lijnen ontwikkeld. Integratie van het transgen werd bepaald dmv PCR op DNA geïsoleerd uit de staart, en bevestigd en gekwantificeerd dmv

Southern blot analyse. LacZ expressie werd bestudeerd dmv RT-PCR, *in situ* hybridizatie, en een β-galactosidase test op weefsels en coupes. Als eerste werd van 26 weefsels lysaat gemaakt en vervolgens werd de β-galactosidase-activiteit in deze lysaten bepaald. In geen van de transgene muizen met het proximale (632 bp) PSA construct kon LacZ activiteit worden aangetoond. Daarentegen kon in alle transgene lijnen met integratie van het 6 Kb PSA promotor-LacZ fragment expressie van het transgen worden aangetoond. Deze expressie bleek specifiek voor de prostaat. Het expressieniveau was vergelijkbaar in alle drie de transgene lijnen en was onafhankelijk van het aantal geïntegreerde kopieën van het transgen. Expressie van het transgen was afwezig in de speekselklier, een orgaan waar veel leden van de muize kallikreine familie hoog tot expressie komen. Ook in mannelijke en vrouwelijke borstklier cellen kon geen transgen expressie worden aangetoond. Expressie van het reportergen bleek alleen aantoonbaar in de laterale prostaat, een van de vier lobben van de muize prostaat. De resultaten konden worden bevestigd dmv RT-PCR met primers specifiek voor het transgen cDNA. X-gal kleuring van de hele prostaat, gevolgd door analyse van weefselcoupes, en daarnaast *in situ* hybridizatie toonden exclusieve expressie van het reportergen in de luminale epithelilale cellen aan, identiek aan expressie van het endogene PSA gen in de humane prostaat. Expressie van het transgen kon niet worden aangetoond in muizen jonger dan 8 weken. Castratie van transgene muizen had een sterke afname van β-galactosidase activiteit tot gevolg, welke hersteld kon worden door toediening van dihydrotestosteron gedurende 40 uur. Zowel het patroon van transgen expressie tijdens de ontwikkeling, alsmede het castratie-experiment duiden op een regulering door androgenen regulering van het transgen, zoals ook gevonden voor het endogene PSA gen. De verkregen resultaten laten duidelijk de prostaat specifieke activiteit van de 6 Kb PSA promotor in normale cellen zien, en suggereren dat de 6 Kb promotor de meeste, zo niet alle elementen bevat, die nodig zijn voor de regulering van expressie van het endogene PSA gen in humane weefsels. De strikte prostaat specificiteit van de 6 Kb promotor is een sterke aanwijzing dat dit grote promotor fragment, of afgeleiden ervan, toegepast kunnen worden in gentherapie voor humaan prostaatkanker. Daarnaast kan dit fragment gebruikt worden voor de expressie van specifieke oncogenen bij de ontwikkeling van nieuwe muize modellen voor prostaatkanker (Hoofdstuk VI).

In het afsluitende Hoofdstuk VII worden de resultaten die beschreven zijn in de voorafgaande hoofdstukken besproken in het licht van recente literatuurgegevens. Tevens worden in dit hoofdstuk de mogelijkheden voor toekomstig onderzoek beschreven.

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Kitty