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Sp1 and Sp3 transcription factors upregulate the proximal promoter of the human prostate-specific antigen gene in prostate cancer cells

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Abstract

The serum level of prostate-specific antigen (PSA) is useful as a clinical marker for diagnosis and assessment of the progression of prostate cancer, and in evaluating the effectiveness of treatment. We characterized four Sp1/Sp3 binding sites in the proximal promoter of the *PSA* gene. In a luciferase assay, these sites contributed to the basal promoter activity in prostate cancer cells. In an electrophoretic mobility shift assay and chromatin immunoprecipitation assay, we confirmed that Sp1 and Sp3 bind to these sites. Overexpression of wild-type Sp1 and Sp3 further upregulated the promoter activity, whereas overexpression of the Sp1 dominant-negative form or addition of mithramycin A significantly reduced the promoter activity and the endogenous mRNA level of PSA. Among the four binding sites, a GC box located at nucleotides −53 to −48 was especially critical for basal promoter activity. These results indicate that Sp1 and Sp3 are involved in the basal expression of PSA in prostate cancer cells.

Keywords: Prostate-specific antigen; Sp1/Sp3; Transcriptional regulation; Promoter; Prostate cancer

Prostate cancer is one of the most frequent cancers in older men. Prostate-specific antigen (PSA)[1] is expressed exclusively in normal, hyperplastic, and malignant prostatic epithelia [1]. The serum level of PSA is a useful clinical marker for the diagnosis and assessment of the progression of prostate cancer, and in evaluating the effectiveness of treatment [2]. After androgen ablation therapy, cancer progression and serum PSA levels are reduced in most patients. However, in most cases, the tumor progresses again despite androgen deprivation, and no longer responds to endocrine therapy. The progression of prostate cancer to androgen independence is commonly associated with a rebound in serum PSA levels [3]. PSA is a 33 kDa chymotrypsin-like serine protease that belongs to the kallikrein family. The well-known biological function of this enzyme is in the liquefaction of seminal coagulum by digestion of the high-molecular-weight seminal–vesicle protein, seminogelin [4]. PSA also degrades insulin-like-growth-factor-binding protein 3 [5]. It is thought to play several roles in tumor biology, such as in the attachment of cancer cells to bone-marrow endothelium [6].

The expression of PSA is tightly regulated by androgens through the androgen receptor (AR) [7–9]. The regulation of the *PSA* gene has been intensively characterized. A region of up to 6 kb upstream from the transcription start site is responsible for its expression [10,11]. The proximal *PSA* promoter has been defined as the fragment to nucleotide −630, which contains a core

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[1] *Abbreviations used:* PSA, prostate-specific antigen; EMSA, electrophoretic mobility shift assay; ChIP assay, chromatin immunoprecipitation assay; AR, androgen receptor; Sp, specificity protein; PBS, phosphate-buffered saline.

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TATA box [10,12], and the enhancer element has been mapped to around the −4 kb region [10,11]. There are several androgen receptor (AR)-binding sites in both the proximal promoter and the enhancer region [10,12]. Other transcription factors also bind in these regions [13–16]. The enhancer region contains most of the elements required for the androgen regulation and prostate-specific expression of the *PSA* gene [10,11,17]. On the other hand, little progress has been made in determining the AR-independent regulation of *PSA* gene expression.

Specificity protein (Sp)1 is a ubiquitously expressed transcription factor that plays an important role in the transcription of many genes containing GC-rich motifs in their promoters [18]. To date, other members of the Sp family, Sp2–Sp8, have been reported [19]. The biochemical properties of Sp5–Sp8 have not been well studied. Sp2 does not recognize the same sequences as Sp1. Sp4 expression is restricted to brain tissue. Both Sp1 and Sp3 are expressed ubiquitously and compete for common target sequences. Sp1 is a transcriptional activator, whereas Sp3 can act as either a transcriptional activator or a repressor. Although the activity of Sp1 is believed to be constitutive, it has recently been shown to participate in activities such as cell differentiation [20], the cell cycle [21], development [22], tolerance of low pH conditions [23], and the enhancement of drug resistance [24]. Sp1 mRNA level and Sp1 DNA binding activity are increased in squamous cell carcinoma in the skin [25], and Sp1 is constitutively overactive in human pancreatic [26] and gastric cancers [27].

In this study, we showed that Sp1 and Sp3 bind in the proximal region of the *PSA* promoter, and contribute to the basal expression in prostate cancer cells.

Materials and methods

Materials

The materials used and their sources were as follows: RPMI 1640 and Mithramycin A, from Sigma (St. Louis, MO); Lipofectamine Plus Reagent System, from Invitrogen (Carlsbad, CA); Dual-Luciferase Reporter Assay System, from Promega (Madison, WI); ECL Plus, from Amersham Biosciences (Piscataway, NJ); ChIP Assay Kit, from Upstate Biotechnology (Lake Placid, NY); and the polyclonal rabbit antibodies against human Sp1 and Sp3, from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells and cell-culture conditions

The human prostate cancer cell line, LNCaP, was purchased from the American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (Sanko Junyaku, Tokyo, Japan) at 37 °C in a humidified 5% CO_2/95% air environment.

Construction of chimeric plasmids

PCR procedures were used to generate 5′ stepwise deletion constructs of the *PSA* promoter. PCR was performed using sets of oligonucleotide primers specific for the human *PSA* gene sequence, of which the forward primer was *Sac*I-site-linked and the reverse primer *Hin*dIII-site-linked (Table 1). LNCaP genomic DNA was used to generate the pGL3 −761/+12 construct, which was used as the template for the other constructs. These PCR products were subcloned into the pGEM-T Easy vector (Promega), then digested with *Sac*I and *Hin*dIII and subcloned into the *Sac*I/*Hin*dIII sites of the pGL3 Basic vector (Promega).

Substitution mutation constructs were generated by site-directed mutagenesis [28] using the pGL3 −761/+12 plasmid as template. For six-base substitutions, we introduced the *Pst*I sequence, CAGCTG, into the substitution sites. The primers used in the PCR amplifications are shown in Table 1. The PCR products were digested with endonuclease and then self-ligated. All mutagenesis plasmids were digested with *Sac*I and *Hin*dIII, and recloned into the *Sac*I/*Hin*dIII sites of the pGL3-Basic vector.

The expression plasmids used were pCMV-Sp1, kindly provided by Dr. Elder [29], and pRC/CMV-Sp3 from Dr. Suske [30]. Both plasmids are under the control of the cytomegalovirus (CMV) promoter. The dominant-negative Sp1 plasmid, pEBG-Sp1, was a generous gift from Dr. Thiel [31].

Transient transfection and luciferase assay

LNCaP cells were plated at a density of 2×10^5/35 mm dish about 18 h before transfection. For transient transfections, 0.5 μg of plasmid DNA was transfected into these cells using the Lipofectamine Plus Reagent System (Invitrogen). The plasmid phRL-TK vector (Promega) was always cotransfected as an internal control for transfection efficiency. After further cultivation under serum-deprived conditions for 24 h, the transfected cells were harvested, lysed, centrifuged to pellet the debris, and subjected to the luciferase assay. Luciferase activity was measured as chemiluminescence in a luminometer (Lumat LB 9507, Perkin–Elmer Life Sciences) using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Cotransfection experiments were performed with 0.5 μg of Sp1 or Sp3 expression plasmids or 1 μg of the dominant-negative Sp1 plasmid. Mithramycin A was incubated with concentration of 5, 25, and 100 nM for 24 h before luciferase assays [32]. All transfections were performed in

triplicate and the results are expressed as means ± standard deviations (SD) of three independent experiments.

Preparation of nuclear extracts

Nuclear extracts from LNCaP cells were prepared according to the method of Dignam et al. with some modifications [33,34]. All buffers contained the protease inhibitors leupeptin (2 μg/ml), aprotinin (2 μg/ml), pepstatin A (2 μg/ml), phenylmethylsulfonyl fluoride (PMSF; 0.5 mM), and dithiothreitol (DTT; 1 mM). Cells (1×10^8) were briefly scraped in ice-cold phosphate-buffered saline (PBS), centrifuged for 5 min at 1500 rpm, and washed with PBS before recentrifugation. The pellets were resuspended in buffer (10 mM Hepes, pH 7.8, 10 mM KCl, 0.1 mM ethylenediaminetetraacetic acid [EDTA], and 0.1% Nonidet P-40), incubated on ice for 10 min, and homogenized. Nuclei were pelleted by centrifugation at 3000 rpm for 10 min at 4 °C, then resuspended in buffer (50 mM Hepes, pH 7.8, 420 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 20% glycerol) and mixed by rotation at 4 °C for 1 h. After centrifugation at 24,000*g* for 30 min at 4 °C, the supernatants were collected and stored at −80 °C until used. The protein concentrations of the nuclear extracts were determined by Bradford assay (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard.

Electrophoretic mobility shift assays

Probes used for electrophoretic mobility shift assays (EMSA) were generated by PCR using each set of *Hin*dIII-site-linked primers (Table 1). All PCR products were subcloned into the pGEM-T Easy vector. All plasmids were digested with *Hin*dIII and the digested fragments were radiolabeled with [α-^{32}P]dCTP using Klenow fragment to fill in the *Hin*dIII overhanging sites.

The binding reaction was carried out for 30 min at 25 °C in 25 μl of binding buffer (50 mM Hepes, pH 7.8, 250 mM KCl, 25 mM $MgCl_2$, 5 mM EDTA, and 50% glycerol) containing 20,000–30,000 cpm of labeled probe, 3 μg of poly(dI–dC), and 15 μg of nuclear extract.

For the competition assays, a double-stranded oligonucleotide containing the consensus Sp1 binding site was generated by annealing equimolar complementary single-stranded oligonucleotides. The consensus Sp1 binding site is 5′-ATTCGATCGGGGCGGGGC GAGC-3′.

For the supershift assay, anti-Sp1 and anti-Sp3 polyclonal antibodies and preimmune goat IgG were purchased (Santa Cruz Biotechnology). For the interference assays, a 20- or 100-fold molar excess of unlabeled competitor or 4 μg of antibody was added to the reaction mixture for 1 h at 4 °C before the addition of radiolabeled probe.

Table 1
Primers used for PCR procedure in the experiments

1. For generation of luciferase constructs of 5′ stepwise deletion (see Fig. 1A)
   pGL −761/+12: (sense; −761) 5′-*gagctc*TATTTGTTGGAGAAGGGGCATTG-3′
   pGL −153/+12: (sense; −153) 5′-*gagctc*CTCTCCCTCCCCTTCCAC-3′
   pGL −100/+12: (sense; −100) 5′-*gagctc*GCAGCATGGGGAGGGCCT-3′
   pGL −81/+12: (sense; −81) 5′-*gagctc*GGTCAGCCTCTGGGTGCC-3′
   Common: (anti-sense; +12) 5′-*aagctt*GGGGCTGGGGAGCCTCCC-3′
   (Italic letters indicate tagged *Sac*I and *Hin*dIII sites in the sense and anti-sense primer for cloning.)

2. For generation of oligonucleotides used for EMSA (see Figs. 2A and 6A)
   −302/−256: (sense) 5′-*aagctt*TATGAAGAATCGGGGATC-3′
   (anti-sense) 5′-*aagctt*AGGATGAAACAGAAACAG-3′
   −147/−101: (sense) 5′-*aagctt*CTCCCCTTCCACAGCTCT-3′
   (anti-sense) 5′-*aagctt*TGGAGGCTGGACAACCCC-3′
   −109/−68: (sense) 5′-*aagctt*CCCAGAGGCTGACCAAGG-3′
   (anti-sense) 5′-*aagctt*CAGCCTCCAGCAGCATGG-3′
   −72/−31: (sense) 5′-*aagctt*CTGGGTGCCAGCAGGGCA-3′
   (anti-sense) 5′-*aagctt*CTTCATTCCCCAGGACTC-3′
   (Italic letters indicate tagged *Hin*dIII sites in the primer for cloning.)

3. For generation of oligonucleotides and luciferase constructs of substitution mutation (see Figs. 6A and 7A)
   M1: (sense M1) 5′-*ctgcag*GAGTCCTGGGGAATAAG-3′
   (anti-sense M1) 5′-*ctgcag*TGCCCTGCTGGCACCCAG-3′
   M2: (sense M2) 5′-*ctgcag*CCTTGGTCAGCCTCTGGG-3′
   (anti-sense M2) 5′-*ctgcag*CCATGCTGCTGGAGGCTG-3′
   M3: (sense M3) 5′-*ctgcag*AGGGGGTTGTCCAGCCTC-3′
   (anti-sense M3) 5′-*ctgcag*CCCAGAGCTGTGGAAGGG-3′
   M4: (sense M4) 5′- *ctgcag*CCTGTTTCTGTTTCATCC-3′
   (anti-sense M4) 5′- *ctgcag*GTACGATCCCCGATTCTT-3′
   (Italic letters indicate tagged *Pst*I sites in the primer for generating mutation.)

The DNA–protein complexes were separated on a 4.5% nondenaturing polyacrylamide gel in 0.25× Tris–borate electrophoresis buffer at 200 V. Following electrophoresis, the gel was transferred onto 3 MM paper (Whatman, Maidstone, England) and dried under vacuum. The dried gel was visualized by autoradiography using the FLA-5000 imaging system (Fuji Film, Tokyo, Japan).

Western blot analysis

Immunoblotting was performed as described by Sambrook et al. [35]. Briefly, proteins were separated on a sodium dodecyl sulfate (SDS)–10% polyacrylamide gel and transferred to a 0.2 μm Pall FluoroTrans W Membrane (Nippon Genetics, Tokyo, Japan). Nonspecific binding was blocked overnight with 5% nonfat milk in PBS. Anti-Sp1 or anti-Sp3 polyclonal antibody (Santa Cruz Biotechnology) was used as the primary antibody at a 1:1000 dilution. Secondary antibody directed against the anti-Sp1 and anti-Sp3 antibodies (horseradish–peroxidase-conjugated anti-rabbit antibody) was used at a 1:3000 dilution. Detection was performed with ECL Plus (Amersham Biosciences).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) assay was performed with a kit (Upstate Biotechnology) according to the manufacturer's protocol, with some modifications. All buffers contained PMSF (1 mM), aprotinin (1 μg/ml), and pepstatin A (1 μg/ml). Briefly, LNCaP cells (2×10^6) were suspended in 1 ml of culture medium and fixed in a final concentration of 1% formaldehyde for 15 min at room temperature. After the addition of 100 μl of 1.5 M glycine and incubation for 10 min at room temperature, the cells were pelleted by centrifugation at 3000 rpm for 5 min at 4 °C, then resuspended in cold PBS and mixed by rotation at 4 °C for 10 min. After centrifugation, the cell pellets were resuspended in SDS lysis buffer, incubated for 10 min at 4 °C, and sonicated four times for 10 s, yielding DNA fragments of 200–1000 bp. After centrifugation, the supernatant was diluted in ChIP dilution buffer, precleared with a salmon sperm DNA (ssDNA)/protein A–agarose slurry, and immunoprecipitated with the indicated antibodies overnight at 4 °C. Immunocomplexes were captured in the ssDNA/protein A–agarose slurry, and washed with low salt wash buffer, high-salt wash buffer, and LiCl wash buffer, then finally washed twice with TE buffer. The immunocomplexes were eluted by incubation for 15 min at 25 °C with 400 μl of elution buffer (1% SDS, 100 mM $NaHCO_3$, and 1 mM DTT). To reverse the DNA cross-linking, the eluates were treated with 16 μl of 5 M NaCl and incubated for 6 h at 65 °C, then treated with proteinase K for 1 h at 45 °C. The DNA fragments were extracted with phenol/chloroform and precipitated with ethanol. PCR was performed for 35 step cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 2 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with ethidium bromide.

RNA extraction, cDNA synthesis, and LightCycler real-time PCR

For the inhibition experiments, the dominant-negative Sp1 plasmid (4 μg) was transfected or mithramycin A (100 nM) was added to each 100 mm dish of subconfluent LNCaP cells for 24 h before the cells were harvested. Total RNA was prepared from the cells using an ISOGEN kit (Nippon Gene, Tokyo, Japan). Total RNA (2 μg) was reverse-transcribed using MuLV reverse transcriptase (Applied Biosystems, Warrington, UK) after random hexamer priming. The mRNA levels of PSA were quantified with the LightCycler System using LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). As an internal control, human ribosomal S26 RNA was also quantified. The primers for PSA were 5′-CCTCC TGAAGAATCGATTCCT-3′ (sense) and 5′-CGTCC AGCACACAGCATGAA-3′ (anti-sense). The primers for S26 were 5′-CGTGCCTCCAAGATGACAAA-3′ (sense) and 5′-TAAATCGGGG TGGGGGTGTT-3′ (anti-sense). The PCR protocol included a denaturation step at 95 °C for 5 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 57 °C for 10 s, and extension at 72 °C for 15 s. The specificity of the PCR products was confirmed with melting curve analysis by the presence of single melting curves and reconfirmed by agarose gel electrophoresis. The fit point method was used to determine the concentration using LightCycler Software version 3.3 (Roche Molecular Biochemicals). The PSA mRNA levels are expressed relative to the levels of S26 RNA in the same samples, as the means ± SD of three independent experiments.

DNA sequencing

Nucleotide sequences were determined by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Foster, CA) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Statistical analysis

An unpaired t test was used to determine statistical significance.

Results

Identification of Sp1/Sp3 binding sites and deletion analysis of the human PSA promoter

Sp1 and Sp3 are involved in the regulation of many aspects of cancer biology. To analyze the role of Sp1 and Sp3 transcription factors in the regulation of the human *PSA* gene, we initially searched for the consensus Sp1/Sp3 binding element in the proximal promoter region using MatInspector (http://www.genomatrix.de) and TFSEARCH (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html). In the −761 to +12 region of the proximal promoter, we found four putative Sp1/Sp3 binding sites that included one GC box and three GT/A-rich motifs (Fig. 1A).

To remove the Sp1/Sp3 binding sites one by one, a series of chimeric 5′ stepwise deletion constructs linked to the luciferase gene were generated. Luciferase assays were performed with LNCaP cells, a human prostate cancer cell line. The longest construct, pGL3 −761/+12, had detectable promoter activity compared with that of the negative control, pGL3 Basic. The activity of the shorter constructs decreased gradually as the Sp1/Sp3 binding sites were lost (Fig. 1B).

Binding of Sp1 and Sp3 to the four consensus elements of the human PSA promoter

To identify Sp1/Sp3 binding to the consensus elements in vitro and in vivo, EMSA and ChIP assay were performed, respectively. For the EMSA, we prepared four oligonucleotides that cover the putative Sp1/Sp3 sites (Fig. 2A). First, the −72/−31 probe bound nuclear protein extracted from LNCaP cells, producing three bands (Fig. 2B). These bands were competitively inhibited with unlabeled excess −72/−31 oligonucleotide or consensus Sp1/Sp3 binding oligonucleotides in a dose-dependent manner, but were not competitively inhibited by random ssDNA.

To determine whether these bands were DNA–Sp1 or DNA–Sp3 complexes, we used an interference assay

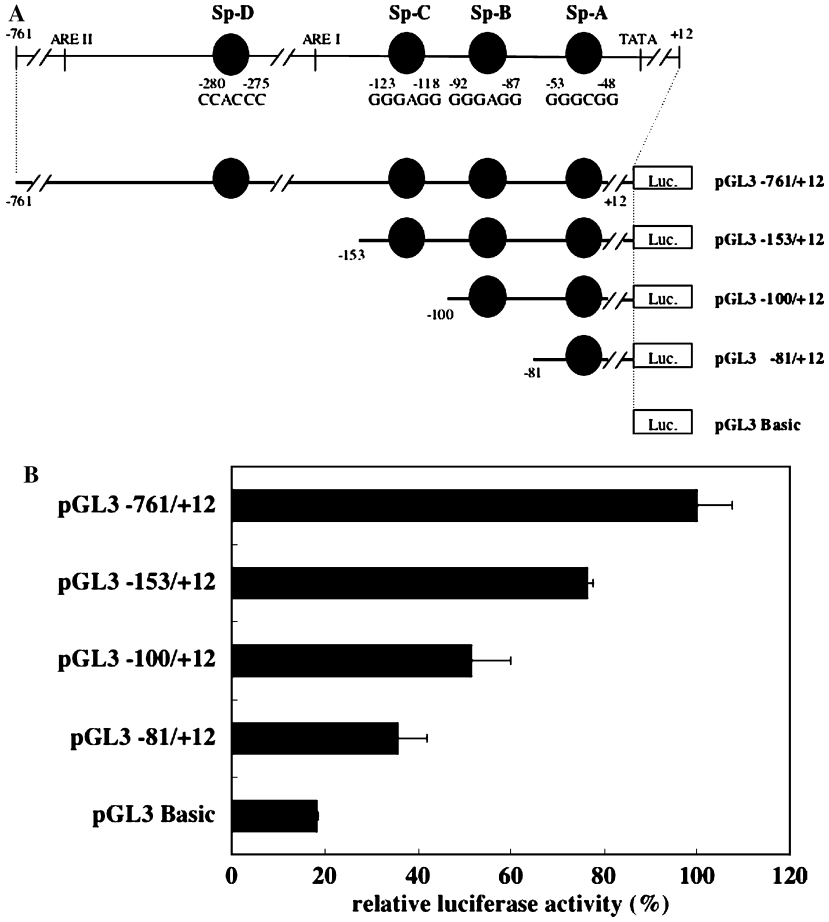

Fig. 1. Identification of Sp1/Sp3 binding sites and deletion analysis of the *PSA* promoter. (A) Schematic representation of the *PSA* proximal promoter and the 5′-deleted promoter-luciferase constructs. Computer analysis revealed four Sp1/Sp3 binding sites, Sp-A, -B, -C, and -D. (B) Luciferase activity in LNCaP cells. All the constructs were cotransfected with the phRL-TK vector as an internal control for transfection efficiency. The histograms indicate activities (%) relative to that of pGL3 −761/+12. Data are means ± SD of three independent experiments.

with specific antibodies. The upper band was supershifted by anti-Sp1 antibody and the middle and lower bands by anti-Sp3 antibody. On the other hand, control normal goat IgG did not supershift any band. These results are consistent with the results of the experiment in which the consensus Sp1/Sp3 binding oligonucleotide was used as probe. We concluded that the upper band was a DNA–Sp1 complex and the middle and lower bands were DNA–Sp3 complexes.

As shown in Fig. 2C, the other probes, oligonucleotides −302/−256, −147/−101, and −109/−68, showed bands similar to those produced with −72/−31, although additional complexes were also seen when these probes were used. To examine whether these bands contained Sp1 or Sp3 complexes, competition assays were performed. Three bands, which migrated to positions similar to those of the Sp1 and Sp3 complexes formed in the experiment with the −72/−31 probe, were competitively inhibited with an unlabeled 100-fold molar excess of the corresponding oligonucleotides or consensus Sp1/Sp3 binding oligonucleotides. However, other bands were inhibited incompletely and no band was ever inhibited with ssDNA. Consistent with these data, the inhibited bands were shifted with specific anti-Sp1 or anti-Sp3 antibody, but the other bands were not (Fig. 2D). These results indicate that Sp1 and Sp3 bind specifically to one GC box and three GT/A-rich motifs in the *PSA* proximal promoter in vitro.

To examine whether Sp1 and Sp3 bind directly to the *PSA* promoter, we performed a ChIP assay. DNA–protein complexes were immunoprecipitated with antibodies, the cross-links were reversed, and the recovered DNA fragments were monitored by PCR using primers for the −302 to −31 bp region of the *PSA* gene. As shown in Fig. 3, DNA fragments immunoprecipitated

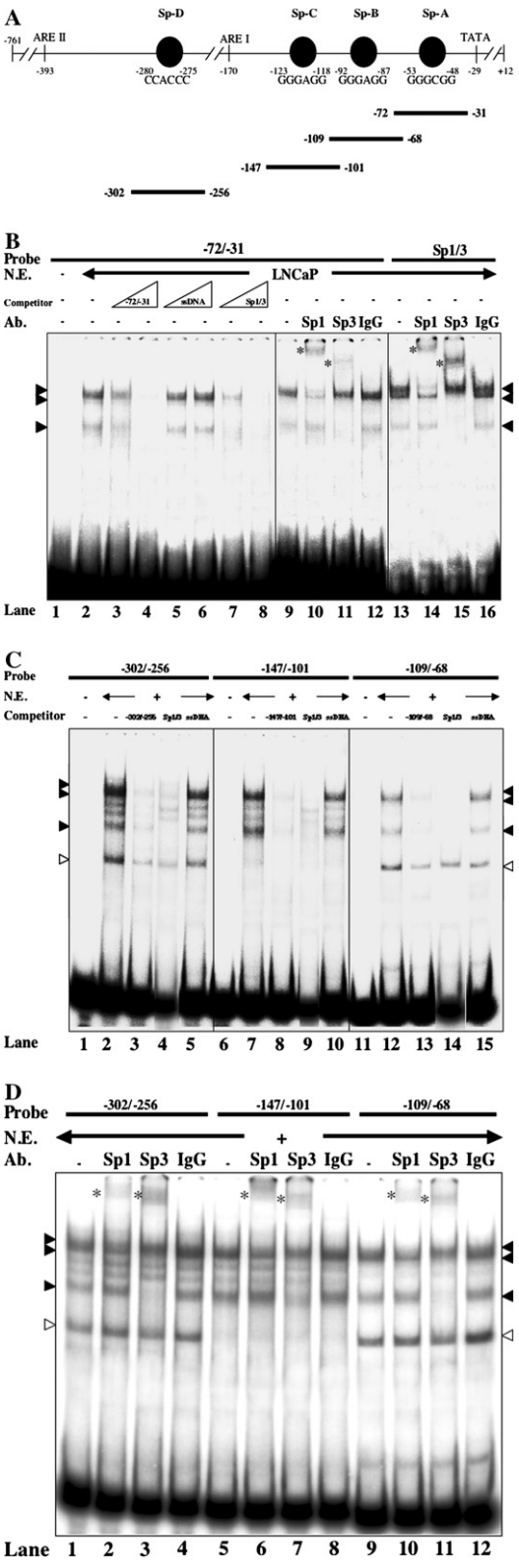

Fig. 2. EMSA analysis of the *PSA* promoter. (A) Schematic representation of four double-stranded oligonucleotides used as probes for EMSA. (B) The ^{32}P-labeled −72/−31 (lanes 1–12) and Sp1/Sp3 consensus oligonucleotide (lanes 13–16) were incubated with nuclear extract from LNCaP cells. Lane 1 lacks nuclear extract. Competition analyses were performed in the presence of a 20- or 100-fold excess of unlabeled −72/−31 (lanes 3 and 4), ssDNA (lanes 5 and 6), or Sp1/Sp3 consensus oligonucleotide (lanes 7 and 8) as competitors. Supershift analysis was performed in the presence of 4 μg of various antibodies, anti-Sp1 (lanes 10 and 14), anti-Sp3 (lanes 11 and 15), or control IgG (lanes 12 and 16). Control assays were performed without competitor or without antibody (lanes 2, 9, and 13). Arrowheads and asterisks indicate the protein–DNA complexes and the supershifted complexes, respectively. (C) Competition analyses were performed using −302/−256, −147/−101, or −109/−68 as probes. The competitor was a 100-fold excess of unlabeled oligonucleotides corresponding to the probe (lanes 3, 8, and 13), Sp1/Sp3 consensus oligonucleotides (lanes 4, 9, and 14), or ssDNA (lanes 5, 10, and 15). Control assays were performed without nuclear extract and competitor (lanes 1, 6, and 11), and with nuclear extract but without competitor (lanes 2, 7, and 12). Arrowheads indicate specific protein–DNA complexes. Note that some nonspecific bands appear, depending on the probes. Open arrowheads indicate the apparently nonspecific bands. (D) Supershift analyses were performed using −302/−256, −147/−101, or −109/−68 as probes. Antibodies used were anti-Sp1 (lanes 2, 6, and 10), anti-Sp3 (lanes 3, 7, and 11), or control IgG (lanes 4, 8, and 12). Control assays were performed without antibody (lanes 1, 5, and 9). Arrowheads, asterisks, and open arrowheads indicate the specific, supershifted, and nonspecific protein–DNA complexes, respectively.

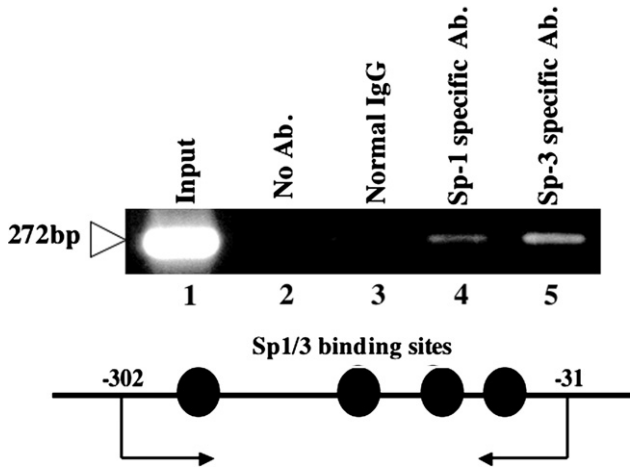

Fig. 3. ChIP assay. A ChIP assay was performed to confirm the binding of Sp1 and Sp3 to the *PSA* promoter in vivo. The protein–DNA complexes were incubated with polyclonal antibodies directed against Sp1 or Sp3, and isolated by immunoprecipitation (lanes 4 and 5). All immunoprecipitated DNA fragments were analyzed by PCR with the indicated primers. Input before immunoprecipitation was used as the positive control (lane 1). As the negative controls, the protein–DNA complexes were incubated without antibodies and with normal IgG (lanes 2 and 3, respectively).

with polyclonal antibodies against Sp1 and Sp3 were amplified by PCR, as was the positive control, whereas those immunoprecipitated with normal IgG were not. These results indicate that Sp1 and Sp3 bind specifically to the expected binding sites in the *PSA* proximal promoter in vivo.

To further confirm the specificity of this binding, we used nuclear extracts from LNCaP cells transfected with Sp1 and/or Sp3 expression vectors. Western blot analysis showed significant increases in Sp1 and Sp3 proteins in experiments using these expression vectors, compared with that in which a mock vector was used, whereas no change in the internal control, β-tubulin, was seen (Fig. 4A). In EMSA, three DNA–protein complexes, which could contain Sp1 or Sp3, were enhanced by transfection with expression vectors encoding both Sp1 and Sp3 in an experiment using all the oligonucleotide probes (Fig. 4B). When the Sp1 or Sp3 expression vector was cotransfected with a luciferase reporter plasmid, luciferase activity increased markedly in parallel with the number of binding sites (Fig. 4C). Conversely, when a dominant-negative Sp1 (DN-Sp1) plasmid was cotransfected with the *PSA* promoter-luciferase construct pGL3

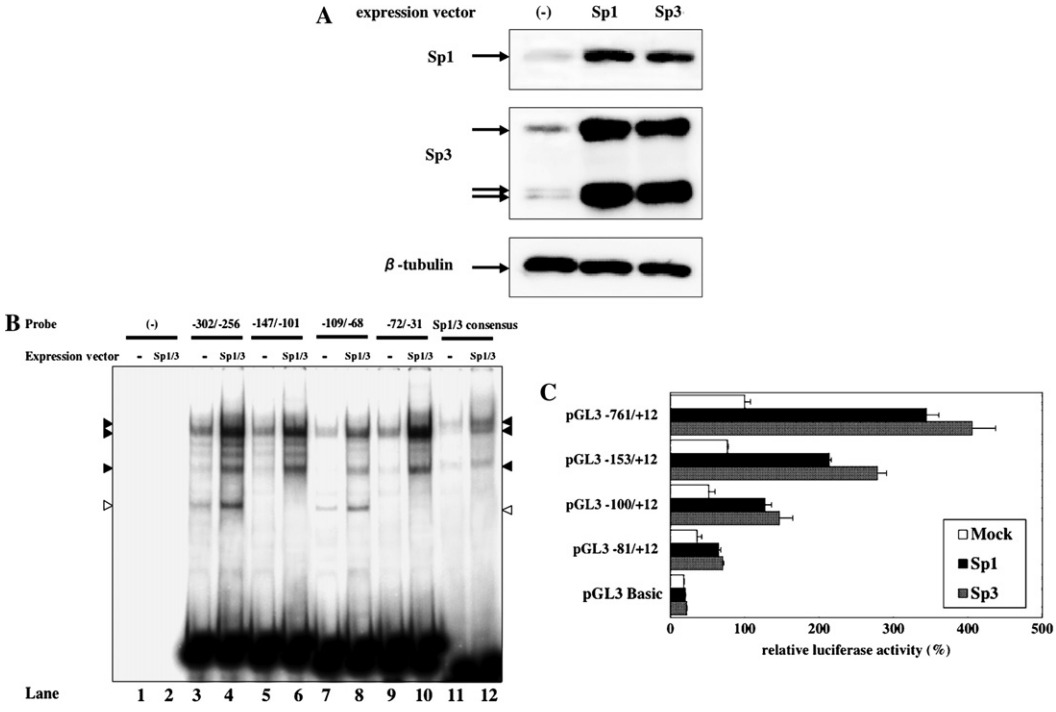

Fig. 4. Overexpression of Sp1 and/or Sp3 in LNCaP cells. (A) Western blot analysis was performed with nuclear extracts (15 μg) from LNCaP cells that had been transfected with the Sp1 (pCMV-Sp1), Sp3 (pRC/CMV-Sp3), or mock expression vector. Sp3 produced three specific bands, which indicate the existence of isoforms [51]. β-Tubulin was used as an internal control. (B) EMSA was performed after overexpression of both Sp1 and Sp3 (lanes 2, 4, 6, 8, 10, and 12) or not (lanes 1, 3, 5, 7, 9, and 11) in LNCaP cells. The ^{32}P-labeled −302/−256 (lanes 3 and 4), −147/−101 (lanes 5 and 6), −109/−68 (lanes 7 and 8), −72/−31 (lanes 9 and 10), or Sp1/Sp3 consensus oligonucleotides (lanes 11 and 12) were used as probes. A control assay was performed without probe (lanes 1 and 2). Arrowheads and open arrowheads indicate the DNA–Sp1/DNA–Sp3 and nonspecific complexes, respectively. (C) LNCaP cells were cotransfected with the promoter–reporter plasmid and the Sp1, Sp3, or mock expression vector. Relative activities (%) were normalized against the activity of the pGL3 −761/+12 cotransfected with the mock expression vector. Data are means ± SD of three independent experiments.

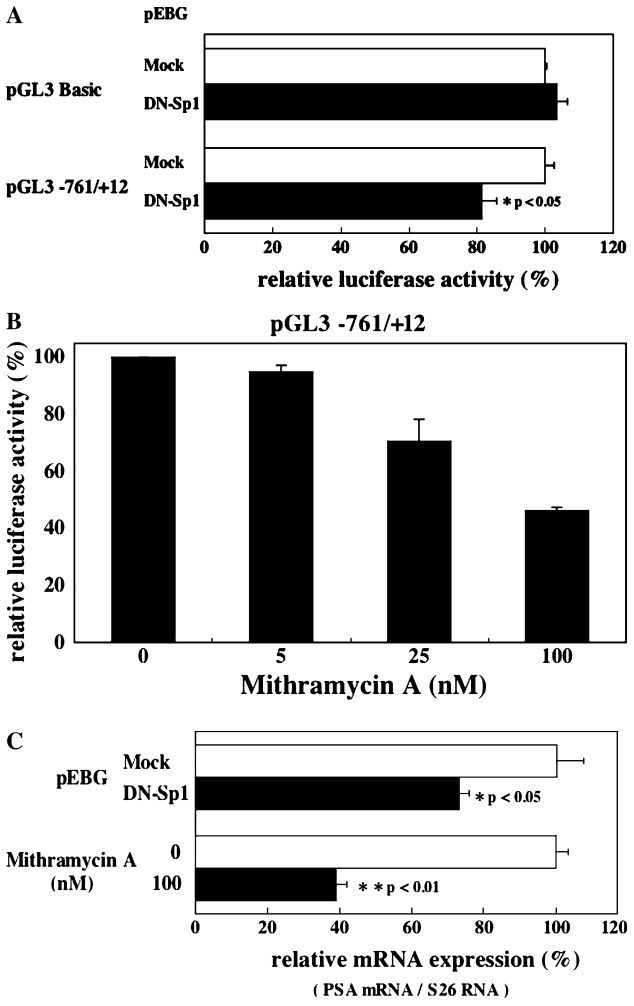

Fig. 5. Inhibition of basal promoter activity and mRNA expression of PSA. (A) LNCaP cells were cotransfected with 0.5 μg of the pGL3 Basic or pGL3 −761/+12 construct and 1 μg of the dominant-negative Sp1 expression vector (pEBG DN-Sp1) or empty vector (pEBG), and a luciferase assay was performed. Relative activities (%) were normalized against the activity of the pGL3 construct cotransfected with the empty vector (pEBG). Data are means ± SD of three independent experiments and differences are statistically significant (*$p < 0.05$). (B) Luciferase assay was performed after LNCaP cells were incubated with various concentrations of mithramycin A for 24 h. Relative activities (%) were normalized against the activity of the pGL3 −761/+12 construct without mithramycin A. Data are means ± SD of three independent experiments. (C) Analysis of PSA mRNA expression using LightCycler real-time PCR. LNCaP cells were transfected with pEBG DN-Sp1 or treated with mithramycin A (100 nM) for 24 h before the cells were harvested. The levels of mRNA were normalized against that of ribosomal S26 from the same preparation of cDNA and expressed relative to the levels in control cells. Data are means ± SD of three independent experiments. A value of *$p < 0.05$ or **$p < 0.01$ when compared with the results for pEBG-Mock or without mithramycin A, respectively.

−761/+12 (Fig. 5A), DN-Sp1 inhibited the basal transcriptional activity of the *PSA* promoter ($p < 0.05$). Mithramycin A, which interferes with the binding of the Sp family of transcription factors to GC-rich promoter regions [32], also decreased the promoter activity in a dose-dependent manner (Fig. 5B). Furthermore, DN-Sp1 or mithramycin A significantly reduced the endogenous mRNA levels of PSA by 27% or by 61% (Fig. 5C). These results indicate that Sp1 and Sp3 transcription factors upregulate the human *PSA* gene through multiple Sp1/Sp3 binding sites in the proximal promoter region.

Sp1/Sp3 binding sites cooperatively regulate the activity of the PSA promoter

To examine whether each sequence at the putative Sp1/Sp3 binding site is critical for binding, we generated mutated oligonucleotides (Fig. 6A). Competition EMSA with the mutated oligonucleotides showed that no band was competitively inhibited by any mutated oligonucleotide, whereas the Sp1 or Sp3 complexes were competitively inhibited with an unlabeled 100-fold molar excess of the corresponding wild-type oligonucleotide (Fig. 6B). These results confirm that Sp1 and Sp3 bind to the predicted Sp1/Sp3 binding sites. To determine which Sp1/Sp3 binding site is functional and most important, a series of substitution mutations in the luciferase construct were prepared (Fig. 7A). LNCaP cells were cotransfected with the mutated pGL3 −761/+12 constructs and the mock, Sp1, or Sp3, expression vector, and the luciferase activity was assayed. Although the activity gradually decreased with stepwise deletions from the 5′ end (Figs. 1B and 4C), the activity of the M1 construct was suppressed to the basal level in transfections with the mock vector (Fig. 7B). The activities of the M2 and M4 constructs were also suppressed by 29 and 54%, respectively. The suppression of the activity of the M3 construct was not statistically significant. These results demonstrate the differences in the activity of the four Sp1/Sp3 binding sites. Furthermore, with overexpression of Sp1 or Sp3, the tendencies were consistent with those of the mock vector. The activity of the M1 construct was significantly lower than that of the other constructs. The activities of M2 and M4 were slightly suppressed, whereas that of M3 was not. These results indicate that the most downstream binding site, Sp-A, located from −53 to −48, is critical for basic promoter activity, and that the other sites also contribute cooperatively to the activity of the *PSA* promoter.

Discussion

In this study, we analyzed the role of Sp1 and Sp3 transcription factors in the regulation of the human *PSA* promoter in prostate cancer cells. We showed that Sp1 and Sp3 transcription factors bind directly to the *PSA* promoter at four binding sites and upregulate the activity of the promoter. Of the four Sp1/Sp3 binding sites in the *PSA* promoter region, the most downstream

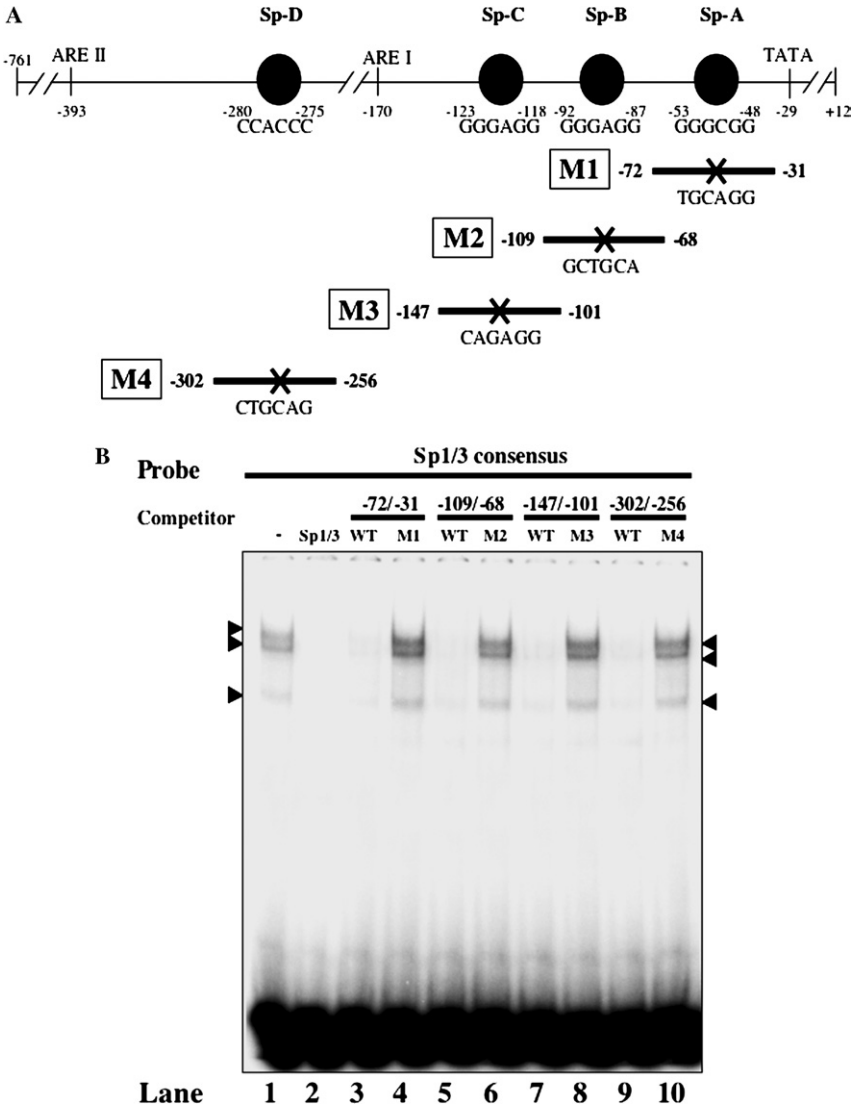

Fig. 6. Mutation analysis of the four Sp1/Sp3 binding sites in the *PSA* promoter. (A) Schematic representation of four double-stranded mutant oligonucleotides used as competitors for EMSA. (B) Competition EMSA with the mutant oligonucleotides. The ^{32}P-labeled Sp1/Sp3 consensus oligonucleotide was used as a probe. The competitor was a 100-fold excess of wild-type or mutant −72/−31 (lanes 3 and 4), −109/−68 (lanes 5 and 6), −147/−101 (lanes 7 and 8), or −302/−256 (lanes 9 and 10). Lane 1 lacks a competitor. Arrowheads indicate Sp1– or Sp3–DNA complexes.

Sp1/Sp3 site, which is a "classical" GC box, is most critical for maintaining basal promoter activity. This finding concurs with Franco et al.'s [36] report that the "B" motif centered at −60 bp is essential for the basal activation of the promoter, analyzed with a luciferase assay. However, they reported that the "B" motif does not interact with Sp family members. Using a footprinting method, Yeung et al. [37] identified a key *cis*-acting element located at −68 to −52. Their EMSA and UV cross-linking experiments indicated that a 45-kDa cell-specific transcription factor associates with this element. However, this element does not contain a complete GC box, and they excluded Sp family members. In same article, they mentioned the P2 element, which is essential for basal activation, deduced from the data of a luciferase assay. This element is located from −62 to −48, where a GC box occurs. Recently, Moorefield et al. [38] reported that PSA expression is stimulated by a subset of Sp family members in prostatic epithelia. Taking these data and our own together, we infer that Sp1/Sp3 and the 45-kDa transcription factor might be involved in this region.

Sp1 is a ubiquitous transcription factor that binds to consensus elements in the proximal promoters of a wide variety of genes and acts as a transcriptional activator. Sp3 is another transcription factor that binds to the same sequences and can act as a transcriptional activator or repressor [18]. We have shown that Sp3 acts as a transcriptional activator, like Sp1, in the proximal region of the *PSA* gene promoter in LNCaP cells (Fig. 4C). Transfection of the Sp1 expression vector increased the expression of Sp3 as well as that of Sp1, and vice versa (Fig. 4A). These results lead us to conjecture that Sp1

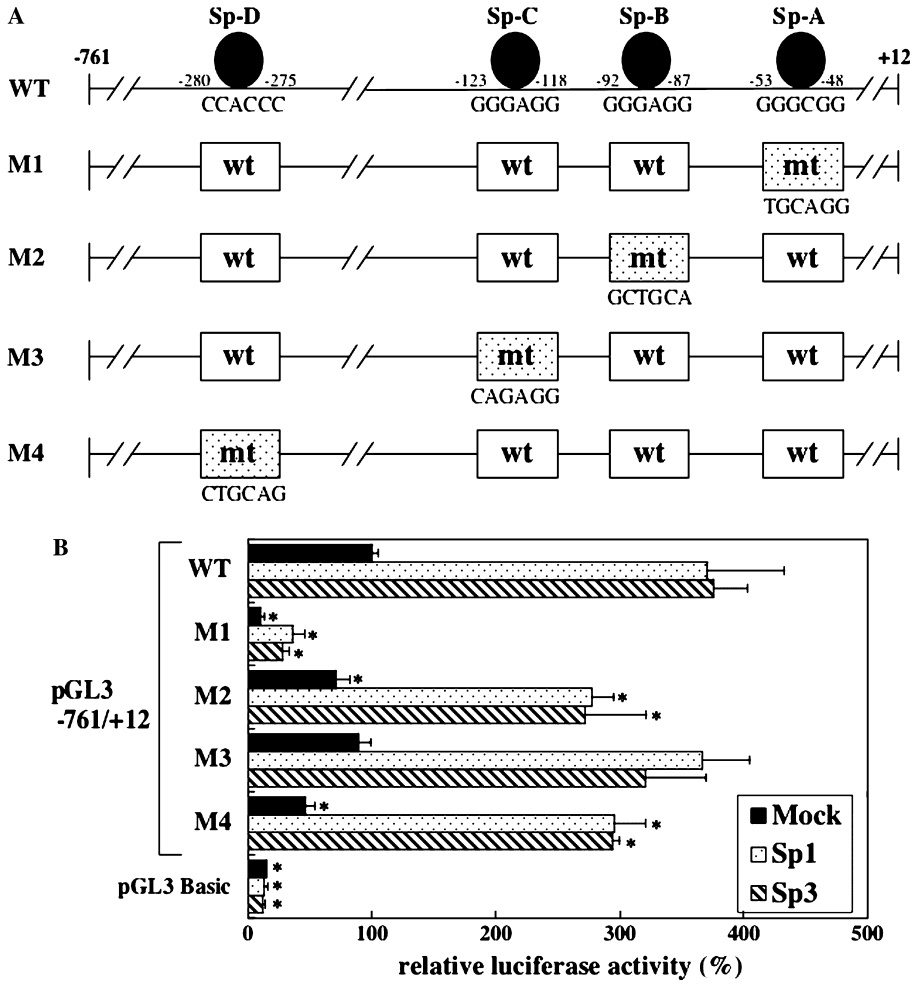

Fig. 7. Functional analysis of the four Sp1/Sp3 binding sites in the *PSA* promoter. (A) Schematic representation of a series of substitution mutations in the Sp1/Sp3 binding sites of the promoter. (B) Luciferase assay was performed with the mutation constructs transfected into LNCaP cells. The Sp1 (pCMV-Sp1), Sp3 (pRC/CMV-Sp3), or mock expression vector was cotransfected. Relative activities (%) were normalized against the activity of the wild-type pGL3 −761/+12 cotransfected with the mock expression vector. Data are means ± SD of three independent experiments. Asterisks indicate statistically significant results ($p < 0.01$) compared with the results for the wild-type pGL3 −761/+12 that was cotransfected with each expression vector. pGL3 Basic was used as the negative control.

and Sp3 cross-regulate the expression of one another in LNCaP cells. This cross-regulation may be partly explained by the fact that Sp3 is able to bind to the *Sp1* promoter [39].

Sp1 and Sp3 are ubiquitous transcription factors, and are involved in the regulation of many aspects of physiological and pathological conditions, including cell growth, apoptosis, angiogenesis, and invasion [26,40–49]. Recent studies have shown that Sp1 expression is related to tumor development and progression in cancer cells including those of epidermal tumors [25], pancreatic cancer [26], and gastric cancer [27]. As shown in Fig. 5C, DN-Sp1 or mithramycin A significantly reduced the endogenous mRNA levels of PSA in LNCaP cells. These findings confirm that Sp1 and Sp3 are involved in the transcriptional regulation of PSA. During tumor development and progression, Sp1 and Sp3 can be overactivated by several mechanisms. Sp1 activity can be modulated by stress factors, such as hypoxia [50] and low pH [23], which are common characteristics of solid tumors. Therefore, Sp1 and Sp3 might play some roles in the tumor biology of prostate cancer through the regulation of the *PSA* gene.

The combination of different transcription factors in regulating gene expression has been widely observed. Like AR, other factors that are activated via different signal transduction pathways might also contribute to the expression of the *PSA* gene. Perez-Stable et al. [13] suggested that GATA-2 and -3 are involved in the androgen-mediated regulation of the *PSA* gene. Wang et al. [16] identified a novel *cis*-acting element, GAGATA, that contributes to the expression of the *PSA* gene in response to androgen. Curiously, there are discrepancies between the data of Fig. 1B (or Fig. 4C) and Fig. 7B. In the former experiment, the constructs were deleted stepwise at the 5′ end, whereas in the latter experiment, all the constructs were the same length but mutated at each Sp1/Sp3 binding site. The 5′ stepwise deletion con-

structs might have lost not only the Sp1/Sp3 binding sites but also other binding sites, which might explain the difference between the data in those experiments. These observations imply that other unidentified positive/negative factors work in the proximal promoter of the *PSA* gene. Clinically, the progression of prostate cancer from androgen-dependent to androgen-independent states is considered a major and serious problem. In the androgen-independent state induced by androgen ablation therapy, a rebound in serum PSA is observed in many patients in parallel with the progression of the cancer. Several studies have shown that transcription factors such as NF-κB [15] and prostate-derived Ets factor (PDEF) [14] are involved in hormone-refractory mechanisms. However, except for the AR pathway, the mechanisms that involve other transcription factors have not been well characterized. In Fig. 7A, the promoter activities of the M1–M4 constructs in the presence of dihydrotestosterone (DHT) decreased in the same proportion as those in the absence of DHT (data not shown). These results support the view that Sp1 and Sp3 directly bind to the *PSA* promoter and do not act via the androgen–AR pathway.

In conclusion, we have shown that Sp1 and Sp3 transcription factors upregulate the human *PSA* gene through multiple Sp1/Sp3 binding sites in the proximal promoter region in LNCaP cells. This study should provide new insight into the functional roles of Sp1 and Sp3 in prostate cancer, such as in the androgen-independent state.

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