Journal of Andrology, Vol. 26, No. 2, March/April 2005

Construction of Prostate-Specific Expressed Recombinant Plasmids With High Transcriptional Activity of Prostate-Specific Membrane Antigen (PSMA) Promoter/Enhancer

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ABSTRACT: To screen different combinations of prostate-specific membrane antigen (PSMA) promoter/enhancer with the strongest transcriptional activity in prostate-specific cells, we used PSMA regulatory elements to control specific expression of the target gene in gene therapy of prostate adenocarcinoma. PSMA promoter and enhancer DNA sequences were amplified from the LNCaP human prostate cancer cell line by polymerase chain reaction, then recombinant plasmids of the enhanced green fluorescent protein (EGFP: pEGFP-$PSMA_{Pro}$, pEGFP-$PSMA_{E-P}$, pEGFP-$PSMA_{E(r)-P}$, pEGFP-$PSMA_{E(d)-P}$, and pEGFP-$PSMA_{E(l)-P}$) were constructed with molecular clonal techniques. At the same time, all experimental cell lines were analyzed for the expression of PSMA with the use of PSMA monoclonal antibody and the ABC immunohistochemical assay kit. After plasmids were transfected via liposome, we observed the expression of the reporter gene (EGFP) under a fluorescent microscope and compared the different levels of EGFP expression with reverse transcriptase polymerase chain reaction and flow cytometry so that we could choose the one with the highest transcriptional activity. Only the LNCaP cell line expressed PSMA positively with immunohistochemical stain. The PSMA promoter/enhancer had transcriptional activity in PSMA(+) cell lines and no activity in PSMA(−) cell lines. $PSMA_{E-P}$ achieved the strongest activity in different PSMA promoter/enhancer combinations. We confirmed the specific expression of PSMA in prostate cells again. Similarly, transcriptional activity of the PSMA promoter/enhancer was prostate specific. $PSMA_{E-P}$ achieved the strongest transcriptional activity among PSMA promoter/enhancer combinations, which could be used in advanced research for tissue-specific treatment.

Key words: Adenocarcinoma, regulatory element.
J Androl 2005;26:215–221

Prostate-specific membrane antigen (PSMA) is a 100-kd transmembrane glycoprotein, an important biomarker of prostate adenocarcinoma (Israeli et al, 1993; Fair et al, 1997). It was initially isolated from prostate cancer cell membrane in 1987 (Horoszewicz et al, 1987). PSMA has been shown to possess four related enzymatic activities: folate hydrolase activity by cleavage of γ-linked glutamates (Pinto et al, 1996); *N*-acetylated α-linked acidic dipeptidase activity by cleavage of α-linked glutamates in the brain (Carter et al, 1996); dipeptidyl peptidase IV α activity by cleavage of the bond between praline and amido methylcoumarin in glycine-praline-7-amido-4-methylcoumarin (Pangalos et al, 1999), and glutamate carboxypeptidase II activity (Meighan et al, 2003). But until now, the role of PSMA in biophysics was not understood.

The high activity of PSMA in prostate adenocarcinoma and its metastasis (Bostwick et al, 1998) and the positive relationship between the pathological grade of the prostate adenocarcinoma and PSMA expression has been confirmed (Kawakami and Nakayama, 1997). Recently, PSMA was discovered to be present in the neovasculature of a range of malignant tumors, whereas there is no expression in the vasculature of normal tissues (Liu et al, 1997; Silver et al, 1997; Chang et al, 1999). Immunohistochemical assay and reverse transcriptase polymerase chain reaction (RT-PCR) have detected the feeble existence of PSMA in some nonprostate tissues (eg, brain, salivary gland, small intestine, kidney tubules, breast; Israeli et al, 1994; Troyer et al, 1995; Wright et al, 1995; Chang et al, 2001). Unlike other prostate-specific proteins (PSA, PAP, probasin), PSMA expression is down-regulated by androgen (Wright et al, 1996), which makes it a more useful tool in the treatment of androgen-independent prostate carcinomas.

The regulatory elements of PSMA expression have been studied. Because of PSMA's folate hydrolase activity, the gene that encodes for PSMA has been designated *FOLH1*, which is located at chromosome 11p11–12 and contains 19 exons and 18 introns (O'Keefe et al, 1998). PSMA promoter has been located 166 bp upstream of the transcriptional start site (O'Keefe et al, 1998). In addition,

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Received for publication July 16, 2004; accepted for publication October 14, 2004.

a leader region upstream of the promoter sequence has little effect on transcription (Noss et al, 2002). Analysis of genetic homology shows that the PSMA promoter sequence is not homologous to other kinds of promoter sequences, which indicates its specificity to prostate. PSMA enhancer has been discovered within the third intron 12 kb downstream from the start site of transcription (Uchida et al, 2000). PSMA enhancer is also up-regulated by androgen deprivation (Watt et al, 2001). Although the most important functional fragment has not yet been identified, O'Keefe et al (2000) reported that the essential fragment of PSMA enhancer was 1290 to 1648 bp. PSMA enhancer was found to be characterized by a 72-bp repeat within a 331-bp core region (Watt et al, 2001).

PSMA promoter could direct gene expression in prostate cells uniquely, and PSMA enhancer could enhance the activity of the promoter. Compared with other strong constitutive promoters (cytomegalovirus [CMV] and Rous virus [RSV]), PSMA promoter/enhancer has high tissue specificity but low regulatory activity. Latham et al (2000) contrasted different combinations of PSA promoter/enhancer and concluded that double PSA enhancer held the best transcriptional activity. Because there are no published studies of PSMA enhancers, we constructed five recombinant plasmids with five different PSMA promoter/enhancer combinations and tried to observe the different regulatory activities among the combinations. Then we tried to screen the best PSMA promoter/enhancer combination with the strongest regulatory activity. We expected to construct a suitable gene therapeutic system, with the PSMA promoter/enhancer controlling expression of the target gene for specific gene therapy of prostate adenocarcinoma.

Materials and Methods

Cell Lines

Human prostate cancer cell lines LNCaP, PC-3, and DU145 and human breast cancer cell line MCF-7 (Soule et al, 1973; Stone et al, 1978; Kaighn et al, 1979; Murphy, 1980) were purchased from laboratory of transplantation and immunology (West China Hospital, Sichuan University). All the cells were maintained in RPMI1640 (GiBco, BRL, Gaithersburg, Md) supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum (FBS) with penicillin-streptomycin.

Reagents and Supplies

LA-*Taq* DNA polymerase kit and RT-PCR kit were from TakaRa Bioteh (DaLian, P.R. China), restriction endonucleases were from New England Biolab (Beverly, Mass), DNA_{ZOL} reagent was from GiBco, and transfection reagent was from jetPEI (Polytransfection, Illkirch, France). PCR purification and gel extraction kits were from Omega (Doraville, Ga).

Number of Primers	Sequence of different primers	Sites
P1	5`-CCCAAGCTTCTACTCAGCTGGCCCATGGC-3`	HindIII
P2	5`-ACGCGTCGACTGTGCTGCTGCTCTACTGCG-3`	SalI
E1-A	5`-GAAGATCTGCCTTCTAAAATGAGTTGGG-3`	BglII
E1-B	5`-CCGCTCGAGGCCTTCTAAAATGAGTTGGG-3`	XhoI
E1-C	5`-CGCGAGCTCGCCTTCTAAAATGAGTTGGG-3`	SacI
E2-A	5`-CGCGAGCTCGGCTACTACATAAGTATAAG-3`	SacI
E2-B	5`-CCGCTCGAGGGCTACTACATAAGTATAAG-3`	XhoI
E2-C	5`-GAAGATCTGGCTACTACATAAGTATAAG-3`	BglII

Figure 1. Table of primers. P indicates promoter; E, enhancer; and 1 and 2, forward and reverse primers, respectively.

PCR Amplification

According to the PSMA sequence (GenBank accession number AF007455) and the principle of primer design, the PSMA promoter ($PSMA_{Pro}$) sequence (nucleotides 1453–2697, accession number AF007455) and PSMA enhancer ($PSMA_{Enh}$) sequence (nucleotides 14703–16351, accession number AF007455) were amplified from template DNA of human prostate cancer cell line LNCaP. *Hin*dIII/*Sal*I linkers were ligated onto the end of the $PSMA_{Pro}$ fragment, whereas *Bgl*II/*Xho*I, *Sac*I/*Xho*I, *Bgl*II/*Sac*I, and *Xho*I linkers were ligated onto the end of the $PSMA_{Enh}$ fragment to amplify enhancers with different restriction sites. Primers are shown in Figure 1.

PCR for $PSMA_{Pro}$ was performed with 30 cycles of denaturing for 30 seconds at 94°C, annealing for 1 minute at 60°C, and primer extension for 4 minutes at 72°C. PCR for $PSMA_{Enh}$ was performed with 30 cycles of denaturing for 30 seconds at 94°C, annealing for 1 minute at 56°C, and primer extension for 4.5 minutes at 72°C. Then, amplified $PSMA_{Enh}$ with different linker sites were ligated by T_4 DNA ligase to form double-enhanced and triple-enhanced PSMA ($PSMA_{E(d)}$ and $PSMA_{E(t)}$, respectively).

Plasmid Construction

Plasmid DNA was harvested from *Escherichia coli* strain DH5α with the plasmid miniprep kit (Omega) and the enhanced green fluorescent protein (EGFP) reporter vector (pEGFP-1) and control vectors (pEGFP-N1 and pCMV-β-gal) with the Clontech kit (BD Biosciences Clontech, Palo Alto, Calif).

The purified $PSMA_{Pro}$ was subcloned as a *Hin*dIII-*Sal*I fragment into the pEGFP-1 vector to construct recombinant plasmid pEGFP-$PSMA_{Pro}$. Then, purified $PSMA_{Enh}$, $PSMA_{E(r)}$, $PSMA_{E(d)}$, and $PSMA_{E(t)}$ were subcloned as a *Bgl*II/*Sac*I fragment into pEGFP-$PSMA_{Pro}$, respectively, to construct recombinant plasmid pEGFP-$PSMA_{E\text{-}P}$, pEGFP-$PSMA_{E(r)\text{-}P}$, pEGFP-$PSMA_{E(d)\text{-}P}$, and pEGFP-$PSMA_{E(t)\text{-}P}$. A diagram of promoter and enhancer constructs are shown in Figure 2. All of recombinants were identified by double enzymatic digestion and DNA sequencing (Shennergy Biocolor BioScience, Shanghai, P.R. China).

Immunohistochemical Assay of Cell Lines

Cell lines were stained with PSMA monoclonal antibody (catalog number MO-T40086B, Yes Biotech, Mississauga, Ont, Canada) according to the manufacturer's instructions, then we observed the expression of PSMA in different cells under an optic microscope.

Cell Transfection and EGFP Assay

Transfections of LNCaP, PC-3, DU145, MCF-7, and T29, cotransfected with pCMV-β-gal as an internal control, were carried out on 35-mm plates (Falcon, St Louis, Mo) with the use of jetPEI according to the manufacturer's instructions. Briefly, 2×10^5 cells were plated per well. When the wells were approximately 60% to 80% confluent, they were washed with fresh RPMI1640 containing 10% FBS. The cells were overlaid with DNA/jetPEI complexes (3 μg with 1 μg control and 6 μL, respectively) in a total volume of 3 mL of RPMI1640 containing 10% FBS. Cells were incubated at 37°C in a humidified incubator with 5% CO_2. After incubation for 24 hours, cells were observed through a fluorescent microscope (Nikon, Tokyo, Japan) successively. Sixty hours after transfection, cells were collected. EGFP expression was measured by flow cytometry (Coulter Elite Esp, San Francisco, Calif) and an RT-PCR assay of green fluorescent protein mRNA. Before flow cytometry measurements, we normalized the instrument with a DNA check, controlling the variable coefficient to less than two. More than 5000 cells were required in each sample for accurate measurement, and results were analyzed with software supplied by Coulter. Forward primer (5′-AGTGCTTCAGCCGCTACCCC-3′) and reverse primer (5′-GATGCCGTTCTTCTGCTTGTC-3′) for RT-PCR were designed for EGFP. Amplification conditions were 30 minutes at 55°C and 5 minutes at 99°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 5 minutes at 72°C. Product concentrations were analyzed with a gel imaging system and β-actin as an internal control. In case of genomic DNA contamination, we added no–transcriptase control for each sample as another control. The EGFP expression in each sample was normalized for variation in transfection efficiency by measuring the level of β-galactosidase expression from the cotransfected pCMV-β-gal plasmid. After incubating the cell lysate at 50°C for 1 hour to inactivate endogenous β-galactosidase, a 50-μL aliquot was mixed with 200 μL of substrate (Clontech) and incubated for another 1 hour at room temperature. β-Galactosidase activity was determined by light emission.

Results

PSMA Expression of Immunohistochemical Assay

Nonprostate cells did not expressed PSMA when they were stained with PSMA monoclonal antibody. In prostate cells, only LNCaP cells expressed PSMA obviously (Figure 3); PC-3 and DU145 cells were not stained. Expression of PSMA focused in the cell plasma; nucleoli were not stained.

Identification of PCR Products

PCR products of PSMA promoter and enhancer were initially identified by electrophoresis. Amplified bands were clear and specific at 1250 and 1650 bp, respectively (Figure 4).

Observation of Transfected Cells and EGFP Analysis

All cells were observed to be fluorescent after transfection with control vector pEGFP-N1 24 hours later, and fluorescence began to weaken progressively at 72 hours. Figure 4A shows the fluorescence of MCF-7 cells. When cells were transfected with recombinants (pEGFP-$PSMA_{Pro}$, pEGFP-$PSMA_{E\text{-}P}$, pEGFP-$PSMA_{E(r)\text{-}P}$, pEGFP-$PSMA_{E(d)\text{-}P}$, and pEGFP-$PSMA_{E(t)\text{-}P}$), fluorescence appeared much later (48–60 hours after transfection) and were expressed more weakly than in the control groups (Figure 5C through G). It is important that fluorescence occurred only in LNCaP cells. In addition, feeble fluorescence was observed in PC-3 cells uniquely transfected with pEGFP-$PSMA_{Pro}$. The rest of the cells did not appear to fluoresce while observed. Density and intensity of fluorescence in different transfected LNCaP cells were evidently different. EGFP assays revealed that a single PSMA enhancer stimulated transcription about 53-fold over the level achieved by the PSMA promoter alone. Reverse PSMA enhancer had a similar effect on transcriptional stimulus. RT-PCR showed that single PSMA enhancer plus promoter has the strongest density, except for the positive control (pEGFP-N1–transfected LNCaP cells), whereas the no-RT controls were negative, which could exclude contamination (data not shown). Double enhancers plus PSMA promoter could only enhance transcription 17-fold, whereas triple enhancers could not stimulate up-regulated transcription. An enhancer had no effect on transcription in the PC-3 cell lines (Figures 6 and 7).

Discussion

In this study, an immunohistochemical assay of the PSMA confirmed LNCaP cells could express PSMA, but non-prostate cells and PC-3; DU145 cells cannot express PSMA. Northern blot analysis of PSMA mRNA proved PSMA expression of LNCaP and C4-2 cell lines, whereas PC-3 cell lines showed a low expression level (Watt et al, 2001). Denmeade et al (2003) compared several kinds of prostate cancer cell lines. Results showed the high expression level in LNCaP and C4-2 cell lines.

Previous analysis of the PSMA promoter region has shown that a 1244-bp fragment directs expression of the reporter gene only in prostate cell lines LNCaP and C4-2 (O'Keefe et al, 1998). Compared with other widely expressed promoters, such as CMV, the PSMA promoter has weaker activity but higher specificity. Good et al (1999) reported that the minimal functional region of PSMA promoter is about 600 bp. Activity of PSMA transcriptional elements of prostate cancer cells are 100 to 1000 times more active than that of normal prostate cells. O'Keefe et al (2000) confirmed that the entire sequence of PSMA

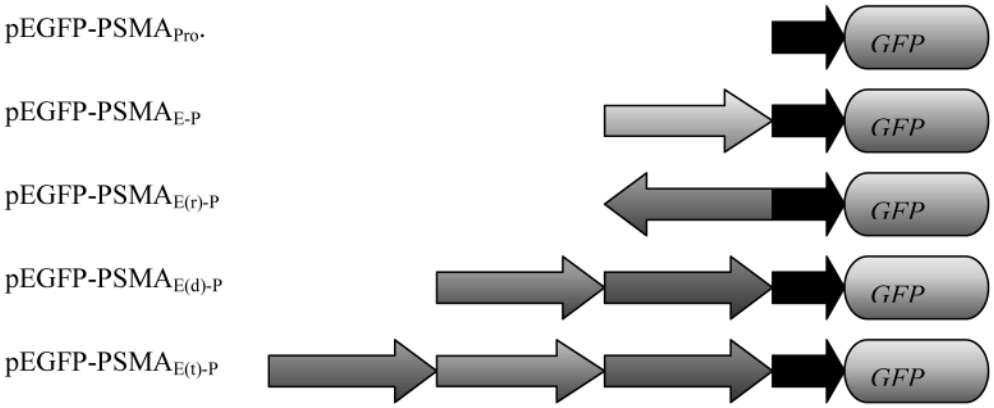

Figure 2. Diagram of promoter and enhancer constructs used to express enhanced green fluorescent protein (EGFP). The black arrow indicates the PSMA promoter.

enhancer is 1913 bp and that the segment 1648 bp from the 5′-flank plays a positive role, whereas the rest of the sequence is a negative regulatory segment.

Therefore, in our research, we obtained PSMA promoter and enhancer sequences directly from LNCaP cell, chose the 1244-bp fragment as PSMA promoter and the 1648-bp fragment as PSMA enhancer amplified by PCR, constructed five recombinant plasmids with five different PSMA promoter/enhancer combinations that successfully controlled reporter gene (EGFP) expression, and tried to screen the strongest controlling element combination. We observed, under a fluorescent microscope after transient transfection, that the transcriptional activity of PSMA-controlling elements had manifested their high specificity. That is to say, PSMA promoter/enhancer combinations only had transcription activity in PSMA-expressing cell lines, such as the LNCaP cell line.

With a PSMA promoter plus a PSMA enhancer, transcription could be enhanced more than 53-fold over the level achieved with the promoter alone. This result is similar to Noss et al (2002). The PSMA enhancer is active in either orientation relative to the PSMA promoter. In its opposite orientation, the PSMA enhancer could increase basal expression of the promoter more than 49-fold in the LNCaP cell line, so PSMA enhancers of both directions have similar incremental activity. O'Keefe et al (1998) reported that PSMA enhancer of the opposite direction was more active than that with the same direction as the promoter. Interestingly, increasing the number of PSMA enhancers progressively decreased the activity of PSMA promoter/enhancer combinations. With double enhancers plus PSMA promoter, activity increased only 17-fold over the basal level. With the addition of triple enhancers, we did not observe any up-regulation of transcription. Latham et al (2000) compared activity among single, double, and triple PSA enhancers, identifying three combinations that could enhance the transcriptional function of the PSA promoter, but the double enhancer was the best. The length of a PSMA promoter and a PSMA enhancer is more than 1200 bp. When the promoter and enhancers

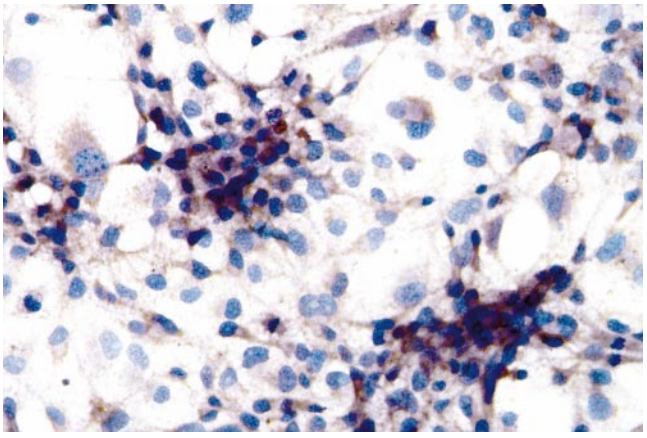

Figure 3. PSMA expression of immunohistochemical assay in the LNCap cell line.

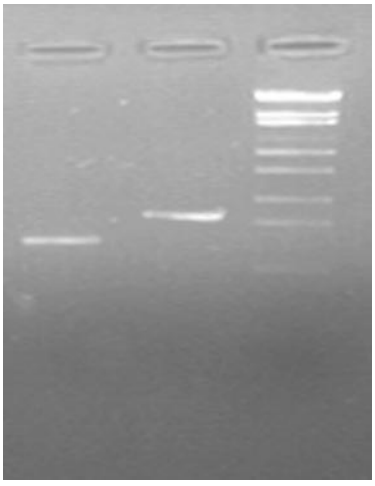

Figure 4. Electrophoresis of the amplified PSMA promoter and enhancer.

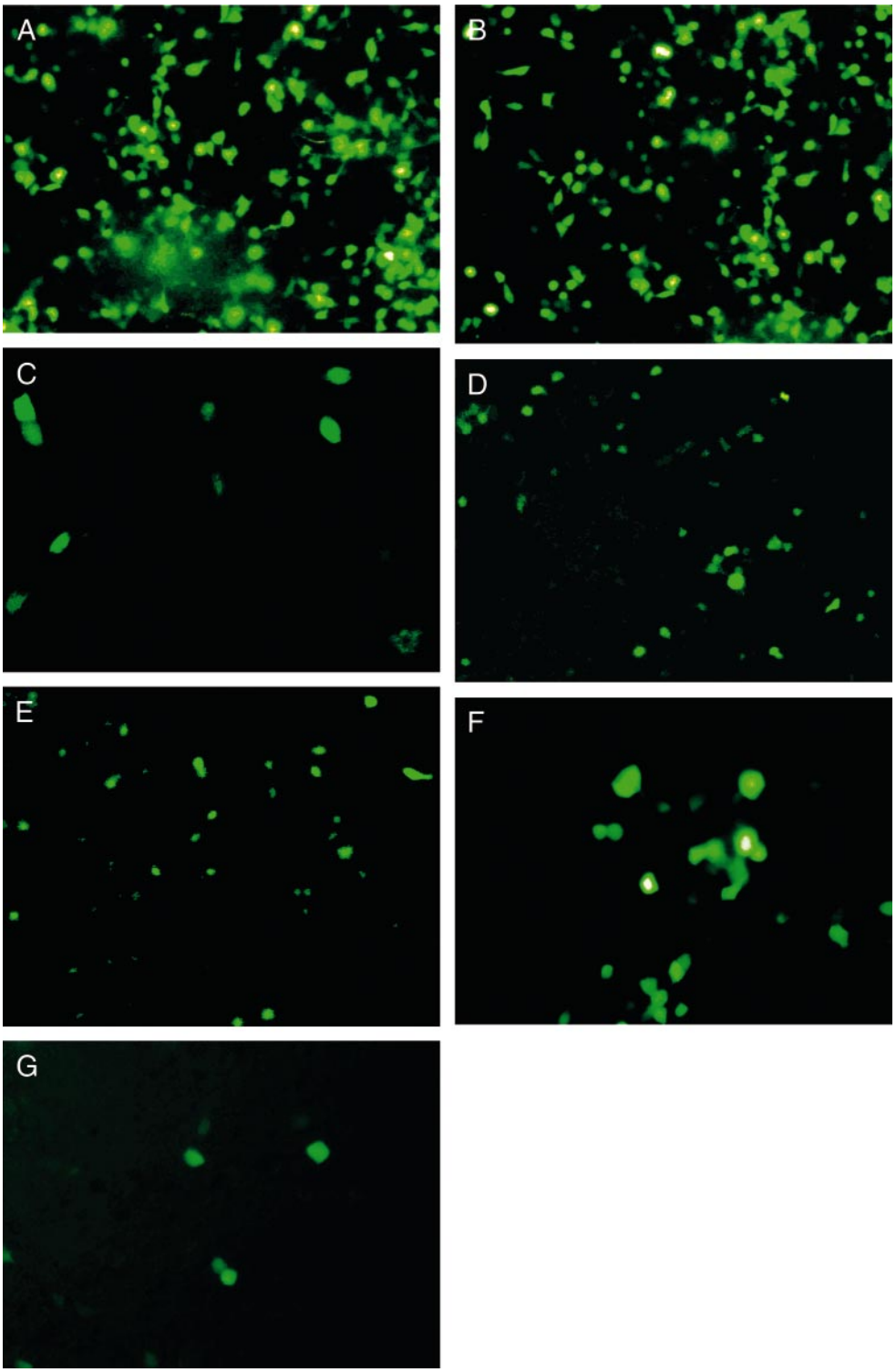

Figure 5. Expression of EGFP in different cell lines.

are added together and inserted into the plasmid, the fragment is too long to enhance transcription completely, and even lost activity, which could explain our observations.

In the PC-3 cell line, we found weak activity of the PSMA promoter alone. A low level of PSMA mRNA in the PC-3 cell line might explain the feeble activity of the PSMA promoter. But, the PSMA enhancer has no activity in PC-3, perhaps because the factors involved in up-reg-

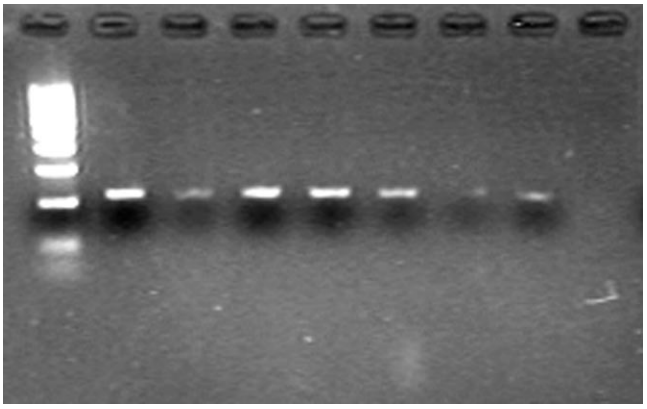

Figure 6. EGFP mRNA expression in LNCaP and DU145 cells transfected with different vectors.

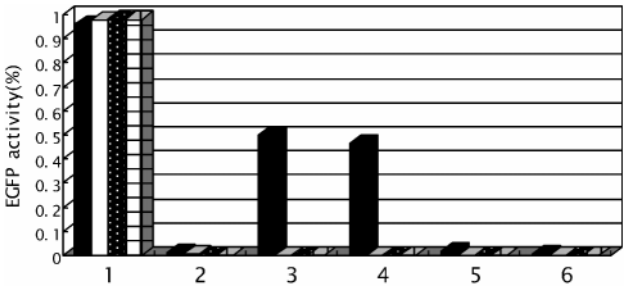

Figure 7. EGFP expression measured by flow cytometry. The results were expressed as the percentage ratio of tested EGFP expression activity to β-galactosidase activity. The black box indicates LNCaP cells; blank box, PC-3 cells; box with dots, DU145 cells; and box with squares, MCF-7 cells. Group 1 indicates transfection with pEGFP-N1; group 2, transfection with pEGFP-PSMA$_{Pro}$; group 3, transfection with pEGFP-PSMA$_{E-P}$; group 4, transfection with pEGFP-PSMA$_{E(r)-P}$; group 5, transfection with pEGFP-PSMA$_{E(d)-P}$; and group 6, transfection with pEGFP-PSMA$_{E(t)-P}$.

ulation via the enhancer are not present in the PC-3 cells or because factors combining with the enhancer in PC-3 cells have very low affinity.

All of the PSMA promoter/enhancer combinations have higher specificity for prostate cells but a lower level of transcription than the control vector. Of the five combinations, PSMA$_{E-P}$ might be the best. Recently, some researchers showed that the PSMA enhancer could increase transcriptional activity of the probasin and SV-40 promoters (O'Keefe et al, 2000; Watt et al, 2001), but compared with the PSMA enhancer plus PSMA promoter, the function of enhancement was weaker, indicating nonspecificity of the PSMA enhancer. To improve the transcriptional activity of PSMA regulatory elements, Ikegame et al (2002) added the Cre-loxP system to the PSMA promoter/enhancer and identified prominent improvement of transcription. A PSMA enhancer combined with a PSA enhancer formed chimeras, which could play a role in both PSA-expressing and PSMA-expressing cells (Lee et al, 2002).

The relationship between PSMA expression and androgen is clear. That is, PSMA expression is up-regulated by androgen deprivation, which indicates its potential utility in hormone refractory prostate adenocarcinoma. The usefulness of the specificity of a PSMA promoter/enhancer in LNCaP cells in enzyme-directed prodrug gene therapy has been investigated in vitro and in vivo (O'Keefe et al, 2000; Uchida et al, 2001). Transcription of the cytosine deaminase gene by PSMA promoter/enhancer produced a 50-fold enhancement of toxicity, which was specific for LNCaP cells versus PC-3 or nonprostate cell lines. Athymic male nude mice receiving C4-2 cells transfected with the cytosine deaminase gene and driven by a PSMA promoter/enhancer exhibited significant reduction in tumor volume and serum PSA concentration. However, in the previous studies, scientists used a plasmid as the transfection vector, with low efficiency of cell transfection, which had an effect on the therapeutic research.

Our study confirmed the transcriptional specificity of the PSMA promoter. We also confirmed that a PSMA enhancer could increase the activity of the PSMA promoter markedly; a single enhancer plus a promoter has the strongest activity among different combinations of controlling elements. In further research, we plan to use a defect adenovirus as the vector system, and we will try to construct a recombinant adenovirus with the PSMA promoter/enhancer to control therapeutic gene expression for advanced gene therapy research.

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