Published online July 7, 2004

3566–3580 *Nucleic Acids Research, 2004, Vol. 32, No. 12*
doi:10.1093/nar/gkh677

Transcriptional activation of the human *prostatic acid phosphatase* gene by NF-κB via a novel hexanucleotide-binding site

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Received as resubmission May 10, 2004; Accepted June 9, 2004

ABSTRACT

Human prostatic acid phosphatase (PAcP) is a prostate epithelium-specific differentiation antigen. Cellular PAcP functions as a neutral protein tyrosine phosphatase and is involved in regulating androgen-promoted prostate cancer cell proliferation. Despite the fact that the promoter of the *PAcP* gene has been cloned, the transcriptional factors that regulate PAcP expression remain unidentified. This article describes our analyses of the promoter of the *PAcP* gene. Deletion analyses of the promoter sequence up to −4893 (−4893/+87) revealed that a 577 bp fragment (−1356/−779) represents the unique positive *cis*-active element in human prostate cancer cells but not in HeLa cervix carcinoma cells. Interestingly, the 577 bp fragment contains a non-consensus nuclear factor κB (NF-κB)-binding site that is required for NF-κB up-regulation in prostate cancer cells, while NF-κB failed to have the same effect in HeLa cells. Conversely, inhibition of the NF-κB pathway stopped p65 NF-κB activation of the p1356 promoter activity. Gel shift and mutation analyses determined that AGGTGT (−1254/−1249) is the core sequence for NF-κB-binding and activation. Biologically, tumor necrosis factor-α (TNF-α) activated endogenous PAcP expression in LNCaP human prostate cancer cells. The data collectively indicate that NF-κB up-regulates PAcP promoter activity via its binding to the AGGTGT motif, a novel binding sequence located inside the *cis*-active enhancer element in human prostate cancer cells.

INTRODUCTION

Prostate cancer has become major a health problem in the male population of the Western world and is the second leading cause of cancer-related death in the United States (1). This is in part due to the lack of effective therapy for advanced prostate cancer (2,3). Therefore, the application of gene therapy to this cancer has attracted great attention. There is considerable interest in achieving a high level of expression in a defined cell population through specific promoters or regulatory elements. Several human genes that exhibit prostate-specific or prostate-enriched expression have been identified, including prostatic-specific antigen (PSA) (4), PSMA (5), Nkx3.1 (6), DD3 (7) and PAcP (8–10). Nevertheless, in part due to the androgen-dependent manner of their expression, except for PAcP and DD3, their potential application to patients undergoing androgen ablation therapy requires further investigation.

Human PAcP has a long history of serving as a marker for prostate cancer due to its prostate-specific manner of expression (11,12). There are two forms of PAcP: the cellular and the secretory forms. The intracellular level of PAcP is decreased, correlating with prostate carcinogenesis (13), but its secreted protein in circulation may serve as a marker for cancer diagnosis (14). The cellular form of PAcP functions as a neutral protein tyrosine phosphatase (15) and is involved in regulating cell growth by dephosphorylating ErbB-2 in human prostate cancer cells (16,17). Cellular PAcP is also involved in regulating non-genomic, androgen-promoted prostate cell proliferation (17,18). The regulation of PAcP expression and secretion has been of long-standing interest. In the past five decades, PAcP secretion has served as a marker for androgen action (19). Nuclear run-on experiments have revealed that androgens can regulate PAcP expression at the transcriptional level (12). Despite intensive studies of androgen regulation of PAcP expression at the post-transcriptional level (20), its transcriptional regulation remains an enigma. It has been proposed that androgen regulation of the PAcP gene is mediated via putative steroid-response elements (SREs) located between −1576 and +336 on the *PAcP* gene (21,22). The results of initial studies, however, show that androgens cannot activate the promoter activity of constructs covering these elements (21). It is possible that the responsibility for androgen regulation lies with the distal SRE(s) in the PAcP promoter, as found in PSA and probasin promoters (23,24). Furthermore, the *cis*-active element located between −1356 and −779 of the PAcP promoter is involved in regulating the high level of prostate-specific promoter activity and exhibits androgen independence (25).

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Taking all this into accounts, the promoter of the PAcP gene represents an interesting candidate for potential application in promoter-based gene therapy for advanced prostate cancer and serves as a model for understanding cell-specific expression.

Nuclear factor κB (NF-κB)/Rel includes a family of related transcription factors that bind to a set of related DNA sequences, i.e. the κB-binding site, for regulating gene expression (26–28). NF-κB forms homo- or heterodimeric complexes of various subunits, and the classical NF-κB is a heterodimer composed of a 50 kDa (p50) subunit and a 65 kDa (p65) subunit (29). Different NF-κB dimers can bind to the known κB sites bearing the consensus sequence GGGRNNYYCC or GGRRNNYCCC (30). The diverse regulation of NF-κB-dependent promoters is due at least in part to the ability of different dimers to bind to the same or distinct κB sites in a cell-type- and/or stimulus-dependent manner. For example, p65/RelA, c-Rel and RelB are positive regulators due to the presence of the potent transcriptional-activation domain. Although those dimers composed solely of Rel proteins lacking transcriptional-activation domains, e.g. p50 and p52, may in general mediate transcriptional repression (29), p50/p50 or p52/p52 homodimers can be transcriptional activators along with the Bcl-3 coactivator (31,32). Interestingly, Bcl-3 is also an inhibitor for these latter homodimers (31,32). In cells, in the absence of stimuli, NF-κB is sequestered in the cytoplasm by association with the inhibitory proteins IκBα and IκBβ (30). Activation of NF-κB occurs through the site-specific phosphorylation of IκB, e.g. serine-32 and -36 of IκBα, by IκB kinases (IKKs) and subsequent degradation of IκB. This activation allows the translocation of the released NF-κB into the nucleus to regulate target gene transcription (27,33). NF-κB can be activated by diverse signals, leading to regulation of various cellular activities (34,35). For example, NF-κB may be involved in the progression of breast cancer cells from the hormone-dependent to the hormone-independent stage (36). NF-κB is also activated in some hormone-refractory prostate cancer cells (37,38). Interestingly, the androgen receptor and NF-κB are mutually antagonistic in transcription assays (39), raising the possibility that the development of androgen independence in prostate cancer cells may be due in part to the aberrant regulation of NF-κB-targeted genes. Clearly, further studies are required to clarify the functional role of NF-κB in prostate cancer progression.

Despite the potential importance of PAcP in prostate cancer biology (10,14,40), little information is available about transcriptional factors that are involved in regulating the expression of this gene (21,41,42). In this article, we describe the characterization of a 5 kb PAcP promoter fragment. Our data reveal that −1356/−779 is the unique positive *cis*-active regulatory element. Within this element, we found a novel hexanucleotide NF-κB-binding site that is required for NF-κB-mediated further activation of the PAcP promoter in human prostate cancer cells.

MATERIALS AND METHODS

Materials

The cell culture medium, fetal bovine serum (FBS), gentamicin, glutamine, Opti-MEM medium and LipofectAMINE PLUS reagent were obtained from Invitrogen, Inc. The Master Amp PCR Optimization kit was purchased from Epicentre Technologies Corp. The Zero Blunt PCR cloning kit and pCR-BluntII vector were obtained from Invitrogen Corp. The pCAT-Basic, pCAT-Promoter, pCAT3-Promoter and pSV-β-galactosidase vectors, hNF-κB(p50) protein, AP1 protein and transcription factor consensus oligonucleotides for AP1, AP2, OCT1, CREB, NF-κB, TFIID, SP1 and the CAT assay kits were bought from Promega Corp. The anti-hNF-κB(p50) and anti-hNF-κB(p65) antibodies (Abs) and the c-Rel (NF-κB p65) protein were from Santa Cruz Biotechnology, Inc. Human tumor necrosis factor-α (TNF-α), IL-1 and the NF-κB inhibitory peptide were purchased from Calbiochem. The NF-κB inhibitory peptide is a cell-permeable synthetic peptide that carries an NF-κB nuclear localization signal and thus can competitively inhibit the subcellular trafficking of the NF-κB protein from the cytoplasm to the nucleus. The pRSV-NF-κB1 and pRSV-RelA plasmids containing inserts encoding NF-κB p50 and p65 proteins, respectively, were obtained from Dr K. T. Jeang at NIH and the NIH AIDS Research Reagent Program. The pFIkBaAA plasmid encoding a dominant-negative form of IκBα was a gift from Dr A. Razzino at the University of Nebraska Medical Center.

Cell culture

LNCaP, PC-3 and DU145 human prostate cancer cells were purchased from ATCC and were maintained in RPMI-1640 medium supplemented with 5% FBS, 1% glutamine and 0.5% gentamicin. HeLa human cervix epitheloid carcinoma cells were grown in DMEM medium supplemented with 10% FBS, 1% glutamine and 0.5% gentamicin (25).

Computer-based sequence analysis

The promoter sequence was analyzed using MatInspector data bank searches (43) to access possible nuclear factor-binding elements using the TRANSFAC 3.5 matrices with a core similarity of 1.00 and a matrix similarity of over 0.90 (http://www.gene-regulation.com).

Construction of reporter plasmids

To analyze the active promoter region of the human PAcP gene, a series of reporter plasmid constructs were made using the backbone of the pCAT-Basic vector. The plasmids p2899, p1668, p1356, p1305, p1258 and p779 were obtained as described previously (12,25). The promoter DNA in plasmid p4893 (−4893/+87) was obtained by PCR using two oligonucleotide primers, 5′-GAG CTC AAG GAA AAA GTA TGT TAT CTC CAG-3′ and 5′-ACT TCG GTC TAG CCA GAA AAA-3′. The amplification was performed for 30 cycles under the following conditions: 1 min at 94°C, 1 min 54°C, 3 min 30 s at 72°C. The DNA fragment was cloned into the pCR-BluntII vector. To analyze the promoter activity, a HindIII/XbaI fragment of the PAcP promoter from the pCR-BluntII vector was cloned into the pCAT-Basic plasmid. The reporter constructs containing the promoter DNA with a deletion region of −3262/−2598 (p4893ΔI), −3262/−1305 (p4893ΔII) and −3888/−1305 (p4893ΔIII) were obtained by digestion of p4893 with KpnI, PpuMI and NheI/KpnI restriction enzymes, respectively, followed by overhead removal (if necessary) by Mung Bean Nuclease and religation. The DNA manipulation of

plasmids was performed using conventional molecular biology techniques (44).

Transfection and reporter assays

For transient transfection experiments, cells were routinely plated at a density of 2.5×10^5 cells per well in a 6-well plate in RPMI 1640 medium containing 5% FBS for PC-3 cells, or in DMEM medium supplemented with 10% FBS for HeLa cells, 48 h before transfection. The adherent cells were transiently transfected using 6 µl of cationic lipid reagent LipofectAMINE PLUS™ (Life Technology) with 1 µg of plasmid DNA for the PAcP promoter/CAT reporter gene constructs in the serum-free Opti-MEM medium. The second plasmid, p-SV-β-galactosidase, was co-transfected at a ratio of 1:10 to the promoter/reporter gene construct, serving as an internal control. After 4 h of incubation, an equal volume of medium containing 10% FBS was added and incubated for an additional 2 h. Cells were then fed with the fresh medium supplemented with 5% FBS. For the CAT and β-galactosidase assays, 48 h after transfection, cells were washed twice with the PBS, scraped and lysed in 1× reporter lysis buffer (Promega). The protein concentration of cell extracts was measured according to Bradford (45) using the Bio-Rad protein assay kit (Bio-Rad Laboratories) with bovine serum albumin as the standard.

Quantitative CAT assays were performed with the same amount of total cell lysate proteins in a reaction volume of 125 µl in the presence of ^{14}C-chloramphenicol (Amersham Life Science, Inc.) as described in the Promega CAT-assay manual accompanying the assay kit. Samples were incubated overnight followed by a single extraction with 300 µl xylene. An aliquot of 250 µl of organic phase was transferred to a scintillation vial containing 2 ml EcoLume™ scintillation fluid (ICN, Corp.) and counted using a Beckman LS 1801 scintillation counter.

β-galactosidase assay

Cultured PC-3 cells were co-transfected with a pSV-β-galactosidase vector containing the β-galactosidase gene driven by an SV40 promoter as described above. Quantitative β-galactosidase assays were performed with the same amount of total cell lysate proteins in a reaction volume of 200 µl, as described in the Promega CAT-assay manual accompanying the assay kit. Accordingly, cell lysate proteins were incubated in 100 mM sodium phosphate buffer (pH 7.3) containing 50 mM β-mercaptoethanol, 1 mM $MgCl_2$ and 0.66 mM *o*-nitrophenyl-β-D-galactopyronoside at 37°C. The optical density was measured at 420 nm.

DNase I footprint assays

For the nuclear protein extract preparations, HeLa, DU 145 and PC-3 cells were plated at a density of 7.5×10^5 cells per T175 flask and harvested when they reached 70–80% confluence. Nuclear protein extracts were prepared as described elsewhere (46). Protein concentration was measured with the Bio-Rad protein assay kit using bovine serum albumin as the standard. DNase I footprinting assays were performed with 364 and 374 bp coding strands of the PAcP 5′ flanking sequences from −1356 to −992 and −1111 to −737 bp from the start codon, respectively. To generate the probes, primers 5′-GTT GAT GAC TAA TAA TATA-3′ and 5′-TCA ATG GAC TCT CCT GCC TCG-3′ were end-labeled using [γ^{32}P]ATP and T4 polynucleotide kinase, and then used in PCR with downstream primers of 5′-GGT CAG GAG TTA AGA CCA GCC-3′ and 5′-ATT CTT ACT CTG TTG GGA GTC-3′, respectively. The plasmid p1356 was used as the template. The corresponding DNA fragment from −1356 to −779 for the non-coding strand was labeled at one end with [α^{32}P]dTTPs and Klenow enzyme, and gel purified as described in (46). DNase I footprint assays were performed in a 20 µl reaction volume containing 50 mM Tris–HCl (pH 8.0), 100 mM KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 1 mM DTT and 2 µg poly(dI–dC). An aliquot of the probe (2×10^4 c.p.m.) was incubated with or without nuclear extract proteins for 20 min at 4°C. Subsequently, 50 µl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ were added, and the reaction mixtures were maintained at room temperature for 1 min. After adding 0.15 U RQ I DNase (Promega), the mixture was incubated for 1 min 30 s at room temperature. The reaction was stopped by the addition of 200 µl of 200 mM NaCl, 1% SDS, 30 mM EDTA and 100 µg/ml yeast RNA. After phenol/chlorophorm extraction and ethanol precipitation, the samples were resuspended in a loading buffer [1:2 (v/v) 0.1 M NaOH:formamide, 0.1% xylene cyanol, 0.1% bromophenol blue], denaturated at 95°C for 2 min and resolved in 6% sequencing gel. The gel was exposed to an X-ray film at −80°C with an intensifying screen.

Electrophoretic mobility shift assay (EMSA)

Reactions were conducted in a total volume of 20 µl. An aliquot of nuclear extracts was added to a reaction buffer containing 20% glycerol, 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris–HCl (pH 7.5) and 0.25 mg/ml poly(dI–dC). The mixture was incubated for 20 min at room temperature, and then 3×10^4 c.p.m. (3000 Ci/mmol) of the [γ^{32}P]ATP-labeled oligonucleotide probe PSD of −1262/−1241, containing the putative sequence for the AP1 protein-binding site (5′-TTG TCT TAA GGT GTG ACT AAA-3′), was added. The control probe for AP1 and NF-κB was labeled with 10^5 c.p.m. (3000 Ci/mmol) of [γ^{32}P]ATP. For the competition assay, a 100-fold molar excess amount of appropriate unlabeled probes was added to the reaction mixture. Samples were electrophoresed in 4% nondenaturing polyacrylamide gel in TBE buffer for 3 h at room temperature. The gel was dried and autoradiography was performed.

PAcP activity determination

PNPP was used as the substrate to quantify the activity of PAcP at pH 5.5 by measuring the absorbance of released *p*-nitrophenol at 410 nm (19).

TNF-α treatment

LNCaP cells were plated at a density of 2.5×10^5 cells per well in a 6-well plate in RPMI 1640 medium containing 5% FBS. After 48 h of incubation, cells were treated with different concentrations of TNF-α for 72 h. PC-3 cells were plated at a density of 1.8×10^5 cells per well in a 6-well plate in RPMI 1640 medium containing 5% FBS for 48 h following transient transfection as described above. Cells were treated,

72 h after transfection, with different concentrations of TNF-α for 48 h.

RESULTS

Functional analyses of the human prostatic acid phosphatase promoter

In order to investigate transcriptional regulation of PAcP expression by identifying the regulatory element(s) of the *PAcP* gene, a 5 kb promoter fragment between −4893 and +87 of the *PAcP* gene was subcloned into a CAT reporter vector. We constructed a series of CAT reporter vectors containing various external and internal deletions (Figure 1) and then transiently transfected them into PC-3 human prostate cancer cells in order to quantify CAT activities. The promoter activity of p779 was arbitrarily assigned the value 1.0 and was used as the reference for normalizing the activities of the other constructs [Figure 1 and (25)].

Deletion analyses of the 5 kb promoter DNA fragment revealed that the p1356 construct exhibited the highest level of activity among the promoter constructs examined (Figure 1). Similarly high levels of activity were observed for p1305 and p1258 (Figure 1). Since p1258, p1305 and p1356 exhibited approximately the same (high) level of activity, these plasmids were used interchangeably for constructing various reporter vectors. The CAT activity of p4893 was <5% of that of p1356. When the region from −3262 to −2598 (p4893ΔI) was deleted, the promoter activity of the resulting plasmid was as low as that of p4893, indicating that there is no putative regulatory element inside this fragment. The promoter activity of p4893ΔII, containing a deletion between −3262 and −1305, was lower than that of p1305, indicating the presence of a negative regulatory element located between −4893 and −3262. Deletion of the region from −3888 to −1305

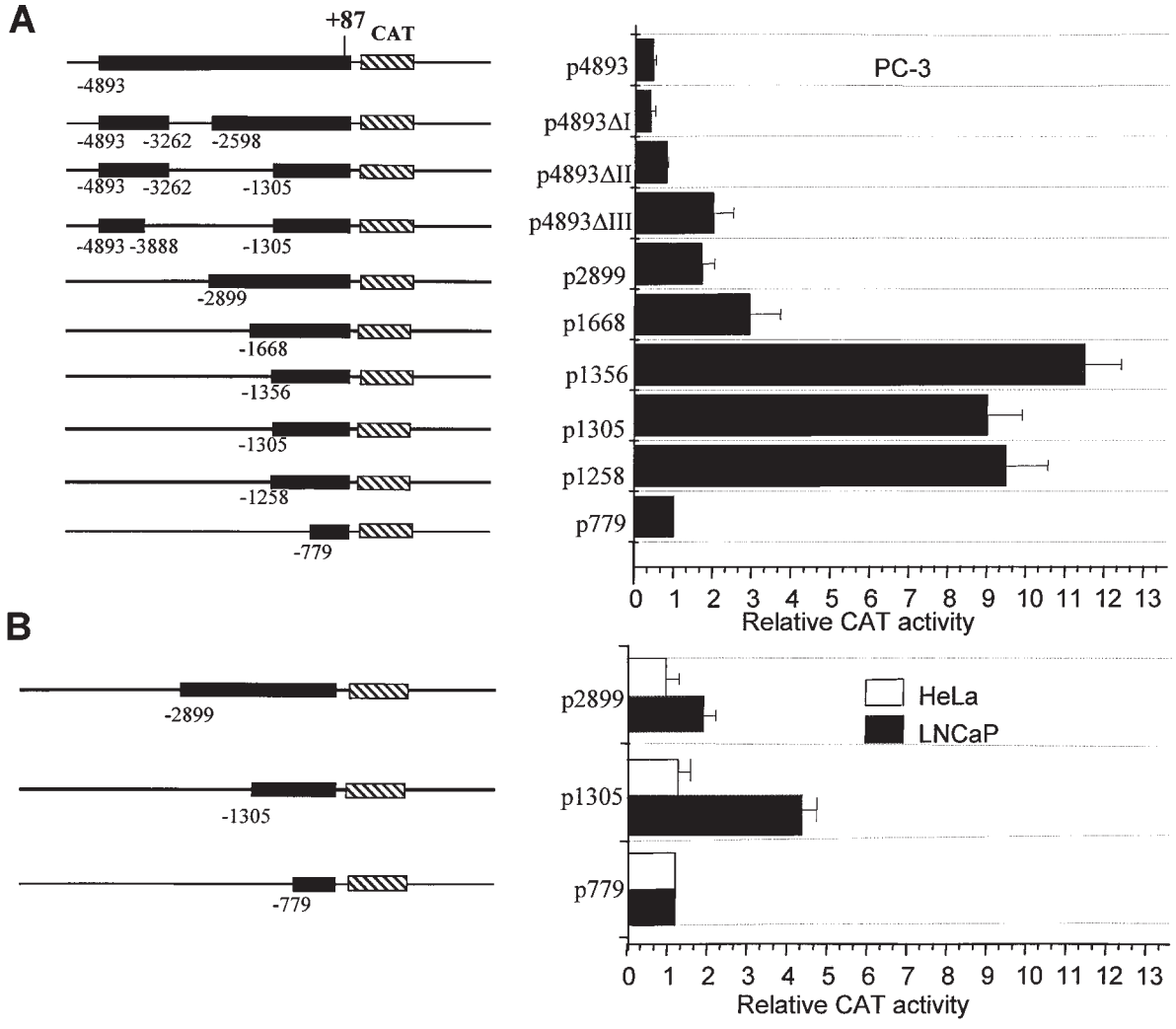

Figure 1. The effect of deletion in the 5′ region of the PAcP promoter on the CAT activity in human prostate cancer cells. (**A**) PC-3 cells and (**B**) LNCaP and HeLa cells. On the left, a schematic representation of the 5 kb PAcP promoter and its deleted variants inserted upstream of the CAT gene in the reporter plasmid pCAT-Basic. The numbers indicate the 5′ and the 3′ ends of the promoter DNA inserts, in relation to the transcription start site (+1). Cells were transfected with 1 μg of the indicated PAcP-CAT reporter constructs and assayed for CAT activity as described under Materials and Methods. On the right, the CAT activity is presented as the ratio of the tested construct to p779. Values were normalized for transfection efficiency by co-transfection with the β-galactosidase expression plasmid. Bars represent the SE of triplicates from at least two sets of independent experiments ($n \geqslant 6$).

(p4893ΔIII) caused an increase in the level of CAT activity, to approximately twice that p4893ΔII, suggesting the presence of a negative regulatory element within the region −3888/−3262. The data also indicate the presence of negative regulatory element(s) between −4893 and −3888, because the promoter activity of p4893ΔIII was ∼20% that of p1305. Previous analyses of the region between −2899 and −1305 revealed the presence of two negative regulatory regions, −2899/−2583 and −1668/−1356 (25).

We also analyzed whether p1305 exhibits a high level of activity in LNCaP human prostate cancer cells that express endogenous PAcP. As shown in Figure 1B, among the three plasmids examined, p1305 exhibited the highest level of promoter activity, as observed in PC-3 cells. The lower activity of p1305 in LNCaP cells than in PC-3 cells is possibly due to competition of transcriptional factors with the endogenous gene. Interestingly, in HeLa human cervix epitheloid carcinoma cells (Figure 1B), WI-38 human lung diploid cells and A431 human epidermoid carcinoma cells [data not shown and (25)], these three plasmids exhibited a similarly low level of promoter activity. The data thus clearly show that the sequences between −1258 and −779 are required for the high level of PAcP expression in prostate cancer cells, although p1356 exhibits an ∼20% higher level of promoter activity than p1305 and p1258. Furthermore, the sequences −4893/−3888, −3888/−3262, −2899/−2583 and −1668/−1356 have negative regulatory effects (Figure 1).

Footprinting analyses of the positive-regulatory region

We characterized the *cis*-active region between −1356 and −779 because this region contains sequences for the high level of cell-specific expression of the *PAcP* gene [Figure 1 and (25)]. To identify the potential protein-binding sites in the *cis*-active region, a DNase I protection assay was carried out using nuclear protein extracts from PC-3, DU145 and HeLa cells. Two partially overlapping subsegments of the enhancer, i.e. from −1356 to −992 and from −1111 to −737, which were synthesized by PCR with end-labeled primers, were used as probes for the positive strand, while a filled-in fragment from −1356 to −779 was used as the probe for the negative strand. The labeled probes were incubated with prostate (DU 145 or PC-3) or non-prostate (HeLa) nuclear proteins and subsequently digested with DNase I. As shown in Figure 2A and B, DNase footprinting analyses identified at least six protected regions in the coding strand from −1300 to −1286 (I), −1277 to −1238 (II), −1228 to −1199 (III), −1082 to −1073 (IV), −1069 to −1047 (V) and −986 to −976 (VI). Two footprints were detected in the non-coding strand from −1266 to −1240 (II′) and from −1226 to −1191 (III′) (Figure 2C). Similar results from footprinting analyses were obtained when LNCaP nuclear proteins were utilized (data not shown). The data indicate that several transcriptional factors interact with the positive regulatory fragment of the PAcP promoter sequence, while no distinct protection region by prostate nuclear proteins, differing from HeLa nuclear proteins, was identified.

Computer analyses of the positive regulatory sequence in the PAcP promoter

We investigated putative transcriptional factor-binding sites in this *cis*-active region using the MatInspector database. As shown in Figure 3, within the *cis*-active region II of −1277/−1238, there was one putative AP1-binding site and one putative Sox-5 protein-binding site. The sequences between −1240 and −1191 were extremely T-rich and contained two putative HNF/FKH-binding sites in each strand. However, there was no significant homolog to the known putative binding site in regions I, IV, V or VI.

EMSA analyses of the −1262/−1242 sequence in the *cis*-active region

To characterize further the *cis*-active region of the PAcP promoter, we carried out EMSA assays. Since region II contains a putative binding site for the AP1 protein, a ^{32}P-labeled oligonucleotide, designated PSD and corresponding to the sequence between −1262 and −1242 of the PAcP promoter (shown in bold in Figure 3), was incubated with nuclear protein extracts from HeLa, DU 145 and PC-3 cells. Purified AP1 protein and its consensus oligonucleotide were used as a positive control (Figure 4A, lane 2). As shown in Figure 4A, there were several bands with reduced mobilities revealed by EMSA using nuclear extracts with the PSD oligonucleotide. These nuclear protein–DNA complexes were outcompeted by the addition of a 100-fold molar excess of unlabeled PSD oligonucleotide, indicating the specific interaction of nuclear proteins with PSD oligonucleotides. Similar patterns of nuclear protein–DNA complex formation were obtained by utilizing LNCaP nuclear proteins, although overall the signal was weaker than for PC-3 nuclear proteins (data not shown). However, no complex formation was observed between the AP1 protein and the ^{32}P-labeled PSD oligonucleotide (Figure 4A, lane 3), and the unlabeled PSD oligonucleotide failed to compete with the AP1 consensus oligonucleotide in complex formation with the AP1 protein (Figure 4A, lane 4). Similarly, the AP1 consensus oligonucleotide could not compete with the PSD oligonucleotide in complex formation with nuclear proteins from PC-3 cells (Figure 4B, lane 3). Therefore, the consensus AP1 sequence on PSD was unable to function as an AP1-binding site, i.e. the AP1 protein could not bind to the PSD oligonucleotide, while several DNA-binding proteins can bind to the PSD fragment of the PAcP promoter *in vitro*.

A novel NF-κB-binding site located in the −1262/−1242 sequence of the *cis*-active region of the PAcP promoter

In order to determine whether the PSD oligonucleotide could interact with known transcription factor(s), several commercially available consensus oligonucleotides were utilized to compete with the PSD oligonucleotide in complex formation with nuclear proteins from PC-3 cells. Unexpectedly, the formation of PSD–protein complexes was outcompeted by an NF-κB consensus oligonucleotide (Figure 5A), but not by AP2, OCT1, CREB, SP1 or TFIID. To confirm that the NF-κB protein could bind directly to the PSD oligonucleotide, we performed an EMSA using the p50 NF-κB protein. The formation of a DNA–protein complex was clearly seen between the NF-κB protein and the PSD oligonucleotide (Figure 5B, lane 1) as well as between the NF-κB protein and the authentic NF-κB consensus oligonucleotide (Figure 5B, lane 3). The specificity of the interaction was confirmed by the successful competition from an excess amount of corresponding, unlabeled oligonucleotides (Figure 5B, lanes 2 and 4). Similarly,

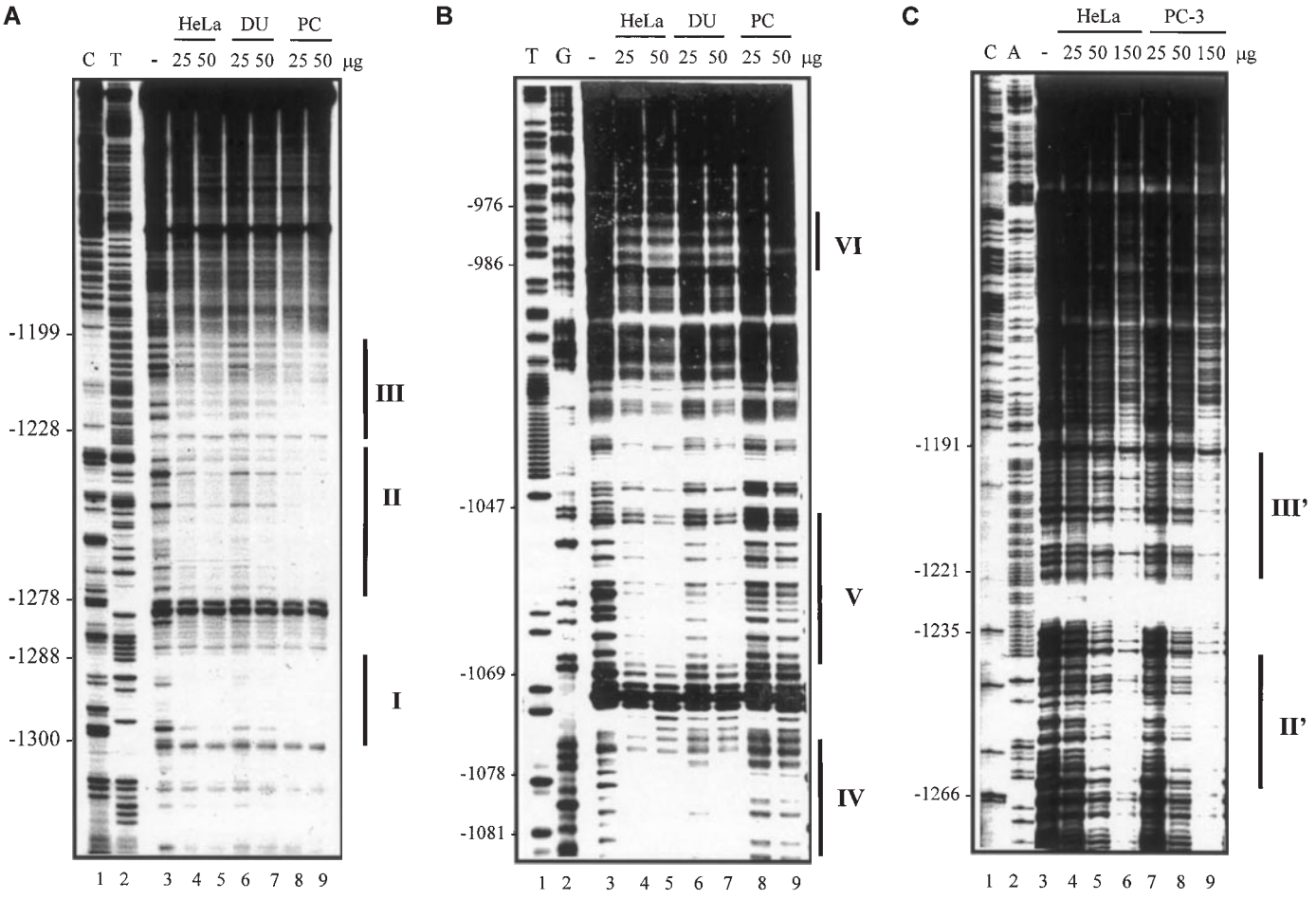

Figure 2. DNase I footprinting analysis of the PAcP promoter. The labeled fragments (as described in Materials and Methods) were reacted with nuclear extracts from HeLa, DU 145 and PC-3 cells, respectively. The data obtained with the coding strand of −1356/−992 bp DNA are shown in the autoradiogram (**A**), those obtained with −1111/−737 bp DNA are shown in (**B**), and those obtained with the protection of the non-coding strand −1356/−731 are shown in (**C**). The protected promoter sequences were determined by a direct comparison with the sequencing reaction. (A) Lanes 1 and 2, sequencing ladders; lane 3, control DNase I reaction of probe DNA without nuclear extracts; lanes 4 and 5, 25 and 50 μg, respectively, of HeLa cell nuclear extracts; lanes 6 and 7, 25 and 50 μg, respectively, of DU 145 cell nuclear extracts; lanes 8 and 9, 25 and 50 μg, respectively, of PC-3 cell nuclear extracts. (B) Lanes 1 and 2, sequencing ladders; lane 3, control DNase I reaction of probe DNA without nuclear extracts; lanes 4 and 5, 25 and 50 μg, respectively, of HeLa cell nuclear extracts; lanes 6 and 7, 25 and 50 μg, respectively, of DU 145 cell nuclear extracts; lanes 8 and 9, 25 and 50 μg, respectively, of PC-3 cell nuclear extracts. (C) Lanes 1 and 2, sequencing ladders; lane 3, control DNase I reaction of probe DNA without nuclear extracts; lanes 4, 5 and 6, 25, 50 and 150 μg, respectively, of HeLa cell nuclear extracts; lanes 7, 8 and 9, 25, 50 and 150 μg, respectively, of PC-3 cell nuclear extracts.

the p65 NF-κB protein formed complexes with the authentic NF-κB consensus oligonucleotide (Figure 5C, lane 1) as well as PSD oligonucleotides (Figure 5C, lane 3). Unlabeled PSD oligonucleotides competed with the formation of the PSD oligonucleotide and p65 NF-κB complexes, indicating the specificity of the interaction (Figure 5C, lane 5). In order to further determine the interaction between the NF-κB subunit and PSD oligonucleotides, we performed super-shift assays with the corresponding Ab. Although the anti-p65 NF-κB Ab had a super-shift effect on the complexes formed by the authentic NF-κB consensus oligonucleotide and the p65 protein (Figure 5C, lane 2), this anti-p65 Ab failed to have a super-shift effect on the PSD oligonucleotide–p65 NF-κB complex (Figure 5C, lane 4). Furthermore, in the presence of the Ab, the PSD oligonucleotide–p65 NF-κB complex disappeared (Figure 5C, lane 4). Similarly, in the presence of the anti-p50 NF-κB Ab, the p50 NF-κB–PSD oligonucleotide complex disappeared (data not shown). The DNASIS program was utilized to analyze the sequence similarity between AP1 or NF-κB consensus oligonucleotides and the PSD sequence. Interestingly, the PSD oligonucleotide exhibited a slightly higher homology with the AP1 oligonucleotide than with the NF-κB consensus oligonucleotide, i.e. 52 versus 46%, in the total alignment window (Figure 5D). The data may indicate a novel binding site for NF-κB in the PAcP promoter.

Effects of TNF-α and IL-1 on the level of PAcP promoter activity

We investigated whether NF-κB can regulate the level of PAcP promoter activity *in vivo*. Since TNF-α can induce NF-κB activation in PC-3 cells (47), PC-3 cells were transiently transfected with p779 or p1356 and then grown in media containing different amounts of TNF-α. Cells were harvested to determine the level of CAT activity. As shown in Figure 6A, in PC-3 cells, a 48 h TNF-α treatment could stimulate the

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-1382  CAAAGTTGATGACTAATAATATAGAATTGTAGGTTTGGGCTTTTTGCTTT

                                                      I
-1332  CTTAAAAAACTCTGTCAGGTTTGGAAAGGGACCTCCAATCTAATTTTGAG

                                 II            AP1
-1282  ATACCACAGGTAATGTCCAATTGTCTTAAGGTGTGACTAAAATTTTTCTT
              AML1                      Sox5  ΔEF1
                          III
         HNF              HNF
-1232  TTGTTTGTTTTTTGTTGTTGTTTGTTTGTTTTGCTGTGTTTTGAGATGGA
         FKH              FKH

-1182  GTCTTGCTCTGTCACTCAGGCTGGAGTGCAGTGGCACAATCTCGGCTCAC

-1132  TGCAACCTCCACCTCCTGGGTTCAAGTGACTCTCCTGCCTCGCCTCCCAG

         IV                V
-1082  GTAGCTGGGACTATAGGCATGTGCCACCACGCCAGGCTAATTTTTTTTTT

                                                     VI
-1032  TTGTATTTTTAGTAGAGATGGGGTTTCACCATGCTGGCCAGGCTGGTCTT

                CREB                                  IK2
 -982  AACTCCTGACCTCAAGTGATCTGCCCACCTCGGCCTCCCAAAGTGCTGGG

                          SREBP1                LYF1
 -932  ATTACAGACATGAGCCACCACGCCCAGCCTAAAATTTTTCAAAATAAACT
                                                    HFH
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Figure 3. Potential transcription factor-binding sites in the enhancer region of the PAcP promoter. The sequences shown are from −1382 to −883 nt, covering the enhancer region of the PAcP gene. Sequence analyses using MatInspector data bank searches identified several region sequences consistent with known nuclear factor-binding sites. The nuclear factors typed above and below a sequence are for its binding to the coding and non-coding strands, respectively. Regions protected in DNase I footprinting experiments shown in Figure 2 are underlined. Double-stranded PSD oligonucleotides used in gel shift experiments are shown in bold.

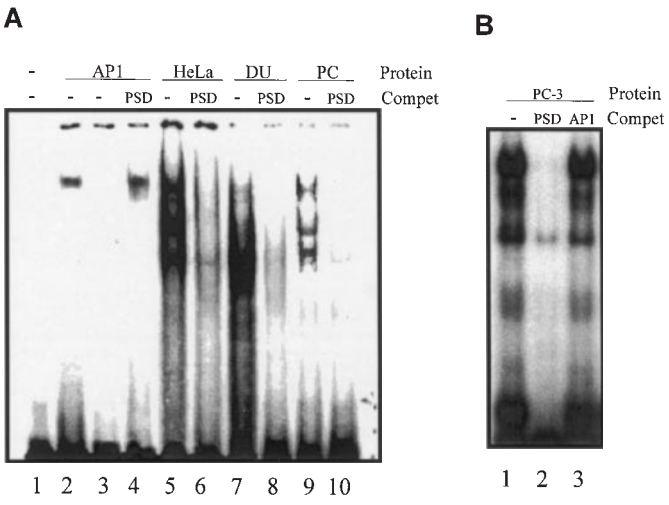

Figure 4. Gel shift analyses of nuclear proteins binding to the PSD oligonucleotide. ^{32}P-labeled oligonucleotide probes were incubated with nuclear extract proteins from HeLa, DU 145 or PC-3 cells. Protein–DNA complexes were resolved on 4% non-denaturing polyacrylamide gel and visualized using autoradiography. (**A**) Lane 1, PSD probe alone; lane 2, end-labeled AP1 consensus oligonucleotide incubated with AP1 protein; lane 3, end-labeled PSD consensus oligonucleotide incubated with AP1 protein; lane 4, end-labeled AP1 consensus oligonucleotide incubated with AP1 protein in the presence of 100-fold molar excess of unlabeled double-stranded PSD oligonucleotide; lanes 5–10, the PSD nucleotide was end-labeled and incubated with HeLa, DU 145 or PC-3 nuclear extracts in the absence or presence of 100-fold molar excess of unlabeled double-stranded PSD; lanes 5 and 6, HeLa extract; lanes 7 and 8, DU 145 extract; lanes 9 and 10, PC-3 extract. Reactions were performed with ~0.03 ng of radiolabeled probe (30 000 c.p.m.) and 5 μg nuclear extracts. (**B**) Lane 1, the PSD probe alone was incubated with 10 μg of PC-3 nuclear extracts; lanes 2 and 3, PSD nucleotide was incubated with 10 μg of PC-3 cell nuclear extract proteins with a 100-fold molar excess of unlabeled PSD or AP1 consensus oligonucleotides.

transcriptional activity of p1356 approximately 2-fold, while it failed to have the same effect for p779. This TNF-α stimulation resulted in an overall activity of p1356 20-fold higher than that of p779 (Figure 6A). However, in HeLa cells TNF-α did not have an effect on the transcriptional activity of p1356 or p779 (Figure 6B). Similar results were obtained when transiently transfected PC-3 cells were treated with IL-1, another NF-κB-activating cytokine (data not shown). These data collectively indicate that TNF-α and IL-1, via NF-κB, up-regulate the level of PAcP promoter activity in PC-3 cells but not in HeLa cells, and that the *cis*-active enhancer region −1356/−779 is essential for this stimulation.

Effects of NF-κB and IκB on the level of PAcP promoter activity

To determine whether NF-κB can directly regulate the level of PAcP promoter activity, the p1356 reporter plasmid and expression vectors encoding NF-κB p65 or p50 subunit proteins were transiently co-transfected into PC-3 cells. Overexpression of the p50 protein, an NF-κB subunit without the transcriptional activation domain, could not activate the PAcP promoter activity (Figure 7A). Instead, higher dosages of p50 expression resulted in suppression of the PAcP promoter activity, similar to previous observations for the p50 subunit (29,31,48). Conversely, overexpression of the p65 subunit, which contains a transactivation domain, increased the level of p1356 PAcP promoter activity by over 2-fold (Figure 7A), which is similar to the effect of TNF-α (Figure 6A). These results showed that the p65 subunit of NF-κB could directly activate the PAcP promoter.

IκBα effectively inhibits NF-κB dimers that contain the p65/RelA or c-Rel subunit and responds to several known NF-κB-inducing signals (34). The role of NF-κB in activating the p1356 PAcP promoter activity was further determined using IκBαAA, a mutant form of IκBα, which can constitutively inactivate the NF-κB dimer that contains the p65 and/or c-Rel subunit. Expression of IκBαAA alone showed only up to an ~20% inhibitory effect on the basal activity of the p1356 promoter construct, indicating the inhibition of endogenous NF-κB activity in PC-3 cells (Figure 7B). Co-transfection of IκBαAA completely abolished the stimulating effect by 0.5 μg p65 NF-κB on p1356 promoter activity, following a dose-dependent response (Figure 7B). Similarly, an NF-κB inhibitory peptide that can competitively inhibit the subcellular traffic of NF-κB from the cytoplasm to the nucleus (49) at 18 μM concentration exhibited the optimal inhibitory activity, abolishing the p65 NF-κB-induced activation of PAcP promoter activity in p1356- and p65 NF-κB-co-transfected PC-3 cells (data not shown). These data thus demonstrate that the p65 NF-κB protein can activate the PAcP promoter activity in prostate cancer cells.

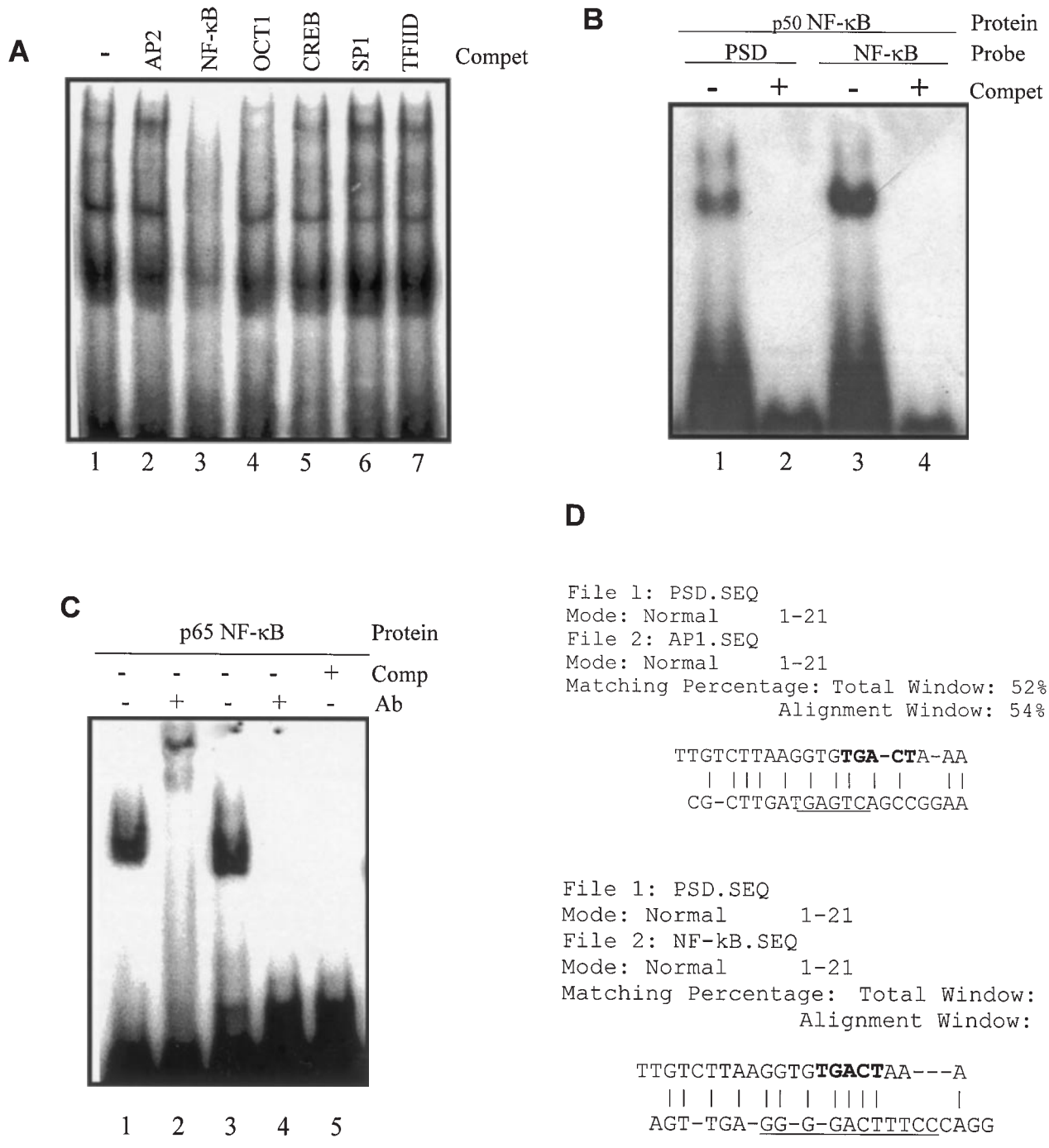

Figure 5. Analysis of transcription factors binding to the PSD oligonucleotide by competitive EMSA. (**A**) Of the end-labeled PSD nucleotide, 0.03 ng (30 000 c.p.m.) was incubated with 10 μg PC-3 cell nuclear extract proteins in the absence (lane 1) or presence (lanes 2–7) of 100-fold molar excess of different unlabeled consensus oligonucleotides. The ^{32}P-labeled PSD DNA–protein complex formation was conducted in the presence of AP2 (lane 2), NF-κB (lane 3), OCT1 (lane 4), CREB (lane 5), SP1 (lane 6) or TFIID (lane 7) consensus oligonucleotides. (**B**) Electrophoretic mobility experiments using ^{32}P-labeled PSD and consensus NF-κB oligonucleotides. Labeled oligonucleotides were incubated with 1×10^{-3} gsu [1 gsu = the amount of protein required to gel shift the NF-κB oligo under defined conditions (Promega)] purified p50 NF-κB protein in the absence (−) or presence (+) of 100-fold molar excess of the unlabeled corresponding oligonucleotide. Lanes 1 and 2, end-labeled PSD oligonucleotide incubated with purified p50 NF-κB protein in the absence (−) or presence (+) of 100-fold molar excess of unlabeled PSD oligonucleotide; lanes 3 and 4, end-labeled NF-κB consensus oligonucleotides incubated with purified p50 NF-κB protein in the absence (−) or presence (+) of 100-fold molar excess of unlabeled NF-κB consensus oligonucleotide. (**C**) Electrophoretic mobility super-shift experiments using labeled PSD and consensus NF-κB oligonucleotides. Labeled oligonucleotides were incubated with the p65 NF-κB protein in the presence or absence of the anti-p65 NF-κB polyclonal antibody. Lanes 1 and 2, end-labeled NF-κB consensus oligonucleotides were incubated with 1×10^{-3} gsu purified p65 NF-κB protein in the absence (−) or presence (+) of the anti-NF-κB Ab; lanes 3 and 4, the end-labeled PSD oligonucleotide was incubated with purified p65 NF-κB protein in the absence (−) or presence (+) of the anti-NF-κB Ab; lane 5, the end-labeled PSD nucleotide was incubated with 1×10^{-3} gsu purified p65 NF-κB protein in the presence of 100-fold molar excess of unlabeled PSD oligonucleotide. (**D**) Computer alignments of the PSD oligonucleotide sequence with the AP1 or NF-κB consensus sequences in the commercial oligonucleotides were analyzed according to the Needleman–Wunsch algorithm using the DNASIS sequence analysis software (Hitachi). The maximal match was achieved by the insertion of gaps, designated as hyphens. Vertical bars indicate matching regions. The highest score is presented. The consensus AP1 and NF-κB-binding sites are underlined. The putative AP1 binding site in the PSD oligonucleotide was shown in bold.

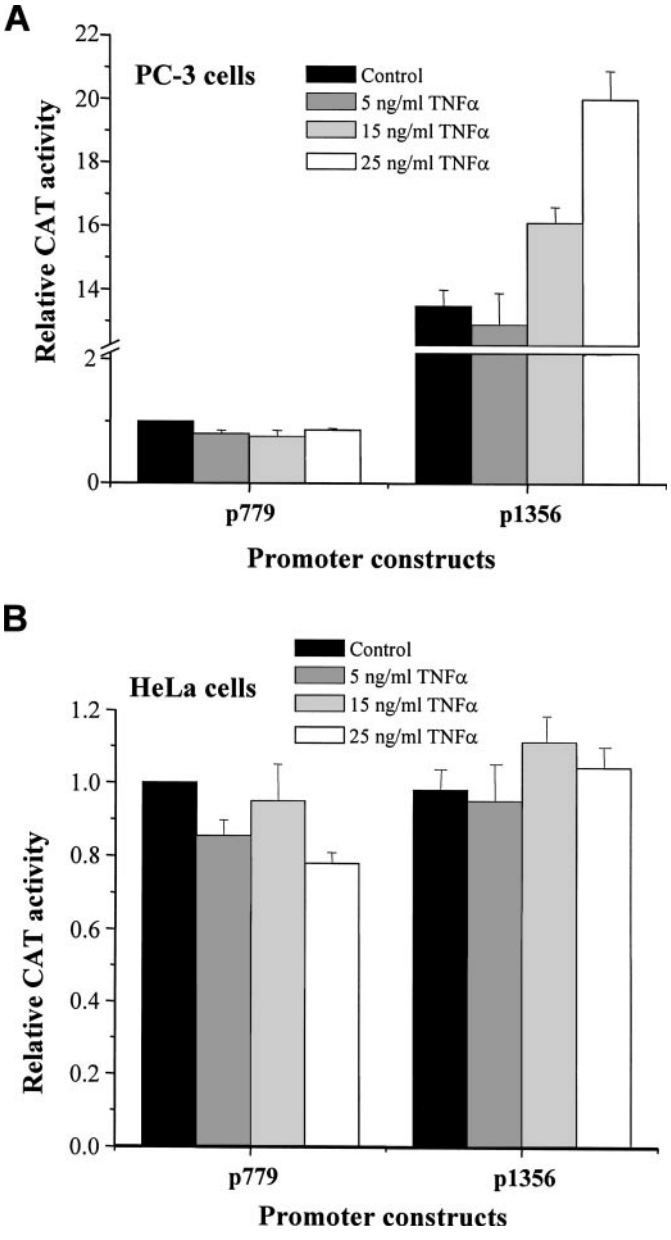

Figure 6. Transcriptional activation of the PAcP promoter by TNF-α in PC-3 and HeLa cells. (**A**) PC-3 cells were transfected with p779 or p1356 promoter constructs. Cells were treated, 24 h after transfection, with 5, 15 or 25 ng/ml of TNF-α for 48 h. Control cells received the solvent alone. CAT assays were performed as described under Materials and Methods. The CAT activity of p779 in control cells was assigned a value of 1.0. The results shown are the average ±SD from two sets of duplicate transfections ($n = 2 \times 2$). (**B**) HeLa cells were transfected with p779 or p1356 promoter constructs. Cells were treated, 24 h after transfection, with 5, 15 or 25 ng/ml TNF-α for 48 h. Control cells received the solvent alone. CAT assays were performed as described under Materials and Methods. The CAT activity of p779 in control cells was assigned a value of 1.0. The results shown are the average ±SD from two sets of duplicate transfections ($n = 2 \times 2$).

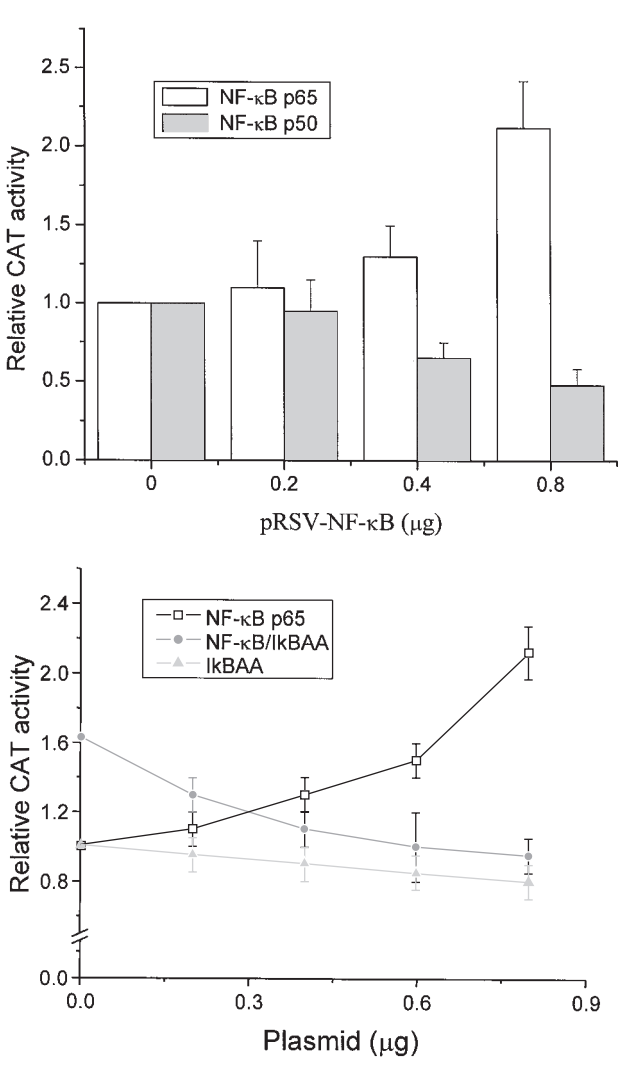

Figure 7. Effect of the NF-κB protein on the PAcP promoter activity. (**A**) PC-3 cells were co-transfected with the reporter construct p1356 (1 μg) plus increasing amounts of human p50 or p65 NF-κB expression plasmids. (**B**) A quantity of 1 μg of p1356 reporter construct was transiently co-transfected into PC-3 cells with increasing amounts of p65 NF-κB (open squares), a dominant-negative mutant of IκBαAA alone (closed triangles), or the combination of 0.5 μg of p65 NF-κB and increasing amounts of IκBαAA (closed circles) as indicated. The CAT activity was measured and then was normalized to β-galactosidase. Results were the average from two sets of duplicate transfections ($n = 2 \times 2$). The bar represents SD.

Determination of the minimal sequence requirement for NF-κB protein-binding to the PSD oligonucleotide

In order to determine the minimal sequence requirement for NF-κB protein-binding, two double-stranded oligonucleotides, in which the 5′- or 3′-PSD oligonucleotide was substituted by random sequences, were generated for gel shift analyses with PC-3 nuclear proteins. Figure 8A (left panel) shows that the 3′-PSD oligonucleotide failed to interact with nuclear proteins, while the 5′-PSD oligonucleotide showed a band of complex formation. The specificity of the interaction between the 5′-PSD oligonucleotide and nuclear proteins was confirmed by the competition between excess amounts of unlabeled probe (Figure 8, left panel). To confirm that the NF-κB protein can bind to the 5′ part of the PSD oligonucleotide, the 5′ probe was incubated with the p65 NF-κB protein. Incubation of the ^{32}P-labeled 5′ probe with the p65 protein formed complexes (Figure 8A, right panel) that were outcompeted by the addition of excess amounts of an unlabeled homologous probe (data not shown). These results indicate that the

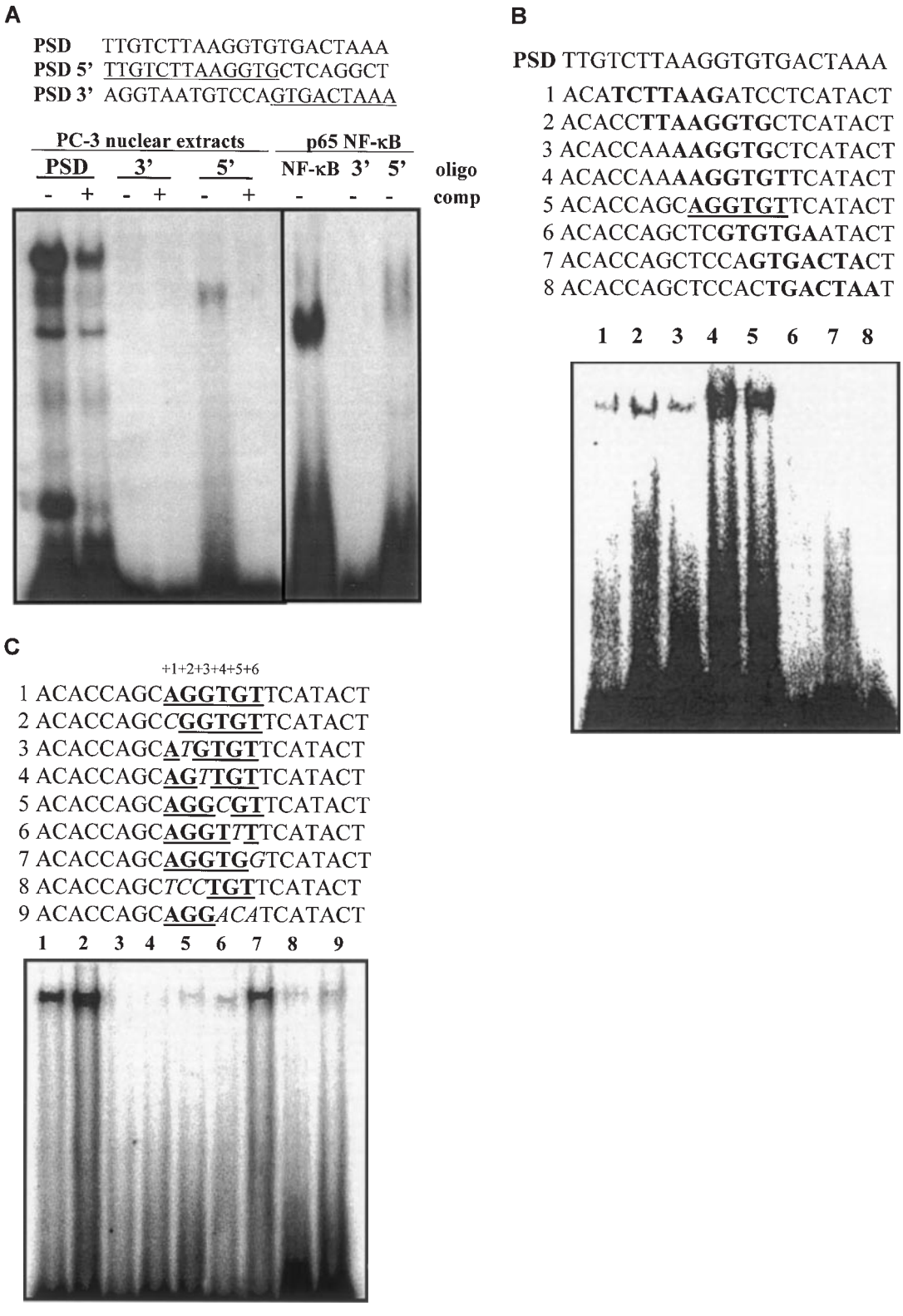

Figure 8. Determination of NF-κB-binding sequences in the PAcP promoter. (**A**) ^{32}P-labeled PSD oligonucleotides and PSD oligonucleotides containing random sequences at the 5′- or 3′-region, or NF-κB consensus oligonucleotides, were incubated with nuclear extracts from PC-3 cells (left panel) or recombinant NF-κB p65 protein (right panel) in the absence or presence of 100 molar excess of unlabeled corresponding probe. (Upper) The authentic PSD oligonucleotide sequence is underlined. (**B**) Oligonucleotides containing different overlapping sequences from the wild-type PSD oligonucleotide (indicated in bold) surrounded by random sequences were tested for *in vitro* binding with p65 NF-κB. (**C**) Effect of point mutation inside the AGGTGT motif on NF-κB-binding. The ^{32}P-labeled oligonucleotide probes containing the wild-type or mutated AGGTGT motifs surrounded with random sequences were incubated with recombinant p65 NF-κB protein. Mutated oligonucleotides are indicated with italic letters. The nuclear extract was prepared and EMSAs were performed as described under Materials and Methods.

5′ sequence of the PSD oligonucleotide contains an element that could be recognized by the NF-κB transcription factor.

In order to identify the core NF-κB-binding sequence within the PSD oligonucleotide, we synthesized eight oligonucleotides containing 6–8 bp overlapping sequences from the PSD oligonucleotide surrounded by random sequences. Incubation of each ^{32}P-labeled probe with the p65 NF-κB protein produced a band of reduced mobility, as seen using EMSA (Figure 8B). The intensity of binding increased when the core sequence moved toward the 3′ end of the PSD oligonucleotide. A clear, pronounced band was seen with the oligonucleotide containing the AGGTGT core sequence (Figure 8B, lane 5). These results reveal that a sequence of AGGTGT is required for NF-κB-binding.

Each nucleotide within the AGGTGT motif was analyzed individually for its impact on p65 NF-κB protein binding using EMSA analyses (Figure 8C). The substitution of A→C at position +1 or T→G at position +6 had only a marginal effect on the binding to p65 (Figure 8C, lanes 2 and 7). When the ^{32}P-labeled oligonucleotides contained a single substitution at positions from +2 to +5, the band disappeared or the intensity was significantly reduced (Figure 8C, lanes 3–6). Moreover, a significant reduction in binding was also observed in substitutions of AGG→TCC (+1/+3) (Figure 8C, lane 8) or TGT→ACA (+4/+6) (Figure 8C, lane 9). These data together show that the presence of all four nucleotides at positions +2/+5 of the (A/C)GGTG(T/G) motif is required for its effective binding to p65 NF-κB.

A functional NF-κB site in the 5′-flanking region of the PAcP promoter

In order to determine the effect of the novel NF-κB binding site on the PAcP promoter activity, PAcP promoter −1356/+86 plasmids with deletion of the putative NF-κB-binding site (ΔAGGTGT) or a single base mutation (A*T*GTGT) were transfected into PC-3 cells following by 24 h of TNF-α treatment. The deletion or the point mutation at the putative NF-κB-binding site in the p1356 promoter had only a minor effect of ∼10–15% on its basal activity, i.e. in the absence of TNF-α (Figure 9A). Nevertheless, the deletion or the point mutation of AGGTGT→A*T*GTGT effectively abolished TNF-α stimulation (Figure 9A). Similar results were obtained for the reporter construct containing triple substitution of AGG→TCC at positions +1/+3, i.e. TCCTGT (data not shown). These results clearly indicate that the novel NF-κB-binding site is critical for the further activation of the *cis*-active element in the PAcP promoter by TNF-α.

To determine the biological significance of the effect of NF-κB on activating the activity of the PAcP promoter, we analyzed whether NF-κB does indeed have an effect on endogenous PAcP expression in LNCaP human prostate carcinoma cells. We exposed these cells to various concentrations of TNF-α for 72 h and determined cPAcP activity, because cPAcP activity in general correlates with its expression level. Interestingly, TNF-α stimulated PAcP expression following a dose–response fashion and a 50 ng/ml concentration could increase PAcP expression up to 3-fold (Figure 9B), as observed in the promoter activity assay in PC-3 cells. Thus, NF-κB activation up-regulates endogenous PAcP expression in LNCaP cells.

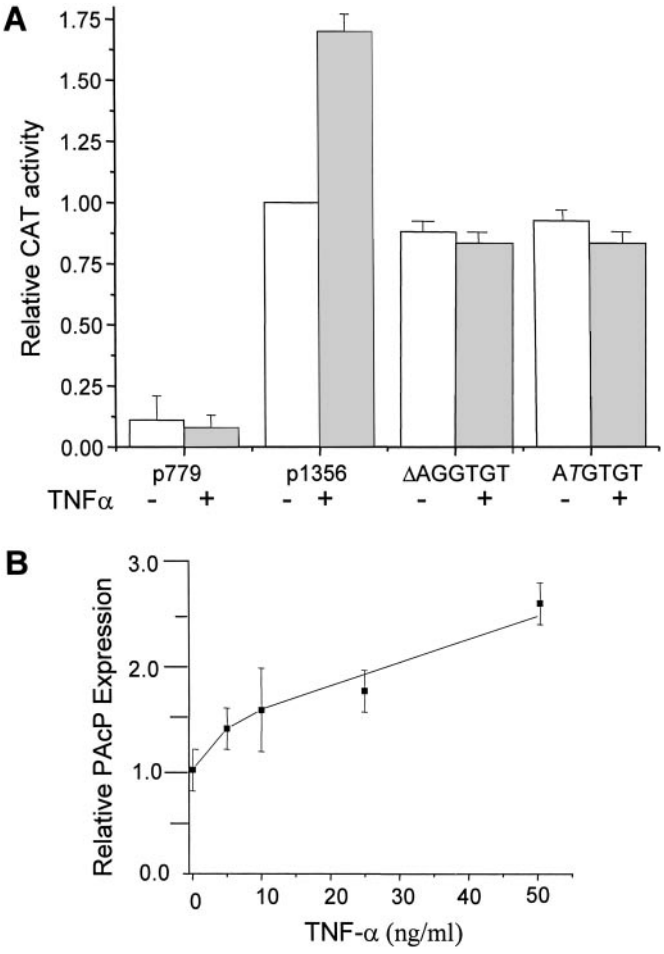

Figure 9. Effect of TNF-α on PAcP promoter activity. (**A**) The effects of deletion or mutation in the AGGTGT sequence on TNF-α-activated PAcP promoter activity. The wild-type p1356, ΔAGGTGT and ATGTGT mutant constructs (1 μg of each) were transfected into PC-3 cells. Transfected cells were incubated for 48 h and subsequently were treated with or without 25 ng/ml of TNF-α for 24 h. CAT activities were determined as described under Materials and Methods. Bars represent the SE of triplicates from at least two sets of independent experiments ($n \geqslant 6$). (**B**) The effect of TNF-α on endogenous PAcP in LNCaP cells. LNCaP cells in tripicates were treated with 5, 10, 25 and 50 ng/ml TNF-α, respectively, for 72 h. Control cells received the solvent alone. The PAcP activity in total cell lysates was determined to represent the PAcP expression. The results shown are the average ± SD ($n = 3$).

Comparative analysis of the novel NF-κB-binding site in prostate-enriched genes

We analyzed the presence of the AGGTGT motif in the promoter regions of several other genes that have high levels of expression in prostate cells, including PSA, Nkx-3.1, MIC-1 and DD3. Computer analyses of an ∼4 kb fragment of the 5′-flanking promoter region upstream of the start codon revealed the presence of AGGTGT motifs within the promoter regions of the PSA, Nkx-3.1 and MIC-1 genes (Table 1). Although no AGGTGT motif was found inside 4 kb of the 5′-flanking promoter region of the DD3 gene, there were four reminiscent motifs with T→G substitution at position +6 that had no significant impact on NF-κB/AGGTG(T/G) interactions (Figure 8C, lane 7). Taken together, the data indicate that

Table 1. Location of the NF-κB-binding motif AGGTGT within the promoter sequences of various prostate-enriched genes

Promoter	Position(s)
PAcP	−1254
PSA	−3984
Nkx-3.1	−1407
MIC-1	−2261
	−3991
DD3[a]	−3642
	−3585
	−2791
	−1895

[a]Computer analyses of the promoter sequence of the DD3 gene revealed the presence of NF-κB-binding motifs with AGGTG*G* sequence that exhibit a similar binding activity to AGGTGT (Figure 8C, lane 1 versus lane 7).

AGGTG(T/G) is a common motif in prostate-enriched genes, and it could potentially participate in the regulation by NF-κB of expression not only for PAcP but also for other prostate-enriched genes in a cell-specific manner.

DISCUSSION

This article describes our finding that the −1356/−779 region represents the unique positive regulatory domain in a 5 kb promoter DNA fragment (−4893/+87) of the *PAcP* gene although there are at least four negative regulatory elements. This positive *cis*-element is also involved in regulating the prostate cell-specific expression of the *PAcP* gene. However, DNase I footprinting analyses on the −1356/−779 fragment do not reveal a discrete binding site for prostate nuclear proteins versus non-prostate nuclear proteins, although there are at least six potential regulatory domains in this fragment. A similar phenomenon is observed in the PAcP promoter region −231/+50 (21). These results may suggest that other weak binding sites or unstable interactions of transcriptional factors with the PAcP promoter are responsible for mediating its prostate-specific expression. Alternatively, cofactors required for the prostate-specific activation of the PAcP promoter are not recruited through their direct interactions with promoter DNA, but rather through the cofactor–cofactor interaction and/or histone modification. For example, the SWI/SNF complex is required for hormone-dependent activation (50). Recruitment of SWI/SNF is mediated through CBP/p300, which itself is recruited through the interaction with the SRC coactivator, which leads to chromatin remodeling with altered DNA topology (50,51). It is also possible that HNF/FKH proteins contribute to the prostate-specific regulation because in the region between −1235 and −1205 bp, there are four consensus-binding sites for HNF/FKH proteins. Forkhead gene proteins interact with chromatin (52,53) and are involved in regulating gene expression in various developmental contexts (54,55), including genes with liver-specific function (56) and in gut development (57,58). Detailed analyses of transcriptional factors interacting with the PAcP promoter may require the utilization of chromatin immunoprecipitation assays. Additionally, it should be noted that there are minor differences in footprinting analyses (Figure 2A and B) and EMSA assays (Figure 4A) between nuclear proteins from PC-3 and DU 145 cells. These differences are apparently due to the nature of the two cell lines, because prostate cancer cells are known to exhibit heterogeneity and these two cell lines were derived from different origins (10,17). Nevertheless, the two cell lines exhibit similar patterns of PAcP promoter activity in transient transfection assays (25). Thus, the effects of these minor differences in regulating the PAcP promoter activity require further investigation.

Unexpectedly, sequence analyses using a MatInspector data bank search for the PAcP positive regulatory fragment from −1356 to −779 reveal that there is no potential binding site for ubiquitous transcription factors, except for the AP1 and CREB proteins. The AP1 protein includes c-*fos* and c-*jun* proto-oncogene products and can be induced by phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). It regulates a diverse range of cellular activities (59) through its interaction with the TPA-responsive element (60,61). Unexpectedly, the putative AP1 binding sequence in the *cis*-active region of the PAcP promoter does not interact with the AP1 protein in EMSA, nor can its consensus oligonucleotides compete with the PSD oligonucleotide in DNA–protein complex formation (Figure 4). The results indicate that the AP1 protein does not directly bind to the PAcP promoter DNA. This notion is parallel to our observations that the effect of TPA on the PAcP mRNA level is a slow response (62). Interestingly, overexpression of c-Jun and c-Fos proteins inhibits androgen-stimulated PSA promoter activity (63,64). Thus, different mechanisms are involved in regulating the expression of PAcP and PSA, two major prostate differentiation antigens (62). Additionally, the putative CREB-binding site is not within the protein interaction domain in footprinting assays. Further analyses of the putative CREB-binding site are required to determine the involvement of the CREB protein in the regulation of PAcP promoter activity.

Based on the results from searching the transcription factors database, the enhancer element of the PAcP promoter between −1356 and −779 does not contain a sequence corresponding to the consensus NF-κB element (Figure 3). Interestingly, the consensus NF-κB oligonucleotide competes with the PSD–protein complex formation in EMSA. Similarly, in the murine tissue inhibitor metalloproteinase-1 gene, the AP1 factor binds to a sequence that exhibits no consensus with the AP1 response element (65,66). Our data further show that the PSD oligonucleotide can directly interact with the NF-κB p50 (Figure 5B) and p65 proteins (Figure 5C), and overexpression of the p65 subunit activates the PAcP promoter activity. The inhibitory effect of p50 is similar to its effect on the PSA promoter (67). In general, it is p65, not p50, that serves as the positive effector of NF-κB-activated genes (68,69). Overexpression of a mutant IκBα (Figure 7B) and treatment with an NF-κB inhibitory peptide (data not shown) effectively abolish p65 NF-κB-induced activation of PAcP promoter construct p1356. The data collectively indicate that NF-κB directly activates the PAcP promoter via a novel binding site specifically in prostate cancer cells. This mode of regulation is biologically significant because the activation of the PAcP promoter activity by NF-κB correlates with elevated expression of endogenous PAcP in LNCaP cells.

Interestingly, the PSD oligonucleotide could form multiple complexes with nuclear proteins (Figures 4 and 5A), while it forms only one major band with the purified NF-κB protein (Figure 5B and C). It seems that NF-κB is directly interacting

with this DNA sequence in the PAcP promoter because NF-κB consensus oligonucleotides effectively compete with the formation of three major complexes. This direct interaction differs from observations in several other systems that NF-κB cooperates with other transcription factors and is not involved in direct DNA binding (70–73). Furthermore, this PSD sequence containing the NF-κB-binding site is required for activating the PAcP promoter activity using TNF-α (Figures 6 and 9) and IL-1 (data not shown) through NF-κB activation in p1356-transfected PC-3 cells but not in HeLa cells (Figure 6). The data collectively indicate that NF-κB-induced PAcP expression exhibits a cell-type-specific manner. However, it should be noted that in the presence of the anti-p65 NF-κB Ab, the complex formation between PSD oligonucleotides and p65 protein disappeared (Figure 5C, lane 4), while the same Ab had a super-shift effect on the NF-κB consensus oligonucleotide and p65 protein (lane 2). Similarly, the anti-p50 Ab abolished the interaction between the p50 NF-κB protein and PSD probes (data not shown). We hypothesize that both Abs have high affinities to corresponding proteins and that after their binding, the corresponding subunit protein undergoes a conformational change, resulting in the loss of their interactions with PSD oligonucleotides, which apparently have low affinity interactions. Of course, there are alternative explanations, e.g. the Abs may recognize the domain that also interacts with PSD probes and thus blocks the interaction. Further experiments are required to identify the mechanism of this Ab effect.

Owing to the potential importance of PAcP expression in prostate cancer as well as the biological significance of NF-κB signaling in general, we examine further the interaction between the DNA sequence AGGTGT and the NF-κB protein. It is known that different NF-κB dimers can interact with vast sequence variations in the κB-binding site. Further analyses of various NF-κB crystal structures reveal that all κB sites consist of two half-sites in which p50 and p52 monomers bind to a 5 bp 5′-GGGRN-3′ consensus, while p65 and c-Rel monomers do not require the first G:C base pair and bind to a 4 bp 5′-GGAA-3′ consensus (74–76). These half-sites are separated by a 1 bp spacer. For example, as shown in Figure 10, p65 NF-κB homodimers can bind to target sites with only one cognate half-site with the consensus sequence GGAA separated by an A–T base pair with highly conservative G at position +2 and a conservative G–C base-pair at position −3 of the other half (75). Significantly, the results of our EMSA analyses of the AGGTGT motif oligonucleotide reveal that the presence of G at positions +2 and +3 is indispensable for binding to the NF-κB p65 protein (Figure 8C). Our observation is thus in agreement with the results of crystal structure analyses of the p65 NF-κB homodimer complex (75). The conserved G–C base pairs at positions +2 and −3 that are present inside κB-like elements regulated by p65 and p50 are also present inside the PSD oligonucleotide (Figure 10) in which GGTG is sufficient and essential for NF-κB-binding (Figure 8A). We therefore hypothesize that GGxxTxxCx is a consensus sequence that can be recognized by both p65 and p50 NF-κB homodimers (Figure 10). Furthermore, the GGTG motif could have a higher binding preference for p65 than for p50 homodimers because it exhibits a slightly higher homology with the p65 consensus than the p50 consensus (Figure 10). Interestingly, the nucleotide polymorphism within the NF-κB motif contributes to the binding affinity of NF-κB proteins toward the target sites, thus regulating different levels of gene transcription (77). This novel NF-κB-binding site may contribute to the differential regulation of gene expression by NF-κB and thus merits further study.

	+2+3+4+5 0 -1-2-3-4
p65 consensus	5'-GGAR T TTCC-3'
PSD sequence	GGTG T GACT
p50 consensus	GGGGAA TCCCC

Figure 10. Sequence alignments of the PSD oligonucleotide sequence with the p65 and p50 NF-κB consensus sequences. The sequence GGTG is sufficient (Figure 8A) and essential (Figure 8B) for NF-κB-binding and thus was used for the comparison with the consensus sequence of the p65 and p50 NF-κB subunits (74–76).

The presence of this new NF-κB-binding site in the positive regulatory region of the PAcP promoter allows the NF-κB signal pathway to further activate the p1356 PAcP promoter activity by >20-fold, higher than the basic p779 promoter activity in a cell-specific manner (Figure 6). It is imaginable that other factor(s), e.g. HNF/FKH, may also interact with this 577 bp fragment to provide additional up-regulations, adding up to an extremely high level of PAcP expression in normal, differentiated prostate epithelia. In addition, NF-κB activation of the p1356 PAcP promoter occurs in androgen-insensitive PC-3 cells in the absence of added androgens, indicating that NF-κB activates PAcP expression via an androgen-independent pathway. In contrast, the PSA promoter contains four low-affinity consensus NF-κB-binding sites. They are either adjacent to or overlapping with androgen-response elements, indicating that possible cooperative interactions between NF-κB and AR contribute to PSA transcription (67). In addition to these low-affinity NF-κB-binding sites, computer analyses reveal the existence of one AGGTGT motif in the PSA promoter (Table 1). Similarly, the promoters of the MIC-1 and Nkx-3.1 genes contain at least one AGGTGT motif. Interestingly, the promoter of the DD3 gene has at least four reminiscent sequences containing one non-essential base pair substitution at position +6, i.e. AGGTGG, although it lacks the AGGTGT motif. The presence of this novel NF-κB-binding motif inside the promoter of prostate-enriched genes may indicate the importance of NF-κB to transactivation of the respective promoters in a prostate cell-specific manner for a high level of expression. In summary, owing to the importance of PAcP expression in prostate cancer biology, further characterizations of its promoter will provide useful information for understanding the regulation of PAcP expression in prostate epithelia and may contribute to new insights into prostate cancer progression.

ACKNOWLEDGEMENTS

We thank Dr Angie Rizzino at UNMC Eppley Cancer Institute for the expression vector of mutant IκBαAA, Dr Kuan-Teh Jeang at the Molecular Virology Section, NIH, and the AIDS Research and Reference Reagent Program, NIH, for NF-κB expression plasmids. This study was supported in part by grants

from NIH R01 CA88184, P20RR017675 and P20RR018759, Nebraska Research Initiative, and Nebraska Department of Health and Eppley Cancer Center LB595.

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