Cooperative Assembly of Androgen Receptor into a Nucleoprotein Complex That Regulates the Prostate-specific Antigen Enhancer*

Weibiao Huang‡§, Yuriy Shostak‡, Paul Tarr‡§, Charles Sawyers‡, and Michael Carey‡**

From the ‡Department of Biological Chemistry, Box 1737, and §Department of Medicine, Box 1678, UCLA School of Medicine, Los Angeles, California 90095

Prostate cancer is characterized by elevated serum levels of prostate-specific antigen (PSA). PSA gene expression is controlled by an androgen-responsive transcriptional enhancer. Our study suggests that formation of a nucleoprotein complex, encompassing 170 base pairs of enhancer DNA, mediates androgen-responsive PSA enhancer activity. The complex is assembled by cooperative binding of androgen receptor to at least four tandem, nonconsensus androgen response elements (AREs). Systematic mutagenesis of the AREs demonstrated that they act synergistically to stimulate androgen receptor-responsive gene expression. We discuss a mechanism whereby a combination of high androgen receptor levels in the prostate and low affinity AREs contribute to the cell type specificity and activity of the enhancer.

Androgen receptor (AR) plays a central role in prostate cancer progression. Depletion of androgens by surgical or chemical treatments slows cancer growth. AR activates transcription of the gene encoding the kallikrein protease prostate-specific antigen (PSA) and other genes involved in secretory epithelial cell metabolism. Serum PSA levels parallel oncogenic growth in the initial androgen-dependent phase and in early stages of androgen-independent cancer. The prostate specificity of the PSA transcriptional regulatory region has made it an ideal reagent for use in gene therapy trials (1–4). We have focused on the role of AR in PSA regulation as a model for understanding how AR regulates prostate gene expression.

Biochemical and genetic studies have led to the cloning and partial genetic dissection of the PSA promoter and enhancer (3, 5–9). Both the enhancer and promoter display androgen responsiveness consistent with the observation that both regions contain androgen response elements (AREs) (3, 5, 7, 8, 10–12). The proximal promoter has been delineated to an approximately 630-bp fragment containing a core TATA box (5) and two AREs, ARE I and ARE II (8). AR activates transcription synergistically from these AREs (8). Although the promoter plays an important role in PSA expression, an experiment in transgenic mice has shown that it is insufficient to confer strong androgen responsiveness and cell type specificity in vivo. In the same experiment, however, a 6-kb region encompassing the promoter and extending further upstream was able to mediate proper regulation (14).

An enhancer element, centered at approximately −4.2 kb, is located within this 6-kb region (6). The enhancer was originally identified as a 1.6-kb fragment containing, by sequence analysis, sites recognized by the androgen receptor, AP-1, cAMP-responsive element binding protein, and Fos (7). Two subsequent studies have further delineated the enhancer to an 822-bp fragment (3) and a 455-bp minimal core region encompassing an androgen-responsive DNase I-hypersensitive site (6). The role of the single identified ARE, termed ARE III, within this minimal core enhancer is not entirely clear. In one study, mutation of ARE III eliminated enhancer activity in transfection assays (6). In a different study, however, mutation of ARE III in combination with ARE I of the promoter was required to abolish androgen responsiveness (11). Although these results may reflect differences in methodology, we will show below that the enhancer is more complex than previously realized. We report that the enhancer contains multiple low affinity, non-consensus AREs, which bind AR cooperatively and act synergistically to stimulate transcription.

AR is a 110-kDa protein containing an amino-terminal transcriptional activation domain (AF-1) spanning amino acids 141–338 (16–22), a zinc finger DNA binding domain from amino acids 556–623, and a carboxyl-terminal hormone/ligand binding domain from amino acids 666–918 (23). AR is a member of the nuclear receptor superfamily. The remarkable homology of the conserved domains suggests that family members will activate transcription using conceptually similar mechanisms (24). The ligand binding domain contains an additional activation domain (AF-2) by virtue of its homology to similar regions in related receptors and its ability to interact with co-activators (25). Interaction between the amino- and carboxyl-terminal activation domains is thought to be important for full activity of AR (16, 26). The regulatory functions of AR in transcription have been demonstrated both in vivo and in vitro (19, 27–30).

The DNA binding domain of AR is a dimer and exhibits strong sequence homology with the progesterone (PR) and glucocorticoid (GR) receptors (31–34). Expression of androgen-responsive genes is regulated by binding of ligand-activated AR to androgen response elements (AREs). Characterization of different AREs indicates that there are two classes of sites (23). One group contains the 15-bp near-dyad, consensus sequence (GGTACACAnnnTGTTCT). The consensus also binds to and
mediates transactivation by GR and PR. A second class of sites, however, has been identified with sequences that diverge considerably from the consensus. It is likely that these non-consensus sites contribute to specific binding by AR (8, 35–37).

AREs are found in promoters of genes expressed in different tissues including prostate, brain, kidney, liver, and testis (23). It is unlikely, therefore, that AR alone regulates cell-type specificity of PSA gene expression. Indeed, DNase I footprinting and gel shift studies have identified several binding sites for prostate-specific and ubiquitous transcription factors within the PSA enhancer and promoter (7, 9, 12). It is likely that a combination of AR and cell-specific factors confers tissue selectivity. One of the hallmarks of such regulation is the formation of nucleoprotein complexes that use cooperative DNA binding and transcriptional synergy to elicit specific patterns of gene expression (38).

In an effort to understand the regulation of the PSA gene as a model for AR-mediated gene expression during prostate cancer, we undertook a biochemical analysis of the core enhancer. We employed DNase I footprinting to demonstrate that the enhancer contains a cluster of six closely spaced putative AREs differing in affinity for recombinant AR DNA binding domain (ARDBD). We systematically mutated these sites to demonstrate that at least four of the six sites were physiologically relevant in conferring response to AR in co-transfection assays into BHK and LNCaP cells. We then generated a retroviral construct encoding a FLAG-tagged version of AR, introduced it into HeLa and LNCaP cells, and isolated the receptor by immunofinity chromatography. We demonstrated by transactivation, nuclear translocation, and DNA binding assays that the FLAG-tagged AR was biologically active in vivo and in vitro. Purified AR bound cooperatively to the four core sites in the enhancer and responded to ARE mutants in a manner that roughly paralleled the in vivo transcriptional analysis. Conversely, PR did not substantially activate transcription from the core enhancer but did activate from a reporter bearing tandem AREs. We discuss how the cooperative binding and synergistic activation of the enhancer by AR can contribute to the strong androgen responsiveness and cell selectivity of PSA gene expression.

EXPERIMENTAL PROCEDURES

Plasmids—pET11dHis6ARDBD was constructed by insertion of a polymerase chain reaction (PCR) DNA fragment, encoding the DNA binding domain of human AR, from amino acids 549–650, into the BamHI site of pET11dHis6, heart muscle kinase (provided by R. Hori). The construct generated a 127-amino acid protein with a His6 tag on the amino terminus and heart muscle kinase phosphorylation site on the carboxyl terminus.

The wild-type PSA enhancer-E4CAT reporter vector was constructed by PCR subcloning a 496-bp PSA enhancer region from the 2.4 kb enhancer described by Belldegrun and colleagues (39) in reverse orientation into HindIII/XhoI sites upstream of E4 TATA box of pE4CAT (39). The PSA enhancer-E4LUC reporter vector was constructed by PCR amplification and subcloning of the PSA enhancer-E4 TATA region (up to +38) into SacII/XhoI sites of pGL2-Basic vector (Promega). All constructs were sequenced to confirm their integrity. The constructs contained several notable point mutations versus the published sequence by Henderson and colleagues (15). These point mutations were present in the original sequence and were not introduced inadvertently by PCR. The DNA sequence of ARE III, −4150GGAAGATATGTATC-4136, was altered to GGAACATatGTTATT, a change that brings the sequence into better alignment with the ARE consensus. Furthermore, the sequence of ARE IV, −3040GATGTCGTCGAGACAGAACTTCGGCTC-4289, was altered to GATGTCGTCGACAGAACTTCGGCTC-4289. Two other positions identical of identifiable AREs were also detected within this region. We have not tested if any of the nucleotide changes affect enhancer activity.

Enhancer mutant vectors were constructed by a two-step overlap PCR method where AREs were replaced by GAL4-binding sites and restriction enzyme cleavage sites to conserve phasing of the DNA (below, the replacement sequence is in capitals).

ARE replacement internal primers are as follows: ARE VI, 5′-CTG-CAGCGGAGTACTGCTCCGCTgttgctactGTCGAC-3′ and 5′-CCGGAGGACAGTACTCCGCGTCCAaagtcttgactgac-3′; ARE V, 5′-GTCGACGAG-TACTGCTCCGCTGCTTGagctgctgactggGAG-3′ and 5′-GCGAGGACAGTACTCCGCGTCCAaagtcttgactgac-3′; ARE IV, 5′-GTCGACGAGTACTCCGCGTCCAaagtcttgactgac-3′ and 5′-GCGAGGACAGTACTCCGCGTCCAaagtcttgactgac-3′; ARE III, 5′-GTCGACGAGTACTCCGCTTCCGATGGTTgatggGAG-3′ and 5′-GCGAGGACAGTACTCCGCGTCCAaagtcttgactgac-3′; ARE II, 5′-GTCGACGAGTACTCCGCTTCCGATGGTTgatggGAG-3′ and 5′-GCGAGGACAGTACTCCGCGTCCAaagtcttgactgac-3′; ARE I, 5′-GTCGACGAGTACTCCGCGTCCAaagtcttgactgac-3′ and 5′-GCGAGGACAGTACTCCGCGTCCAaagtcttgactgac-3′. Flag-tagged AR was constructed in two steps. In the first step a primer encoding the FLAG peptide fused to the sequences encoding the amino-terminal 5 amino acids of AR (5′-GCTTGTGACCAACTGGAC-TACAAAGGCGACAGGCAAGCCGCGGAACTGAGTTGTAAGTGGG-3′) was used in combination with an internal AR primer (5′-CCCTCTAGACGCGGCAGGATGTGCAAGAC-3′) to encode a FLAG-tagged version of AR. This fragment was digested with XhoI and inserted into the XhoI site of pBluescript KS I(Stratagene). The resulting plasmid was digested with EcoRI, and an EcoRI/XbaI fragment containing the PSA enhancer-E4LUC reporter vector was ligated to the remaining sequences from amino acids 10–919, was cloned in. The FLAG-tagged full-length AR (cDNA) was then excised from the pBluescriptI vector by XhoI digestion and placed into the XhoI site of pSRa (40) to generate pSRaARDBD.

ARDBD Purification—The Escherichia coli strain BL21(DE3) was transformed with pET11dHis6ARDBD. 0.1 liter of cells was grown to an Abs600 of 0.6 and induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside and 10 μM ZnCl2 for 3 h. After harvesting, cells were resuspended in 5 ml of Buffer A (20 mM HEpes, pH 7.9, 20% glycerol, 10 μM ZnCl2, 0.5 mM phenylmethylsulfonyl fluoride) containing 0.1 mM KCI and lysed by sonication. The supernatant was incubated with 1.5 ml of Ni-NTA agarose (Qiagen) resin in batch, rocked gently for 30 min at 4 °C, and then loaded on to a small Bio-Rad Econo-column. After successive washing the resin with 15 ml of Buffer A containing 0.1 mM KCI, and 15 ml of Buffer A containing 0.1 mM KCI and 20 mM imidazole, ARDBD was eluted with Buffer A containing 0.1 mM KCI and 150 mM imidazole. Peak fractions were combined and loaded onto a 1-mL ARE affinity column prepared by binding 1 mg of a biotinylated ARE containing double-stranded oligonucleotide (5′-gccaggtttgcaagttTGAAcagt-3′ and 5′-gcaggtttgcaagttTGAAcagt-3′) to 1 ml of packed streptavidin beads. The column was washed with 10 ml of Buffer A containing 0.1 mM KCI, 10 ml of Buffer A containing 0.5 mM KCI, and 15 ml of Buffer A containing 0.1 mM KCI and 20 mM imidazole. ARDBD was eluted with Buffer A containing 0.8 mM KCI. The fractions were analyzed by SDS-polyacrylamide gel electrophoresis and Coomasie Blue staining. Peak fractions were combined, dialyzed against Buffer B (20 mM HEpes, pH 7.9, 20% glycerol, 0.1 mM KCI, 0.2 M NaCl, 0.5 mM phenylmethylsulfonyl fluoride) and concentrated on Centricon-10 columns. The final preparation contained 100 μM arDBD.

Expression and Purification of FLAG-tagged AR—20 μg of pSRaAR was transfected with helper virus ϕ into a 10-cm dish of 293T cells at 50% confluency using calcium phosphate transfection (41). One day after transfection, virus was collected in Iscove media containing 10% FBS at 12-h intervals for 48 h. Virus was isolated by filtration of the cleared virus through a 0.45 μM filter. 2–3 ml of virus supernatant was incubated with HeLa and LNCaP cells at 10–30% confluency (LNCaP cells 20–30%, HeLa cells 10–20%) in the presence of 8 μg/ml Polybrene for 4 h or overnight. 10 ml of medium (DMEM for HeLa cells, RPMI 1640 for LNCaP cells) was added, and cells were passed for 24 h. 800 μg/ml G418 (Life Technologies, Inc.) was then added. After 2 weeks, individual clones were isolated and expanded, and levels of viral expression were determined by immunoblotting with FLAG monoclonal antibodies (Sigma). The clone expressing the highest level of fAR was chosen for large scale preparation.

Nuclear extracts from both HeLa and LNCaP cells expressing fAR were prepared as described previously (42). Although the scale of the procedure was increased, 1 mg of nuclear extract was incubated with 20 μl of agarose beads conjugated with FLAG monoclonal antibodies (Sigma) at 4 °C for 6 h. The beads were washed three times with 0.5 ml of Buffer D containing 0.3 M KCI, 0.05% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM diithiothreitol and eluted twice, 20 min each, with 20 μl of Buffer D containing 0.2 mg/ml FLAG peptide (Eastman Kodak Co.).
Identification of a Cluster of Low Affinity AREs in the PSA Enhancer—We first subjected the enhancer region to DNase I footprinting analysis using the AR DNA binding domain (ARDBD), amino acids 549–650. The ARDBD was expressed as a His$_6$ fusion protein from a modified pET11d (Novagen) T7 expression vector in *E. coli*. The *E. coli* lysate was bound to a Ni-NTA resin, and ARDBD was eluted with imidazole as described under “Experimental Procedures.” To purify active ARDBD away from inactive protein, we subjected the Ni-NTA eluate to an ARE-DNA affinity column. The final protein was >95% homogeneous as measured by Coomassie Blue staining of SDS-polyacrylamide gels (Fig. 1A). The peak fractions in Fig. 1A were pooled and employed in the experiment of Fig. 1B.

Our initial objective was to confirm the presence of a single published AR-binding site in the PSA enhancer using DNase I footprinting analysis. This site, termed ARE III, is centered at -4200 (6) or -4143 (7, 11) depending on the numbering system used in different laboratories. The high specific activity of our ARDBD, however, allowed us to detect five additional binding sites within the enhancer region, each footprint approximately 23 bp in size. The sites are visible on the autoradiograph in Fig. 1B. We have denoted the additional sites AREs IIIb, IIIia, IV, V, and VI with respect to their position relative to ARE III.

The affinity of the sites varied considerably. Among the six sites, site III had the highest affinity consistent with the study by Trapman and colleagues (6). Overall, however, the AREs were 15–60-fold lower affinity than the consensus (see Fig. 1B, legend). This observation may bear on the tissue specificity of PSA expression, an issue we will cover under “Discussion.” Fig. 1C summarizes the positions of all six AR-binding sites in the context of the minimal AR-responsive PSA enhancer. The DNA sequences and relative positions of the sites are shown in Fig. 1D.

The observation that the affinities of AREs in the enhancer are significantly lower than the consensus raised the issue of their physiological relevance. Indeed, inspection of the DNA sequences within the footprinted region revealed few matches to the consensus ARE. Even ARE III diverged from the consensus by two nucleotides. To investigate this issue in more detail, we performed three experiments. First, we subjected the enhancer AREs to substitution mutagenesis to determine which sites were important in co-transfection experiments with AR into BHK cells. We then compared the key constructs by transfection into a more physiological prostate cancer cell line, LNCaP. Finally, we developed a procedure to purify intact AR to homogeneity from mammalian cells and employed the intact AR to study binding to the enhancer and its substitution mutants.

Enhancer Mutagenesis and Transfection Analysis—To establish the physiological validity of the new AR-binding sites, we mutagenized them individually and in select pairwise combinations. The AREs were replaced by GAL4-binding sites using PCR mutagenesis techniques. The substitutions were constructed based on the positioning of the footprints. In creating the substitution mutants, we attempted in some cases to remove precisely the site and in others we attempted to disrupt both the target site and putative adjacent sites (see regions marked by dark bars in Fig. 1D). The rationale for this approach will be justified below, where we show that intact AR binds over the entire region between ARE IIb and ARE V. In all cases we preserved the original spacing by replacing the enhancer sequences with an equal number of bases. We employed the 17-bp GAL4 sites because GAL4 is a yeast transcriptional activator, and numerous studies have shown that in the absence of GAL4, or its derivatives, the sites have no endogenous transcriptional activity in mammalian cells (39). Fig. 2A shows a representative DNase I footprint demonstrating that mutants lacking either ARE III, ARE IV, or ARE V no longer bind the ARDBD but instead bind to the GAL4 DNA binding domain.

To determine whether the AREs contributed to the AR response, we first performed co-transfection assays into the baby hamster kidney (BHK) cell line. We chose BHK cells because...
they contained low amounts of endogenous AR (i.e. relative to LNCaP) as measured by immunoblotting (data not shown) and were unlikely to contain prostate-specific transcription factors. This experimental scenario permitted us to assess directly the contribution of co-transfected AR, and its interaction with AREs, to enhancer activity. Fig. 2B is a bar graph showing the average of three independent co-transfection experiments of an effector plasmid, expressing AR from the CMV enhancer (22), and wild-type and mutant PSA-E4-CAT enhancer constructs. The transcriptional activity of the enhancer was dependent upon co-transfected AR and the addition of 1 nM R1881 (data not shown). Sites IIIA, III, IV, and V contributed significantly to enhancer activity because, when these sites were mutated, transcription decreased by 50–75% relative to wild type. In contrast, mutation of sites IIIB and VI had only marginal effects. For the remainder of the paper we will focus solely on AREs IIIA, III, IV, and V. This is not to say that sites IIIB and VI are unimportant. However, the experimental design only allowed us to confidently attribute androgen responsiveness to AREs IIIA-V.

The wild-type and mutant enhancer constructs containing a luciferase reporter were transfected into an LNCaP cell line (Fig. 2C). We reasoned that by differing the concentration of ligand we might observe differences in the effects of particular mutants, i.e. different mutants might be sensitive to the concentration of active AR. At 1 nM R1881 the individual site mutants all retained low activity averaging about 20% that seen with wild type. However, the effects of S-III and S-IV mutants were consistently more severe at 0.3 nM ligand. Furthermore, the residual, but reproducible, activity of the four-site mutant (S-All) at 1 nM ligand disappeared at 0.3 nM. Remarkably, the S-IIIA and S-V mutants retained much of their activity at the lower concentration of ligand. In all cases the sites appeared to act synergistically in LNCaP cells as removal of any site elicited a disproportional decrease in activity. We noted that the transcriptional activities of the mutants

![Diagram of PSA enhancer](attachment:image.png)
were similar to those observed in BHK cells, although the deleterious effects of individual mutations were more pronounced in LNCaP. The more severe effect in LNCaP might be attributed to the fact that the LNCaP line expresses physiological levels of AR, whereas the CMV-driven AR is likely to accumulate at high levels in BHK cells. Alternatively, the presence of prostate-specific factors may modulate the response to AR in LNCaP.

To verify further that AR binding to individual AREs is responsible for induction of the enhancer activity, we created three point mutations targeted to site V, a novel site identified in this study. We chose site V because its sequence matched the consensus more clearly than sites IIIA and IV, and site III has already been validated as an authentic ARE by point mutagenesis in other studies. Furthermore, AR apparently binds sites III and V in footprinting experiments with LNCaP extracts (data to be presented below). Among the three point mutants, mutants MV1 and MV3 were inactive for AR binding whereas mutant MV2 retained AR binding activity comparable with that of wild-type site V as determined by DNase I footprinting (Fig. 3A, compare lanes 3, 6, 9, and 12) and gel shift assays (data not shown). When tested for enhancer activity by transfection into LNCaP cells, the activity of these mutants correlated with their AR binding activity. As shown in Fig. 3B, the activity of MV1 and MV3 is significantly reduced while the activity of MV2 is comparable with that of the wild-type enhancer.

Since hormone-responsive elements are conserved among
different nuclear receptors, it is possible that the PSA enhancer activity might not be AR-specific. To address the receptor specificity issue, we tested the responsiveness of this enhancer to progesterone receptor (PR). We performed the experiment in BHK cells as the AR in LNCaP cells contains a mutation in AF-2 that renders it activable by numerous ligands. Such an effect would preclude us from measuring the specificity and contributions of PR to enhancer activity. Both AR and PR, when co-transfected with a reporter template containing four ARE sites upstream of the luciferase gene, were able to strongly induce activity in a ligand-dependent fashion (data not shown). However, co-transfection of AR but not PR had a significant effect on PSA enhancer activity (Fig. 4).

Synthesis and Purification of Intact AR—The tandem arrangement of low affinity AREs was reminiscent of many eukaryotic promoters where cooperative DNA binding by activators is an important mechanism for ensuring specificity in gene expression. The DNA binding experiments of Figs. 1–3 were performed with ARDBD which, we reasoned based on previous studies of GR and AR, could be missing domains necessary for cooperativity. Indeed Fig. 1B showed that ARDBD filled the enhancer sites at widely varying concentrations, whereas cooperative binding is generally characterized by simultaneous site occupancy.

To understand the binding profile of intact AR it was necessary to purify AR to homogeneity. Although AR had been purified previously from the insect baculovirus system (44), we chose to express and purify AR from mammalian cells in the event that AR was post-translationally modified in subtle ways that might influence its activity. AR had also previously been expressed in the mammalian vaccinia system (28). We initially tested this system but felt that the high level of overexpression and cytotoxicity of the virus might also preclude important modifications, and prevent AR from being used for certain applications.

We therefore chose to stably express AR in both HeLa cells, where AR has been shown to be active in co-transfection assays, and in LNCaP cells, where AR is normally expressed and is essential for normal metabolic growth. We employed an approach previously used in the purification of the thyroid receptor (45). We first generated a subclone of AR containing the 8-amino acid FLAG epitope at its amino terminus. The FLAG-tagged AR (fAR) was then introduced into an amphotropic retroviral vector pSRα, packaged into virus, and used to stably transform HeLa and LNCaP cells (40, 41).

Stable HeLa cell lines were selected by resistance to G418. Clones expressing fAR were identified by immunoblotting cell extracts against the FLAG epitope (Fig. 5A). Clone 2, which expressed the highest level of fAR, was chosen for further analysis and scale-up. First, we transfected a plasmid expressing fAR into BHK cells and showed that it stimulated PSA enhancer activity to the same extent as native AR (data not shown). Although this test confirmed the efficacy of fAR, we thought it was important to determine whether the stably expressed fAR was functional because we would be purifying it from the cell lines for biochemical analysis. To address this issue we transfected a luciferase reporter containing the entire PSA promoter/enhancer region, previously shown to be highly AR-responsive (3), into clone 2 HeLa cells. HeLa cells are an ideal assay system because they do not contain appreciable amounts of endogenous AR and therefore allow us to measure directly the effects of the fAR on transcription.

The bar graph of Fig. 5B demonstrates that the PSA transcriptional activity was 5-fold higher in the fAR-HeLa cells in the presence of 1 nM R1881 than in its absence. In contrast, R1881-activatable enhancer-mediated transcription was undetectable in the parental HeLa cell line. Although the absolute level of stimulation was 5-fold lower than that observed in LNCaP cells, this might be explained by the fact that fAR-HeLa cells do not contain prostate-specific factors that might augment enhancer activity. Nevertheless, the
data indicate that fAR is functional for transcriptional activation in vivo. We also compared the fAR levels in nuclear extracts prepared from clone 2 HeLa cells in the absence or presence of R1881 (Fig. 5C). As expected, R1881 was able to stimulate fAR nuclear translocation resulting in approximately 10-fold more fAR in extracts from R1881-treated clone 2 cells versus untreated cells.

To test further the in vivo function of LNCaP fAR, we performed an immunofluorescence assay using FLAG monoclonal antibodies. Fig. 6A shows that fAR localizes to the cytoplasm in steroid-depleted medium. However, after treatment with 1 nM R1881, the majority of fAR translocated into the nucleus. Because LNCaP cells contain endogenous AR, we wished to determine the ratio of fAR to the endogenous AR. We subjected similar amounts of nuclear extract from the parental and fAR-LNCaP cells to immunoblot analysis using antibodies against AR. There appeared to be approximately twice as much AR in fAR-LNCaP cells as in the parental cell line (Fig. 6B, upper panel, compare lane fLNCaP NE with lane LNCaP NE), indicating that the expression levels of fAR and endogenous AR are approximately equal. This result was further verified by subjecting the fAR-LNCaP nuclear extracts to immunoprecipitation with anti-FLAG antibody to remove the fAR. Only half of the total AR-specific signal was depleted under conditions where all of the fAR was removed (Fig. 6B, lower panel).

We next employed anti-FLAG immunoadfinity chromatography to purify fAR from the extracts. The major advantage of the FLAG approach versus standard immunoprecipitation is that the AR can be eluted from the resin with FLAG peptide for subsequent biochemical analyses. Fig. 7A shows a silver-stained gel of the purified FLAG-tagged AR from both fAR-LNCaP and fAR-HeLa cells (Fig. 7A, lanes f-LNCaP and f-HeLa). A prominent band at 110 kDa, the size predicted for AR, was observed. To ensure that the band was indeed fAR, we subjected extracts from the parental LNCaP and HeLa cell lines to a mock immunopurification (Fig. 7A, lanes LNCaP and HeLa). The 110-kDa band was not observed, and only a low background binding of contaminant proteins was evident on the gel. Furthermore, the highly pure fAR is immunoreactive to polyclonal AR antibodies in blotting analysis (data not shown).

Purified fAR was then subjected to DNA binding assays to examine its ability to bind the ARE sequence. The gel shift experiment of Fig. 7B shows that 10 ng of fAR, purified from nuclear extracts of fAR-HeLa and fAR-LNCaP lines treated with R1881, gives rise to specific gel shift complexes on a 32P-labeled consensus ARE oligonucleotide (lanes 5 and 6) but not on a mutant ARE containing a scrambled arrangement of bases in one of the dyad half-sites (lanes 17 and 18). Further addition of 100 nM R1881 had little effect on the binding activity either in this experiment (lanes 11 and 12) or in a parallel experiment, where more limiting amounts of AR were added (data not shown). Mock protein preparations from wild-type HeLa (HeLa NuC) and LNCaP (LNCaP NuC) nuclear extracts did not generate shifted complexes nor did protein prepared from cytoplasmic extracts of the FLAG-tagged cells (f-HeLa Cyt).

Cooperative Binding of fAR to the PSA Enhancer—In cases such as the mammalian interferon-β promoter (46), the yeast GAL4-controlled GAL1–10 promoters (47), and others, it has been found that small changes in the concentration of limiting activators can cause large changes in transcriptional output. Occupancy of those promoters by their respective activators occurs in a cooperative fashion. The cooperative occupancy is a result of protein-protein interactions between activators bound to adjacent sites. Cooperative binding of AR has previously been observed on the rat probasin and the mouse sex-limited regulatory regions (36, 37). These studies prompted us to investigate whether AR binds cooperatively to the sites within the PSA enhancer.

A 32P-labeled PCR fragment from −4309 to −4011 of the enhancer was subjected to DNase I footprinting studies. Increasing concentrations of ARDBD led to gradual protection of the AREs such that 75% occupancy of ARE III and ARE V occurred at 350 nM and 1.4 μM protein, respectively (Fig. 8, lanes 10–15). In contrast, LNCaP fAR occupied several of the sites simultaneously. A 2-fold increase in concentration from 7 to 14 nM led to 60% protection of AREs III, IV, and V (Fig. 8, lanes 4 and 5). ARE IIIA filled at the slightly higher concentration of 56 nM AR and coincided with greater than 80% occupancy of sites III, IV, and V. More remarkably, fAR protected not only AREs IIIA, III, IV, and V from DNase I cleavage but also the regions between the sites (Fig. 8, lane 6). The binding is specific because mutation of all four AREs abolished the entire DNase I footprint (Fig. 8, lanes 8 and 9). Furthermore, one striking similarity between the protection patterns of AR and ARDBD is a distinct DNase I-hypersensitive site upon protection of site V (indicated by an arrow in Fig. 8). This observation indicated that AR was binding in a manner similar to that of ARDBD. We also noticed a weak footprint below site V that we had not detected with ARDBD in earlier experiments. This footprint may represent binding of another molecule of AR, an issue we will return to below.

It has been reported that regions outside of the DNA binding domain of AR can contribute to its affinity, and possibly specificity, although the mechanism has not been determined (48). To eliminate the possibility that intact AR has different specificity requirements than the ARDBD alone, we compared by gel shift the affinities of fAR for AREs III, IV, and V. We found that although the affinity of ARDBD was >10-fold lower than that of AR, both proteins exhibited the same relative affinities for different individual sites (data not shown). Taken together, the data suggest that the ability of fAR to bind simultaneously to the AREs within the enhancer is an indication that AR binding is cooperative.

We further investigated how each individual ARE contributes to the recruitment of fAR using the enhancer mutants from Fig. 2. Fig. 9A is a DNase I footprint comparing binding of
ARDBD to individual mutants in AREs IIIA, III, IV, and V and a mutant removing all four sites (S-All). The individual mutants revealed a significant reduction of ARDBD binding only to the mutated site, whereas binding of ARDBD to other sites was unaffected. S-All, on the other hand, completely abolished binding to all four sites.

The binding profiles of fAR in Fig. 9B were far more complex. Each of the individual site mutants abolished tight binding to that site, although a weaker nonspecific protection over the mutated site was observed in some cases. For example, mutation of AREs III, IV, and V led to a decrease in protection of the site from 75 to 25%. Mutations of ARE IIIA or V had only a
small quantitative effect on binding to sites III and IV. The mutation to site V did, however, abolish the additional footprint below site V again arguing for it relevance as an AR-binding site. In contrast, mutations in ARE III and IV clearly diminished cooperative binding to all sites at lower concentrations of fAR (Fig. 9B, compare intensity of protection in lanes 8 and 9, 11 and 12 with lanes 2 and 3). The ARE IV result was surprising because individually it is a much weaker site for AR than ARE III. This observation suggests that the intrinsic affinity of individual AREs does not necessarily correlate with their role in cooperative binding. However, when all four sites were mutagenized, AR binding to the enhancer was largely abolished (Fig. 9B, lanes 17–18). The disappearance of the footprint in the S-All mutant suggests that the partial protection seen in the individual site mutants, even over the mutant sites themselves, may be due to AR binding nonspecifically through interactions with AR bound specifically at intact neighboring sites.

To demonstrate further the physiological relevance of AR binding to this region, we employed nuclear extracts prepared from LNCaP cells with or without androgen treatment to perform footprinting assays on the enhancer. As shown in Fig. 10, the footprint of LNCaP extracts treated with R1881 was remarkably similar in some respects to that obtained with intact fAR (Fig. 9B, compare lanes 2 and 3). The disappearance of the footprint in the S-All mutant suggests that the partial protection seen in the individual site mutants, even over the mutant sites themselves, may be due to AR binding nonspecifically through interactions with AR bound specifically at intact neighboring sites.

To demonstrate further the physiological relevance of AR binding to this region, we employed nuclear extracts prepared from LNCaP cells with or without androgen treatment to perform footprinting assays on the enhancer. As shown in Fig. 10, the footprint of LNCaP extracts treated with R1881 was remarkably similar in some respects to that obtained with intact fAR (Fig. 9B, compare lanes 2 and 3). The disappearance of the footprint in the S-All mutant suggests that the partial protection seen in the individual site mutants, even over the mutant sites themselves, may be due to AR binding nonspecifically through interactions with AR bound specifically at intact neighboring sites.

DISCUSSION

We have employed the PSA gene as a model to understand how AR binds to and activates transcription of a natural target gene expressed during prostate cancer. Although previous studies had revealed the existence of two ARE sites within the proximal PSA promoter, the effect of the proximal promoter alone on the activity and tissue specificity of PSA gene expression is minor (3, 6, 7, 15). More recent studies led to the view that the major control region is an enhancer centered at 4.2 kb upstream of the PSA gene. Deletion mutagenesis around this region revealed that the androgen responsiveness of the enhancer is conferred by a 455-bp fragment bearing a single, moderate affinity ARE referred to as ARE III (centered at −4143). We cloned this region upstream of a reporter gene and studied its response to AR. Our DNase I footprinting results, using both AR DNA binding domain and full-length AR, revealed that there are multiple AREs with varying but low affinities when compared with the consensus ARE. These sites were termed ARE IIIB, IIIA, III, IV, V, and VI and were
because mutagenesis of the four main AREs abolishes bothomerization, however, is dependent upon the identified AREs stable androgen-responsive nucleoprotein complex. This oli-
specifically and nonspecifically along the DNA to provide a
that multiple AR molecules are cooperatively binding both
age between the sites. The mutagenesis data support the idea
ARDBD might simply lead to steric inhibition of DNase I cleav-
tions of AR with the intervening DNA, again mediated by
explanations including the following: (i) the existence of addi-
ty, and further work will be required to identify the proteins
mutation of many regions of the enhancer decreases its activ-
(data not shown). It is clear from our study and others that
ARE IIIB and VI also decreases transcription in LNCaP cells
fAR binds cooperatively to these sites in vitro. We have not
roles for the other two sites because mutagenesis of ARE IIIB and VI also decreases transcription in LNCaP cells (data not shown). It is clear from our study and others that
mutation of many regions of the enhancer decreases its activ-
ity, and further work will be required to identify the proteins
that bind these regions.

The large difference in the size of the protected region by AR
versus ARDBD was surprising. This effect could have multiple
explanations including the following: (i) the existence of addi-
tional AREs that bind cooperatively to intact AR (i.e., such as
the weakly protected site below site V); (ii) nonspecific interac-
tions of AR with the intervening DNA, again mediated by
cooperative interactions; (iii) finally, either a general distortion
of the enhancer fragment or the larger mass of AR versus
ARDBD might simply lead to steric inhibition of DNase I cleav-
age between the sites. The mutagenesis data support the idea
that multiple AR molecules are cooperatively binding both
specifically and nonspecifically along the DNA to provide a
stable androgen-responsive nucleoprotein complex. This oli-
gomerization, however, is dependent upon the identified AREs
because mutagenesis of the four main AREs abolishes both

Cooperative binding of activators to multiple, adjacent low
affinity sites and the resulting synergistic effects on gene ex-
pression are a common mechanism for ensuring specificity in
the transcriptional response (38). In the case of the PSA en-
hancer the low affinity AREs may ensure that binding occurs
only at a higher concentration of AR. In situ hybridization
studies on rat tissues, for example, have shown that prostatic
secretory epithelial cells are among two or three cell types
expressing the highest levels of AR in the body (23). The com-
bination of the low affinity AREs and the high concentrations
of AR may therefore provide a partial explanation for the cell type
specificity of PSA expression.

The presence of multiple AREs also augments the androgen
responsiveness of the enhancer through synergy. Synergy is
defined as the greater-than-additive transcriptional response
to increasing numbers of bound activators. The current view is
that synergy results from simultaneous interaction of multiple
activators with the transcriptional machinery. These interac-
tions lead to cooperative recruitment of the general machinery
and the ensuing synergistic transcriptional response. In the
case of the PSA enhancer, we imagine that simultaneous bind-
ing of multiple AR molecules is in part responsible for the
synergistic androgen-dependent effect on PSA gene expression
(8). This effect, when superimposed with a parallel synergistic
effect of AR on AREs I and II within the proximal promoter (13)
would, in principle, generate a sensitive and potent response of
the gene to androgens.

The use of nonconsensus sites to regulate gene expression
has been widely observed in nature. The consensus is an arti-
ficial amalgam of sites from various steroid receptor-responsive
elements and, as such, binds and mediates response to several
related steroid receptors (i.e., AR, GR, mineral corricoid recep-
tor, and PR). The specificity for a particular receptor is likely
conferred by contextual interactions between a single site or
combinations of sites and the receptor. Results shown in Fig. 4
support this notion. In line with previous observations that AR
and PR share near identical consensus hormone response ele-
ments, we demonstrated that both AR and PR were able to
efficiently induce the activity of a construct containing four
consensus AREs. However, when tested on a construct contain-
ning the PSA enhancer, only AR was able to stimulate the
activity. This leads us to conclude that nonconsensus sequences
may play a pivotal role in conferring receptor specificity. Stud-
ies on GR, for example, have also established that the DNA
sequence of the site influences nuclear receptor activity, possi-
bly through conformational changes in the ligand binding do-
main (49, 50). Furthermore, natural low affinity sites that bind
AR are commonplace. In one case, the ARE in the rat probasin
gene was shown to display an inherent preference for AR over
GR suggesting that nonconsensus changes in DNA sequence
may represent a basic mechanism for conferring receptor spec-
ificity (35).

Contextual cooperative interactions involving interplay be-
tween multiple AREs and domains of AR also regulate speci-
ficity. In an elegant study that emphasized the complex nature
of receptor DNA binding specificity, Robins and colleagues(36)
studied the DNA binding properties of domain-swap chimeras
of AR and GR. The target promoters included both a standard
array of consensus steroid (hormone) response elements and
the natural AR-responsive enhancer of the sex-limited protein
(slp) gene of mice. Whereas the different chimeras bound to
and functioned from standard steroid response elements (36), AR
bound tightly and cooperatively to the nonconsensus slp en-
hancer AREs. The most efficient cooperativity required the AR
DNA binding domain and its natural ligand and amino-termi-
nal domains. The ligand and amino-terminal domains could not be substituted by the analogous domains of GR, which appeared to, in fact, suppress specific enhancer binding. The study implied that contextual AR-AR and AR-DNA interactions occurring within the nucleoprotein complex on the slp enhancer are the key determinants of specificity. Such contextual protein-protein interactions may also explain the inability of PR to activate the PSA enhancer.

Although contextual, cooperative AR-AR interactions are likely to contribute to the specificity of PSA gene expression, other mechanisms must exist to ensure an accurate, cell-specific transcriptional response. Indeed, DNase I footprinting studies have identified binding sites for prostate-specific and ubiquitous transcription factors within the PSA enhancer and promoter (7, 9, 12). Henderson and colleagues (7) used gel shift analysis to identify several complexes, which were specific to extracts prepared from LNCaP cells. It was speculated that some of these complexes located between −4150 and −4000 were due to binding of AR, a finding supported by our data since this region contains site IIIA. However, other complexes

Fig. 9. Binding of fAR to PSA enhancer mutants. A, binding of ARDBD to the mutant enhancers. 100 ng of ARDBD was used to confirm replacement mutations on various ARE sites. Sites that are mutated are indicated by S for substitution. Wild-type is included as a control. B, binding of intact AR to the mutant enhancers. 40 and 80 ng of fAR were incubated with different enhancer mutants in binding reactions. Individual ARE sites are identified to the right. Mutant sites are denoted by S. The DNase I-hypersensitive site on ARE V is indicated by an arrow.
within the enhancer appeared unique and may represent binding of prostate-specific factors. Indeed, Farmer and Freedman\(^2\) recently identified a prostate-specific factor, which appears to bind adjacent to our ARE IV. Our footprint assay using crude LNCaP nuclear extracts also demonstrates that in addition to regions around ARE IV, other regions indicated by asterisks in Fig. 10 are protected from both R1881-treated and untreated cells. Our data combined with others strongly suggest that additional factors exist to modulate the enhancer activity. These factors may function by interacting with AR in the context of the nucleoprotein complex, an issue we are currently investigating.

Mechanistic studies on nuclear receptors suggest that they stimulate transcription by direct or indirect communication with the general factors (51). Direct interactions between the general factors and several nuclear receptors including AR have been detected biochemically (52–55). In addition to general factor interactions the current view is that the receptor also interacts with ancillary factors called co-activators to increase the recruitment of the transcriptional machinery. For example, CBP/p300 has been found to support activation by many nuclear receptors including AR (56). In addition, co-activators for individual nuclear receptors have been isolated using both biochemical assays and yeast two-hybrid screens. Examples include the TRAPs for the thyroid hormone receptor (45), ARA70 for the androgen receptor (57), SRC-1/p160 (58) and RIP 140 (59) for the progesterone, estrogen, and other steroid receptors, and GRIP1 for the glucocorticoid and androgen receptors (18). The yeast two-hybrid systems have identified individual interacting molecules, whereas biochemical studies have shown that several co-activators may simultaneously associate with a receptor to form a large multiprotein complex (56). For example, the thyroid receptor-associated proteins or TRAPs contain at least 9 polypeptides, which form a transcriptionally active complex with thyroid hormone receptor and play a role as positive co-activators in vitro (45). Vitamin D receptor has also recently been shown to interact with multiple polypeptides, the DRIPs, which are very similar in mass and may be identical to the TRAPs (60). Other systems have revealed that co-activators may also be gene- and site-specific (61, 62). It is therefore likely that co-activators will play a role in gene- and tissue-specific transcription by AR. The ability to isolate and study AR from natural sources should now permit us to examine whether AR is naturally associated with many of the co-activators identified in two-hybrid screens and other analyses.

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