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Androgen dependent transcription of a mouse prostatic protein gene, PSP94: Involvement of estrogen receptors☆

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ABSTRACT

Prostatic secretory protein 94 (PSP94) is a prostatic protein found in both humans and rodents. As with other prostatic proteins, expression of this protein is regulated by androgens. In order to understand the androgen-responsive transcriptional regulation mechanisms involved, the present study aimed to identify and characterize the promoter activity of the gene. The 5′flanking (5′f) region of mouse PSP94 (mPSP94) gene was cloned and introduced into a vector upstream of the luciferase reporter gene. A Chinese hamster ovarian cell line, CHO, and a human prostate adenocarcinoma cell line, LNCaP, were transiently transfected with our reporter constructs along with an androgen receptor expression vector, and treated with dihydrotestosterone. Reporter gene assay revealed that the 5′f region of mPSP94 gene was indeed responsible for the androgen-dependent transcription. Subsequent deletion and mutation analysis indicated that the androgen responsive element (ARE)-like sequence at position −93 from the transcription start site was primarily responsible for androgen dependency. Interestingly, when estrogen receptor (ER) α was co-transfected, the androgen-dependent transcription was substantially increased. However, ERα-dependent enhancement of androgen responses was not observed when estrogen responsive element (ERE)-like motifs of the promoter region were deleted. Administration of estrogen did not influence the enhancement associated with ERα, although an anti-estrogen suppressed such effects. Collectively, these data suggest that the androgen-dependent transcription of the mPSP94 gene was co-regulated/modulated by the presence of ERα via ERE-like motifs.

1. Introduction

Prostatic secretory protein 94 (PSP94), or β-microseminoprotein, is one of the major proteins along with PSA (prostate specific antigen) and PAP (prostatic acid phosphatase) that are secreted by the human prostate gland [1–3]. PSP94 is also secreted in abundance by the rodent prostate although the composition of prostatic proteins differ significantly between rodents and primates [4,5]. The rodent prostate consists of anatomically separate prostatic lobes including ventral, lateral, dorsal and anterior lobes. PSP94 is known to be highly expressed in dorsal and lateral regions of the rat prostate but localized to ventral and dorso-lateral prostatic lobes in mice. PSP94 may function as an immunoglobulin binding protein and is involved in the regulation of immune response in the female reproductive tract [6]. In addition, PSP94 is known to inhibit motility of sperm and the acrosome reaction [7]. PSP94 homologues have been identified in several other mammals and non-mammalian species including ostrich and Japanese viper [8]. The PSP94 family of proteins share ten highly conserved cysteine residues.

The expression of PSP94 is regulated by androgens, in a manner similar to other prostatic proteins such as human PSA and rat probasin [9–11]. In the human PSA gene, an androgen-responsive element (ARE) motif capable of inducing transcription in response to androgens via androgen receptors (AR) was identified in the promoter region at position −156 upstream of the transcriptional starting site [12]. Another functional ARE was additionally identified at approximately −4 kbp [13]. In the rat probasin gene, two functional AREs were identified in the 5′flanking (5′f) region. Interestingly, these were androgen selective AREs rather than responsive to androgens and glucocorticoids [14,15]. In the case of PSP94, it was demonstrated that the 5′f region of the mouse PSP94 (mPSP94) gene exhibited promoter activity which conferred prostatic-specific expression in transgenic mice [16]. However, the androgen-responsive transcription mechanism responsible for such promoter activity has yet to be identified.

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At position −93 from the transcription start site of mPSP94 gene, there is an ARE-like sequence, TACCTAnnnTGTTCT that contains half site of consensus ARE, TGTTCT. We demonstrated that this site is indeed a functional ARE for androgen-dependent transcription in the present study. Interestingly, the promoter region also contains sequences similar to estrogen responsive element (ERE) which enhance androgen-dependent promoter activity of the gene in the presence of the estrogen receptor (ER) α.

2. Materials and methods

2.1. Construction of reporter plasmids and transient transfection

The 5′f region of the mPSP94 gene (GenBank AF087140) was cloned by precise PCR. Specific primers at positions −1202 and +21 from the transcription start site were designed (5′-AGCAACCTCACTTGTTCCTCAGA and 5′-GGTACCTCCAGCAAAGTCCTTG). PCR was performed with PrimStar Taq (Takara Bio., Otsu, Japan) and genomic DNA of C57BL mouse liver following the manufacturer's recommended conditions. After adding an adenosine residue, the PCR fragment was cloned into the pCR2.1-TOPO TA cloning vector (Invitrogen, Carlsbad, CA, U.S.A.) and the sequence confirmed with a capillary sequencer, ABI PRISM 310 (Applied Biosystems, Foster City, CA, U.S.A.). Truncated fragments of the 5′f region were also prepared by PCR with LA-Taq (Takara Bio.) between positions −450, −358, −200, −118, −95, −76 and +21 from the cloned 5′f region −1202/+21. Each fragment was cloned into the PCR2.1-TOPO vector. Sac I/Xho I-digested fragments were then inserted into the corresponding restriction enzyme sites of the pGL3-basic luciferase reporter plasmid (Promega, Madison, WI, U.S.A.), and designated as mPSP94p-1202, -450, -358, -200, -118, -95, -76.

Mutations and deletions were introduced into pGL3 promoters using a QuickChange Site-Directed Mutagenesis Kit (Agilent Technology, Santa Clara, CA, U.S.A.). For ARE analysis, nucleotides between positions −85 and −79, and between −44 and −31 were deleted from mPSP94p-118. In addition, the TGT sequence at position −84 was changed to GAG. For ERE analysis, estrogen responsive element (ERE)-like sequences between positions −435/−421 and −216/−202 were deleted from the PSP94p-450 reporter. Construction of the hAR and hER expression plasmids, pSG5-hAR, pSG5-hERα, pSG5-hERβ were as described previously [17]. PhRL-CMV (Promega) was utilized as an internal control.

CHO cells were plated at a concentration of 2×10^4/well in 48-well plates and transiently transfected with 300 ng of a reporter, 30 ng of pSG5-hAR and 2 ng of phRL-CMV with Hilymax transfection reagent containing a synthetic cationic lipid (Dojindo Laboratories, Kumamoto, Japan), following the manufacturer's protocol. The weight ratio of the reagent to DNA was 1:1. After 24 h of incubation, cells were harvested with 25 μl of cell lysis buffer (Promega) and firefly and renilla luciferase activities determined with a Dual Luciferase Assay Kit (Promega) by measuring luminescence with a Lumino/Fluro meter. Firefly luciferase reporter activity was normalized to renilla luciferase activity from phRL-CMV. For transfection into the LNCaP cell line, a concentration of 4×10^4 cells was plated into 48-well plates; the ratio of reagent to DNA was 3:1.

DNA motif searches for ARE and ERE were performed using TRANSFAC at http://www.genome.jp/tools/motif/.

2.2. Cell culture

The CHO cell line was maintained in DME medium (Sigma Chemical Co., St. Louis, Mo., U.S.A.) containing penicillin and streptomycin with 5% FBS (Biosolutions Japan Co., Osaka, Japan). The LNCaP cell line was maintained in RPMI-1640 medium (Sigma Chemical Co.) with 10% FBS and penicillin/streptomycin. For hormone treatment experiments, cells were maintained for a week in phenol red-free medium (Sigma Chemicals) containing the same antibiotics along with dextran-charcoal treated sera. Dihydrotestosterone (DHT), hydroxy flutamide (OH-flutamide), 17β-estradil (E_2) and ICI182780 were purchased from Wakojunyaku K.K., Osaka, Japan, Toronto Research Chemicals Inc., North York, ON, Canada, Sigma Chemicals and Tocris Bioscience, Ellisville, MI, U.S.A., respectively.

3. Results

3.1. DHT-dependent promoter activity of the 5′f region of mPSP94 gene

Data concerning promoter activity of the cloned 5′f region −1202/+21 and the successive truncated regions in response to DHT are summarized in Fig. 1A. Position numbers are assigned based upon the transcription start site (+1). Significant induction of luciferase activity by DHT was noted in all constructs but mPSP94p-76, which suggested that the region between positions −95 and −76 was essential for androgen-dependent transcription. The induction appeared lower with reporter constructs containing longer distal 5′f regions of the promoter, although these remained significant in terms of induction.

Since there is a half conserved ARE sequence between positions −84 and −79, luciferase activities of the mPSP94p-118 reporter deleting this region and a reporter exhibiting mutations within this site converting TGT to GAG at position −84 were examined and are shown in Fig. 1B. Those constructs disrupting the half ARE site showed no induction of luciferase activity in response to DHT, while a reporter constructing deleting elsewhere (Δ-44/-31) exhibited DHT-dependent transcription.

Induction of mPSP94p-118 reporter activity was DHT-dependent, and significant responses were identified with 10^{-11} M of DHT with maximal response at 10^{-9} M (Fig. 1C). An anti-androgen, OH-flutamide, antagonized the DHT-induced responses (Fig. 1D).

3.2. Enhancement of DHT responsive transcription in the presence of ERα

The observed increase in mPSP94-p1202 activity treated with DHT at 10^{-9} M was about 2.5 fold of the control, which was lower than that with mPSP94p-118. When an ERα expression vector was co-transfected in CHO cells along with the AR expression vector, the mPSP94p-1202 reporter activity in response to DHT increased significantly, but did not alter PSP94-p118-luc activity. Increased levels of ERα transfection resulted in higher PSP94-1202 activity in response to androgens (Fig. 2A). Transfection of ERβ, on the other hand, did not alter response to androgens. ICI 182780 suppressed the androgen-induced promoter activity of mPSP94 while E_2 administration did not alter activity (Fig. 2B).

3.3. ERE-like motifs in the 5′f region of mPSP94 gene

The enhancing effects of ERα were examined with successive truncated luciferase reporters (Fig. 3A). Significant enhancement was observed with PSP94p-1202 and -450 but not with -200 or -118. Since there are two ERE-like motifs at positions −435 and −216 in the promoter region, it is possible that these sequences may be involved in the ER-dependent enhancement of testosterone responses. When both ERE-like motifs were deleted in the PSP94p-450 reporter, the expected enhancement with ERα was lost, while deletions of only one of the EREs did not affect the enhancing effect (Fig. 3B).

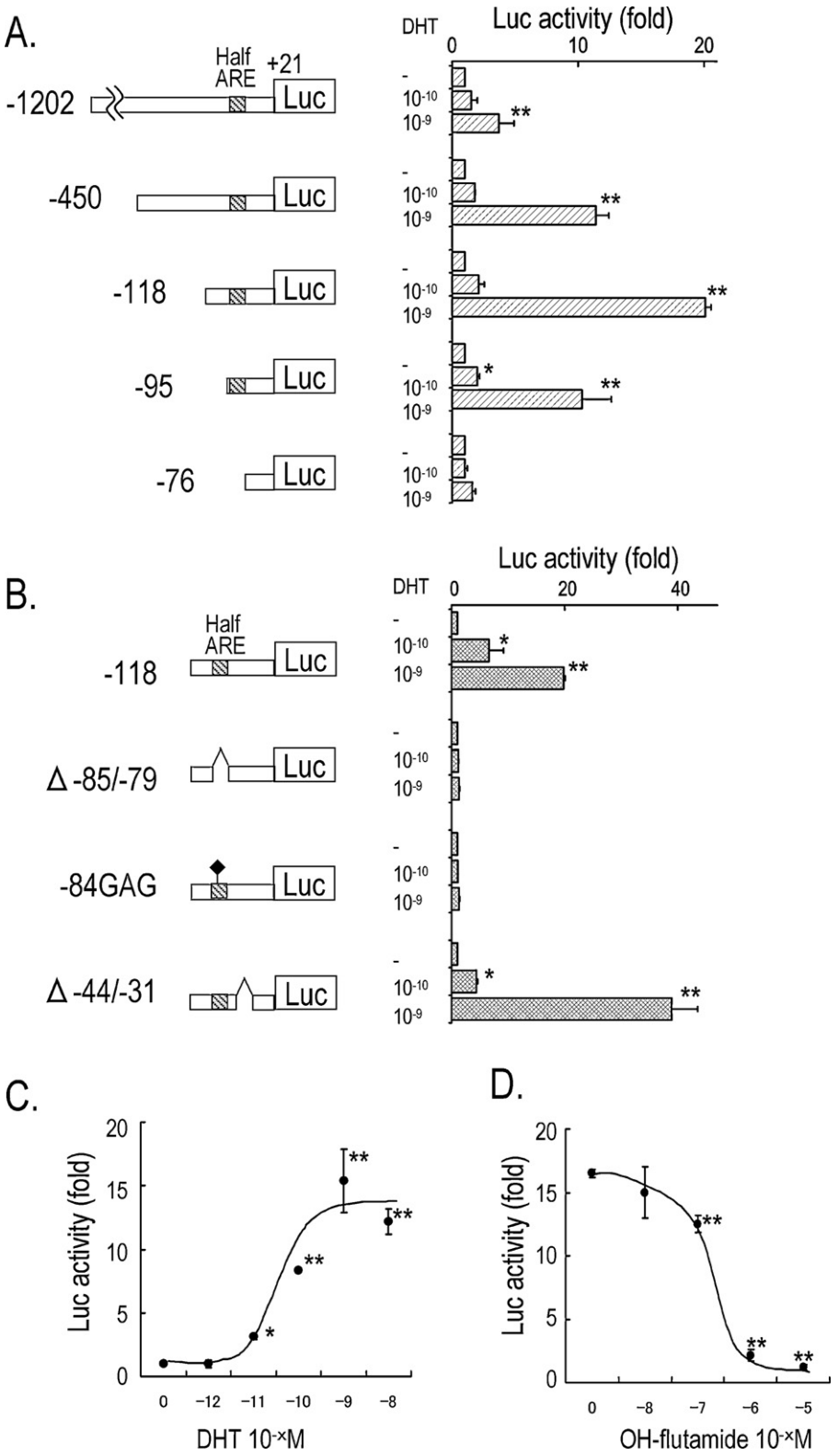

Fig. 1. DHT-dependent promoter activity of the mPSP94 gene in CHO cells. (A) Successive truncated fragments of the 5′f region from mPSP94 were inserted into a luciferase reporter. Position numbers were assigned based on the transcription start site (+1). Reporter plasmids (300 ng) and 30 ng of pSG5-hAR were transfected. DHT was administered at concentrations of 10^{-10} and 10^{-9} M. (B) DHT-induced luciferase activities of deletion and point mutants from the mPSP94p-118 reporter. (C) DHT-dose dependent induction of luciferase activity from the mPSP94p-118 reporter. (D) Dose dependent inhibition by OH-flutamide against DHT (10^{-9} M) induced activity. Bar indicates mean ± SEM, $n = 5$. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

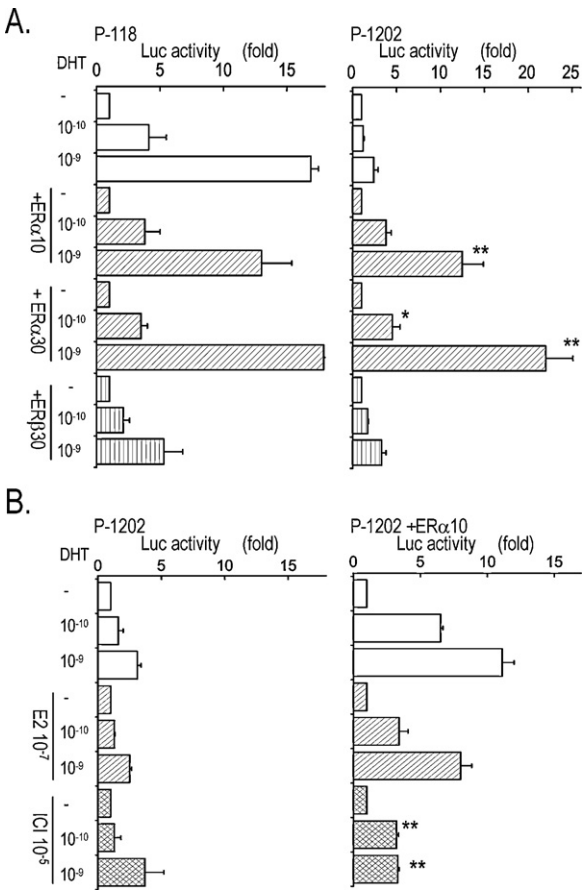

Fig. 2. Effects of ERα transfection upon DHT-dependent promoter activity of the mPSP94 gene in CHO cells. (A) Reporters containing p-118 or p-1202 were transfected along with 30 ng of pSG5-hAR and/or pSG5-hERα (10 or 30 ng) or pSG5-hERβ (30 ng). DHT was administered at concentrations of 10^{-10} or 10^{-9} M. (B) A reporter containing p-1202, pSG5-hAR and/or pSG5-hERα were transfected. DHT was administered at concentrations of 10^{-10} or 10^{-9} M with/without estradiol (E2) at 10^{-7} or ICI182178 at 10^{-5} M. Bar indicates mean ± SEM, $n = 5$. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control.

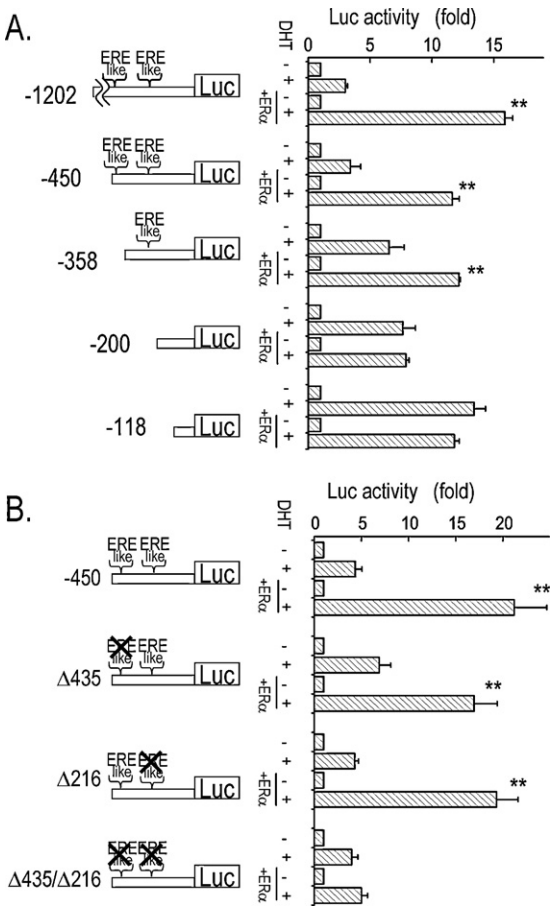

Fig. 3. Requirement of ERE-like motifs for ERα-dependent enhancing effects of androgen responses of the mPSP94 gene in CHO cells. (A) Successive truncated fragments of the mPSP94 promoter were transfected along with pSG5-hAR and pSG5-hERα. DHT was at 10^{-9} M. (B) Deletion mutants of pGL3-mPSP94p-450 were transfected with pSG5-hAR and pSG5-hERα. Bar indicates mean ± SEM, $n = 5$. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. ER negative control.

3.4. Promoter activity in LNCaP cells

Responses of reporter genes to DHT with successive truncated fragments of the mPSP94 promoter in the LNCaP cell line are summarized in Fig. 4A. Significant inductions were noted in all reporter constructs except mPSP94p-76. We also examined the effect of co-transfection with an ERα expression vector (Fig. 4B). With the mPSPp-118 reporter, ERα did not alter DHT responses, while mPSP94p-450 responses were significantly increased by co-transfection with the ERα expression vector. Co-transfection with the ERα expression vector did not change the response of mPSPp-450(Δ435/Δ216).

4. Discussion

PSP94 is a non-glycosylated and cysteine-rich protein composed of 94 amino acids [3,18,19]. First isolated from human seminal plasma, PSP94 was found to be one of the major prostatic proteins in humans [1]. Although the composition of rodent prostatic proteins is very different from that of humans, PSP94 is commonly expressed in rodents [5,20]. The function of this protein has yet to be fully determined. PSP94 exhibits immunoglobulin-binding capability in order to suppress the activation of B cells [7]. PSP94 may also function as an inhibitor of sperm motility [8].

Prostatic proteins are generally regulated by androgens. When the expression of proteins secreted from the mouse prostate was examined in our previous study, we found that all proteins were indeed significantly reduced just one week following castration of the animal but increased following androgen administration [21]. Some genes may be regulated by the direct interaction of liganded AR and promoter/enhancer regions of the gene, while other changes would be secondary or tertiary events along with involution and regeneration of the prostate tissue. In the case of PSP94, testosterone administration increased mRNA levels in castrated mice by a factor of 38 in just 24 h, suggesting that gene expression was directly controlled by androgens. PSP94 mRNA is expressed in ventral prostate as well as dorso-lateral prostate in the mouse, but is localized specifically in the dorso- and lateral-prostate lobes in the rat. In humans, the expression of PSP94 was not restricted to the prostate but was additionally detected in secretions from the respiratory tract, gastric fluid and other secretory tissues [18]. Since expression was very specific to the prostate in rodents, the promoter/enhancer structure of mPSP94 gene has drawn significant interest as potential gene targeting tools. It has been demonstrated that a 3.8 kb of the 5′f region was capable of directing gene expression in a prostate tissue specific mode, that was additionally ventral- and dorso-lateral lobe specific, in a transgenic mouse model [16].

Androgens regulate their responsive genes via intracellular ARs. Upon ligand binding, ARs interact with specific DNA sequences,

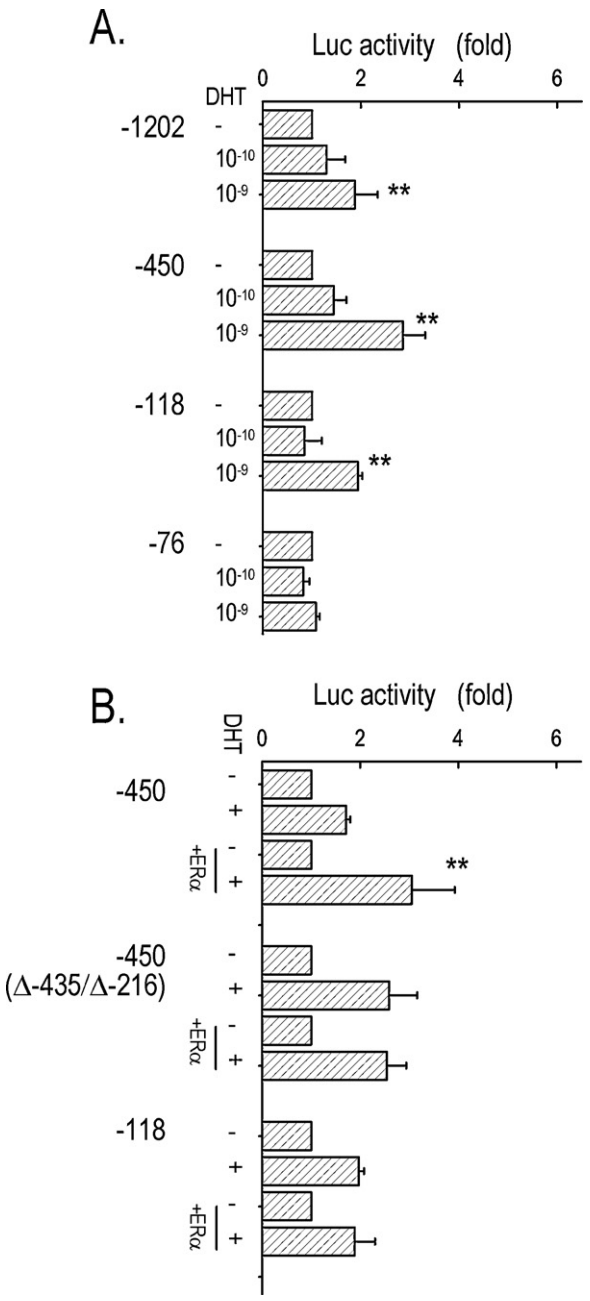

Fig. 4. DHT-dependent promoter activity of the mPSP94 gene in LNCaP cells. (A) Luciferase reporter constructs containing truncated fragments of the mPSP94 promoter were transfected and DHT was administered at concentrations of 10^{-10} and 10^{-9} M. (B) Reporter constructs containing p-450, ERE deleted p-450(Δ435/Δ 216) and p-118 were transfected with pSG5-hERα. DHT was at 10^{-9} M. Bar indicates mean ± SEM, $n = 5$. $^{**}p < 0.01$ vs. DHT negative control (A) and vs. ERα negative control (B).

known as AREs, and thus regulate the transcriptional activity of genes [22,23]. Steroid hormone receptors generally bind to DNA elements consisting of inverted repeats of six base pairs by three nucleotide spacers. The consensus hexametric sequence for AR is TGTTCT, which is identical to the glucocorticoid and progesterone responsive element. Previous investigations have revealed that AREs are divided into two categories; one interacts with glucocorticoids, mineralocorticoids and progesterone receptors, in addition to ARs, while the other is specific to ARs [24,25]. Previously described sequence data suggested that AR specific AREs tend to be organized as partial direct repeats rather than inverted repeats of the consensus hexametric repeat [22]. In mPSP94, we found an ARE candidate, TACCTAnnnTGTTCT that contains a half consensus ARE at position −93. Since androgen-dependent luciferase reporter activity was completely lost when this element was deleted or mutated, this motif between positions −93 and −79 appears to represent the functional ARE, although further studies are needed to determine if there are any direct interactions between this ARE-like sequence and AR. In the present study we primarily utilized CHO cells for the promoter assay since it is easy to perform transient transfection experiments in high efficiencies with this mammalian cell line. In addition, we used LNCaP, a human prostate adenocarcinoma cell line, to confirm the biological relevance of the promoter activity. LNCaP is an AR positive cell line that grows responding to testosterone and has been frequently used to study the androgen responsive gene regulation [26].

It has been demonstrated that combined administration of androgen and estrogen synergistically enhanced the development of prostatic hyperplasia and cancer in Noble rats as well as in chemical carcinogen-treated F344 rats [27,28]. In the previous study, we examined the expression of prostatic protein genes such as probasin and kallikrein S3 to understand the molecular mechanism underling the androgen plus estrogen effect [29]. We found that estradiol enhanced the androgen dependent expression of prostatic genes along with increase in ERα expression, which suggested that the elevated prostatic ERα may contribute to the enhancing effects. In the present study, we tested this hypothesis with PSP94 promoter and found that the androgen-dependent induction was enhanced by the presence of ERα while administration of estradiol did not change the transcription levels. In the PSP94 promoter, there are two ERE-like motifs that potentially interact with ERs at positions −435 and −216, ctannAnnnTGACCT and gGGnncnnnTGACCa, where the consensus ERE is ARGnnAnnnTGACCY. Results arising from deletion mutants indicated that at least one ERE similar motif is enough for the enhancement of androgen responses. These enhanced responses, however, did not exceed the degree of responses with the mPSP94p-118 reporter. Since reporters containing longer 5′f region displayed lower responses, it appears that the distal region of the promoter has suppressive function on the transcription and the presence of ERα may release the suppression. In normal adult prostate tissue, ERα expression is localized to stromal cells and ERβ is in epithelia [30]. In PC, however, epithelial expression of ERα increases while ERβ expression is reduced or lost [31–33]. During the development of PC, increasing ERα expression might enhance some critical androgen responsive genes related to prostatic carcinogenesis by the similar mechanisms found in the present study.

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