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TITLE: Characterization of Prostate-Specific Membrane Antigen (PSMA) for use in Therapeutic and Diagnostic Strategies Against Prostate Cancer

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Cleveland, Ohio 44195

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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Prostate-Specific Membrane Antigen (PSMA) appears to be an ideal prostate cancer marker and potential therapeutic target, however there have been reports of PSMA expression in non-prostatic tissues, including brain, kidney and liver. Such expression of PSMA could weaken the potential of the gene as a prostate cancer marker or at least produce confusing and conflicting data. Prior to this project, we had demonstrated that there was another human gene, 97% identical to PSMA, which we termed the PSMA-Like gene. The first aim of this project was to characterize the differences between the non-prostatic and prostatic forms of PSMA and to determine strategies to specifically target PSMA expressed in prostate or prostate tumors. The second aim of the project was to define the minimal regulatory regions of the PSMA gene, for future utilization in gene therapy strategies, by analyzing deletion constructs of the gene regulatory regions. To date we have cloned and characterized the PSMA-Like gene, and determined ways of distinguishing the two genes at the DNA, RNA and protein levels which will aid in designing new diagnostic and therapeutic anti-prostate cancer strategies. In addition, we have characterized the promoter and enhancer region of PSMA, and developed gene therapy vectors that specifically kill human prostate cancer tumors growing in mice.

14. SUBJECT TERMS
Prostate-specific enhancers, tumor markers, gene expression, Promoters, PSMA, Folate

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Table of Contents

Introduction

Prostate-Specific Membrane Antigen (PSMA) appears to be an ideal prostate cancer marker and potential therapeutic target, however there have been reports of PSMA expression in non-prostatic tissues, including brain, kidney and liver. Such expression of PSMA could weaken the potential of the gene as a prostate cancer marker or at least produce confusing and conflicting data. Prior to the onset of this project, we had demonstrated that there was another human gene, approximately 97% identical to PSMA, which we termed the PSMA-Like gene. Therefore the first aim of this project was to characterize the differences between the non-prostatic and prostatic forms of PSMA at the nucleic acid, protein, and functional levels and to determine strategies to specifically target PSMA expressed in prostate or prostate tumors. The second aim of the project was to define the minimal regulatory regions of the PSMA gene, for future utilization in gene therapy strategies, by analyzing deletion constructs of the gene regulatory regions and by comparison with the PSMA-Like gene regulatory regions.

Specific Aim 1. *To clone and sequence the "PSMA-Like" gene, and comparatively analyze tissue expression and function of the PSMA and PSMA-Like genes.*

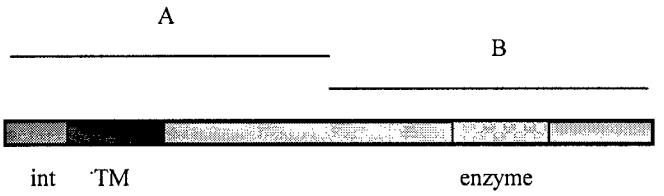
As described in my first report, I have cloned and characterized the tissue distribution and enzymatic activity of the PSMA-Like gene (now designated Folh1B by Genbank; accession number AF261715). The genomic sequence of the PSMA and PSMA-Like genes is 97% identical, however the PSMA-Like gene has undergone a deletion of the region corresponding to the PSMA promoter, exon one and part of intron one. Therefore, the PSMA-Like gene uses an alternative promoter which is found in intron five of the gene. Interestingly, when I attempted to develop RNase protection assays and RT-PCRs assuming that the transcribed region of intron five in PSMA-Like would differentiate it from PSMA, I found that in fact PSMA also initiates transcription from this promoter, although this promoter is significantly weaker than the original PSMA promoter. I was able to distinguish the two genes using RT-PCR followed by restriction enzyme digestion and using this method determined that the PSMA-Like gene is expressed in kidney and liver. The PSMA gene is expressed in prostate, prostate cancer, tumor neovasculature, the brain and small intestine, and in addition we see PSMA RNA in kidney and liver. The cDNA sequence of the PSMA-Like gene is 1992 bp, and translation predicts a protein of 443 amino acids or 49kD in size, as well as a cytosolic location in the cell.

Functional Analysis of the PSMA-Like gene

These studies have been somewhat difficult. Last year, I reported that we had expressed the PSMA-Like gene in a cell line and shown the cell line gained NAALADase activity, one of the enzymatic activities of PSMA. However this data was based on one experiment, which I attempted to repeat a significant number of times before publishing. I was unable to repeat this result. In addition, I was unable to establish stable cell lines expressing PSMA-Like, although I repeatedly had no problem establishing stable cell lines with my control experiments (vector alone and vector containing PSMA). I next optimized transient transfections of PC3 cells so as NAALADase activity could be examined after transfection of a PSMA containing vector. Repeatedly I was unable to demonstrate PSMA-Like enzymatic activity. Western blot analysis

with antibodies against PSMA demonstrated PSMA protein present after transient transfection, but was not able to show PSMA-Like protein. This could be because antibodies such as Cyt-351 bind to the N-terminus of PSMA or other regions that are missing in PSMA-Like. Because the amino-acid differences between the two proteins in the presumed enzymatic region are so few, it was possible that folding of the PSMA-Like protein was affected by the lack of a transmembrane domain, leading to loss of enzymatic activity. I next created a "hybrid" molecule, containing the first 307 amino acids of PSMA, followed by all of PSMA-Like (see figure 1).

Figure 1: Diagrammatic representation of the PSMA/PSMA-Like hybrid molecule. The Cyt-351 antibody binds the internal domain (int) of PSMA. TM refers to the transmembrane domain, enzyme to the enzymatic pocket. A indicates the first 307 amino acids of PSMA, B indicates the entire protein sequence of PSMA-Like, together they form the hybrid.



Following transient transfection into PC-3 cells, I was able to demonstrate appropriate expression of the PSMA/PSMA-Like hybrid molecule via western analysis using the Cyt-351 antibody which binds the amino-terminal of PSMA. Surprisingly, this construct did not have NAALADase activity. This is surprising as PSMA and the PSMA/PSMA-Like only differ by nine amino-acids. Of these, six are similar amino acids. In addition, these six amino acids that differ are all found in at least one of the other species for which NAALADase homology have been cloned (e.g. rat, mouse, pig), that do exhibit NAALADase activity and therefore we do not think these changes are responsible for the loss in activity. Of the three other differences, two are found near the zinc-ligand binding regions of the enzyme, and also the glutamic acid that is thought to be necessary for catalysis. These changes are:

a.a. 398 isoleucine→threonine (PSMA→PSMA-Like)

a.a. 437 glutamic acid→aspartic acid (PSMA→PSMA-Like)

We consider that these changes might be important when designing drugs to inhibit PSMA, and therefore are testing these two amino acids (individually) by site-directed mutagenesis to determine if the changes are critical to the enzymatic activity of the protein. These are the final experiments we plan to do to complete specific aim one of the project.

Specific Aim Two. *To define the minimal promoter/enhancer regions of the PSMA gene and determine the specific sequence elements responsible for activity.*

In my last report I stated that the DNA sequence in the PSMA-Like gene that corresponds to the promoter, exon one and part of intron one of the PSMA gene, has been deleted. As such, PSMA-Like uses an alternative promoter found in the region corresponding to intron five of the gene. This promoter is entirely different to the PSMA promoter. The enhancer regions of the two genes however, are 95% identical. If the PSMA-Like enhancer was not able to drive activity in prostate cells, we could use the differences to determine the critical regions for activity in the PSMA enhancer. However, we found that in combination with the PSMA promoter, the PSMA-Like enhancer was in fact able to drive prostate-specific reporter gene activity, with over 80% of

the activity of the PSMA enhancer. However, in collaboration with Dr. Peter Molloy's laboratory in Australia, we were able to determine that the PSMA-enhancer is able to drive prostate-specific expression using heterologous promoters, including the PSA and probasin promoters which are prostate derived promoters, and the non-tissue specific herpes virus TK promoter. In addition, the most active portion of the PSMA enhancer is a 331bp region, which, like the native PSMA gene, is upregulated by androgen deprivation (refer to attached manuscripts by O'Keefe et al., 2000 and Watt et al., 2001).
In our aim to design a gene therapy approach against prostate cancer, utilizing the PSMA enhancer, I last reported that we had generated such a vector. The vector drives the *E.coli* Cytosine deaminase gene and we were able to demonstrate cytotoxicity of this vector in prostate cancer but not control cell lines treated with the non-toxic prodrug 5-fluorocytosine (5-FC). In collaboration with Dr. Atsushi Uchida, a clinical fellow whom I have been assisting in his research training in the lab, we were able to show that treatment with this vector and 5-FC in nude mice with LNCaP-C42 prostate cancer cell xenografts resulted in significantly smaller, necrotic and non-hemorrhagic tumors in the treatment group (see figure 2; in press in *Urology*). We are currently attempting to improve these results by enhancing the activity of the cytosine deaminase "suicide" gene.

In the final six months of this project, I am assisting a Urology resident in his research which will examine ways of improving the efficiency of transfer as well as activity of the gene therapy construct. In addition, isolation of the enhancer binding factors necessary for activation of the PSMA-enhancer still remains to be examined. I expect this part of the project to be particularly difficult, as from my experiments with deletion constructs of the PSMA enhancer (see the attached O'Keefe et al., 2000), there seems to be a number of regions over a 1,648 nucleotide segment of DNA that provide both upregulation and down regulation of our reporter gene.

Key Research Accomplishments

- I have cloned and functionally characterized the PSMA-Like gene.
- I have identified several ways of differentially targeting the PSMA versus the PSMA-Like gene using antibodies, specific primers and enzymatic activity.
- In collaboration, we have shown the core enhancer region of the PSMA gene, and demonstrated that it is upregulated by androgen deprivation.
- I have shown via analysis of deletion constructs of the PSMA enhancer that a 1.648 kb region (which includes the core region), is able to drive expression of the highest activity of the luciferase reporter gene *in vitro*.
- I have generated a gene therapy construct that is able to kill prostate cancer cells both in vitro, and in a mouse prostate cancer xenograft model.
- I have identified three amino acids in the PSMA protein that appear to be either individually or in concert responsible for NAALADase enzymatic activity. This is a finding that if confirmed, may be able to give clues as to the best region of PSMA to target with small molecule inhibitors.

Reportable Outcomes

Manuscripts / Book Chapters

1. **O'Keefe, D.S.,** Bacich, D.J. and Heston, W.D.W. Prostate Specific Membrane Antigen,

pp 307-326 in "Prostate Cancer: Biology, Genetics and the New Therapeutics", edited by L.W.K. Chung, W.B. Isaacs and J.W. Simons. Humana Press Inc., Totowa, NJ, 2000.

2. Gong MC, Chang, SS, Watt, F, **O'Keefe, D.S.,** Bacich, D.J., Uchida, A., Bander, NH., Reuter, VE., Gaudin, PB., Molloy, PL., Sadelain, M. and Heston, W.D.W. Overview of Evolving Strategies Incorporating Prostate-Specific Membrane Antigen as a Target for Therapy (2000). Molecular Urology 4:217-22.
3. **O'Keefe, D.S.,** Uchida, A., Bacich, D.J., Watt, F.B., Martorana, A., Molloy P.L. and Heston, W.D.W. Prostate-Specific Suicide Gene Therapy using the Prostate-Specific Membrane Antigen Promoter and Enhancer (2000). Prostate 45:149-57.
4. Watt, F. B., Martorana, A., Brookes DE., Ho, T., Kingsley, E., **O'Keefe, D.S.** Russell, P.J. Heston, W.D.W. Molloy, P.L. A Tissue-specific Enhancer of the Prostate-Specific Membrane Antigen Gene, FOLH1. (2001) Genomics 73:243-254.
5. Uchida, A. **O'Keefe, D.S.**, Bacich, D.J., Molloy, P.L. and Heston, W.D.W. *In Vivo* Suicide Gene Therapy Model Using a Newly Discovered Prostate-Specific Membrane Antigen (PSMA) Promoter/Enhancer: A Potential Alternative Approach to Androgen Deprivation Therapy *(Urology, in press).*

Abstracts/Presentations

O'Keefe, D.S. and Heston, W.D.W. Characterization of the prostate-specific membrane antigen like gene. Proceedings of the Hormones and Cancer Conference (2000), Port Douglas, Queensland, Australia (a satellite meeting of the International Congress in Endocrinology).

Other Training Accomplishments

I have recently been promoted to Project Scientist in the Department of Cancer Biology, at the Cleveland Clinic Foundation. I have just finished assisting one clinical research fellow in his training at the Clinic, and I am beginning to train a resident from the Department of Urology in his research year. I attended an international conference in Australia, on Hormone Dependent cancers. This allowed me to see the objectives of research not only carried out in the U.S., but also overseas. Interestingly, there are significant differences in how different cultures approach this research. Also, following my career development plan, I recently applied for a tenure-track assistant professor position at two distinguished cancer centers, and am shortly planning on using the research described in this proposal as a basis for a research grant aimed at developing a gene therapy protocol that could be tested in humans in the near future.

Personnel receiving compensation from this award:

Denise S. O'Keefe

Conclusions

This project set out to determine the differences in sequence and function of the PSMA and PSMA-Like genes. PSMA is a very important clinical target for prostate cancer, and the presence of another highly similar gene needed to be investigated to maximize the utility of PSMA as a tumor marker and target. We have cloned and characterized both the PSMA-Like gene and it regulatory regions, and carried out a comparative analysis with the PSMA gene. We have shown that antibodies used for clinical imaging, such as the cyt351 antibody (Prostascint) will not cross-react with PSMA-Like protein. In addition, because PSMA-Like is not on the surface of the cell, it is not available for treatment methods designed to target PSMA. Clinical

tests which were based on RT-PCR of PSMA from peripheral blood or urine could be resurrected using oligonucleotide primers that will not detect the PSMA-Like gene. Previously, it was thought that PSMA transcripts were non-specifically found in cells isolated from blood and urine, with no clinical relevance. As PSMA-Like is expressed in the kidney, it is especially likely that cells expressing PSMA-Like could escape into the urine, possibly confounding these results.

The second part of this project is essentially aimed at defining the regulatory regions of PSMA for use in future gene therapy constructs. We have defined the best region of the enhancer for prostate-specific expression, and generated a gene therapy vector that combined with a "suicide" gene and non-toxic prodrug therapy, this enhancer is able to kill both prostate cancer cells in culture, and in an (immunodeficient) mouse model, *in vivo*. Future studies are aimed at maximizing the suicide gene activity by genetically enhancing it, and also on delivery methods that could be used in humans to treat prostate cancer.

Appendix

Curriculum Vitae

Name: **Denise Susan O'Keefe, PhD.**
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Education:

1987-1989: Bachelor of Science awarded by La Trobe University, Melbourne, Australia. Double major in Human Genetics and Microbiology.

1990: Honors degree in Genetics and Human Variation awarded by La Trobe University, Melbourne, Australia.

1991-1995: Doctorate of Philosophy, in the Department of Hematology-Oncology, the Faculty of Medicine, at The Queen Elizabeth Hospital, The University Of Adelaide, Adelaide, Australia. The degree was awarded in May, 1996.

Training:

1996-1997: Post-Doctoral Fellow, Department of Pathology, Columbia University College of Physicians and Surgeons, New York, NY.

1997- 1999: Post-Doctoral Fellow, Department of Urology, Memorial Sloan-Kettering Cancer Center.

1999- 2000: Research Associate, Department of Cancer Biology, The Cleveland Clinic Foundation.

October 2000 - present: Project Scientist, Department of Cancer Biology, The Cleveland Clinic Foundation.

Scientific and Medical Societies:

Member of the American Association for Cancer Research (1997-present).

Patent Applications: "DNA Encoding the Prostate-Specific Membrane Antigen-Like Gene and Uses Thereof." #D6230; O'Keefe, D.S. and Heston, W.D.W. (Domestic & International Application).

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1. **O'Keefe DS**, Dobrovic A: A rapid and reliable method for genotyping the ABO blood group (1993). Human Mutation 2:67-70.
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8. **O'Keefe D**, Dao D, Zhao L, Sanderson R, Warburton D, Weiss L, Anyane-Yeboa K, Tycko B. Coding mutations in p57KIP2 are present in some cases of Beckwith-Wiedemann syndrome but are rare or absent in Wilms tumors (1997). American Journal of Human Genetics. 61:295-303.
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21. **O'Keefe, D.S.,** Bacich, D.J. and Heston, W.D.W. Evolutionary comparison of the PSMA gene family yields some surprising insights *(in preparation).*

Book Chapters

22. **O'Keefe DS,** Bacich DJ, Heston WDW. Prostate-Specific Membrane Antigen (2000). (Chapter 18, pp307-326) in "Prostate Cancer: Biology, Genetics and New Therapeutics", Eds. Leland Chung, John Issaacs and Johnathon Simons, Humana Press; ISBN: 0896038688

Selected Presentations for the year 2000

23. **O'Keefe, D.S.,** Bacich, D.J., and Heston, W.D.W. Characterization of the Prostate-Specific Membrane Antigen-Like Gene. Abstract 313, Keystone Symposia on Advances in Human Breast and Prostate Cancer, Lake Tahoe, NV., March 2000.
24. Balaji, K.C., Bacich, D.J., **O'Keefe, D.S.**, and Heston, W.D.W. Differential Gene Expression between Androgen Dependent and Independent Prostate Cancer Cell Lines. Abstract 1978, 91 St. AACR Annual Meeting, San Francisco, April 2000.
25. Uchida, A., **O'Keefe, D.S.**, Bacich, D.J., Watt, F., Molloy, P.L. and Heston, W.D.W. Prostate-Specific Suicide Gene Therapy Using the Newly Discovered Prostate-Specific Membrane Antigen (PSMA) Enhancer. Abstract 2413, 91st. AACR Annual Meeting, San Francisco, April 2000.
26. Balaji, K.C., Bacich, D.J., **O'Keefe, D.S.** and Heston, W.D.W. Combination Gene Therapy Using Antisense Strategy For Advanced Prostate Cancer. Abstract 2414, 91 St. AACR Annual Meeting, San Francisco, April 2000.
27. Bacich, D.J., **O'Keefe, D.S.,** Watt, F.B., Molloy, P.L. and Heston, W.D.W. Prostate-Specific Membrane Antigen (PSMA) Promoter and Enhancer Driven Green Fluorescent Protein (GFP) Expression in Transgenic Mice. Abstract 122, 91 St. AACR Annual Meeting, San Francisco, April 2000.

28. **O'Keefe, D.S.**, Bacich, D.J. and Heston, W.D.W. Cloning and Characterization of the Prostate-Specific Membrane Antigen-Like Gene. Abstract 251, 91 St. AACR Annual Meeting, San Francisco, April 2000.
29. Richter, F., **O'Keefe, D.S.,** Bacich, D.J., Uchida, A., Bisognia, M. and Heston, W.D.W. Breast Cancer Cell Line Stimulates PSMA Expression in Microvascular Endothelial Cell Lines. Abstract 5051, 91 St. AACR Annual Meeting, San Francisco, April 2000.
30. Richter, F., **O'Keefe, D.S**., Bacich, D.J., Uchida, A., Bisogna, M., Heston, W.D.W. Co-incubation of Microvascular Endothelial Cells with a Breast Cancer Cell Line Stimulates PSMA-Expression *in vitro*. Abstract 127, American Urological Association Annual Meeting, Atlanta, GA, May 2000.

Figure 2 (next page)

LNCaP-C42 xenograft tumors following gene therapy treatment utilizing the PSMA promoter and enhancer driving expression of the *E.coli* cytosine deaminase gene in a Sense or Antisense (negative control) orientation, with or without 5-fluorocytosine treatment (5-FC). Parental tumor is from non-DNA treated LNCaP-C42 cells.

(a) Macroscopic appearance of tumors. Tumors on the upper, the middle, and the lower two rows are from Group 1 (Parental tumor, 5-FC), Group 3 (Sense, 5-FC), Group 4 (Sense, saline), respectively. All tumors in Group 3, (Sense, 5-FC) showed soft and whitish tumors without hemorrhage, whereas majority in the other groups were highly hemorrhagic,

Microscopic appearance of representative tumors (HE staining, 400X original magnification). Tumors shown in (b), (c), (d), and (e) are from Group 1 (Parental, 5-FC), 3 (Sense, 5-FC), 4(Sense, saline) and 6 (antisense, saline) respectively. Cancer cells in almost the whole mass from Group 3 (Sense, 5-FC) were eliminated and substituted with the non-cellular component (hyaline degeneration). On the other hand, the other tumors showed the typical findings of poorly differentiated adenocarcinoma with hemorrhage (a), central necrosis (c), and fibrosis (a), (d).

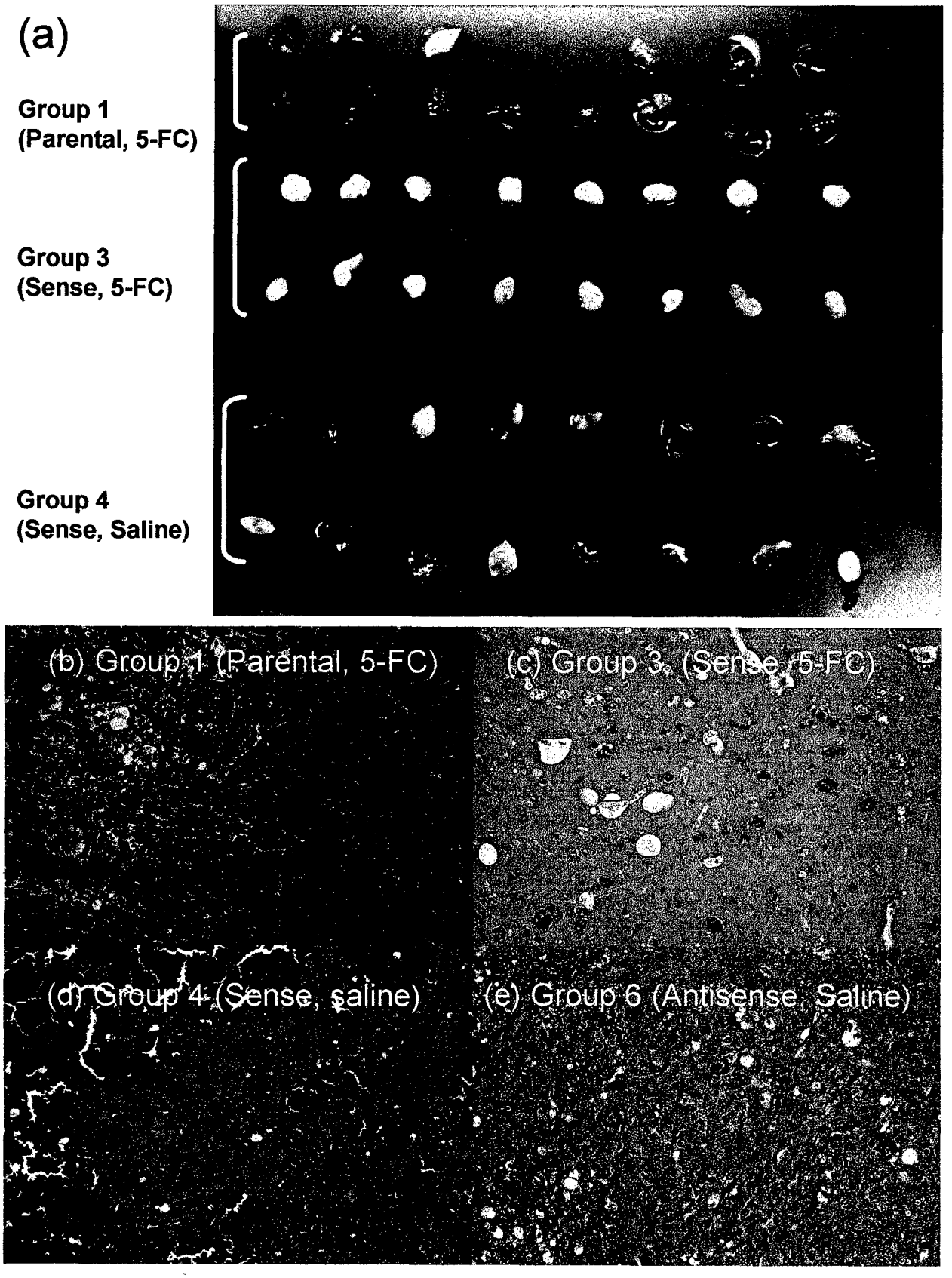
(a)
Group 1
(Parental, 5-FC)
Group 3
(Sense, 5-FC)
Group 4
(Sense, Saline)
(b) Group 1 (Parental, 5-FC)
(c) Group 3 (Sense, 5-FC)
(d) Group 4 (Sense, saline)
(e) Group 6 (Antisense, Saline)

Figure 2

PSI-27

Characterization of the prostate-specific membrane antigen like gene

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Prostate-Specific Membrane Antigen (PSMA) is a 100kD type II transmembrane protein with folate hydrolase and NAALADase activity. PSMA is highly expressed in prostate cancer and the vasculature of most solid tumors, and is currently the target of a number of diagnostic and therapeutic strategies. However, northern analyses using probes directed against PSMA have shown expression of PSMA or a similar gene in some non-prostatic tissues, including brain. We recently determined the PSMA gene was subject to a duplication even 22 MYA and that a gene homologous to PSMA resides on chromosome 11q14.3. In order for specific targeting of PSMA for clinical and therapeutic methods, it was necessary to clone and characterize this novel gene, and to determine which of the two PSMA-related genes is expressed in various tissues. Although the major coding regions of the two genes are 97% identical, we were able to examine the mRNA tissue distribution pattern of the two genes using assays that specifically distinguish between them. The "PSMA-Like" (PSMAL; Genbank accession AF261715) gene is expressed in kidney and liver, but not in the prostate. We have also been able to show that the PSMA gene and not the PSMA-Like gene is expressed in tumor-associated neovasculature. The different expression pattern of the two genes is not surprising, as the promoter region of PSMA has been lost from the duplication event that formed both the PSMA and PSMA-Like genes. Instead, the PSMAL gene utilizes a promoter corresponding to a region from within intron 5 of the PSMA gene, including a novel exon. As such, antibodies such as Cyt-356 which is the active portion of the imaging agent Prostascint specifically detect the PSMA gene. The PSMAL gene is found cytosolically, and therefore is not available to reagents used to target PSMA expressed on the outside of the cell membrane. However, we have been able to show that the PSMAL gene also possesses the same NAALADase enzymatic activity as the PSMA gene. Interestingly, NAALADase activity is altered in Schizophrenia Disorder II patients, and the PSMAL gene is the closest known gene to a translocation breakpoint that co-segregates with Schizophrenia in a large Scottish kindred. A second useful feature of the PSMAL gene is the fact that the PSMA prostate-specific enhancer region was also subject to the duplication event. The two "enhancer" regions are 95% identical, however the PSMAL enhancer region is unable to increase reporter gene activity when in combination with either the SV-40 or PSMA promoter. This finding will allow us to more quickly isolate the region(s) in the PSMA enhancer responsible for prostate-specificity, which in turn will aid production of gene therapy vectors targeting prostate cancer.

The Prostate 45:149–157 (2000)

Prostate-Specific Suicide Gene Therapy Using the Prostate-Specific Membrane Antigen Promoter and Enhancer

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BACKGROUND. Prostate-specific membrane antigen (PSMA) is abundantly expressed in virtually 100% of prostate cancers and metastases. In addition, unlike prostate-specific antigen (PSA), PSMA is upregulated under conditions of androgen deprivation. Therefore, PSMA is an attractive therapeutic target for advanced prostate cancer. Recently, both the promoter and the enhancer driving prostate-specific expression of the PSMA gene were cloned. We describe here our analysis of the PSMA enhancer for the most active region(s) and present a way of using the enhancer in combination with the *E. coli* cytosine deaminase gene for suicide-driven gene therapy that converts the nontoxic prodrug 5-fluorocytosine (5-FC) into the cytotoxic drug 5-fluorouracil (5-FU) in prostate cancer cells.

METHODS. Deletion constructs of the full-length PSMA enhancer were subcloned into a luciferase reporter vector containing either the PSMA or SV-40 promoter. The most active portion of the enhancer was then determined via luciferase activity in the C4-2 cell line. We then replaced the luciferase gene with the *E. coli* cytosine deaminase gene in the subclone that showed the most luciferase activity. The specificity of this technique was examined in vitro, using the prostate cancer cell line LNCaP, its androgen-independent derivative C4-2, and a number of nonprostatic cell lines. The toxicity of 5-FC and 5-FU on transiently transfected cell lines was then compared.

RESULTS. The enhancer region originally isolated from the PSMA gene was approximately 2 kb. Deletion constructs revealed that at least two distinct regions seem to contribute to expression of the gene in prostate cancer cells, and therefore the best construct for prostate-specific expression was determined to be 1,648 bp long. The IC_{50} of 5-FC was similar in all cell lines tested (>10 mM). However, transfection with the 1648 nt PSMA enhancer and the PSMA promoter to drive the cytosine deaminase gene enhanced toxicity in a dose-dependent manner more than 50-fold, while cells that did not express the PSMA gene were not significantly sensitized by transfection.

CONCLUSIONS. Suicide gene therapy using the PSMA enhancer may be of benefit to pa-

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tients who have undergone androgen ablation therapy and are suffering a relapse of disease.
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KEY WORDS: **enhancer elements; promoter regions; gene therapy; prodrugs; flucytosine; PSMA**

INTRODUCTION

Prostate cancer is the second leading cause of cancer death [1]. Although the most recent data suggest that the incidence of death from this disease is on the decline due to the advent of the prostate-specific antigen (PSA) test and subsequent early intervention, once a patient becomes hormone-refractory, there are few treatment choices available [1]. One approach currently being considered is prostate-specific gene therapy followed by prodrug administration. The prodrug 5-fluorocytosine (5-FC) is converted by the bacterial enzyme cytosine deaminase (CD) to 5-fluorouracil (5-FU) [2,3]. 5-FU has already been used for treatment of metastatic prostate cancer, while 5-FC is used in the treatment of fungal infections and is nontoxic to humans. Prostate-specific membrane antigen (PSMA) is highly expressed in prostate cancer and normal prostate [4–6], and more recently, expression of PSMA was observed in the tumor-associated neovasculature of nearly all solid tumors [7–10].

Expression of PSMA or a "PSMA-Like" gene has also been seen in some other tissues including kidney, liver, and brain, although at a much lower level than in the prostate or prostatic carcinoma, as determined by Northern blot analyses [6] (also Bacich et al., unpublished observations). Furthermore, expression of PSMA is upregulated under conditions of androgen deprivation, which makes it a useful marker for patients who have undergone hormonal ablation [6]. Accordingly, PSMA is currently being used as a therapeutic and clinical target in a number of strategies against prostate cancer. Recently, our group cloned the PSMA promoter and an enhancer from intron 3 of the PSMA gene that drives prostate-specific expression of reporter genes [11–13]. To further our aim of generating a way of targeting prostate cancer and cancer metastases using gene therapy, we designed a genetic construct that places expression of the bacterial CD gene under the regulatory control of the PSMA promoter and enhancer.

MATERIALS AND METHODS

Cell Culture

The prostate cancer cell line LNCaP, the breast cancer cell line MCF-7, colorectal cancer line HCT8, and lung cancer cell line H157 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The androgen-independent subline of LNCaP, C4-2, was purchased from UroCor Laboratories (Oklahoma City, OK). LNCaP and HCT8 cells were grown in RPMI-1640 (Life Technologies, Rockville, MD) containing 10% fetal bovine serum (FBS). C4-2 and MCF-7 were grown in DMEM (Life Technologies, Rockville, MD) containing 10% FBS. H157 cells were grown in RPMI-1640 with 2 mM L-glutamine, adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate with 10% FBS. All cells were grown in the absence of antibiotics.

Cloning of Enhancer Deletion Constructs

The promoter of the PSMA gene was cloned into the pGL3-Basic vector (Promega, Madison, WI) as previously described, to form pGL3-B-PSM [11]. Deletion constructs of the PSMA enhancer were produced using primers with an artifically incorporated *Bam*HI restriction endonuclease recognition site. The following primer sets were used to amplify genomic DNA NA1944 (Coriell Cell Repositories, Camden, NJ). This DNA was derived from a somatic cell hybrid containing retaining human 11pter→cen translocated to a hamster chromosome, and therefore does not contain the PSMA-like gene. PCR was carried out under standard conditions. Following PCR, the resultant products were digested with *Bam*HI and subjected to gel electrophoresis followed by gel purification. The gel-purified products were cloned into pGL3-B-PSM or pGL3-promoter vector (containing the SV-40 promoter and no enhancer; Promega, Madison, WI), which had been digested with *Bam*HI and treated with alkaline phosphatase. The primer sets used were: S 14704 5′ CGCGGATCCGCCTTCTAAAATGAGTTGGG 3′, with each of the following primers: AS 15205 5′ CGCGGATCCCAACATAGTGGAACCACGTC 3′ (501 bp), AS 15573 5′ CGCGGATCCTGAGAAAAGATTGCCAACGC 3′ (869 bp), AS 15994 5′ CGCGGATCCATTAGGTTCCAAAGGAAGCC 3′ (1,290 bp), AS 16352 5′ CGCGGATCCGGCTACTACATAAGTATAAGTC 3′ (1,648 bp), and AS 16617 5′ CGCGGATCCATGACACCAAAGCTTTAGGG 3′ (1,913 bp). The artifically incorporated *Bam*HI restriction sites are underlined.

Analysis of PSMA Enhancer Constructs

Activity of the PSMA enhancer constructs was determined using luciferase as a reporter gene following transient transfection. C4-2 and MCF-7 cells were used as positive and negative control cells, respectively, to determine activity since tissue specificity of the entire enhancer region has already been demonstrated [13]. The day before transfection, 50,000 C4-2 and 75,000 MCF-7 cells were plated into 24-well plates. Cells were then transfected with 400 ng of each enhancer construct, using Lipofectamine Plus Reagent (Life Technologies), according to the manufacturer's instructions, with the exception that the DNA/lipofectamine complexes were allowed to remain on the cells overnight. Approximately 40 hr after transfection, cells were harvested and analyzed for reporter activity, using the Dual Luciferase Assay Kit (Promega). All experiments were carried out in triplicate, and each well was cotransfected with 100 ng of pBIND (Promega), which is a plasmid carrying the *Renilla* luciferase gene under the control of the SV-40 promoter and enhancer, to control for transfection efficiency.

Cloning of the Cytosine Deaminase Gene Construct

The plasmid pCD containing the cytosine deaminase gene from *E. coli* was kindly provided by Dr. I. Yoshimura (Department of Urology, National Defense Medical College, Saitama, Japan). The gene was modified to possess a eukaryotic translation initiation signal. The luciferase gene in the enhancer construct showing the most reporter gene activity, pGL3-B-PSM-Enh1648, was removed by restriction enzyme digestion with *Xba*I and replaced with the CD gene from pCD (the 1.5-kb CD gene from pCD was excised using *Xba*I and *Spe*I digestion). *Xba*I digestion actually removes some of the PSMA promoter sequence, but the removal of this region has no effect on promoter activity (O'Keefe et al., unpublished observations). Subsequent clones were sequenced to determine the orientation of the CD gene with respect to the PSMA promoter. The clones used for the following experiments were named pPSM/CD-sense and pPSM/CD-antisense, where sense and antisense refer only to the direction of the CD gene.

Cytotoxicity/Proliferation Inhibition Assay

Initially, the cytotoxicity of 5-FC and 5-FU in nontransfected cell lines was examined. Cells (2.5×10^3 C4-2, H157, and HCT8, and 4.0×10^4 LNCaP and MCF-7 cells) were plated in triplicate for each time point, in 24-well plates. The cells were then exposed 24 hr later to various concentrations of 5-FC (Sigma, St. Louis, MO) or 5-FU (Sigma). Relative cell numbers were assayed after 3 days, using the MTS assay (Cell Titer 96 AQ_{ueous}, Promega), and the inhibitory concentration (IC_{50}) was determined by figuring the concentration at which the cell number was 50% of the number of control (untreated) cells grown for the same length of time.

Cytotoxicity of 5-FC on Transiently Transfected Cells

The cytoxicity of 5-FC on cells transiently transfected with pPSM/CD-sense or pPSM/CD-antisense or liposome only was determined. Cells were plated in 24-well plates and transfected as described above. Transfection efficiency was determined by in situ X-gal staining replica wells transfected with pSV-β-galactosidase (Promega). Two days after initiation of transfection, cells were exposed to various concentrations of 5-FC for 3 days, and cell viability was measured.

Enzymatic Activity of Cytosine Deaminase in the Transfected Cells

The enzymatic activity in the cell lysate was measured with spectrophotometry [14]. H157, LNCaP, and C4-2 cells were plated in a 100-mm dish and preincubated for 24–48 hr. At 50% confluency, the cells were transfected with pPSM/CD-sense, pPSM/CD-antisense, or pSV-β-galactosidase. Two days after initiation of transfection, the cells were collected and preserved at −20°C. On the initial day of the assay, 110 μl of PBS were added to each cell pellet. Pellets were then sonicated and centrifuged at 14,000*g* at 4°C. Ten microliters of each supernatant were used to measure the protein concentration, using the BCA protein assay (Pierce, Rockford, IL). The remaining 100 μl of cell lysate were added to 900 μl of 3 mM 5-FC in PBS. The reaction mixtures were incubated at 37°C. At various time points, 50 μl of each aliquot were removed and added to 950 μl of 0.1M HCl. The concentration of 5-FC was measured by determining the absorbance at 290 nm (A290) and 255 nm (A255). The concentration of 5-FC was calculated as follows:

$$\text{5-FC (mM)} = 0.119 \times \text{A290} - 0.025 \times \text{A255}$$ [14].

The enzymatic unit of cytosine deaminase was defined as nmol of 5-FC catalyzed per min per mg of protein from whole-cell supernatant.

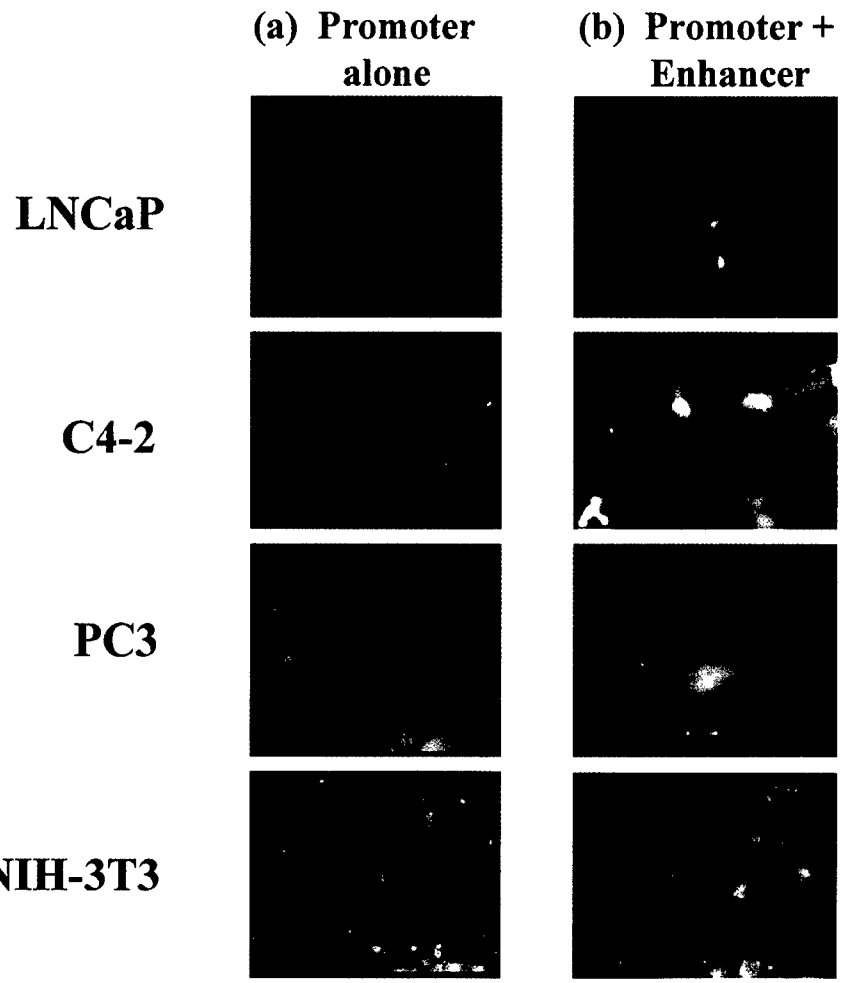

Fig. 1. PMSA promoter and promoter/enhancer-driven expression of GFP **a:** PSMA promoter alone. **b:** PSMA promoter and enhancer, driving GFP expression in LNCaP, C4-2, and PC-3 (prostatic cell lines) and in NIH 3T3 cells (used as a negative control). Addition of the PSMA enhancer to the PSMA promoter clearly results in a large increase in GFP reporter expression in LNCaP (magnification ×200, 30-sec exposure) and C4-2 (magnification ×400, 30-sec exposure) cells, while no expression is seen in NIH-3T3 (magnification ×400, 60-sec exposure) cells. Note the fluorescence of the promoter alone in PC-3 (magnification ×400, 30-sec exposure) cells, which do not express PSMA.

RESULTS

PSMA Promoter and Promoter/Enhancer-Driven GFP Expression in Various Cell Lines

Green fluorescent protein (GFP) expression was used to compare the activity of the PSMA promoter with that of the PSMA promoter and the PSMA enhancer that was identified by Watt et al. [13]. These constructs were transiently transfected into LNCaP, C4-2, PC-3, and NIH-3T3 cell lines, and the cells were examined for green fluorescence (see Fig. 1). Minimal GFP expression was seen in LNCaP, C4-2, and NIH-3T3 cells with the promoter alone, though PC-3 cells had moderately high levels of GFP expression with the PSMA promoter alone. Addition of the PSMA enhancer to the construct resulted in significantly increased levels of GFP expression in the LNCaP and C4-2 cell lines, although there appeared to be no enhancement of GFP expression in the PC-3 and NIH-3T3 cells over that seen with the PSMA promoter alone.

Analysis of the PSMA Enhancer

Reporter plasmids carrying various portions of the PSMA enhancer and driven by either the PSMA promoter or the viral SV-40 promoter were analyzed for luciferase expression in the prostatic cell line C4-2 and the breast cancer cell line MCF-7. The entire enhancer sequence is shown in Figure 2A, as well as the constructs indicated by the primer positioning, so that all deletions were made progressively from the 3′ end of the enhancer (see Fig. 2B). All the enhancer deletion constructs were tested with the enhancer placed in both orientations (downstream of the luciferase reporter gene) with respect to the PSMA or SV-40 pro-

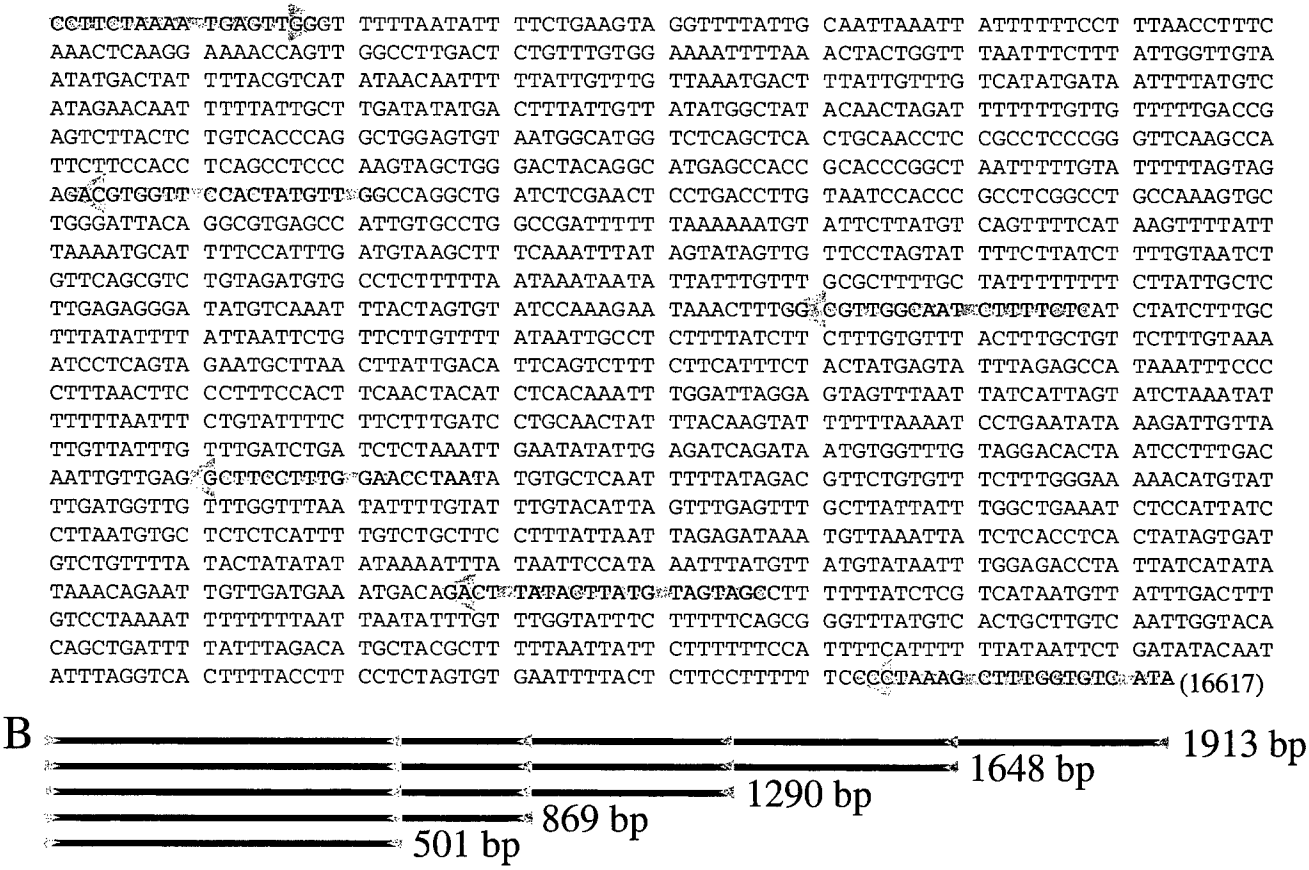

Fig. 2. **A:** PSMA enhancer sequence; grey arrows indicate positioning of primers used to create the deletion constructs. Numbering refers to Genbank accession number AF007544. **B:** Pictorial representation of the PSMA enhancer region of the deletion constructs.

moter. All results were normalized for transfection efficiency, and the mean and standard deviation of the experiments in triplicate were expressed as a percentage of the pGL3-control (SV-40 promoter and enhancer). All the enhancer deletions were able to drive prostate-specific expression of luciferase in both orientations, although with varying amounts of activity (see Fig. 3). The most active constructs were the pGL3-B-PSMA1648 and pGL3-B-PSMA1290 plasmids, which in the antisense orientation showed approximately 20-fold expression over the pGL3-control vector, and 200-fold over the PSMA promoter alone in C4-2 cells. Conversely, when the same constructs were transfected into breast cancer MCF-7 cells, the enhancer provided no additional activity over the PSMA-promoter alone. These results are consistent with those reported for the original characterization of the PSMA enhancer [13]. When the enhancer constructs were driven by the SV-40 promoter, the activity of each deletion was consistent with that seen in combination with the PSMA promoter, although the overall activity of the two best constructs, pGL3-B-PSMA1648 and pGL3-B-PSMA1290, was increased to about 25-fold of that of the pGL3 control vector in C4-2 cells. However, when the SV-40 promoter was tested with the enhancer deletions in MCF-7 cells, these same two constructs showed up to 20% of the activity of the pGL3-control. This suggests that there may be some elements of the PSMA promoter itself that contribute to prostate-specific expression. Given these results, we next took the pGL3-B-PSMA1648 plasmid and replaced the luciferase reporter gene with the *E. coli* CD gene to form pPSM/CD (sense or antisense, dependent on the orientation of the CD gene relative to the PSMA promoter).

Cytotoxicity/Proliferation Inhibition of 5-FU and 5-FC

In order to determine the sensitivity of each cell line to 5-FU, cytotoxicity/proliferation inhibition assays were performed (see Fig. 4 and Table I). The IC_{50} of 5-FU for all cell lines was similar, ranging between

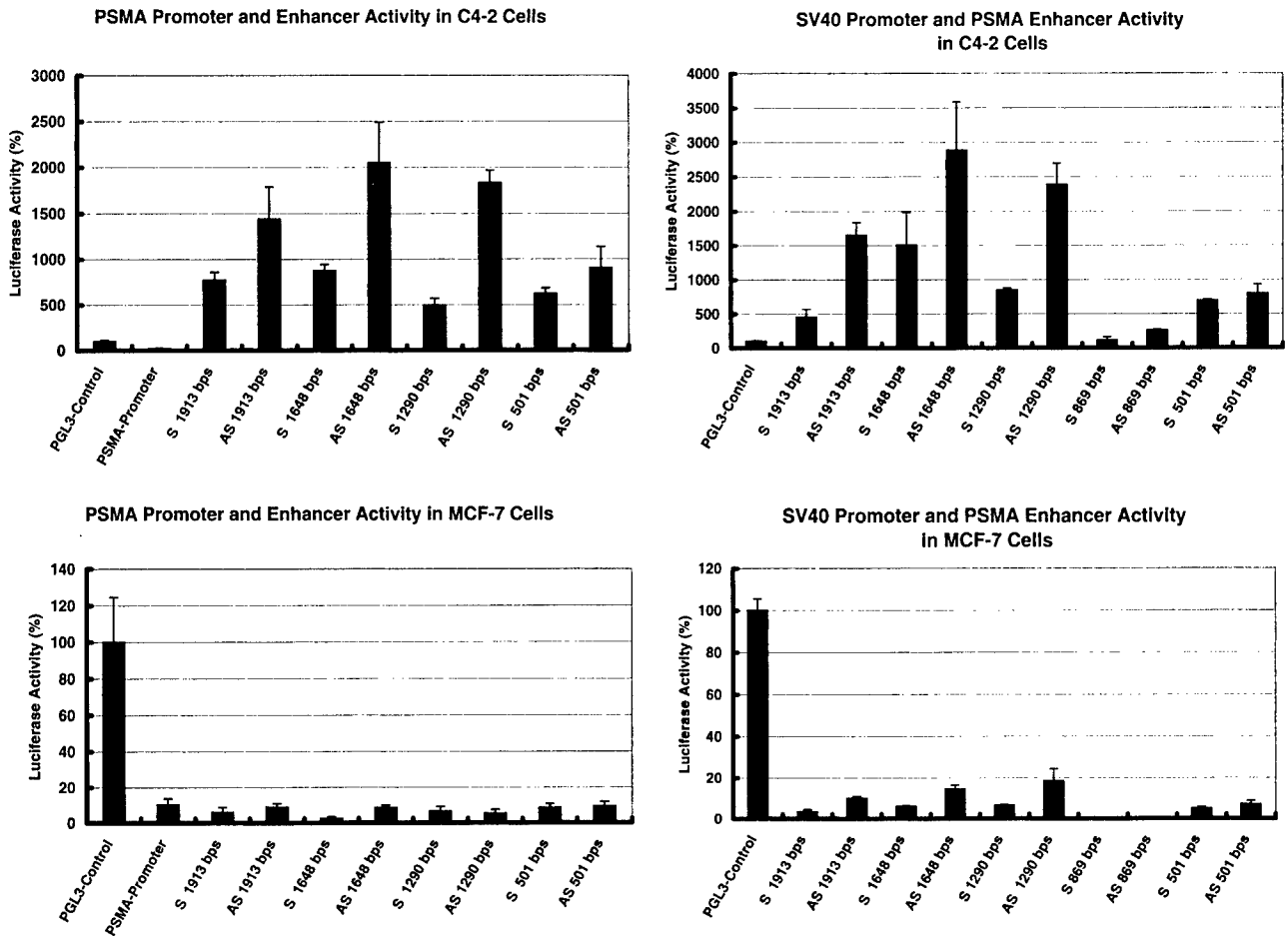

Fig. 3. Deletion analysis of the PSMA enhancer. Luciferase activity after normalization for transfection efficiency, expressed as percentage of positive control (pGL3-control), where the control was set at 100% activity. Enhancer deletions are indicated. S, enhancer in the sense orientation with respect to the promoter; AS, antisense orientation. Addition of PSMA enhancer to either the PSMA promoter or the SV40 promoter resulted in a significant (up to 300-fold) increase in reporter gene expression over the pGL3 control vector in the prostate cancer cell lines C4-2 and LNCaP, while minimal expression was seen in breast cancer cell line MCF-7. The most active deletions are the antisense 1,648-bp and 1,290-bp constructs, respectively.

1–10 μM according to several independent experiments. Compared to 5-FU, 5-FC is much less toxic for all cell lines. The IC_{50} of 5-FC for all cell lines was over 10 mM (more than 1,000 times that of 5-FU).

Cytotoxicity/Proliferation Inhibition in Transiently Transfected Cells

Transient transfection of LNCaP and C4-2 cells with pPSMA/CD-sense resulted in sensitization against 5-FC. In C4-2 cells, the 5-FC at 200, 300, and 400 μM inhibited cell growth to 67%, 39%, and 38%, respectively, compared to the control (nontransfected cells without 5-FC). The IC_{50} of 5-FC on transfected C4-2 was between 200–300 μM.

Compared to the IC_{50} of nontransfected cells (>10 mM), C4-2 was sensitized nearly 40-fold by transfection with pPSMA/CD-sense. It should be noted that the transfection efficiency of C4-2 cells was only 8% as determined by X-gal staining, and exposure to 5-FC was only 3 days. The enhanced cytotoxic effect of 5-FC was also noted in LNCaP cells when transfected with the pPSMA/CD-sense plasmid. However, the sensitization in LNCaP cells (IC_{50} 1–5 mM), was not as great as that in C4-2 cells. This could be because LNCaP cells are less sensitive to 5-FU treatment (see Table I), probably due to genetic differences between the two cell lines. Another cause could be that the recovery period of LNCaP after transfection is much longer than that of C4-2. Therefore, there is less chance of LNCaP cells incorporating 5-FU as their cell cycle is longer. In H157, HCT8, and MCF-7 cells, there was no significant difference in cell numbers between those transfected with pPSMA/CD sense and antisense constructs, demonstrating the specificity of the PSMA promoter/enhancer construct for prostate cells.

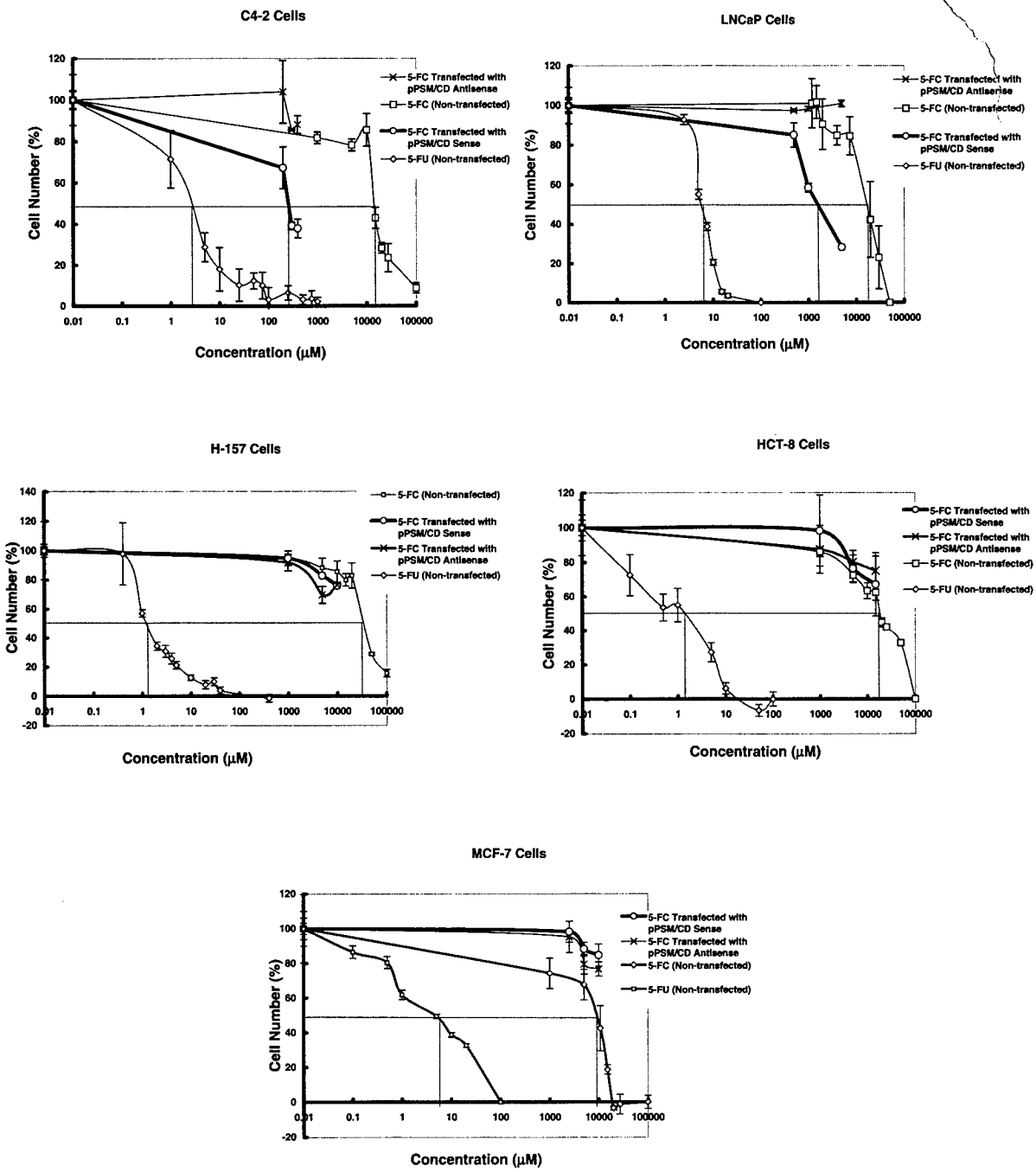

Fig. 4. Determination of IC_{50} of 5-fluorocytosine (5-FC) and 5-fluorouracil (5-FU) in nontransfected cells, and cells transfected with both sense and antisense cytosine deaminase, driven by the PSMA promoter and the 1,648-bp enhancer from the PSMA gene. The vertical line dropped from the 50% horizontal indicates the IC_{50}. It can be seen that transfection with the sense cytosine deaminase construct sensitizes LNCaP and C4-2 cells significantly to 5-FC treatment, while the nonprostatic cell lines H-157, HCT-8, and MCF-7 are not sensitized to 5-FC, demonstrating the specificity of this approach for prostate cancer.

Cytosine Deaminase Enzymatic Activity in Transfected Cells

The cytosine deaminase activity in C4-2 cell lysate was dependent on transfection efficiency. When 5% and 1% of C4-2 and 5% of LNCaP cells in a 100-mm dish were transfected with pPSM/CD-sense plasmid, the lysate showed an enzymatic activity of 2.4, 1.6, and 0.7 nmol/min/mg protein, respectively (data not shown). The enzymatic activity in the lysate of cells transfected with pPSM/CD-antisense plasmid, as well as H157 transfected with the pPSM/CD sense construct, did not show any detectable enzymatic activity of cytosine deaminase.

TABLE 1. Comparison of Sensitivity to 5-Fluorouracil (5-FU) and 5-Fluorocytosine (5-FC) in Each Cell Line*

Cell line	Tissue origin	PSMA expression	IC_{50} 5-FU (μM)	IC_{50} 5-FC parental (μM)	IC_{50} 5-FC PSMA/CD (μM)	Ratio IC_{50} of 5-FC (parental)/ PSMA/CD)	Transfection efficiency (%)
C4-2	Prostate	+	1–5	15,000	200–300	>50	8
LNCaP	Prostate	+	1–10	15,000	1,000–2,000	10	5
H-157	Lung	–	1–10	>20,000	>10000	<2	10
HCT-8	Colon	–	1–5	20,000	>15000	<2	6
MCF-7	Breast	–	1–5	10,000	>10000	<2	15

*The inhibitory concentration 50% (IC_{50}) of 5-FU and 5-FC in all cell lines was very similar. However, when cells were transfected with the pPSMA/CD-sense plasmid, C4-2 and LNCaP cells were sensitized to 5-FC, 50- and 10-fold more, respectively, than the parental nontransfected cells, whereas the other nonprostatic cells were not significantly sensitized. Note that sensitization was achieved with only 8% transfection efficiency in C4-2 cells.

DISCUSSION

Gene therapy is in the process of becoming an acceptable treatment method for patients for whom there is no other effective treatment. Control of drug activation using prodrug therapy in combination with gene therapy is theoretically a way of staging a maximal attack on the targeted tumor, while controlling cytotoxicity to noncancerous tissue. To date, the two promoter/enhancers most often considered for use in prostate-specific therapy have been those of the rat probasin gene, and the human PSA gene. Both of these promoter/enhancers are positively regulated by androgens, and therefore would be of little use to patients who are undergoing androgen ablative therapy [15,16]. However, the PSMA gene is upregulated in the absence of androgens and is highly expressed in virtually all tumors and their metastases examined so far. As such, we chose to use regulatory elements from the PSMA gene to develop a construct that could be used to control expression of a "suicide gene" in a gene therapy approach against this disease. We recently cloned the promoter of the PSMA gene, which is able by itself to drive prostate-specific expression of a reporter gene; albeit at relatively low levels [11]. More recently, we isolated a prostate-specific enhancer from the third intron of the gene, which is upregulated in the absence of androgens [13]. Addition of the full-length enhancer to the PSMA promoter in a reporter construct results in approximately a 100-fold increase in expression. These data demonstrate that the enhancer retains full activity when positioned downstream of the gene; previous results were obtained with the enhancer upstream of the promoter [13]. As the full-length enhancer had not been tested in C4-2 cells, we initially compared its ability to drive the GFP reporter gene in a number of cell lines. While the enhancer clearly shows a significant increase in activity over the PSMA promoter alone in LNCaP and C4-2 cells, we were surprised to see more activity of the PSMA promoter alone in PC-3 cells than in either LNCaP or C4-2. PC-3 cells do not express PSMA; however, expression of the PSMA promoter would suggest that while the transcription factors necessary to drive the promoter's expression are available in PC-3 cells, there is some kind of defect in the PSMA promoter in these cells. Such a defect might be due to homozygous deletion or methylation of the PC-3 PSMA promoter. In addition, the enhancer did not seem to significantly increase expression over the promoter alone in PC-3 cells. This may be due to a number of reasons, e.g., the factors involved in upregulation via the enhancer are not present in PC-3 cells, and fluorescence microscopy is less quantitative than luciferase assays.

Interestingly, analysis of the deletion constructs revealed that there appear to be at least two distinct positive-regulatory regions within the enhancer region, as the smallest construct (501 bp) still exhibited more than a 50-fold increase in expression over the promoter alone. However, the most active constructs were clearly the 1,648-bp and 1,290-bp fragments, which showed a 175- to more than 200-fold increase in expression over the PSMA promoter alone. As this is more than the entire enhancer, it would suggest that there may be a negative regulator between nt 1648 and 1913 of the originally defined enhancer region. The identity of either of the positively or negatively regulating elements is unclear, as there are no consensus enhancer elements present in the PSMA enhancer sequence. There also appeared to be an effect of the orientation of the enhancer with respect to the promoter. While all the constructs showed significantly increased activity over both the SV-40 control and the PSMA-promoter alone, the constructs with the enhancer in the antisense orientation with respect to

their orientation in the native PSMA gene seemed to have significantly more activity, and the reason for this is unknown.

We tested the most active PSMA-promoter/enhancer/CD gene construct in vitro using a transient transfection approach, because it probably most resembles the principal problem with genetic therapy at the present time, i.e., delivery of the vector to the target tissue. One advantage of the cytosine deaminase/5-FC therapy approach is the bystander effect, in which cells close to the actual cell producing the engineered enzyme (in this case CD) are affected by the converted prodrug and destroyed. All results in this study were normalized for transfection efficiency, so that a logical comparison could be made between the different cell lines. However, even with the average transfection efficiency of C4-2 cells of around 8%, significant conversion of 5-FC to 5-FU and subsequent cell death were seen. Transfection with the suicide construct sensitized C4-2 cells to 5-FC nearly 40-fold, with an IC_{50} of between 200–300 μM. The usual oral dose of 5-FC for fungal treatment in humans is 37.5 mg per kg body weight every 6 hr, which results in peak serum concentrations of 540–620 μM in adults. The specificity of this method was shown by the lack of sensitization to 5-FC by transfection in the breast-, colon-, and lung-derived cell lines. However, it still remains to be determined if this enhancer is active in other tissues in vivo where PSMA is reportedly expressed. Other enhancers could be responsible for this nonprostatic expression, or the expression might be due to activation of another gene such as the PSMA-like gene. Furthermore, it will be important to determine if this PSMA enhancer is responsible for the expression of PSMA-protein seen in tumor neovasculature [10], as this would provide a much wider use for gene therapy constructs utilizing the PSMA regulatory control regions described here. We are attempting to answer both of these questions, in part by generating a transgenic mouse model carrying the human PSMA promoter and full-length enhancer-driving expression of green fluorescent protein.

CONCLUSIONS

We have identified the PSMA promoter and enhancer regulatory regions responsible for the strong expression of PSMA in prostate tumor cells such as LNCaP and C4-2. Furthermore, we have demonstrated that this PSMA-promoter/enhancer construct is capable of selectively driving expression of therapeutic genes, and we are exploring which gene or genes would be most useful along with ways to increase the effectiveness of vector delivery to the tumor or metastatic site. Suicide gene therapy for prostate cancer using the PSMA promoter and enhancer holds promise for the treatment of prostate cancer.

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Prostate Specific Membrane Antigen

Denise S. O'Keefe, PhD, Dean J. Bacich, PhD, and Warren D. W. Heston, PhD

1. INTRODUCTION

The molecular basis of prostate carcinoma has always been less understood than that of other cancers, despite its high incidence in the population. One of the reasons for this is that the molecular pathways leading to prostate cancer do not seem to parallel that of other cancers, and until recently there have been few markers for this tumor. One of the most exciting recent findings in prostate cancer was the discovery of prostate specific membrane antigen (PSMA). PSMA is a glutamate carboxypeptidase that switches from a cytosolically located protein in the normal prostate to a membrane-bound protein in prostatic carcinoma. The majority of PSMA expression appears to be restricted to the prostate, with some expression seen in the brain, salivary glands, and small intestine. Intriguingly, our group recently found that PSMA is expressed in the endothelial cells of the neovasculature of nearly all solid tumors examined. The membrane-bound nature of this protein—and the limited sites of expression as well as expression in tumor-associated neovasculature—makes PSMA an ideal marker and therapeutic target for clinical studies and treatment of not only prostate cancer, but of other solid tumors as we progress into the 21st century.

2. THE DISCOVERY OF PSMA

The antigen itself was discovered by Horoszewicz et al. *(28)*, who isolated LNCaP cell membranes and immunized mice with the mixture, producing the antibody known as 7E11C5.3. The LNCaP cell line is derived from a Lymph Node metastasis from a Carcinoma of the Prostate, and is considered the most relevant of the few prostatic cell lines available, because it retains expression of prostate specific antigen (PSA), prostatic acid phosphatase (PAP) and the androgen receptor, among other characteristics typical of human prostate cancers in vivo *(23,25,29,30)*. Characterization of the antibody revealed that it specifically bound epithelial cells of normal prostate, benign prostatic hypertrophy (BPH), and prostatic carcinoma specimens, making PSMA an attractive prostate-specific marker *(28)*. The rights to the 7E11C5.3 antibody were then bought by a biotechnology company called Cytogen Corporation (Princeton, NJ).

Cytogen modified the antibody so that it could be labeled with ^{111}Indium while retaining its specificity, and renamed it Cyt-356. The radiolabeled antibody was then

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administered to nude mice carrying tumors established from the LNCaP cell line. After 3 d, 30% of the injected dose had localized to the LNCaP xenograft, with no significant amounts found in other tissues *(42)*. This occurred despite the fact that the same investigators had noted that immunohistochemical staining using this modified antibody against normal human tissues showed weak reactivity with cardiac muscle, proximal kidney tubules, and sweat glands. There was also strong binding to a subset of skeletal muscle cells. A phase I clinical study using radiolabeled Cyt-356 as an imaging agent for metastatic deposits was then carried out in patients with prostate cancer and known distant metastases *(78)*. No adverse affects of the agent were noted in the patients, and the results showed promise for use of the immunoconjugate as an imaging agent. At this stage it was clear that a deeper understanding of the molecular basis and function of PSMA expression in prostate cancer was needed.

3. CLONING AND EXPRESSION PATTERN OF PSMA

The complementary DNA (cDNA) sequence encoding PSMA was cloned in 1993 using a classic textbook approach *(33)*. The monoclonal antibody Cyt-356 was used to immunoprecipitate PSMA from LNCaP cell membranes, and the protein was then electrophoresed on and isolated from a polyacrylamide gel. PSMA was then subjected to proteolytic digestion and the subsequent peptide fragments were microsequenced to determine their amino acid composition. Based on the amino acid sequence, degenerate oligonucleotide primers were designed that could theoretically amplify the PSMA cDNA sequence from LNCaP reverse-transcribed mRNA. The resulting PCR product was cloned and used to probe a LNCaP cDNA library and isolate the full-length PSMA transcript of 2653 nucleotides (the sequence can be found in Genbank under the accession number M99487).

Translation of the cDNA sequence predicted that the protein consists of 750 amino acids with a molecular wt of 84 kDa before posttranslational modifications *(33)*. It was later shown that in vitro translation of the PSMA cDNA sequence with and without dog pancreatic microsomal membranes (which permit glycosylation of proteins to occur in vitro) produces proteins of 100 and 84 kDa, respectively. This is consistent with the 100 kDa molecular wt of PSMA seen in LNCaP cells *(32)*. PSMA is a Type II integral membrane protein, and as such the short N-terminal of the protein is located on the cytoplasmic side of the membrane, with the majority of the protein located on the extracellular side of the membrane, making it available for clinical and therapeutic targeting *(33)*. Northern analyses using the PSMA cDNA probe and ribonuclease protection assays using a probe corresponding to nucleotides 242–588 of the PSMA cDNA sequence showed no expression of PSMA mRNA in the prostatic cell lines PC-3 and DU145, and no expression in normal tissues from kidney, liver, lung, mammary gland, pancreas, placenta, skeletal muscle, spleen, and testis. However, there was high expression in normal prostate and prostatic carcinomas, and barely detectable expression in salivary gland, whole brain, and small intestine. Expression of PSMA mRNA in BPH specimens was either reduced relative to that of normal prostate, or absent altogether *(32)*, which is most likely an indication of the major cell type that constitues BPH (stromal cells that do not express PSMA), rather than a biologic phenomenon. Interestingly, our group and others recently found that PSMA is expressed in the

endothelial cells of neovasculature associated with almost all solid tumors, but not in normal vasculature *(40,61)*. Immunohistochemistry using five different antibodies against PSMA has confirmed this, as have *in situ* hybridization and RT-PCR results *(8,9)*. In fact, the only tumor which does not seem to consistently express PSMA in the associated vasculature is that of the prostate (2/12 prostate cancer specimens expressed PSMA in the vasculature), perhaps providing a clue to the function of PSMA in these cells *(9)*.

As PSA expression is modulated by androgens, Israeli et al. examined the effect of various steroids on PSMA expression in LNCaP cells *(32)*. In contrast to PSA expression, PSMA is downregulated in the presence of androgens, with the highest amount of PSMA expressed in LNCaP cells grown in charcoal-stripped (and therefore steroid-reduced) media. This finding was later supported both in vitro in LNCaP cells, and in vivo by Wright et al., *(76)*, who found that in 55% (11 of 20) and 100% (4 of 4) primary and metastatic tumor specimens, PSMA expression was significantly upregulated in patients who had undergone some form of hormonal deprivation, relative to matched specimens from the patients before treatment. These findings are particularly significant, because of the implication that PSMA can be a highly useful clinical and therapeutic target for patients with recurrent disease.

4. REGULATION OF PSMA EXPRESSION: CLONING OF THE PSMA PROMOTER AND ENHANCER

To obtain more information about the genetic regulation of PSMA expression, we set out to determine the complete sequence of the gene. A bacteriophage P1 library containing fragments of DNA from normal human lymphocytes approx 60–80 kb in size was screened using PCR. Two sets of oligonucleotide primers were used—one set corresponding to the 5′ end of the PSMA cDNA sequence, and one set corresponding to the 3′ end of the sequence. The advantage of this method of screening was that the gene spanned more than 60 kb of DNA, and two P1 clones that overlapped by about 5.6 kb had to be analyzed to acquire the entire sequence *(50)*.

Comparison of the genomic and cDNA sequences of the PSMA gene revealed 19 exons ranging in size from 64 to 379 nucleotides, and 18 introns from 300 to 7363 base pairs (Fig. 1). The entire genomic sequence of the gene can be found in Genbank, under accession number AF007544. One of the most striking features of the genomic sequence was the presence of a CpG island at the 5′ end of the gene. From nucelotides 2661–2990 of the genomic sequence—which extends from exon 1 into the first intron of the gene—the observed/expected ratio of the CpG dinucleotide was 1.85, which is significantly greater than the ratio for bulk human DNA (0.25)*(1,50)*. CpG islands are substrates for DNA methyltransferase, and the presence of a CpG island in the 5′ region of the PSMA gene suggests a role for DNA methylation in the regulation of PSMA expression.

Once we had the genomic sequence of the 5′ portion of the gene, we were able to clone the promoter controlling transcription of PSMA mRNA. To confirm the transcription start site indicated by the initial PSMA cDNA sequence *(33)*, we carried out 5′ Rapid Amplification of cDNA Ends (5′ RACE). 5′ RACE is a form of PCR that uses one primer based in the known cDNA sequence of the gene, and one primer that binds to 5′ ends of all mRNA transcripts. Thus, only one primer is specific to the gene of

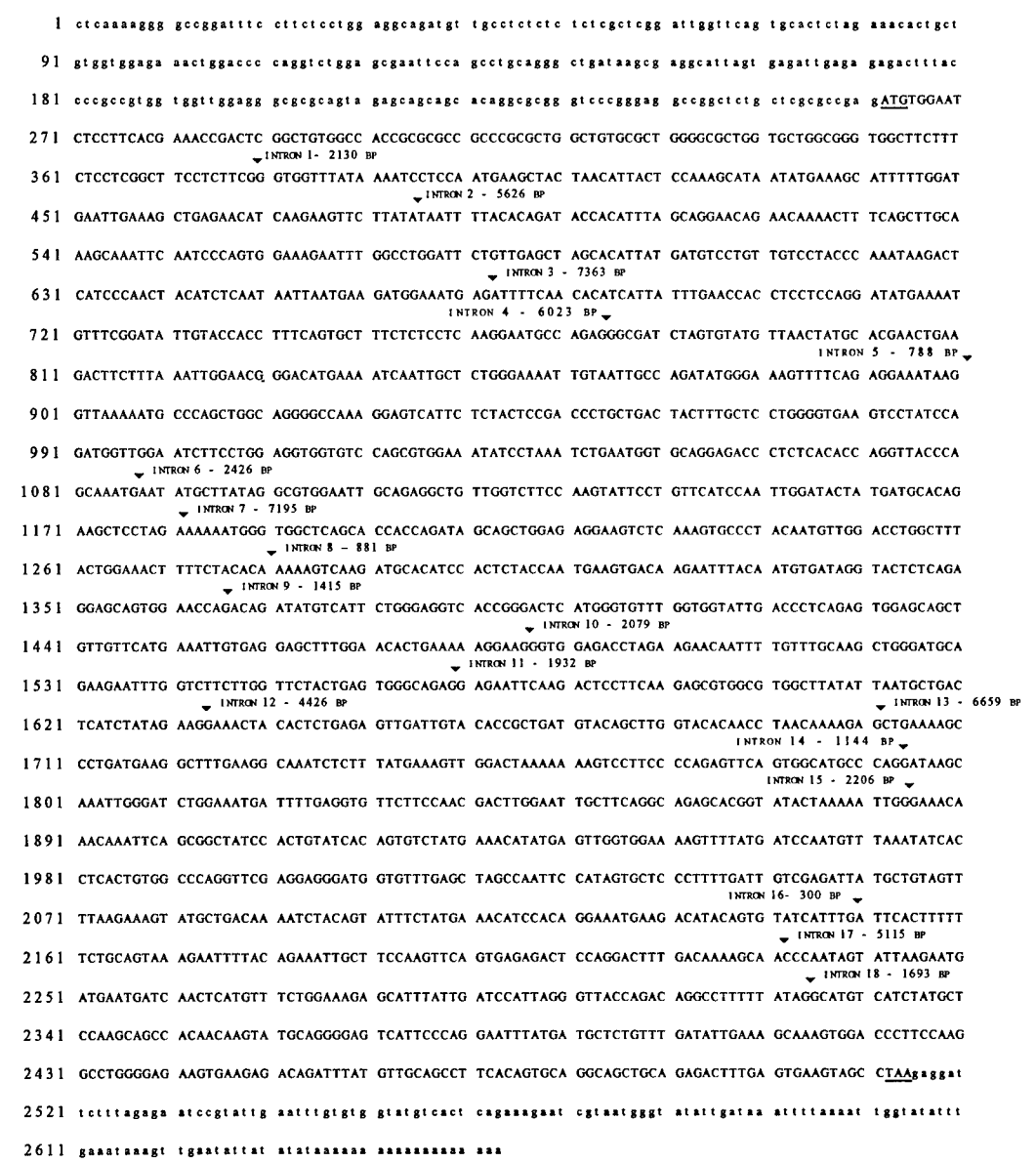

Fig. 1. The cDNA sequence of the human PSMA gene. The 5′ and 3′ untranslated regions are shown in lower case; the coding region is in upper case. The start and stop codons are underlined, and intron positions and sizes are indicated by the arrowhead.

interest, but by using this method it is possible to determine the exact start sites of transcription and therefore predict the sequence encompassing the promoter region of the gene. Our 5′ RACE experiments confirmed the original start site (+1 of the cDNA sequence), but also showed other start sites within this region, at –195 and –235, in addition to the original start site at –262 relative to the translation initiation codon of the gene. Such heterogeneity of transcription start sites is not uncommon in genes where the promoter lacks a TATA box. Consistent with this finding, the DNA sequence upstream of these start sites has no typical TATA boxes *(50)*. We next cloned the 1244

base pair region of genomic DNA spanning the start sites, and approx 1 kb of the region 5′, into a reporter vector to test whether the region was capable of driving transcription of the firefly luciferase gene. To our surprise, not only did this region of the gene have significant activity/mg of cell protein relative to the strong SV-40 viral promoter/enhancer in LNCaP cells, but it also appeared to be prostate specific, in that we could not detect significant levels of luciferase in the DU145 and MCF-7 cell lines (prostate and breast carcinoma lines). These cell lines do not express PSMA, and therefore presumably do not contain the appropriate trancription factors for activation of the PSMA promoter.

Interestingly, we did see luciferase expression driven by the PSMA promoter in PC-3 cells—another prostatic cell line that does not express PSMA—at levels corresponding to about 10% of that of the control SV-40 promoter/enhancer *(50)*. This led us to examine the genomic region of PC-3 DNA containing the CpG island for hypermethylation by Southern analysis with methylation sensitive restriction enzymes. We were able to demonstrate that while DNA from normal male lymphocytes and the LNCaP cell line was not hypermethylated, DNA from PC-3 cells was at least partially hypermethylated in this region. We next treated PC-3 cells with the demethylating agent 5-azacytidine, but were unable to detect expression of PSMA after treatment with the drug (O'Keefe et al., unpublished data), and we are still investigating this phenomenon.

In further experiments to delineate the minimal promoter region of the gene, we discovered that our original estimation of the strength of the PSMA promoter was somewhat high. We had calculated the strength of the PSMA promoter relative to that of the usually strong SV-40 promoter/enhancer in the luciferase-reporter experiments. However, when we transfected various cell lines with the SV-40 promoter alone—and after adjusting for transfection efficiency compared these transfections to those with the SV-40 promoter/enhancer combination—we were surprised to find that in LNCaP cells, addition of the SV-40 enhancer to the basal SV-40 promoter did not enhance reporter gene transcription and in some cases repressed it. In contrast, in other cell lines such as PC-3, the SV-40 promoter/enhancer combination significantly increases reporter gene transcription (O'Keefe et al., unpublished data). Other laboratories have reported similar findings in LNCaP cells when combining the SV-40 enhancer with other basal promoters such as the PSA minimal promoter (Peter Molloy, personal communication) although the reason for this remains unclear.

Deletion constructs of the promoter region allowed us to localize the minimal promoter to between bases 461–1097. The original promoter construct had contained an *Alu* repeat sequence, and we also found that once this region was deleted, reporter gene expression increased (Horiguchi et al., unpublished data). Although PSMA expression is regulated by androgens *(32,76)*, there are no typical androgen-response elements in the promoter region or in the entire PSMA genomic sequence. However, because there might be novel androgen response elements, we tested the deletion constructs and the original promoter construct for androgen responsiveness in LNCaP cells.

Although the minimal PSMA promoter described here appeared to exhibit prostate-specificity, it could only promote basal levels of reporter gene expression *(75)*. Watt et al. *(75)* cloned the PSMA enhancer region (PSME) using an "enhancer trap" system. The enhancer trap library was created by partial digestion of the P1 bacteriophage clones containing the PSMA genomic sequence, and subcloning the resultant fragments

into a vector containing the PSMA promoter-driving expression of the Green Fluorescent Protein (GFP) gene. Screening of the library for DNA fragments able to increase GFP expression over that seen by the promoter alone was carried out in LNCaP cells and a number of other non-PSMA expressing cell lines. Using this method, a fragment of DNA that was able to increase transcription from the PSMA promoter by 250-fold was identified. When the enhancer was linked to other stronger basal promoters instead of the PSMA promoter, transcription levels were increased by at least 10-fold; in the most impressive experiment, the PSME was linked to the herpes virus thymidine kinase (TK) promoter and transcription was nearly threefold that of the Rous-Sarcoma Virus promoter/enhancer, which in itself is a strong viral promoter. The PSME retains prostate-specificity even when linked to the TK promoter—which is not prostate restricted in expression—and the PSME also shows repression by androgens *(75)*. As such, the PSME shows excellent promise for use in gene therapy approaches targeting prostate cancer in the near future.

5. ALTERNATIVE SPLICING OF THE PSMA GENE

Using RT-PCR of normal prostate tissue, Su et al. *(64)* discovered the first reported mRNA splice-variant of the PSMA gene. The variant, PSM′ (PSM-prime), transcribes from the regular PSMA promoter and uses an alternative 5′ splice donor site within exon one of the gene, deleting bases 114–379, which includes the translation start codon for PSMA. Initiation of translation of the PSM′ protein begins at nucleotide 427, producing a glycoprotein of about 95 kDa that lacks the intracellular and transmembrane domains of PSMA. As such, PSM′ is located within the cytoplasm, but still retains the enzymatic activity of PSMA *(21)*. RNase protection assays differentiating PSMA from PSM′ mRNA transcripts revealed that in normal prostate PSM′ is the dominant isoform, whereas in prostate tumors and the LNCaP cell line PSMA is more prevalent *(36,64)*. Compilation of the data to form a "tumor index" comparing the ratio of PSMA:PSM′ resulted in a score of 9–11 for LNCaP cells, 3–6 for prostate carcinoma, 0.75–1.6 for BPH, and 0.075–0.45 for the normal prostate. Unfortunately, further analysis of the ratio of PSMA:PSM′ in clinical specimens has not been reported, so it remains unclear whether or not this tumor index could have a clinical impact.

Another alternative splice form of PSMA that was isolated from the human brain, prostate, and liver deletes amino acids 657–688 of the protein *(5)*, and creates an amino acid substitution (Asn→Lys). These amino acids correspond to the entire 18th exon of the gene, so the splicing event probably occurs by "exon skipping." We have also seen this splice form in cDNA derived from a colon tumor. Further investigation of this variant is required to determine whether it retains the activity of the full length PSMA protein, and whether it exists in significant levels relative to PSMA and PSM′.

Finally, when we implemented 5′ RACE of the PSMA gene using LNCaP cells, we discovered a number of novel transcripts. The first, which we have called PSM-C, begins transcription at the same nucleotides as the PSMA and PSM′ transcripts, then uses the same splice donor site as PSM′ (nt 114), but uses an alternative splice acceptor site located within intron one. Nucleotides 3270–3402 of the genomic PSMA sequence are transcribed, followed by exon 2 and exon 3. Translation of this variant containing a previously unidentified exon, which we have termed exon 1b, would result in a protein

identical to PSM′. Another variant, PSM-D, again uses the same splice donor site as PSM′, and a unique splice acceptor site in intron one, including another novel exon (exon 1c) which is from nucleotides 4289–4389 of the genomic PSMA sequence. The putative translation of this protein reveals a new translation-initiation start site located in exon 1c, followed by 42 novel amino acids and the rest of the PSMA protein in-frame. Interestingly, a motif in the novel region consisting of the peptide Ala-Ala-Tyr-Ala-Cys-Thr-Gly-Cys-Leu-Ala is similar to that seen in the growth-factor cys-knot family of proteins. Using RT-PCR, we were able to demonstrate the existence of this variant in normal prostate and LNCaP cells; however, we were unable to demonstrate significant amounts of this mRNA splice variant via RNase protection assays on these tissues.

At least one other group has also found splice variants arising from novel exons in intron one of the gene; one exon continuing on from the 3′ end of exon 1 for 68 nucleotides, and another extending for 97 nucleotides. All three variants include exon 1 (nt 1–379), and thus would be predicted to translate into a protein with a transmembrane domain. One of the variants contains both new exons aligned in tandem, and the three variants are expected to produce proteins between 40 and 805 amino acids in length *(53)*. Novel variants such as those described here have not been proven to contribute to or be functionally involved in prostate cancer. Therefore, their clinical significance remains uncertain.

6. MAPPING OF THE PSMA GENE AND IDENTIFICATION OF THE PSMA-LIKE GENE

Chromosomal localization of the PSMA gene has proven to be controversial. Initial mapping by two independent research groups using Fluorescent *In Situ* Hybridization (FISH) and the full-length cDNA sequence as a probe indicated two regions for the gene—11p11-12, and 11q14 *(38,56)*. To identify the true location of the PSMA gene, Leek et al. *(38)* used PCR of somatic cell hybrids containing various regions of chromosome 11, assigned the gene to 11p, and suggested that the 11q14 locus represented a PSMA pseudogene. Rinker-Schaeffer et al. used two P1 clones containing approx 120 kb of the PSMA gene and surrounding sequence to repeat the FISH experiment, and under conditions of high stringency, assigned the gene to 11q14 *(56)*. Later, it became apparent that FISH can be subject to artifact under conditions of high stringency when one of the regions involved is close to a centromere, which in this case is the 11p11 locus. We therefore mapped the gene again, using a number of sets of oligonucleotide primers designed to bind both intronic and exonic sequences of the gene, and PCR against a panel of somatic cell hybrids containing various regions of chromosome 11. We found that the PSMA gene does map to 11p11, approx 7 Mb from D11S1350. We also established that the "PSMA pseudogene" sequence reported on Genbank as mapping to 11q14 (accession number HSU93599) did not exist, but instead a gene that is highly homologous to the PSMA gene resides on 11q14.3 *(50)*. Further analysis of the gene at the 11q14 locus (which we have termed the "PSMA-like" gene, Genbank accession number AF261715), revealed that exons 2–19 of the PSMA gene are duplicated on the long arm of chromosome 11, along with their corresponding introns. We have been unable to detect duplication of the promoter region, or of exon

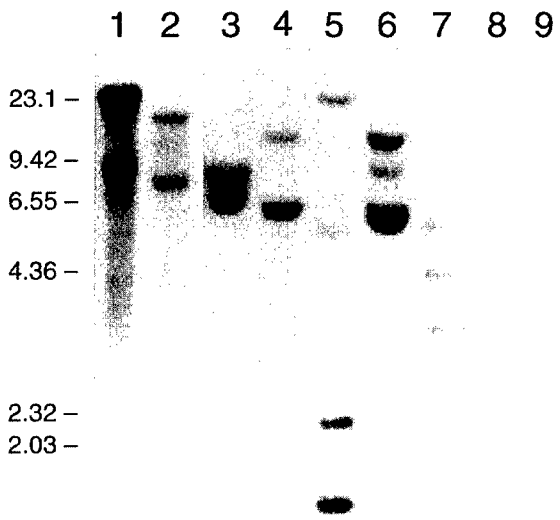

Fig. 2. Zoo-Blot using PSMA cDNA as a probe. Genes homologous to PSMA exist in many other species, including yeast. Lanes 1–9 are hybridized with genomic DNA from: (1) human, (2) monkey, (3) rat, (4) mouse, (5) dog, (6) bovine, (7) rabbit, (8) chicken, and (9) yeast.

one *(50)*. This would suggest a different mechanism of regulation, and therefore a different tissue expression pattern of the PSMA-like gene. The exonic sequences of the two genes are highly conserved (97% identical), and we have determined that the PSMA-like gene is transcribed and expressed in kidney and liver tissue, but not in prostate samples (O'Keefe et al., unpublished data). Complete characterization of the PSMA-like gene is needed. Thus, clinical and therapeutic strategies targeting PSMA can be designed to avoid PSMA-like expressing tissues or targets, and subsequently limit lack of specificity and unecessary toxicity.

7. MOLECULAR EVOLUTION OF THE PSMA FAMILY OF PROTEINS

Comparison of the intronic sequence differences of the PSMA and PSMA-like genes allowed us to calculate that evolutionary period in which the original gene duplicated was 22 million years ago *(50)*. This data is consistent with the report that the tyrosinase gene, which is closely linked to the PSMA-like gene on chromosome 11q14, was subject to duplication to 11p 24 million years ago *(17)*. It would therefore seem that the locus at 11q14 contained the original gene, and that a little more than 20 million years ago—after the divergence of man and rodent, but before the separation of man and chimp—this locus was duplicated on 11p11. Both genes then evolved further, with the PSMA gene gaining expression in the prostate. Southern blot analysis has shown that PSMA homologs exist in many species, and this is supported by the finding of homologs as far back in evolution as yeast and *C. Elegans* (Fig. 2; Heston et al., unpublished; *[55]*). Similarly, it is interesting to note that although PSMA homologs have been cloned in several of these species, there are no reports of PSMA expression in the prostate *(2,5,22)*. Instead, these PSMA homologs seem to be primarily expressed in the

Table 1
Homology Between Human PSMA and Selected Homologs and Paralogs

Species	Protein	Size (aa)	AA/NT homology to PSMA	Folate Hyd. activity	DPP IV activity	NAALA-Dase activity	GenBank accession number	Reference
Human	PSMA	750	100/100%	+	+	+	M99487	*(8)*
	NAALADase II	740	67/74%	nt	+	+	AJ012370	*(30)*
	NAALADase L	740	35/ns%	nt	+	–	AJ012371	*(30)*
	PGCP	542	27/ns%	nt	nt	+	AF119386	*(29)*
	DPP IV	766	29/ns%	nt	+	nt	M80536	*(33)*
	Transferin Receptor.	760	31/ns%	–	–	–	M11507	*(34)*
Mouse	MoPSM (PSMA homolog)	752	86/85%	+	nt	+	AF026380	*(2)*
Rat	NAALADase (PSMA homolog)	752	86/85%	+[a]	nt	+	U75973	*(21)*
	I100[b]	746	41/ns%	nt	+	–	AF009921	*(35)*
Pig	Folypoly-γ-glutamate Carboxypeptidase (PSMA homolog)	751	91/88%	+	nt	+	AF050502	*(27)*

[a] Bacich et al. unpublished observations;
[b] rat homolog of NAALADase L protein; The amino acid (AA) and nucleotide (NT) homologies to PSMA are shown; NT homologies were determined by a BLAST 2 sequences alignment using standard parameters. "ns" refers to a nonsignificant alignment (BLOSUM 62). "nt" refers to enzymatic activities that have not been tested.

small intestine, brain, and kidney of other species. In the species which is most often used as an experimental model resembling man—the mouse—there is clearly no prostatic expression of the murine homolog of PSMA (termed MoPSM), as determined by northern analysis and RT-PCR *(2)*. Furthermore, there is only one gene in the mouse, and the MoPSM gene maps to mouse chromosome 7D1-2, which is syntenic with human 11q14 *(2)*. Considering these facts, it is tempting to suggest that the PSMA-like gene contributes to the extraprostatic expression currently attributed to PSMA, and that expression of PSMA in the prostate may somehow put the prostate at high risk for developing mutations and subsequent carcinogenesis.

8. ENZYMATIC ACTIVITY OF PSMA AND RELATED GENES

Recently, PSMA homologs have been cloned from mouse *(2)*, rat *(5)*, and pig *(22)*, in addition to the cloning of more distantly related paralogs of PSMA in humans *(18,51)*. The cloning and comparison of these homologs and paralogs has shed considerable light on some of the activities of PSMA, and provided some insight into which amino acid sequences appear to be crucial in these activities. PSMA has three known activities: a folate poly γ glutamyl carboxypeptidase (folate hydrolase) *(54)*, a NAALADase *(7)* and a dipeptidyl peptidase IV *(51)*. A comparison of these three activities in the PSMA homologs and paralogs is summarized in Table 1.

The folate hydrolase activity of PSMA describes the sequential removal of γ-linked glutamates from conjugated folates and folate analogs such as methotrexate γ-glutamate

and pteroylpentaglutamate, as shown in Fig. 3A *(54)*. The folate hydrolase activity of PSMA is maintained in the presence of sulfhydryl reducing agents and *p*-hydroxymercuribenzonate, in contrast to an unrelated folate hydrolase enzyme that is located in lysosomes.

PSMA also possesses NAALADase activity, because it is able to hydrolyze the neuropeptide N-acetyl-L-aspartyl-L-glutamate (NAAG) to form *N*-acetyl-L-aspartate and glutamate. This is hydrolysis of the aspartyl α linkage, as shown in Fig. 3B. This activity was first reported by Robinson et al. *(57)* in 1986 in the rat brain, and was demonstrated to be inhibited by quisqualate. NAALADase and its neuropeptide substrate NAAG have been implicated in the regulation of excitatory signaling in the nervous system *(14,77)*. Altered activity of NAALADase has been associated with various neurolgical disorders, including schizophrenia *(11,73)*, Alzheimer's disease, and Huntington's disease *(52)*. In addition, increased levels of NAALADase have been observed in animal models for epilepsy *(45–47,52)* and amylotrophic lateral sclerosis *(58,72,74)*.

Carter et al. *(7)* used antisera to purified rat NAALADase to screen a rat-brain expression library, resulting in the isolation of a partial 1428 nt cDNA clone that had 86% homology to part of the human PSMA cDNA sequence. The entire rat PSMA/NAALADase sequence was subsequently cloned by *(5)*, and when transiently transfected into PC-3 cells (which are NAALADase-negative), they gained NAALADase activity that could be inhibited by quisqualic acid. Human PSMA was also demonstrated to have NAALADase activity *(7)*. Further characterization by Luthi-Carter et al. demonstrated that the human brain NAALADase could be immunoprecipitated with the MAb 7E11-C5 *(44)*. As this antibody binds to residues not conserved in PSMA-like (O'Keefe et al., unpublished), it suggests that human NAALADase and PSMA are derived from the same gene. In addition, Luthi-Carter amplified RNA by RT-PCR, a sequence identical to the LNCaP-derived PSMA sequence, from human cerebellum, indicating that this RNA is present, but in itself not proving that all of the NAALADase activity found in the brain is from PSMA.

Recently, it was shown that PSMA also has dipeptidyl peptidase IV activity, which refers to the ability to hydrolyze Glycine-Proline-7-amido-4-methylcoumarin *(51)*. This amino dipeptidyl peptidase IV activity cleaves the bond between the proline residue and amido methylcoumarin molecule, as shown in Fig. 3C. It was first reported that PSMA possesses this activity when Pangalos et al. *(51)* transiently transfected COS cells with PSMA cDNA and assayed for the dipeptidyl peptidase IV activity. Although the mock-transfected COS cells had dipeptidyl peptidase IV activity, the PSMA-transfected COS cells had significantly more activity. The physiolgical role of the dipeptidyl peptidase IV activity of PSMA is unclear; however, it may play a role in the regulation of various biologically active peptides, including collagen, neuropeptide Y, and growth hormone releasing factor *(51)*. As such, it would appear that PSMA is a multifunctional enzyme, possessing both amino and carboxy-peptidase activities as a mono and dipeptidase.

Rawlings and Barrett predicted the secondary structure of PSMA using a number of protein prediction and protein alignment programs, modifying the results so that the potential zinc ligand binding sites and other blocks of secondary structures were aligned

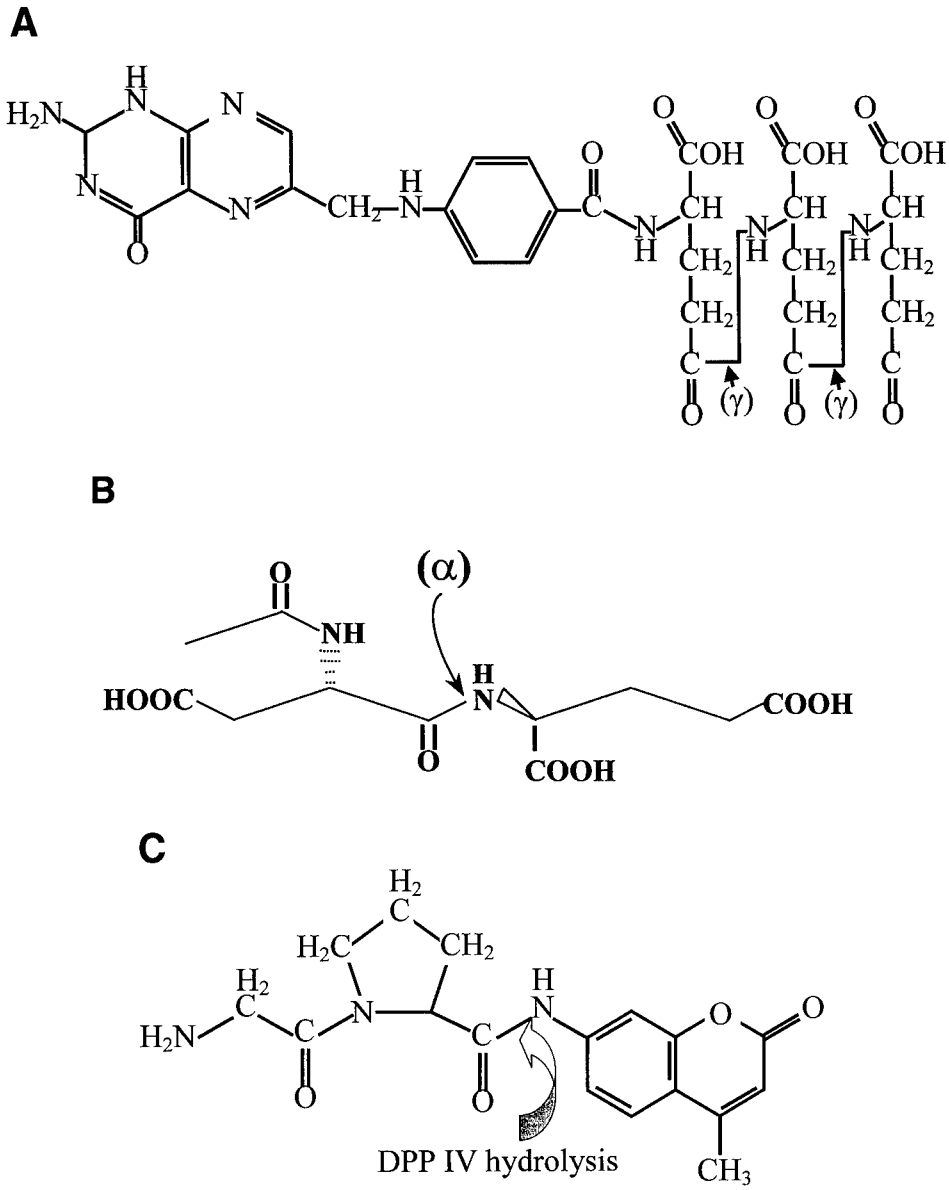

Fig. 3. PSMA substrates. **(A)** folic-acid polyglutamate, **(B)** *N*-acetyl-aspartylglutamate (NAAG), and **(C)** glycine-proline-7-amido-4-methylcoumarin. Arrows indicate the bond that is hydrolyzed by PSMA.

(55). They predicted that PSMA is made up of six organizational domains, with domain E (a.a. 273–587) responsible for the catalytic activity. They were then able to assign this catalytic domain to the M28 peptidase family, and predicted that Asp^{377}, Asp^{387}, Glu^{425}, Asp^{453} and His^{553} are ligands for two atoms of zinc required for catalytic activity *(55)*. Speno et al. performed site-directed mutagenesis experiments alter-

ing these residues resulting in severely reduced NAALADase activity, and were also able to demonstrate that substitution of some amino acids near the putative zinc ligands has a major impact on enzyme structure and/or function *(63)*.

9. THE ROLE OF PSMA IN PROSTATE CARCINOGENESIS AND PROGRESSION

PSMA is believed to be involved both in glutamatergic signaling and folate metabolism. Although there is no evidence that the PSMA substrate NAAG is present in the prostate, our laboratory has demonstrated the existence of Glu 2/3 and Glu 4 glutamate receptors using immunohistochemistry *(26)*. This would suggest that when PSMA is expressed on the surface of the prostate epithelial cell—and particularly when the membrane-bound form of PSMA is upregulated in cancer—sufficient quantities of glutamate could be released to stimulate these receptors, leading to oxidative stress and subsequent cell and DNA damage *(12)*, and further enhancing the ability of the cell to mutate and the cancer to progress.

Another possible function of PSMA was recently reported by Liu et al. *(41)*, who observed a di-leucine motif in the amino terminal of the protein, indicating a possible role in internalization of ligands via PSMA. Incubation of LNCaP cells with antibodies against PSMA followed by laser scanning confocal microscopy revealed that the antibodies were internalized and remained in endosomes within the cell. The endocytosis occurred via clathrin-coated pits and was shown to occur constitutively, although it was enhanced by the presence of antibodies, suggesting a role for PSMA in the internalization of as yet undefined ligand(s) *(41)*.

Our most favored theory is based on the folate hydrolase activity of PSMA and PSM′, which releases the terminal gamma-linked glutamates from folates. To appreciate how the folate hydrolase activity of PSMA might be involved in carcinogenesis and the progression of prostate cancer, it is first necessary to understand the role of folate in this tissue. Dietary folates are generally polygammaglutamated. However, folate can only enter the cell by passive diffusion if it has been deglutamated (although most extracellular folate is monoglutamated). Within the cell, folate is polyglutamated so that it cannot diffuse out of the cell. The presence of PSM′ in the cell would lead to deglutamation of the polyglutamated folate, and subsequent loss of folate from the cell. In the prostate, there is an increased need for folate relative to that of other tissues *(24)*. Folate hydrolase would be expected to deglutamate folate, and allow it to be in a form that could easily diffuse out of the cell—thus placing the cell at risk of becoming folate deficient *(54)*. Folate deficiency is associated with DNA damage and carcinogenesis *(13,34)*.

Folic-acid deficiency can lead to DNA damage via increased uracil incoporation, resulting in single-stranded DNA breaks and decondensation of chromosomes *(4,37)*. Folate deficiency may also lead to carcinogenesis by reducing DNA methylation, which in turn has been proven to lead to the overexpression of certain genes, including a number of oncogenes *(3,16,35)*. Folate is integral to a number of basic metabolic processes in the cell, including DNA synthesis, DNA methylation, and the formation of methionine and polyamines. The prostate is the major organ responsible for polyamines, producing between eightfold and 100-fold greater amounts than other polyamine-producing tissues *(24)*. This high production of polyamines places stress on the folate-

methionine pathway, and as a result the prostate is at greater risk of DNA damage induced by a low-folate environment.

Therefore it is our hypothesis that PSM′, the cytosolic version of PSMA expressed in normal prostate epithelial cells, would be a "catalyst" of DNA damage, and subsequently carcinogenesis, by depleting the prostate of intracellular folate. PSMA, the membrane-bound isoform highly expressed in the tumor and metastatic deposits, could also be expected to hydrolyze poly-γ-glutamated folates, allowing them to diffuse into cells in the local microenvironment. Although poly-γ-glutamated folates are not typically considered extracellular substances, there are a large number of dead or dying cells that can liberate these polyglutamated folates within an environment such as a prostate tumor. Therefore, cells expressing a membrane folate hydrolase such as PSMA would have a growth and survival advantage over nonexpressing cells, especially if the levels of PSM′ decreased in the PSMA-expressing cells. A possible extension of this hypothesis might explain why the neovasculature of most solid tumors express PSMA, tumors which characteristically have an inadequate blood supply may be able to sequester folate from dead cells if they can induce the endothelial cells of the vasculature to express such a folate hydrolase.

After the switch of mRNA splicing to predominantly form PSMA—the membrane-bound isoform of the protein—folate uptake by the cell would be enhanced. This in turn would lead to a greater proliferation rate for the cell, and could possibly also lead to enhanced mutation rates via glutamate receptors and oxidative stress on the cell. In this case, increased expression of PSMA could assist in evolution of the tumor and tumor growth, and progression of the cancer.

To assess this theory in the laboratory, we are currently using the transgenic mouse model. This is significantly assisted by the fact that the murine prostate does not express the homolog of PSMA, MoPSM. We have created transgenic mice expressing human PSMA and/or PSM′ under the control of a prostate-specific promoter, and are examining the effect of folate deficiency on the rate of DNA damage in the presence of the PSMA isoforms (Bacich et al., unpublished).

10. CLINICAL UTILITY OF PROSTATE SPECIFIC MEMBRANE ANTIGEN

The potential of PSMA as a marker of clinical progression was first noted during characterization of the 7E11C5.3 antibody *(28)*. Sera from 20 of 43 patients with prostate cancer appeared to carry molecules reactive with the 7E11C5.3 antibody. However, none of the 30 normal blood donors or seven patients with BPH exhibited such reactivity. The authors also reported that prostate cancer patients who tested positive were more likely to be in progression ($p < 0.05$) *(28)*, although other groups have been unable to detect PSMA in the serum of any patients with metastatic disease *(69)*. The possibility that PSMA could be used as a marker of circulating prostate cancer cells was quickly examined by a number of groups. Israeli et al. *(31)* developed a highly sensitive technique using reverse-transcriptase PCR with nested primer sets to amplify PSMA sequences from patient blood samples. Using similar nested, "enhanced," or radioactive PCR-based methods, the consensus appears to be that there is no correlation between PSMA-positive results and clinical stage, pathological stage, or tumor

grade *(6,49,62)*. There are other reports of PSMA-mRNA expression in normal lymphocytes, urine, and bone marrow (including specimens from female controls), as the result of "illegitimate transcription"—insignificant numbers of PSMA transcripts produced to have any functional effect, but that are able to be detected by sensitive PCR techniques *(10,15,39,80)*.

As with all PCR-based methods to detect circulating cancer cells, the technique needs to be standardized between laboratories. It is clear from the literature that in the hands of different researchers, significant variation is found in test results. Furthermore, the presence of circulating cells does not appear to be directly related to metastatic potential of the primary tumor. For example, Loric et al. *(43)* have shown that patients with inflamed prostates also exhibit circulating prostate cells. However, new technologies currently available may be able to solve these problems. The advent of "Real-Time" PCR, which allows sensitive quantitation of PCR products and the expanding access to custom-designed "Gene Chips" should make it easier to quantitate one or a number of prostate specific transcripts. It has already been shown that a combination of RT-PCR methods for PSMA and PSA-expressing cells is more accurate than either technique alone *(20,79)*. In addition to combining several different markers for analysis, it might also be worthwhile to examine relative amounts of the PSMA mRNA splice variants—particularly PSM′ vs PSMA.

PSMA RT-PCR—in combination with PSA RT-PCR—has also been used to determine the "molecular surgical margins" at radical prostatectomy, by examining five biopsy specimens from the prostatic fossa *(65)*. The results, although preliminary, are promising. The authors found a perfect correlation between a positive PCR result and histopathological determination of positive margins or extracapsular extension. Furthermore, control biopsy specimens taken from men undergoing radical cystoprostatectomy for bladder cancer or abdominoperineal resection for rectal cancer were all negative for the test. Interestingly, in four of 16 cases with histopathologically negative surgical margins, the molecular margins were positive *(65)*. Validation of this unique method requires larger, longer, and multi-institutional studies.

At the present time, clinical imaging using PSMA-directed immunoconjugates utilize the Cyt-356 antibody, commercially known as the "Prostascint Scan™". While the results are promising, the test is probably not optimally designed. In studies localizing the target epitope of Cyt-356, Troyer et al. *(71)* found that the antibody binds to the short cytoplasmic domain of the protein. As such, Cyt-356 binds efficiently to dead cells, and not viable cells *(40,70,71)*. The ability of Cyt-356 to image metastatic deposits is most likely caused by necrotic cells in the tumors, and therefore the sensitivity of imaging would be expected to be enhanced through the use of antibodies directed against the external domain of PSMA. A number of groups have developed such "second generation" antibodies *(40,48)*, and are currently carrying out phase I trials using the antibodies as both imaging agents and therapeutic vectors.

11. THE FUTURE OF CLINICAL AND THERAPEUTIC STRATEGIES UTILIZING PSMA

In an immunotherapeutic approach, Tjoa et al. *(66)* showed that T-cell proliferation could be induced in vitro by autologous dendritic cells pulsed with peptides from the PSMA amino acid sequence. Dendritic cells are professional antigen-presenting cells

that can induce T-cell proliferation and cytotoxicity against specific antigens. This study was followed by Phase I and II clinical trials, which showed positive results, with partial responders identified in groups of patients with both metastatic and suspected local recurrent disease. Follow-up of the responsive patients nearly 300 d later revealed that more than 50% of the subjects were still responding *(59,60,67,68)*. While these results indicate a promising future for immunotherapeutic strategies against prostate cancer, there are several intrinisic problems. The therapy described here is restricted to patients of major histocompatibility antigen type A2 (HLA-A2) tissue type. Furthermore, HLA antigens are downregulated by tumor cells, and thus would not be available for immuno-targeting.

Using an innovative approach to avoid these restrictions, Gong et al. *(19)* devised an immunotherapeutic method that completely circumvents the need for MHC-mediated presentation of peptides. An artificial T-cell receptor was generated by cloning the DNA sequence responsible for recognition of PSMA by the J-591 antibody described here *(40)*, followed by a linker region and the zeta chain receptor, into a retroviral vector *(19)*. T-cells (CD4+ and CD8+) from prostate cancer patients were then transduced with the vector, and their response to cells expressing PSMA was examined. The transduced cells efficiently and specifically lysed PSMA-expressing cells, and also released cytokines in response to PSMA, suggesting that a prolonged response might be feasible *(19)*. If these results are as impressive in vivo as they are in vitro, such an approach should be able to target both the primary tumor and metastatic deposits, as well as the neovasculature of other solid tumors.

Other therapeutic approaches targeting PSMA currently being investigated by our and other laboratories include the use of prodrug strategies and gene therapy. To investigate prodrug strategies against prostate cancer, NIH3T3+/– PSMA and PC-3+/– PSMA-transfected cells and LNCaP cells were grown in the presence of methotrexate triglutamate *(27)* and Heston et al. (unpublished). In the cells expressing PSMA, the drug was converted into its cytotoxic derivative methotrexate, and cell growth was inhibited. However, in the non-PSMA-expressing cells, the drug was nontoxic. While these results show promise, we are currently using the LNCaP xenograft model to determine whether toxicity is specifically targeted to the tumor, or affects other cells expressing the murine homologs of the genes described in Table 1.

Cloning of the PSMA promoter, and more particularly the enhancer, has made the use of gene therapy constructs carrying either therapeutic or cytotoxic genes a viable alternative to those described here *(50a)*. In this approach, cytotoxic genes such as the cytosine deaminase (CD) or herpes virus TK genes are linked to the PSME and a compatible promoter, and the patient is treated with cytotoxic prodrugs such as 5-fluorocytosine or gancyclovir. However, the first challenge is to demonstrate prostate-specificity of the PSME in the transgenic mouse model, and we are currently evaluating this process (Bacich et al., unpublished).

12. INTO THE 21ST CENTURY

The PSMA story has yielded several unexpected surprises so far, but we still do not know whether expression of this gene influences the development or progression of prostate cancer and if so, how. The possible role of PSMA in the angiogenic pathway of tumors is intriguing, but it also suggests that we have much to learn about this fasci-

nating protein. The expression of PSMA in tumor-associated vasculature, as well as its high expression in virtually all prostate tumors and metastases, and particularly in hormone refractory disease for which there is currently no efficient treatment, indicates that targeting of PSMA may be highly valuable as a treatment for not only prostate cancer, but several types of solid tumors.

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Overview of Evolving Strategies Incorporating Prostate-Specific Membrane Antigen as Target for Therapy

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ABSTRACT

Prostate-specific membrane antigen (PSMA) is a potential target in prostate cancer patients because it is very highly expressed and because it has been reported to be upregulated by androgen deprivation. This overview addresses the expression of the PSMA gene in terms of the promoter and enhancer and how that may play a role in gene therapy. We also review PSMA as a target for antibodies for imaging and treatment and the development of a novel hybrid T-cell receptor that combines the specificity of anti-PSMA antibodies with that of T-cell receptor activation when introduced into primary lymphocytes by retroviral-mediated gene transfer. We also discuss our recent findings on the expression of a PSMA-like gene and how that understanding allows specific targeting of PSMA.

INTRODUCTION

PROSTATE-SPECIFIC MEMBRANE ANTIGEN (PSMA) is an excellent target for therapeutic drug development because it is highly expressed in prostate cancer and its metastases.[1–3] In evolutionary terms, PSMA is the result of a gene duplication event that occurred about 25 million years ago. This event has resulted in two highly homologous genes, on chromosome 11p and 11q, but it is the gene on 11p that has evolved to be highly expressed in the prostate, with upregulation of expression in tumors. We have found PSMA to be expressed in the neovasculature of all solid tumors but not in the established vasculature of normal cells unassociated with cancer.

PHYSICAL PROPERTIES OF THE PROTEIN

The PSMA gene product is a type II membrane protein of approximately 100 kD. Being a type II membrane protein, it possesses a short amino terminus of 19 amino acids inside the cell, a membrane-spanning domain, and a large extracellular domain. Rawlings and Barrett have predicted the secondary structure of PSMA using a number of protein prediction and alignment programs, editing the results so that the potential zinc-binding ligand sites and other blocks of secondary structures were aligned.[4] They predicted that amino acids Asp377, Asp387, Glu425, Asp453, and His553 are responsible for zinc-ligand catalytic activity as a peptidase. Indeed, the protein has evolutionary homology with a number of carboxy and amino peptidases and has been placed in the category of the M-28 peptidases, which have dicatalytic adjacent sites of hydrolytic activity.[4] The finding of homologous proteins in bacteria, yeast, and plants suggests that the function of the protein is of very ancient origin and important to the economy of the cell.

Another protein related evolutionarily to PSMA is the transferrin receptor.[5,6] There are many areas of the PSMA amino acid sequence that have some homology with the transferrin receptor. However, PSMA does not bind transferrin, and the signal sequence for internalization of the transferrin protein is not found in FSMA. It is thought that PSMA is internalized, as there

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is a dileucine motif in the intracellular portion of the protein; the dileucine moiety has been found in proteins that are internalized and sorted to the endosomal compartment. Also, Liu and associates reported that PSMA is internalized,[7] and antibody binding will induce internalization. Whether peptide substrates for PSMA will induce internalization has not been clearly established.

EMZYMATIC ACTIVITY

We have identified PSMA as a unique folate hydrolase; that is, a glutamate carboxypeptidase that will hydrolyze the gamma-linked peptide glutamates of polygammaglutamated folate.[8,9] We arrived at this conclusion from two observations. First, it has been known for a long time that semen contains folate-binding proteins of approximately 100 kD. Second, we found that the proximal small intestine expresses a protein that is highly homologous to PSMA. We knew that the proximal small intestine had a protein of approximately 100 kD that was a folate hydrolase. Therefore, we tested whether PSMA had folate hydrolase activity and determined that it did.[8] At nearly the same time, workers in Coyle's laboratory found the PSMA is able to hydrolyze the alpha-peptide linkage of the postulated neurotransmitter N-acetylaspartylglutamate.[10] In addition, we observed that PSMA does not hydrolyze the alpha-linked glutamate in folate.[8] Thus, PSMA is a unique glutamate-preferring carboxypeptidase and has been designated glutamate carboxypeptidase II (EC 3.4.17.21) by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology.[11]

PRODRUG ACTIVATION

Because the hydrolytic activity of PSMA resides outside the cell, we explored whether PSMA might serve as a prodrug-activating enzyme. We incubated LNCaP cells with the potentially toxic methotrexate trigammaglutamate (MTXglu3). This compound should not be toxic, as it should not be able to gain access to the cell because of its negative charge, and it is not a substrate for the folate transporter. In Figure 1, it can be seen that when 0.5 μM MTXglu3 is added to LNCaP cells, the cells decrease their growth over time and begin to die relative to the untreated control cells. However, if we add an inhibitor of PSMA's enzymatic activity, quisqualic acid, we can completely block the cytotoxicity of MTXglu3. Interestingly, quisqualic acid exhibits inhibitory activity toward LNCaP cells, but it is not clear whether PSMA functions in the growth regulation of LNCaP cells. We also incubated cells with methotrexate (MTX), a drug that utilizes the folate transporter to gain access to the cell's cytoplasmic compartment, where it blocks folate metabolism and interferes with nucleic acid generation. As expected, MTX was toxic for LNCaP cells, and this toxicity was not reversed by inhibition of PSMA enzymatic activity (Fig. 1). This result demonstrates that PSMA may serve as a prodrug-activating enzyme.

In treating prostate cancer, however, we have to take into account that we have observed two forms of PSMA: a membrane form and an intracellular form (PSM′).[12,13] The PSMA will help enable the removal of polygammaglutamated material from the prodrug, but once inside the cell MTX has to be polygammaglutamated for retention to maintain its cytotoxic activity. With the alternatively spliced cytosolic form PSM′ inside the cell, MTX is not likely to be polygammaglutamated and re-

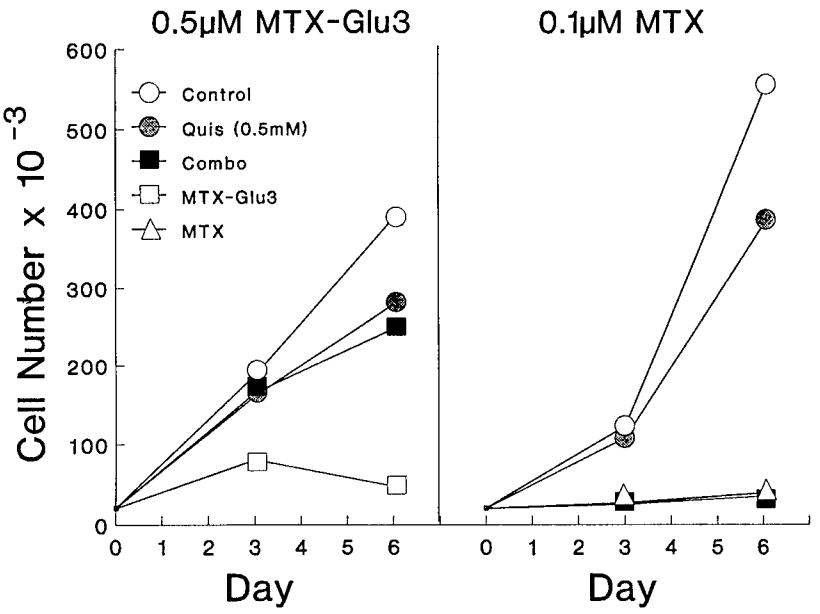

FIG. 1. Effect of methotrexate trigammaglutamate (MTX-Glu3) or methotrexate (MTX) on growth of LNCaP cells with or without quisqualate (0.5 mM). The LNCaP cells (5×10^4) were plated, and following day, either MTX-Glu3 or MTX with or without quisqualate was added. Number of cells was determined at times shown.

tained and thus would be inactive, so we are currently identifying prodrugs that need activation in relation to the cell surface of PSMA-expressing cells but do not require polygammaglutamation of the intracellular drug for retention or expression of cytotoxic activity.

PSMA EXPRESSION IN TUMOR NEOVASCULATURE

The extracellular portion of PSMA appears to be a unique target. As with any target, there are positives and negatives. The upside of PSMA as a target is that it is very strong expressed in prostate cancer. An added upside centers on the serendipitous finding of PSMA's strong expression in the neovasculature of all solid tumors that we have examined to date (Table 1). This discovery was accomplished with five different antibodies, each of which binds to a different region of the PSMA molecule. This development of other antibodies was necessary because the original antibody to PSMA, Cyt-356, is cross-reactive with a protein that is not PSMA but is similar in molecular weight. All of the antibodies demonstrated the same strong immunoreactivity with tumor-associated vasculature.[14]

To make certain that it was PSMA being expressed in the vasculature and not a PSMA-like protein that would cross-react with these antibodies, we performed *in situ* hybridization studies with reverse transcriptase-polymerase chain reaction on the RNA. Indeed, it was PSMA and not a PSMA-like protein whose mRNA was present in the tumor-associated vasculature.[15] Currently, we do not know the reason for the induction of PSMA expression during the angiogenic response to tumors. It is of interest that an exception to the expression of PSMA in the neovasculature of all solid tumors is prostate cancer, where only about 20% of the cancers appear to induce expression of PSMA. It remains to be determined why PSMA is not strongly expressed in the tumor-associated vasculature of all carcinomas of the prostate, but, as there is such a broad spectrum of malignant potential for prostate carcinomas, it is interesting to speculate that those cancers inducing expression are the ones with greater malignant potential.

TABLE 1. NEOVASCULAR EXPRESSION OF PSMA IN SOLID TUMORS

Renal-cell carcinoma	11/11
Transitional-cell carcinoma	6/6
Testicular embryonal Ca	1/1
Colon adenocarcinoma	5/5
Neuroendocrine carcinoma	5/5
Glioblastoma multiforme	1/1
Melanoma	5/5
Pancreatic duct carcinoma	4/4
Non-small-cell lung Ca	5/5
Soft tissue sarcoma	5/6
Breast carcinoma	5/6
Prostatic carcinoma	2/12

All tissues were prepared for examination from frozen sections. The monoclonal antibodies used were 7E11C5 (Cytogen; internal domain), PM2J004.5 (Hybridtech; internal domain), PEQ226.5 (Hybridtech; external domain), and J591 and J415 (Bander; external domain). Each of the antibodies bound to a different antigenic site on PSMA. Data from reference 14.

PSMA-LIKE GENE

A potential downside for targeting of PMSA is that there is a gene that is highly homologous called the PSMA-like gene.[12] When we were determining the chromosomal location of PSMA, we identified not one but two sites on chromosome 11 by *in situ* hybridization. We have since determined that PSMA resides on the short arm at 11p11-p12 approximately 7 Mb from marker D11S1350, with the other gene being on the long arm at 11q14.[12] We have sequenced the entire genomic region encompassing PSMA, which has been deposited in GenBank (Accession No. AF007544). This GenBank entry consists of 93,525 basepairs, 2484 of which are upstream of our published cDNA sequence NM 004476 (*Homo sapiens* folate hydrolase [prostate-specific membrane antigen], [FOLH1] mRNA).[12] The entry further contains 31,208 nucleotides of downstream sequence after the polyadenylation site. The PSMA gene consists of 18 introns and 19 exons and has 60,133 bases beginning from the published cDNA sequence. Because we have the entire PSMA sequence, we have been able to compare it with the sequence of the PSMA-like gene on 11q14 and have found high homology in both intronic and exonic regions until one approaches the 5′ region of the cDNA. We examined intronic sequences and found that they were 97% identical between the PSMA and PSMA-like genes. We were able to calculate that PSMA was likely the result of an endoduplication event that occurred a short time ago (in evolutionary terms) of approximately 22 million years. It has been found by others working on chromosome 11q14 that a fragment containing the tyrosinase gene was involved in a duplication event. The calculations from base-substitution rates suggested that it likewise occurred about 22 to 24 million years ago.[16] This means that if the genomic information on chromosome 11q produces a protein, it is likely to be highly homologous to PSMA.

In mice, we have observed that the ortholog of PSMA resides on the chromosome that is syntenic with human chromosome 11q14 (7D1-2). Indeed, the mouse PSMA ortholog is not expressed in the prostate but rather in the kidney, brain, testes, and ovaries; and we hypothesize that this expression would likely be areas in which the PSMA-like gene would be expressed in the human as well. Therefore, we have been examining nonprostatic tissue immunohistochemically for expression of PSMA because the presence of protein would be more important than RNA expression, which does not always correspond to the level of protein expression. If PSMA and PSMA-like proteins truly are nearly identical, then expression in nonprostatic tissues or tumor vasculature should be very telling. As noted, we have used five antibodies, which bind to different regions of the PSMA protein. In all of these experiments, we observed PSMA expression to be the strongest in prostate, prostate cancers, tumor-assisted vasculature, and proximal small intestine epithelium. Weak expression was noted in breast ductal cells and in the proximal tubule cells in the kidney. No expression was observed by any of the PSMA antibodies in the brain.[3,14] This result is really different from what would be expected in the mouse and human PSMA-like proteins had sim-

ilar expression, in that there would be strong and equal expression in the brain and kidney. In humans, only weak expression is observed in the kidney proximal tubules, and no expression was detected in the brain. Only the Cyt-356 antibody exhibited reactivity with skeletal muscle, which is in keeping with the fact that the Cytogen antibody demonstrates some nonspecific immunoreactivity. These studies of immunoreactivity of multiple antibodies would suggest that PSMA is a reasonably restricted target.

PSMA PROMOTER ENHANCER

Why is PSMA expressed in the human prostate and not in the mouse prostate? We have found that the promoter region of PSMA has a number of potential sites for transcription factor binding, none of which by itself would be responsible for such high expression in the prostate. Usually, the strong activation in selected tissues is attributable to an enhancer element. Using an enhancer trap strategy in which restriction digests of the PSMA gene were shotgun cloned into a reporter vector containing the PSMA promoter and Green Fluorescent Protein, we have identified an enhancer in the third intron of the PSMA gene (Watt et al, manuscript submitted). This enhancer causes a 250-fold increase in expression of the reporter in LNCaP cells over the level seen without a promoter enhancer construct, and the expression nearly equals that driven by a viral RSV construct. We have found that PSMA is decreased in expression in the presence of androgens. Likewise, the reporter associated with the enhancer is decreased threefold in expression in response to androgens in the LNCaP cell line. In patients receiving neoadjuvant hormone deprivation, however, we did not find a significant increase in PSMA expression in the remaining cancer.[17] This may be because of the initial high level of expression. Using the corresponding genomic region from the PSMA-like gene, which is 97% identical in sequence, the 11q region did not perform as an enhancer in LNCaP cells, and there was no increase in reporter expression. Expression of PMSA appears differ from what is found for the PSA enhancer and the probasin enhancer, as these enhancers work in both human and mouse prostates.

TABLE 2. EFFECT ON CELL PROLIFERATION OF TRANSFECTION OF C4-2 CELLS AND MCF-7 CELLS AND TREATMENT WITH 250 μM 5-FLUOROCYTOSINE

	% of Control
C4-2 cells	
Sense	30
Antisense	102
MCF7 cells	
Sense	100
Antisense	99

Cells were plated and the following day were transfected with plasmid vector. After 48 hours, they were exposed to 5-fluorocytosine for 72 hours, and the number of cells was determined with an MTS assay. Not shown are the results with HC-157 and HCT-8 cells, which were like MCF-7 cells and were not responsive to the PSMA promoter/enhancer.

PSMA ENHANCER-DRIVEN GENE THERAPY

The PMSA enhancer can be used to drive expression of genes that have potential therapeutic benefit in prostate cancer cells. We have done this initially with cytosine deaminase (Table 2).[18] Basically, when we transfect either the LNCaP derivative line C4-2 (prostate cancer) or MCF-7 (breast cancer) cells with the PSMA promoter/enhancer construct linked to cytosine deaminase in either the sense or antisense orientation and expose the cells to the prodrug 5-fluorocytosine 48 hours later, the C4-2 cells are killed with the cytosine deaminase is expressed (sense construct) but not when the cytosine deaminase is in the antisense orientation and the protein thus is not expressed. There

TABLE 3. EFFECT OF PZ-1 (ANTI-PSMA) AND NTP (NEGATIVE CONTROL) RETROVIRAL-TRANSFECTED PERIPHERAL BLOOD LYMPHOCYTES FROM PATIENTS WITH PROSTATE CANCER IN INDUCING LYSIS OF PSMA-POSITIVE AND -NEGATIVE CELLS

	Effector:target ratio	
Cell line	*3:1 % lysis*	*50:1 % lysis*
LNCaP		
Pz-1		
Pt 1	30	90
Pt 2	25	67
Pt 3	22	72
NTP		
Pt 1	0	5
Pt 2	0	5
Pt 3	18	30
E14 (PSMA positive)		
Pz-1		
Pt 1	50	100
Pt 2	25	70
Pt 3	20	50
NTP		
Pt 1	0	0
Pt 2	0	5
Pt 3	0	5
E14 (PSMA negative)		
Pz-1		
Pt 1	0	0
Pt 2	0	5
Pt 3	0	5
NTP		
Pt 1	0	0
Pt 2	0	5
Pt 3	0	5

The PBL from three patients with prostate cancer were transduced with either Pz-1 or NTP. Transduced PBL were incubated for 4 hours at effector:target ratios of either 3:1 or 50:1 with 10,000 ^{51}Cr-labeled target cells at 37°C. The chromium released was measured as a percentage of total releasable chromium relative to that spontaneously released. Triplicate supernatant samples were counted using an automated gamma counter. The PC-3 cells, transfected with PSMA or not, exhibited a lytic response similar to that exhibited by E14 murine thymoma cells. (Modified from Gong et al[20]).

also is tissue specificity, in that the same construct, when transfected into MCF-7 cells or, indeed, into a number of other cell lines, is not toxic. What remains to be determined is whether the strong expression of PSMA in the tumor-associated neovasculature is driven by the same transcription factor and the same enhancer region. We are currently developing models that will allow us to ask this question directly. They appear to require human cells because to date, we have not observed PSMA expression in the neovasculature of tumors in mice. The available antibodies that react with the cell surface of the highly expressed prostate cancer antigen could also enhance the delivery of DNA-based gene therapy to tumors, furthering antitumor specificity.

ANTIBODY TARGETING-T BODIES

Antibodies to the external domain of PSMA have a further potential utility: they can be used for the creation of T bodies against PSMA antigen. In current immune strategies, many investigators are using tissue-specific antigens to enhance the development of a cellular or humoral response by the patient against his tumor. To induce a cellular response, many investigators are using peptides and dendritic cells to generate a specific immune response, and initial studies using PSMA-specific peptides have produced responses.[19] There are some drawbacks to the cellular approach in that it requires the tumor cells to present a peptide fragment in an MHC-specific context. Some tumors downregulate MHC expression, and different peptides will be presented with the different MHC molecules in different patients. However, T bodies overcome both of these difficulties. Basically, in a T body, the T-cell receptor is modified so that the signaling no longer requires presentation of a peptide but is engineered to be activated by recognition of a cell-surface protein. In a T body, the gene encoding the protein region responsible for the specificity of the antibody is used to generate a single-chain antibody. This antibody is then used to generate a chimeric protein in which the external domain has the cloned single-chain antibody plus the internal signaling domain of the T-cell receptor for activation and killing of the cell bearing the antigen recognized by the antibody.

We have therefore created a retroviral vector, Pz-1, with the single-chain J-591 antiPSMA antibody, the hinge and transmembrane domain of CD-8, and the T-cell receptor zeta-chain cytoplasmic domain.[20] This construct was transduced into patients' peripheral blood lymphocytes (PBL) with gibbon ape leukemia virus envelope pseudotyped virions. Gene transfer was found to be 20% to 50% efficient in both CD4 and CD8 cells, as monitored by fluorescence-activated cell sorting. Monitoring of these anti-PSMA T bodies demonstrated selective and effective toxicity toward PSMA-expressing cells, as human LNCaP cells were lysed, as were human PC-3 cells and murine E14 cells that had been transfected with PSMA (Table 3). Control cells did not demonstrate similar levels of lysis when the controls were PSMA-negative cells or PBL transfected with a low-affinity nerve growth factor receptor, NTP. The antibody J-591 has been used clinically and exhibited no toxicity. Studies with this antibody linked to toxins such as radionuclides have demonstrated that the antibody localizes to tumor *in vivo,* even to sites of bony metastatic disease.[21] Thus, it is likely that these strategies employing anti-PSMA antibodies, whether linked with toxins or in the form of T bodies, will provide a potential approach to the treatment of prostate cancer.

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A Tissue-Specific Enhancer of the Prostate-Specific Membrane Antigen Gene, *FOLH1*

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Prostate-specific membrane antigen (PSMA) is an integral membrane protein that is highly expressed on the surface of prostate epithelial cells. It is also expressed on the vascular endothelium of a number of tumor types. We have used an enhancer trap approach with randomly cleaved overlapping DNA fragments from an approximately 55-kb P1 cosmid insert encompassing the 5′ half and upstream sequences of the PSMA gene (*FOLH1*) to isolate an enhancer that strongly activates the *FOLH1* core promoter region. The enhancer (PSME) is located in the third intron about 12 kb downstream from the start site of transcription and is characterized by a 72-bp direct repeat within a 331-bp core region. The PSME activates transcription from its own and heterologous promoters in prostate cell lines; enhancement is greatest in the PSMA-expressing cell line LNCaP (>250-fold). The PSME shows essentially no activity in five nonprostate cell lines. PSME-enhanced expression is repressed in the presence of androgen, mimicking the repression of the endogenous *FOLH1* gene. The data demonstrate that both cell-type specificity and androgen regulation are intrinsic properties of the enhancer. These properties make the PSME an excellent candidate for regulation of gene expression in gene therapy approaches to prostate cancer. © 2001 Academic Press

INTRODUCTION

Prostate-specific membrane antigen (PSMA) is a 100-kDa glycoprotein that is an important biomarker of prostate epithelial cells (Israeli *et al.*, 1993; Fair *et al.*, 1997) and has more recently been found to be present in the neovasculature of a range of tumors (Liu *et al.*, 1997; Silver *et al.*, 1997; Chang *et al.*, 1999). PSMA was first identified as the antigen interacting with a prostate-specific monoclonal antibody, 7E11-C5.3, that had been raised against a membrane fraction of the prostate cancer cell line, LNCaP (Horoscewicz *et al.*, 1987). PSMA has been shown to possess two related enzymatic activities: it acts as a carboxypeptidase (folate hydrolase) on poly-γ-glutamated folates (Pinto *et al.*, 1996) and as a peptidase on the acidic neuropeptide *N*-acetylaspartyl glutamate (Carter *et al.*, 1996). This latter activity is consistent with the expression of PSMA or a related protein in the brain (Israeli *et al.*, 1994). The role of PSMA in prostate biology is not yet understood.

The cDNA and gene encoding PSMA (*FOLH1*, for folate hydrolase 1, see http://www.gene.ucl.ac.uk/nomenclature/) have been cloned, and their sequences and the exon/intron structure of the gene have been determined (O'Keefe *et al.*, 1998). The *FOLH1* gene is located at chromosome 11p11–p12, includes 19 exons, and stretches over approximately 60 kb. A homologous but not identical gene is located at chromosome 11q14 (O'Keefe *et al.*, 1998; Rinker-Schaeffer *et al.*, 1995; Leek *et al.*, 1995). The mRNA encoding PSMA is 2.65 kb long, and a variant, Psm′, that has spliced out part of the first exon has also been identified (Su *et al.*, 1995). The protein encoded by the Psm′ splice variant lacks the membrane anchor domain and has been shown to be cytoplasmically located (Troyer *et al.*, 1995).

The 7E11-C5.3 antibody that binds to an internal epitope of PSMA (Troyer *et al.*, 1995, 1997) and others that bind to external epitopes (Liu *et al.*, 1997; Murphy *et al.*, 1996) have been used in immunohistochemical studies to identify sites of PSMA expression. In addition to its major site of expression in prostate epithelium, expression has been seen in the duodenum brush border/small intestine, in a subset of proximal tubules in the kidney, and in rare cells in the colon (Silver *et al.*, 1997; Wright *et al.*, 1995; Lopes *et al.*, 1990). All

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession No. AF007544.

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other normal tissues studied have been negative for expression, except for striated muscle, which stains with the 7E11-C5 antibody, but not with antibodies to the external domain of PSMA (Liu *et al.,* 1997). RNA expression has been found largely to parallel the protein expression data. RNase protection analysis identified PSMA mRNA in the prostate, salivary gland, and brain and sometimes in the small intestine (Israeli *et al.,* 1994). More sensitive assays using RT-PCR have allowed detection of PSMA mRNA in a number of other tissues but the levels have not been quantified (Renneberg *et al.,* 1999). The identification of PSMA RNA in the brain is consistent with the cloning of a closely related cDNA from rat brain (Carter *et al.,* 1996). More recently, both 7E11-C5 and external domain antibodies have been shown to react with the vasculature of a wide range of tumor types (Liu *et al.,* 1997; Silver *et al.,* 1997; Chang *et al.,* 1999). PSMA expression has not been identified in any normal vasculature, indicating a specific induction of expression in tumor neovasculature.

Unlike other prostate-specific proteins that have been studied (PSA, prostatic steroid binding proteins, prostatic acid phosphatase, and probasin), PSMA expression is not induced by androgens. Indeed, it has been shown to be down-regulated in the presence of androgens in cell culture, and expression is generally elevated in late-stage prostate cancer and in patients undergoing androgen deprivation or ablation therapies (Israeli *et al.,* 1994; Wright *et al.,* 1996). Among prostate-expressed genes, the regulatory regions of the PSA and rat probasin genes are the best studied. The proximal promoter of the probasin gene is able to direct prostate-specific expression both in cell culture and in transgenic animals (Kasper *et al.,* 1994; Greenberg *et al.,* 1994; Brookes *et al.,* 1998), while a longer, 11-kb region upstream of the gene provides for much stronger prostate-specific expression (Yan *et al.,* 1997). Expression directed by the core probasin promoter is strongly dependent on androgens (Rennie *et al.,* 1993). The core promoter region of the PSA gene is also strongly androgen dependent (Reigman *et al.,* 1991) but shows little specificity of expression (Brookes *et al.,* 1998; Schaffner *et al.,* 1995; Schuur *et al.,* 1996); a variant promoter isolated from prostate cancer tissue has been shown to be specifically active in prostate cells (Pang *et al.,* 1995). Strong prostate specificity that is also androgen-dependent is conferred by an enhancer (prostate-specific enhancer, PSE) that is located about 4 kb upstream of the PSA transcription start site (Schuur *et al.,* 1996; Cleutjens *et al.,* 1997).

We have previously reported that the proximal 1-kb promoter of the *FOLH1* gene can direct reporter gene expression in prostate cells (O'Keefe *et al.,* 1998) but this has proved to be low relative to strong constitutive promoters such as those of Rous sarcoma virus (RSV) and human cytomegalovirus (CMV). Here we report the identification and characterization of a strong prostate-specific enhancer in the third intron of the *FOLH1* gene. The enhancer appears to be the major regulatory element for prostate-specific expression of the *FOLH1* gene and should prove to be particularly useful for targeting gene expression to prostate cells.

MATERIALS AND METHODS

DNAs

Enhancer trap vector. pPSMentrap (Fig. 1) contains the plasmid backbone including the ampicillin resistance gene and origin of replication from the pCI vector (Promega, Madison, WI). It was made by first replacing the SV40 polyadenylation signal with that of the bovine growth hormone gene (bGH, construct kindly provided by P. Porronik). The green fluorescent protein gene (GFP) was inserted between the intron sequence and the bGH polyadenylation region, and the human CMV immediate-early enhancer/promoter was replaced by the *FOLH1* promoter region stretching from base 1386 to base 2560 (*Xba*I site) of the *FOLH1* sequence (GenBank Accession No. AF007544). Finally, a polylinker containing restriction sites for the enzymes *Kpn*I, *Hin*dIII, *Sal*I, *Mfe*I, *Nsi*I, *Bgl*I, *Nhe*I, and *Spe*I was cloned immediately upstream of the PSM promoter. Further details are available from the authors.

pCAT3SAT plasmids. The pCAT3SAT vector contains a modified bacterial CAT reporter gene for determining promoter activity and a reference reporter gene, SAT, under the control of the RSV promoter to normalize CAT expression for transfection efficiency. It was prepared by cloning the serine acetyl transferase reporter gene from the pCATSAT plasmid (Brookes *et al.,* 1998) as a *Sal*I/*Bam*HI fragment into *Bam*HI-, *Sal*I-cut pCAT3 vector (Promega). Constructs pPSM1k-C3S and pEn4PSM1k-C3S, containing the *FOLH1* promoter with or without the *FOLH1* enhancer fragment 4 (En4), were prepared by cloning the *FOLH1* enhancer/promoter fragments as *Sal*I/*Pst*I fragments from the pPSMentrap vector into pCAT3SAT cut at the *Xho*I and *Pst*I sites in the polylinker upstream of the CAT gene (Fig. 2). A control construct containing the RSV promoter, pRSV-C3S, was also prepared by blunt end ligation of a *Nae*I to *Sac*I fragment from pCATSAT (Brookes *et al.,* 1998) into the *Nhe*I site of pCAT3SAT (Fig. 2).

pGL3 plasmids. Because of the low level of activity of the *FOLH1* 1-kb promoter in the CAT assay system, promoter and enhancer sequences were cloned into the pGL3 vector (Promega), which contains the luciferase reporter gene. The structure of the clones is shown in Fig. 2. pPSM1k-GL3 and pEn4PSM1k-GL3 were prepared by cloning *Kpn*I to *Xba*I fragments from pPSM1k-C3S and pEn4PSM1k-C3S, respectively, into pGL3 cut with *Kpn*I and *Nhe*I. pEn3PSM1k-GL3 was prepared by cloning the *Kpn*I to *Nhe*I enhancer fragment of pEn3PSMentrap into pEn4PSM1k-GL3 cut with *Kpn*I and *Nhe*I. Details of the construction of plasmids pEn3+4PSM1k-GL3 (where En3 + 4 refers to the entire region spanned by enhancer clones 3 and 4) (Fig. 2), pEn3/4aPSM1k-GL3, and pEn3/4bPSM1k-GL3 (where En3/4 refers to the region common to both enhancer clones 3 and 4, and where a and b represent opposite orientations of En3/4) (Fig. 2) can be obtained from the authors. A series of deletion mutants of En3/4, the region overlapped by enhancer clones 3 and 4, was prepared to determine the minimum region required for transcriptional activation of the *FOLH1* 1-kb core promoter (Fig. 3). The deletion series of clones pEnb770PSM1k-GL3, pEnb614PSM1k-GL3, pEnb592PSM1k-GL3, pEnb445PSM1k-GL3, pEnb331PSM1k-GL3, and pEnb168PSM1k-GL3 was initially constructed using En3/4b in pBluescript. Existing restriction sites were used to delete sequences from one end of En3/4b, generating a series of clones with progressively shortened inserts. The shortened inserts were then subcloned in front of the *FOLH1* 1-kb core promoter. The deletion clones pEna430PSM1k-GL3, pEna722PSM1k-GL3, and pEna886PSM1k-GL3 were similarly constructed except that En3/4a was used instead of En3/4b to delete sequences from the opposite end of the insert.

Constructs with heterologous promoters. For the PSA and probasin constructs, the enhancer region, En4, was cloned as an *Nhe*I fragment from the pEn4PSM1k-C3S plasmid into the *Xba*I-cut plasmids pPSA630 CATSAT and pPb430 CATSAT, respectively (by partial digestion with *Xba*I for the probasin construct). pPSA630CATSAT and pPb430CATSAT have been described previously (Brookes *et al.,* 1998). The plasmid pTKCATSAT.1 was prepared by cloning the TK promoter region, bases −101 to +59, as a *Sal*I to *Xho*I fragment into the *Sal*I-cut vector pCATSAT.1 (Brookes *et al.,* 1998). pEn4TKCATSAT was prepared by cloning the *Sal*I to *Bgl*II enhancer-containing fragment from pEn4PSMentrap into pTKCATSAT.1 cut with *Sal*I and partially cut with *Bam*HI.

DNA Preparations

DNA was isolated by the alkaline lysis method followed by either CsCl/ethidium bromide gradient centrifugation or purification using BRESApure columns (Bresatec, Adelaide, South Australia, Australia), according to the manufacturer's protocols. For pGL3 constructs, master mixes of each plasmid and the reference plasmid, pRSVCAT, were prepared. DNA concentrations were determined by image analysis of ethidium bromide-stained gels and master mixes prepared in the ratio of 1.5 μg of pGL3 construct to 1 μg of pRSVCAT. The same master mixes were used for transfections into all cell lines.

Cell Lines and Transfections

The cell lines used and their conditions for growth have been described previously (Brookes *et al.,* 1998). Here 3.5-cm dishes were transfected with 2.5 μg of either individual plasmids for the pCATSAT and pCAT3SAT clones or plasmid mixes for the pGL3 clones. Transfection reagents for each cell line were as described previously (Brookes *et al.,* 1998) except that transfection of MRC-5 cells was achieved using Superfect (Qiagen). Transfections and subsequent incubations were performed using serum that had been charcoal-stripped to remove androgens, except in the case of LAPC-4, for which normal serum was used. Where indicated, the synthetic androgen analogue R1881 (Dupont, Boston, MA) was added back to the medium during the transfection and subsequent culture of cells. Two days following transfection, cells were harvested and extracts were prepared for reporter gene assays. For luciferase constructs, cell lysates were prepared, and luciferase activity was measured using the Luciferase Assay System (Promega). An aliquot of the lysate was taken for determination of CAT activity. Luciferase expression levels were normalized with respect to the pRSVCAT internal control. For pCATSAT and pCAT3SAT plasmids, cells were harvested and assays for CAT and SAT activity were performed as described previously (Brookes *et al.,* 1998). CAT activities were normalized with respect to SAT expression.

RESULTS

Analysis of the region upstream and encompassing the transcription start site of the *FOLH1* gene (O'Keefe *et al.,* 1998) has shown that a 1-kb region directs expression of reporter genes in the prostate cell line LNCaP. Within this a 600-bp region has been shown to act as a minimal promoter (Good *et al.,* 1999). Since expression from this proximal promoter was low, a strategy was developed to allow screening of cosmid clones encompassing the *FOLH1* gene for DNA fragments that could enhance transcription directed by its 1-kb proximal promoter.

The 1-kb promoter was cloned in front of the GFP gene in the plasmid vector pPSMentrap shown in Fig. 1. The polylinker in the vector includes a range of sites for cloning complete or partial restriction enzyme digestion products upstream of the promoter. The vector

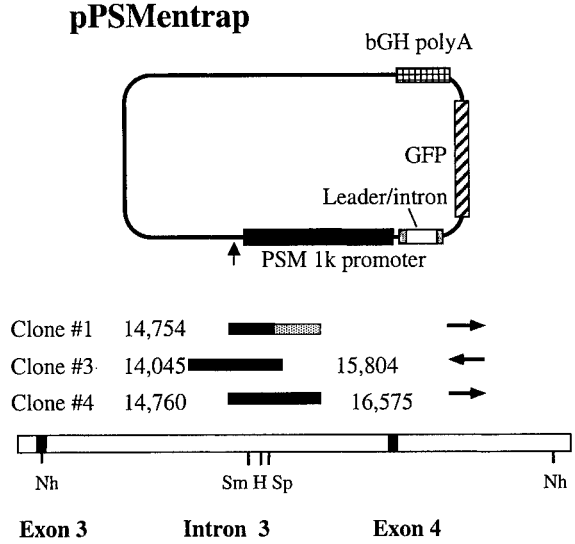

FIG. 1. pPSMentrap vector and cloned enhancer fragments. Key features of the vector are shown in the upper part of the figure. The vertical arrow indicates the location of the polylinker containing *Kpn*I, *Hin*dIII, *Sal*I, *Mfe*I, *Nsi*I, *Bgl*I, *Nhe*I, and *Spe*I restriction sites. Fragments of the PSM gene were cloned into the *Mfe*I site upstream of the PSM promoter (indicated by the vertical arrow). In the lower part of the figure is shown the location of the cloned enhancer fragments within intron 3 of the *FOLH1* gene. Base numbers (GenBank Accession No. AF007544) are indicated for the ends of the cloned segments; intron 3 spans bases 10,812 to 18,274. The locations of the restriction sites *Sma*I (Sm), *Hin*dIII (H), and *Spe*I (Sp) within the intron and for two *Nhe*I (Nh) sites are shown. The arrows indicate the orientation of the cloned sequences within the pPSMentrap vector. The right-hand end of enhancer clone 1 is shown as a stippled box since this end of the clone has undergone rearrangement. The *Sma*I, *Hin*dIII, and *Spe*I sites are present in all three cloned regions.

alone gives only very weak fluorescence following transfection into LNCaP cells, in comparison with a control plasmid in which the GFP gene is under the control of the human CMV enhancer/promoter. DNA of the bacteriophage P1 cosmid P1-683, which contains the 5′ half and upstream flanking sequence of the *FOLH1* gene (O'Keefe *et al.,* 1998), was digested for various times with the enzyme *Tsp*509I, which cuts at AATT sites, generating a range of partial digestion products. These were separated by agarose gel electrophoresis and fragments in the size range 1 to 2 kb were recovered and cloned into the *Mfe*I site of the pPSMentrap vector. A library of about 600 individual clones was assessed by transfection into LNCaP cells and fluorescence microscopy. After three rounds of screening matrices of pooled clones, three clones (1, 3, and 4) showing cells with the strongest GFP expression relative to the *FOLH1* 1-kb promoter alone were isolated and further characterized.

Location and Sequence Analysis of Enhancing Fragments

Sequence analysis allowed identification of the ends of the cloned segments. All three clones were found to originate from the third intron of the *FOLH1* gene 12

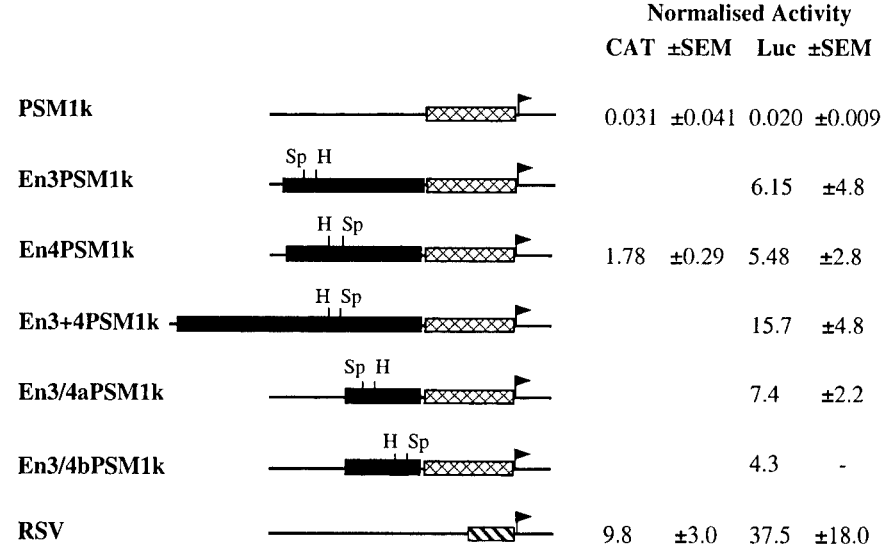

FIG. 2. PSM enhancer function in LNCaP cells. Maps to the left show the positions of the PSM1k promoter (▩), PSM enhancer sequences (■), and the RSV (▨) promoter directing expression of either the CAT or luciferase reporter genes in the pCAT3SAT and pGL3 vectors, respectively. To the right of the promoters and transcription start sites (arrows) is the leader sequence and chimeric intron followed by the CAT or luciferase reporter gene. Restriction enzyme sites for *Hin*dIII (H) and *Spe*I (Sp) in the enhancer indicate its orientation. Expression levels determined following transfection into LNCaP cells are normalized using the cotransfected reporter genes (see Materials and Methods) and expressed in arbitrary units. Results were determined from one to four experiments in which each construct was assayed in duplicate; standard errors are indicated.

to 14 kb downstream of the transcription start sites as shown in Fig. 1. The inserts in clones 3 and 4 were aligned in opposite orientations relative to the *FOLH1* promoter in the pPSMentrap vector. The clones share a common overlapping sequence of 1044 bp and extend in total over 2530 bp. Sequence analysis and restriction mapping data verify that they correspond to the parent cosmid sequence. The third clone, No. 1, was derived from the same region and also contained the *Sma*I, *Hin*dIII, and *Spe*I sites common to clones 3 and 4. Its 1.8-kb insert comprises two smaller fragments containing sequences at the 5′ end of clone 4, one with an end identical to that of clone 4 and the other with an end 6 bp upstream of the end of clone 4. As the sequences in clone 1 are fully contained within those of clones 3 and 4, it has not been further studied.

Function of PSMA Enhancer Region

To provide for quantitative determination of promoter and enhancer function, enhancers 3 and 4 (hereafter designated En3 and En4) in combination with the *FOLH1* 1-kb promoter (PSM1k) were recloned into the pGL3 vector that carries the luciferase reporter gene or pCAT3SAT that carries the chloramphenicol acetyl transferase (CAT) reporter gene and the transfection control reporter gene, serine acetyl transferase (SAT) under the control of the RSV promoter. The orientation of the enhancer fragments (Fig. 2) is indicated by the relative position of the *Hin*dIII and *Spe*I sites; En4 is in the same orientation as the enhancer in its natural position in the third intron of the *FOLH1* gene. Following transfection of plasmids into a number of cell lines, reporter gene expression was measured at 48 h (Figs. 2 and 3). Expression levels were normalized by dividing by the activity of the cotransfected internal control plasmid pRSV-CAT or by SAT activity as appropriate. Since maximal expression of PSMA is seen in the absence of androgens, during and after transfection cells were maintained in medium containing serum that had been charcoal-stripped to remove androgens, except for the androgen-dependent LAPC-4 cells.

In LNCaP cells (Fig. 2), an enhancement of expression of approximately 60-fold relative to the PSM1k promoter was seen when the En3 or En4 fragment was placed adjacent to the PSM1k promoter in the pCAT3SAT vector. Data obtained using the more sensitive luciferase reporter gene showed similar high levels of enhancement of expression from the PSM1k promoter (300- and 270-fold) by En3 and En4 with the absolute level of expression reaching 15 to 16% of that of the RSV promoter. Expression directed by the full 2.5-kb region encompassing En3 and En4 (pEn3+4PSM1k-GL3) was about 2-fold higher than that of either enhancer alone, while activity of the overlapping 1-kb region of the two (pEn3/4PSM1k-GL3) was similar to that of either En3 or En4. The enhancing activity of an 11-kb *Nhe*I fragment encompassing all of intron 3 and extending into intron 4 was also assessed and found to be about half that of the En3 + 4 region (data not shown), indicating that the cloned region included the full enhancer. The oppositely oriented En3 and En4, as well as both orientations of the overlapping region of the two, En3/4, strongly enhanced expression.

Mapping the Core Enhancer Region in En3/4

The 1044-bp overlapping region between En3 and En4, En3/4, was shown to retain a level of transcrip-

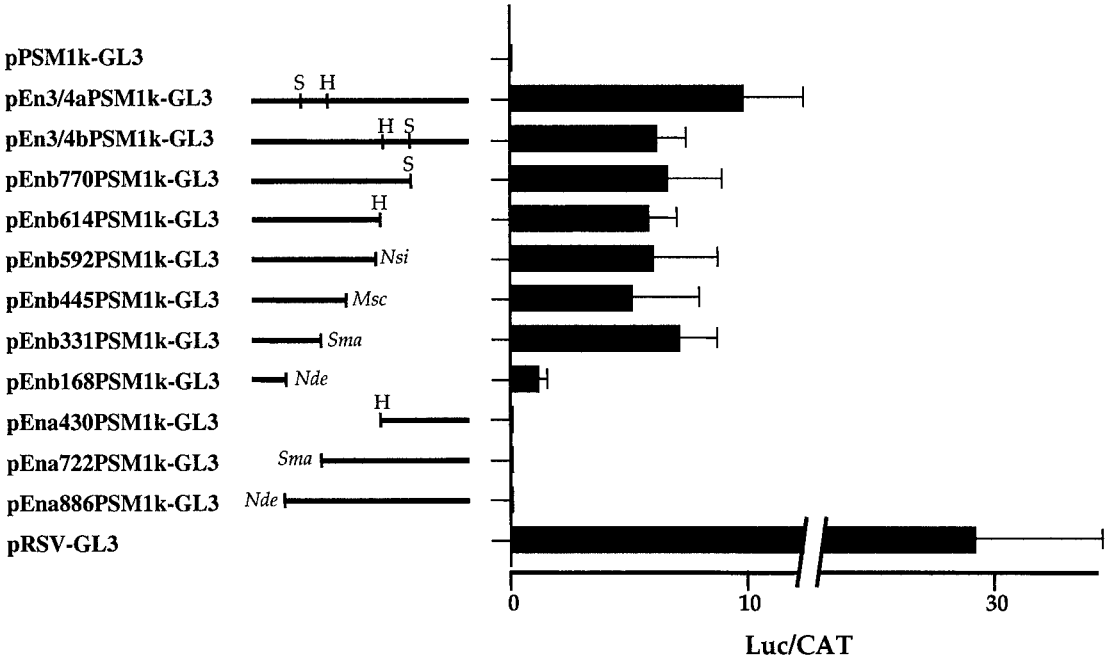

FIG. 3. Assessment of enhancer deletion mutants in LNCaP cells. A series of deletion mutants of En3/4 was placed immediately in front of the PSM1k promoter in pGL3 to determine the minimum region required for transcriptional enhancement. Luciferase pGL3 reporter constructs were cotransfected into LNCaP cells with an RSV–CAT control plasmid, and reporter gene activities were measured 48 h later. Data are expressed as luciferase activity normalized with respect to the cotransfected RSV–CAT gene expression. S, *Spe*I; H, *Hin*dIII.

tional enhancement of the PSM1k promoter similar to that of En3 or En4 alone, in LNCaP cells. To define the smallest region in En3/4 that is sufficient for transcriptional activation of the PSM1k promoter, a series of deletion mutants of En3/4 was constructed, and their activity was measured following transfection into LNCaP cells (Fig. 3). Successive deletions from the right-hand end of En3/4 showed that most of the enhancer activity is contained within a 331-bp region extending from the left end of En4 to the *Sma*I restriction site (pEnb331PSM1k-GL3). Further reducing the size of this region by half to 168 bp (pEnb168PSM1k-GL3) is deleterious as enhancer activity is diminished to less than 20% of that of the 331-bp region. Deletion from the left end of the overlap of either the 331-bp fragment (pEna722PSM1k-GL3) or the 168-bp fragment (pEna886PSM1k-GL3) completely eliminated enhancer activity. The first half of the 331-bp region (pEnb168PSM1k-GL3) is essential, therefore, to effect transcriptional enhancement from the PSM1k promoter, and the second half, while not active on its own (pEna886PSM1k-GL3), confers a high level of transcriptional enhancement in conjunction with the first half (pEnb331PSM1k-GL3). The 331-bp fragment therefore comprises a core region that is essential to enhancer activity in LNCaP cells.

Enhancer Function in Different Cell Types

Activation of expression showed a high level of specificity for prostate cells (Fig. 4A). Greatest activation was seen in LNCaP cells that express PSMA (>250-fold), while a significantly lower level of activation, 8.5-fold, was seen in LAPC-4 cells (Klein *et al.*, 1997) that express low levels of PSMA. Similar low-level activation, 3.7- and 5.2-fold by En3 and En4, respectively, was seen in the PC-3 prostate cell line in which PSMA expression can be detected only by reverse transcription-PCR (O'Keefe *et al.*, 1998; and unpublished data). In another prostate cell line, DU145, in which no PSMA expression can be detected, no enhancer function, and indeed a level of suppression, was seen. The 331-bp core enhancer performed similarly to En3 and En4 in the cell lines in which it was tested. The same high level of activation was observed in LNCaP cells, and a low but significant level of activation was maintained by the shortened enhancer in PC3 cells. In addition, the suppression of expression seen in DU145 cells with En3 and En4 was also observed with the 331-bp core enhancer.

When tested in a number of nonprostate cell lines, no enhancer function was evident for either En3 or En4 (Fig. 4B). Lines tested included MCF-7, a breast cancer line, HepG2 hepatocarcinoma, BL13 bladder carcinoma, human embryonic kidney HEK293 cells, and MRC-5, a primary lung fibroblast line. For HEK293, MCF-7, and BL13 cells, a significant basal level of expression of the PSM1k promoter was observed but this was not increased by either En3 or En4; in fact, small decreases in expression were observed. No expression above background was seen in MRC-5 and HepG2 cells. Likewise, the full enhancer region (En3 + 4) that shows higher activity than En3 or En4 alone in LNCaP cells is completely inactive in MRC-5, BL13, MCF-7, and HEK293 cells (data not shown). Enhancer

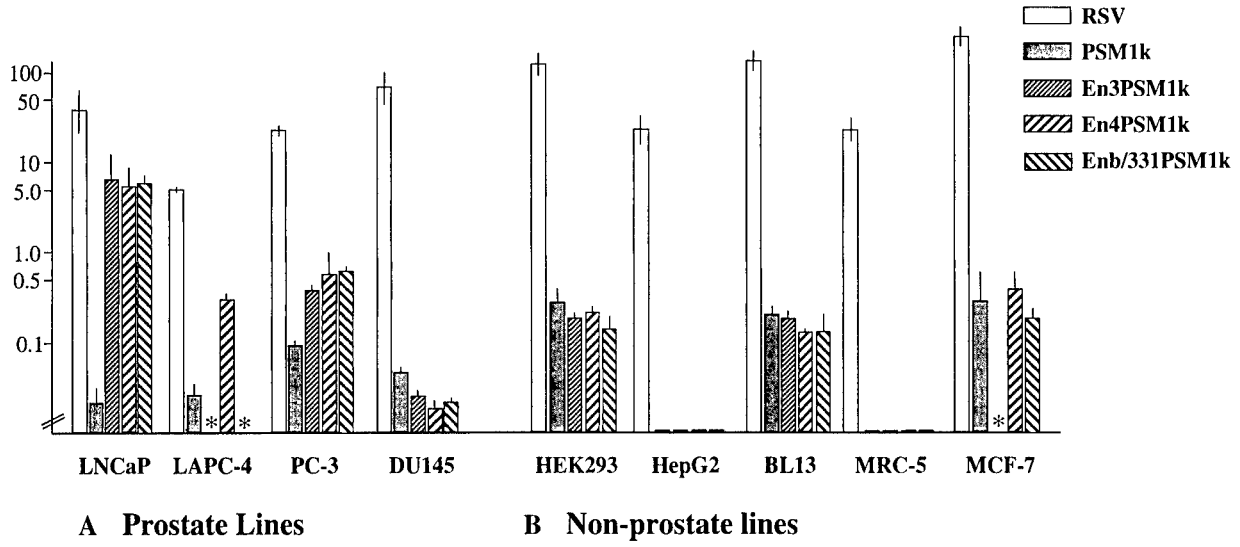

FIG. 4. Enhancer function in different cell types. Activities of different constructs containing the PSM1k promoter in the presence or in the absence of enhancer sequences were determined following transfection into the cell lines. Activities are normalized with respect to the cotransfected RSV–CAT gene expression. Expression in prostate and nonprostate cell lines is shown in **A** and **B,** respectively. Transfections of constructs were performed in duplicate in one to three experiments for each cell line; lines indicate standard errors. The asterisk indicates not done.

activity of the 331-bp region was tested in the five nonprostate cell lines. The profile of expression shown by the 331-bp enhancer was the same as that shown by En3 and En4 in these cell lines, demonstrating that cell-type specificity is maintained by the smallest region encompassing the core enhancer. The control RSV-GL3 plasmid was active in all cell lines.

PSMA Enhancer Activity on Heterologous Promoters

The properties of the enhancer (PSME) were further assessed by linking En4 sequences to other promoters, both those from genes active primarily in prostate cells, PSA, and probasin and from a non-tissue-specific promoter, that of the herpesvirus thymidine kinase gene (TK). The different PSME/promoter combinations directed expression of the CAT reporter gene contained in a plasmid, pCATSAT, that also contains a second reporter gene (SAT) under the control of the RSV promoter as an internal standard. pCATSAT plasmids containing each promoter alone or in combination with En4 were transfected into a number of cell lines, and reporter gene expression was assayed. After transfection, cells were maintained in androgen-depleted medium prior to cell harvesting (hence the low-level expression from the probasin promoter in LNCaP cells). Normalized expression levels are presented in Table 1.

In LNCaP cells, strong enhancement of the PSA, probasin, and TK promoters was seen (Table 1). The ratios of expression in the presence and in the absence of the enhancer were of the same order (56- to 250-fold) as seen with the homologous PSM1k promoter (Fig. 2), but expression levels were at least 10-fold higher, reflecting the greater basal activity of these promoters in LNCaP cells. Expression from all three PSME/promoter combinations was significantly greater than that from the strong RSV promoter. In PC3 prostate cells, which express minimal levels of PSMA, a much lower level of enhancement was seen, being 5- to 16-fold for

TABLE 1

Activation of Heterologous Promoters by PSM En4 Sequences

	LNCap	PC-3	MRC-5	HEK293	HepG2	BL13
Probasin	1.1 ± 1.3	3.63 ± 0.82	0 —	1.6 ± 0.18	0.06 ± 0.06	0.62 ± 0.25
PSMEn4/Probasin	273 ± 120	28.6 ± 1.64	0.05 ± 0.07	14.9 ± 1.7	0.06 ± 0.03	0.92 ± 0.19
PSA	2.9 ± 1.0	0.85 ± 0.23	0.04 ± 0.02	0.43 ± 0.29	1.35 ± 0.81	0.61 ± 0.25
PSMEn4/PSA	163 ± 48	9.58 ± 2.47	0.09 ± 0.04	0.65 ± 0.32	1.1 ± 0.48	2.0 ± 1.0
TK	3.54 ± 0.7	20.0 ± 5.6	8.44 ± 1.2	26.5 ± 10.1	35.1 ± 11.1	97 ± 4.7
PSMEn4/TK	325 ± 57	88.8 ± 44.6	5.53 ± 1.5	16.1 ± 1.0	12.3 ± 7.5	53 ± 0.95
RSV	109 ± 10.1	274 ± 149	291 ± 49	263 ± 52	300 ± 101	241 ± 13

Note. The different promoter and enhancer combinations in the pCATSAT vector, as indicated in the left-most column, were transfected into cell lines as shown, and promoter activities were determined. CAT expression is normalized with respect to expression of the internal control SAT gene under the control of the RSV promoter. Results represent averages of at least two experiments; standard errors are shown.

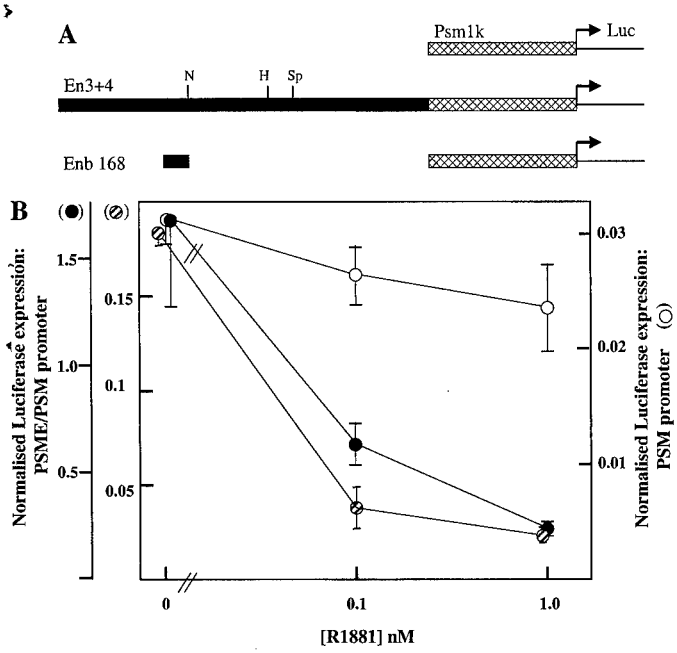

FIG. 5. Androgen repression of PSME activity. (**A**) The promoter/enhancer regions contained in the plasmids pPSM1k-GL3, pEn3+4PSM1k-GL3, and pEnb168PSM1k-GL3 are shown. Enhancer sequences encompass bases 14,045 to 16,575 (En3 + 4) or bases 14,760 to 14,928 (Enb168) of the *FOLH1* gene. Arrow and Luc indicate the direction of luciferase transcription. (**B**) The plasmids were cotransfected with pRSVCAT into LNCaP cells. Cells were maintained in medium with charcoal-stripped serum and the indicated levels of R1881 for 48 h prior to cell harvesting and reporter gene assays. Normalized expression is shown for all plasmids; separate scales for the promoter alone (right side; ○) and promoter plus PSME (En3+4 left side, outer, ●; Enb168, left side, inner, ⊘) allow for direct comparison of the effects of R1881.

the different promoters. This is similar to the results seen in PC-3 cells when the PSME was joined with its own *FOLH1* promoter.

For the nonprostate cell lines, HepG2 (liver), BL13 (bladder), HEK293 (embryonic kidney), and MRC-5 (lung fibroblasts), no or little enhancement of expression from any of the promoters was seen in almost all cases. However, enhancement of probasin promoter activity by nearly 10-fold was seen in embryonic kidney HEK293 cells. This finding was reproduced in two independent experiments. No enhancement of the PSA or TK promoters by En4 (Table 1) or of its homologous PSM1k promoter (Fig. 3) was seen in HEK293 cells.

Effect of Androgen on PSME Activity

The responsiveness to androgen of the PSME coupled to the PSM1k promoter and of the PSM1k promoter alone was examined following transfection into LNCaP cells (Fig. 5). A modest reduction (about 20%) in normalized activity was seen for the promoter alone with increasing levels of the synthetic, nonmetabolizable androgen analogue, R1881. A much greater concentration-dependent reduction in normalized luciferase expression directed by constructs containing the full enhancer (pEn3+4PSM1k-GL3; Fig. 5) or its 331-bp core region (not shown) was seen. Further, a similar proportionate decrease in activity was seen when expression was directed by the partially active 168-bp left end of the PSME (pEnb168PSM1k-GL3; Fig. 5). In different experiments, we have found expression directed by the PSME to be 3.3- to 6.7-fold reduced in the presence of 0.1 or 1 nM R1881. The data indicated that the nucleotide sequences conferring transcriptional repression by androgen are located within the left 168 bp of the PSME core region.

The effect of androgen on expression when the PSME (En4) was linked to either the probasin or the TK promoter was also evaluated. In LNCaP (Fig. 6A) cells, a strong suppression of expression from the PSME/TK promoter (>10-fold) was seen in the presence of 0.3 nM R1881. Expression from the probasin promoter alone was strongly stimulated in response to androgen. However, in the presence of the PSME, androgen no longer activated expression, and there was actually a slight decrease.

Since the androgen receptor in LNCaP cells is mutated, we further examined androgen responsiveness in the presence of wildtype androgen receptor. The androgen receptor was cotransfected along with reporter plasmids into the PC-3 cell line, which does not express androgen receptor and in which the PSME is partially active (Fig. 6B). This lower level of up-regulation of the TK promoter by the PSME in PC-3 cells was completely lost in the presence of androgen, and, as in LNCaP cells, expression from the probasin pro-

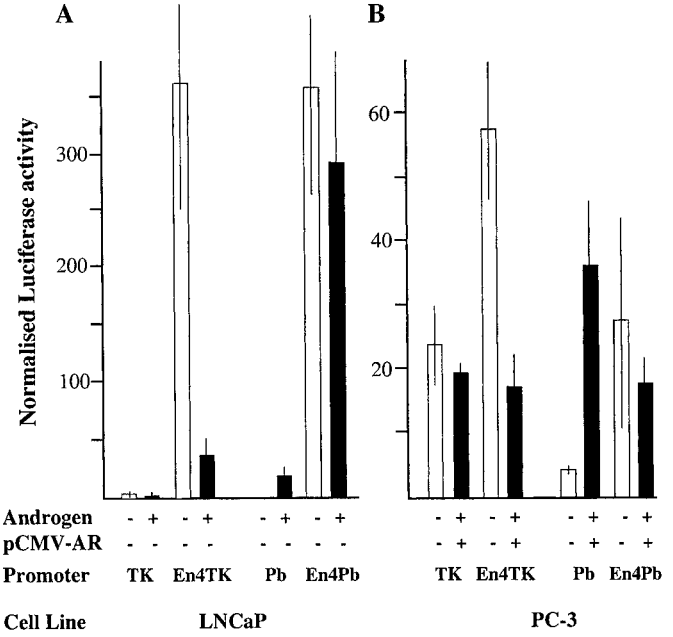

FIG. 6. Effect of androgen on enhancement of heterologous promoters. Reporter plasmids were transfected into either LNCaP or PC-3 cells, and expression of reporter genes was assayed after 48 h of incubation in androgen-depleted medium either in the absence (−) or in the presence (+) of 0.3 nM R1881. For PC-3 cells, a plasmid expressing the AR was also cotransfected. Normalized expression levels for LNCaP cells are shown on the left axis and for PC-3 cells on the right axis.

moter was no longer androgen induced when it is linked to the PSME.

DISCUSSION

Previous analysis of the region upstream and encompassing the transcription start site of the *FOLH1* gene (O'Keefe *et al.,* 1998) has shown that a 1-kb region directs expression of reporter genes in the prostate cell line LNCaP. This expression showed specificity for prostate cells when compared to that directed by the SV40 enhancer/promoter, with expression in LNCaP cells being about 75% of that directed by the SV40 enhancer/promoter. However, comparison with another widely expressed promoter, RSV, has indicated that the SV40 enhancer/promoter is only very weakly active in LNCaP cells (unpublished data). When compared with the RSV promoter/enhancer, the proximal promoter region shows little specificity (Fig. 4). Its level of activity is unrelated to PSMA expression levels in different prostate cell lines, and while the promoter is completely inactive in liver and lung fibroblast cells, it is active in three cell lines of epithelial origin (breast, bladder, and kidney). Good *et al.* (1999) have also reported that the promoter is active in two nonprostate lines that they tested. In addition to the proximal 1-kb promoter region and the 2-kb region studied by Good *et al.* (1999), we have cloned regions encompassing up to 11 kb of sequences 5′ to the *FOLH1* transcription start site and tested their ability to provide increased reporter gene expression; no increased activity was seen relative to the 1-kb promoter region (unpublished data).

To identify regions that might be important in the control of *FOLH1* gene expression, we have applied an enhancer trap approach that has allowed us to clone and identify an enhancer located within the third intron of the *FOLH1* gene about 13 kb downstream of the start sites of transcription. Use of the GFP reporter gene in the trap vector provides for rapid screening of clones and can be readily adapted for fluorescence-based cell sorting and plasmid recovery for larger libraries. The library of fragments screened was derived from a P1 cosmid that covered the first six exons of the *FOLH1* gene (about 25 kb) and approximately 30 kb of 5′ flanking sequences. That the three highest-expressing clones identified from the screen were all derived from the same region suggests that this region contains the major regulatory elements responsible for directing prostate expression of the *FOLH1* gene. The PSME increases basal expression of the *FOLH1* proximal promoter by more than 250-fold in LNCaP prostate cancer cells. The cloned region encompasses about 2.7 kb of the 7.5-kb intron 3, with a sequence of about 1 kb shared in common between En3 and En4 (En3/4a or En3/4b). The higher level of expression directed by the 2.5-kb region that includes all En3 and En4 sequences suggests, however, that interactions of regions outside the overlapping sequences contribute to maximal expression. Analysis of a DNA fragment encompassing all of intron 3 indicates that there are no further enhancing functions in this region. It remains possible that there may be other regions within or around the *FOLH1* gene contributing to its expression in the prostate or in other cell types.

The PSME is active in either orientation relative to the proximal promoter. En3 and En4 sequences were initially cloned and identified in the pPSMentrap vector in opposite orientations and subsequently assayed in these opposite orientations in the pGL3 vector. The core 1-kb region was also active in either orientation. O'Keefe *et al.* (2000) used the PSME to direct expression of the cytosine deaminase gene in an approach to gene therapy for prostate cancer. Here the enhancer was shown to also be active in both orientations when positioned at the 3′ end of the expression cassette rather than upstream of the promoter. In this case, as also evident in the data of Fig. 2, reverse-orientation constructs showed higher activity.

Within the 1-kb region of overlap between En3 and En4 sequences, a series of deletion constructs further defined a 331-bp core sequence that contained all its enhancer activity. The sequence and features of the PSME 331-bp core are shown in Fig. 7. Overlapping the *Sma*I site that marks the right end of the enhancer is an *Alu* repeat element. About 90 bp consisting of the 3′ end of the *Alu* repeat and remnant poly(A) tail lie within the 331-bp enhancer core. Positioned centrally within the fragment is a 72-bp direct repeat that overlaps tail to head by 10 nucleotides, with the sequence of the two repeats differing at 9 positions (Fig. 7). Located 15 nucleotides from the start of the second repeat is the *Nde*I restriction site used to generate the 168-bp deletion mutant (pEnb168PSM1k-GL3). This 168-bp left end of the enhancer core shows partial but significant activity by itself, and its deletion from the 1-kb enhancer region completely eliminates activity. This essential enhancer region contains the full first 72-bp repeat.

A number of potential binding sites for known transcription factors were identified using MatInspector (www.gsf.de/BIODV/matinspector.html) and Signalscan (bimas.dcrt.nih.gov/molbio/signal/) software and databases. Only sites that showed essential identity to consensus sequences or corresponded to well-established binding sites are shown in Fig. 7. Within the 72-bp repeat sequences are a number of potential binding sites for members of the SOX/SRY gene family. Proteins of these families are involved in a number of developmental pathways, including testis determination (Wegner, 1999), and SRY transcripts have been reported to be present in prostate cells (Tricoli *et al.,* 1993). They are thus candidates for a role in developmental control of *FOLH1* gene expression. Good candidate sequences for the binding of ATF/CREB and CEBP are found within the first repeat and for GATA-1/3 and CEBP within the second repeat. Outside the repeat region, binding sites for AP1, AP3, a further

FIG. 7. Sequence analysis of the 331-bp enhancer core. The PSME core extends from the left end of En4 331 bp to the *Sma*I restriction site and is characterized by a 72-bp direct repeat. The individual repeats are highlighted by overlapping gray boxes, and the nine nucleotide differences between the first and the second repeats are indicated in boldface type. Fifteen nucleotides into the second repeat is the *Nde*I site used to separate the two repeat units. Underlined are putative transcription factor binding sites identified by the MatInspector and Signalscan software packages with matrix similarity scores of at least 0.9. The arrowed line shows the position of the partial *Alu* repeat that continues beyond the *Sma*I restriction site of En4. *rpt 1,* repeat 1; *rpt 2,* repeat 2.

potential SRY site, and a consensus androgen receptor element (ARE) half-site were identified.

Cell Type Specificity of the PSME

Among the prostate cancer cell lines that we have examined, LNCaP cells are the only ones that maintain good expression of the endogenous *FOLH1* gene, and they support the highest level of PSME activity (from En3, En4, or 331-bp core sequences). LAPC-4 cells that express low levels of PSMA (unpublished data) show an intermediate level of enhancer activity (about 15-fold induction of the *FOLH1* promoter). PC-3 cells express minimal levels of PSMA mRNA and the PSME also showed an intermediate capacity to enhance expression in PC-3 cells, between about 4- and 17-fold in different promoter combinations. DU145 cells show no detectable endogenous PSMA mRNA, and the PSME is completely inactive. Thus the enhancer activity reflects the pattern of expression of the endogenous *FOLH1* gene. It appears that LAPC-4 and PC-3 cells contain some factors that can interact with the PSME to activate transcription, but lack others, or do not have sufficient levels, to enable full enhancer function as is seen in LNCaP cells. DU145 cells must lack a factor or factors essential for enhancer function. The different prostate cell lines should prove useful in dissecting sequence elements contributing to enhancer function and for identifying proteins that interact with them.

Activity of the PSME is highly specific for prostate cells, and no activation of basal promoter activity was seen in any nonprostate cell line using En3, En4, or 331-bp core sequences. When coupled to other promoters, the PSME (En4 sequences) retains its activity and its high specificity for prostate cells. The herpes virus TK promoter is expressed in a range of cell types, though in many cases not strongly. The PSME highly up-regulates the TK promoter in LNCaP cells while it has no enhancing effect and actually decreases expression in the four nonprostate cell types assayed. As is common with other enhancers, the PSME may harbor repressor elements that act in nonexpressing cell types. It is noteworthy that the PSME strongly enhances expression from the promoters of two other prostate-specific genes, PSA and probasin, again maintaining a high level of cell specificity. The PSME/Pb and PSME/PSA combinations are highly active in LNCaP cells, being about two- to threefold stronger than the highly active RSV promoter. The only significant activity of the PSME in nonprostate cells is its activation of the probasin promoter in HEK293 cells. The partial enhancement of the probasin promoter by the PSME in this embryonic kidney cell line may be biologically relevant as PSMA expression is seen in some kidney proximal tubule cells (Silver *et al.,* 1997). PSME function in HEK293 cells was not observed, however, with either the *FOLH1* promoter or the other two heterologous promoters, PSA and TK.

Androgen Repression

Previous data indicate that expression of the endogenous *FOLH1* gene is repressed by androgens (Israeli *et al.,* 1994; Wright *et al.,* 1996). As reported by Good *et al.* (1999), we found little effect of androgen on expression directed by the proximal 1-kb promoter region. However, expression directed by the PSME when linked to either the *FOLH1* or the TK promoter was found to be repressed in the presence of androgen. The PSME thus not only contains sequences responsible for prostate cell-specific enhancement of transcription but includes regulatory sequences that are responsible for repression of PSMA expression by androgens. Further, this property was conferred by the partially active 168-bp left-hand end of the PSME core region. In other systems, transcriptional repression by androgen has

been found to occur either through AREs similar to those involved in gene activation (Song *et al.,* 1998) or through indirect means that do not involve typical AREs (Cairns *et al.,* 1993). There is no full binding site for the androgen receptor (AR), within either the PSME or the *FOLH1* promoter region, such as are present in the PSA promoter and enhancer (Cleutjens *et al.,* 1996, 1997) and the probasin promoter (Rennie *et al.,* 1993). There is a good match to an ARE half-site (Fig. 7), but this is in the right half of the PSME core. At least in some cases, indirect repression (or suppression of androgen-mediated activation) still involves the AR (Kallio *et al.,* 1995) but appears to occur through competition with other regulatory proteins such as AP1 and CREB (Heckert *et al.,* 1997; Burgos-Trinidad *et al.,* 1997; Murtha *et al.,* 1997; Sato *et al.,* 1997), with CREB binding protein a likely key mediator (Aarnisalo *et al.,* 1998; Fronsdal *et al.,* 1998); such regulation may or may not require DNA binding by AR. Within the 168-bp region of the PSME shown to maintain androgen responsiveness, there are sequences with good matches to both AP1 and CREB sites (Fig. 7). We are currently attempting to define the role of these sites and to characterize the mechanism of responsiveness to androgen.

Comparison with Other Prostate Enhancers

Like the PSME, the previously described enhancer of the PSA gene (Schuur *et al.,* 1996; Cleutjens *et al.,* 1997) and that of the closely related kallikrein 2 gene (Yu *et al.,* 1999) are highly active in LNCaP cells and show no activity or repress basal promoter activity in a number of nonprostate cell lines. The PSE contrasts with the PSME in that it shows a very strong dependence on androgen for activity in LNCaP cells (Schuur *et al.,* 1996). The level of expression from the PSME/PSM1k promoter is similar to that from the PSE/PSA promoter in LNCaP cells, but when combined with either the probasin or the PSA promoters, the PSME gives much higher levels of expression. While the PSME shows partial activity, the PSE is not active in PC-3 cells (which display features of very advanced prostate cancer) nor is activity restored by cotransfection of an AR-expressing plasmid (Brookes *et al.,* 1998). This suggests that the factor(s) present in PC-3 cells that leads to partial activation of the PSME cannot similarly activate the PSE even in the presence of androgen and AR. Further dissection of both enhancer regions will be necessary to ascertain whether any of the factors responsible for the prostate-specific expression directed by PSE and PSME are the same.

Dissection of the regulatory elements of the PSME will help to separate the mechanisms responsible for prostate-specificity and developmental control of expression from those involved in androgen regulation. Tissue-specific gene regulatory sequences have important potential application in gene therapy approaches to disease treatment as they enable the expression of introduced genes to be limited to target cells, minimizing side effects due to expression at other sites. This is of particular importance in targeting of cell-killing systems in cancer treatment (O'Keefe *et al.,* 2000). The high level of promoter activation and cell-type specificity displayed by the PSME make it a very promising regulatory sequence for use with its own or heterologous promoters for targeting expression of therapeutic genes for the treatment of both androgen-dependent and -independent prostate cancer or other prostatic diseases.

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DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF

MCMR-RMI-S (70-1y) 15 May 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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