Regions of Prostate-specific Antigen (PSA) Promoter Confer Androgen-independent Expression of PSA in Prostate Cancer Cells*

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Prostate-specific antigen (PSA) is expressed primarily by both normal prostate epithelium and the vast majority of prostate cancers. Increases in serum PSA during endocrine therapy are generally considered as evidence for prostate cancer recurrence or progression to androgen independence. The mechanisms by which PSA up-regulation occurs in androgen-refractory prostate cancer cells are unknown. In this study, by using LNCaP and its lineage-derived androgen-independent PSA-producing subline, C4-2, we identified two cis-elements within the 5.8-kilobase pair PSA promoter that are essential for the androgen-independent activity of PSA promoter in prostate cancer cells. First, a previously reported 440-bp androgen-responsive element enhancer core (AREc) was found to be important for the high basal PSA promoter activity in C4-2 cells. Both mutation analysis and supershift experiments demonstrated that androgen receptor (AR) binds to the AREs within the AREc and activates the basal PSA promoter activity in C4-2 cells under androgen-deprived conditions. Second, a 150-bp pN/H region was demonstrated to be a strong AR-independent positive-regulatory element of the PSA promoter in both LNCaP and C4-2 cells. Through DNase I footprinting and linker scan mutagenesis, a 17-bp RI site was identified as the key cis-element within the pN/H region. Data from electrophoretic mobility shift analysis and UV cross-linking experiments further indicated that a 45-kDa (p45) cell-specific transcription factor associates with RI in prostate cancer cells and may be responsible for driving the PSA promoter activity independent of androgen and AR. Furthermore, by juxtaposing AREc and pN/H, we produced a chimeric PSA promoter (supra-PSA) that exhibits 2–3-fold higher activity than the wild type PSA promoter in both LNCaP and C4-2 cells.

Prostate-specific antigen (PSA) is a chymotrypsin-like serine protease synthesized primarily by normal, hyperplastic, and malignant prostate (1–3). PSA expression is tightly regulated by androgen through the action of androgen receptor (AR) (2, 4, 5). Upon binding to androgen, AR translocates into the nucleus and binds to the androgen response elements (AREs) on the PSA promoter, where it interacts with other transcription factors and activates PSA gene transcription. Cleutjens et al. (6, 7) have identified three AREs within the 5.8-kb PSA promoter: ARE-I and -II are located within the proximal region of the promoter, whereas ARE-III is contained within a 440-bp strong enhancer element core (AREc) located at ~4.2 kb of the promoter (7–9). Recently, additional non-consensus AREs have been identified within the AREc enhancer element. This study proposes that androgen regulation of the AREc in prostate cells might involve the formation of AR and prostate-specific factor nucleoprotein complexes on the multiple non-consensus AREs in the enhancer region (10).

PSA is a serum marker for prostate cancer. It has been shown that serum PSA is proportional to tumor volume and correlates positively with the clinical stage of the disease (11). Progression of prostate cancer to androgen independence is commonly associated with a rebound of serum PSA (12). PSA elevation in hormone-refractory prostate tumors has been attributed to: 1) mutations and/or amplifications of AR (13–18) that broaden its ligand specificity and/or enhance tumor cells’ responsiveness to androgen, respectively (19); 2) androgen-independent (AI) activation of the AR by growth factor signaling pathways like insulin-like growth factor-1 and keratinocyte growth factor, which would elicit AR-mediated transcriptional activation (20, 21). It has also been demonstrated that AR could “cross-talk” with protein kinase A (PKA) and/or protein kinase C signaling pathways (22–25), and/or the direct stimulatory action by soluble prostate-specific autocrine factor(s) (PSAF) secreted by hormone-refractory prostate cancer cells (26).

To understand the molecular pathways that may regulate PSA expression by androgen in normal prostate epithelium, and dysregulation during AI progression, much effort has focused on delineating the activities of AR in mediating androgen regulation of PSA expression in prostate cancer cells (2, 6–8, 27, 28). For the limited availability of AI yet PSA-producing prostate cancer cell lines, little is known about the androgen-independent regulation of PSA expression in hormone-refractory prostate cancer cells. The development of the androgen-independent PSA-producing C4-2 cell line from androgen-dependent parental LNCaP cells (29) has allowed us to study how PSA expression is regulated in an androgen- and growth factor-deprived environment. Like hormone-refractory prostate cancer, C4-2 was shown to be androgen-independent, derepress chain reaction; CMV, cytomegalovirus; PKA, cAMP-dependent protein kinase; RLA, relative luciferase activity.
were obtained from Dr. Jan Trapman (6, 7). The p61/pGL3 and the AREIII used in this study were generated by inserting a HindIII fragment of the p61-Luc and ARE-III-Luc, respectively, in the multiple cloning site of pGL3-basic vector (Promega). Digesting p61/pGL3 with BglII and BamHI generated p61-1. Construct p61/pGL3 containing the ARE-I was generated by digesting the p61/pGL3 with BstEII and XhoI. p61-4 was constructed by digesting p61/pGL3 with PacI and XhoI. The 440-bp AREc was amplified first by PCR using sequence-specific primers at both ends followed by TA cloning into a PCR vector (Invitrogen, Carlsbad, CA). AREc was then cut out with PacI/XhoI and subcloned into a pGL3/TATA basic vector by digesting the p61/pGL3 with EcoRI and KpnI. p61/pGL3 with NheI construct were generated by digesting the p61/pGL3 with PacI and NheI. p61/P2 is created by ligating NheI-digested p61/pGL3 and P2. P2/TATA is generated by ligating annealed oligos (5'-ctagtcgacagcagcagggg-3' and 5'-ctagtcgacgtcgtgca-ga-3') to the KpnI- and NheI-digested pGL3/TATA.

**Linker-scanning Mutagenesis**—Both L4 and A3a were generated by replacing the respective AREs with the GAL4 sequence (egggatgactcAGT) and GLprimer2 (ctttatgtttttggcgtcttcc) of the pGL3-basic vector. All clones were confirmed by DNA sequence.

**Cell Culture and Transfection—**LNCaP and C4-2 were cultured in T-medium (33) supplemented with 5% fetal bovine serum. For transfection, cells were grown in phenol red-free and serum-free RPMI 1640 (Life Technologies, Inc.). LNCaP and C4-2 were plated at 3.5 × 10⁵ cells/well in six-well plates 2 days before transfection. Plasmid DNAs were transfected into cells by complexing with DOTAP (Roche Molecular Biochemicals). Briefly, 2.5 μg of the tested DNA constructs and 0.5 μg of the internal control CMV/β-galactosidase DNA were mixed with 27 μl of 20 mM HEPES (pH 7.4) and added to separate tube containing 8 μl of DOTAP in 16 μl of HEPES with gentle mixing. DNA-lipid complexes were allowed to form for 15 min at room temperature prior to their addition to each well containing 1 ml of serum-free and phenol red-free RPMI 1640 medium. The cells were incubated with the complexes at 37 °C for 15–30 min. The β-galactosidase activity was determined by plate reader at 405 nm wavelength. Data are expressed as relative luciferase activity (RLA), which is defined as luciferase activity normalizing to internal control CMV/β-galactosidase activity for transfection efficiency. RLA is expressed as the mean ± standard error of the mean of at least three independent experiments.

**DNase I Protection Assay—**Bromodeoxyuridine-substituted probe was prepared according to Current Protocols (36). Briefly, 100,000 cpm of labeled probe and 5–10 μg of nuclear extracts were incubated with 20 μl of buffer containing 12.5 mM HEPES, pH 7.9, 12.5% glycerol, 5 mM MgCl₂, 70 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 1 μM of poly(dI-dC) (Amersham Pharmacia Biotech), and 1 mM dNTP at 30 °C for 30 min. The samples were subjected to electrophoresis at room temperature on a 4% nondenaturing polyacrylamide gel in 0.5% TBE at 35 mA for 2 h. For experiments using AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 4 μg of antibody was added to the reaction mixture for 30 min before addition of the probe and nuclear extracts were incubated for experiments using AR antibody (CW2), nuclear extract and 2 μg of antibody were pre-incubated at 37 °C for 1 h before the addition of probe. In competition experiments, competitor oligos were incubated with nuclear extracts for 30 min at 30 °C before the addition of the probe.

**Western Blot and PSA Immunoassay—**Immunoblotting was performed as described in Sambrook et al. (34). Briefly, proteins were separated on a 7.5% SDS-polyacrylamide gel and transferred to a 0.42-μm nitrocellulose membrane. Nonspecific binding was blocked with 5% nonfat milk in phosphate-buffered saline for 30 min. Antibody (PG-21-21A) against the amino terminus of AR was used as the primary antibody. Secondary antibody was constant for the anti-AR IgG (horse-radish peroxidase anti-rabbit antibody) (Amersham Pharmacia Biotech). PSA proteins were detected by the IMX (Abbott Laboratory, Chicago, IL) immunoassay method as described previously (35).

**Construction of Plasmids—**All plasmid constructs were prepared using standard methods (34). The original p61-Luc and ARE-III-Luc were fine mixed with 100 m Spars, MD). For luminometer (Monolight 2010, Analytical Luminescence Laboratory, with fresh serum-free and phenol red-free RPMI 1640 medium. Cells were allowed to form for 15 min at room temperature prior to their addition to each well containing 1 ml of serum-free and phenol red-free RPMI 1640 medium. The cells were incubated with the complexes at 5% CO₂, 37 °C for 5 h. DNA-lipid complexes were allowed to form for 15 min at room temperature prior to their addition to each well containing 1 ml of serum-free and phenol red-free RPMI 1640 medium. Cells were collected after 36–48 h of additional incubation. All the transfections were carried out in the above serum-free conditions, unless specified otherwise.

**Luciferase Assay—**Cells were washed with 1 ml of phosphate-buffered saline/well and lysed in 300 μl of 1× lysis buffer (Promega, Madison, WI). Cell lysates were vortexed for a few seconds and spun for 3 min. For luciferase activity detection, 20 μl of the supernatant was mixed with 100 μl of luciferase substrate (Promega) and measured by a luminometer (Monolight 100, Analytical Luminescence Laboratory, Sparks, MD). For β-galactosidase activity detection, 100 μl of the supernatant was mixed with an equal volume of 2% β-galactosidase substrate (Promega) and incubated at 37 °C for 15–30 min. The β-galactosidase activity was determined by plate reader at 405 nm wavelength. Data are expressed as relative luciferase activity (RLA), which is defined as luciferase activity normalized to internal control CMV/β-galactosidase activity for transfection efficiency. RLA is expressed as the mean ± standard error of the mean of at least three independent experiments.

**Bromodeoxyuridine-substituted probe was prepared according to Current Protocols (36).** The binding reactions for DNase I footprinting were described previously (37, 38). 20,000 cpm of labeled probe and 10 μg of nuclear extracts from LNCaP or C4-2 cells were incubated in 13 μl of buffer containing 12.5 mM HEPES, pH 7.9, 12.5% glycerol, 5 mM MgCl₂, 70 mM KCl, 0.2 mM EDTA, 0.5 mM mercaptoethanol, 0.5 mg/ml bovine serum albumin, and 200 ng of poly(dI-dC). After 30 min at 30 °C, 2 μl of DNase I (Promega) was added to the reactions. The cleavage reactions were terminated after 1 min by addition of 100 μl of stop buffer containing 400 μg sodium acetate (pH 5.2), 0.2% SDS, 10 mM EDTA, 50 μg/ml yeast RNA, and 100 μg/ml proteinase K (Roche Molecular Biochemicals). The mixtures were incubated at 50 °C for 15 min, extracted with phenol/chloroform, and precipitated with ethanol. Precipitates were dissolved in formamide loading buffer (Ambion, Austin, TX) and analyzed on 6% polyacrylamide gels.

**UV Cross-linking—**Bromodeoxyuridine-substituted probe was prepared according to Current Protocols (36). Briefly, a 50-bp synthetic oligo (containing the RI site) was annealed to a complementary 15-bp synthetic oligo and filled in with Klenow fragment (New England Biolabs, Beverly MA). Nuclear extracts were prepared from cells growing under 3-day complete serum-free condition (36). 100,000 cpm of labeled probe and 5–10 μg of nuclear extracts were incubated with binding buffer containing 10 mM Tris-Base (pH 7.5), 50 mM NaCl, 0.5 mM EDTA, 0.5 mM dithiothreitol, 4% glycerol, 1 μM of poly(dI-dC) (Amersham Pharmacia Biotech), and 1 mM KCl at 30 °C for 30 min. The samples were subjected to electrophoresis at room temperature on a 4% nondenaturing polyacrylamide gel in 0.5% TBE at 35 mA for 2 h. For experiments using AR antibody (Santa Cruz Biotechnology, Santa Cruz, CA), 4 μg of antibody was added to the reaction mixture for 30 min before addition of the probe and nuclear extracts were incubated for experiments using AR antibody (CW2), nuclear extract and 2 μg of antibody were pre-incubated at 37 °C for 1 h before the addition of probe. In competition experiments, competitor oligos were incubated with nuclear extracts for 30 min at 30 °C before the addition of the probe.

**Androgen-independent Regulation of PSA Promoter**

40847
and 2 µg of poly(dI-dC). The reaction was then incubated at 30 °C for 30 min, followed by UV irradiation (320–350 nm) at 4 °C for 30 min. The samples were subjected to electrophoresis on a 4–12% SDS-gradient gel (Invitrogen) at room temperature for 1 h, fixed with 30% methanol, 3% glycerol solution for 20 min, and then dried. For competition experiments, 400 ng of the competitor was incubated with nuclear extracts for 30 min at 30 °C prior to the addition of probe.

RESULTS

Up-regulation of PSA Gene Expression in an Androgen-independent Prostate Cancer Cell Line, C4-2—

C4-2, a lineage-derived LNCaP subline, was shown previously to be able to grow in castrated hosts and exhibit metastatic potential in vivo (30–32). This cell line synthesizes and secretes a higher basal level of PSA than LNCaP cells in the absence of androgen stimulus in vitro (32). This unique feature of C4-2 cells provides an opportunity to study PSA promoter regulation in prostate cancer cells with characteristics mimicking hormone-refractory status. To compare PSA secretion between parental LNCaP and its C4-2 subline in vitro, cells were first cultured in T-medium supplemented with 5% fetal bovine serum and switched to serum-free and phenol red-free RPMI 1640 medium when they reached 80% confluence. After a 3-day incubation period, cells were counted and medium was collected for immunoassay of the PSA protein. C4-2 cells consistently secreted 4–5-fold (14.9 ± 1.12 versus 2.89 ± 0.22 ng/ml/10^6 cells) more PSA protein than LNCaP cells into the medium in the complete absence of exogenous androgen and growth factor stimulus over a 3-day period. To investigate whether the up-regulation of PSA protein in C4-2 cells occurs at the transcriptional level, a 5.8-kb PSA promoter was inserted upstream to a luciferase reporter gene (p61/pGL3) and transfected into LNCaP and C4-2 cells for transient expression analysis. In parallel with PSA secretion results, the basal PSA promoter activity in C4-2 is 18 ± 3.7-fold higher than that observed in LNCaP cells. Thus, up-regulation of PSA protein expression in C4-2 is due to a high intrinsic basal PSA promoter activity.

Since the PSA promoter contains multiple AREs, the up-regulation of PSA promoter activity in C4-2 may be explained by an elevated AR protein level in this AI cell line. A Western blot analysis of AR was performed with the total cell lysate of LNCaP and C4-2 (Fig. 1). Consistent with our previously published results (32), AR protein was found to be expressed by these two cell lines at comparable levels. Moreover, we have sequenced both the ligand-binding domain and the DNA-binding domain of AR from LNCaP and C4-2 cell lines and have identified the reported single point mutation in the ligand-binding domain (39) in both of these cell lines without additional mutations.2 Comparison of AR binding affinity and ca-

2 H. Yang, H. Y. E. Zhau, and L. W. K. Chung, unpublished results

Fig. 1. Western blot analysis demonstrated equal expression of AR protein level in C4-2 and LNCaP cells. Cells were grown in complete serum-free RPMI 1640 for 3 days before collection. Two concentrations of the whole cell lysate (10 and 5 µg) were used to compare the AR protein levels in LNCaP and C4-2 cells.

Fig. 2. Deletion analysis of the PSA promoter. A. AREc is essential for the androgen-independent activity of PSA promoter in C4-2 cells. The hatched box represents the 440-bp AREc. Experimental details of the transfections are described under “Materials and Methods.” The activity of the 5.8-kb PSA promoter (p61/pGL3) construct is set at 100% in both LNCaP and C4-2 cells. Relative activities of various constructs to the p61/pGL3 are presented as a bar graph. The luciferase activity of p61/pGL3 in LNCaP and C4-2 were approximately 3,700 and 64,000 light units, respectively. B. ARc exhibits higher activity in C4-2 than in LNCaP cells. The 440-bp ARc was inserted upstream of an artificial TATA box (AREc/TATA). The RLA of AREc/TATA of each cell lines has been corrected to the basal activity of the vector backbone (pGL3/TATA). The RLA of AREc/TATA in LNCaP was set to be 100%; its luciferase activity was approximately 1022 light units after correction.
pacity between LNCaP and C4-2 cells revealed that both of these cell lines contain high affinity and capacity AR (32). Thus, neither the steady-state level of AR expression nor additional AR mutations play a role in the up-regulation of the PSA promoter activity in C4-2 cells.

Identification of AREc as an Essential Element for the Basal PSA Promoter Activity in C4-2 Cells—Deletion analysis was performed to dissect out the cis-element(s) essential for conferring high PSA promoter activity in C4-2 cells under androgen-deprived conditions. Various deletion constructs were generated by restriction enzyme digestion (Fig. 2A). The P61-2 construct containing a deletion between ARE-II and ARE-III retains all of the wild-type promoter activity. In contrast, a 1.5-kb terminal deletion immediately proximal to AREc (p61-5) caused a 50% drop in promoter activity only in C4-2, and not in LNCaP cells (Fig. 2A). It is possible that the deletion has partially destroyed the 5’ end of the AREc element resulting in a sharp drop of PSA promoter activity in C4-2 cells. A further decrease of promoter activity was observed in C4-2 cells when the terminal deletion extended downstream to include the AREc (p61-4). The 500-bp AREc region was then removed in p61-3 to demonstrate the effect of AREc deletion in PSA promoter activity. As indicated in Fig. 2A, p61-3 has a similar activity to p61-4. The AREc deletion reduced the promoter activity to 14% of the 5.8-kb promoter in C4-2 cells, but again without appreciably affecting the basal PSA promoter activity in LNCaP cells. Hence, AREc is a crucial cis-element for maintaining the androgen-independent PSA promoter activity in C4-2 cells.

AREc was shown to be important for androgen induction of PSA promoter activity in LNCaP cells (7, 8). However, AREc does not seem to have an important role in regulating the PSA basal promoter activity in LNCaP cells (Fig. 2A). On the contrary AREc is indispensable for the maintenance of high basal PSA promoter activity in LNCaP cells (Fig. 2A). The differential activity of AREc between the two cell lines was demonstrated by inserting AREc upstream to an artificial TATA box (AREc/TATA). We chose a simple TATA box promoter because transfection experiments indicated that this promoter alone did not respond to AR or androgen in the absence of AREs in prostate cancer cells. Consistent with the above observations, the AREc element has about 10 times higher activity in C4-2 than LN-
CaP cells (Fig. 2B). The 440-bp AREc was shown to be a strong tissue-specific enhancer element that exhibits high androgen responsiveness only in PSA-positive cells. In addition to containing multiple AREs, the AREc potentially contains other prostate-specific regulatory elements (7, 8). It has been hypothesized that liganded AR binds co-operatively to the cluster of non-consensus AREs in the enhancer core, where it assembles into a nucleoprotein complex with prostate-specific factor(s).

Fig. 4. The activity of a short proximal PSA promoter in prostate cancer cells. A, a 150-bp pN/H element confers high activity in LNCaP and C4-2 cells. The activities of several p61/pGL3 deletion constructs were shown here as relative activities to the wild-type p61/pGL3. The p61/pGL3 activity in LNCaP was set to be 100%. B, pN/H activity is independent of androgen. 1 nM R1881 (+ group) was added to the transfected cells for 36 h. The basal activity of pN/H was set to be 100% in LNCaP with approximately 4500–5500 RLA. C, AREc and pN/H could co-operate synergistically in activating PSA promoter in prostate cancer cells. In sPSA, the 440-bp AREc was inserted directly upstream to the pN/H region of pN/H construct. Experiments were carried out as described under "Materials and Methods."
and acts synergistically to activate PSA enhancer activity in PSA-producing prostate cancer cells (10, 40).

**Functional AREs Are Required for the High Basal AREc Activity in C4-2 Cells—** To address the role of AREs in the differential regulation of AREc/TATA activity in LNCaP and C4-2 cells, two ARE mutants were generated by replacing specific regions of ARE in the AREc enhancer core with a 17-bp GAL4 sequence. Mutant L4/TATA has the ARE-III sequence replaced, while mutant A3a/TATA has the A3a sequence (38) replaced. Fig. 3A demonstrates that mutation of ARE-III caused a 70% decrease in the basal AREc/TATA activity, while mutation of A3a resulted in an 85% decrease in the basal AREc/TATA activity in C4-2 cells. None of the AREs mutations appreciably affect the basal AREc/TATA activity in LNCaP cells. These results were further confirmed when multiple point mutations at ARE-III in p61/pGL3 (AREmIII) mimicked the effect of ARE deletion (p61-3) on the basal PSA promoter activity in C4-2 cells (Fig. 3B). Therefore, we concluded that the AREs within the enhancer core are essential for its activity in C4-2 cells.

We employed two approaches to investigate whether AR is involved in activating the AREc in C4-2 by binding to the

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**Location of P2 site.**

AGCATCCCGGAGGCTTGGTCAGCCTCTGGTGCCAGCAGGGCAGGGCAGGTCTTG
GGGAATGAGGTTTATA GGGCT

**Fig. 5. Identification of the RI element.** A, P2 mutation abolished most of the pN/H and p61/pGL3 activity in both LNCaP and C4-2. The P2 site mutation in P2 and p61/P2 constructs is depicted as a filled box in the figures, and it is underlined in the pN/H sequence. B, P2 element exhibits specific and differential activity between LNCaP and C4-2 cells. The activity of P2/TATA was set to 100% in LNCaP, and the RLU of each cell line was corrected with the vector (pGL3/TATA) RLU. The RLU of PC3 is ~1100 light units after correction. C, DNase I protection of pN/H demonstrated two footprint regions. Experimental details are listed under "Materials and Methods." The pN/H fragment that used in the experiment contains −155 to −30 bp. The RI and RII regions are underlined, and the P2 element is overlined. The core sequence is boldface. C4-2 nuclear extracts were used in the competition experiments.
AREs. First, anti-androgen Casodex (23) was used to block the AR transcriptional activity in C4-2 cells. Casodex was able to bring AREc/TATA activity down to 11.78 ± 1.75% of the control level. Second, by using AR antibody in EMSA, we demonstrated that AR indeed is the transcription factor in C4-2 cells that binds to ARE-III and A3a sites. When radiolabeled ARE-III and A3a were used to perform EMSA with C4-2 nuclear extracts (Fig. 3C), DNA-protein complexes were observed that could be supershifted by anti-AR antibody (CW2) into two higher molecular weight species. Although two bands were observed with ARE-III oligo, the bottom band appears to be nonspecific because it also diminished with control SP-3 antibody. Together these results showed that the AR in C4-2 cells is highly active and the co-operative binding of AR to the multiple AREs on the enhancer core allows the core to be transcriptionally active even in the absence of androgen.

The PSA Proximal Promoter Contains a 150-bp Positive Regulatory Element—The fact that p61-3 has substantial activity in C4-2 cells and still displays differential activity between LNCaP and C4-2 cells (Fig. 4A) implies that AREc is not the only cis-element that supports the AI PSA promoter activity in C4-2 cells. Detailed analysis of the deletion construct data led us to look for additional positive regulatory element(s) within the 600-bp proximal PSA promoter region. To test this possibility, we first compared the activity of the short PSA promoter, pPA8 (6), which contains the 632-bp proximal PSA promoter region including ARE-I and ARE-II, between LNCaP and C4-2 cells. As shown in Fig. 4A, pPA8 exhibits 6-fold higher basal activity in C4-2 than in LNCaP cells. Through terminally deleting the pPA8 until 150-bp upstream of the TATA box (just beyond the ARE-I), pNH was generated. In comparison to the full-length PSA promoter (p61/pGL3), pNH was able to retain a substantial basal PSA promoter activity in C4-2 cells (~40% that of the full-length PSA promoter). pNH construct also demonstrated differential activity (Fig. 4B) between LNCaP and C4-2 cells (~5-fold higher activity in C4-2 than LNCaP cells). These results strongly suggest that like AREc, pNH is a positive regulatory cis-element that contributes to the AI PSA promoter activity in C4-2 cells. In contrast to AREc, the activity of pNH is not regulated by androgen, for the addition of R1881 did not further enhance pNH activity in either LNCaP or C4-2 cell lines (Fig. 4B). By juxtaposing these two positive-regulatory elements together, we not only reconstituted the activity of the full-length promoter, we have created a 590-bp sPSA promoter that exhibits 2–3-fold higher activity than the wild-type promoter (Fig. 4C). The superior activity of sPSA implies that AREc could co-operate with pNH in maintaining the high steady state basal PSA promoter activity in C4-2 cells.

The Identification of RI Site—To locate the crucial cis-element within the pNH contributing to its high basal activity in C4-2 cells, we first performed linker-scanning mutagenesis. Briefly, a 17-bp fragment in the pNH was replaced systematically with a GAL4 binding site, a panel of eight mutant constructs (P1–P8) were generated, with P1 being the closest to the TATA box and P8 being the most upstream to the TATA box. Among the eight mutant constructs, only P1 and P2 showed significant activity decrease in LNCaP and C4-2 cells. P1 has about 50% of the wild type pNH activity (data not shown), while P2 has only 2–7% of the pNH activity (Fig. 5A). In addition, when a single copy of the P2 element was inserted upstream to a simple TATA box (P2/TATA), it was able to exhibit significant activity in both LNCaP and C4-2 cells, with 5–6-fold higher activity in C4-2 than in LNCaP cells (Fig. 5B). However, P2/TATA showed no activity in the PSA-negative prostate cancer cell line (PC3) when compared with the PSA-positive LNCaP and C4-2 cells (Fig. 5B). These results indicated that P2 element is able to exhibit high activity only in PSA-positive prostate cancer cells. Therefore, we hypothesized that P2 element is associated with a cell-specific transcription factor.

In order to precisely map the binding site of this cell-specific transcription factor, we then performed DNase I footprinting assay with the pNH fragment. In Fig. 5C, two regions (RI and RII) are distinctly protected by a protein factor present in LNCaP (lane 3) and C4-2 (lane 2), but not in PC3 (lane 1) nuclear extracts. The specificity of the protection was confirmed by successful competition with cold P2 element (lane 7), whereas other nonspecific DNA (lanes 4–6) was not able to compete away the protections observed. RI region coincides with the P2 element over a 11-bp sequence (agcagggcagg), so we have identified a core sequence within the RI region that is crucial for its activity in prostate cancer cells. RII coincides with the region covered by P5 mutant; as mentioned before, this mutation did not affect the pNH activity significantly. It is unclear at this point why both footprints could be competed away by P2 element. The RI binding factor may complex with transcription factor that binds to RII, so by squalching RI binding factor with cold P2 element, the RI binding factor was also prevented from binding to RII. Together, these results showed that RI site is occupied by a cell-specific transcription factor present in PSA-positive prostate cancer cells, and it regulates PSA promoter activity in an androgen- and AR-independent manner.

To further characterize the transcription factor that binds RI site, EMSA was performed to investigate the mobility pattern of the RI-binding protein. Two protein-DNA complexes were observed to associate with RI site (Fig. 6A); only one appeared to be specific (complex A), for it was competed away by specific competitor RI, but not affected when nonspecific competitor AP-1 was used. Moreover, complex A seems to be more abundant in C4-2 nuclear extracts (lane 5) than in LNCaP nuclear extracts (lane 2), and it was not observed when PC3 nuclear extracts were used (lane 8). Since a single copy of RI site (P2/TATA) shows higher activity in C4-2 than in LNCaP, and it
has no significant activity in PC3 (Fig. 5C), we concluded that complex A is a strong candidate for regulating the RI activity in LNCaP and C4-2 cells. A data base consensus site search indicated that RI site shares high homology with the SP-1 transcription factor family binding site. Therefore, we compared the mobility patterns of complex A to the SP-1 binding factors (Fig. 6B). With either LNCaP or C4-2 nuclear extracts, the SP-1 consensus site consistently yielded three distinctive bands, which corresponded to SP-1, SP-2, and SP-3, protein factors (lanes 2 and 8). They appeared to have a completely different migration pattern than complex A. Since complex A migrated closely to where the SP-3 factor is on the gel, supershift experiments were carried out with SP-3 antibody to determine whether complex A is SP-3. In the positive control (lanes 4 and 10), SP-3 protein was completely supershifted by the antibody, while complex A was not. Furthermore, unla- beled SP-1 competitor cannot compete away the complex A band (lanes 6 and 12). Therefore, it appears that the factor that binds to RI may not belong to the SP-1 transcription factor family.

Next, we determined the molecular weight of RI binding factor by covalently linking it to a radiolabeled RI site in UV cross-linking experiments. In Fig. 6C, an intense band was observed at 60 kDa; by subtracting the mass of the probe (15 kDa), RI binding factor has an apparent mass of 45 kDa. The specificity of the photoadduct was rigorously determined by competition with both specific and nonspecific competitors, nonspecific competitors like AP-1 and SP-1 were not able to compete away the RI-protein complex, while specific competitor RI could successfully compete away the DNA-protein complex. In conclusion, we have identified a novel regulatory element (RI site), which is associated with a 45-kDa cell-specific transcription factor (p45) in prostate cancer cells. The increased association of p45 to RI site in C4-2 cells could account for the higher basal PSA promoter activity in AI prostate cancer cells in the absence of androgen.
Androgen-independent Regulation of PSA Promoter

**DISCUSSION**

Hormone-refractory prostate cancer is one of the most detrimental diseases affecting men in the United States. Until now, the progression of prostate cancer from an androgen-dependent to an androgen-independent status has been poorly understood. Rebound of serum PSA is most consistently observed in prostate cancer patients with evidence of androgen-independent progression. Among the gene products specifically expressed by the human prostate, the transcription regulation of the PSA gene has been widely studied (6–8, 10). However, in most of the studies conducted, investigators have focused mainly on the androgen regulation of the PSA promoter in an androgen-dependent/responsive prostate cancer cell line, LNCaP (2, 4). By doing so, they have identified several AREs in various regions of the PSA promoter (6–8, 28). In the present report, we describe for the first time the identification of cis-elements in the PSA promoter whose up-regulation is associated with AI progression of prostate cancer.

In this study, we provide evidence to indicate that the androgen-independent activation of PSA promoter in androgen-independent prostate cancer cells (C4-2) involves two distinct regions, AREc and pN/H (Figs. 2 and 4). The requirement of these two regions suggested there are two different pathways involved in the up-regulation of PSA promoter activity in C4-2 cells. One pathway clearly requires AR because the binding of AR to the AREs within the AREc appears to be a prerequisite for the high activity of the AREc in C4-2 cells (Fig. 3). It is possible that AR is activated through a ligand-independent pathway where growth factors might be involved. It has been reported that in DU145 prostate cancer cells (with co-transfection of AR), insulin-like growth factor could stimulate AR-mediated reporter gene transcription to the same extent as a synthetic androgen, methyltrienolone (R1881). In the same study, Culig et al. (20, 21) demonstrated that keratinocyte growth factor and epidermal growth factor could also activate artificial promoter with two AREs. Moreover, activation of the protein kinase C and/or the PKA pathway has also been reported to stimulate AR activity in the absence of androgen (22–24). AR was shown to be activated by a PKA activator (forskolin) in the absence of androgen. This activation can be blocked by a PKA-specific inhibitor or anti-androgen (Casodex, flutamide). The two pathways just mentioned are not necessarily mutually exclusive. It is conceivable that growth factors like insulin-like growth factor would signal through the protein kinase cascade, which in turn could increase the transactivating activity of AR, either by modification of the protein itself (25, 41) or by enhancing the interactions between AR and its co-activators (42, 43) (Fig. 7). The fact that AREc is highly tissue-specific (7, 8) suggests that in addition to AR, other prostate-specific co-activators are also involved in the androgen-independent regulation of AREc in C4-2 cells. Activated AR is one of the key factors that interacts with prostate-specific transcription factor(s), and together they associate with the AREc and assemble into a highly active AREc enhanceosome complex (10, 40) in C4-2 cells. Thus, the aberrant activation of AR and/or its co-activators may be one of the mechanisms that contribute to the up-regulation of PSA promoter activity in androgen-refractory prostate cancer cells.

The second pathway mediated by pN/H element appears to be androgen- and AR-independent, for pN/H activity is not affected by the addition of androgen at all (Fig. 4B). Through DNase I footprinting (Fig. 5C) and linker-scanning mutagenesis (Fig. 5A), we have mapped the location of the first AI regulatory element (RI) in the PSA promoter. We believe that RI regulates the basal PSA promoter activity by the binding of a 45-kDa (p45) unknown transcription factor in prostate cancer cells. The absence of p45 in PC3 cells (Figs. 5C and 6A) indicates that its expression is restricted to PSA-positive cells like LNCaP and C4-2. The fact that C4-2 nuclear extracts consistently showed a higher level of RI-p45 complex in EMSA suggested that increased association of p45 to RI site is the mechanism by which PSA promoter is activated in C4-2 cells independent of androgen. The increased association observed in C4-2 cells could be explained as follows: 1) increased gene transcription of p45 in C4-2 cells, resulting in a higher level of p45 in the cells, or 2) p45 is activated in C4-2 by unknown growth signals, so it could bind the RI site better. This activation could be contributed by any modification (e.g. phosphorylation) or mutation of the protein that allows it to associate with DNA with higher affinity, or translocate into the nucleus more efficiently. In conclusion, p45 may represent a new class of androgen-independent prostate-specific transcription factor that regulates PSA expression in prostate cancer cells.

Previously, our laboratory demonstrated that a PSAF secreted by C4-2 cells could up-regulate PSA production in LNCaP cells (26). We observed that PSA synthesis and secretion were induced in LNCaP cells upon the addition of C4-2 conditioned media. It is possible that autocrine factor(s) like PSAF secreted by C4-2 cells might have similar effects to the growth factors in activating AR and its interaction with co-activators (42–44), hence creating a highly transcriptionally active AREc even in the absence of exogenous androgen and growth factors. At the same time, the autocrine factor(s) could also enhance the activity of p45 in C4-2 cells (Fig. 6A). The synergistic effect observed in the chimeric sPSA promoter (Fig. 4C) indicates that AR and p45 could work cooperatively in activating the PSA promoter in an androgen-independent manner in C4-2 cells (Fig. 7). In addition to PSA promoter regulation, ligand-independent activation of AR has long been implicated as a culprit in the androgen-independent prostate cancer progression (45); therefore, it is conceivable that activated AR together with p45 in AI prostate cancer cells might give them growth advantages in an androgen-deprived condition. Hence, prostate cancer cells through the production of autocrine factors could have bypassed the requirement of androgen for growth and survival. This then allows them to progress into a hormone refractory stage and become androgen-independent. Further efforts to identify p45 may provide additional insights into...
prostate cancer progression and PSA regulation in men with hormone refractory prostate cancer.

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