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# Two Androgen Response Regions Cooperate in Steroid Hormone Regulated Activity of the Prostate-specific Antigen Promoter*

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**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-base pair 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 promoter. Both AR binding and transcriptional activity resided in a 20-base pair 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 was able to stimulate PSA promoter activity. Both the ARE and ARR are involved in dexamethasone regulation of the PSA promoter. Both the AR and glucocorticoid receptor were 20–100-fold more active on ARR-PSA and ARR-thymidine kinase promoter constructs in LNCaP cells than in other cell types (COS, HeLa, Hep3B, and T47D cells), indicating (prostate) cell specificity.**

Androgens exert their function via the intracellular androgen receptor (AR),[1] which is a member of the family of the steroid hormone receptors (see for reviews, Refs. 1 and 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-base pair spacer. The consensus sequence of the response element for the glucocorticoid receptor (GR), progesterone receptor, and AR is identical: GGTACAnnnTGTTCT (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 hormone response elements has also been observed (7, 8). At present it is unclear how specificity of the AR/GR/progesterone receptor response operates. Several mutually not exclusive mechanisms have been proposed, including subtle differences in sequences flanking the hormone response element, 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 kilobases 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 modified 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 (DuPont NEN) 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) 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 (*Hin*dIII, *Pvu*II, *Nhe*I, *Bgl*II, and *Nco*I) in the *Hin*dIII and *Nco*I sites of PSLA3. PSA-LUC constructs, PSA-4-LUC (*Eco*RI/*Hin*dIII; −632/+12), PSA-5-LUC (*Bgl*II/*Hin*dIII, −539/+12), PSA-6-LUC (*Xho*II/*Hin*dIII; −324/+12), and PSA-7-LUC (*Nhe*I/*Hin*dIII; −157/+12) were generated by ligation of the appropriate fragments in the multiple

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*The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) X92553.*

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[1] The abbreviations used are: AR, androgen receptor; GR, glucocorticoid receptor; PSA, prostate-specific antigen; ARE, androgen response element; ARR, androgen response region; TK, thymidine kinase; AR(DBD), AR DNA-binding domain.

cloning site of pLUC.

Constructs PSA-8 to PSA-11-LUC were obtained by exonuclease III digestion of PSA-2-CAT (−632/+12)(23) from the *Sal*I site. After exonuclease III incubation according to the "Erase a base" protocol (Promega, Madison, WI), the plasmid was digested with *Hin*dIII and the derived promoter fragments were ligated in the multiple cloning site 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 *Pst*I site at position −174 by polymerase chain reaction. The polymerase chain reaction product was digested with *Pst*I and *Hin*dIII (+12) and isolated from a 1.5% agarose gel. The isolated fragment was ligated in the *Pst*I and *Hin*dIII sites of the pLUC multiple cloning site. 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 *Bam*HI 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 *Sal*I compatible ends were inserted in the *Sal*I 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 $1 \times 10^6$ cells per 25-cm$^2$ flask, 5 μg of the appropriate PSA-LUC construct, and where indicated 2.5 μg of 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, containing 15% glycerol (incubation for 90 s 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 of cell extracts. Luciferase activities and relative induction factors are expressed as mean and standard error (S.E.).

*Luciferase Assay*—Cells were washed once with phosphate-buffered saline and lysed in 300 μl of lysis buffer (25 mM Tris phosphate, pH 7.8, 8 mM $MgCl_2$, 1 mM dithiothreitol, 1% Triton X-100, 15% glycerol). Next, 100 μl of 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 s (according to supplier), the light emission during 5 s 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 (Promega). AR synthesis was in the presence of 10 μM $ZnCl_2$. Production in *Escherichia coli*, and purification of AR(DBD) was done as described previously (27).

Double stranded oligonucleotide probes used in gel retardations are as follows.

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

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

Oligonucleotides 1–10

Probes were filled in with Moloney murine leukemia virus-reverse transcriptase in the presence of [α-$^{32}$P]dATP, and subsequently isolated from nondenaturing polyacrylamide gel. For gel retardation assays, $20–50 \times 10^3$ cpm of each probe was added to 20 μl of reaction mixture, containing 2 μg of poly(dI-dC), 2 μg of bovine serum albumin, 10 μM $ZnCl_2$, 1 mM dithiothreitol, and 2 μl of 10 × 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 of 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 room temperature. 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 glucocorticoid response element, 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 room temperature. Subsequently, gels were fixed, dried, and exposed to x-ray film.

## RESULTS

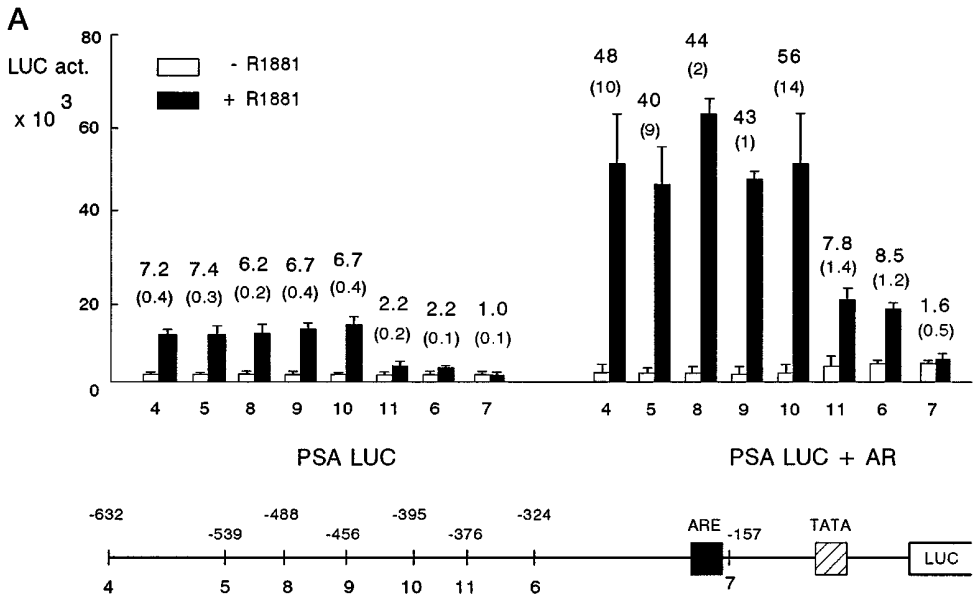
*Deletion Mapping of the PSA Promoter: Effect of Androgen Receptor Overexpression*—In a previous study we analyzed androgen regulation of the PSA promoter in COS cells which were co-transfected with several different PSA promoter-chloramphenicol acetyltransferase reporter gene constructs and the AR expression 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 (Fig. 1*A*). Transfection of PSA-4-LUC (−632/+12) or PSA-5-LUC (−539/+12) resulted in an approximately 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 approximately 4 kilobases (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).

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 Fig. 1*B* 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 (6–7-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 considerably 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 (Fig. 1*A*). 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 (Ref. 23, and data not shown), indi-



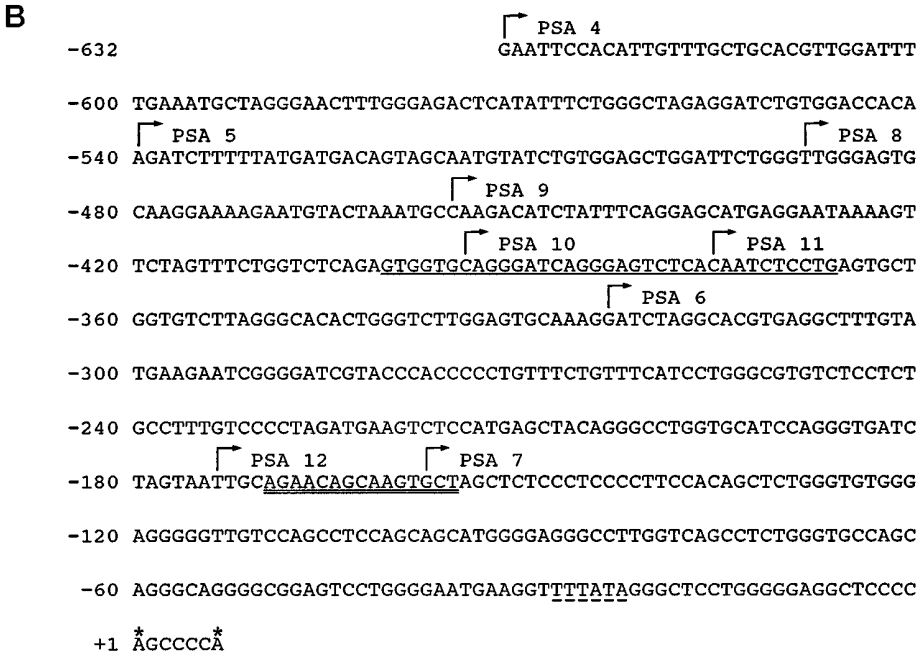




FIG. 1. **Androgen regulation of the PSA promoter in LNCaP cells.** *A*, LNCaP cells were transfected with PSA-LUC constructs (*left panel*) and with PSA-LUC constructs plus the AR expression plasmid pAR0 (*right panel*) as described under "Materials and Methods." Start positions of PSA promoter constructs are indicated below the figure and in *B*. 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*. S.E. of absolute activity is represented by a *vertical stripe*; S.E. 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*.

cating 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* (Fig. 2, *A-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 Fig. 2*A*, *lanes 1–4*). The retarded complexes visible in the absence of antibody Sp197 (*lane 1*) are considered to be nonspecific because a 100-fold molar excess of unlabeled 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 (Fig. 2*B*). Application of the AR(DBD) in the gel retardation assay revealed a clear AR(DBD)·ARE(−170) complex in the absence of antibody (Fig. 2*C*).

*Effect of the −400 to −366 Fragment (ARR) on Basal PSA*

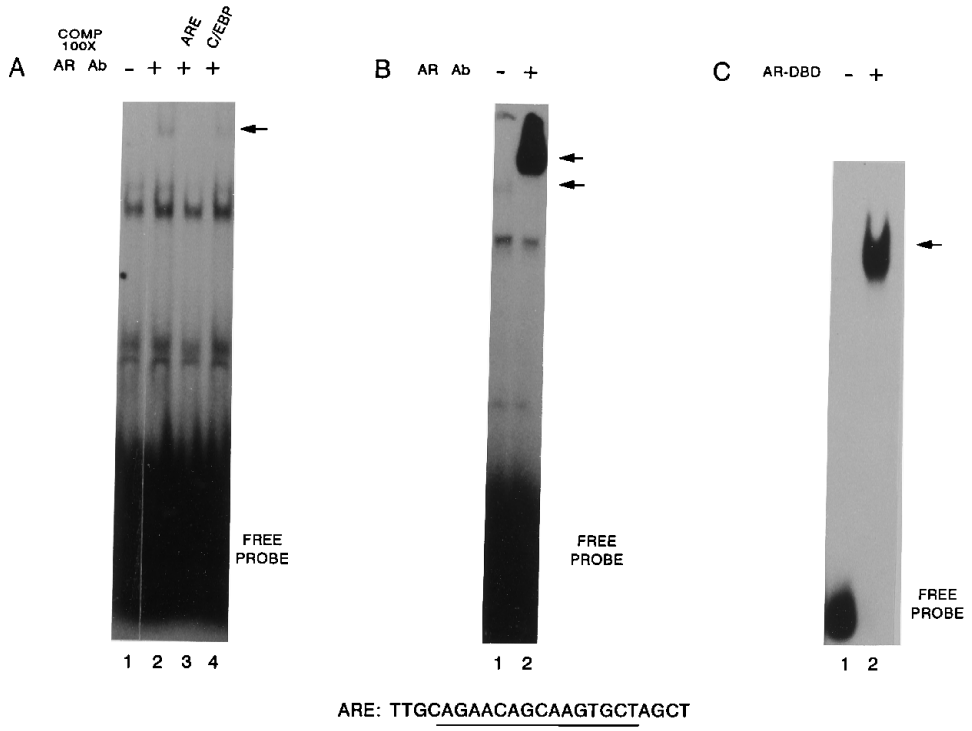



FIG. 2. **Gel retardation analysis of AR·ARE(−170) complexes.** *A*, $20 \times 10^3$ cpm of ARE(−170) was incubated with LNCaP nuclear extract, and complexes were analyzed by polyacrylamide gel electrophoresis as described under "Materials and Methods." Incubations were in the absence (*lane 1*) or presence (*lanes 2–4*) of antibody Sp197. 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 nonspecific C/EBP competitor (*lane 4*) are given under "Materials and Methods" (see for the −170 ARE also the *underlined* sequence below the figure). *B*, $40 \times 10^3$ cpm of ARE(−170) was incubated with *in vitro* synthesized full-length AR, and AR·ARE(−170) complexes were analyzed by polyacrylamide gel electrophoresis as described under "Materials and Methods." *Lanes 1* and *2*, ARE with reticulocyte lysate, containing *in vitro* synthesized AR. Incubations were in the absence (*lane 1*) and presence (*lane 2*) of antibody Sp197. *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 ($50 \times 10^3$ cpm). The *arrow* indicates the position of the AR(DBD)·ARE complex. *Lane 2*, $50 \times 10^3$ cpm of ARE(−170) was incubated with AR(DBD) expressed in *E. coli*, and purified as described under "Materials and Methods." Subsequently, the reaction mixture was analyzed by polyacrylamide gel electrophoresis.

*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 Fig. 1*B*) 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 approximately 6 times more active in R1881-treated, pARO co-transfected LNCaP cells than in the absence of hormone (Fig. 3*A*). Constructs PSA-12.1s-LUC and PSA-12.1as-LUC, containing one ARR copy in sense and antisense orientation, respectively, gave rise to an increased R1881-induced activity of the promoter (22–23-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.

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 (Fig. 3*B*). 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.

*Gel Retardation Analysis of the 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 (Fig. 4*A*, *lanes 1–3*). Incubation with antibody Sp197 did not result in a visible supershift (Fig. 4*A*, *lane 2*; compare Fig. 2*A* for ARE(−170) shifts), nor could one of the complexes be competed with an excess of unlabeled ARE(−170) (Fig. 4*A*, *lane 4*). Identical complexes were formed with extracts from cells grown in the absence or presence of hormone, indicating that the expression or activity of none of the proteins visualized in Fig. 4*B* 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 (Fig. 4*C*), suggesting the presence of a low affinity AR binding site in the ARR. In agreement with this observation, a 100-fold excess of ARR could partially compete the formation of an AR·ARE(−170) complex (Fig. 4*C*, *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 (Fig. 4*D*).

*Analysis of the AR-binding Segment in ARR*—To find the precise sequence in ARR which was responsible for AR binding,

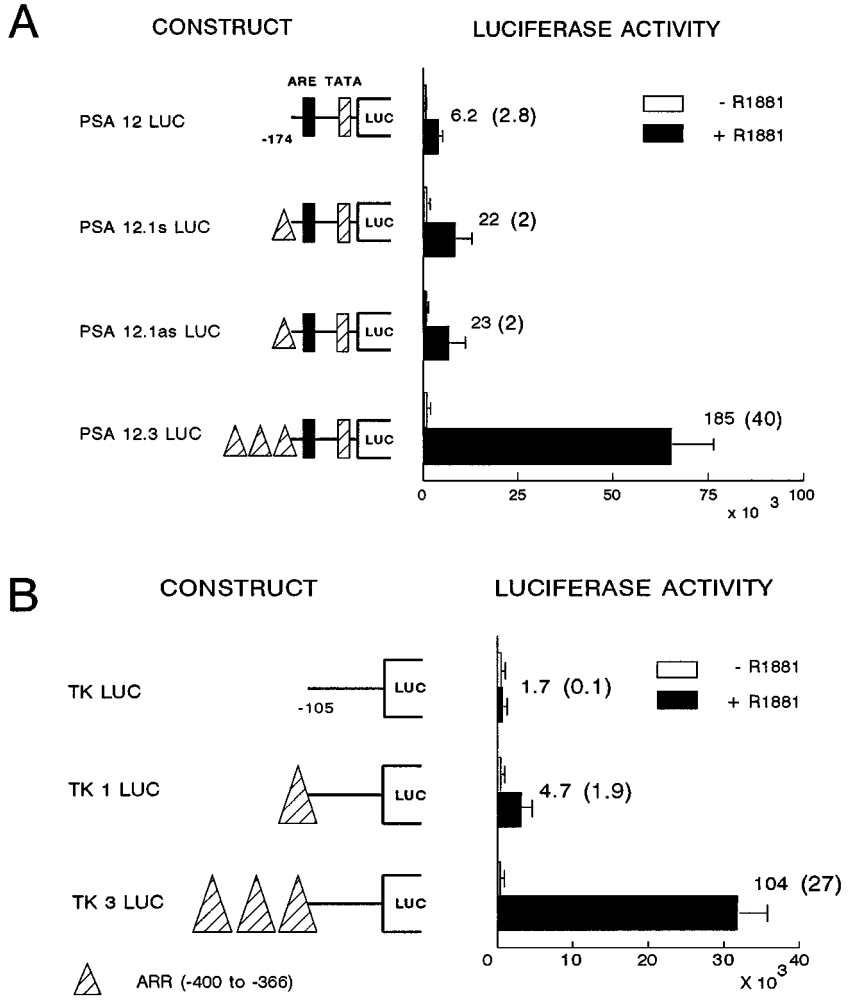



FIG. 3. *A*, effect of the −400 to −366 region (ARR) on basal PSA promoter activity in LNCaP cells overexpressing the AR. The ARR is represented by a *hatched triangle*, the ARE by a *black box*, and the TATA element by a *hatched box*. *B*, effect of the −400 to −366 region of the PSA promoter (ARR) 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 Fig. 1*A*. The ARR sequence is *single underlined* in Fig. 1*B*.

the ARR was subdivided into three partially overlapping fragments. Gel retardation assay with AR(DBD) and the three ARR subfragments 1S (GTGGTGCAGGGATCAGGGAG (−400 to −381)), 2S (CAGGGATCAGGGAGTCTCAC (−394 to −375)), and 3S (GAGTCTCACAATCTCCTG (−383 to −366)) showed that 2S contained the AR binding sequence, no or extremely weak binding was detected with 1S and 3S (Fig. 5*A*).

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 (Fig. 5*B*). 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 (Fig. 6*A*). 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.

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 (Fig. 6*B*). 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

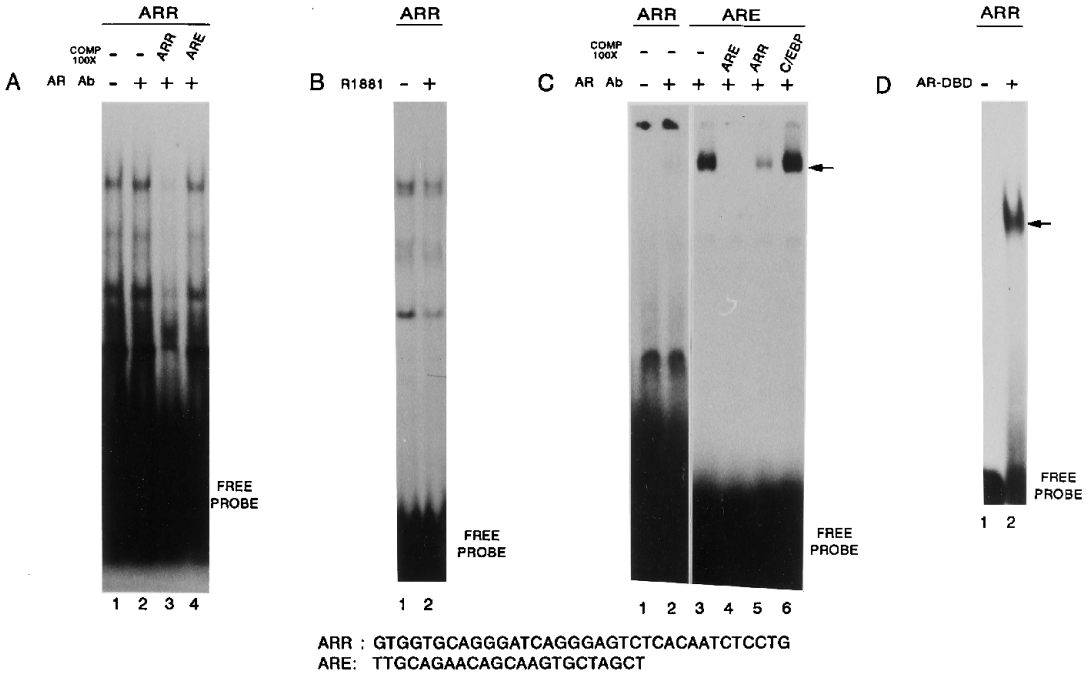



FIG. 4. **Gel retardation analysis of AR·ARR complexes.** *A*, $40 \times 10^3$ cpm of ARR was incubated with LNCaP nuclear extract, and complexes were analyzed as described under "Materials and Methods." Complex formation was in the absence (*lane 1*) or presence (*lanes 2–4*) of antibody Sp197. Incubation 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*, $40 \times 10^3$ cpm of 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*, $20 \times 10^3$ cpm of ARR was incubated with *in vitro* synthesized full-length AR, in the absence (*lane 1*) and presence (*lane 2*) of antibody Sp197, and subsequently analyzed by polyacrylamide gel electrophoresis as described under "Materials and Methods." *Lanes 3–6*, $40 \times 10^3$ cpm of ARE incubated with *in vitro* synthesized AR. Complexes were analyzed by gel retardation assay as described under "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*, $50 \times 10^3$ cpm ARR was incubated with AR(DBD) expressed in *E. coli* and purified as described under "Materials and Methods" and subsequently analyzed by polyacrylamide gel electrophoresis. The *arrow* indicates the position of the AR(DBD)·ARR complex.

shown). As depicted in Fig. 7*A*, the pattern of PSA promoter activity induced by dexamethasone in the presence of GR turned out to be identical to AR mediated activity (compare with Fig. 1*A*): the ARE at position −170 could function as a 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 (Fig. 7*B*). 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 Figs. 3 and 7*B*) 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 the activity in LNCaP cells. Essentially identical results were obtained in 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: 42–97-fold; GR/PSA-12.3-LUC: 26–48-fold; AR/TK-3-LUC: 18–54-fold; GR/TK-3-LUC: 27–78-fold). Our data indicate that LNCaP cells contain one or more factors, which positively affect PSA minimal promoter activity and ARR(−400) activity. It is tempting to speculate that this factor(s) directly or indirectly interacts with a steroid receptor bound to its response elements.

## 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 Figs. 1B and 2). Comparison of this sequence with a GGTACAnnnTGTTCT ARE/glucocorticoid response element 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 (approximately 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)

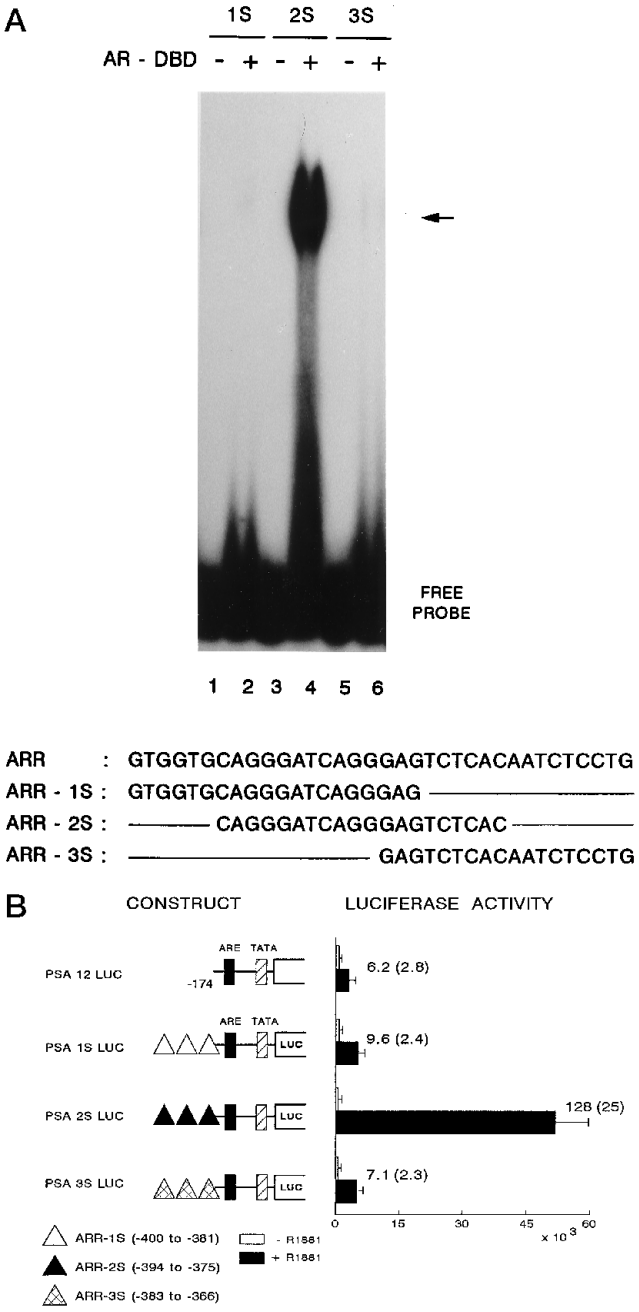



FIG. 5. **Analysis of the AR binding segment in ARR.** *A*, gel retardation analysis of AR(DBD)·ARR subfragment complexes. 50 × $10^3$ cpm of ARR-1S (*lanes 1* and *2*), -2S (*lanes 3* and *4*), and -3S (*lanes 5* and *6*) were incubated with AR(DBD) and analyzed by gel retardation assay as described under "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 S.E. are from three independent, duplicate experiments. Further experimental details are identical to those described in the legend to Fig. 1*A*.

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 mouse vas deferens protein 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 (Ref. 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 hormone response element-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-base pair fragment (N39) which shows AR binding and confers weak androgen responsiveness 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. Cotransfection 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.[2] 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

[2] K. B. J. M. Cleutjens, unpublished data.

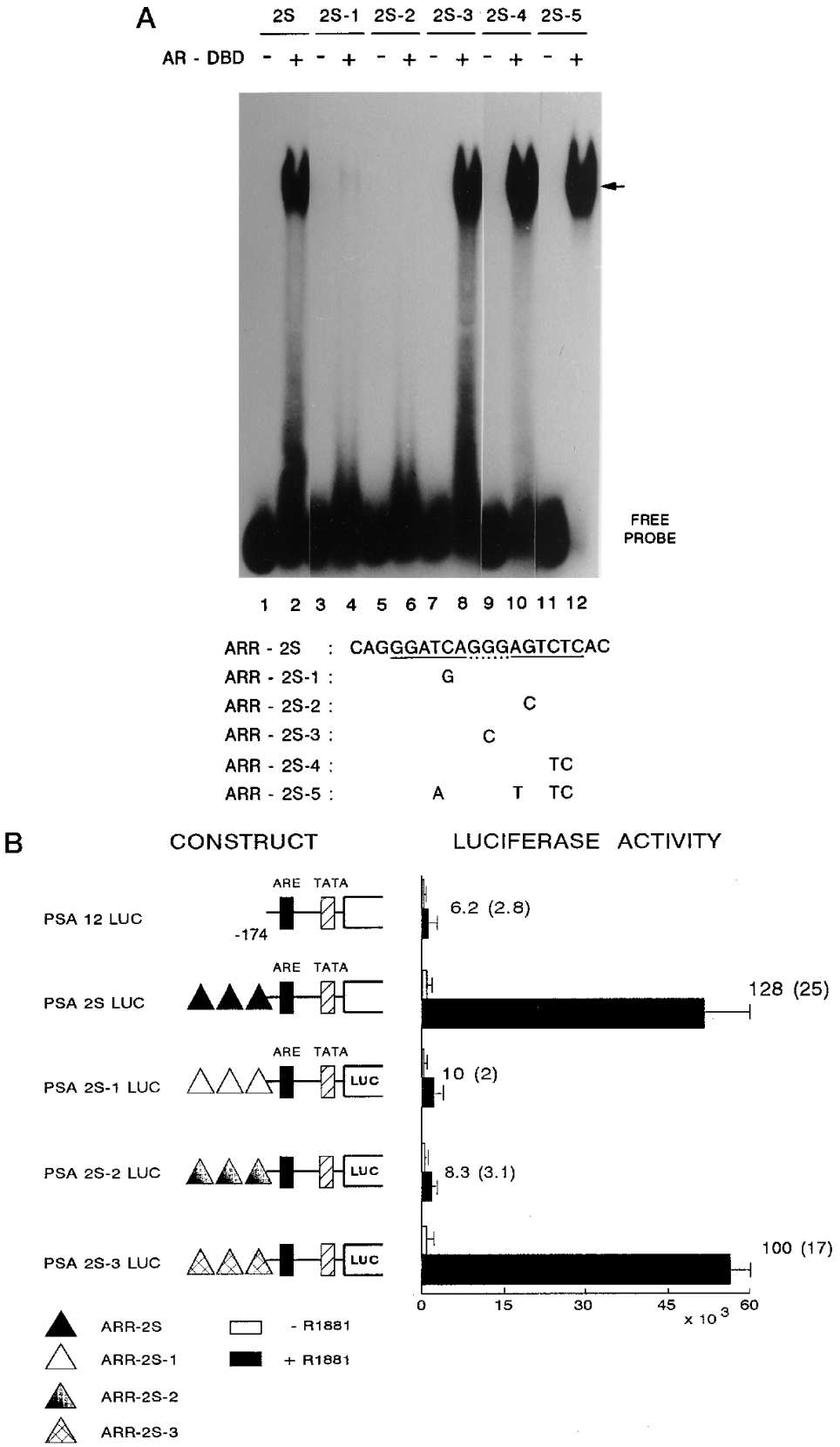



FIG. 6. **Mutation analysis of the ARR-2S segment.** *A*, gel retardation analysis of ARR-2S and ARR-2S mutants with AR(DBD). $50 \times 10^3$ cpm of ARR-2S (*lanes 1* and *2*), ARR-2S-1 (*lanes 3* and *4*), -2S-2 (*lanes 5* and *6*), -2S-3 (*lanes 7* and *8*), -2S-4 (*lanes 9* and *10*), and -2S-5 (*lanes 11* and *12*) were incubated with AR(DBD) and analyzed by gel retardation assay as described under "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 S.E. are from three independent, duplicate experiments. Experimental details are as described in the legend to Fig. 1*A*. *Closed bar*, activity in the presence of R1881 (1 nM); *open bar*, activity in the absence of hormone.

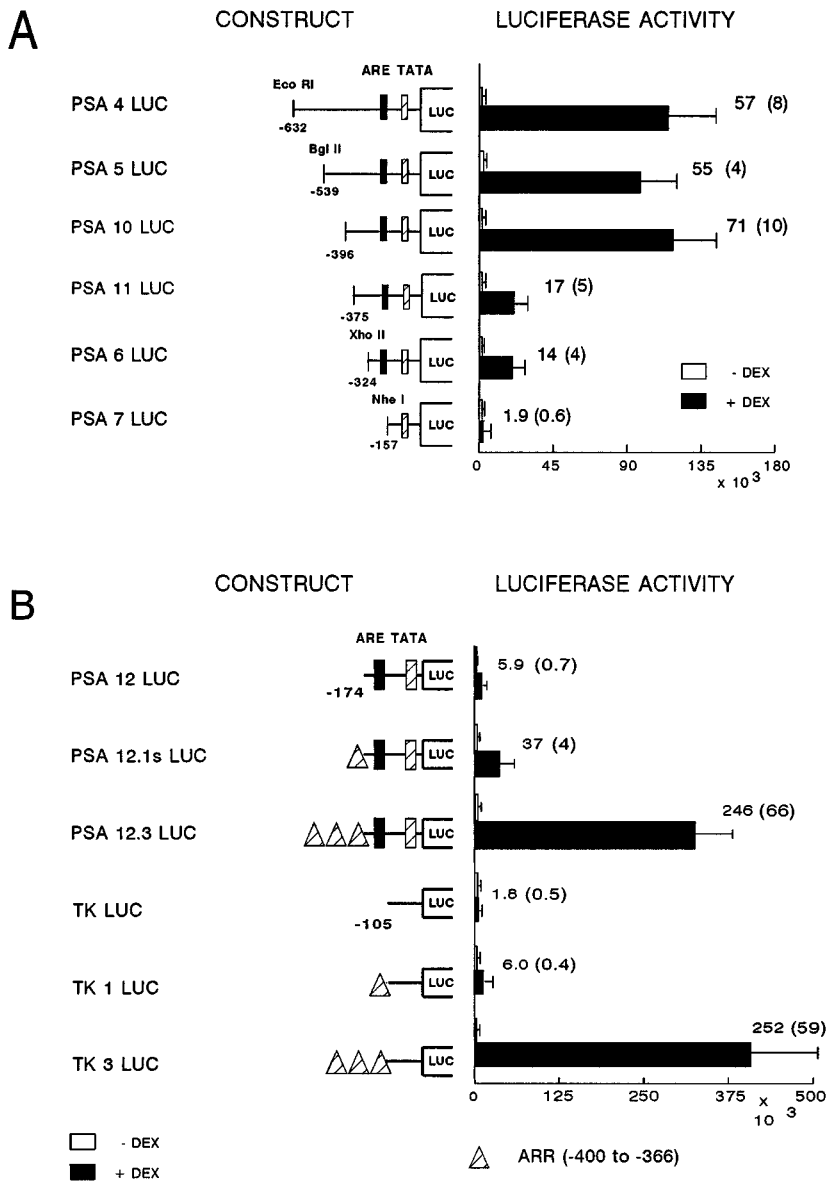



FIG. 7. **Effect of glucocorticoid receptor overexpression on PSA promoter activity in LNCaP cells.** *A*, LNCaP cells were transfected with PSA promoter constructs in the presence of the GR expression plasmid PSTC-GR (see "Materials and Methods" for experimental details). After transfection, cells were 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. S.E. of luciferase activity is given by the *horizontal bar*; S.E. 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 *A*.

TABLE I

*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 pARO and (B) glucocorticoid receptor expression plasmid PSTC-GR*

| | Construct | Relative induction (± hormone)[a] | | | | |
|---|---|---|---|---|---|---|
| | | LNCaP | COS | HeLa | Hep3B | T47D |
| A | PSA-12-LUC[b] | 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 |
| B | 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 |

[a] Induction factor is the mean of three to seven independent, duplicate experiments ± S.E.
[b] Constructs are as described under "Materials and Methods."

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 (Fig. 4*A*). However, these factors are not androgen regulated (Fig. 4*B*) 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). Al-

ternatively, specific factors affect AR and GR activity by protein-protein interaction.

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Nucleic Acids, Protein Synthesis, and Molecular Genetics:

# Two Androgen Response Regions Cooperate in Steroid Hormone Regulated Activity of the Prostate-specific Antigen Promoter

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