Blackwell Publishing

Asian J Androl 2007; 9 (1): 41–50
DOI: 10.1111/J.1745-7262.2007.00226.x

AJA
www.asiaandro.com

·*Original Article*·

Dual androgen-response elements mediate androgen regulation of MMP-2 expression in prostate cancer cells

Ben-Yi Li[1,2,3,4], Xin-Bo Liao[1], Atsuya Fujito[1], J. Brantley Thrasher[1,4], Fang-Yun Shen[5], Ping-Yi Xu[6]

Departments of [1]Urology, [2]Molecular and Integrative Physiology, [3]Pathology and Laboratory Medicine, and [4]Kansas Masonic Cancer Research Institute, University of Kansas Medical Center, Kansas City, KS 66160, USA
[5]Department of Urology, The Affiliated Shenzhen Sun Hospital, Dalian Medical University, Shenzhen 518001, China
[6]Department of Neurology, Sun Yat-Sen University, Guangzhou 510080, China

Abstract

Aim: To characterize the matrix metalloproteinases (MMP)-2 promoter and to identify androgen response elements (AREs) involved in androgen-induced MMP-2 expression. **Methods:** MMP-2 mRNA levels was determined by reverse transcription-polymerase chain reaction (RT-PCR). MMP-2 promoter-driven luciferase assays were used to determine the fragments responsible for androgen-induced activity. Chromatin-immunoprecipitation assay and electrophoretic mobility shift assays (EMSA) were used to verify the identified AREs in the MMP-2 promoter. **Results:** Androgen significantly induced MMP-2 expression at the mRNA level, which was blocked by the androgen antagonist bicalutamide. Deletion of a region encompassing base pairs -1591 to -1259 (relative to the start codon) of the MMP-2 promoter led to a significant loss of androgen-induced reporter activity. Additional deletion of the 5'-region up to -562 bp further reduced the androgen-induced MMP-2 promoter activity. Sequence analysis of these two regions revealed two putative ARE motifs. Introducing mutations in the putative ARE motifs by site-directed mutagenesis approach resulted in a dramatic loss of androgen-induced MMP-2 promoter activity, indicating that the putative ARE motifs are required for androgen-stimulated MMP-2 expression. Most importantly, the androgen receptor (AR) interacted with both motif-containing promoter regions *in vivo* in a chromatin immunoprecipitation assay after androgen treatment. Furthermore, the AR specifically bound to the wild-type but not mutated ARE motifs-containing probes in an *in vitro* EMSA assay. **Conclusion:** Two ARE motifs were identified to be responsible for androgen-induced MMP-2 expression in prostate cancer cells. *(Asian J Androl 2007 Jan; 9: 41–50)*

Keywords: androgen; androgen receptor; androgen response element; matrix metalloproteinases-2; promoter; prostate cancer

Correspondence to: Ben-Yi Li, MD, PhD, Department of Urology, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS 66160, USA.
Tel: +1-913-588-4773 Fax: +1-913-588-4756
E-mail: bli@kumc.edu
Dr Ping-Yi Xu, Department of Neurology, Sun Yat-Sen University, Guangzhou 510080, China.
Tel: +86-20-8733-4438
Email: pxu@kumc.edu
Received 2006-05-12 Accepted 2006-07-12

1 Introduction

Matrix metalloproteinases (MMP) are enzymes implicated in various steps of cancer development and metastasis [1]. The expression of MMP in the prostate is related to normal and pathological tissue organization changes. In animal experiments, only those sublines of prostate cancer cells that produce high levels of MMP

are capable of distant metastasis when tumors are generated orthotropically. MMP-2, also called gelatinase A, has been localized by immunohistochemistry to basal and to a lesser extent secretory epithelial cells, but not stromal cells of normal and benign prostatic hyperplastic (BPH) tissues [2, 3]. It has been shown that higher expression levels of MMP-2 correlated with tumor metastasis and aggressive behavior of prostate cancer [1, 3].

Transcription of the human *mmp-2* gene is regulated in cell- and stimulus-specific manner, and sequence analysis of the MMP-2 promoter has revealed some potential *cis*-acting regulatory elements including p53, AP-1, Ets-1, C/EBP, CREB, PEA3, Stat3, GATA-2, Sp1 and AP-2 that could be involved in the regulation of MMP-2 expression. Many factors including cytokines, growth factors and extracellular matrix proteins have been reported to promote expression of MMP-2 in human prostate tissue. Although some MMPs (MMP-1, -3, -7) are downregulated by androgen treatment *in vitro* via androgen receptor (AR)-Ets protein interaction and increased collagen content was found in the ventral prostate of the rat after castration, increased expression of MMP-2, -7 and -9 was observed in both premalignant and malignant tissues after androgen treatment in the Noble rat [3]. Recently, we demonstrated that MMP-2 expression is increased upon androgen treatment in prostate cancer cells via a transcriptional mechanism [4].

Androgen-induced gene regulation typically occurs through AR interaction with specific DNA sequences termed as androgen response element (ARE). The location, sequence and number of ARE motifs associated with a given androgen target gene varies, although androgen-responsive regions typically contain multiple nonconsensus ARE sequences 5'-tgttct-3' [5]. In the present study, we characterized the MMP-2 promoter and identified two ARE motifs that are responsible for androgen-induced MMP-2 expression in prostate cancer cells.

2 Materials and methods

2.1 Cell culture and reagents

The human prostate cancer LNCaP, PC-3, PC-3/AR and PC-3/Neo cells have been described previously [4, 6]. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics. Antibodies against human AR and secondary antibodies were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Other reagents were supplied by Sigma (St. Louis, MO, USA). Charcoal-stripped fetal bovine serum (cFBS) was obtained from Atlanta Biologicals (Norcross, GA, USA).

2.2 mRNA expression analysis and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared using TriZol™ reagent (Invitrogen, Carlsbad, CA, USA). To assess mRNA expression, a semiquantitative RT-PCR method was used as described previously [6]. RT-PCR was carried out using an RETROscript kit from Ambion (Austin, TX, USA) following the manufacturer's manual. The primers and PCR conditions were described as follows: for human *mmp-2* gene (forward 5'-ctgacattgaccttggcacc-3'; backward 5'-tagccagtcggatttgatgc-3'), for human *PSA* gene (5'-agaacagcaagtgctagctc-3' and 5'-aggtggtaagcttgggg gctg-3'). 28S ribozyme RNA (forward 5'-gttcacccac taatagggaacgtg-3'; backward, 5'-gattctgacttagaggcgttcagt-3') was used as an internal control [7]. The primers were synthesized by IDT Inc. (Coralville, IA, USA). The amplification profile was as follows: 95ºC for 30 s, 56ºC for 30 s, and 72ºC for 1 min running in a total of 25 cycles. After 25 amplification cycles, the expected PCR products were size fractionated onto a 2% agarose gel and stained with ethidium bromide. The band density was quantitatively measured with a Gel Logic 100 system (Kodak, New Haven, CT, USA).

2.3 Site-directed mutagenesis of the human MMP-2 promoter

Site-directed mutagenesis was used to mutate the putative ARE motifs in the MMP-2 promoter using a commercial QuickChange kit (Stratagene, La Jolla, CA, USA). Two pairs of PCR primers were used in these experiments: for ARE-1 (wild-type [WT] 5'-tgtatct-3'), forward 5'-gctctatttcccaaggCgCGCctagcatctcgcactatacg-3' and backward 5'-cgtatagtgcgagatgctagGCGgGccttggga aatagagc-3'; for ARE-2 (WT 5'-tgttcct-3'), forward 5'-cccccacaagtataGgGGccGgattctttcagcccctg-3' and backward 5'-caggggctgaaagaatcCggCCcCatatacttgtggggg-3'. Mutated nucleotides were shown as capital letters. First, two individual site-directed mutants on each ARE motifs (termed as ARE-1M and ARE-2M, respectively) were generated using the WT promoter construct as template. And then, another construct (ARE-1/2M) containing mutations within both ARE motifs was generated by mutating the ARE-2 motif with the ARE-1M construct as a template. Successful mutation was confirmed by direct

sequencing, and the constructs were used in luciferase reporter assays.

2.4 Luciferase and secreted alkaline phosphatase reporter (SEAP) assay

The luciferase reporters controlled by the WT (1 716 bp) and various truncated forms of the human MMP-2 promoter were obtained from Dr Etty N. Benveniste [8]. The reporter vector pCMV-SEAP, expressing SEAP under the control of the cytomegalovirus (CMV) promoter, was described previously [4, 6] and was used as an internal reference control. The reporter assays were carried out as described in our previous studies [4, 6]. Briefly, cells plated in 6-well tissue culture plates were transfected in the following day with different MMP-2 reporter constructs (WT, truncated or mutant promoters) together with pCMV-SEAP construct using the Cytofectene reagent (BioRad, Hercules, CA, USA) according to the manufacturer's protocol. After 24 h, cells were serum-starved for another 24 h and then treated with R1881 (1.0 nmol/L) in 2% cFBS or fibroblast growth factor-2 (FGF-2) (10 ng/mL) in serum-free media. Culture supernatants were collected 24 h later and assayed for SEAP activity. Cell lysates were used for luciferase assay and protein assays as described in our previous studies [4, 6]. The luciferase activity of each sample was normalized against the corresponding SEAP activity before the fold induction value relative to control cells was calculated.

2.5 Chromatin immunoprecipitation (ChIP) assay

Cells were maintained in 10-cm dishes in medium without serum for at least 16 h and treated with or without 1.0 nmol/L R1881 for 12 h. The androgen antagonist bicalutamide was added 30 min before R1881 treatment where indicated. The ChIP assay was carried out by using a ChIP assay kit and the polyclonal antibody against AR were obtained from upstate according to the manual (Charlottesville, VA, USA). Normal rabbit serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as a negative control. The primers for the PCR reactions were listed as follows: for ARE-1 region (-784/-576), forward 5'-agtgcagcccagcaggtctc-3' and backward 5'-gagacagtggaaggtcccag-3'; for ARE-2 region (-1676/-1460), 5'-ccaccagacaagcctgaact-3' and backward 5'-gcccagagatgaaaaacagc-3'; for the region beyond Exon 1 (the third pair), forward 5'-ccaccgtttgcaagagactc-3' and backward 5'-ctcaggcggtggctggaggctgc-3' (based on gene bank sequence NC_000016). The PCR products were run on 1% agarose gel and stained with ethidium bromide for visualization.

2.6 Nuclear extract and electrophoretic mobility shift assay (EMSA)

Essentially, nuclear extracts were prepared using a NE-PER kit, oligonucleotide probes were biotin-labeled with a Biotin 3' labeling kit and EMSA were carried out with the LightShift kit. All these kits were purchased from Pierce Inc. (Rockford, IL, USA) and the experiments were carried out according to the manufacturer's manuals. Briefly, cells grown in 100-mm cell culture dishes were serum-starved for 24 h and then treated with R1881 (1.0 nmol/L) for another 6 h. After washed in cold phosphate-buffered saline (PBS), cells were harvested and nuclear proteins were extracted using the buffer system from the NE-PER kit. Protein concentrations were determined using Bio-Rad protein assay as described [4, 6].

EMSA was carried out using the following oligonucleotides as probes and/or competitors: positive AR binding probes containing the consensus ARE sequence (5'-CTAGAAGTCTGGTACAGGGTGTTCTTTTTGCA-3') was purchased from Santa Cruz Biotech Inc. The oligonucleotide probes are listed as follow: WT ARE-1: 5'-ctctatttcccaaggtgtatctagcatctcgcacta-3'; WT ARE-2: 5'-cccccacaagtatattgttcctgattctttcagcccc-3'; mutant ARE-1: 5'-ctctatttcccaaggCgCGCctagcatctcgcacta-3'; mutant ARE-2: 5'-cccccacaagtatatGgGGccGgattctttcagcccc-3'. The putative ARE sequences are underlined and the mutations are indicated by capital letters. DNA probes were synthesized by IDT Inc. (Coralville, IA, USA). The probes were 3'-end labeled with biotin using a kit as mentioned above. In EMSA analysis, 20 fmol/L biotin-labeled probes were incubated with 5 μg nuclear proteins for 30 min at room temperature in a volume of 20 μL containing 1 μg of poly (dI-dC). For competition assay, a 100-fold molar excess of unlabeled probes was incubated with the nuclear extracts at 4ºC for 20 min before addition of labeled probe. Bound and free probes were resolved by electrophoresis through 0.5% agarose gel and then transferred to nylon membrane (Hybond-N^+; Amersham Bioscience, Piscataway, NJ, USA). Biotin-labeled DNA probes were visualized by chemiluminescence protocol provided by the kit.

2.7 Statistical analysis

All experiments were carried out in triplicates and repeated two or three times. The RT-PCR results are pre-

sented from a representative experiment (Figure 1). The mean and SD from two or three separate experiments for luciferase assay are shown (Figures 2 and 3). The significant differences between groups were analyzed using SPSS computer software (SPSS, Chicago, IL, USA). $P < 0.05$ was considered significantly different.

3 Results

3.1 Androgen stimulates the transcription of mmp-2 gene via the androgen receptor

We have recently shown that androgen treatment increases the MMP-2 protein level and activity in prostate cancer cells [4] in which the mechanism at a gene transcriptional level was proposed. To further determine the mechanism at the transcriptional level, we carried out a RT-PCR assay to measure the MMP-2 mRNA level after androgen treatment. A well-known androgen target, human prostate specific antigen (PSA), was included as a positive control. LNCaP cells were serum-starved for 24 h and then stimulated with a synthetic androgen R1881 for 6 h. Total cellular RNA was extracted for RT-PCR assay. As shown in Figure 1, R1881 treatment dramatically increased the mRNA level of *mmp-2* gene, which was completely abolished by a pretreatment of the cells with an androgen antagonist bicalutamide. The same inhibition was also seen for *PSA* gene, indicating that androgen-induced MMP-2 expression is an AR-dependent genomic effect. These data confirmed our previous observation that androgen stimulates MMP-2 expression via the AR at the transcriptional level (Figure 1).

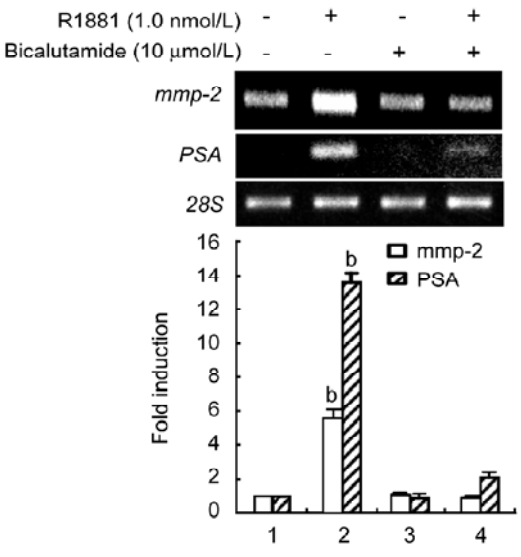

Figure 1. Androgen stimulates *mmp-2* gene expression via an androgen receptor (AR)-dependent mechanism. After serum starvation for 24 h, LNCaP cells were left untreated (lanes 1 and 3) or treated (lanes 2 and 4) with R1881 (1.0 nmol/L) in the present (lanes 3 and 4) or absent (lanes 1 and 2) of bicalutamide (10 μmol/L) for 24 h. Cells were harvested and the mRNA levels of *mmp-2* gene were determined by reverse transcription-polymerase chain reaction (RT-PCR) assay. *28S* gene served as internal control. The upper panel shows a representative image from one of two separate experiments and the relative band densities of PCR results for *mmp-2* gene and *PSA* from two experiments were illustrated in the bottom panel after normalization against *28S* control. $^bP < 0.05$, compared with the *28S* control. PSA, prostate specific antigen.

3.2 Two putative ARE motifs in the MMP-2 promoter

As mentioned earlier, androgen-induced genomic effects usually act through ARE motifs located in the promoter of AR-target genes. To determine if there are any putative ARE motifs responsible for androgen-induced MMP-2 up-regulation, we utilized a series of 5'-deletional MMP-2 promoter constructs (Figure 2A) in a luciferase assay. Because the basal activities of these truncated promoters were studied by another group [8], we directly went on to compare the androgen-induced activities between WT and the truncated promoters. LNCaP cells plated in 6-well plates were transfected with WT or various truncated forms of MMP-2-Luc reporters. After serum starvation, cells were incubated with R1881 for 24 h. Cellular extracts were assayed in triplicate for luciferase activities. As shown in Figure 2A, deletion of the 5'-fragment up to -1 259 (D4) resulted in a dramatic reduction of androgen-stimulated MMP-2 promoter activity. Deletion of the 5'-region up to -562 (D6) led to additional reduction of MMP-2 promoter activity compared to the WT promoter; although the difference between D4 and D6 was not significant. These data suggest that there are putative ARE motifs localized within the deleted regions in D4 and D6 that are responsible for androgen-stimulated MMP-2 promoter activity.

Next, we analyzed the MMP-2 promoter sequence and noticed that there were two putative ARE-like motifs corresponding to the 5' deletion data, as shown in Figure 2B. These elements are very similar to either consensus ARE or some natural ARE motifs, as shown in Figure 2C [8].

Because truncation on a promoter sequence also removes other regulatory elements in addition to those putative ARE-like motifs, the specificity to androgen-in-

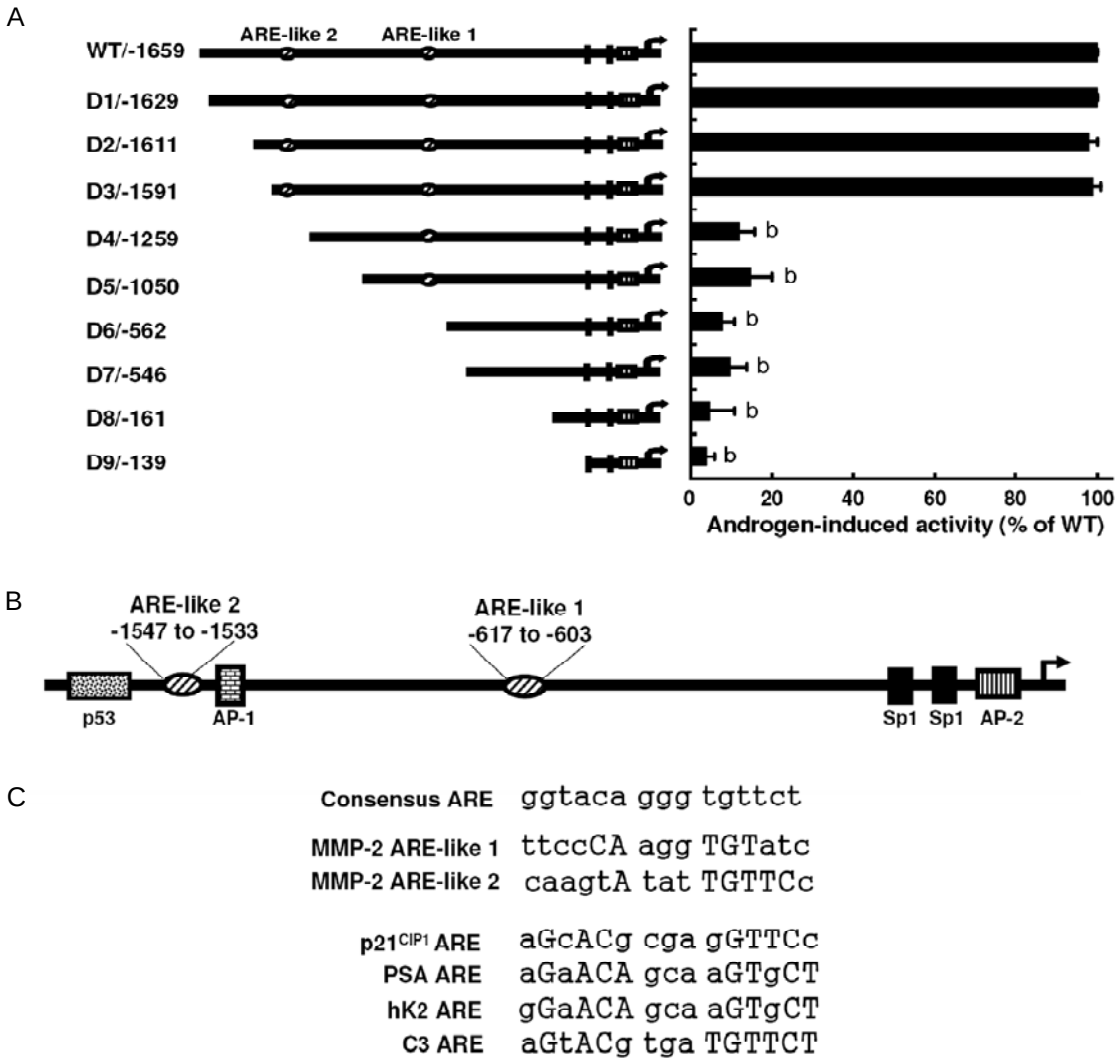

Figure 2. Effect of 5'-deletion on androgen-induced matrix metalloproteinases (MMP)-2 promoter activity in prostate cancer cells. (A): Following transfection with the wild-type (WT) or individual 5' truncated MMP-2 promoter constructs and the internal control reporter as described in the text, LNCaP cells were treated with R1881 (1.0 nmol/L) and then harvested 24 h later for reporter assays (Luciferase and secreted alkaline phosphatase [SEAP] activities). The luciferase activity from the WT promoter was set up as 100% after normalization with protein level and SEAP activity. The relative fold reduction from different truncated promoters was compared with the WT one. $^{b}P < 0.05$, compared with the WT promoter. Data represent triplicate data from two independent experiments. (B): Putative ARE-like motifs identified within the MMP-2 promoter are indicated. (C): Comparison of consensus, several published androgen response element (ARE) sequences and MMP-2 ARE-like motifs. Matched residues with consensus sequence are shown in capital letters.

duced promoter activity needs to be verified by additional approaches. Therefore, we mutated four nucleotides (multiple site-directed point mutations) within the core sequences of the two putative ARE-like motifs using the WT MMP-2 promoter as a template. ARE-1 (5'-*tgtatct*-3') was mutated to 5'-C*g*CGC*ct*-3', termed as ARE-1M and ARE-2 (5'-*tgttcct*-3') was mutated to 5'-G*g*GG*cc*G-3', termed as ARE-2M. In the third construct, both ARE motifs were mutated simultaneously, termed as ARE-1/2M. Then, we examined their responses to androgen-stimulated promoter activity. Growth factor FGF-2 was used to examine the specificity of the mutations to an-

drogen stimulation. The WT and mutated MMP-2 promoter constructs were transfected into LNCaP cells and their responsiveness to androgen stimulation was evaluated in a luciferase reporter assay. As shown in Figure 3, compared with the WT one, mutations on either one ARE-like motif or both motifs did not result in any significant change in terms of their basal activities of the promoters at the culture condition with either charcoal-stripped FBS (Figure 3A) or serum-free (Figure 3B) media. However, the mutated promoters completely lost the responsiveness to androgen stimulation, indicating that both ARE-like motifs are required for androgen regulation of MMP-2 expression. These results are consistent with the observation from the experiments with the truncated promoters (D4 and D6). To determine if these ARE-like motifs are only specific to androgen stimulation, we tested another MMP-2 inducer, FGF-2, in the next experiments. FGF-2 has been demonstrated to induce MMP-2 expression by our group and others [4, 9]. After transfection of the cells with the mutated MMP-2 promoter constructs, cells were treated with FGF-2 and luciferase assay was carried out thereafter. As shown in Figure 3B, compared with the WT promoter, mutations on either one of the ARE-like motifs had no significant effect on FGF-2-induced MMP-2 promoter activities. These data suggest that these ARE-like motifs are specific to androgen-induced MMP-2 promoter activity.

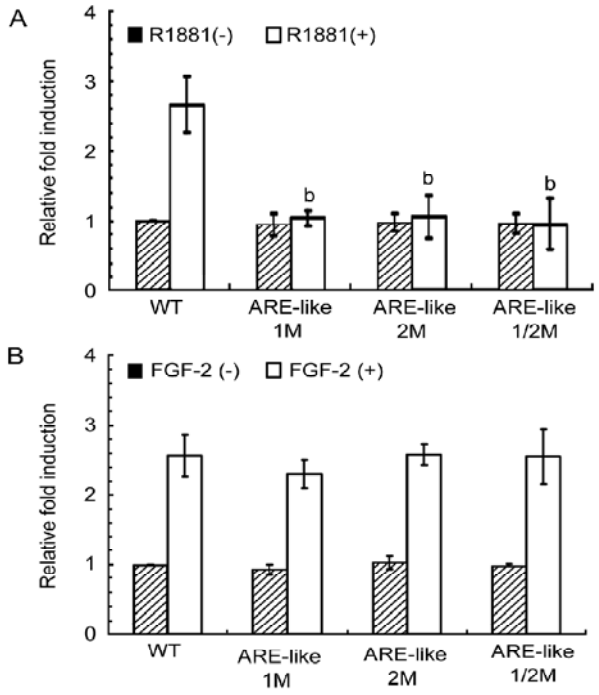

Figure 3. Effect of androgen-response element (ARE) mutations on androgen-induced matrix metalloproteinases (MMP)-2 promoter activity in prostate cancer cells. Following transfection with the wild-type (WT) or individual ARE-mutated MMP-2 promoter constructs as indicated and control reporter as described in the text (ARE-like 1M, ARE-like 2M and ARE-like 1/2M), LNCaP cells were treated with the solvent as control, R1881 1.0 nmol/L in 2% cFBS-containing media (A), or fibroblast growth factor-2 (FGF-2) (10 ng/mL) in serum-free media (B). Cells were harvested for reporter assays (Luciferase and SEAP activities) 24 h later. The luciferase activity was presented as fold induction against control samples after normalization with protein level and SEAP activity. $^{b}P < 0.05$, compared with the WT promoter. Data represent three independent experiments.

3.3 AR binds to the putative ARE-like motifs in responding to androgen stimulation in vivo

To determine if the AR binds to the putative ARE motifs of the MMP-2 promoter in response to androgen stimulation, we carried out an *in vivo* protein-DNA binding assay (ChIP assay) in LNCaP cells. In this assay, androgen-induced AR-DNA binding was first fixed and the genomic DNAs were then broken down to ~500 bp fragments by sonication. After immunoprecipitation with anti-AR antibodies, the AR-bound DNA fragments were released from the AR binding, and then amplified by a PCR reaction. Two pairs of PCR primers were designed to amplify the fragments spanning on either ARE motifs. The primer binding sites for the ARE-like motifs are illustrated in Figure 4A. After treatment with R1881, LNCaP cells were harvested and nuclear extracts were used for the ChIP assay. FGF-2 treatment was used to verify the AR specificity. As shown in Figure 4B, using the primer pairs spanning either ARE-like motif-containing sequence, a PCR product was obtained after R1881 treatment but not FGF-2 treatment. In addition, to rule out the possibility that the AR only binds to one ARE site within the MMP-2 promoter, we carried out a PCR reaction using a pair of two far-distant primers, P1 and P4 (Figure 4A), which amplifies the DNA sequence spanning both putative ARE motifs. As shown in Figure 4B (lower panel), as expected, no PCR product was obtained when the primers of P1/P4 were used. However, a positive PCR product was obtained when a plasmid construct bearing the WT MMP-2 promoter was used as a template (data not shown), indicating that the P1/P4 primers are functional. Finally, when anti-AR antibody was replaced with an anti-Actin antibody, or a third pair

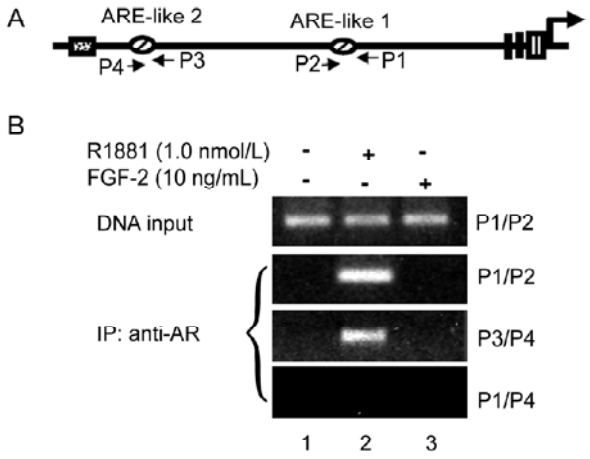

Figure 4. Androgen promotes androgen receptor (AR) binding to the matrix metalloproteinases (MMP)-2 promoter *in vivo*. (A): The putative androgen (ARE)-response element-like motifs and the primer binding sites used in ChIP assay are indicated. (B): LNCaP cells were serum-starved for 24 h and then left untreated or treated with R1881 (1.0 nmol/L) or fibroblast growth factor-2 (FGF-2) (10 ng/mL) for 18 h. Binding of AR to the MMP-2 promoter was determined with the ChIP assay as described in the text. The upper panel represents input signals obtained from 1% input chromatin. IP, immunoprecipitation; P, primer. Data represent three independent experiments.

of primers designed to amplify a region after Exon 1 within *mmp-2* gene was used for the PCR reaction as a negative control in the ChIP assay, no PCR product was obtained, indicating the specificity of AR binding to the ARE-like motifs (data not shown). These results demonstrated that the positive results from the PCR reactions using P1/P2 or P3/P4 primers were not to the result of contamination of a longer DNA fragment containing both ARE motifs. These data suggest that androgen treatment promotes AR binding to two putative ARE motifs of the MMP-2 promoter.

3.4 The AR interacts specifically with the putative ARE motifs in vitro

Next, we carried out an *in vitro* EMSA assay (also called gel retardation assay) to determine AR specific binding to the putative ARE-like motifs. Nuclear extracts were obtained from R1881-stimulated LNCaP cells and used in the experiments. Two oligonucleotide probes spanning each of the ARE-like motifs from MMP-2 promoter were synthesized. A consensus ARE-containing oligonucleotide probe was used as a positive control. All the probes were labeled with biotin for non-isotopic detection. As expected, a band shift was seen when the positive probe was added to the reaction (Figure 5A, right panel). Similarly, when a probe containing either the putative ARE motifs was mixed with the nuclear extracts, a protein-DNA binding complex was detected (Figure 5A, left panel). Then, to verify the specificity of AR binding to the ARE-containing probes, we prepared nuclear protein extracts from a pair of prostate cancer cell lines, PC-3/Neo and PC-3/AR after R1881 treatment. PC-3/Neo is an AR-null cell line stably transfected with an empty vector, and PC-3/AR is a stable subline expressing a human AR gene. The rationale to use this pair of cell lines is that once a protein-DNA complex is detected in a reaction using PC-3/AR nuclear extract but not PC-3/Neo, it will indicate to us that the AR binds to the putative ARE motif. As expected, a band-shift was detected when the labeled probes carrying the putative ARE motifs were mixed with PC-3/AR nuclear extract but not PC-3/Neo (Figure 5B). These results indicate that the putative ARE-like motifs in MMP-2 promoter are *bona fide* AR binding sites. We also noticed that the binding efficiency was much higher when the ARE-2-containing probes were used compared with the ARE-1 ones (also from the data shown later).

To further verify that the AR binding specificity was not a non-specific protein-DNA interaction, we used two mutated probes, which contained the same mutations used in luciferase assay. As shown in Figure 5C, mutations in both ARE motifs abolished the AR binding abilities (lane 4 *vs.* 2, and lane 8 *vs.* 6). In a competition experiment, unlabeled probes were used to examine the AR binding specificity again. As shown in Figure 5D, adding a 100-fold molar excess of unlabeled wild-type ARE probes (Figure 5D, lanes 3 and 7) significantly inhibited AR interaction with the labeled wild-type probes. However, adding a 100-fold molar excess of unlabeled mutated ARE probes (Figure 5D, lanes 4 and 8) did not cause any reduction of AR interaction with the wild-type probes. Taken together, these data demonstrated that the putative ARE motifs are *bona fide* AR binding sites responsible for androgen-stimulated *mmp-2* gene expression.

4 Discussion

It has been shown that MMP-2 expression is regu-

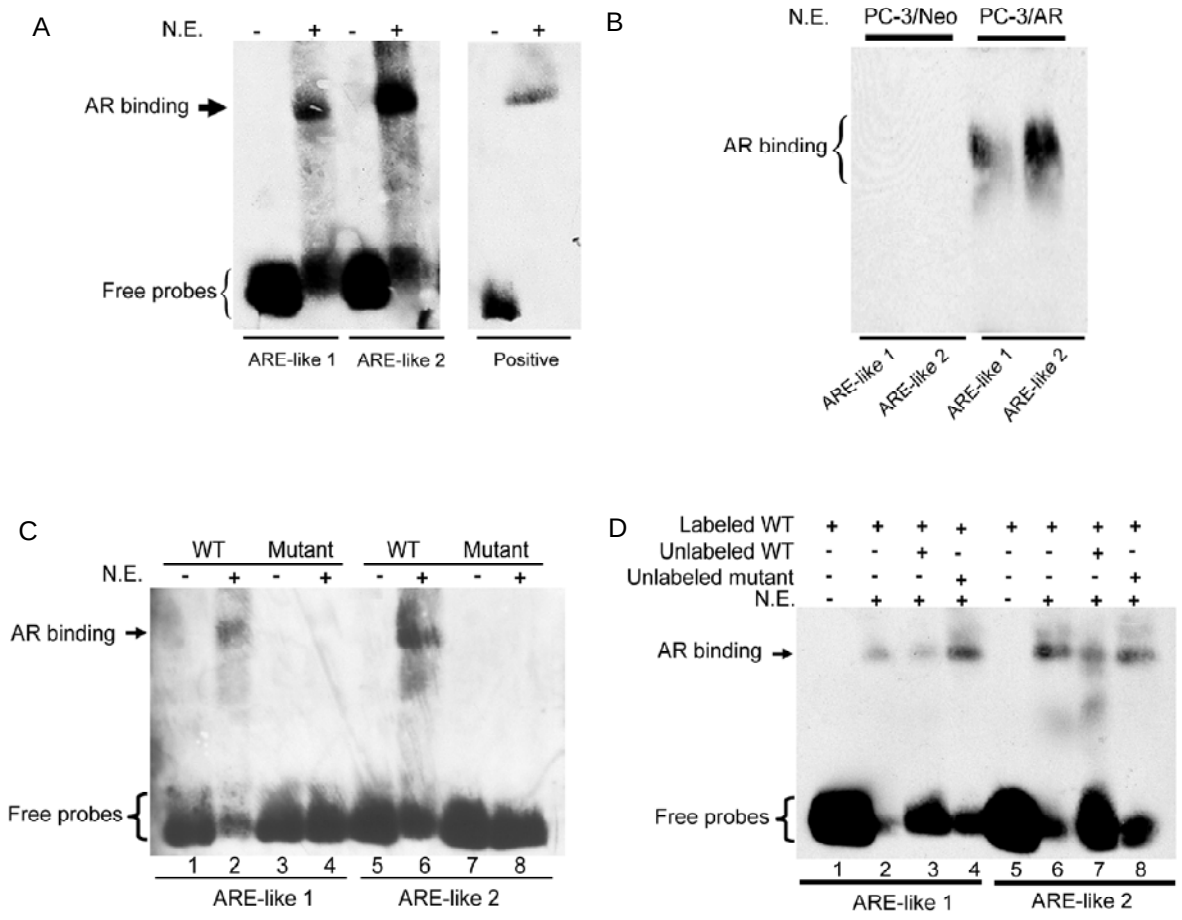

Figure 5. Androgen receptor (AR) specifically binds to the putative androgen-response element (ARE) motifs *in vitro*. LNCaP, PC-3/Neo or PC-3/AR cells were treated with R1881 (1.0 nmol/L) overnight, and then nuclear extracts (N.E.) were prepared. EMSA assay was carried out with biotin-labeled wild type (WT) probes as indicated using LightShift kit (described in the text). (A): LNCaP N.E. were used and a WT ARE probe was used as the positive control (right panel). (B): EMSA assay was carried out using PC-3/Neo and PC-3/AR N.E.. (C): EMSA assay was carried out with LNCaP N.E. and biotin-labeled WT or mutated-ARE probes as indicated using the LightShift kit (described in the text). (D): Competition analysis was carried out with WT ARE probes in the presence or absence of 100-fold molar excess of unlabeled WT or mutated-ARE probes as indicated. Data shown are representative of three separate experiments.

lated at both transcriptional and post-transcriptional levels. Although a large body of information is available for the regulation of MMP-2 activity at the protein level, the transcriptional mechanisms are not well characterized. We have previously shown that androgen increased MMP-2 production and promoter activity in human prostate cancer cells, indicating a mechanism at the transcription level [4]. Analysis of the MMP-2 promoter was only carried out in a few cell types of both rat and human origin [3, 8].

The objective of the present work was to analyze the MMP-2 promoter for androgen-regulated activity. By screening the published sequence of human MMP-2 promoter [8], we noticed that there were two putative ARE-like motifs in the promoter region. Functional analysis of the promoter was initially carried out using serial 5'-deletion constructs in human prostate cancer LNCaP cells, which endogenously express a functional AR protein and produce more MMP-2 proteins on androgen stimulation [4]. Deletion of the distal ARE motif (ARE-like 2, D4 construct) caused a dramatic decrease of androgen-induced promoter activity compared with the WT promoter. Whereas, additional deletion of the MMP-2 promoter until

the proximal ARE-like motif (ARE-like 1, D6 construct) caused a further reduction of the promoter activity compared with D4 deletion. Meanwhile, in a mutagenesis assay, mutations on the core sequences of the two ARE-like motifs separately or simultaneously caused a similar reduction of androgen-induced promoter activity compared with the WT promoter. Furthermore, the AR bound to the two ARE-like motifs in response to androgen *in vivo* and *in vitro* using two different approaches, ChIP and EMSA assays. These results clearly indicate that the two ARE motifs are *bona fide* AR binding sites responsible for androgen-stimulated *mmp-2* gene expression.

Androgens act through their cognate AR, which is a member of the steroid nuclear receptor superfamily, to induce genomic effects. On androgen stimulation, the AR translocates from the cytoplasm to nuclear compartment and then interacts with specific ARE motifs within androgen target genes. Previous reports have shown that AR binds to multiple ARE in the target gene promoter or enhancer regions. ARE motifs have been found in regions within or proximal to the promoter, or even several kilobases away upstream from the promoter, in some cases, within introns or exons of the target genes [9, 11–19]. Consensus steroid response element (SRE) usually exists as a semipalindromic sequence of 5'-TGTTCT-3', whereas natural ARE motifs in androgen-regulated genes display suboptimal binding sites and show weaker affinity to the AR compared with the consensus SRE sequence [5, 20]. Recently, a proposal divided AR binding sites into two groups; class I and II. The class I site displays a conventional pattern in terms of guanine contact, whereas the class II site shows an unusual pattern to facilitate cooperative binding to the adjacent class I site [20]. Either type alone cannot fully mediate androgen responses, whereas a combination of class I and II sites synergistically increase the DNA binding affinity, hormone sensitivity and levels of transcription in comparison to a singular site. Thus, cooperation among multiple ARE motifs might be necessary for selective stimulation, as shown for regulation of the sex-limited protein (Slp), the probasin, the PSA and 20-kDa protein genes [14–18]. In addition, studies have shown that interaction between different ARE motifs is essential to achieve maximal responses at suboptimal DNA-binding sequence [18, 20, 21]. Consistent with these notions, we also identified two ARE motifs in the MMP-2 promoter, in which mutation on either one of them resulted in complete loss of androgen responses. The ARE-like 2 motif is very similar to the class I; however, the ARE-like 1 motif could not fit into either class I or II [20]. Nonetheless, their similarity to the consensus core sequence plus our functional analysis data suggest that the two ARE motifs are working together in androgen-induced MMP-2 promoter activity, as proposed for androgen regulation of PSA promoter [22].

In conclusion, we identified two ARE motifs in the MMP-2 promoter. Mutagenesis analyses, including 5'-sequential truncation and multi-point mutations, indicated that they are responsible for androgen-induced MMP-2 expression in prostate cancer cells. These indications were further supported by AR-specific binding assays, ChIP and EMSA. Our data also indicated that these two ARE motifs are involved in mediating androgen-induced MMP-2 expression in prostate cancer cells. Further investigation of possible differences between these two ARE motifs in recruiting cofactors after AR binding is under way by our group. Future studies will determine how these two ARE motifs are working together in response to androgen stimulation in prostate cancer cells and other cell types, as well as the role of androgen-induced MMP-2 expression in organ development and tumor metastasis.

Acknowledgment

We thank Dr Etty N. Benveniste (University of Alabama at Birmingham, Birmingham, AL, USA) for the truncated MMP-2 promoter-driven luciferase constructs and Ms Donna Barnes for excellent secretarial assistance. This study was supported by KU William L.Valk Endowment and Kansas Mason's Foundation, and a grant from KUMC Lied Foundation to Dr Ben-Yi Li. This study was also partially supported by grants from the National Natural Science Foundation of China (No. 30370509 and No. 30370645) to Dr Ping-Yi Xu.

References

1 Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science 2002; 295: 2387–92.
2 Sato H, Takino T, Okada Y, Cao J, Shinagawa A, Yamamoto E, *et al*. A matrix metalloproteinase expressed on the surface of invasive tumor cells. Nature 1994; 370: 61–5.
3 Lokeshwar BL. MMP inhibition in prostate cancer. Ann N Y Acad Sci 1999; 878: 271–89.
4 Liao X, Thrasher JB, Pelling J, Holzbeierlein J, Sang QX, Li B. Androgen stimulates matrix metalloproteinase-2 expres-

sion in human prostate cancer. Endocrinology 2003; 144: 1656–63.
5 Verrijdt G, Haelens A, Claessens F. Selective DNA recognition by the androgen receptor as a mechanism for hormone-specific regulation of gene expression. Mol Genet Metab 2003; 78: 175–85.
6 Liao X, Tang S, Thrasher JB, Griebling TL, Li B. Small-interfering RNA-induced androgen receptor silencing leads to apoptotic cell death in prostate cancer. Mol Cancer Ther 2005; 4: 505–15.
7 Goffin F, Munaut C, Frankenne F, Perrier D, Hauterive S, Beliard A, *et al*. Expression pattern of metalloproteinases and tissue inhibitors of matrix-metalloproteinases in cycling human endometrium. Biol Reprod 2003; 69: 976–84.
8 Qin H, Sun Y, Benveniste EN. The transcription factors Sp1, Sp3, and AP-2 are required for constitutive matrix metalloproteinase-2 gene expression in astroglioma cells. J Biol Chem 1992; 274: 29130–7.
9 Nelson CC, Hendy SC, Shukin RJ, Cheng H, Bruchovsky N, Koop BF, *et al*. Determinants of DNA sequence specificity of the androgen, progesterone, and glucocorticoid receptors: evidence for differential steroid receptor response elements. Mol Endocrinol 1999; 13: 2090–107.
10 Miyake H, Hara I, Yoshimura K, Eto H, Arakawa S, Wada S, *et al*. Introduction of basic fibroblast growth factor gene into mouse renal cell carcinoma cell line enhances its metastatic potential. Cancer Res 1996; 56: 2440–5.
11 Dai JL, Burnstein KL. Two androgen response elements in the androgen receptor coding region are required for cell-specific up-regulation of receptor mRNA. Mol Endocrinol 1996; 10: 1582–94.
12 Celis L, Claessens F, Peeters B, Heyns W, Verhoeven G, Rombauts W. Proteins interacting with an androgen-responsive unit in the C3(1) gene intron. Mol Cell Endocrinol 1993; 94: 165–72.
13 Ho KC, Marschke KB, Tan J, Power SG, Wilson EM, French FS. A complex response element in intron 1 of the androgen-regulated 20-kDa protein gene displays cell type-dependent androgen receptor specificity. J Biol Chem 1993; 268: 27226–35.
14 Devos A, Claessens F, Alen P, Winderick J, Heyns W, Rombauts W, *et al*. Identification of a functional androgen-response element in the exon 1-coding sequence of the cystatin-related protein gene *crp2*. Mol Endocrinol 1997; 11: 1033–43.
15 Cleutjens KB, van der Korput HA, van Edkelen CC, van Rooij HC, Faber PW, *et al*. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen gene. Mol Endocrinol 1997; 11: 148–61.
16 Riegman PH, Vlietstra RJ, van der Korput JA, Brinkmann AO, Trapman J. The promoter of the prostate-specific antigen gene contains a functional androgen responsive element. Mol Endocrinol 1991; 5: 1921–30.
17 Rennie PS, Bruchovsky N, Leco KJ, Sheppard PC, Mc-Queen SA, Cheng H, *et al*. Characterization of two *cis*-acting DNA elements involved in the androgen regulation of the probasin gene. Mol Endocrinol 1993; 7: 23–36.
18 Kasper S, Rennie PS, Bruchovsky N, Sheppard PC, Cheng H, Lin L, *et al*. Cooperative binding of androgen receptor to two DNA sequences is required for androgen induction of the probasin gene. J Biol Chem 1994; 269: 31763–9.
19 Schuur ER, Henderson GA, Kmetec LA, Miller JD, Lamparski HG, Henderson DR. Prostate-specific antigen expression is regulated by an upstream enhancer. J Biol Chem 1996; 271: 7043–51.
20 Reid KJ, Hendy SC, Saito J, Sorensen P, Nelson CC. Two classes of androgen receptor elements mediate cooperativity through allosteric interactions. J Biol Chem 2001; 276: 2943–52.
21 Shaffer PL, Jivan A, Dollins DE, Claessens F, Gewirth DT. Structural basis of androgen receptor binding to selective androgen response elements. Proc Natl Acad Sci USA 2004; 101: 4758–63.
22 Wang Q, Carroll JS, Brown M. Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell 2005; 19: 631–42.

Edited by Prof. Chawnshang Chang