Two-step transcriptional amplification–lipid-based nanoparticles using PSMA or midkine promoter for suicide gene therapy in prostate cancer

Yoshiyuki Hattori and Yoshie Maitani

Institute of Medicinal Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501, Japan

(Received March 13, 2006/Revised April 14, 2006/Accepted April 24, 2006/Online publication June 16, 2006)

A two-step transcriptional amplification system (TSTA) was used to enhance the efficacy of suicide gene therapy for treatment of prostate cancer. We designed a TSTA system and constructed two types of plasmid: one containing GAL4–VP16 fusion protein under the control of a tumor-specific promoter, the other containing luciferase or herpes simplex virus thymidine kinase (HSV-tk) under the control of a synthetic promoter. The TSTA systems using nanoparticles based on lipids were evaluated by measuring the amount of induced luciferase activity as a function of prostate-specific membrane antigen (PSMA) and midkine (Mk) promoters, specific for LNCaP and PC-3 prostate cancer cells, respectively. In LNCaP cells that were PSMA-positive, the TSTA system featuring the PSMA enhancer and promoter exhibited activity that was 640-fold greater than a system consisting of one-step transcription with the PSMA promoter. In contrast, this difference in activity did not occur in PSMA-negative PC-3 cells. In Mk-positive PC-3 cells, the TSTA system with the Mk promoter exhibited a five-fold increase in activity over one-step transcription, but such activity was not induced in Mk-negative LNCaP cells. When using HSV-tk for suicide gene therapy, TSTA systems featuring the PSMA or Mk promoter inhibited in vitro cell growth in the presence of ganciclovir. Furthermore, the TSTA system featuring the Mk promoter suppressed in vivo growth of PC-3 tumor xenografts to a greater extent than one-step transcription. These findings show that TSTA systems can enhance PSMA and Mk promoter activities and selectively inhibit PC-3 cell growth in tumors. This suggests that TSTA systems featuring tumor-specific promoters are suitable for cancer treatment by gene therapy. (Cancer Sci 2006; 97: 787–798)

Currently, many kinds of gene therapy research are being carried out, especially in the field of cancer treatment. The most difficult aspect of developing an in vivo approach is correctly targeting cancer cells. The random delivery of a therapeutic gene damages normal organs such as the liver, lung, kidney and spleen, and can cause death. Many vectors target tumors for gene delivery, including viral and synthetic vectors (liposome and emulsion), whereas others that use tumor-specific promoters to regulate expression transcriptionally in target cancer cells have also shown promise. It is essential to use a strong and tissue-specific promoter region if a suicide gene is to be expressed selectively in the cancer cells. Prostate cancer is the most frequently diagnosed cancer and the second leading cause of death in men in the USA, after lung cancer. The prostate-specific antigen (PSA) test is carried out routinely in men to detect the presence of prostate cancer by immunoassaying the level of PSA in serum. Currently, androgen deprivation is the most effective treatment for advanced prostate cancer, but it reduces PSA serum levels, affecting the utility of PSA as a prostate tumor-specific promoter. Like PSA, prostate-specific membrane antigen (PSMA) has elevated expression in prostate cancer and has been reported to accumulate under conditions of androgen deprivation, potentially making it a more useful tool when tracking a patient’s response during prostate cancer treatment. This suggested that the PSMA promoter appears to be highly suitable for gene therapy. Midkine (Mk) is a heparin-binding growth factor whose expression is regulated developmentally. Its biological function during tumorigenesis remains unclear but Mk is expressed in various types of human cancer, including prostate cancer. Several groups have reported that Mk promoter-mediated suicide gene therapy effectively produces cytotoxic effects in cancer cells.

Both the PSMA and Mk genes have been identified as factors expressed specifically in cancer cells; however, the promoter region has one disadvantage in that it does not have strong promoter activity, which, in turn, limits the cells’ ability to express the suicide gene. Suicide gene therapy with a PSA enhancer and promoter in the LNCaP model also had no significant effect with a one-step transcription system. It is essential to find a way to enhance the transcriptional activity of such promoters. Several methods can potentially be used to increase levels of reporter or therapeutic proteins in prostate cancer. One of the amplification approaches, referred to as a two-step transcriptional amplification system (TSTA), can potentially be used to improve the transcriptional activity of cellular promoters with the GAL4–VP16 fusion protein, which comprises the DNA-binding domain of the yeast transcriptional activator GAL4 and the activation domain of the herpes simplex virus 1 activator VP16 (Fig. 1). GAL4 is a transcriptional factor that regulates gene transcription tightly by binding its responsive elements. A potent transcriptional activator, GAL4–VP16, which is driven by the cell-specific promoter of an effector plasmid, acts on the promoter of a second expression plasmid (reporter plasmid), which encodes the reporter or therapeutic protein (Fig. 1). For temporally regulated expression, a tetracycline-repressible transactivator system for inducible gene expression was developed using tet-repressor fused to VP16. TSTA
systems for the amplification of tumor-specific gene expression has been reported in PSA promoter for prostate cancer,\(^{(15,16)}\) Muc-1 promoter for colon carcinoma\(^{(20)}\) and carcinoembryonic antigen (CEA) promoter for lung cancer and colon adenocarcinoma, respectively.\(^{(21)}\) However, TSTA systems with PSMA and Mk promoters have not been reported, and the TSTA system has not been applied to suicide gene therapy with herpes simplex virus thymidine kinase (HSV-tk).

In the present study, we modified the TSTA system using a reporter plasmid with a combination of the adenoviral E1B minimal promoter, SV40 enhancer and an effector plasmid with the PSMA enhancer and promoter or Mk promoter to achieve novel tumor-specific transcriptional amplification for prostate cancer, and evaluated selectiveness to drive gene expression in LNCaP and PC-3 cancer cells. In PSMA-positive LNCaP cells and Mk-positive PC-3 cells, the TSTA system with each promoter and enhancer showed greater activity than one-step transcription with each promoter, as confirmed by growth inhibition of the cells and PC-3 tumor xenografts on suicide gene therapy.

**Materials and Methods**

**Plasmid construction**

pGL3-control, pGL3-enhancer and pGL3-basic plasmids were purchased from Promega (Madison, WI, USA). pFR-luc plasmid for expression of the luciferase gene controlled by a synthetic promoter that contains the yeast GAL4-binding sites in front of the E1B minimal promoter was obtained from Stratagene (La Jolla, CA, USA). The HSV-tk cDNA fragment was amplified as described previously.\(^{(22)}\) This cDNA was then subcloned into the Neol and XbaI restriction enzyme sites of the pGL3 enhancer vector, and pGL3-tk was constructed.

For amplification of the GAL4–VP16 fusion protein, the cDNA encoding the GAL4–VP16 fusion protein was generated as follows by site-directed mutagenesis by overlap extension using polymerase chain reaction (PCR).\(^{(23)}\) The yeast GAL4 cDNA was amplified by PCR using the primer set GAL4-forward primer (FW), 5′-ATCCATGGaccATGAAGCTACTGTCTTCTAT-3′ and GAL4-reverse primer (RW), 5′-CGGTCGGGGG-GGCCTCGAGACAGTCAACTGTCT-3′. The GAL4-FW contained a 3-bp optimal Kozak sequence (in lowercase letters) together with a Neol restriction site (underlined). The GAL4-RW coded for the N-terminal region of the activation domain of VP16 (underlined), followed by the C-terminal region of GAL4. The cDNA coding for the activation domain of VP16 was amplified by PCR using the primer set VP16-FW, 5′-ACAGTTGACTGTATCGACGCGGCCCCCGAGCAT-3′ and VP16-RW, CATACTAGCTATCCCGGACCCGGAATCC-3′. The forward primer, VP16-FW, coded for the C-terminal
region of GAL4 (underlined), followed by the N-terminal region of the activation domain of VP16. The reverse primer, VP16-RW, coded for the VP16 sequence with an N-terminal restriction enzyme sites for PCR amplification according to findings reported previously. The forward and reverse primers, respectively, contained KpnI and HindIII restriction sites (underlined). The amplified PCR fragment of the PSA enhancer located between −1283 and −39. The forward and reverse primers, respectively, contained XhoI and HindIII restriction sites (underlined).

The PSA enhancer fragment was cloned by nested-PCR amplification. The first PCR primer set was PSA(E)-FW, 5′-GGAATTCGAGGAAATATTTGCAGTTTGTCTAT-3′ and PSA(E)-RW, 5′-CTTAAGCTTGGCTGCTACTTGTGCCTGCCG-3′ to amplify a DNA fragment for the PSA promoter located between −1283 and −39. The forward and reverse primers, respectively, contained XhoI and HindIII restriction sites (underlined).

The PSA enhancer fragment was cloned by nested-PCR amplification. The first PCR primer set was PSA(E)-FW, 5′-GGAATTCGAGGAAATATTTGCAGTTTGTCTAT-3′ and PSA(E)-RW, 5′-GCTTCGCTTTCTGTTGGCC-3′, and PSA(E)-I-FW, 5′-GCTTCGCTTTCTGTTGGCC-3′, and PSA(E)-RW, 5′-GCTTCGCTTTCTGTTGGCC-3′ to amplify a DNA fragment for the PSA promoter located between +11958 and +13606. The forward and reverse primers, respectively, contained HindIII and Ncol restriction sites (underlined).

For the construction of reporter plasmids, the primer set was Syn-FW, 5′-CCAGCTTGGCATGCTTGGC-3′ and Syn-RW, 5′-ATCCATGGTACACACGTACCGA-3′. (13) The primer set was Mk(P)-FW, 5′-GAAAGTTACCCTGCTGACCTTGCC-3′ and Mk(P)-RW, 5′-GCTTCCAGGGCTGGCC-3′ to amplify a DNA fragment for the Mk promoter stretching from bp −559 of the 5′ upstream flanking region to bp 27 of exon 1 of the human Mk gene. The forward and reverse primers, respectively, contained KpnI and HindIII restriction sites (underlined). The amplified PCR fragment of the Mk promoter (−0.6 kb in length) was subcloned into the KpnI and HindIII restriction sites of the pGL3 enhancer and pGL3-tk and pGL3-GAL/VP, and pMk(P)-luc, pMk(P)-tk and pMk(P)-GAL/VP, respectively, were constructed.

For the construction of reporter plasmids, the primer set was Syn-FW, 5′-CCAGCTTGGCATGCTTGGC-3′ and Syn-RW, 5′-ATCCATGGTACACACGTACCGA-3′. (13) The primer set was Mk(P)-FW, 5′-GAAAGTTACCCTGCTGACCTTGCC-3′ and Mk(P)-RW, 5′-GCTTCCAGGGCTGGCC-3′ to amplify a DNA fragment for the Mk promoter stretching from bp −559 of the 5′ upstream flanking region to bp 27 of exon 1 of the human Mk gene. The forward and reverse primers, respectively, contained KpnI and HindIII restriction sites (underlined). The amplified PCR fragment of the Mk promoter (−0.6 kb in length) was subcloned into the KpnI and HindIII restriction sites of the pGL3 enhancer and pGL3-tk and pGL3-GAL/VP, and pMk(P)-luc, pMk(P)-tk and pMk(P)-GAL/VP, respectively, were constructed.

The plasmid pCMV-tk encoding HSV-tk under the control of the cytomegalovirus (CMV) promoter was constructed as described previously. (22) The plasmid pCMV-luc, encoding luciferase under the CMV promoter, was constructed by insertion of the CMV promoter into the pGL3 enhancer, as described previously. (22) A protein-free preparation of the plasmid was purified following alkaline lysis using maxiprep columns (Qiagen, Hilden, Germany).

Cell culture
LNCaP cells were supplied by the Department of Urology, Keio University Hospital (Tokyo, Japan). PC-3 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). Human cervix carcinoma HeLa cells were kindly provided by Toyobo (Osaka, Japan). All of the cell lines used in this study were grown in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies) and kanamycin (100 μg/mL) at 37°C in a humidified atmosphere with 5% CO2.

RNA isolation and reverse transcription–polymerase chain reaction
Total RNA was isolated from LNCaP, PC-3 and HeLa cells, using NucleoSpin RNA II (Macherey-Nagel, Düren, Germany). Total RNA from normal and malignant prostate cells were purchased from Ambion (First Choice Tumor/Normal Adjacent Prostate Total RNA and First Choice Prostate Tumor Total RNA; Austin, TX, USA). First-strand cDNA was synthesized from 5 μg of total RNA as described previously. (22) Reverse transcription (RT)-PCR was carried out in a 25-μL reaction volume containing the following: 1 μL of synthesized cDNA, 10 pmol of each specific primer pair, and 0.25 units of Ex Taq DNA polymerase (Takara Shuzo, Kyoto, Japan) with a PCR buffer containing 1.5 mM MgCl2 and 0.2 mM of each dNTP. The
profile of PCR amplification consisted of denaturation at 94°C for 0.5 min, primer annealing at 58°C for 0.5 min, and elongation at 72°C for 1 min for 25 cycles. PCR of the housekeeping gene β-actin, Mk and PSMA were carried out during the same cycle run for all samples. The PCR products for Mk, PSMA and β-actin were analyzed by 1.5% agarose gel electrophoresis in a Tris-Borate-ethylenediamine tetraacetate acid (TBE) buffer. The products were visualized by ethidium bromide staining.

Real-time PCR was carried out on the corresponding cDNA synthesized from each sample described above. The optimized settings were transferred to real-time PCR protocols on iCycler MyiQ detection systems (Bio-Rad Laboratories, Hercules, CA, USA) and SYBR Green I assay (iQ SYBER Green Supermix, Bio-Rad Laboratories) was used for quantification. Samples were run in triplicate and the expression level of Mk and PSMA mRNA was normalized for the amount of β-actin in the same sample. The difference of one cycle was calculated as a two-fold change in gene expression.

In vitro transfection

Cholesteryl-3β-carboxymidoethylen-N-hydroxyethylamine (OH-Chol) was synthesized as reported previously. The nanoparticle (NP) as a gene transfection reagent was prepared with lipids (OH-Chol : Tween 80 [NOF, Tokyo, Japan] 95 : 5, molar ratio = 10 : 1.3, weight) in 10 mL of water using a simplified ethanol injection method as described previously. Based on preliminary experiments with the cotransfected plasmids, the optimized ratio (w/w) of effector : reporter plasmid was determined as 1 : 1. The nanoplex at a charge ratio (+/-) of cationic lipid to DNA of 3/1 was formed by addition of NP to 2 μg of DNA (e.g. 1 μg of effector plasmid and 1 μg of reporter plasmid in the TSTA system) in 50 mM NaCl with gentle shaking and left at room temperature for 10 min. For transfection, the nanoplexes were incubated in 1 mL of medium supplemented with 10% serum and then incubated for 24 h. Androgen stimulation of transfected cells was carried out by adding 10 nM dihydrotestosterone (DHT; Sigma, St Louis, MO, USA) to the culture medium.

Luciferase assay

Cell cultures were prepared by plating cells in a 35-mm culture dish 24 h prior to each experiment. The cells at 70% confluence were transfected as described above. Luciferase expression was measured as counts per s (cps)/μg protein using the luciferase assay system (Pica gene; Toyo Ink Manufacturing, Tokyo, Japan) and bicinecinic acid (BCA) reagent (Pierce, Rockford, IL, USA) as reported previously.

In vitro sensitivity to the ganciclovir assay

LNCaP, PC-3 and HeLa cells were seeded separately at a density of 1 × 10^4 cells per well in 96-well plates and maintained for 12 h before transfection in RPMI-1640 medium supplemented with 10% serum. The cells were transfected with the nanoplexes at 0.2 μg plasmid/well. After 12 h incubation, the culture medium was replaced with medium containing various concentrations of ganciclovir (GCV; Glaxo Smith Kline, Helis, UK) ranging from 0.1 to 1000 μg/mL. The number of surviving cells was determined with a WST-8 assay (Dojindo Laboratories, Kumamoto, Japan) after 3 days’ exposure to GCV as described previously.

Immunoblotting

LNCaP, PC-3 and HeLa cells were transfected with various plasmids and then incubated for 24 h. The cells were suspended in lysis buffer (0.5% Triton-X 100 in phosphate-buffered saline pH 7.4), then centrifuged at 15 000 r.p.m. (20 000 × g) for 10 min. The supernatants were resolved by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane (FluoroTrans W; PALL Gelman Laboratory, Ann Arbor, MI, USA). HSV-tk was identified using a specific rabbit antiserum (kindly provided by the Department of Virology, Toyama Medical and Pharmaceutical University) with antirabbit IgG peroxidase conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as the secondary antibody and detected with peroxidase-induced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate; Pierce).

Assessment of PC-3 xenograft tumor growth in vivo

To generate PC-3 tumor xenografts, 1 × 10^7 cells suspended in 50 μL of RPMI-1640 medium containing 60% reconstituted basement membrane (Matrigel; Collaborative Research, Bedford, MA, USA) were inoculated subcutaneously into the flank ing region of male BALB/c nu/nu mice (6 weeks of age; CLEA Japan, Tokyo, Japan). The tumor volume was calculated using the formula:

\[ \text{tumor volume} = 0.5ab^2, \]

where \( a \) and \( b \) are the larger and smaller diameters, respectively. When the average volume of PC-3 xenograft tumors reached 150 mm^3 (day 0), these mice were divided into four groups: group I, pGL3-basic (10 μg) as a control; group II, pMk(P)-tk (10 μg); group III, pMk(P)-GAL/VP (5 μg) plus pRep-tk (5 μg); and group IV, pCMV-tk (10 μg). Each experimental group consisted of four tumors. Based on a preliminary experiment of gene expression by intratumoral injection, the optimized ratio of cationic lipid to DNA was determined as 1 : 1. The nanoplex at a charge ratio (+/-) of 1/1 of cationic lipid to DNA was formed by addition of NP to 15.8 μL) to 10 μg of DNA with gentle shaking and incubation at room temperature for 10 min. The nanoplexes of 10 μg of plasmid per tumor were injected directly into xenografts on days 0, 3 and 6. GCV at a dose of 25 mg/kg was administered intraperitoneally 12, 24 and 36 h after the injections of nanoplexes. The tumor volume was measured at days 0, 3, 6, 8, 10, 12 and 14.

Statistical analysis

The statistical significance of differences between mean values was determined using Welch’s t-test. Multiple measurement comparisons were carried out by analysis of variance followed by the Bonferroni/Dunn test. For the animal study, statistical comparisons were carried out using Fisher’s exact test. \( P \)-values less than 0.05 were considered significant.

Results

Expression of midkine and PSMA mRNA

First, we investigated the expression of Mk and PSMA in human prostate malignant biopsy and LNCaP, PC-3 and HeLa cells using the quantitative PCR and RT-PCR method (Fig. 2). HeLa cells were used as a Mk-positive control.
the human prostate biopsy, two kinds of RNA from normal prostate and three prostate tumors were used. Elevated expression of PSMA and/or Mk mRNA was observed in prostate tumor (Fig. 2A), indicating that upregulated expression of PSMA and Mk could be utilized as a marker for prostate tumor. Mk mRNA was expressed strongly in PC-3 cells and weakly in HeLa cells, but was not detected in LNCaP cells (Fig. 2B). Quantitative PCR analysis showed that the amount of Mk mRNA in PC-3 cells was 31-fold higher than in HeLa cells (data not shown). PSMA mRNA was detected in LNCaP cells, but not in PC-3 or HeLa cells. This result suggested that PC-3 and HeLa cells could be utilized with the Mk promoter for tumor-specific expression, and LNCaP cells could be utilized with the PSMA promoter.

**Analysis of PSMA and PSA enhancer/promoter activity**

In a preliminary study, we evaluated the activities of the PSA and PSMA promoters as they have been well characterized and determined to be tissue specific. PSMA promoter activity was increased with a combination of SV40 enhancer in LNCaP cells. Therefore, we used the combination of PSA or PSMA enhancer and promoter and SV40 enhancer to enhance prostate-specific gene expression (Fig. 1). To assess the transcriptional activity of our cloned promoter and enhancer regions, we constructed two PSA promoter-based plasmids, pPSA(P)-luc and pPSA(EP)-luc coding for the luciferase gene under the control of the PSA promoter and PSA enhancer and promoter, respectively, and two PSMA promoter-based plasmids, pPSMA(P)-luc and pPSMA(EP)-luc coding for the luciferase gene under the control of the PSMA promoter and PSMA enhancer and promoter, respectively (Table 1). Four kinds of plasmids (2 µg) were transfected into LNCaP cells cultured in the absence or presence of DHT or into PC-3 cells, and a luciferase assay was carried out 24 h after transfection. We normalized each experiment using SV40 constructs (pGL3-control) (Fig. 3). It has been reported that LNCaP cells are androgen responsive, showing a decrease in PSMA mRNA levels and increase in PSA mRNA levels with increasing androgen concentrations in culture media. In the medium without DHT, PSA-related plasmids, pPSA(P)-luc and pPSA(EP)-luc, exhibited weak transfectional activity (11 and 24% of the luciferase activity with pGL3-control) in LNCaP cells. However, in the medium with DHT, pPSA(EP)-luc, but not pPSA(P)-luc, increased the luciferase activity 6.3-fold compared to that in medium without DHT (151% of pGL3-control). Among the PSMA promoter-based plasmids, pPSMA(P)-luc showed weak transfection activity (28.8% and 45.9% of pGL3-control in medium without or with DHT, respectively). In contrast, pPSMA(EP)-luc induced relatively strong transfection activity (28.8% and 45.9% of pGL3-control in medium without or with DHT, respectively). We confirmed that our cloned promoter and enhancer regions of PSA and PSMA had prostate-specific transcriptional activity consistent with findings reported previously.

**Two-step transcriptional amplification system using the PSMA promoter**

The PSMA promoter appeared to be suitable for gene therapy because the expression of PSMA was not strongly affected...
by the presence or absence of androgen. However, the PSMA enhancer and promoter do not have strong promoter activity even when combined with the SV40 enhancer. Therefore, to enhance the PSMA promoter activity, we constructed the

Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Transcription</th>
<th>Plasmid</th>
<th>Upstream of expression gene</th>
<th>Expression gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>One-step</td>
<td>pGL3-control</td>
<td>–</td>
<td>SV40</td>
</tr>
<tr>
<td></td>
<td>pCMV-luc</td>
<td>CMV</td>
<td>CMV</td>
</tr>
<tr>
<td></td>
<td>pCMV-tk</td>
<td>CMV</td>
<td>HSV-tk</td>
</tr>
<tr>
<td></td>
<td>pPSA(P)-luc</td>
<td>PSA (0.6 kb)</td>
<td>PSA (0.6 kb)</td>
</tr>
<tr>
<td></td>
<td>pPSA(EP)-luc</td>
<td>PSMA (1.2 kb)</td>
<td>Luciferase</td>
</tr>
<tr>
<td></td>
<td>pPSMA(P)-luc</td>
<td>–</td>
<td>PSMA (1.2 kb)</td>
</tr>
<tr>
<td></td>
<td>pPSMA(EP)-luc</td>
<td>PSMA (1.6 kb)</td>
<td>Luciferase</td>
</tr>
<tr>
<td></td>
<td>pPSMA(EP)-tk</td>
<td>PSMA (1.6 kb)</td>
<td>HSV-tk</td>
</tr>
<tr>
<td></td>
<td>pMk(P)-luc</td>
<td>–</td>
<td>Midkine (0.6 kb)</td>
</tr>
<tr>
<td></td>
<td>pMk(P)-tk</td>
<td>–</td>
<td>HSV-tk</td>
</tr>
<tr>
<td>Two-step</td>
<td>Effector pPSMA(EP)-GAL/VP</td>
<td>PSMA (1.6 kb)</td>
<td>GAL4/VP16</td>
</tr>
<tr>
<td>Plasmid</td>
<td>pMk(P)-GAL/VP</td>
<td>Midkine (0.6 kb)</td>
<td>GAL4/VP16</td>
</tr>
<tr>
<td>Reporter</td>
<td>pRep-luc</td>
<td>–</td>
<td>5 × GAL4 bs + Minimal</td>
</tr>
<tr>
<td>Plasmid</td>
<td>pRep-tk</td>
<td>–</td>
<td>HSV-tk</td>
</tr>
</tbody>
</table>

All plasmids contained SV40 enhancer in downstream of expression gene. GAL4 bs, GAL4 binding site; HSV-tk, herpes simplex virus thymidine kinase; minimal, adenoviral E1B minimal promoter; Mk(P), midkine promoter; PSA, prostate-specific antigen; PSA(EP), PSA enhancer and promoter; PSA(P), PSA promoter; PSMA, prostate-specific membrane antigen; PSMA(EP), PSMA enhancer and promoter; PSMA(P), PSMA promoter.

Fig. 3. Prostate-specific antigen (PSA) or prostate-specific membrane antigen (PSMA) enhancer and promoter activities in the absence or presence of dihydrotestosterone (DHT) in LNCaP and PC-3 cells. The prostate cancer cell lines, PSA-positive and PSMA-positive LNCaP cells and PSA-negative and PSMA-negative PC-3 cells were transfected with the plasmids indicated (2 µg). The promoter and enhancer activities were determined with a luciferase expression assay: 100% luciferase activity was taken as that of the pGL3-control. The results were expressed as the mean ± SD (n = 4). Statistical significance of the data was evaluated using the Bonferroni/Dunn test. *P < 0.05.
synthetic promoter composed of $5 \times$ GAL4-binding sites and the adenoviral E1B minimal promoter with the SV40 enhancer (Table 1), and evaluated luciferase activity by conducting cotransfection assays in LNCaP and PC-3 cells.

Strong luciferase activity was observed when the combination of pPSMA(EP)-GAL/VP and pRep-luc was transfected into LNCaP cells (Fig. 4A), but not when either pPSMA(EP)-GAL/VP or pRep-luc alone was transfected into the cell lines (1.7% and 3.8% of pGL3-control, respectively; data not shown). The paired plasmid, pPSMA(EP)-GAL/VP and pRep-luc (13 500% of pGL3-control, equivalent to approximately 45 000 cps/µg protein) showed 85-fold and 640-fold more luciferase activity than pPSMA(EP)-luc and pPSMA(P)-luc, respectively, in LNCaP cells (Fig. 4A), but showed comparatively less promoter activity, approximately 61% of pGL3-control in PC-3 cells (Fig. 4B). Luciferase activity by the paired plasmid, pPSMA(EP)-GAL/VP and pRep-luc, in LNCaP cells was 2.7% of that by CMV promoter (data not shown). This suggested that the TSTA system induced strong activity in PSMA-positive LNCaP cells but comparatively less activity in PC-3 cells.

Two-step transcriptional amplification system using the Mk promoter
To examine the TSTA system using another promoter, we constructed Mk promoter-based plasmids, pMk(P)-luc and pMk(P)-GAL/VP coding for the luciferase gene and GAL4–VP16 fusion protein, respectively, under control of the Mk promoter and SV40 enhancer (Table 1). We cloned a DNA fragment for the Mk promoter stretching from bp 27 of exon 1 to bp 559 of the 5′ flanking region of the human Mk gene. The 2.3-kb genomic fragment in the 5′ region of the Mk gene contained the elements responsible for promoter activity. (33) The Mk enhancer is composed of two elements, which are located between bp $−1006$ and $−895$ and between bp $−901$ and $−794$. (34) However, Yoshida et al. reported that the transcriptional activity mediated by a fragment spanning bp $−559$ to $+50$ was stronger than that mediated by a fragment stretching from bp $−2285$ to 50. (13) Therefore, this fragment would be suitable for the Mk promoter. In Mk-positive PC-3 and HeLa cells, the paired plasmid, pMk(P)-GAL/VP and pRep-luc, showed 5.0-fold and 2.6-fold higher luciferase activity.
activities, respectively (270 000 and 22 400 cps/µg protein, equivalent to approximately 9100% and 5500% of the pGL3-control), than the plasmid pMk(P)-luc (54 000 and 8500 cps/µg protein, equivalent to approximately 1800% and 2100% of the pGL3-control) (Fig. 5). Luciferase activities by the paired plasmid, pMk(P)-GAL/VP and pRep-luc, in PC-3 and HeLa cells were 22.1% and 3.7%, respectively, of that by the CMV promoter, (data not shown). However, strong luciferase activity was not observed when either pMk(P)-GAL/VP (0.1% and 4.2% of pGL3-control in PC-3 and HeLa cells, respectively) or pRep-luc alone (8.1% and 11.1% of pGL3-control, respectively) was transfected into the cells (data not shown). The paired plasmids did not induce luciferase activity in LNCaP cells (Fig. 5), suggesting that the TSTA system with the Mk promoter did not induce promoter activity in Mk-negative LNCaP cells.

**In vitro suicide gene therapy model in PSMA-positive LNCaP cells and Mk-positive PC-3 and HeLa cells**

Next, we applied the TSTA system to suicide gene therapy with the HSV-tk gene, pPSMA(EP)-tk or a paired plasmid, pPSMA(EP)-GAL/VP and pRep-tk, was transfected into LNCaP and PC-3 cells, and the inhibitory effect on cell growth was investigated in the presence of various concentrations of GCV. pCMV-tk and pGL3-basic were used as positive and negative controls, respectively. In LNCaP cells, the paired plasmids significantly inhibited cell growth (Fig. 6A). However, pPSMA(EP)-tk did not inhibit cell growth, having a similar effect to pGL3-basic. The therapeutic effects were also tested in PSMA-negative PC-3 cells, and the paired plasmids showed no inhibitory effect (Fig. 6B). pCMV-tk exerted inhibitory effects on LNCaP and PC-3 cells (Fig. 6A,B). These results indicated that a combination of pPSMA(EP)-GAL/VP and pRep-tk would be better for gene therapy against PSMA-positive cells.

In PC-3 and HeLa cells, the paired plasmids pMk(P)-GAL/VP and pRep-tk showed significant inhibitory effects, but pMk(P)-tk did not (Fig. 7A,B). The paired plasmids showed very similar inhibition to pCMV-tk in PC-3 and HeLa cells. In Mk-negative LNCaP cells, the paired plasmids did not actually inhibit cell growth (Fig. 7C). It appears that a combination of pMk(P)-GAL/VP and pRep-tk would be better for gene therapy against Mk-positive cells.

**Western blot analysis**

We investigated whether the observed inhibitory effects in the TSTA system with the Mk promoter corresponded with the expression level of HSV-tk protein (Fig. 7D). HSV-tk expression on transfection of pCMV-tk was observed clearly in all cell lines. The paired plasmids pMk(P)-GAL/VP and pRep-tk more markedly expressed HSV-tk than pMk(P)-tk in PC-3 and HeLa cells. In Mk-negative LNCaP cells, the paired plasmid showed a similar expression level to pCMV-tk. In Mk-negative LNCaP cells, pMk(P)-tk and the paired plasmids did not induce HSV-tk expression.

**In vivo suicide gene therapy in PC-3 tumor xenografts**

The TSTA system with the Mk promoter in PC-3 cells (22% of CMV promoter) induced stronger luciferase activity than that with the PSMA promoter in LNCaP cells (2.7% of CMV promoter). Therefore, we evaluated the antitumor effect by direct injection of the nanoplexes into PC-3 tumor xenografts. The average growth rate of tumors was suppressed significantly in the mice treated with the nanoplex of the paired plasmid, pMk(P)-GAL/VP and pRep-tk, compared with the control mice (Fig. 8). The paired plasmid showed similar growth inhibition to pCMV-tk. These results indicated that the TSTA system with the Mk promoter could induce greater inhibition of tumor growth than one-step transcription with the Mk promoter.

**Discussion**

The activity of a prostate tumor-specific promoter is generally weak in comparison to that of a universal promoter such as the CMV promoter.(14,35) Therefore, in the present study, a TSTA system was used to enhance the efficacy of suicide gene therapy for treatment of prostate tumor. The TSTA systems showed greater activity than one-step transcription, as confirmed by the growth inhibition of PSMA-positive LNCaP cells and Mk-positive PC-3 cells, and PC-3 tumor xenografts on suicide gene therapy. This suggests that TSTA systems featuring tumor-specific promoters are suitable for cancer treatment by gene therapy.
The metastatic prostate cancer within an individual is composed heterogeneously of clones of both androgen-dependent and androgen-independent cancer cells. At present, the androgen-dependent and androgen-independent prostate cancer cells available are LNCaP and PC-3, respectively. PSMA mRNA was expressed in LNCaP cells, but not in PC-3 cells. Mk mRNA was expressed strongly in PC-3 cells, but not in LNCaP cells. These findings suggest that the PSMA promoter could be utilized with androgen-dependent prostate cancer (e.g., LNCaP cells), and the Mk promoter with androgen-independent prostate cancer (e.g., PC-3 cells) for tumor-specific gene therapy. The PSMA and Mk promoters have already been cloned and used for suicide gene therapy with the one-step transcription system. However, usage of the TSTA system without the PSA promoter has not been reported in prostate cancer.

In the present study, we modified the TSTA system for use with the SV40 enhancer in reporter and effector plasmids to amplify the transcriptional activity, and tried to apply the PSA or Mk promoter to the TSTA system. We confirmed that our cloned PSA or PSMA promoter and enhancer regions had prostate-specific transcriptional activity based on findings reported previously in which androgen-responsive LNCaP cells showed increasing PSMA promoter activity and decreasing PSA promoter activity when DHT was not added into the culture medium (Fig. 2). Thus, the PSMA promoter system seems to offer an advantage under conditions of androgen ablation.

In PSMA promoter-related plasmids, the TSTA system with the paired plasmids pPSMA(EP)-GAL/VP and pRep-luc amplified the expression of the luciferase gene 640-fold and 85-fold more than one-step transcription with pPSMA(P)-luc and pPSMA(EP)-luc, respectively, in LNCaP cells. However, the paired plasmids showed weak promoter activity in PC-3 cells (Fig. 4B), whereas PSMA mRNA could not be detected by RT-PCR analysis (Fig. 2). Recently, Laidler et al. reported that basic fibroblast growth factor (bFGF) and estradiol (E2) induce weak expression of PSMA in PC-3 cells. A growth factor such as bFGF is included in the serum for the culture medium. Therefore, in our study, the PSMA promoter activity in PC-3 cells may be induced weakly by bFGF or E2 in the culture medium and be amplified by the TSTA system. In Mk promoter-based plasmids, TSTA with the paired plasmids pMk(P)-GAL/VP and pRep-luc resulted in 5.0-fold and 2.6-fold higher luciferase activity than one-step transcription with pMk(P)-luc in PC-3 and HeLa cells (Fig. 5). The difference in the degree of amplification by the TSTA system between the PSMA and Mk promoters might be attributed to different transcriptional activity by the promoters.

In cancer gene therapy, the TSTA system with the PSA promoter used to drive the expression of expanded polyglutamine (ex-polyQ) for induction of apoptosis selectively eliminated PSA-positive LNCaP to an extent comparable with that of CMV promoter-driven ex-polyQ. The TSTA system with the CEA promoter used to control the expression of Bax for induction of apoptosis can suppress in vitro and in vivo tumor growth in lung cancer and colon adenocarcinoma. In the present study, we applied the TSTA system to suicide therapy with HSV-tk and demonstrated that the paired plasmids, pPSMA(EP)-GAL/VP and pRep-tk, and pMk(P)-GAL/VP and pRep-tk, enhanced cytotoxic activity in LNCaP cells, and in PC-3 and HeLa cells, respectively. In PSMA-positive LNCaP cells, the inhibitory effect was dependent on the concentration of GCV in the cells transfected with the paired plasmids, pPSMA(EP)-GAL/VP and pRep-tk (Fig. 6A). In PC-3 cells, the paired plasmids showed comparatively less
promoter activity (Fig. 4B); however, the activity was apparently non-toxic as long as the level of HSV-tk expression by the paired plasmid remained low (Fig. 6B). In PC-3 and HeLa cells, inhibitory effects were observed with paired plasmids, pMk(P)-GAL/VP and pRep-tk (Fig. 7A,B), but not in Mk-negative LNCaP cells (Fig. 7C). HSV-tk expression with the TSTA system with the Mk promoter was stronger than that with one-step transcription in PC-3 and HeLa cells, being equivalent to that with one-step transcription with the CMV promoter in PC-3 cells (Fig. 7D). Furthermore, in in vitro suicide gene therapy, the TSTA system with the Mk promoter suppressed the growth of PC-3 tumor xenografts greatly, compared with one-step transcription (Fig. 8). These characteristics can be used most powerfully in prostate cancer when combining the TSTA system with the PSMA or Mk promoter, in which strong expression is restricted to target cells, and would minimize the side-effects in non-targeted cells.

The TSTA system has been applied to gene therapy mediated by adenovirus,(20,21,40,41) lentivirus(42) and cationic liposomes.(15,16) Adenoviruses and lentiviruses can evoke non-specific inflammation, and readily induce strong antivirus immune responses. The mixing of cationic liposomes with DNA often results in large aggregated lipoplexes, which cannot be injected into blood vessels and yield very low levels of transfection efficiency.
in vivo. Recently, we developed a lipid-based nanoparticle that formed injectable-sized nanoplexes (200–300 nm). When the nanoplexes were injected directly into PC-3 xenografts, they showed approximately 6.5-fold higher luciferase activity than in vivo-Jet PEI-Gal, a commercially available cationic polymer transfection reagent (PolyPlus-transfection; ILLKIRCH, Illkirch, France; data not shown). Therefore, in the present study, we used this nanoparticle to induce strong expression in the TSTA system in vitro and in vivo. Furthermore, we reported recently that a folate-linked nanoparticle could deliver DNA with high transfection efficiency and selectivity into human nasopharyngeal and prostate cancer cells. The combination of this folate-linked nanoparticle with the TSTA system has great potential as a tumor-specific vector for in vivo cancer gene therapy.

In conclusion, we developed an ideal gene expression amplification system with the PSMA and Mk promoters. This system is a promising tool with which to create targeted gene-based therapeutic applications.

Acknowledgments

We thank Dr Kenji Moriyama (Kyoto University) for valuable advice. This project was supported in part by a grant from the Promotion and Mutual Aid Corporation for Private Schools of Japan, and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References


© 2006 Japanese Cancer Association