

## RESEARCH ARTICLE

# Intracellular production of DNA enzyme by a novel single-stranded DNA expression vector

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A set of single-stranded DNA (ssDNA) expression vectors, which can generate intracellularly any ssDNA or oligodeoxynucleotide (ODN) molecules, have been developed in our laboratory. Studies from our laboratory as well as our collaborators demonstrated that these ssDNA expression vectors are capable of producing: (1) 10-23 DNA enzyme for downregulating *c-raf* kinase gene expression and (2) triplex-forming oligodeoxynucleotide (TFO) for inducing genomic recombination. We report here the construction of a new version of ssDNA expression vector. A  $\beta$ -galactosidase ( $\beta$ -gal) reporter gene was used as a test target so that the alteration of gene expression can be easily measured using  $\beta$ -gal activity assay. We designed a 10-23 DNA enzyme molecule that specifically cleaves  $\beta$ -gal mRNA at protein translation starting site (ATG). Using a cell-free RNA cleavage assay, we confirmed that this DNA enzyme

molecule could effectively cleave  $\beta$ -gal RNA. However, a single substitution from T to G in the catalytic domain of this DNA enzyme molecule abolished its RNA cleavage activity. We also constructed an expression vector that can generate DNA enzyme molecules in cells. A549 lung carcinoma cells were cotransfected with both DNA enzyme expression vector and the  $\beta$ -gal reporter gene. Compared to the cells that were transfected with the mutated DNA enzyme expression vector, significant reduction of  $\beta$ -gal gene expression (up to 76%) was observed in the cells transfected with DNA enzyme expression vector as indicated by the protein expression level as well as its enzyme activity. These results further suggest that the ssDNA expression vector has potential applications in the study of gene function and target validation. Gene Therapy (2003) 10, 1776–1780. doi:10.1038/sj.gt.3302068

**Keywords:** single-stranded DNA expression vector; DNA enzyme; gene regulation;  $\beta$ -gal

## Introduction

In the last few years, there has been increasing interest in antisense oligonucleotides as tools for gene target validation and ultimately as potential therapeutic agents.<sup>1</sup> Oligonucleotides, such as antisense oligodeoxynucleotides (ODNs), ribozymes or triplex-forming oligodeoxynucleotides (TFOs), modulate gene expression by interacting with mRNA or genomic DNA in sequence-specific manners.<sup>2–4</sup> The completion of human genome sequencing project has created a tremendous opportunity for the development of oligonucleotide-based drugs since any nucleic acid sequence information can be used directly for the design of oligonucleotides.

Recently, several novel ODNs have been demonstrated to catalyze the direct cleavage of target mRNA.<sup>5</sup> These RNA-cleaving ODNs employ distinct catalytic mechanisms and require different kinds of cofactors such as magnesium, histidine or copper. One such catalytic ODN referred as 10-23 DNA enzyme has the potential to cleave almost any RNA target containing purine–pyrimidine junctions.<sup>6,7</sup> This 10-23 DNA enzyme, isolated by *in vitro* selection using a combinatorial ODN library, consists of a 15 deoxynucleotide catalytic domain flanked by two target RNA-binding domains of seven or eight deoxy-

nucleotides each.<sup>6,7</sup> Unlike antisense ODNs that mainly rely on cellular Ribonuclease H activity to destroy target mRNA, the 10-23 DNA enzyme is responsible for both target recognition and cleavage of target mRNA.<sup>6,7</sup> This 10-23 DNA enzyme has been evaluated in a number of studies both *in vitro* and *in vivo* for the suppression of various target gene expression and has shown promising results.<sup>8</sup>

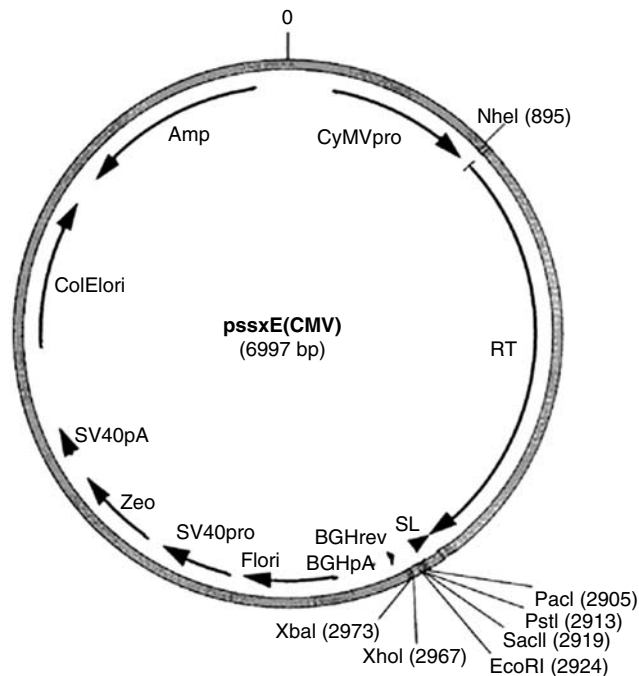
Instead of delivering oligonucleotides externally into cells, we have developed a vector-mediated approach to produce ODNs or single-stranded DNA (ssDNA) molecules intracellularly.<sup>9</sup> In our first-generation ssDNA expression vector, two plasmids were utilized to produce *c-raf* mRNA-cleavage DNA enzyme molecules in cells.<sup>9</sup> This first-generation expression system has also been successfully applied to generate TFO molecules to induce genomic recombination.<sup>10</sup> Since both plasmids need to be present within a desired cell simultaneously, potentially resulting in low copy numbers of ssDNA expression, we then constructed the second-generation ssDNA expression vector by integrating all the essential components into a single plasmid construct.<sup>11</sup> Chen *et al*<sup>11</sup> demonstrated that this single vector system was able to generate sufficient copies of DNA enzyme molecules to reduce the level of *c-raf* mRNA in cells significantly.<sup>11</sup> Furthermore, suppression of *c-raf* gene expression induced cell apoptosis as indicated by increasing genomic DNA fragmentation in cells, which agrees with the observation by others using an antisense approach.<sup>12,13</sup>

In this study, we report the construction of an improved version of single vector system. We tested this new ssDNA expression vector for generating DNA enzyme molecules that specifically cleaves  $\beta$ -gal mRNA at protein translation starting site (ATG). We found that this new vector could generate enough DNA enzyme molecules to suppress  $\beta$ -gal gene expression in cells up to 76%.

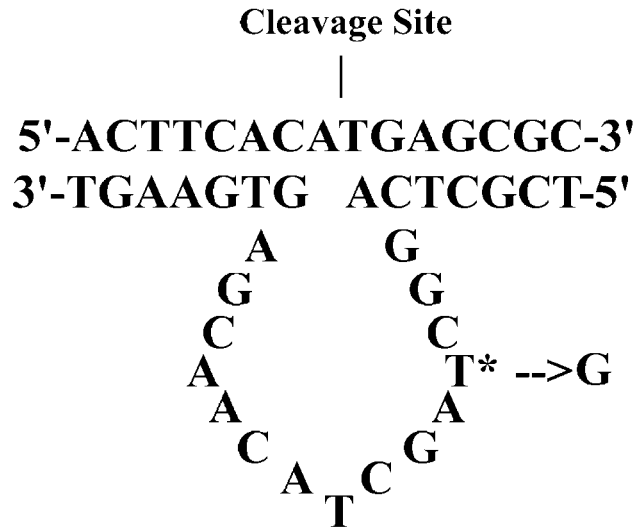
## Results

### Construction of the pssXE ssDNA expression vector

In our earlier study, we have developed a two-vector as well as a single vector ssDNA expression system.<sup>9,11</sup> We demonstrated that *c-raf* mRNA-cleaving 10-23 DNA enzyme molecules could be produced using both ssDNA expression systems and DNA enzyme molecules generated in cells could suppress the level of *c-raf* mRNA.<sup>9,11</sup> In this new pssXE construct, instead of using the rous sarcoma virus (RSV) promoter, a stronger cytomegalovirus (CMV) promoter was inserted. All the essential elements are transcribed into a single RNA transcript (Figures 1 and 2) that includes: (1) a mouse Moloney leukemia viral reverse transcriptase (MoMuLV RT) gene coding for a truncated but fully active RT;<sup>14</sup> (2) a primer binding site (PBS) along with some flanking regions of the promoter that are essential for the reverse transcription initiation by MoMuLV RT;<sup>15</sup> (3) a coding sequence



**Figure 1** Design of the ssDNA expression vector, pssXE. RT: reverse transcriptase; SL: stem-loop structure; PBS: RT primer binding site. The coding sequence for  $\beta$ -gal RNA-cleavage DNA enzyme (DZ7) was inserted into Pacl and EcoRI sites after annealing two ODNs, 5'-Gal1(Pac/Eco) (5'-TAAACTTCACTCGTTGTAGCTAGCCTGAGCGAG) and 3'-Gal1(Pac/Eco) (5'-AATTCTCGCTCAGGCTAGCTACAACGAGTGAAGTTAAT). A control plasmid that codes for a mutated DNA enzyme, DZ7m, was similarly constructed. The sequences of the two ODNs coding for DZ7m are 1. 5'-Gal1m(Pac/Eco) (5'-TAAACTTCACTCGTTGTAGCTCGCTGAGCGAG) and 2. 3'-Gal1m(Pac/Eco) (5'-AATTCTCGCTCAGGCGAGCTACAACGAGTGAAGTTAAT).



**Figure 2** Design of the DNA enzymes and sequence of binding area of template  $\beta$ -gal RNA. Cleavage occurs at the position indicated by arrow. Substitution of T by G in the catalytic domain causes a loss of catalytic activity.

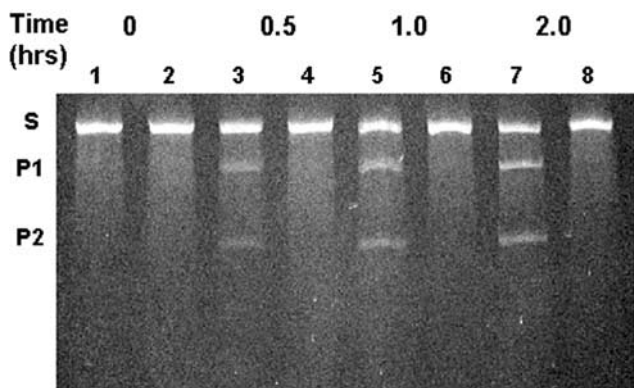
for  $\beta$ -gal RNA-cleaving DNA enzyme and (4) a stem-loop structure designed for the termination of the reverse transcription reaction. RT expressed in cells uses endogenous tRNA<sup>PRO</sup> as a primer, which binds to the PBS on the 3' end of the RNA transcript, for ssDNA synthesis.<sup>16</sup> After reverse transcription, ssDNA is released when the template mRNA is degraded either by endogenous RNase H or the RNase H activity of RT.<sup>14</sup>

### In vitro cleavage of template $\beta$ -gal RNA

Before examining the pssXE vector for the production of DNA enzyme molecules in cells, we investigated the activity and specificity of a synthetic DNA enzyme molecule, DZ7, which was designed to specifically target the  $\beta$ -gal protein translation site (ATG) (Figure 2), in a cell-free system. A 950 nt  $\beta$ -gal RNA, produced by *in vitro* transcription, was used to test the cleavage activity of DZ7. The cell-free cleavage assay was performed with 0.1  $\mu$ M  $\beta$ -gal RNA and 100  $\mu$ M DZ7 or control DZ7m in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 150 mM NaCl and 10 U RNasin at 37°C. As shown in Figure 3, DZ7 effectively cleaved  $\beta$ -gal RNA to produce the products with the expected sizes (650 nt and 300 nt) (Figure 3). It has been reported that the 10-23 DNA enzyme is almost completely intolerant of sequence changes within the catalytic domain<sup>7</sup> and a single substitution from T to G in the catalytic domain of DNA enzyme abolishes target RNA cleavage activity.<sup>17</sup> As shown in Figure 3, DZ7m with the same substitution had no cleavage activity. These results indicate that DZ7 is an active and target-specific DNA enzyme.

### DZ7-mediated suppression of $\beta$ -gal expression in cells

Since DZ7 was shown to cleave  $\beta$ -gal RNA in a cell-free assay (Figure 3), we then constructed an expression vector that could generate DZ7 DNA enzyme molecules in cells. Both DZ7 and DZ7m coding ODNs were inserted into Pacl and EcoRI sites of the pssXE vector and the resulting plasmids were designated as pssXE( $\beta$ -gal) and pssXE( $\beta$ galm), respectively. A549 cells were

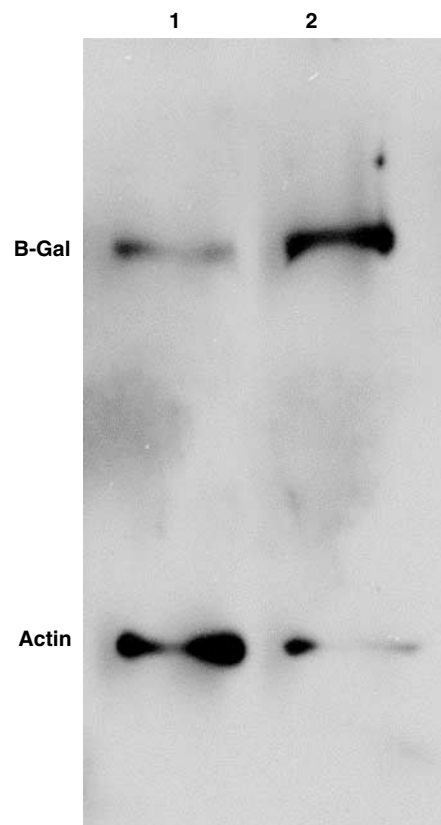


**Figure 3** *In vitro* RNA cleavage assay. *In vitro* cleavage assay was performed by incubating 0.1  $\mu$ M of template  $\beta$ -gal RNA, prepared by *in vitro* transcription, and 100  $\mu$ M DZ7 or DZ7m in a solution containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 150 mM NaCl and 10 U RNasin at 37°C for 0.5, 1, or 2 h. The reactions were stopped by adding 83 mM EDTA and then analyzed on 8 M urea 10% polyacrylamide gels. S: substrate; P1 and P2: products. lanes 1, 3, 5, 7, DZ7; lanes 2, 4, 6, 8, DZ7m.

cotransfected with pssXE(bgal) or pssXE(bgalm) along with a pSV  $\beta$ -gal vector at a ratio of 3/1(w/w). Cell lysates were prepared 72 h after transfection. As shown in Figure 4, DZ7 generated by pssXE vector significantly reduced the level of  $\beta$ -gal protein compared to the cells transfected with pssXE(bgalm). In order to determine the effect of gene downregulation more quantitatively, a  $\beta$ -gal enzyme activity assay was performed. Compared to the cells that were transfected with pssXE(bgalm), reduction of  $\beta$ -gal activity (63, 76 or 73% after 24, 48 or 72 h, respectively) could be observed in cells expressing DZ7 molecules (Figure 5).

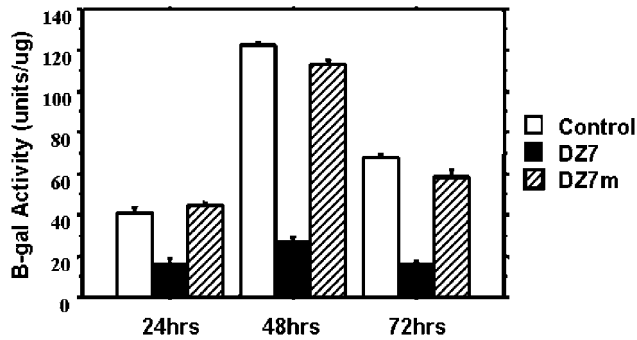
## Discussion

Although a range of viral vectors has been designed to express ribozymes or antisense RNA molecules endogenously in target cells, the application of ODNs, mainly antisense ODNs, has been restricted to exogenous delivery.<sup>18</sup> Inouye *et al* have utilized a bacterial retron system to generate ssDNA as a form of msDNA in bacteria, yeast and mammalian cells.<sup>19–21</sup> Retrons, isolated from Gram-negative bacteria such as *Myxococcus xanthus*, *Stigmatella aurantiaca* and *Escherichia coli*, are genetic elements responsible for the synthesis of multi-copy single-stranded DNA (msDNA).<sup>22</sup> msDNA molecules are actually complexes of DNA and RNA. The single-stranded DNA fragment of these peculiar molecules synthesized by a reverse transcriptase is linked to an internal guanosine residue of its RNA fragment by a 2',5'-phosphodiester linkage.<sup>22</sup> Data from Inouye's group demonstrated that antisense ODNs generated as forms of msDNA in bacteria can effectively inhibit the expression of a lipoprotein gene in *E. coli*.<sup>19</sup> However, using a similar approach, only limited copies (estimated about 25) of msDNA molecules were generated in mammalian cells.<sup>21</sup> It is unlikely that these limited copies of msDNA could have any effects on gene expression in mammalian cells. This may be because of the lower efficiency of bacteria reverse transcriptase in eucaryotic cells. Moreover, the potential side effects of the unique structure of msDNA are still unknown.



**Figure 4** Western blot analysis. A549 cells were transfected with 1.5  $\mu$ g of pssXE, pssXE(bgal) or pssXE(bgalm) along with 0.5  $\mu$ g of pSV-galactosidase control vector using GenePORTER 2 transfection reagent as directed by the manufacturer. At 72 h after transfection, cells were lysed with 0.1 M phosphate buffer (pH 7.5) containing 0.02% Triton X-100. Aliquots of cell lysates, containing about 20  $\mu$ g of protein, were resolved by 15% SDS-polyacrylamide gel electrophoresis and proteins were then transferred to Hybond ECL nitrocellulose membrane. The membrane was first incubated with the blocking buffer (25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20 and 5% nonfat milk) for 1 h at room temperature and then probed with antibody against  $\beta$ -galactosidase (1:5000) and antibody against actin (1:10 000). Subsequently, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:10 000) and signal was detected using the SuperSignal West Pico chemiluminescence substrate kit from Pierce according to the manufacturer's instructions. Lane 1: pssXE(bgal) transfected cells; lane 2: pssXE(bgalm) transfected cells.

The ssDNA expression vectors we developed have the potential to generate any ssDNA molecules or ODNs in cells. In this study, we identified a RNA-cleaving DNA enzyme molecule, DZ7, which specifically targets the  $\beta$ -gal protein translation site (ATG) using a cell-free RNA cleavage assay. We then demonstrated that a new version of the ssDNA expression vector with CMV promoter was capable of generating RNA-cleaving DNA enzyme molecules in cells to effectively suppress a  $\beta$ -gal reporter gene expression. Moreover, gene suppression remains at high level (approximately 70%) as long as 72 h in our transient transfection assay. The low efficacy of DZ7 in the cell-free RNA cleavage assay compared to the results in whole cells is likely because of the different folding structure that makes *in vitro* transcribed target RNA less accessible to DNA enzyme molecules. Since the ssDNA expression vector we constructed expresses high level of reverse transcriptase in transfected cells, the potential deleterious effects remains to be investigated.



**Figure 5**  $\beta$ -gal activity assay. Cell lysates were prepared similarly as described in the legend of Figure 4 after cells were transfected with 1.5  $\mu$ g pssXE, pssXE(bgal), or pssXE(bgalm) along with 0.5  $\mu$ g pSV-galactosidase control vector.  $\beta$ -Gal activity was quantified using *p*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG) as a substrate. Cell extracts (50  $\mu$ g protein) were mixed with 0.01 M phosphate buffer (pH 7.5) containing 0.1 M MgCl<sub>2</sub>, 0.01 mM ONPG and 45 mM  $\beta$ -mercaptoethanol. After incubation for 1 h at 37°C, absorbance at 410 nm was measured by spectrophotometry.

In conclusion, the results demonstrated here as well as our earlier data<sup>10–12</sup> suggest that this ssDNA expression technology may offer an important research tool in gene function study and has potential applications in gene target validation and drug development.

## Materials and methods

### Plasmid construction

The new version of ssDNA expression vector used in this study was constructed based on the early version of the expression vector systems as described.<sup>10,11</sup> Both pssXB<sup>10</sup> and pssXD<sup>11</sup> were double-digested with restriction enzymes, *NheI* and *XhoI*. The *NheI*–*XhoI* DNA fragment obtained from pssXD that contains all the essential components for ssDNA synthesis was then ligated into the *NheI*- and *XhoI*-digested pssXB vector. The new plasmid was designated pssXD(CMV). In order to insert multiple cloning site<sup>a</sup> (MCS), the pssXD(CMV) vector was double-digested with restriction enzymes, *PacI* and *XhoI*, and the resulting *PacI*–*XhoI* fragment was replaced with a double-stranded ODN linker which was formed by annealing two ODNs, 5'E/S/P/P-linker (5'-TCGA GCGCCAGGGTCTCCCGATCCCGGACGAGCCCC CAAAGAATTCCGCGGCTGCAGTTAAT) and 3'E/S/P/P-linker (TAACTGCAGCCGCGGAATCTTTGGGG GCTCGTCCGGGATCGGGAGACCCCTGGCCGC) (Integrated DNA technologies, Inc., Coralville, IA, USA). This double-stranded ODN linker contains several unique subcloning sites including *EcoRI*, *SallI*, *PstI*, and *PacI* as well as nucleotides that were removed from the pssXD(CMV) by *PacI* and *XhoI*, double-digestion. The newly created ssDNA expression vector was designated as pssXE.

A 10-23 DNA enzyme sequence was designed to specifically target the translation starting site (ATG) of  $\beta$ -gal mRNA (see below). The DNA enzyme coding ODN inserts were prepared by annealing two ODNs, 5'-Gal1(Pac/Eco) (5'-TAACTTCACTCGTTGTAGCTAGCTGAGCGAG) and 3'-Gal1(Pac/Eco) (5'-AATTCTCGCTCAGGCTAGCTACAACGAGTGAAGTTAAT). The double-stranded ODNs were then ligated into *PacI* and

*EcoRI* sites of the pssXE vector. The new plasmid was designated as pssXE(bgal). A control plasmid that codes for a mutated DNA enzyme, pssXE(bgalm), was similarly constructed. The sequences of the two ODNs coding for the mutated DNA enzyme are: 1. 5'-Gal1m(Pac/Eco) (5'-TAACTTCACTCGTTGTAGCTCGCTGAGCGAG) and 2. 3'-Gal1m(Pac/Eco) (5'-AATTCTCGCTCAGGCGAGCTACAACGAGTGAAGTTAAT).

### In vitro RNA cleavage assay

To prepare the template  $\beta$ -gal RNA for *in vitro* cleavage assay, a 950 bp  $\beta$ -gal gene fragment was amplified from a pSV-galactosidase control vector (Promega Corp., Madison, WI, USA) by PCR. The sequences of two PCR primers corresponding to nucleotides 193–212 and 1141–1150 were 5'bgal(t)*EcoRI* (5'-GGAATTCCAGCAGGCA GAAGTATGCAA) and 3'bgal(t2)*BamHI* (5'-CGGGAT CCGTTCACCCACAGATGAAACG). *EcoRI* and *BamHI* restriction sites were introduced so that the amplified fragment could be digested and subcloned into *EcoRI* and *BamHI* sites of pTRLamp18 vector (Ambion, Austin, TX, USA). The new construct, designated as pTRLamp18(b-gal) was linearized and used in an *in vitro* transcription reaction to prepare template  $\beta$ -gal RNA using MEGAscript kit (Ambion, Austin, TX) according to the manufacturer's instructions.

An *in vitro* cleavage assay was carried out according to Kurreck *et al.*<sup>23</sup> with some modifications. Briefly, template  $\beta$ -gal RNA (0.1  $\mu$ M), produced by *in vitro* transcription as described above, and DNA enzyme (100  $\mu$ M), DZ7, which specifically binds to the translation starting site (ATG) of  $\beta$ -gal mRNA (5'-TCGCTCAGGCGAGCTA CAACGAGTGAAGT-3') or a mutated DNA enzyme, DZ7m (5'TCGCTCAGGCGAGCTACAACGAGTGAAG T-3'), were incubated in a solution containing 50 mM Tris-HCl, pH 7.5, 10 mM MgCl<sub>2</sub>, 150 mM NaCl and 10 units RNasin (Promega, Madison, WI) at 37°C for various periods of time. The reactions were stopped by adding 83 mM EDTA and then analyzed on 8 M Urea 10% polyacrylamide gels.

### Cell culture and transfection

Human A549 lung carcinoma cells were obtained from the American Type Tissue Collection (ATCC). A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, USA), 100 U/ml penicillin G sodium and 100  $\mu$ g/ml streptomycin sulfate (Invitrogen, Carlsbad, CA, USA) and incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator.

A549 cells were seeded in six-well plates at a density 1  $\times$  10<sup>5</sup> cells per well the day before transfection. GenePORTER 2 transfection reagent (Gene Therapy System, San Diego, CA, USA) was used for cell transfection as directed by the manufacturer. Briefly, 1.5  $\mu$ g of pssXE, pssXE(bgal), or pssXE(bgalm) along with 0.5  $\mu$ g of pSV-galactosidase control vector (Promega Corp., Madison, WI, USA) were diluted with 50  $\mu$ l of DNA diluent B and then incubated at room temperature for 5 min. DNA solution was then mixed with 7  $\mu$ l of GenePORTER 2 reagent and incubated for another 5 min at room temperature to form GenePORTER 2/DNA complexes. These GenePORTER 2/DNA complexes were added directly to the cells that were in serum-free DMEM medium. After 4 h, serum-free medium was

replaced with regular DMEM medium in addition to 30  $\mu$ l of Booster 3. After incubation for various periods of time, cells were washed once with PBS and lysed with 0.1 M phosphate buffer, pH7.5 containing 0.02% Triton X-100. Cell debris was removed by centrifugation at 12,000 g for 10 min. Protein concentration of cell lysates was determined using DcProtein Assay kit from Bio-Rad (Richmond, CA, USA) according to the manufacturer's instructions.

#### Western blot analysis

Aliquots of cell lysates, containing about 20  $\mu$ g of protein, were resolved by 15% SDS-polyacrylamide gel electrophoresis and proteins were then transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was first incubated with the blocking buffer (25 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween-20 and 5% nonfat milk) for 1 h at room temperature and then probed with monoclonal antibody against  $\beta$ -galactosidase (1:5000) (Promega Corp., Madison, WI, USA). Subsequently, the membrane was incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:10 000) (Pierce, Rockford, IL, USA) and the signal was detected using a SuperSignal West Pico chemiluminescence substrate kit from Pierce (Rockford, IL, USA) according to the manufacturer's instructions. The same blot was also probed with antibody against actin (1:10 000) (Calbiochem, San Diego, CA, USA).

#### $\beta$ -Gal activity assay

$\beta$ -Gal activity was quantified using a substrate, *p*-nitrophenyl  $\beta$ -D-galactopyranoside (ONPG). Briefly, cell lysates (50  $\mu$ g protein) were mixed with 0.01 M phosphate buffer (pH 7.5) containing 0.1 M MgCl<sub>2</sub>, 0.01 mM ONPG and 45 mM  $\beta$ -mercaptoethanol. After incubation for 1 h at 37°C, absorbances at 410 nm were measured by spectrophotometry.

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