doi:10.1006/mthe.2002.0682, available online at http://www.idealibrary.com on IDEAL

ARTICLE

Novel Prostate-Specific Promoter Derived from PSA and PSMA Enhancers

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The expression of prostate-specific membrane antigen (PSMA) and prostate-specific antigen (PSA), two well characterized marker proteins, remains highly active in the hormone refractory stage of prostate cancer. In this study, an artificial chimeric enhancer (PSES) composed of two modified regulatory elements controlling the expression of PSA and PSMA genes was tested for its promoter activity and tissue specificity using the reporter system. As a result, this novel PSES promoter remained silent in PSA- and PSMA-negative prostate and non-prostate cancer cell lines, but mediated high levels of luciferase in PSA- and PSMA-expressing prostate cancer cell lines in the presence and absence of androgen. To determine whether PSES could be used for *in vivo* gene therapy of prostate cancer, a recombinant adenovirus, Ad-PSES-luc, was constructed. Luciferase activity in prostate cancer cell lines mediated by Ad-PSES-luc was 400- to 1000-fold higher than in several other non-prostate cell lines, suggesting the high tissue-specificity of the PSES promoter in an adenoviral vector. Finally, recombinant virus Ad-PSES-luc was injected into mice to evaluate the tissue-discriminatory promoter activity in an experimental animal. Unlike Ad-CMV-luc, the luciferase activity from systemic injection of Ad-PSES-luc was fairly low in all major organs. However, when injected into prostate, Ad-PSES-luc drove high luciferase activity almost exclusively in prostate and not in other tissues. Our results demonstrated the potential use of PSES for the treatment of androgen-independent prostate cancer patients.

Key Words: PSA, PSMA, prostate, gene therapy, adenovirus

INTRODUCTION

Prostate cancer is the second leading cause of cancer death in men annually [1]. Initially, prostate cancer development is androgen-dependent. However, it evolves to an androgen-independent stage by unknown molecular mechanisms and becomes refractory to hormone ablation therapy. Currently, there are no effective therapeutic agents available for patients with hormone-refractory prostate cancer.

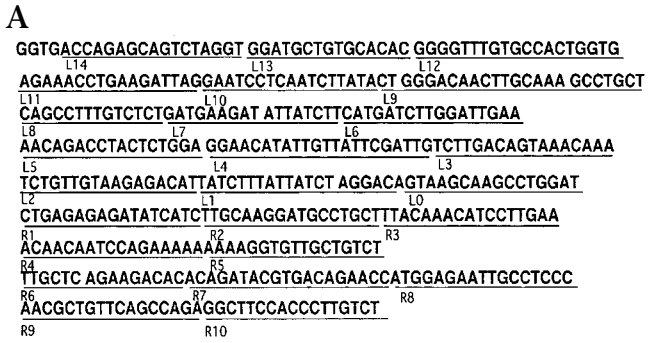
Among several prostate-specific proteins, prostate-specific antigen (PSA) and prostate-specific membrane antigen (PSMA) are sensitive markers for prostate cancer diagnosis and progression. PSA is a serine protease produced by the prostatic ductal epithelial cells and secreted into the seminal plasma. The basement membrane acts as a barrier to block PSA from escaping into systemic circulation. Prostate cancer or benign hyperplasia disrupts the membrane, eliciting significant increases in serum PSA. Therefore, serum PSA is used as a molecular marker for prostate abnormalities.

PSA expression is modulated by androgens, acting through androgen receptor (AR) like other nuclear receptor families [2]. Upon binding androgen, primed AR is translocated into the nucleus and binds the proximal promoter and enhancer core region (AREc) 4.2 kb upstream of the PSA gene [3–5]. The PSA regulatory element or its modification exhibits strong tissue-specific activity and has been used to drive therapeutic gene expression targeting prostate cancer [6,7]. The transcriptional levels mediated by PSA promoters are, however, significantly lower in the absence of androgen, which potentially limits its application in patients under androgen ablation therapy. Transcription levels must be improved for effective therapeutic results; a highly active promoter with stringent tissue-specificity is crucial for delivering foreign DNA to target tissue.

PSMA was discovered as a membrane antigen reactive to monoclonal antibody 7E11-C5 [8]. The physiological role of PSMA in prostate remains unknown. It has hydrolase

1525-0016/02 $35.00

doi:10.1006/mthe.2002.0682, available online at http://www.idealibrary.com on IDEAL



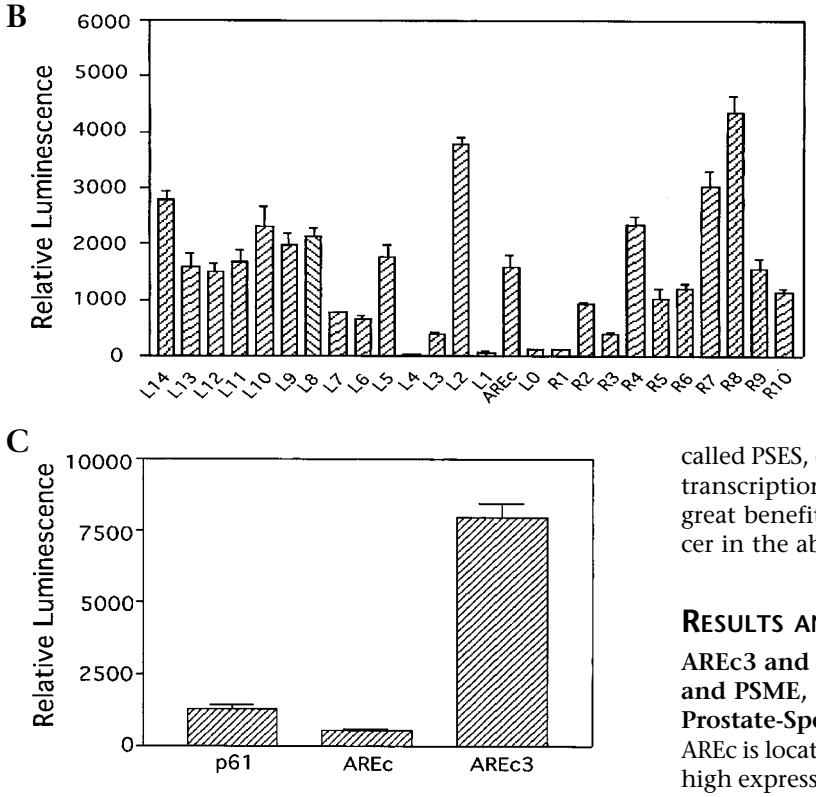


FIG. 1. Localization of a strong enhancer region within the AREc of the PSA promoter. (A) Sequence of the 440-bp AREc in the 4.2 kb upstream of PSA promoter. Linker-scanning mutations were introduced in the pGL3/AREc/TATA construct [16] by substituting 17 bp with a GAL4 binding sequence (underlined). Mutants were named L14 to R10. (B) LNCaP (2×10^5 cells) seeded in 12-well plates were transfected with 0.5 μg of each construct. We added 3 nM of androgen R1881 when the transfection medium was changed 5 hours after transfection. We cotransfected 1 ng of *Renilla* luciferase vector, pRL-SV40 (Promega), to monitor transfectional efficiency. Firefly and *Renilla* luciferase activities were determined using the Dual Luciferase Reporter System (Promega). Results are presented as mean ± SD of three transfections. (C) The region ranging from L7 to R3 was placed upstream of an artificial TATA box to make a new construct AREc3 (pGL3/AREc3/TATA). The transcriptional activity of pGL3/AREc3/TATA was compared with PSA promoter (pGL3/p61/TATA) and AREc (pGL3/AREc/TATA) constructs [16].

enzymatic activity with the substrates of folate and *N*-acetylaspartylglutamate (NAAG) [9,10]. Serum PSMA in prostate cancer is significantly higher than for benign prostate hyperplasia or normal prostate, suggesting enhanced expression of PSMA as prostate cancer progresses [11]. Furthermore, because the level of PSMA expression is downregulated by androgen, patients undergoing hormone ablation therapy exhibit increasing expression of serum PSMA [12].

PSMA expression is controlled by two characterized regulatory elements. The proximal 1.2-kb promoter upstream of the gene that encodes PSMA (*FOLH1*) drives reporter gene expression but with less tissue-specificity than the PSA promoter [13]. Recently, PSMA enhancer (PSME) was found to be located within the third intron of *FOLH1,* conferring the prostate-specific expression of PSMA [14]. Its tissue-specificity and upregulation in the absence of androgen enabled PSME to be used for expressing a toxic gene exclusively in the prostate in an androgen-depleted environment [15].

We investigated the hypothesis that factors that transactivate AREc and PSME could function together synergistically. Through deletion and linker scan mutagenesis, we located the minimal sequences, AREc3 and PSME (del2) in AREc and PSME, respectively, that retained high prostate-specific activity. A combination of AREc3 and PSME (del2), called PSES, demonstrated synergistic and prostate-specific transcriptional activity. This novel promoter could be of great benefit to gene therapy for metastatic prostate cancer in the absence of androgen.

RESULTS AND DISCUSSION

AREc3 and PSME (del2), Derived from AREc and PSME, Respectively, Exhibit Strong Prostate-Specific Activity

AREc is located upstream of the PSA promoter, allowing the high expression of PSA in a tissue-specific manner [3,4,16]. We constructed 25 linker scanning mutants within the AREc region to analyze important *cis*-elements (Fig. 1A). Several linker mutants (L1, L3, L4, L6, L7, R1, R2, and R3) showed a clear and significant decrease in transcriptional activity (Fig. 1B), indicating the existence of an activator element located between positions L7 and R3. To evaluate the promoter activity mediated by the fragment (AREc3) containing GATA sites within the L7 to R3 region, AREc3 was cloned in the luciferase reporter system and compared with AREc and the 5.8-kb PSA promoter p61 [16]. AREc3 activity was 5.7-fold higher than that of p61 and 24-fold higher than that of AREc in the presence of androgen (Fig. 1C). However, all constructs had a basal promoter activity in the absence of androgen (data not shown). AREc3 appears to have high enhancer activity due to deletion of potential silencers outside the AREc3 region. AREc3 has

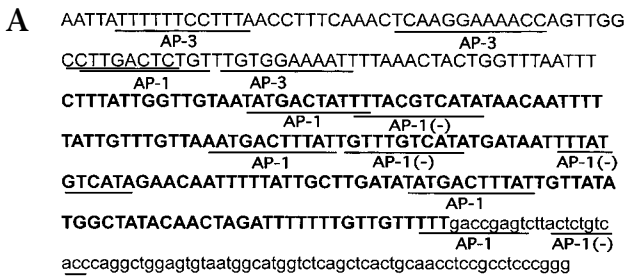
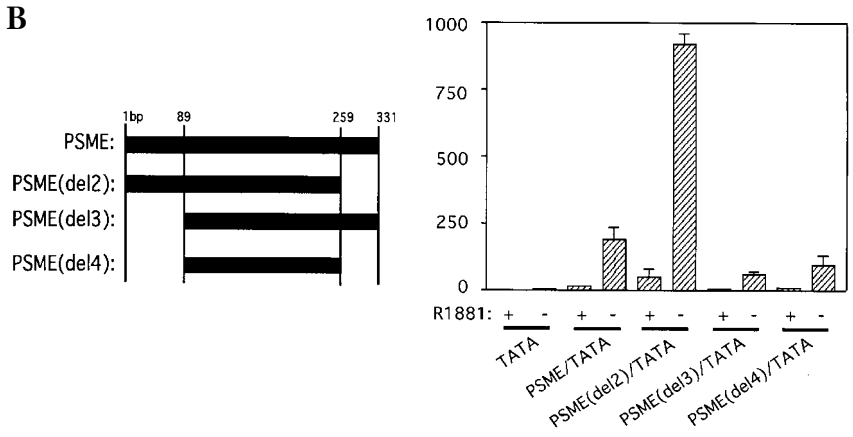


FIG. 2. Deletion analysis of PSME. (A) Sequence of PSME located in the third intron in the PSMA gene (*FOLH1*). PSME contains nine AP-1 sites, a repeat sequence (bold), and an *Alu* sequence (lower case). (B) Left panel schematically illustrates the deletions of PSME used in this study. The shortened versions of PSME were inserted upstream of TATA in pGL3/TATA. The transcriptional activities of these constructs were then evaluated by transient reporter assay as described in Fig. 1. Results are presented as mean ± SD.



three AR binding sites and six GATA transcription factor binding sites, suggesting that GATA is involved in the optimal androgen induction.

PSME, which is within the third intron of the PSMA-encoding gene, enables the prostate-specific gene expression of PSMA in the absence of androgen [14]. We constructed a series of deletions to further locate prostate-specific enhancers within PSME (Fig. 2A) and evaluated their enhancing activities. PSME (del2), which lost the *Alu*-repeat sequence (262 to 327 bp) located at the end of PSME, had four- to fivefold higher activity than PSME, suggesting that the *Alu* repeat contains a suppressive regulatory element (Fig. 2B). The *Alu* repeat belongs to the SINE (short interspersed element) family of human repetitive sequences [17]. Although the function of *Alu*-repeat sequences is not well understood, several studies have identified transcriptional silencers within *Alu*-repeat sequences and suggested that *Alu*-repeat sequences might be involved in transcriptional regulation [17]. This study provides additional data suggesting a role for *Alu*-repeat sequences in gene regulation.

In contrast to PSME (del2), PSME (del3), which lacks bp 1–90, had much lower promoter activity compared with the full-length PSME, indicating that this region upstream of direct repeat sequences (Fig. 2A) harbors one or more enhancer elements. According to sequence analysis, this upstream region of PSME, bp 1–90, includes an activator protein 1 (AP-1) binding site and three AP-3 binding sites (Fig. 2A). These potential regulators may be involved in the transcriptional activation of PSMA gene expression.

Mutant PSME (del4), which lacks both the 90-bp upstream enhancer and the *Alu* repeat silencer (Fig. 2B), had activity that was moderately higher than that of PSME (del3) but lower than that of PSME (del2), reflecting the overall level of promoter activity without silencing or enhancing regulatory elements in the regions of deletion of bp 1–90 and bp 262–327, respectively.

All PSME deletions shown in Fig. 2B showed downregulation in the presence of androgen, suggesting that the regulatory element responsible for androgen-mediated downregulation of PSME activity resides in the direct repeat sequence (Fig. 2A). Interestingly, AR was demonstrated to inhibit c-Jun/AP-1 site interaction by forming a complex with c-Jun without directly binding to the AP-1 site [18]. Because there are nine AP-1 sites within and upstream of the direct repeat sequence, AP-1s may act as positive regulators and their activities may be downregulated by androgen.

PSA promoter, which has been investigated for prostate cancer gene therapy [6,7], is a tissue-specific promoter whose activity heavily depends on androgens. We reported that PSA promoter had higher activity in androgen-independent C4-2 cells than in androgen-dependent LNCaP cells in the absence of androgen [16]. However, its activity in C4-2 cells in the absence of androgen is still much weaker than that of the commonly used simian virus 40 (SV40) or Rous sarcoma virus (RSV) promoters. This lower level of promoter activity potentially hampers its application in patients for androgen ablation therapy. On the other hand, the enhancer activity of PSME (del2) was higher in the absence of androgen but still significantly lower compared with constitutively active viral promoters. We believed that both AREc3 and PSME (del2) were likely to function weakly in patients under androgen ablation therapy due to the patients' low levels of androgen and to AR mutation or amplification, which resulted in partial activation of AREc3 and suppression of PSME (del2) activity (unpublished data). We hypothesized that the combination of AREc3 and PSME (del2) might have a synergistic enhancer effect, balance out the positive and negative regulatory effect of androgen, and retain tissue specificity.

doi:10.1006/mthe.2002.0682, available online at http://www.idealibrary.com on IDEAL

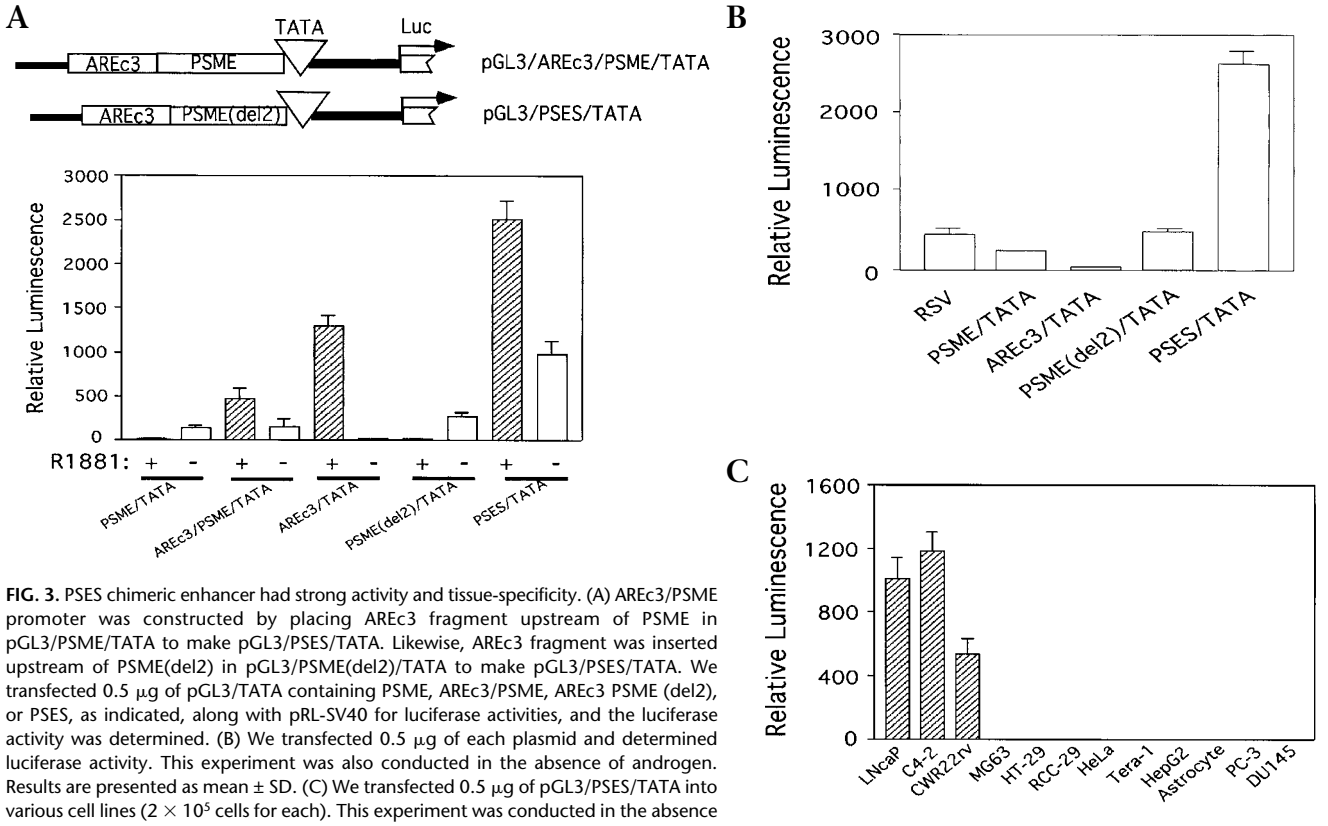


FIG. 3. PSES chimeric enhancer had strong activity and tissue-specificity. (A) AREc3/PSME promoter was constructed by placing AREc3 fragment upstream of PSME in pGL3/PSME/TATA to make pGL3/PSES/TATA. Likewise, AREc3 fragment was inserted upstream of PSME(del2) in pGL3/PSME(del2)/TATA to make pGL3/PSES/TATA. We transfected 0.5 μg of pGL3/TATA containing PSME, AREc3/PSME, AREc3 PSME (del2), or PSES, as indicated, along with pRL-SV40 for luciferase activities, and the luciferase activity was determined. (B) We transfected 0.5 μg of each plasmid and determined luciferase activity. This experiment was also conducted in the absence of androgen. Results are presented as mean ± SD. (C) We transfected 0.5 μg of pGL3/PSES/TATA into various cell lines (2×10^5 cells for each). This experiment was conducted in the absence of androgen. The luciferase activity was determined by division by the basal activity represented by transfection of pGL3/TATA. Results are presented as mean ± SD.

PSES Synergistically Drives Gene Expression in a Prostate-Specific Manner

We constructed a chimeric promoter composed of AREc3 and PSME (del2), called prostate-specific enhancing sequence (PSES; Fig. 3A). Evaluation of its promoter activity is shown in Fig. 3B. The level of luciferase activity driven by PSES was approximately fivefold higher than RSV promoter-mediated luciferase activity. As expected, promoter activity of PSES was greater than the sum of the promoter activities of AREc3 and PSME (del2), suggesting that transcription regulators in AREc3 and PSME (del2) cooperated synergistically to drive a high level of gene expression. Yet, the addition of PSME to AREc3 elicited a negative effect on the promoter activity of AREc3. The *Alu* repeat appears to interfere with the AR-induced transcriptional enhancement of AREc3 by an unknown mechanism.

Although the potency of a promoter is important for gene-therapy purposes, tissue specificity is also critical for avoiding potential nonspecific side effects. The tissue specificity of PSES was tested in several different cell lines. PSES drove a high level of luciferase expression in the PSA- and PSMA-expressing prostate cancer cells LNCaP, C4-2, and CWR22rv (Fig. 3C). However, its activity in non-prostate cancer cells and the PSMA-negative prostate cancer cells PC-3 and DU145 was negligible, suggesting that PSES retained tissue specificity like PSA and PSME enhancers.

PSES Retained Tissue Specificity in an Adenoviral Vector

Adenoviral vectors have been extensively investigated as anti-tumor reagents, demonstrating real promise for prostate cancer gene-therapy [19]. To evaluate the potential application of PSES for gene-therapy, we constructed a recombinant adenovirus, Ad-PSES-luc, carrying the luciferase gene under the control of PSES (Fig. 4A). We tested whether PSES would exhibit prostate-specific activity in a recombinant adenovirus form. Ad-PSES-luc was prepared and used to infect several different cell types. PSES was more active in PSA- and PSMA-positive prostate cancer cells by 400- to 1000-fold compared with PSA- and PSMA-negative prostate cancer cells (for example, PC-3 and DU145) or with non-prostate cancer cells, demonstrating that PSES retained tissue specificity in a recombinant adenoviral vector (Fig. 4B).

To evaluate the tissue-discriminatory promoter activity of PSES in an experimental animal, BALB/c nude mice were injected with 2.0×10^9 virus particles of Ad-CMV-luc,

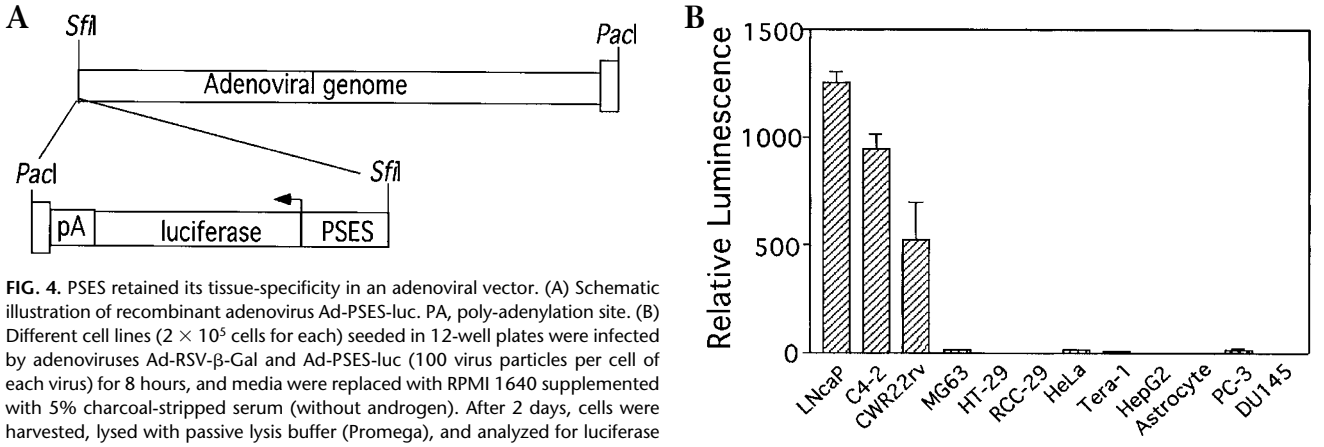


FIG. 4. PSES retained its tissue-specificity in an adenoviral vector. (A) Schematic illustration of recombinant adenovirus Ad-PSES-luc. PA, poly-adenylation site. (B) Different cell lines (2×10^5 cells for each) seeded in 12-well plates were infected by adenoviruses Ad-RSV-β-Gal and Ad-PSES-luc (100 virus particles per cell of each virus) for 8 hours, and media were replaced with RPMI 1640 supplemented with 5% charcoal-stripped serum (without androgen). After 2 days, cells were harvested, lysed with passive lysis buffer (Promega), and analyzed for luciferase and β-galactosidase activities. The luciferase activities are normalized with β-galactosidase activity. Results are presented as mean ± SD.

carrying the luciferase gene under the control of the cytomegalovirus (CMV) promoter, or Ad-PSES-luc. After 3 days, we measured the luciferase expression in different mouse organs. High levels of luciferase activity were detected predominantly in liver, spleen, and lung, but levels were negligible in other tissues obtained from mice injected with Ad-CMV-luc(Fig. 5A). Unlike Ad-CMV-luc, Ad-PSES-luc was inactive in all organs tested. However, when injected directly into prostate (Fig. 5B), Ad-PSES-luc was highly active in prostate. These data demonstrated that PSES in the form of Ad-PSES-luc was active in normal mouse prostate with high tissue specificity.

In this study, we developed a novel prostate-specific chimeric enhancer, PSES, from the enhancer cores of PSA promoter, AREc3, and PSME (del2). This PSES was highly active in PSA- and PSMA-positive prostate cancer cells in the presence and absence of androgens, exhibiting strong tissue-specificity as an adenoviral vector. Its strong androgen-independent promoter activity makes PSES superior to PSA and PSMA promoters for the treatment of patients undergoing androgen ablation therapy.

We are currently investigating the use of PSES to control the replication-competent oncolytic adenovirus for the treatment of androgen-independent prostate cancer. Although PSA enhancer and promoter were used to drive the prostate-specific replication of oncolytic adenoviruses [20], we believe that PSES-based replication-competent adenovirus would be more efficacious for the treatment of androgen-independent prostate cancer.

MATERIALS AND METHODS

Cells and cell culture. LNCaP, C4-2, CWR22rv, PC-3, and DU145 prostate cancer cell lines were maintained in T medium supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). C4-2 was purchased from Urocore (Oklahoma City, OK). CWR22rv was obtained from Liang Cheng (Indiana University). LNCaP was obtained from Leland Chung (Emory University). Human liver carcinoma HepG2, human cervix cancer cell line HeLa, human testicular cancer cell line Tera-1, human kidney cancer cell line RCC-29, human colon cancer cell line HT-29, human osteoblast-derived osteosarcoma cell line MG63, and human prostate cancer cell lines PC-3 and DU145 were purchased from American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FBS and 1% P/S.

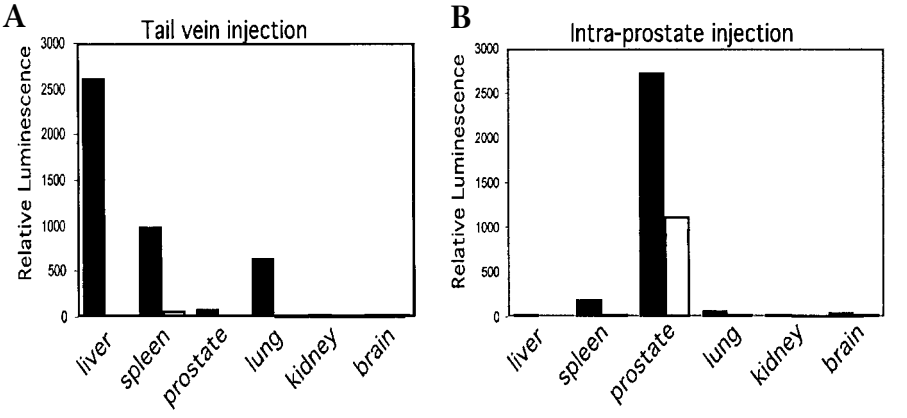


FIG. 5. PSES promoter in the form of Ad-PSES-luc remains specifically active in the prostate of *in vivo* mice. (A) We injected 7×10^{10} virus particles of recombinant virus Ad-CMV-luc (filled bar) or Ad-PSES-luc (open bar) into tail veins of male athymic mice. After 2 days, organs were harvested from sacrificed mice and homogenized. Protein extracts (0.48 mg) were used to determine luciferase activities. (B) We injected 1.4×10^{10} virus particles of recombinant virus Ad-CMV-luc (filled bar) or Ad-PSES-luc (open bar) into prostates of male athymic mice. Protein extracts were obtained and used as described for luciferase activities.

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Plasmid construction. Full-length PSME was obtained by PCR-amplification using a BAC clone from Genome Systems (St. Louis, MO) as a substrate and two primers, 5′-TTAGGCTAGCAATTATTTTTTCCTTTAACCTT-3′ and 5′-ATCCCCCGGGAGGCGGAGGTTGCAGTGAGC-3′. PCR-amplified fragments were digested with *Nhe*I and *Sma*I and ligated into the same sites of pGL3/TATA [16]. PSME deletions were generated by PCR technology. Forward primers for PCR amplification were 5′-TTAGGCTAGCAATTATTTTTTCCTTTAACCTT-3′ for PSME (del2), and 5′-TTAGGCTAGCCTTTATTGGTTGTAATATTGACT-3′ for PSME (del3) and PSME (del4). Backward primers were 5′-ATCCCCCGGGAGGCGGAGGTTGCAGTGAGC-3′ for PSME (del3), and 5′-ATCCCCCGGGAAAACAACAAAAAAATCTAG-3′ for PSME (del2) and PSME (del4). PCR-amplified fragments were digested with *Nhe*I and *Sma*I and ligated into the equivalent site of pGL3/TATA vector (Promega, Madison, WI). PSES was constructed by digesting PSME (del2) with *Nhe*I and *Sma*I and inserting the resultant DNA fragment into pGL3/AREc3/TATA digested with *Nhe*I and *Sma*I restriction enzymes.

PSA promoter derivatives were constructed. AREc DNA fragments were prepared by PCR-amplification of p61 [16] using two primers, 5′-GGTACCCCTAGGGGTGACCAGAGCAGTCTA-3′ and 5′-GCTAGCAGACAAGGGTGGAAGCCT-3′, subcloned into a TOPO cloning vector (Invitrogen, Carlsbad, CA). The resulting TOPO vector containing AREc was digested with *Kpn*I and *Nhe*I and ligated into pGL3/TATA cut with *Kpn*I and *Nhe*I. The pGL3/AREc3/TATA was prepared by digesting PCR-amplified fragments with *Kpn*I and *Nhe*I and inserting them into pGL3/TATA cut with the same enzymes. Amplification was performed using pGL3/AREc/TATA as a template and two primers, 5′-GGTACCCCTAGGAGATATTATCTTCATGATC-3′ and 5′-GCTAGCTTCAAGGATGTTTGTAAGC-3′. All constructs used in this study were confirmed by sequence analysis.

Linker scanning mutagenesis. For linker scanning analysis of AREc, 25 linker mutants, derived from pGL3/AREc/TATA, were generated as described [16]. These plasmids carry linker-scan mutants that were introduced in the pGL3/AREc/TATA by replacing 17 bp with GAL4-binding site (5′-CGGAGTACTGTCCTCCG-3′). The forward primers and reverse primers contain half of the GAL4-binding site, 5′-GTCCTCCG-3′ and 5′-AGTACTCCG-3′ at the 5′ end, followed by 20 bp of AREc adjacent to the mutagenized bases. PCR reactions were carried out with 100 pg of pGL3/AREc/TATA as a template. The PCR products were purified from a 0.8% agarose gel and ligated with a rapid ligation kit (Roche, Indianapolis, IN) following the manufacturer's protocol. Clones were screened by PCR for the presence of a GAL4-binding site and Glprimer2 from the pGL3-basic vector (Promega). If a mutant demonstrated a 20% higher or lower activity than AREc, it was subcloned back to pGL3/TATA to exclude the possibility that the effect was due to nonspecific PCR mutation.

Transient transfection for luciferase and β-galactosidase assays. For transient transfections, 1×10^5 cells/well were plated in 12-well plates for 24 hours. Plasmid DNAs were delivered into cells with DOTAP (Roche) following the manufacturer's protocol. Briefly, 0.5–1 μg of tested plasmid DNA was mixed with lipid at room temperature before addition to a well containing 1 ml of serum-free and phenol red-free RPMI 1640 medium. After 15 minutes, DNA-lipid complexes were added to the well and incubated for 5 hours at 5% CO_2 and 37°C. DNA-lipid containing medium was then replaced with 1 ml of serum-free and phenol red-free RPMI 1640 medium containing 5% charcoal stripped serum and 1% P/S, with or without 3 nM of androgen (R1881). After 2 days, cells were collected and lysed in 250 μl passive lysis buffer (Promega). Cell lysates were vortexed for a few seconds and spun for 3 minutes. For dual luciferase activity detection, 10 μl of the supernatant was mixed with 50 μl of luciferase substrate (Promega) and measured with a femtometer (Zylux, Germany). Subsequently, 50 μl of stop solution (Promega) was added to the tube, mixed, and measured with a luminometer for the *Renilla* luciferase activity. β-Galactosidase activity was determined with a femtometer by mixing 20 μl of the supernatant with 200 μl of substrate (Tropix, Bedford, MA) and incubating at 37°C for 30 minutes (Zylux, Germany). Data are expressed as relative luciferase activity, defined as luciferase activity normalized to internal control CMV/β-galactosidase activity for viral luciferase activity or to SV40/*Renilla* for dual luciferase activity. Relative luminescence is expressed as the mean ± standard error of the mean of at least three independent experiments.

Virus construction. Recombinant Ad-PSES-luc was constructed (Fig. 4A). Briefly, a *Kpn*I/*Bam*HI fragment containing a PSES-luc expression cassette from pGL3/PSES/TATA was subcloned into pAd1020sfidA (a gift from Xavier Denthinne, OD 260, Inc., Boise, ID) digested with the same restriction enzymes. pAd1020sfidA contains adenovirus left ITR and packaging signal (bp 1–358). After cloning the expression cassette into pAd1020sfidA, the left arm of the adenovirus with the expression cassette was cut out by digestion with *Sfi*I and *Pac*I and ligation to the *Sfi*I-*Pac*I adenovirus left arm called AdenoZapsfi.2 (OD 260) in the presence of *Pac*I. The ligation mixture was precipitated and transfected into 293 cells with lipofectamine (GIBCO, Rockville, MD) following the manufacturer's protocol. The cells were passed from 24-well to 6-well growth conditions and covered with 0.6% agarose 2 days after transfection. Several selected clones were obtained by at least three rounds of plaque isolation as described [21]. One clone was propagated in 293 cells, purified by $CsCl_2$ gradient centrifugation, and dialyzed against 10 mM Tris-HCl (pH 7.5)/1 mM $MgCl_2$ buffer supplemented with 10% glycerol. The viral titer was determined by measuring the optical density at 260 nm after lysing viral particles in 5% SDS. The control viruses Ad-RSV-β-Gal and Ad-CMV-luc were prepared as described [21].

In vivo animal experiments. BALB/c nude mice were injected through the tail vein with 7.5×10^{10} virus particles of Ad-CMV-luc or Ad-PSES-luc. For intra-prostatic injection, 7.5×10^9 virus particles of Ad-CMV-luc or Ad-PSES-luc were used. Animals were sacrificed and their major organs were collected. We homogenized organs in 1 ml of 1× passive lysis buffer (Promega) supplemented with protease inhibitor cocktails using a PowerGen 125 (Polytron Kinematica, Switzerland) 3 days after virus injection. We analyzed the resultant 50 μl of lysate for luciferase activity.

ACKNOWLEDGMENTS

We thank Xavier Danthinne (OD 260, Inc.) for the pAd1020sfidA vector and Gary Mawyer (Journal of Urology) for editorial comments. This work was supported by National Institutes of Health Grant CA74042, United States Army Prostate Cancer Research Program DAMD 17-98-1-8643, and Thomson Prostate Cancer Research Fund (to C.K.).

RECEIVED FOR PUBLICATION APRIL 24; ACCEPTED JULY 23, 2002.

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