

Cloning and Characterization of the Prostate-Specific Membrane Antigen Promoter

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Abstract Prostate-specific membrane antigen (PSMA) is a protein that is expressed predominantly in normal prostate epithelial cells and in most adenocarcinomas of the prostate (Cap) and in virtually all Cap metastases. In this article we describe the cloning of a 2-kb human genomic DNA fragment containing the 5' upstream untranslated region of the PSMA gene and present evidence that it provides promoter activity. When the DNA fragment was cloned into transient expression vectors to examine promoter activity, the vectors were functional in promoting expression in several prostate and nonprostate cell lines in transient transfection assays. A 614-bp fragment derived from the 3' end of the 2-kb fragment may represent the minimal PSMA promoter as determined by deletion mutagenesis. The 2-kb fragment compared with the 614-bp fragment provided higher expression levels when using prostate-derived cell lines (DU 145 and LNCaP). The increased transcription using the 2-kb fragment was not as great in non-prostate cell lines. Little or no transcription over basal levels was seen with a 232-bp promoter fragment. When the concentration of dihydrotestosterone was depleted or supplemented in the growth medium, no significant effect was seen on PSMA-promoted transient expression in LNCaP cells, a prostate cell line. *J. Cell. Biochem.* 75:395–405, 1999. Published 1999 Wiley-Liss, Inc.†

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Prostate-specific membrane antigen (PSMA) is a 750-amino acid type II transmembrane glycoprotein with an apparent molecular weight of approximately 100 kDa [Israeli et al., 1993]. PSMA has been shown to be a unique folate hydrolase-carboxypeptidase that can release glutamate with either γ - or α -linkage [Heston, 1997]. It has been shown to have homology to

the brain protein NAALADase, and it exhibits *in vitro* neuropeptidase activity [Troyer et al., 1995; Carter et al., 1996]. A 140-amino acid extracellular region of PSMA has significant homology to the transferrin receptor [Troyer et al., 1995; Carter et al., 1996].

PSMA was originally defined by the monoclonal antibody 7E11.C5 and has been shown to be highly prostate specific [Horoszewicz et al., 1987]. In histological studies using monoclonal antibodies that recognize PSMA, the protein was shown to have specificity for prostate epithelial tissue [Horoszewicz et al., 1987]. Weak expression was seen in salivary ducts and the proximal small intestine, as well as in smooth muscle [Murphy, 1995; Troyer et al., 1995]. The

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protein has been detected in a subset of proximal renal tubules and intense staining was observed in endothelial cells of capillary vessels in peritumoral and endotumoral areas of certain malignancies [Silver et al., 1997].

PSMA mRNA has been detected in the LNCaP prostate cancer cell line, but not in DU-145 or PC-3 cell lines in Northern blot analyses [Israeli et al., 1993]. Ribonuclease protection revealed a significant level of PSMA mRNA only in prostate. Much lower steady-state levels were detected in brain and salivary gland [Israeli et al., 1994b]. Nevertheless, the precise function of PSMA in the prostate, in prostate cancer, and in these various other tissues remains unknown.

The PSMA gene has been cloned [Israeli et al., 1993] and has been shown to be located on chromosome 11 [Leek et al., 1995; Rinker-Schaeffer et al., 1995]. Two molecular forms of the protein, designated PSMA and PSMA', have been identified [Su et al., 1995]. PSMA', which is derived from an alternatively spliced transcript that lacks the transmembrane portion of PSMA, is located in the cytoplasm and its level is typically higher in normal cells [Su et al., 1995]. Recently, a 1.2-kb portion of the 5' promoter region of the PSMA gene was reported to be functional in driving expression of a reporter gene [O'Keefe et al., 1998].

Cancer of the prostate (CaP) is the most frequently diagnosed malignancy in men. It is estimated that there will be more than 184,000 newly diagnosed cases of CaP and 39,000 deaths in 1998. This disease constitutes a major health problem at both its early and advanced stages, with no cure available for the latter. PSMA promises to be useful as a diagnostic target for prostatic cancer detection [Abdel-Nabi et al., 1990; Wynant et al., 1991; Babaian et al., 1994; Murphy et al., 1995; Haseman et al., 1996]. Western blot assays of serum derived from prostatic cancer patients using the 7E11.C5 antibody have demonstrated increased levels of 7E11.C5 reactive PSMA antigen compared to normal subjects [Murphy et al., 1995, 1997]. In addition to the successful use of these antibodies for prostate cancer detection, there is a potential for their use in treatment of prostate cancer.

Previous observations revealed an increase in PSMA expression in prostate tumors of patients after chemical or surgical androgen abla-

tion [Wright et al., 1996], suggesting that androgens may be involved in downregulating transcription by the PSMA promoter. There is a diminished steady-state level of PSMA mRNA in LNCaP cells treated with physiological concentrations of dihydrotestosterone. In these experiments, PSMA mRNA levels were highest in charcoal-stripped medium [Israeli et al., 1994b]. However, the promoter has neither been defined completely nor examined for regulation by androgens. Because of the potential value of the PSMA gene, we felt that it was important to clone the PSMA promoter. Furthermore, it was important to demonstrate that the cloned promoter fragment was functional in promoting transcription of a reporter gene and to determine whether the promoter was responsive to changes in the concentration of dihydrotestosterone in transient transfection assays.

MATERIALS AND METHODS

Cloning of PSMA cDNA

Total cellular RNA was extracted from LNCaP cells (ATCC, Rockville, MD) by a single-step method [Tjoa et al., 1997]. The primer pair CAR2 (5'-AGC CAC GCC ACG CTC TTG-3') and CAR4 (5'-TCT TTC TGA GTG ACATAC-3'), specific for human PSMA, was used to prepare cDNA from 5 µg of total RNA, using avian myeloblastosis virus reverse transcriptase in two separate reactions following the specifications of the manufacturer (Promega, Madison, WI). The cDNA from these reactions was amplified in two separate polymerase chain reaction (PCR) reactions using the primer pair CAR1 (5'-TGC AGG GCT GAT AAG CGA G-3') and CAR 2 or CAR3 (5'-TCA TCC AAT TGG ATA CTA TG-3') and CAR4 (Roche Diagnostic Systems, Branchburg, NJ). PCR was conducted for 30 cycles heating 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. The PCR amplification with CAR1 and CAR2 resulted in a 1,472-bp product spanning nucleotides 134–1,605 (based on GenBank sequence M99487); PCR amplification with CAR3 and CAR4 resulted in a 1,427-bp product spanning nucleotides 1143–2569. These products contained a 463-bp overlap. A 2,436-bp PCR product spanning the entire PSMA cDNA was created using the products of CAR1 and CAR2 amplification (1) and the products of CAR3 and CAR4 amplification (1) as template together with primers CAR1 and CAR4. The

2.4-kb product was TA cloned into pCR2.1 (Invitrogen, Carlsbad, CA).

Human BAC Library Screening and Analysis

PSMA cDNA served as a PCR template, and its published sequence was used to design PCR oligonucleotide primers. Primer pairs were tested so that PCR amplification from both human genomic DNA and PSMA cDNA yielded products with the same predicted size, ensuring amplification of a segment without introns. A primer pair satisfying these requirements (5' TAC CAC ATT TAG CAG GAA CA 3'); (5' ATG AGT CTT ATT TGG GTA GG 3') amplified the fragment between nucleotides 500 and 614 of the published PSMA cDNA. These primers were used to screen a commercially available pooled human bacterial artificial chromosomal (BAC) library (Research Genetics, Huntsville, AL) by PCR. The pooled library is 9-fold redundant, so screening with a single PCR amplification should result in the identification of multiple clones. Three putative positive BAC clones were identified. The one (BAL 519 M3) exhibiting the strongest PCR amplification was chosen for further studies.

Total BAC DNA and PSMA cDNA were digested with *Bgl*I and analyzed by Southern blot using random primed cDNA as a probe to detect BAC restriction fragments that contain 5' flanking regulatory sequences for the PSMA gene. Southern blots were exposed to Kodak XAR-5 film and processed as previously described [Zhou et al., 1994].

Subcloning the PSMA Promoter

BAC Clone 96012 519 M3 was partially digested with *Sau*3AI and fragments of 10–12 kb were excised from a 0.7% low melting point agarose gel (SeaPlaque GTG, FMC BioProducts, Rockland, ME) and subcloned into *Bam*H I-digested and dephosphorylated pBluescript SK⁺ vector (Stratagene, La Jolla, CA). The ligations were introduced into host *Escherichia coli* cells plated and screened by colony hybridization, using the PCR product from the 5' UTR of the PSMA gene as a probe. A portion of the 5' untranslated region (5' UTR) of PSMA was amplified using the primer pair 5' CAG AGC TAA GAG CTG AAG CAT AGG C 3' and 5' CAG TAG TCA CAT TTA GTG GTG GCG 3'. Thermocycler settings were 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and

72°C for 1 min. Separation by electrophoresis on a 2% agarose gel provided a fragment of the expected size (640 bp). This PCR fragment was excised, purified, and [³²P]-labeled with random hexamer primers (Stratagene) to serve as probe for colony hybridization. Three positive clones were identified. Subsequent restriction endonuclease cleavage with *Eco*RI exhibited similar restriction patterns, suggesting that the clones contained identical sequences. Southern blot analysis using the 5' UTR of PSMA as a probe showed positive hybridization signals from two *Eco*RI fragments of 2.1 kb and 1.0 kb. These fragments were excised, purified, and subcloned into *Eco*RI digested pBluescript SK⁺.

Sequence Analysis

After restriction digestion of the 2.1-kb insert, overlapping fragments were subcloned into pUC19 and sequenced (McConnell Research, San Diego, CA). The sequence of the promoter fragment derived from the BAC library has accession number AF044684. The 2.1-kb *Eco*RI fragment was also subcloned and sequenced from an independently screened human PAC clone PAC-280-17L (Genome Systems, St. Louis, MO) (Accession number AF061571). Both clones were used for expression studies with similar results.

Tissue Culture and Transient Transfection Analysis

All cell lines except ALVA-31 [Loop et al., 1993] and PPC-1 [Brothman et al., 1989] were obtained from ATTC and were grown at 37°C in 5% CO₂. Media was supplemented with 10% fetal bovine serum (FBS). Tissue culture reagents were obtained from Gibco (Gaithersburg, MD). The following media was used in the respective cell lines: RPMI for LNCaP, EMEM for DU45, and Dulbecco's modified Eagle's medium (DMEM) for 911 and HeLa cells. The PZ line was grown in completely defined Keratinocyte-SFM medium (serum-free medium, Gibco). Within 24 h of passage, cells were transfected with LipofectAMINE (Gibco) in 6-well plates. Cells were transfected when 90% confluent. All cells were analyzed for reporter gene expression 48 h after completion of the transfection procedure following the recommendations of the manufacturer for *lacZ* (Galactolite, Tropix, Medford, MA) and luciferase gene (Promega) expression, on a Perkin-Elmer luminometer (2-s

delay time and 5-s integration time). Protein concentration of each sample was determined by a Bichinoic acid/Cu-based detection system (Pierce, Rockford, IL) and all measurements are expressed as relative light units (RLU) per mg protein.

Statistical Analysis

Means of data were calculated by analysis of variance with Fisher's follow-up testing (Statview, San Diego, CA). Statistical significance is assumed at $P < 0.05$. Alignment and comparison of nucleotide sequences and evaluation of transcription factor binding sites were performed with the MacVector computer program. In addition, the web-based software program TRANSFAC (<http://agave.humgen.upen.edu/utess/tess32>) was used to help identify transcription factor binding sites.

Construction of Reporter Plasmids

Fragments of various sizes (232, 614, and 1,915 bp) containing the upstream portions of the PSMA gene and 185 bp of transcribed sequence were cloned into mammalian gene expression vectors containing a luciferase reporter gene (the pGL3 series from Promega). These primers are listed below and the locations of the three upstream primers are underlined in Figure 1: PSM 167, 5' TTACTGGGT-GATCCACGTTTTAC 3' (used to produce the 1,915-bp fragment); PSM 1468, 5' GAT GGG GTT TCA CCA TGT TGG3' (used to produce the 614-bp fragment); and PSM 1860, 5' GCA AGA GCT GGA CAT TTT CCA AG3' (used to produce the 232-bp fragment). The same lower primer (PSM + 70, 5' GCG GGG TAA AGTC TCT CTC AAT C 3') was used with each of the three upper primers.

PCR amplification reactions yielded fragments of the PSMA promoter with the predicted sizes. PCR conditions were followed as recommended by the manufacturer of the reagents (Promega). Thermocycle settings were denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by heating at 72°C for 10 min.

The three resulting fragments were separated by gel electrophoresis and purified and initially TA cloned into pCR2.1 (Invitrogen). Clones were excised from pCR2.1 with *KpnI* and *XhoI*. The three fragments were moved

into the *KpnI* and *XhoI* sites of the polylinker in pGL3-Enhanced (pGL3EV) and in pGL3-Basic (pGL3BV), both of which contain a luciferase reporter gene. The resulting mammalian expression vectors were named EV300, EV600, EV2000, and BV2000. The corresponding promoterless constructs were pGL3-Control (designated EV-Control) and pGL3-Basic (BV-Control). The constructs labeled EV (derived from pGL3-Enhanced) differ from those designated BV (derived from pGL3-Basic) by the presence of an SV40 enhancer downstream of the reporter in the enhanced vector. These constructs are represented diagrammatically in Figure 2.

In one experiment, *EcoRI-HindIII* linkers were added to the ends of the *EcoRI* fragment derived from the PAC clone, the sample was digested with *HindIII*, and the entire fragment was cloned into the *HindIII* site within the polylinker region of pGL3-Basic and pGL3-Enhanced. Thus, the promoter used contained the entire *EcoRI* sequence shown in Figure 1. This promoter construct contained 128 bp of sequence downstream from the mRNA.

RESULTS

Cloning and Sequencing the 5' Flanking Region of the PSMA Gene

In this study two genomic DNA libraries were screened (a BAC library and a PAC library), and three clones from each library containing the PSMA gene sequence were identified. A 2.1-kb *EcoRI* restriction fragment located immediately upstream of the PSMA gene was identified from one of the clones from each library and was subcloned. The sequences of the promoter fragment derived from the BAC library and the fragment derived from the PAC library are depicted in Figure 1, and the positions of the *EcoRI* restriction sites are shown. Differences between the two sequences are marked as shaded nucleotides in Figure 1. Nucleotide 86 in the PAC derivative is A rather than T. Nucleotide 642 in the PAC derivative is A rather than G. In the PAC sequence, the G at nucleotide 687 is omitted. These minor differences may be the result of cloning artifacts or sequencing errors, but more likely they are due to polymorphism within the human PSMA gene. An arrow indicates the transcription start site at position 1898 based on the start site of the published PSMA cDNA sequence. We have not identified an associated TATA box. Although

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1  gaattcttca aatacatcct cttcaccata gcgtcttatt aattgaatta ttaattgaat
61 aaattctatt gttcaaaaat cacttttata ttttaactgaa atttgccttac ttataatcac
121 atctaaccctt caaagaaaac acatttaacca actgtactgg gtaatgttac tggtgatcc
181 cacggtttac aaatgagaag atatatcttg gtaagttgaa tacttagcac ccaggggtaa
241 tcagcttgga caggaccagg tccaaagact gttaagagtc ttctgactcc aaactcagtg
301 ctccctccag tgccacaagc aaactccata aaggatcct gtgctgaata gagactgtag
361 agtggtagaa agtaagacag acattatatt aagtcttagc tttgtgactt cgaatgactt
421 acctaattcta gctaaatttc agttttacca tgtgtaaattc aggaagagta atagaacaaa
481 ccttgaaggg tccaatggg gattaaatga ggtgatgtac ataacatgca tcactcataa
541 taagtgtctt ttaaataatta gtcactatta ttagccatct ctgattagat ttgacaatag
601 gaacattagg aaagatatag tacattcagg attttgtag agagagatga agaaattccc
661 ttcttctctg ccctagggtca tctagggagt tgtcatgggt cattgttgac aaattaattt
721 tcccaaattt ttcactttgc tcagaaagtc tacatcgaag cacccaagac tgtacaatct
781 agtccatctt tttccactta actcatactg tgcctcctct ttctcaaagc aaactgtttg
841 ctattccttg aatacactct gagttttctg cctttgccta ctcagctggc ccatggcccc
901 taatgtttct tctcatctcc actgggtcaa atcctacctg taccttatgg ttctgttaaa
961 agcagtgtct ccataaagta ctctagcaa atgcacggcc tctctcacgg attataagaa
1021 cacagtttat tttataaagc atgtagctat tctctccctc gaaatacgat tattattatt
1081 aagaatttat agcagggata taattttgta tgatgattct tctggttaat ccaaccaaga
1141 ttgattttat atctattacg taagacagta gccagacata gccgggatat gaaaataaag
1201 tctctgcctt caacaagttc cagtattctt ttctttctct cctccctcct cctccctcct
1261 cctccctcct cctccctcct cctccctcct cctttcttct ttgagggagt ctcactctgt
1321 caccaggctc cagtgcagtg gcgctatctt ggctgactgc aacctccgcc tccccgggtc
1381 aagcgattct cctgcctcag cctcctgagt agctgggact acaggagccc gccaccacgc
1441 ccagctaatt tttgtatttt tagtagagat gggtttcacc atgttgcca ggatgggtctc
1501 gatttctcga cttcgtgatc cgctgtctg ggctcccaa agtgctggga ttacaggcgt
1561 gagccaccac gcccggtttt aaaaaatggg tttgtaatgt aagtggagga taatacccta
1621 catgtttatt aataacaata atattcttta ggaaaaaggg cgcgggtggtg atttactctg
1681 atgacaagca ttcccgaacta tggaaaaaaa ggcagctttt ttctgctctg cttttattca
1741 gtagagattt gtagagattg tatagaattt cagagttgaa taaaagttcc tcataattat
1801 aggagtggag agaggagagt ctctttcttc ctttcatttt tatatttaag caagagctgg
1861 acattttcca agaaagtttt ttttttttaa ggcgcttctc aaaaggggcc ggatttctct
1921 ctctggaggg cagatgttgc ctctctctct cgctcggatt gggtcagtgc actctagaaa
1981 cactgctgtg gtggagaaac tggacccag gtctggagcg aattccagcc tgcagggctg
2041 ataagcgagg cattagttag attgagagag actttacccc gccgtggtgg ttggagggcg
2101 cgcagtagag cagcagcaca ggcggggtc ccgggaggcc ggctctgctc gcgccgagatg

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Fig. 1. Sequence of the prostate-specific membrane antigen (PSMA) promoter. The cloned promoter derived from the BAC library (Genbank accession number AF044684) and from the PAC library (Genbank accession number AF061571) are identical, except for single base changes at positions 85, 642, and 687 (highlighted) (see under Results, for description). The mRNA start site based on the published cDNA sequence is indicated by

an arrow at position 1898. The positions of the three polymerase chain reaction (PCR) primers used to produce the promoter deletion constructs are double underlined at positions starting at 167, 1468, and 1859. The lower PCR primer is single underlined at 2061. The *EcoRI* sites are underlined at positions 1 and 2020 and the ATG start codon is underlined at position 2159.

there are multiple potential TATA boxes in the promoter, they are all located further upstream.

The Cloned PSMA Promoter Is Not Derived From the PSMA Pseudogene

Work performed by Leek et al. [1995] indicates the existence of a PSMA pseudogene, which was mapped in close proximity to the

authentic PSMA gene on chromosome 11. To determine whether the isolated BAC clone contained the pseudogene or the authentic genomic PSMA sequence, Southern blot analysis using radiolabeled PSMA cDNA as a probe was performed on *Bgl*I-digested BAC DNA and *Bgl*I-digested PSMA cDNA. The digested BAC genomic DNA samples produced hybridizing bands considerably larger than fragments obtained

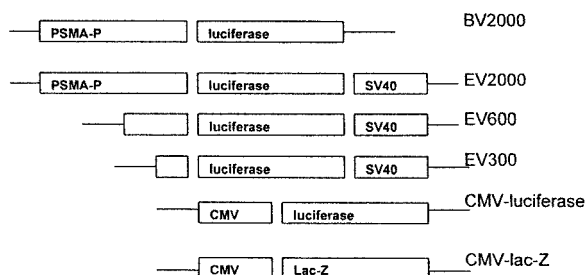


Fig. 2. Promoter and control constructs. The luciferase reporter gene under control of the 2-kb prostate-specific membrane antigen (PSMA) promoter sequence is designated BV2000. The same construct with an SV40 enhancer sequence at the 3' end of PSMA promoter is named EV2000. Analogous deletion constructs with the SV40 enhancer sequence consisting of the 614-bp and 232-bp 3' PSMA promoter fragments respectively are referred to as EV600 and EV300. The promoterless control EV-Control is not shown. The CMV lac-Z construct was used for cotransfection to normalize transfection efficiency as described under Materials and Methods.

from the PSMA cDNA digest (data not shown), because of introns present within the authentic PSMA gene. These results indicate that the BAC clone contains a copy of the authentic PSMA gene, and not the pseudogene.

Transcriptional Analysis of the PSMA Promoter

Experiments were conducted to examine transcription driven by the PSMA promoter. In the first experiment, four human cell lines were transfected by expression vectors using the PSMA promoter derived from the BAC library. The cell lines used include two prostate lines (DU 145, a human prostate cancer line and LNCaP, a human PSMA-expressing prostate cancer line) and two non-prostate lines (HeLa, a human cervical cancer line, and 911, a human retinoblastoma line). Transcription of a luciferase reporter gene construct was driven by the 2-kb clone PSMA promoter fragment (BV2000). This vector and the other expression vectors used in this experiment are represented diagrammatically in Figure 2.

A significant level of transcription was provided by the 2-kb PSMA fragment in all cell lines tested (Fig. 3A, column 1 (BV2000)). In the LNCaP, DU 145, and HeLa cell lines, the PSMA promoter in an expression vector with an SV40 enhancer sequence (column 2 (EV2000) marked with an asterisk) provided a significantly higher level of transcription compared with the PSMA promoter alone in BV2000 (a 2 logfold increase in the LNCaP cell line and 1 logfold increase in DU 145 cell line) (Fig. 3A,

BV2000 vs EV2000). Reporter gene transcription provided by these two constructs were relatively low compared with transcription provided by a cytomegalovirus (CMV) promoter construct that served as a positive control with very high promoter activity in this assay (Fig. 3A, column 3 (CMV)). Similar results were obtained in two subsequent experiments.

In another experiment (Figure 3B), several prostate cell lines (PPC-1, ALVA, PC3, DU 145, PZ, and LNCaP), as well as the non-prostate HeLa cell line, were transfected with luciferase expression vectors to examine activity of the PSMA promoter derived from the PAC clone. Five different firefly luciferase expression vectors were used in this experiment. Three of these were controls (pGL3-Control with an SV40 promoter and enhancer, pGL3-Basic with no promoter or enhancer, and pGL3-Enhanced with no promoter but with an SV40 enhancer), and two were test expression vectors (PSMA-promoted PSMA-pGL3-Basic, and PSMA-promoted PSMA-pGL3-Enhanced). All cell lines were cotransfected with a *Renilla* luciferase reporter construct as a transfection efficiency control vector using the dual luciferase assay system. In this experiment, the value of the promoterless vector was not subtracted, but the data were normalized by dividing firefly luciferase activity by *Renilla* luciferase activity.

The 2-kb PSMA promoter was active in driving transcription of the luciferase gene in all prostate cell lines tested, as well as in HeLa cells (Fig. 3B). Transcription was seen in all cell lines with all the expression vectors. The low transcriptional activity provided by the promoterless pGL3-Basic vector represents basal transcription of the luciferase gene in the absence of a functional promoter. The PSMA-promoted PSMA-pGL3-Basic test vector consistently provided much higher transcription levels than did the promoterless pGL3-Basic control vector. The PSMA-pGL3-Basic vector promoted transcription at a higher rate than the control pGL3-Enhanced vector in ALVA, PC3, and LNCaP prostate cell lines. By contrast, PSMA-pGL3-Basic provided similar levels of activity in PPC-1 and DU 145 cell lines.

The PSMA promoted PSMA-pGL3-Enhanced vector provided significantly higher transcription levels than pGL3-Enhanced (without the PSMA promoter) in all cell lines tested except the PZ prostate cell line. The large error in this

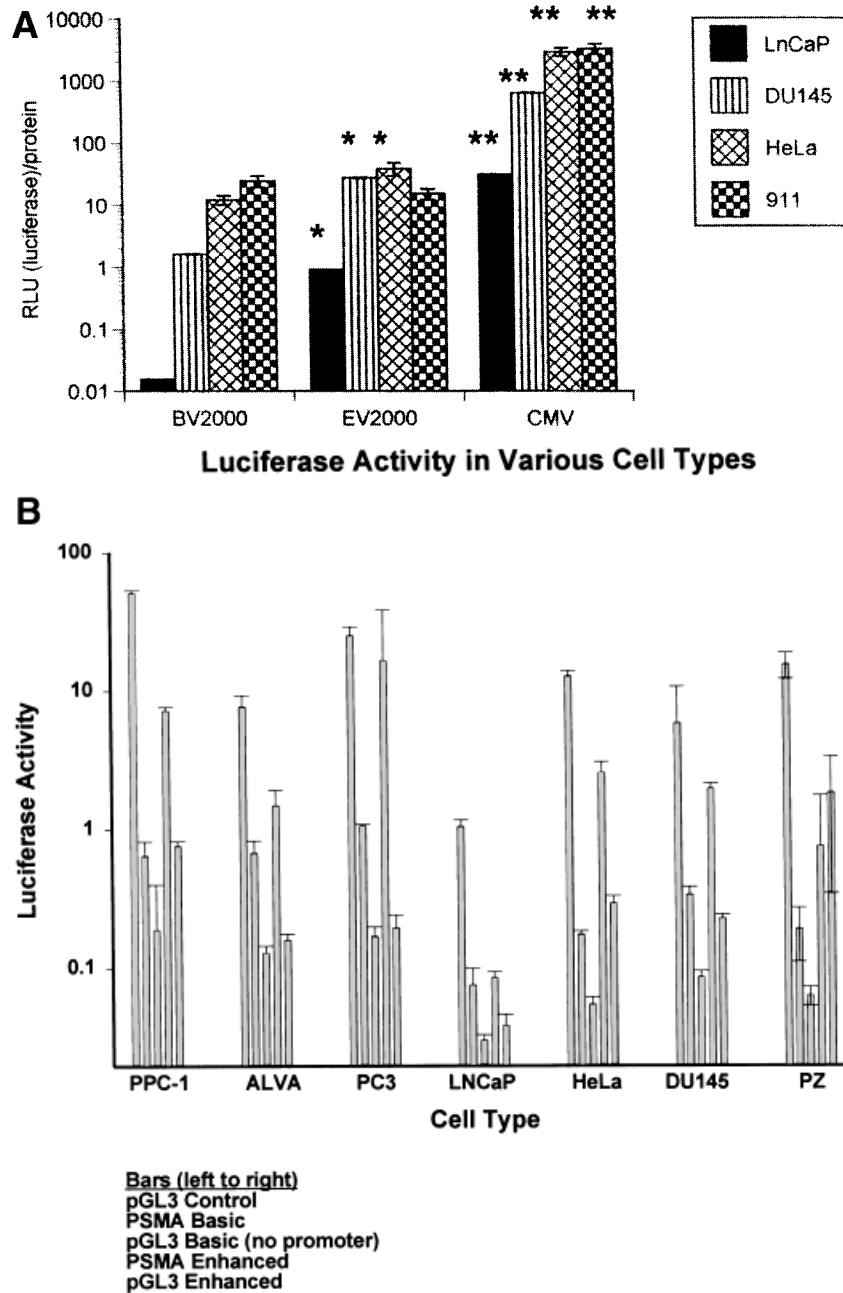


Fig. 3. Transient expression assays with the prostate-specific membrane antigen (PSMA) promoter. **A:** Activity of the BAC-derived PSMA promoter was tested by transient expression analysis in different cell lines. Human cell lines used in this experiment included prostate cancer cell lines (LNCaP and DU 145) and non-prostate lines (HeLa and 911). Cells were transfected with plasmids encoding the luciferase reporter gene. Transcription was driven by the 2-kb PSMA promoter in pGL3-Basic (BV2000) and in pGL3-Enhanced (EV2000), or it was driven by the CMV promoter. Results are expressed as relative light units (RLU) per mg of protein with a correction made by subtracting the basal activity generated by the promoterless control vector (pGL3-Basic). Values are corrected for basal luciferase activity by subtracting the activity of a promoterless control vector. *, statistically significant increase of gene expression of EV2000 over expression by the BV2000 construct ($P < 0.05$); **, statistically significant increase of gene expres-

sion by the CMV control construct over the EV2000 construct ($P < 0.05$). Results are the mean of three individual values \pm SEM. **B:** Activity of the PAC-derived PSMA promoter was tested in several cell lines in this experiment. The luciferase expression vectors used include the controls pGL3-Basic, pGL3-Enhanced, and pGL3-Control Luciferase expression vectors (Promega), as well as the PSMA-promoted PSMA-pGL3-Basic and PSMA-pGL3-Enhanced vectors. Prostate cell lines transfected in this assay included PPC-1, ALVA, PC3, DU 145, PZ, and LNCaP. One non-prostate line (HeLa) was also tested. The activities of the five vectors for each of the cell types are shown in the figure from left to right (pGL3-Control, PSMA-pGL3-Basic, pGL3-Basic, PSMA-pGL3-Enhanced, and pGL3-Enhanced). These data were collected using reagents in Promega's Dual Luciferase kit and are expressed as firefly luciferase activity divided by *Renilla* luciferase activity to correct for transfection efficiency in each cell line.

PZ data set does not allow us to see a significant difference in PSMA-pGL3-Enhanced versus pGL3-Enhanced. The data shown in Figure 3B confirm that the PSMA promoter drives expression in both prostate and non-prostate cell lines in transient expression assays.

We conclude that the cloned PSMA promoters derived from both the BAC and PAC clones are functional in six prostate and two non-prostate cell lines. When the PSMA promoter is placed in a vector with an SV40 enhancer sequence (PSMA-pGL3-Enhanced), the transcriptional activity is elevated compared with transcription using vector without an SV40 enhancer sequence (PSMA-pGL3-Basic).

Deletion Mutagenesis of the PSMA Promoter

In the previous experiments we observed relatively low luciferase activity driven by the PSMA promoter in the BV-2000 (PSMA-pGL3-Basic) construct. Therefore, promoter deletion constructs were made with plasmids containing the SV40 enhancer that gave higher levels of transcription. No increase in transcription over the basal transcription level was observed with the 232-bp construct compared with the promoterless control EV-Control (Fig. 4, cf. EV300 with EV-Control). Thus, important sequence elements that provide enhanced transcriptional over the basal rate are most likely located upstream from the proximal 232-bp region of the promoter. Cells were transfected

with the EV-300, the EV-600, or EV-2000 promoter constructs and they were all co-transfected with CMV-lacZ control. The data are expressed as the ratio of RLU (luciferase)/RLU(X-Gal).

In all cell lines tested, the 614-bp fragment (EV600) produced a significantly higher level of transcriptional activity in all cell lines than that of the promoterless control (EV-Control) or the 232-bp construct (EV300). Transcriptional activity with the 2-kb fragment was significantly higher than with the 614-bp fragment in the prostate cell lines (LNCaP and DU 145). In the non-prostate cell lines (HeLa and 911), there was no significant increase in activity of the 2-kb fragment compared with the 614-bp fragment.

We conclude that PSMA promoter sequence elements that enhance transcription are located within the promoter region at 232–614. Nevertheless, gene expression was highest with the full-length 2-kb construct in the prostate cancer cell lines. This finding points to the existence of additional regulatory sequence elements located in the region between 614 and the 5' end of the 2-kb PSMA promoter fragment.

PSMA Promoter Activity Does Not Appear to Be Directly Regulated by Androgens

Previous observations indicated that PSMA expression increased in prostate cell lines and

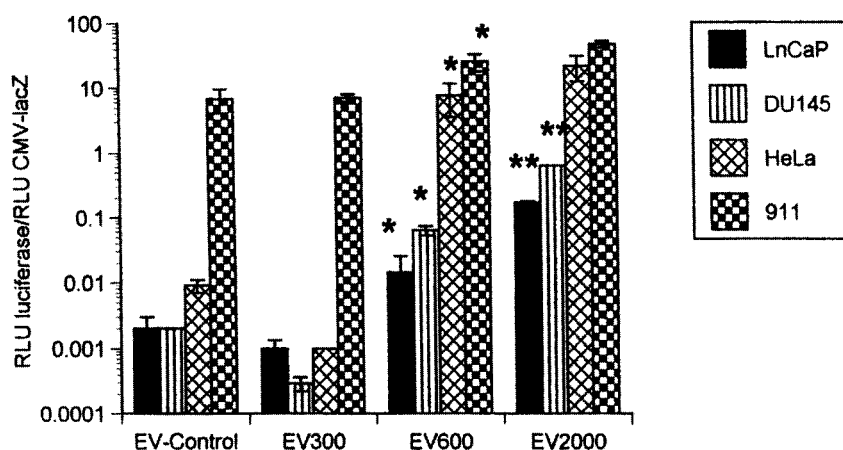


Fig. 4. Prostate-specific membrane antigen (PSMA) promoter deletion analysis. Cells were transfected with plasmids expressing the luciferase reporter gene driven by the BAC derived 2-kb PSMA promoter EV2000 or by the deletion constructs EV600 and EV300. Cells were cotransfected with the lacZ reporter gene under control of the CMV promoter at a ratio of 40:1. Results are expressed as relative light units RLU-luciferase corrected for transfection efficiency by dividing by the activity of

the cotransfected control vector CMV-lacZ. Data are presented as the mean ratio \pm SEM of three different values. *, statistically significant increase of gene expression over the promoterless EV-Control construct ($P < 0.05$); **, statistically significant increase of gene expression of EV2000 over the EV600 construct ($P < 0.05$). Results are reported as the mean of three individual values \pm SEM.

in tumors of patients after removal of androgen [Israeli et al., 1994b; Wright et al., 1996]. The data suggest that androgens downregulate expression by the PSMA promoter. Therefore, PSMA promoter activity was examined in an experiment to determine the effect of supplementing androgen-depleted media with dihydrotestosterone. The data presented in Figure 5 show that removal of androgens (column 1) and addition of dihydrotestosterone at concentrations of 10 (column 2) or 100 μM (column 3) to depleted medium do not affect luciferase expression by PSMA-promoted PSMA-pGL3-Basic in LNCaP cells. The experiment was conducted twice with the same results. Therefore, androgen depletion does not upregulate expression, and androgen supplementation does not downregulate expression by the subcloned 2-kb PSMA promoter.

DISCUSSION

Research on the PSMA gene has been initiated to define the promoter and to determine the degree to which this gene is regulated at the transcriptional level. There is a strong association of PSMA with CaP, making this gene an attractive molecular target for further study.

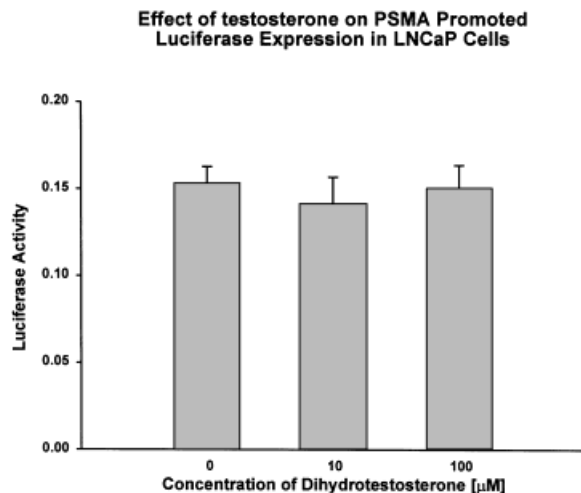


Fig. 5. Dihydrotestosterone does not appear to affect prostate-specific membrane antigen (PSMA) promoted transient expression. This transient expression assay was designed to examine the effect of supplementing androgen-depleted media with dihydrotestosterone on PSMA-promoted luciferase expression in LNCaP cells. The experiment was conducted twice with the similar results. The data show that removal of androgens using commercially "stripped" serum (column 1) and supplementing androgen-depleted medium with dihydrotestosterone at concentrations of 10 μM (column 2) or 100 μM (column 3) do not significantly alter luciferase expression by PSMA-promoted PSMA-pGL3Basic.

Proteins that are diagnostic for cancer through increased serum levels, such as prostate-specific antigen (PSA) or prostate acid phosphatase (PAP), have proved useful in disease screening and measurement of treatment response [Chomczynski and Sacchi, 1987; Brawer, 1995; Carter et al., 1996; Douglas et al., 1997]. PSMA, however, appears to be a more sensitive and specific marker for CaP [Horoszewicz et al., 1980, 1987; Gittes, 1991; Grizzle et al., 1994]. PSMA levels are also closely associated with disease progression [Horoszewicz et al., 1980, 1987; Gittes, 1991; Israeli et al., 1993, 1994a; Grizzle et al., 1994]. Therefore, a better understanding of mechanisms regulating transcription of the PSMA gene may contribute to development of strategies to eradicate prostate cancer cells selectively *in vivo* by using the promoter to express cytotoxic or growth regulatory genes in prostate tumors.

PSMA was originally defined by a monoclonal antibody raised against LNCaP cells [Israeli et al., 1994a, 1994b, 1995; Maroulakou et al., 1994]. Israeli and colleagues characterized the protein and cloned the corresponding 2.65-kb cDNA [Israeli 1994a, 1995]. Since then, PSMA has been further characterized as an integral membrane glycoprotein that is detected by immunological methods in most prostate cancers. The immunogenicity of PSMA is being exploited in clinical trials by vaccine protocols [Murphy et al., 1995].

Since PSMA is useful as a diagnostic tool and may be used as a therapeutic tool in the future, it was important to clone and characterize the PSMA promoter. In this study, we cloned and sequenced a 2-kb genomic DNA fragment located in the 5' upstream region of the PSMA gene. Multiple consensus sequence elements, some of which may be potential sites for binding transcription factors, have been identified in the PSMA promoter using TRANSFAC, a software program designed to identify consensus sequence elements. For example, 53 consensus AP1 sites and 12 consensus c-jun sites were identified in the 2-kb promoter fragment. However, we have no evidence indicating which, if any, of these sequence elements are functional. Therefore, it will be important to identify elements within the PSMA promoter that contribute to regulation of transcription of the gene in future studies.

We have not identified a TATA box that is associated with the presumptive mRNA start site. However, when we used the cloned 2-kb

EcoRI fragment as a probe in Northern blot assays, PSMA mRNA of the expected length was detected in total cellular RNA derived from the LNCaP cell line but not from DU145 and PC-3 cell line (data not shown). This confirms the published pattern of PSMA steady-state mRNA levels in these cell lines [Israeli et al., 1993]. These data also indicate that a portion of the mRNA leader region is located within the 2-kb *EcoRI* fragment.

This study demonstrates that the cloned 2-kb promoter is functional in a number of cell lines by transient transfection assays. Furthermore, the 2-kb PSMA promoter activity increased to a greater degree compared with the 614-bp promoter in the presence of an enhancer provided by pGL3-Enhanced in prostate cell lines versus non-prostate cell lines (Fig. 4). Although the enhanced activity appears to be specific for prostate cell lines, there is nevertheless a relatively high level of expression in non-prostate cell lines. Identification of specific promoter elements that provide enhanced transcriptional activity in prostate cells requires further investigation. It was recently reported that a 1.2-kb promoter fragment of the PSMA gene was functional in driving expression in prostate cell lines but not in breast-derived cell lines [O'Keefe et al., 1998]. We have not examined breast-derived cell lines, but our study shows that both 614-bp and 2-kb promoter fragments are functional in driving expression in several prostate and at least two non-prostate cell lines.

Previous observations revealed that PSMA expression increased in prostate tumors of patients after chemical or surgical androgen ablation [Wright et al., 1996]. The results of those studies suggested that androgens may be involved in downregulating transcription by the PSMA promoter. Therefore, we tested the effect of androgen concentration on PSMA promoter activity in transient expression assays in the LNCaP cell line. We were surprised to find little or no effect of androgen deprivation or androgen supplementation of "androgen stripped" serum upon PSMA-promoted transient expression (Fig. 5). It is possible that transcriptional control elements responsive to androgens are located upstream from the cloned 2-kb promoter fragment used in this study. If the PSMA promoter is to be used as a tool for gene therapy, upregulation of the promoter when androgen concentration is lowered may be advantageous over promoters such as the PSA promoter that

are known to be activated by androgens. Upon androgen ablation, the cloned PSMA promoter should remain active, whereas activities of androgen-dependent promoters are reduced. Consequently, if PSMA promoter activity is found to increase in the absence of androgen, the promoter is potentially even more useful.

In summary, the results of the experiments presented in this article provide a rationale for continued research on the PSMA promoter. There is a need to identify specific sequence elements that enhance transcription in prostate epithelial cells and in prostate cancer cells and that silence transcription in most non-prostate cells. In the transient expression assays described in this study, the PSMA promoter does not appear to provide enhanced prostate-specific transcription. It is possible that enhancers and other elements that lead to enhanced expression in prostate epithelial cells and in prostate cancer are located farther upstream or downstream from the 2-kb fragment examined in this study. Likewise, androgen-responsive elements within the promoter have not been identified. Therefore, future experiments will be directed toward the identification of these elements.

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REFERENCES

- Abdel-Nabi HH, Ortman-Nabi JA, See W, Lee J, Ireton R, Boileau M, Unger MW, Halverson C. 1990. Clinical experience with intra lymphatic administration of ¹¹¹In-labelled monoclonal antibody PAY 276 for the detection of pelvic nodal metastases in prostatic carcinoma. *Eur J Nucl Med* 16:149-156.
- Babaian RJ, Sayer J, Podotoff DA, Steelhammer LC, Bhadkamkar VA, Gulfo JV. 1994. Radioimmunosintigraphy of pelvic lymph nodes with ¹¹¹indium-labeled monoclonal antibody CYT-356. *J Urol* 152(6 Pt 1):1952-1955.
- Brawer MK. 1995. How to use prostate-specific antigen in the early detection or screening for prostatic carcinoma. *CA Cancer J Clin* 45:148-164.

- Brothman AR, Lesho LJ, Somers KD, Wright GL, Jr., Merchant DJ. 1989. Phenotypic and cytogenetic characterization of a cell line derived from primary prostatic carcinoma. *Int J Cancer* 44:898-903.
- Carter RE, Feldman AR, Coyle JT. 1996. Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. *Proc Natl Acad Sci USA* 93:749-753.
- Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156-159.
- Douglas TH, Morgan TO, McLeod DG, Moul JW, Murphy GP, Barren R, 3rd, Sesterhenn IA, Mostofi FK. 1997. Comparison of serum prostate specific membrane antigen, prostate specific antigen, and free prostate specific antigen levels in radical prostatectomy patients. *Cancer* 80:107-114.
- Gittes RF. 1991. Carcinoma of the prostate [see comments]. *N Engl J Med* 324:236-245.
- Grizzle WE, Myers RB, Arnold MM, Srivastava S. 1994. Evaluation of biomarkers in breast and prostate cancer. *J Cell Biochem Suppl* 19:259-266.
- Haseman MK, Reed NL, Rosenthal SA. 1996. Monoclonal antibody imaging of occult prostate cancer in patients with elevated prostate-specific antigen. Positron emission tomography and biopsy correlation. *Clin Nucl Med* 21:704-713.
- Heston WD. 1997. Characterization and glutamyl preferring carboxypeptidase function of prostate specific membrane antigen: a novel folate hydrolase. *Urology* 49(3A suppl):104-112.
- Horoszewicz JS, Leong SS, Chu TM, Waisman ZL, Friedman M, Papsidero L, Kim U, Chai LS, Kakati S, Arya SK, Sandberg AA. 1980. The LNCaP cell line—a new model for studies on human prostate carcinoma. *Prog Clin Biol Res* 37:115-132.
- Horoszewicz JS, Kawinski E, Murphy GP. 1987. Monoclonal antibodies to a new antigenic marker in epithelial prostatic cells and serum of prostatic cancer patients. *Anticancer Res* 7:927-935.
- Israeli RS, Powell CT, Fair WR, Heston WD. 1993. Molecular cloning of a complementary DNA encoding a prostate specific membrane antigen. *Cancer Res* 53:227-230.
- Israeli RS, Miller WH Jr, Su SL, Powell CT, Fair WR, Samadi DS, Huryk RF, DeBlasio A, Edwards ET, Wise GJ, et al. 1994a. Sensitive nested reverse transcription polymerase chain reaction detection of circulating prostatic tumor cells: comparison of prostate-specific membrane antigen and prostate-specific antigen-based assays. *Cancer Res* 54:6306-6310.
- Israeli RS, Powell CT, Fair WR, Heston WD. 1994b. Expression of the prostate specific membrane antigen. *Cancer Res* 54:1807-1811.
- Israeli RS, Miller WH Jr, Su SL, Samadi DS, Powell CT, Heston WD, Wise GJ, Fair WR. 1995. Sensitive detection of prostatic hematogenous tumor cell dissemination using prostate specific antigen and prostate specific membrane-derived primers in the polymerase chain reaction. *J Urol* 153(3 Pt 1):573-577.
- Leek J, Lench N, Maraj B, Bailey A, Carr IM, Andersen S, Cross J, Whelan P, MacLennan KA, Meredith DM, et al. 1995. Prostate-specific membrane antigen: evidence for the existence of a second related human gene. *Br J Cancer* 72:583-588.
- Loop SM, Rozanski TA, Ostenson RC. 1993. Human primary prostate tumor cell line, ALVA-31: a new model for studying the hormonal regulation of prostate tumor cell growth. *Prostate* 22:93-108.
- Maroulakou IG, Anver M, Garrett L, Green JE. 1994. Prostate and mammary adenocarcinoma in transgenic mice carrying a rat C3(1) simian virus 40 large tumor antigen fusion gene. *Proc Natl Acad Sci USA* 91:11236-11240.
- Murphy GP. 1995. Radioscintiscanning of prostate cancer. *Cancer* 75:1819-1822.
- Murphy G, Ragde H, Hall MC, Zhao LS, Troncoso P, Pollack A, Chung LW. 1995. Comparison of prostate specific membrane antigen, and prostate specific antigen levels in prostatic cancer patients. *Anticancer Res* 15:1473-1479.
- Murphy GP, Maguire RT, Rogers B, Partin AW, Nelp WB, Troychak MJ, Ragde H, Kenny GM, Barren RJ, 3rd, Bowes VA, Gregorakis AK, Holmes EH, Boynton AL. 1997. Comparison of serum PSMA, PSA levels with results of Cytogen-356 ProstaScint scanning in prostatic cancer patients. *Prostate* 33:281-285.
- O'Keefe DS, Su SL, Bacich DJ, Horiguchi Y, Luo Y, Powell CT, Zandvliet D, Russell PJ, Molloy PL, Nowak NJ, Shows TB, Mullins C, Vonder Haar RA, Fair WR, Heston WDW. 1998. Mapping, genomic organization and promoter analysis of the human prostate-specific membrane antigen gene. *Biochim Biophys Acta* 1443:113-127.
- Rinker-Schaeffer CW, Hawkins AL, Su SL, Israeli RS, Griffin CA, Isaacs JT, Heston WD. 1995. Localization and physical mapping of the prostate-specific membrane antigen (PSM) gene to human chromosome 11. *Genomics* 30:105-108.
- Silver DA, Pellicer I, Fair WR, Heston WDW, Cordon-Cardo C. 1997. Prostate-specific membrane antigen expression in normal and malignant human tissues. *Clin Cancer Res* 3:81-85.
- Su SL, Huang IP, Fair WR, Powell CT, Heston WD. 1995. Alternatively spliced variants of prostate-specific membrane antigen RNA: ratio of expression as a potential measurement of progression. *Cancer Res* 55:1441-1443.
- Tjoa BA, Erickson SJ, Bowes VA, Ragde H, Kenny GM, Cobb OE, Ireton RC, Troychak MJ, Boynton AL, Murphy GP. 1997. Follow-up evaluation of prostate cancer patients infused with autologous dendritic cells pulsed with PSMA peptides. *Prostate* 32:272-278.
- Troyer JK, Beckett ML, Wright GL. 1995. Detection and characterization of the prostate-specific membrane antigen (PSMA) in tissue extracts and body fluids. *Int J Cancer* 62:552-558.
- Wright GL Jr, Grob BM, Haley C, Grossman K, Newhall K, Petrylak D, Troyer J, Konchuba A, Schellhammer PF, Moriarty R. 1996. Upregulation of prostate-specific membrane antigen after androgen-deprivation therapy. *Urology* 48:326-334.
- Wynant GE, Murphy GP, Horoszewicz JS, Neal CE, Collier BD, Mitchell E, Purnell G, Tyson I, Heal A, Abdel-Nabi H, et al. 1991. Immunoscintigraphy of prostatic cancer: preliminary results with ¹¹¹In-labeled monoclonal antibody 7E11-C5.3 (CYT-356). *Prostate* 18:229-241.
- Zhou HE, Pisters LL, Kenny G, Barren R, 3rd, Erickson S, Tjoa B, Boynton A, Holmes E, Gilbaugh J, Douglas T. 1994. Biomarkers associated with prostate cancer progression. *J Cell Biochem Suppl* 19:208-216.