Identification of two novel cis-elements in the promoter of the prostate-specific antigen gene that are required to enhance androgen receptor-mediated transactivation

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ABSTRACT

A monomeric androgen responsive element (ARE) is not sufficient to mediate significant androgen induction of the prostate-specific antigen (PSA) gene. Co-transfection experiments using a series of 5′ deletion fragments of the proximal promoter region of the PSA gene linked to bacterial chloramphenicol acetyltransferase (CAT) as a reporter have identified two motif sequences which are indispensable for androgen receptor (AR)-mediated transactivation of the PSA promoter and have been designated as motifs A and B respectively. Of note, motif B alone has very little independent enhancer activity regardless of the presence or absence of androgen, whereas multicopies of motif A exert androgenic inducibility for a heterologous promoter independent of the presence of ARE. Nucleotide substitutions in either motif significantly decrease the androgen inducibility and the nuclear protein binding ability. Furthermore, gel band shift experiments consistently demonstrate that nuclear proteins can bind these motifs, and they are non-receptor factors. Our data indicate that these two DNA motifs are novel cis-regulatory elements and exhibit different mechanisms in cooperation with ARE for AR-mediated transactivation.

INTRODUCTION

Androgens are endocrine factors that require an intracellular mediator such as the androgen receptor (AR) for their actions in target cells. Androgens play an important role in male sexual development and maturation (1) and defect or deficiency in either androgens or AR may result in a number of disorders (1–4). Moreover, androgens play a crucial role in the development and progression of prostate malignancy (5).

AR is a transcription factor that belongs to the steroid hormone/thyroid-retinoid receptor superfamily (1,6,7). Ligand-activated AR can bind to a specific DNA sequence in order to enhance gene transcription. Therefore, the specificity of the enhanced transcription relies on at least three components. These components include ligands such as androgens, the AR and the androgen responsive element (ARE). Recent cloning and use of cDNA for AR (8–11) provide unequivocal evidence to support the above notion. Furthermore, in addition to the binding sequences for the receptors, the function of each class of steroid receptors may largely depend on the context of non-receptor binding sites in the promoter of a particular gene (1,6,12,13). Non-DNA binding proteins are also required for specific actions of the receptors via protein–protein interaction (6,14–18). However, compared to other steroid receptors, much less is known about the function of AR in this regard because fewer androgen receptor regulated genes have been characterized.

Prostate-specific antigen (PSA) is a differentiation marker for the human prostate. It has become the most sensitive marker for monitoring and detecting prostate cancer (19). Seminogelin has been suggested to be the physiologic substrate for PSA during the liquefaction process of semen coagulation (20). Recent studies have shown that PSA can proteolytically activate growth factor related proteins which might be related to the advancement of prostate cancer (19).

We and others have recently demonstrated (21,22) that the expression of PSA is mainly induced by androgens at the transcriptional level. Previous studies on mouse sex-limited protein (Slp), rat probasin, PSA and human glandular kallikrein-1 (hKLK2) genes have shown that ARE is necessary but not sufficient to confer the inducibility of gene expression by androgens (21–25). Many of the androgen regulated genes have been reported (23,25–28) to contain a complex ARE with more than one copy of ARE in the first intron or the 5’ far upstream flanking region of these genes.

The Slp gene has been studied (23,24,27) in great detail with respect to the function of its AREs and the surrounding auxiliary sequences in an 120 bp DNA enhancer region. The androgen-dependent enhancer is located 2 kb upstream of the Slp gene and resides within a proviral long terminal repeat. The ARE unit in the
enhancer consists of one canonical ARE and two degenerate AREs. The degenerate AREs, unlike the canonical ARE, seem to be unable to bind AR in vitro. However, transfection experiments have shown that they are functional when neighboring sequences are present. Although the ARE unit decides steroid hormonal response for the Slp gene expression, the studies of Adler et al. (23,24) have suggested that multiple non-receptor factors binding sites are critical for determining androgen specificity. Another study has also indicated (26) that the androgen-dependent expression of a rat prostate gene requires a combinational effect of multiple AREs and other transcription factor/binding elements. Without doubt, completely defining these non-receptor factor/binding elements will help us better understand the mechanism of androgen action.

In this report, we characterize two novel non-receptor binding sequences that are required in AR-mediated transactivation of the PSA gene in human prostate cells. Gene transfer, mutagenesis and in vitro DNA binding assays have defined two sequences within ~15 bp in the proximal promoter of the PSA. Together with these two elements, a simple ARE exhibits significant androgen inducibility of the proximal promoter of the PSA gene.

**MATERIALS AND METHODS**

**Plasmid constructs**

To generate a series of 5′ deletion fragments of the PSA promoter, PSA-624 was used as a template with a 3′ oligodeoxynucleotide (ODN) and a number of 5′ ODNs to produce many different lengths of PSA promoter using the polymerase chain reaction (PCR). Similar means were also used in PCR to produce PSA promoter DNA fragments containing an internal deletion or nucleotide substitution mutations. The sequences of these ODNs are listed in Table 1.

**Table 1. Oligonucleotide sequences for PSA promoter–CAT constructs by PCR**

<table>
<thead>
<tr>
<th>Primers for PSA promoter constructiona,b</th>
<th>5′ primers:</th>
<th>3′ primer:</th>
</tr>
</thead>
<tbody>
<tr>
<td>3′ primer: (+31/+6)</td>
<td>5′CTTCCGGGGTGAGGTTGTTAAAGCTTG3';</td>
<td></td>
</tr>
<tr>
<td>5′ primers:</td>
<td>5′GGTTGGAAGAATGCAAAGGAAAGAG3';</td>
<td></td>
</tr>
<tr>
<td>-484:</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407:</td>
<td>5′CTGATGCTGTCTGTTAGGCG3';</td>
<td></td>
</tr>
<tr>
<td>-371:</td>
<td>5′CTTGGATGCAAGGATCTAG3';</td>
<td></td>
</tr>
<tr>
<td>-342:</td>
<td>5′GCCAGTGAAGCTTTGATAAGA3';</td>
<td></td>
</tr>
<tr>
<td>-320:</td>
<td>5′GTCCTCACTGACCTAACAGG3';</td>
<td></td>
</tr>
<tr>
<td>-222:</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407(A-1):</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407(A-2):</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407(A-3):</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407(A-4):</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407(B-3):</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407(B-1):</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407(B-2):</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
<tr>
<td>-407(B-3):</td>
<td>5′CACAGTGGTGACAGGGATCAGG3';</td>
<td></td>
</tr>
</tbody>
</table>

a Both 3′ and 5′ primers contain BamHI and XbaI restriction sequence at their respective 5′ ends.
bNumbers for the 3′ antisense primer indicate the 5′ and 3′ ends respectively, relative to the cap site of the PSA transcript.
Nuclear extracts

PC-3 and LNCaP human prostate cancer cells were grown in the same conditions as described above, except that prior to nuclear extraction LNCaP cells were treated with 3 nM Mib for 12 h. Nuclear extracts were prepared as described (29). Briefly, cells were collected and centrifuged at 1000 r.p.m., were resuspended with Ca\(^{2+}\), Mg\(^{2+}\) free phosphate buffered saline (PBS) containing 1 mM EDTA and centrifuged at 4\(^{\circ}\)C in Beckman JA 20 rotor for 30 min. The supernatant was collected and dialyzed against 100 vol of a buffer containing 20 mM HEPES, pH 7.5, 5% glycerol, 100 mM KCl, 0.2 mM EDTA, 5 mM MgCl\(_2\) and 2 mM DTT, 0.5 mM 2-mercaptoethanol and 0.5 mM PMSF and 3 nM of Mib for 4 h with one change of buffer. The dialyzed nuclear extract was centrifuged at the same speed for 20 min to remove insoluble matter and stored frozen at −100\(^{\circ}\)C in small aliquots. The protein concentration of nuclear extracts was measured using Bio-Rad protein assay kit (Bradford assay).

Gel band shift experiments

Double-stranded ODNs (ds-ODNs) corresponding to sequences within the A or B region in the PSA promoter and containing XbaI 5′ protruding ends, were labeled with \([\alpha\text{-}^{32}P]dCTP (3000\text{ Ci/mmol}; Amersham corp., Arlington Heights, IL) by Klenow enzyme to a specific activity of \(8 \times 10^{7} - 8 \times 10^{8}\) c.p.m./µg. The sequences for these ds-ODNs are shown in Table 2.

The ds-ODNs of NF-κB, AP1 and SP1 were purchased from Promega (Madison, WI). In vitro DNA binding was performed by incubating the above nuclear extract (5–8 µg) in a buffer containing 20 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl\(_2\), 0.1 mM EDTA, 12% glycerol, 4 mM DTT and 1 µg poly(dI:dC) with or without unlabeled ds-ODNs or a random ODN (AN\(_{17}\)) in a 100–200-fold molar excess for 10–30 min at room temperature or on ice prior to receiving 20–30 fmol of a labeled ds-ODN probe for an additional 10 min incubation.

For super-shift assay, 1 µg of specific mouse anti-AR antibody (PharMingen, San Diego, CA) was incubated with the LNCaP nuclear extract 30 min prior to in vitro DNA binding. Non-immune mouse IgG was included as a control. Finally, the above reaction mixtures were electrophoresed in a pre-run 5% polyacrylamide (29:1 of acrylamide:bisacrylamide) with TG buffer (12.5 mM Tris and 85 mM glycine) or 0.5x TBE (1x TBE = 0.089 M Tris–borate, 0.089 M boric acid and 2 mM EDTA, pH 8.0) at 250 V for 1–1.5 h. Gels were dried and autoradiographed.

Statistics

Student’s t-test, one-way Anova test and Duncan’s multiple range test were used for analyzing transfection data. A value of \(P < 0.05\) was considered statistically significant. Mean values of CAT activities with no Mib of each construct shown in Figures 1, 2 and 4 are not significantly different as analyzed by one-way Anova test and Duncan’s multiple range test. Therefore, the mean values of the CAT activities without Mib in each of the above figures were considered as an equivalent background. CAT activities of each construct with Mib treatment were subtracted by CAT activities of pBLCA T3 with no Mib and used for comparison.

RESULTS

PSA glycoprotein is one of the main secretory proteins produced by the prostate. Our previous study indicated that androgens via the cognate receptor are the main factors that up-regulate the
expression of the PSA gene (30,31). Although deletion of the ARE completely abolishes androgen inducibility of the proximal promoter region of the PSA gene, it seems that an ARE alone is not enough to bring about significant androgenic induction. To understand how sequences other than ARE can influence androgen inducibility, a series of 5′ deletion fragments of the PSA promoter was generated (Fig. 1) for transient co-transfection with an AR expression vector in an AR lacking human prostate cancer cell line, PC-3. As seen in Figure 1, the CAT activities of constructs –624, –484 and –407 show no significant difference. The CAT activity of the –407 construct is significantly different from that of the –371 and –342 constructs (P < 0.05) whereas the latter two are not significantly different. The CAT activities between –342 and –320 constructs are significantly different (P < 0.05). Thus, there are at least two regions in addition to the ARE in the PSA promoter examined that seem to have a positive effect on AR-mediated transactivation. These two regions are at approximately –407 to –371 and –342 to –320 and designated as regions A and B respectively. Furthermore, to assure that region B contributes to androgen induction, a PSA –407 promoter construct containing an internal deletion of B (–340–326) was made for co-transfection. As expected, lack of this region does diminish the androgen induction [407 versus 407 (BΔ), P < 0.05]. The third region (from –484 to –407) seemed to have some effect on the PSA promoter. However, the difference of CAT gene induction between the constructs –484 and –407 is not statistically significant. For this reason we chose to concentrate on regions A and B in the following studies.

First, to examine region A we made nucleotide substitution mutations in several positions of this region in the PSA –407 construct (Fig. 2, lower panel). Note that there are two direct repeats (i.e., CAGGGGA) with one nucleotide as a spacer in this region. Three of four nucleotide substitutions are in the direct repeats. Indeed, these mutant constructs no longer exhibit androgen induction of CAT gene in the presence of activated AR

![Figure 1](image1.png)

![Figure 2](image2.png)

**Figure 1.** 5′ deletion analysis of the PSA promoter using transfection assays. PC-3 cells in duplicate plates were co-transfected with designated PSA 5′ deletion promoter–pBLCA T3 construct (10 µg/plate) and a human AR expression vector (0.4 µg/plate) using DEAE-dextran-chloroquine followed by treatment with (+) or without (−) 3.2 nM Mib for 40 h. Cell extracts were prepared from transfected cells and used for protein and CAT activity assays. The left panel of the diagram is a schematic representation of the PSA promoter fragment of each construct; numbers denote base pair position relative to the cap site of the gene. The right panel of the diagram shows the results of the CAT assay as expressed in (c.p.m./min)/mg protein. Error bars indicate the standard error of the mean of three separate experiments.

**Figure 2.** Characterization of the effect of mutations in region A of PSA promoter on androgen inducibility. PC-3 cells in duplicate plates were cotransfected with designated PSA promoter–pBLCA T3 constructs (4 µg/plate) and a human AR expression vector (0.2 µg/plate) using Lipofectamine (12 µg/plate). Cells were then treated with or without 3.2 nM Mib for 24 h. Cell extracts were prepared and used for protein and CAT activity assays. The results are expressed in (c.p.m./min)/mg protein. Error bars indicate the standard error of the mean of three separate experiments. The lower panel represents the actual nucleotide mutations in the corresponding constructs shown in the upper panel.
Figure 3. Band-shift analysis of prostatic nuclear protein which binds to region A of PSA promoter. 32P-labeled double-stranded PSA –396/–382 was incubated with LNCaP or PC-3 cell nuclear extracts which had been preincubated with or without a 100-fold molar excess of designated ds-ODN under the conditions described in Materials and Methods.

Figure 4. Effects of deletion or nucleotide substitution mutations in region B of PSA promoter on androgen inducibility. Transient co-transfection experiments were performed as described in the legend to Figure 2, except the use of PSA promoter–pBlCAT constructs as indicated in this figure. The data were collected from four separate assays. The results are expressed in (cpm/min)/mg protein. Error bars indicate the standard error of the mean of three separate experiments. The lower panel represents the actual nucleotide mutations in the corresponding constructs shown in the upper panel.

Figure 5. Interaction of prostatic nuclear protein with DNA in the region B of the PSA promoter by band-shift analysis. 32P-labeled double-stranded PSA –340/–326 was incubated with PC-3 cell nuclear extract which had been preincubated with or without a 100-fold molar excess of designated double-stranded oligonucleotides under the conditions described in Materials and Methods.

Therefore, we were suspicious that PSA –340/–326 might contain an MRE. However, the experiment (Fig. 5) has shown this is unlikely, because hMTIIA–MREa cannot inhibit the formation of radioactive complexes.
To further study the properties of regions A and B, a series of constructs containing monomeric or multimeric A or B were generated for the following transfection experiments in both PC-3 and LNCaP cells. As shown in Figure 6, motif B has very little transactivation activity regardless of the presence or absence of androgen, indicating that B itself may not be an enhancer motif. However, surprisingly, three copies of motif A seem to exert androgenic inducibility for a heterologous promoter without ARE, which is consistent with the result in Figure 2. The same nucleotide substitution mutation also diminishes transactivation activity of multimeric A under androgen influence. The above studies suggest that A and B motifs exert different mechanisms in assisting AR–ARE-mediated transactivation of the PSA gene.

Because of the above result, we performed additional band-shift experiments to reaffirm that motif A is not a direct binding site for AR. As can be seen in Figure 7, 32P-PSA ARE and AR from LNCaP cells can form a complex which is not only competeable by unlabeled ARE but also super-shifted by a specific AR antibody. It is also evident that the super-shifted complex is able to be competed out by unlabeled ARE and that either specific antibody alone or non-immune IgG with nuclear extract does not form a complex or super-shift with the labeled ARE. In addition, the ARE–AR complex cannot be competed by a ds-ODN containing one or three copies of motif A. Conversely, the complex formed by nuclear protein and 32P-motif A is only competeable by itself but not by PSA ARE. The formation of this complex cannot be altered by anti-AR antibody. Thus, a non-AR nuclear protein forms the complex with the A motif.

DISCUSSION

PSA is one of three members of the human kallikrein gene family (19). The other two are human glandular kallikrein-1 (hKLK2) and renal/pancreatic kallikrein (hKLK1). Interestingly, both PSA and hKLK2 are expressed almost exclusively in the prostate of males and under androgen regulation. In contrast to human counterparts, rodents may have 12–24 kallikrein genes with differential expression in a variety of tissues (32). Many of these genes are also under control of steroid hormones.

Although the expression of the PSA gene is mainly controlled by androgens, intrinsic regulation may also exist because sequence analysis shows that the PSA promoter contains TATA box, CACCC box, SP1 and AP2 regulatory sequences downstream of the putative ARE. It has been demonstrated (21,22) that...
a functional ARE located at –170 to –155 plays a role in androgen induction of PSA expression in prostate cells. Moreover, Riegman et al. (22) have inferred from their study that the region –539 to –320 in PSA promoter may cooperate with the ARE for androgen induction. The present study seems to suggest that there are at least two regions at –396 to –382 and –340 to –326 acting synergistically with the ARE for androgen induction.

The initial study, as shown in Figure 1, indicates that removal of motifs A and B by 5’ deletion of the PSA promoter only causes step-wise reduction of androgen inducibility of the promoter. However, as shown in Figures 2 and 4, internal deletion mutations and most of those nucleotide substitution mutations almost completely diminish their androgen inducibility. At the present time, we cannot offer clear explanation for the discrepancy between the results from 5’ deletion mutants and nucleotide substitution mutants. However, the results from the above experiments and gel bandshift assays (Figs 3 and 5) show that the sequences in regions A and B are indeed specific nuclear protein binding sites and involved in ARE/AR-mediated induction of the PSA gene. Furthermore, the aforementioned discrepancy might be an indication of a complex interaction of motif A/motif A binding protein, motif B/motif B binding protein and ARE/AR for in vivo androgen regulation of expression of the PSA gene.

It is generally true that multimeric interaction of homologous steroid hormone receptors or of steroid receptors and other transcription factors is required for the manifestation of steroid hormone action (6,7,12). It has been demonstrated that factors binding to SP1, NF1, OTF and CACCC-box have strong synergistic effects on progesterone or glucocorticoid receptor-mediated transactivation. Although cooperativity between different cis-acting elements is common, very limited information regarding cooperativity between ARE and other cis-acting elements is available. Among androgen-regulated genes containing functional AREs, most studies have concentrated on delineating the function of ARE. Much less attention has been paid to the detailed study of AR-cooperating factors and cognate elements.

AR/ARE is required for androgen induction of PSA and other androgen regulated genes (1). However, in order to exhibit androgen inducibility, cooperating factors/DNA elements are also needed. As shown in Figures 2 and 4, nucleotides were mutated or deleted in region A or B; subsequently, androgen induction was abolished or largely reduced even though the ARE remained intact. Gel band shift assays further demonstrated that these two regions were nuclear protein binding sites. The binding patterns produced by using PC-3 or LNCaP cell nuclear extract are the same. The result from the band shift assays suggests that these two DNA binding proteins exist commonly in prostate cells. Since PC-3 cells do not produce AR and PSA, and ARE cannot compete out the band formation by motif A or B with nuclear extracts in the band shift assays, we can conclude that the sequences in the regions A and B are two different cis-acting elements which are not AR binding sites. Moreover, those nucleotide substitution mutations seem to demonstrate the importance of certain nucleotides in these regions for protein binding and transactivation functions. Our study certainly warrants more detailed and comprehensive investigation into the role of nucleotide sequences in the above regions for protein binding.

It has been shown that many protein factors can interact with steroid hormone receptors and may influence receptor-mediated transactivation. Some of these factors are known DNA binding transcription factors such as c-Fos, c-Jun and octamer factor (12.33–35). They exhibit interference or enhancement effects on receptor-mediated transactivation. Others have been shown to be associated with the receptors in a ligand-dependent manner. These receptor associated factors include the estrogen receptor-associated proteins ERAP160, RIP160 and RIP80 (36,37), the thyroid hormone receptor interacting proteins Trips and TRIP1 (38), the human homolog of the adaptor Sug1p (39), a mouse bromodomain-containing protein, TIF1 (40), the human steroid hormone receptor coactivator-1 (SRC-1) (34) and an AR-specific coactivator, ARAP70 (18). In addition, the insulin degrading enzyme is also a known non-receptor, non-DNA binding factor that can directly interact with the androgen receptor protein (41). However, its role in the AR-mediated transactivation of specific genes remains to be elucidated.

Data presented in this paper implies that nuclear proteins for motifs A and B can interact with AR either directly or indirectly and enhance AR’s transactivation function in a ligand-dependent manner. Moreover, it is very intriguing that motif A and its binding protein show androgenic inducibility in the presence of ligand activated AR but independent of ARE. Our observation seems to suggest that the function of motif A and its binding protein may present a novel mechanism by which AR can activate transcription rates in the absence of an ARE. This implies that if the promoter of a particular gene possesses suitable number of motif A or A-like sequences, the expression of such a gene would be regulated via ligand-activated AR at transcriptional levels.

Of note, these two motifs have little basal enhancer activities suggesting that these motifs and their binding proteins may act as accessory factors to AR. Additionally, in transfection experiments multi-copies of motif A exert androgenic induction independent of ARE, whereas multi-copies of motif B cannot. Indicating that the mechanisms by which the two motifs influence AR’s function may be different from each other. Isolation of these proteins and their cDNAs in the future will greatly facilitate the understanding of how both protein–protein interaction and DNA–protein interaction influence the AR-mediated transactivation.

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