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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Prostate-specific antigen (PSA) 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. 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 ~−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 nonprostate cell lines showed largely LNCaP cell specificity of the upstream enhancer region, although some activity was found in the T47D mammary tumor cell line. (Molecular Endocrinology 11: 148–161, 1997)

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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 nonprostate 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 prox-

imal promoter might contribute to androgen-regulated PSA gene expression. For several strong, tissue-specific promoters, like those of the β-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 ARE-I and ARE-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 DHSs in the PSA Upstream Region

In a previous study we identified two regions in the PSA proximal promoter that 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 (~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 *Eco*RI and evaluated for location of DHSs by Southern blot analysis with the appropriate hybridization probes. With two different probes, DHSs could be found (Fig. 1). No other DHSs were detected over the 31-kb region with any of the probes tested (data not shown). Hybridization of *Eco*RI-digested DNA from LNCaP cells with a 1.1-kb *Hin*dIII-*Eco*RI fragment, spanning exon 1 and intron 1 of the PSA gene, showed one DHS (DHSIV), which was most prominent in the presence of R1881 (Fig. 1C). This DHS mapped to the proximal promoter region. Hybridization of genomic DNA from R1881-treated LNCaP cells with a 0.5-kb *Eco*RI-*Hin*dIII probe (−6 kb) revealed the presence of a cluster of three DHSs, approximately 4 kb upstream of the PSA gene (Fig. 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 (Fig. 1B). In contrast, DHSII was clearly absent in HeLa cell chromatin, indicating cell specificity.

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 (Fig. 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.

To map this upstream region in more detail, a 2.2-kb *Xba*I-*Stu*I fragment (see Fig. 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 *Eco*RI site at −632 and contains ARE-I (−170) and ARE-II (−394) (13) (Fig. 3A), giving rise to construct PSA-64-s-LUC. Transfection of PSA-64-s-LUC to LNCaP cells resulted in an even higher (>6000-fold) induction of promoter activity upon R1881 activation (Fig. 3A), indicating that the upstream enhancer activity resides within this 2.2-kb fragment. The 2.2-kb *Xba*I-*Stu*I fragment in the opposite orientation gave rise to a similar high activity (PSA-64-as-LUC in Fig. 3A).

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 *Pst*I-*Bam*HI) and PSA-74-LUC (0.9-kb *Pst*I-*Pst*I) 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 (*Pst*I-*Eco*RV) resulted in a 4-fold decrease in activity upon R1881 treatment. Similarly, deletion of the distal end (PSA-83-LUC) resulted in an 8-fold reduction in activity. The 440-bp

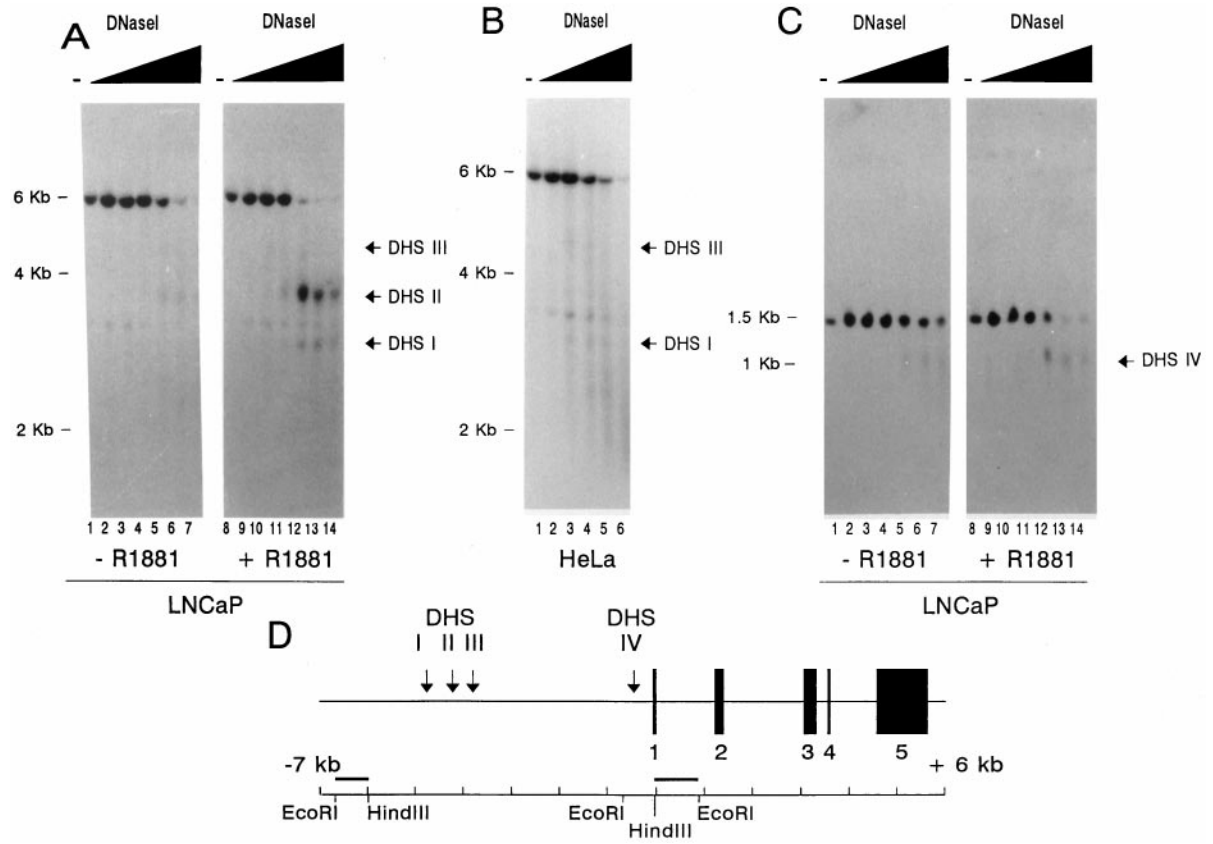


Fig. 1. DHSs in Chromatin Upstream of the PSA Gene

Southern blot analysis of genomic DNA from nuclei of LNCaP (A and C) and HeLa (B) cells incubated with increasing amounts of DNaseI (lanes 2–7 and 9–14 in panel A; 2–6 in panel B; and 2–7 and 9–14 in panel C; lanes 1 and 8 in panel A, lane 1 in panel B, and lanes 1 and 8 in panel C are controls without DNAseI treatment), and digested with *Eco*RI. Hybridization was with an *Eco*RI-*Hin*dIII probe at −6 kb (A and B) or a *Hin*dIII-*Eco*RI probe at +1 kb (C). Nuclei were isolated from LNCaP cells grown in the absence (panel A, lanes 1–7; panel C, lanes 1–7) or presence of R1881 (panel A, lanes 8–14; panel C, lanes 8–14), or from HeLa cells grown in 5% complete FCS (panel B). D, Schematic representation of the PSA gene. *Black squares* 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).

*Bst*EII-*Pst*I fragment (PSA-85-LUC) turned out to be the smallest fragment with strong enhancer activity (Fig. 3A). We defined the *Bst*EII-*Pst*I fragment as the core enhancer. The sequence of this core enhancer is shown in Fig. 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 DHSI, -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 *Pst*I site at −4.8 kb, and DHSIII is located close to the *Bam*HI site at −3.8 kb (see Fig. 2B).

To determine whether core enhancer activity was directly androgen regulated, the *Bst*EII-*Pst*I 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 (Fig. 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 (Figs. 2A and 3, A and C, and data not shown).

Identification of an ARE 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′-ACTCTGGAGGAA-CATATTGTATCGATT-3′, directly upstream of the *Cla*I

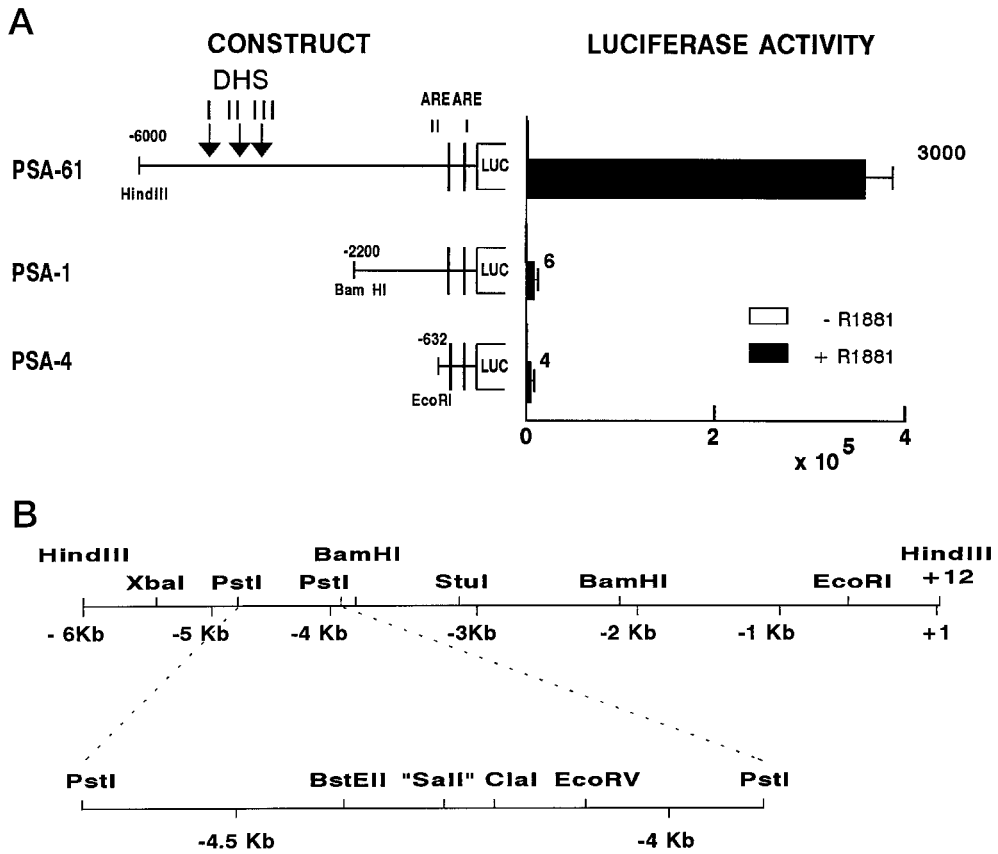


Fig. 2. Androgen Regulation of the PSA Promoter in LNCaP Cells

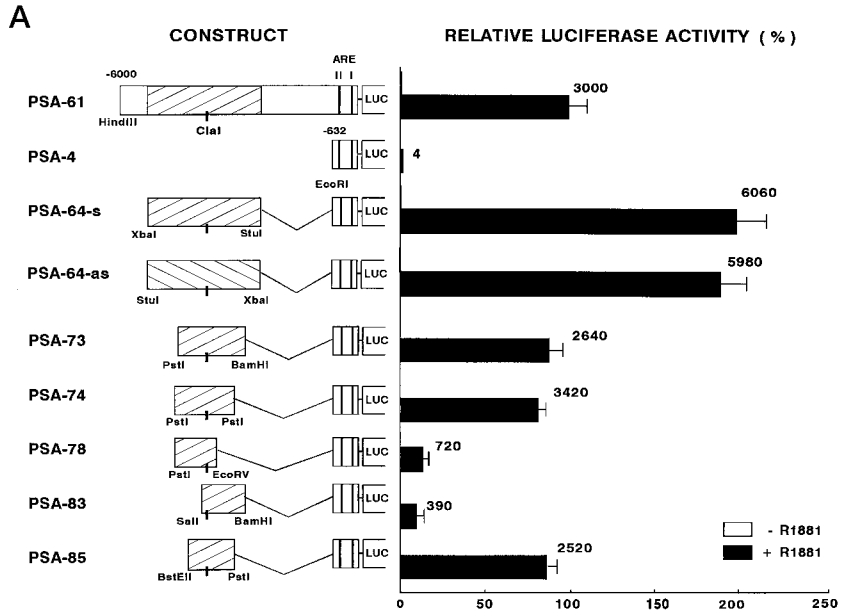
A, LNCaP cells were transiently transfected with PSA-LUC constructs as described in *Materials and Methods*. After 4 h incubation with the plasmid precipitate, transfected cells were cultured for 24 h in the absence or in the presence of R1881 (1 nM). The absolute activity and relative induction factor were calculated as the mean of four or more independent experiments, which were done in duplicate. *Solid 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 squares*. Positions of DHS I, II, and III are indicated by *arrows*. B, Partial restriction map of the PSA promoter. "*Sal*I" represents the position of an artificial *Sal*I site derived from the border of a human genomic DNA fragment in λ EMBL3.

site (Fig. 4A). The protected area contained the sequence GGAACAtatTGTATC, which shows high homology (overall 9 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 (Fig. 4A, lanes 4 and 5), indicating specificity of the interaction. Although both the *Bst*EII-*Sal*I and *Eco*RV-*Pst*I subfragments contributed to maximal activity of the core enhancer (Fig. 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 (Fig. 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 (Fig. 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.

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 (Fig. 5A). ARE-I and ARE-III turned out to be high-affinity AR-DBD-binding sites, with comparable AR-binding affinity.



B

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1
BstEII
ggtgaccaga gcagtctagg tggatgctgt gcagacgggg tttgtgccac tggtgagaaa

61
cctgagatta ggaatcctca atcttatact gggacaactt gcaaacctgc tcagcctttg

121                    "SalI"
tctctgatga agatattatc ttcatgatct tggattgaaa acagacctac tctggaGGAA

181     ClaI
CAtatTGTAT Cgattgtcct tgacagtaaa caaatctgtt gtaagagaca ttatctttat

241                                    EcoRV
tatctaggac agtaagcaag cctggatctg agagagatat catcttgcaa ggatgcctgc

301
tttacaaaca tccttgaaac aacaatccag aaaaaaaaag gtgttgctgt ctttgctcag

361                     NcoI
aagacacaca gatacgtgac agaaccatgg agaattgcct cccaacgctg ttcagccaga

421            PstI
gccttccacc cttgtctgca g
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C

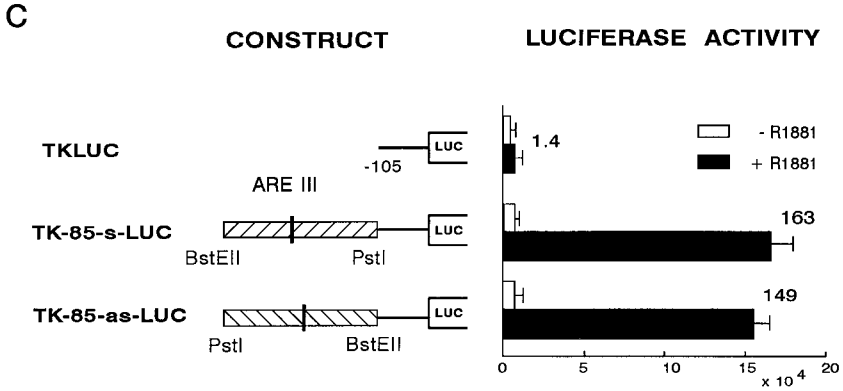

Fig. 3. Identification of the Androgen-Regulated Core Enhancer Region, Upstream of the PSA Gene

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 Fig. 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 *Bst*EII-*Pst*I upstream core enhancer fragment. Important restriction sites are indicated above the sequence. The ARE-III sequence is *underlined*, with the two half-sites in *capital letters*. 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 Fig. 2A.

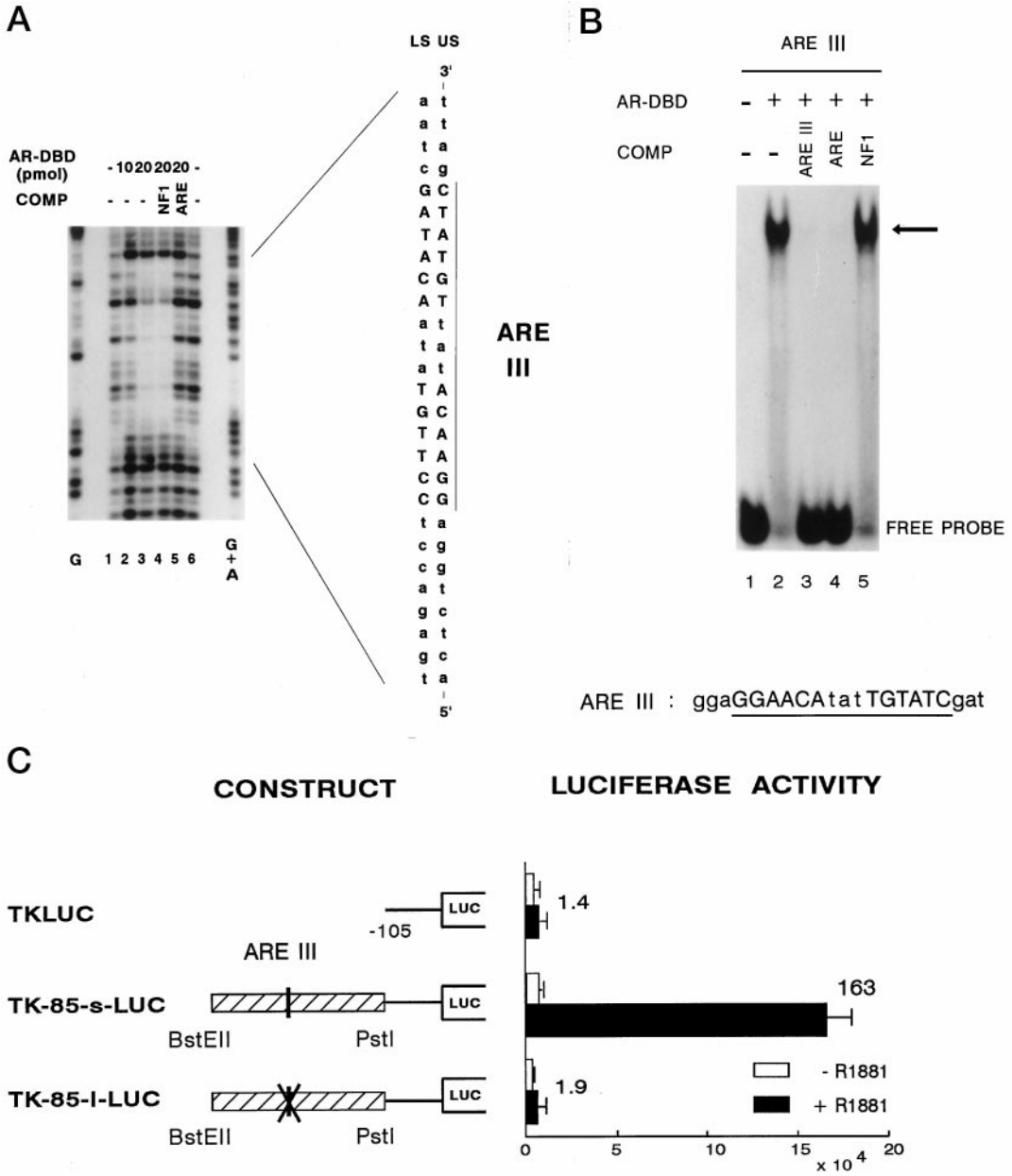

Fig. 4. Identification of a Functionally Active AR-Binding Site in the Core Enhancer Region

A, DNaseI footprint analysis over ARE-III. The lower strand of pHS2 ("*Sal*I"-*Eco*RV fragment in the core enhancer) was ^{32}P-end-labeled, 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 nonspecific (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 *capital letters*. 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 Fig. 2A. *Solid 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 square*. The inactivated ARE-III is represented by a *cross*.

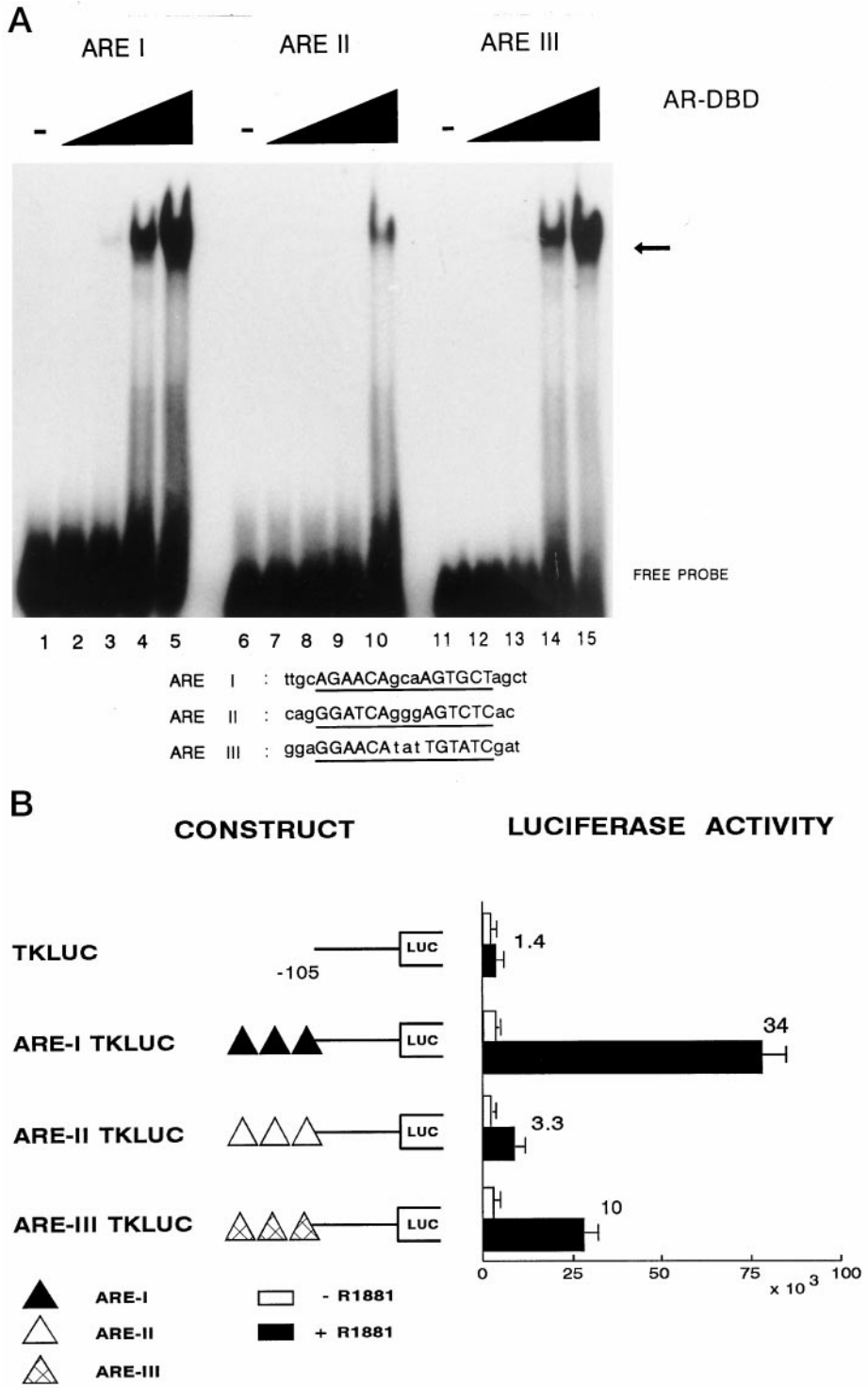

Fig. 5. Comparison of ARE-I, ARE-II, and ARE-III *in Vitro* AR(DBD) Binding and Functional Activity

A, Gel retardation analysis of ARE-I, ARE-II, and ARE-III complexed with AR(DBD). ARE-1 (lanes 1–5), ARE-II (lanes 6–10), and ARE-III (lanes 11–15) (50×10^3 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 Fig. 2A.

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 functionally active, albeit less than ARE-I: 10-fold and 34-fold induction upon R1881 stimulation, respectively (see Fig. 5B). However, both ARE-I and ARE-III were more active than ARE-II.

Mutational Analysis of ARE-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 (Fig. 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 with 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 Fig. 4C, but also in the context of the 6-kb PSA promoter.

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 nonprostate cells (13). To study whether the androgen-induced activity of the upstream core enhancer also showed cell specificity, reporter constructs PSA-61-LUC (Fig. 2), TK-85-s-LUC, and the TK-LUC control (Fig. 3C) were cotransfected with the AR expression plasmid pSVARo to a series of AR-negative prostate and nonprostate 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 1). 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.

The only tested cells, other than 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

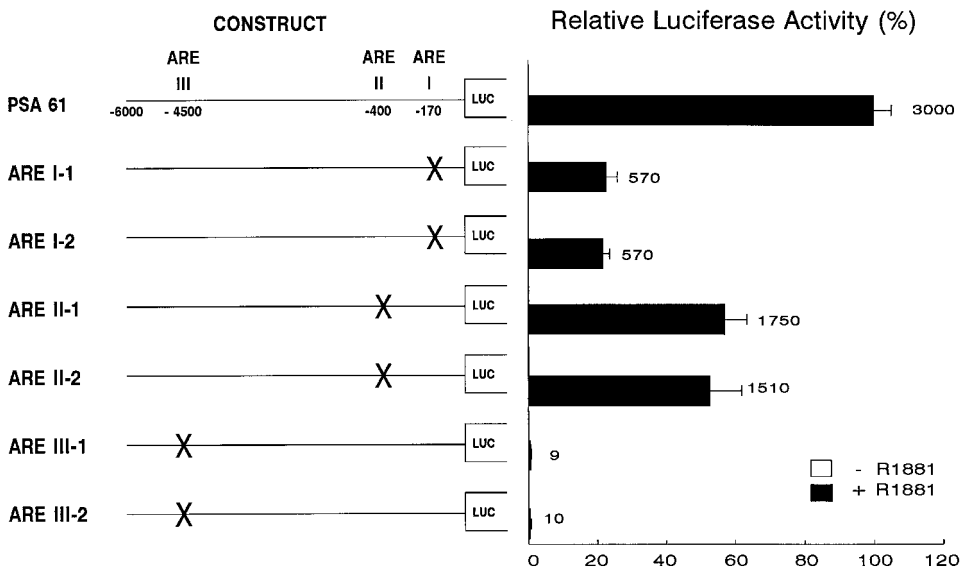

Fig. 6. The Effect of the Inactivation of ARE-I, ARE-II, and ARE-III on Androgen Regulation of the PSA Promoter

Experimental details are as described in *Materials and Methods* and in the legend to 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*.

Table 1. Effect of R1881 on PSA-61-LUC, TK-85-LUC, and MMTV-LUC Activity in PC3, DU145, COS, and Hep3B Cells, Cotransfected with the AR Expression Plasmid pSVARo, as Compared to LNCaP Cells

Construct	Relative Induction (+/− R1881)[a]				
	PC3	DU145	COS	Hep3B	LNCaP
TK-85-s-LUC[b]	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

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

Table 2. Effect of R1881, DHT, and R5020 on PSA-61-LUC, TK-85-LUC, and MMTV-LUC Activity in T47D Cells, without (A) and with Cotransfection of the AR Expression Plasmid pSVARo (B)

Construct	Relative Induction (+/− Hormone)[a]					
	A	R1881	DHT	R5020	B	DHT
TK-85-s-LUC[b]		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

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

2A). 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 2B). 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 1 and 2) still indicates a strong preference of the PSA promoter for LNCaP cells.

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 with the latter study, two major differences seem obvious: 1) In the experiments described in the present study, approximately 50% of the activity of the upstream enhancer region is retained within the 440-bp *Bst*EII-*Pst*I (−4.3 to −3.9) fragment (see Fig. 3A), whereas in the work of Schuur *et al.* essentially all activity is lost by deletion of sequences downstream of the *Xba*I site at −5.3 kb. At the 3′-border of the enhancer region the differences are less dramatic. Deletion from the *Pst*I site to the *Eco*RV site in constructs PSA-73 and PSA-74 results in an approximately 5-fold loss of activity (see Fig. 3A); the comparable constructs in Ref. 18 (CN70 and CN71) show a 2-fold decrease in activity. In summary, 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. 2) 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 with 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 Fig. 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 *Pst*I site, which is at the 5′-border of PSA-73-LUC; DHSIII maps close to the *Bam*HI 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 *Cla*I 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 Fig. 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 *Sal*I-*Eco*RV 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 attached 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 in the recruitment of the RNA polymerase II holoenzyme to the PSA promoter (20, 21). In this respect the PSA upstream enhancer might function as a classic 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 may be of great help in the identification of prostate-specific transcription factors and in 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 inter-

actions 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 synergistic interaction between multiple ARE sequences has also been 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 (Fig. 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 nonprostate 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 2). 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 determine 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 MEMα, 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 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 before use). The cells were disrupted by passing five to 10 times through a 0.5 $\times$ 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 5×10^7 nuclei/ml. Limited DNaseI digestion was carried out in a final volume of 0.5 ml HS buffer containing 5×10^6 nuclei, 5 mM $MgCl_2$, and DNaseI (0–800 U; Boehringer Mannheim, 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 *Eco*RI. 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 Fig. 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 *Hin*dIII/*Hin*dIII (−6 kb/+12) fragment of the PSA promoter in the multiple cloning site (MCS) of pLUC. PSA-1-LUC was generated by ligation of the *Bam*HI/*Hin*dIII (−2.2 kb/+12) fragment in the MCS of pLUC. PSA-64-s-LUC and PSA-64-as-LUC (*Xba*I-*Stu*I, −5.4/−3.2 kb), PSA-73-LUC (*Pst*I-*Pst*I, −4.7/−3.9 plus *Pst*I-*Bam*HI −3.9/−3.8 kb), PSA-74-LUC (*Pst*I-*Pst*I, −4.7/−3.9 kb), PSA-78-LUC (*Pst*I-*Eco*RV, −4.8/−4.1 kb), PSA-83-LUC (*Sal*I-*Bam*HI, −4.25/−3.8 kb), and PSA-85-LUC (*Bst*EII-*Pst*I, −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 *Sal*I 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 *Bst*EII-*Pst*I 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 *Sal*I site of TKLUC.

```
ARE-I:    5′ GATCCTTGCAGAACAGCAAGTGCTAGCTG      3′
          3′      GAACGTCTTGTCGTTCACGATCGACCTAG 5′
3ARE-II:  5′ TCGACAGGGATCAGGGAGTCTCACCAGGGATCA-
          3′      GTCCCTAGTCCCTCAGACTGGTCCCTAGT-
             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 *Bst*EII/*Cla*I, *Sal*I/*Eco*RV, *Eco*RV/*Pst*I, and *Cla*I/*Nco*I fragments, respectively, into the *Sma*I 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 that 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:

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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 1×10^6 cells per 25-cm^2 flask and 5 μg of the appropriate PSA-LUC construct. After 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 dithiothreitol, 1% Triton X-100, 15% glycerol). Next, 0.1 ml luciferin (0.25 μM) (Sigma, St. Louis, MO)/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 for 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 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, pHS2, pHS3, and pHS4 with *Xba*I and *Sac*I, or with *Sph*I and *Eco*RI, in order 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 nondenaturing polyacrylamide gel. The DNaseI footprinting experiments were performed essentially according to Lemaigre *et al.* (34). Labeled 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 oligos (consensus ARE or consensus NF-1; 5′-GATCCAGGGAACAGGGTGTTC-TACG-3′, and 5′-ATTTTGGCTTGAAGCCAATATG-3′, respectively)) was added. Digestion with 0.04 U of 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 of DNAseI was used. After 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 (7 M 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′ GATCCAGGGATCAGGGAGTCTCAGG 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 nondenaturing 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 were added. After incubation for 20 min, samples were run on a 4% nondenaturing polyacrylamide gel. Subsequently, gels were fixed, dried, and exposed to x-ray film.

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