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Identification of Androgen-Responsive Element (ARE) and Sp1 Element in the *Maspin* Promoter

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Abstract

Maspin **is a serine protease inhibitor (serpin) with tumor-suppressing function in mammary gland. It is down-regulated in primary prostate cancer cells and lost in metastatic cells. To better understand the transcriptional regulation of *maspin* gene, the 860bp (-765~+95) of its promoter sequence was amplified by PCR from the human genomic DNA. Then this 860bp sequence and a series of deletions from 5' and 3' ends were inserted into the upstream of luciferase reporter gene respectively. Results from dual luciferase reporter assay and electrophoretic mobility shift assay indicated that there were a negative androgen-responsive element (ARE) in the region of -277 to -262 and a positive Sp1 element in the region of +14 to +35, respectively. In addition, androgen receptor (AR) can recognize and bind to the ARE element, and then inhibit the activity of *maspin* promoter.**

Key Words: *maspin* promoter, prostate cancer, androgen responsive element, Sp1 element

Introduction

Prostate cancer is one of the major life-threatening diseases in men, but its invasion and metastasis at the molecular level are poorly understood. *Maspin* is a member of serine protease inhibitor family with tumor suppressing capability for prostate cancers, hence playing an important role in tumor invasion and metastasis (10, 12, 13). Functional studies have demonstrated that *maspin* inhibits tumor invasion and motility of human prostate cancer cells *in vitro* (14). Tumor growth and metastasis are repressed as well in the nude mice assay (19). Moreover, *maspin* is an effective inhibitor of angiogenesis. When prostate cancer LNCaP cells were implanted into a xenograft mouse model, treatment with recombinant GST-*maspin* inhibited tumor growth as well as vascularization (18). *Maspin* suppressed tumor progression not only at the step of invasion and motility, but also by regulating tumor cell apoptosis. Endogenous *maspin* in mammary carcinoma cells MDA-MB-435 enhances staurosporine (STS)-induced apoptosis (5). All of these data indicated that *maspin* could be developed into a potently therapeutic anticancer factor. Therefore, it is important to elucidate the mechanism underlying the regulation of *maspin* gene in prostate cancer cells.

The *maspin* gene is located at human chromosome 18q21.3 and encodes a 3.0kb mRNA transcript and translates into a 42kDa protein, which is located in cell membrane and extracellular matrix (ECM) of epithelial cells of multiple organs. *Maspin* cDNA was isolated from a library-prepared normal human mammary epithelial (76N) cells. The cDNA sequence contains 2584 nucleotides, including untranslated sequence of 75 nucleotides at 5'-end and 1381 nucleotides at 3' end. Sequence analysis of cDNA and genes from normal and tumor cell lines show that the sequence and structure of *maspin* gene are not altered in the tumor cells. It indicates that *maspin* is possibly down-regulated or silenced rather than mutated in cancer cells (2). In this study, we have cloned 860bp of *maspin* promoter, and carried out the deletion, point-

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mutation and electrophoretic mobility shift assay (EMSA) to define the elements regulating the *maspin* gene expression. Our results showed that a negative element is located at -277 to -262, and a positive element is at +14 to +35 in the *maspin* promoter. In addition, AR may recognize and bind to the ARE element, hence inhibit the transcription of *maspin* gene.

Materials and Methods

Cell Culture

The human prostate cancer cell lines LNCaP and PC-3M were obtained from the American Type Culture Collection. The LNCaP cell line was established from a lymph node metastasis of a prostate cancer patient, and PC-3M cells were isolated from metastases in lumbar vertebra. LNCaP cells express AR, but PC-3M cells do not. LNCaP and PC-3M cells were routinely grown at 37°C in 5% CO_2 incubator with RPMI 1640 media containing 100 units/ml of ampicillin and 100 units/ml streptomycin, and 10% fetal bovine serum (FBS) and 4% FBS (GIBCO BRL Grand Island, NK, USA), respectively.

Reverse Transcription-PCR (RT-PCR) Analysis

Total RNA was isolated from LNCaP and PC-3M cells by TRIzol reagent (MBI) following the manufacturer's instructions. A portion of total RNA (2 μg) was transcribed reversibly with the M-Mulv reverse transcriptase in the presence of random hexamer primer. The resulting cDNA preparation was subjected to PCR amplification using PCR kit from TaKaRa Biotech, China. The primers used for *maspin* were sense 5'-TGCTGCCTACTTTGTTGGCAAGT- 3' and antisense 5'-TGATACTGTCAATGTTTCCCATACAGA-3', and for the housekeeping gene *β-actin*, sense 5'-GTGGGGCGCCCAGGCACCAC-3' and antisense 5'-CTCCTTAATGTCACGCACGATTT-3'.

Western Blot Analysis

LNCaP and PC-3M cells were harvested and lysed as described previously (4). Cell extracts were quantified by BCA method. For Western blot analysis, 40 μg of cell extracts were separated on 10% SDS/PAGE and transferred to nitrocellulose membrane. Then, the nitrocellulose membrane was immediately blocked with 5% non-fat milk in PBS buffer for 1 h at room temperature. After blocking, the membrane was incubated with human specific anti-*maspin* antibodies (BD Biosciences, San Diego, CA, USA) at 4°C for 12 h, followed by the incubation with peroxidase-labeled second antibody for 1 h, and immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). β-tubulin (Sigma, St. Louis, MO, USA) was used to normalize the quantity of the protein on the blot.

Isolation of Maspin Promoter

Human genomic DNA was extracted from white blood cells following the method of rapid isolation of mammalian DNA. The region from -765 to +95 of *maspin* promoter was isolated by PCR using primer F and R. The sequences of primers were as follows:

F: '-GAGACTCGAGAGGCTGAAGTA-CAGTGGTT - 3' (with *XhoI* site at 5' end) R: 5'-GAGAAAGCTTGGCAGAAGCAGCGGTGGCTC - 3' (with *HindIII* site at 5' end) PCR conditions were 94°C for 4 min, followed by 30 cycles of 94°C for 50 s, 60°C for 50 s, and 72°C for 1 min. The products of PCR were cloned into pMD18-T vector (TaKaRa Biotech Co, Dalian, China) and sequenced (Bioasia Biotech, Shanghai, China), then the clone containing *maspin* promoter was designated as pMD-860.

Construction of pGL3-860 Plasmid and its Deletion Mutants

The 860bp fragment was released from pMD-860 recombinant with *XhoI* and *HindIII* cutting and inserted into pGL3-basic vector (Promega Co, Madison, WI, USA) to construct the pGL3-860 (A). A series of deletions of *maspin* promoter, including 5' and 3' deletions, were generated by PCR using pGL3-860 as a template. To insert the amplified fragments into pGL3-basic vector, *XhoI* and *HindIII* sites were created at the 5' and 3' ends of the fragments, respectively. The plasmids containing deletions were named B (-312~+95), C (-277~+95), D (-261~+95), F (-765~+13), and W (-765~+35). The plasmid M includes a mutated Sp1 site in the W construct. All resultant constructs were confirmed by *XhoI* and *HindIII* digestion and sequencing by using the general primers Rvprimer3 and GLprimer2.

Transient Transfection

Cells were seeded in 24-well plates at 1.5×10^5 cells/well. After the cells reached 80-90% confluence, they were transfected with plasmids DNA using lipofectimine™ 2000 (Invitrogen). Each well included 0.8 μg pGL3 construct DNA, 0.04 μg internal control vector pRL-TK DNA, 2 μl lipofectimine™ 2000 and 500 μl RPMI 1640 media without serum and antibiotics. PC-3M cells were co-transfected with human androgen receptor expression vector and pGL3 construct containing different fragment of *maspin* promoter. After 6 h incubation, these cells were

replaced with RPMI1640 medium containing 2% charcoal-stripped FBS (GIBCO BRL) and 10^{-8} mol/l synthetic androgen R1881 (Sigma). R1881 was dissolved in dimethyl sulfoxide (DMSO) which was also a control vehicle. All cells were harvested for dual-luciferase activity assay after 48 h of transfection.

Dual-Luciferase Reporter Assays

The activities of firefly luciferase in pGL3 and Renilla luciferase in pRL-TK were determined by the dual luciferase reporter assay following the protocol from Promega. The cells were rinsed with PBS and then harvested by manually scraping the cells from culture plates using 1×PLB (passive lysis buffer). 20 µl of cell lysate were transferred into the luminometer tube containing 100 µl LAR II, and the firefly luciferase activity (M1) was measured first. Then, the Renilla luciferase activity (M2) was determined after adding 100 µl of Stop & Glo Reagent. M1/M2 was taken as the relative activity of pGL3 construct. The luminometer was programmed to have a 2 seconds of premeasurement delay followed by a 10-second measurement period for each assay.

Nuclear Extracts

LNCaP and PC-3M cells were grown to 90% confluence before being harvested. 2×10^9 cells were pelleted and resuspended in 1 ml cold hypotonic buffer (10 mmol/l HEPES, pH 7.9, 1.5 mmol/l $MgCl_2$, 10 mmol/l KCl, 0.5 mmol/l DTT, 0.5 mmol/l PMSF). Following a 15-min incubation on ice, the cells were lysed by adding 50 µl of 10% NP-40 and centrifuged at 7000 rpm for 5 min at 4°C. Then, the pellets were resuspended in 70 µl of cold hypertonic buffer (20 mmol/l HEPES, pH 7.9, 1.5 mmol/l $MgCl_2$, 420 mmol/l NaCl, 0.2 mmol/l EDTA, 25% glycerol, 10 µg/ml aprotinin, 0.5 mmol/l PMSF). The resuspended cells were stirred gently at 4°C for 30 min and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was extensively dialyzed against the dialysis buffer (20 mmol/l HEPES, pH7.9, 50 mmol/l KCl, 25% glycerol, 0.5 mmol/l DTT, 0.5 mmol/l PMSF) for 2 h at 4°C. The protein concentration of the dialyzed material was determined by BCA method and stored at -80°C in small aliquots.

Electrophoretic Mobility Shift Assay

Equal amounts of sense and antisense oligonucleotides were mixed and annealed in 10 mmol/l Tris·HCl, pH 8.0, 200 mmol/l NaCl, 1 mmol/l EDTA by heating to 95°C for 5 min and cooling to room temperature for over 3 h. The corresponding oligonucleotides were labeled with DIG. The following oligonucleotides were used for EMSA experiments:

Maspin Sp1:
(sense 5'-TGCCGCCGAGGCGGGGCGGGGC-GGGGCGTGGAG-3')
(antisense 5'- GCTCCACGCCCCGCCCCGC-CCCGCCTCGGCGGCA-3');

Maspin ARE:
(sense 5'-AAGAATGGAGATCAGAGTACTT-3')
(antisense 5'-AAGTACTCTGATCTCCATTCTT-3')

Binding reactions were carried out at room temperature for 30 min in a mixture containing 4% glycerol, 1 mmol/l $MgCl_2$, 0.2 mmol/l EDTA, 0.5 mmol/l DTT, 50 mmol/l NaCl, 10 mmol/l Tris·HCl, 2 µg poly (dI-dC), 10 µg nuclear extracts, and DIG-labeled oligonucleotide probe. Then the reaction mixture were subjected to electrophoresis in 5% non-denaturing polyacrylamide gels in 0.25× Tris/borate/EDTA buffer. Based on the instruction of DIG Gel Shift Kit (Roche Co., Indianapolis, IN, USA), electro-blotting and chemiluminescent detection were performed.

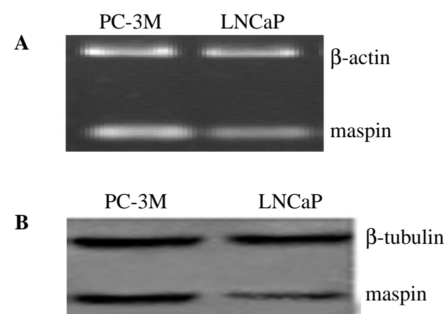

Fig. 1. The profile of *maspin* expression in LNCaP and PC-3M cells. A: RT-PCR analysis for *maspin* in PC-3M and LNCaP cells, in which β-actin was used as an internal control. B: Western blot analysis for *maspin* in PC-3M and LNCaP cells, in which β-tubulin was used as an internal control for protein loading and transfer efficiency.

Results

The Profile of Maspin Expression in LNCaP Cells and PC-3M Cells

Initially, RT-PCR was carried out to determine the status of *maspin* expression in LNCaP and PC-3M cells. The results showed that the *maspin* expression in PC-3M cells was higher than in LNCaP cells (Fig. 1A). To confirm the results of RT-PCR, western blot analysis was carried out for *maspin*. As expected, *maspin* expression was strongly detected in PC-3M cells compared with in LNCaP cells (Fig. 1B).

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-765   AGGCTGAAGTACAGTGGTTAGCTCACGGCTCACTGCAGCTTTGACCTCCCAGGCTCAAGTGATCCTCTCG
-695   TCTCAGCTTTCCAAGTAACTGGGACCACAGGCATGCATCACCACACTAGGCTATTGTTTTACATTTTTTG
-625   TAGAGATGGGGTCTCACCATGTTGCCCAGGTTGGTCTCAAACTCCTGGGCTCAAGCAATCCGCTCACGTC
-555   AACCTCCCCAAATGCTGGGATTACAGGCGTGAGCCACCGCGCCAGGCCTGAGTAATCCTAATCACAGGAT
-485   TTTAAAAAGAAACTTCCTGCGCCACCCATTAAACAATATCTCCTACCAATTTGGTAGTAAATATTTTGCT
-415   AATAGTACCTAATTTTTAGGTAGGCACTGTGTTTATACATATATCCATTCCTTCTTTTTTGATTGTCTTT
-345   CTGTTTAATGGGCAGCTACCTCTCTTGGCATCTAGCAGAATGAGCTGCTGCAGTTTACACAAAAAGAATG
-275   GAGATCAGAGTACTTTTTGTGCCACCAACGTGTCTGAGAAATTTGTAGTGTTACTATCATCACACATTAC
         ARE
-205   TTTTATTTCATCGAATATTTCACCTTCCGGTCCTGCGTGGGCCGAGAGGATTGCCGTACGCATGTCTGTA
-135   CGTATGCATGTAACTCACAGCCCCTTCCTGCCCGAACATGTTGGAGGCCTTTTGGAAGCTGTGCAGACAA
-65    CAGTAACTTCAGCCTGAATCAATTTCTTTCAATTGTGGACAAGCTGCCAAGAGGCTTGAGTAGGAGAGGA
+5     GTGCCGCCGAGGCGGGGCGGGGCGGGGCGTGGAGCTGGGCTGGCAGTGGGCGTGGCGGTGCTGCCCAGGT
                     Sp1
+75    GAGCCACCGCTGCTTCTGCC
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Fig. 2. Sequence of *maspin* promoter. Nucleotides are numbered on the left side, with the transcriptional start site designated +1 and boxed. The putative elements are double underlined.

Cloning of Maspin Promoter

To isolate 860bp *maspin* promoter, routine PCR was used according to the reported DNA sequence. The specific DNA fragment was amplified and sequenced (Fig. 2). Software (TRANSFAC) analysis of the *maspin* promoter revealed the presence of androgen receptor element (ARE) and Sp1 element in the region of (-277~-262) and (+14~+35), respectively.

5' Deletions Analysis in LNCaP Cells

In order to localize cis-element in *mapsin* promoter, 5' deletion mutants were constructed and transfected into LNCaP cells (androgen-resposive prostate cancer cell lines). The relative LUC activities were assayed by dual-luciferase reporter assays. SV40 enhancer-LUC and pGL3-basic vector were used as a positive and a negative control, respectively. The activity of full length promoter (A) was about 2-fold higher than positive control and about 100-fold higher than the negative one. Deletion from -765bp to -313bp decreased the activity about 2-fold, and further deletion from -312bp to -278bp did not alter the activity significantly, but deletion from -277bp to -262bp, which removed ARE (androgen responsive element) homologous sequence, reversed the activity to almost as high as full length promoter (Fig. 3). The results indicated that the ARE site in the *maspin* promoter was a negative element for *maspin* expression.

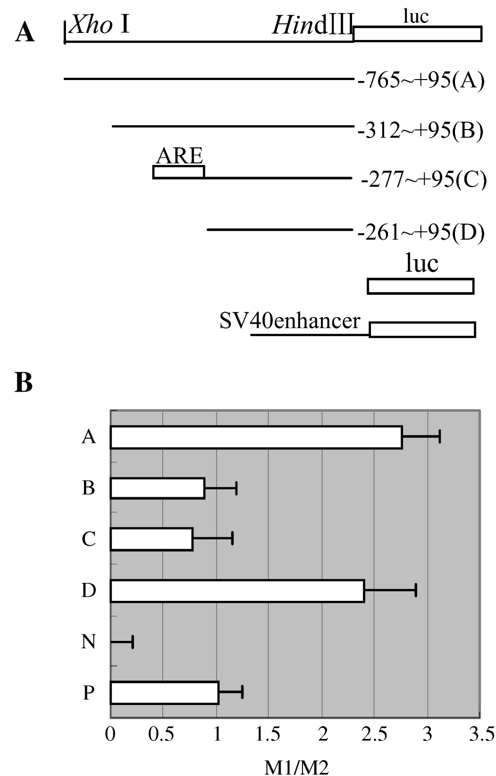

Fig. 3. Maspin constructs and LUC assays. A: LUC constructs and a schematic representation of *maspin* promoter with putative transcription factor binding sites. B: LUC assays in LNCaP cells, along with a negative control N and a positive control P. Relative activity was represented by normalizing to pRL-TK. Values were obtained from at least five experiments.

5' Deletions Analysis in PC-3M Cells

Androgen and its receptor (AR) play a significant role in the development and progression of prostate cancer. To investigate whether AR was bound to ARE in the *maspin* promoter, we cotransfected 5' deletions and human androgen receptor expression vector into androgen-refractory prostate cancer cells

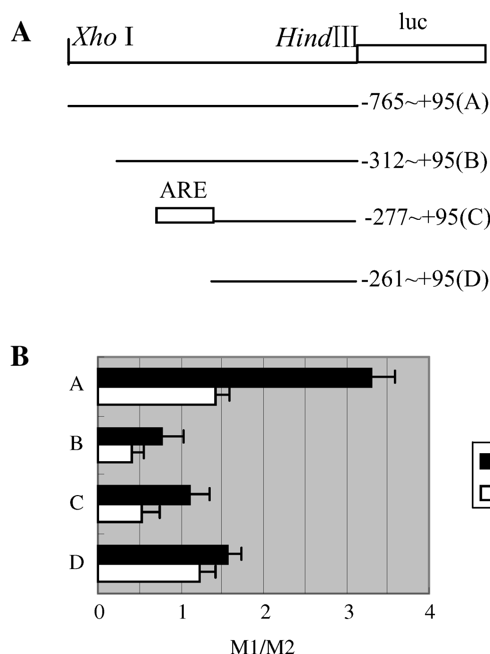

Fig. 4. Maspin constructs and LUC assays in PC-3M cells. A: LUC constructs and a schematic representation of *maspin* promoter with putative transcription factor binding sites. B: LUC assays in PC-3M cells. Relative activity was represented by normalizing to pRL-TK. Values were obtained from at least five experiments.

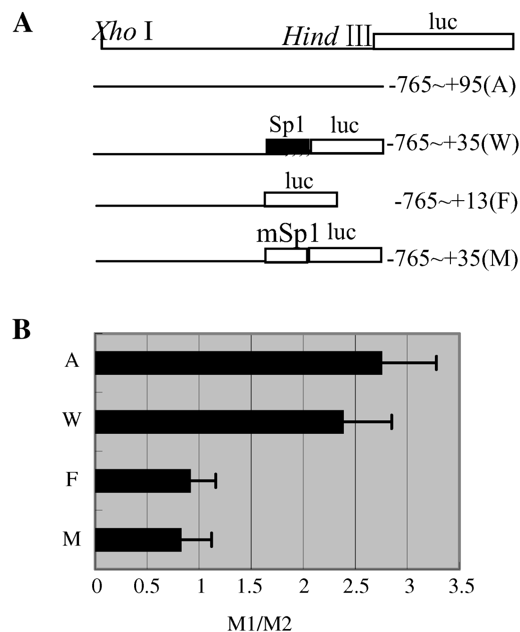

Fig. 5. LUC assays of 3' deletion in LNCaP cells. A: LUC constructs and a schematic representation of *maspin* promoter with putative transcription factor binding sites. M construct includes a mutated Sp1 site (mSp1). B: LUC assays in LNCaP cells. Relative activity was represented by normalizing to pRL-TK. Values were obtained from at least three experiments.

PC-3M (AR^-) with 10^{-8} mol/l synthetic androgen R1881. As shown in Fig. 4, AR inhibited the activity of *maspin* promoter (A, B, C) containing ARE element for up to 50%, but only 20% inhibition of the activity of *maspin* promoter (D) without ARE element was observed. This indicated that AR might bind to ARE element in the *maspin* promoter and inhibit the transcription of *maspin* gene.

3' Deletion Analysis in LNCaP Cells

The Sp1 element in the *maspin* promoter shares 94% homology with the consensus sequence. When Sp1 element (CCGCCCCGCCCCGCCCCGCAC) was deleted in F construct, the activity decreased about 2-fold. To further confirm the function of Sp1 element in the *maspin* promoter, Sp1 element was mutated in M construct. As shown in Fig. 5, the mutation (CCGC*T*C*T*GCCC*A*GTCC*T*G*T*AC) abolished the activity of Sp1 element. This might be an implication that Sp1 element was involved in the transcriptional activation of *maspin*.

AR Recognized and Boud to the ARE Element in the Maspin Promoter

To confirm the binding specificity of AR to ARE of the *maspin* promoter in prostate cancer cells, the ARE oligonucleotides was DIG-labeled and reacted with nuclear extracts from LNCaP and PC-3M cells, respectively. A specific DNA-protein complex was identified with LNCaP nuclear extracts (AR^+), but not with PC-3M nuclear extracts (AR^-). The band was competed by unlabeled ARE oligonucleotides, and by unlabeled ARE oligonucleotides in the basic promoter of prostate specific antigen (PSA). In addition, the monoclonal antibody against AR in the reaction mixture blocked the formation of the DNA-protein complex (Fig. 6). The results indicated that AR recognized and was bound to the ARE element in the *maspin* promoter.

Sp1 Element in the Maspin Promoter Binds to Some Proteins in Prostate Cancer Cells

To investigate the interaction of Sp1 element with some proteins in prostate cancer cells, the oligonucleotides corresponding to the Sp1 site were labeled with DIG and used in EMSA experiments with nuclear extracts from LNCaP and PC-3M cells. Specific DNA-protein complex were identified with both LNCaP and PC-3M nuclear extracts (Fig. 7), and the band could be competed by unlabeled Sp1 oligonucleotides, but not by nonspecific

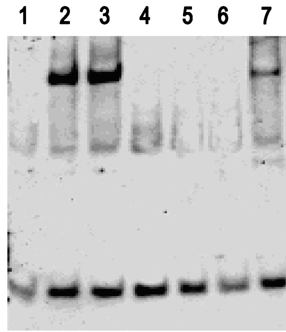

Fig. 6. EMSA experiment for ARE. DIG-labeled ARE oligonucleotides were incubated without extracts (lane 1) or with extracts from LNCaP cells (lanes2-5, 7) or PC-3M cells (lane 6). Competition was carried out with nonspecific oligonucleotides (lane 3), 125 × unlabeled wild-type ARE oligonucleotides (lane 4), 300 × unlabeled ARE oligonucleotides of PSA promoter (lane 5); After 20-min incubation of AR antibody and the reaction mixture, DIG-labeled ARE oligonucleotides was added into the mixture (lane 7).

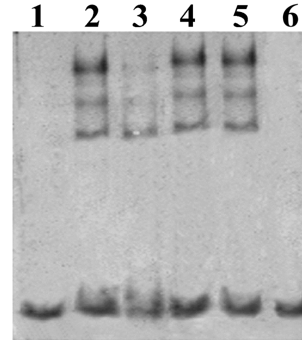

Fig. 7. EMSA experiment for Sp1. DIG-labeled Sp1 oligonucleotides were incubated without extracts (lane 1) or with extracts from LNCaP cells (lanes 2-4) or PC-3M cells (lane 5); Competition was carried out with 125 × unlabeled wild-type Sp1 oligonucleotides (lane 3); nonspecific oligonucleotides (lane 4). mSp1 oligonucleotides were incubated with extracts from LNCaP cells (lane 6).

oligonucleotides. Moreover, mSp1 (mutation of Sp1 elements) oligonucleotides did not form DNA-protein complex with nuclear extracts from LNCaP cells. These results revealed that some proteins in prostate cancer cells had high affinity for the Sp1 element.

Discussion

Maspin gene is a tumor-suppressing gene and closely related to the metastasis of prostate cancer, which makes this gene a promising target for therapeutic intervention in prostate cancer. To understand the regulation of *maspin* gene in prostate cancer cells, the promoter activities of *maspin* in LNCaP and PC-3M cells were analyzed. Its activity decreased with the progressive deletion from -765 bp to -278 bp, and there exists a positive element related to epidermal growth factor (EGF) in the region of (-604~-589) (paper accepted). This study showed that unexpectedly, AR could bind to ARE of the *maspin* promoter and inhibit the activity of *maspin* promoter. As shown in Fig. 4, after deletion of ARE (-277~-262) in the *maspin* promoter, the activity of *maspin* promoter in PC-3M cells transfected with AR expression vector just slightly increased and could not recover as high as in LNCaP cells. PC-3m cells are more aggressive than LNCaP cells. It may have acquired additional changes that bypass the normal regulation of *maspin* expression. Androgens and AR are generally thought to contribute to the development and progression of prostate cancer. They play their roles by regulating the expression of some genes (9). AR accelerates the progression of prostate cancer maybe in part by inhibiting the *maspin* gene expression.

Maspin gene is located at chromosome 18q21.3. It consists of 8 exons and 7 introns, and exon 1 consists of nucleotides from +1 to +184 (17). However, exon 1 is not coded. Sp1 element was found in the region of (+14~ +35) by software (TRANSFAC). Sp1 is one of the cellular transcription factors. It was identified and cloned in virtue of its binding to a GC-rich motif in the SV40 early promoter. Sp1 are ubiquitously expressed and are required for the constitutive and inducible expression of a variety of genes (1, 15, 16). More reports showed that 5' untranslated region of mRNA participate in the regulation of gene expression (7, 11). In order to test whether Sp1 element is involved in the regulation of *maspin* gene, we deleted and mutated Sp1 element, and then found corresponding promoter activities decreased about 2-fold. In addition, three specific DNA-complexes were identified with both LNCaP and PC-3M nuclear extracts by EMSA experiments. It implied that Sp1 element might play a positive role in the transcription of *maspin*. Sp1 has two other closely related members of a gene family which encodes proteins with very similar structure but different molecular weights, Sp3 and Sp4. Most significantly, the DNA binding domains of Sp1, Sp3, and Sp4 are highly conserved and can recognize the GC box with identical affinities (3).

We have detected the promoter activities of *maspin* in both androgen-responsive (LNCaP) and androgen-refractory prostate cancer cells (PC-3M). The results showed that the repression function of ARE of the *maspin* promoter was inactive in PC-3M cells, while the activation function of Sp1 was intact in both LNCaP and PC-3M cells. We have also

demonstrated that *maspin* gene expression is higher in PC-3M cells than in LNCaP cells at the mRNA and protein level by using RT-PCR and Western blot (Fig. 1). It speculates that this result may result from the impaired repression of ARE and the intact activation of Sp1 in PC-3M cells. However, the reason of up-regulated *maspin* gene expression in advanced prostate cancer cells still awaits further study.

There is no effective therapy for the metastasis of prostate cancer at present. Reexpression of *maspin* gene in the prostate tumors offers great hope for the therapy of prostate cancer metastasis. *Maspin* expression is directly regulated by the *p53* gene. *p53* induces *maspin* expression in prostate cancer cells and suppresses the tumor metastasis (20). The manganese-containing superoxide dismutase might imply the up-regulation of *maspin* in human breast cancer cells (8). γ-Linolenic acid, an essential fatty acid with anticancer properties, is reported to induce *maspin* expression and inhibit the cell motility (6). Our discovery of ARE-mediated repression and Sp1-mediated activation provides another approach to increase the expression of *maspin* in prostate tumors, which in turn reduce the metastasis of prostate cancer. Future studies will focus on the identification of the upstream molecular pathways influencing the expression of AR and Sp1 protein.

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