A novel two-step transcriptional activation system for gene therapy directed toward epithelial cells

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Abstract
The two-step transcriptional activation (TSTA) mechanism in gene therapy amplifies cell type–specific promoter activity, allowing for increased levels of gene expression in target tissues. In this system, the specific promoter drives expression of a strong transcriptional activator that binds to DNA target sequences located upstream from a second promoter controlling the expression of the therapeutic gene. The majority of previous studies have exploited a fusion between the DNA binding domain of the yeast transcriptional activator Gal4 fused to the VP16 activation domain of herpes simplex virus 1 as the transcriptional activator. In this report, an alternative to this system is described based on a fusion protein containing the DNA binding domain of the bovine papillomavirus 1 transcriptional activator E2 fused to VP16 that induces target gene expression following binding to a minimal bovine papillomavirus 4 promoter containing upstream E2 binding sites and only 3 bp of promoter sequence upstream from the TATA box. VP16-E2 is superior to Gal4-VP16 as the transcriptional activator in a TSTA system driven by either of the two potentially cancer-specific promoters telomerase RNA and telomerase reverse transcriptase in several cell lines. Results also suggest that this new system has an advantage in epithelial cells and is therefore ideal for potential targeting of carcinomas. By incorporating the TRAIL gene as a transgene in the VP16-E2 TSTA system, selective killing of telomerase-positive cells occurs. We propose that our new system should be considered in future TSTA, particularly when targeting epithelial-derived cells. [Mol Cancer Ther 2009;8(12):3244–54]

Introduction
One of the key properties of any successful gene therapy is to specifically express the delivered gene in the target cell type while restricting expression in nontarget cells. For example, therapeutic gene expression can be targeted to cancer cells by using promoters shown to be more active in those cells than normal cells (1). Although many gene promoters have cell type–specific transcriptional activity and have been proposed for gene therapy, the activity of these promoters is often too low to induce expression of a delivered gene at a therapeutic level. To overcome this limitation, while retaining the specificity of the promoter, two-step transcriptional activation (TSTA) systems have been developed for gene therapy. This technology involves exploiting a tissue- or disease-specific promoter with specificity to the target cell type to drive expression of a strong transcriptional activator that in turn binds to target sequences upstream from a promoter-driven expression of the therapeutic gene.

The transcriptional activator that is commonly used in TSTA systems is a fusion between the DNA binding domain of the yeast transcription factor Gal4 (2) and the VP16 transcriptional activation domain from herpes simplex virus 1 (3). Combined with prostate-specific promoters, Gal4-VP16 has been used in TSTA systems to improve imaging of reporter genes and to improve delivery of therapeutic genes to prostate lines and tissues (4–8), and results show that the incorporation of the two-step technology greatly enhances expression of the reporter/therapeutic gene compared with using the prostate promoters alone. Gal4-VP16 has been used to target additional disease tissues in TSTA protocols using other promoters, such as the carcinoma-embryonic antigen promoter for targeting colorectal and lung cancer cells (9), the vascular endothelial growth factor promoter to target expression to cells involved in wound healing (10), and the tumor-specific survivin promoter to direct gene expression to cancer cells (11).

Alternative Gal4 fusion proteins have also been used in TSTA systems. A fusion with a part of the NF-κB p65 protein has targeted gene expression to neuronal cells in combination with the human SYN promoter (12) and for delivery of therapeutic genes for the treatment of diabetes (13). In addition, alternative transcriptional activators have been used for the development of TSTA systems; for example, thyroid transcription factor 1 driven by the human telomerase reverse transcriptase (hTERT) promoter and binding to thyroid transcription factor 1 target sequences driving expression of reporter genes have been used to show selective activity in lung cancer cells (14). However, the majority of TSTA systems use Gal4 fusion proteins.

Several steps have been taken to improve the Gal4-VP16 system. Increasing the number of Gal4 target sequences used in the TSTA system can improve therapeutic gene delivery of reporter genes to prostate lines and tissues (4–8), and results show that the incorporation of the two-step technology greatly enhances expression of the reporter/therapeutic gene compared with using the prostate promoters alone. Gal4-VP16 has been used to target additional disease tissues in TSTA protocols using other promoters, such as the carcinoma-embryonic antigen promoter for targeting colorectal and lung cancer cells (9), the vascular endothelial growth factor promoter to target expression to cells involved in wound healing (10), and the tumor-specific survivin promoter to direct gene expression to cancer cells (11).

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expression; however, this effect plateaus at a certain number of Gal4 binding sites. Novel bidirectional promoter systems have also been used for gene delivery (11, 15). In such a system, the Gal4-VP16 activator is used to activate two promoters driving expression of a reporter gene and an independent therapeutic gene: the reporter gene can be used to estimate delivery, whereas the therapeutic gene is used for the treatment of the target cell type. Increasing the number of VP16 domains fused to the Gal4 DNA binding domain can also improve the TSTA system (6) with the optimum number of VP16 domains reported to be two; however, multiple shorter VP16 repeat sequences have also shown to improve efficacy and to decrease squelching in TSTA systems. To move the TSTA systems into more clinically relevant delivery vehicles, several studies have combined viral delivery vectors with the TSTA system. For example, a lentiviral vector has been developed for the delivery and analysis of bidirectional TSTA systems (16), although more commonly adenovirus vectors have been used (8, 9, 11, 14, 17–19).

What is consistent in almost all TSTA systems is that the Gal4 DNA binding domain is used in the activating fusion protein. The Gal4 protein has not evolved to operate in mammalian cells as it is derived from an intracellular yeast protein. Here, we present an alternative fusion protein based on a viral transcription factor designed to operate in mammalian cells, which is better than Gal4 in a TSTA system. The protein is the DNA binding domain of the bovine papillomavirus type 1 (BPV1) E2 protein fused to the VP16 activation domain. This protein recognizes 12-bp palindromic target sequences in the promoter regions of papillomavirus genomes and regulates transcription (20). Using the human telomerase RNA (TR) and TERT promoters (21) to drive expression of the transcriptional activators, our results show that VP16-E2 is better than Gal4-VP16 in a TSTA system in telomerase-positive cell types. In telomerase-negative cells, the TR promoter is more active than the TERT promoter, and in telomerase-positive cells, there is not much difference between the two promoters, suggesting more specificity by using the TERT promoter for this application. The optimization of this new TSTA system by incorporating E2-responsive promoters more active in epithelial cells into the system along with the TERT promoter is discussed (22, 23). Finally, we describe efficient and selective cell killing of telomerase-positive cells with incorporation of the full-length tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) gene into this new TSTA system.

Materials and Methods

Plasmids

Reporter Plasmids. The CMV-VP16E2 vector contains the cytomegalovirus (CMV) promoter, which transcribes a fusion protein made up by amino acids 410 to 487 of the full-length VP16 protein and 161 amino acids of the BPV1 E2 gene including the full-length DNA binding domain. This plasmid was a gift from Dr. Mart Ustav (Estonian Biocenter). The pTK-5xGal4-6xE2-Luc vector is based on pTK6E2Luc (22), with the five Gal4 sites from pG5Luc inserted immediately upstream from the E2 sites. This plasmid was kindly prepared by Dr. Roni Wright (Gene Regulation Program, Center for Genomic Regulation, Barcelona, Spain) by cloning five Gal4 binding sites, which were amplified by PCR from the pG5Luc vector, into the pTK6E2Luc vector using the KpnI and Nhel restriction sites. The p6xE2-3bp-Luc vector has been described previously (23).

TERT- and TR-Driven Transcriptional Activator Plasmids. The plasmids pLhTERT 19 and pLh2023 (24) were digested with XbaI and HindIII to remove the luciferase gene, and the TERT- and TR-containing vector fragments were purified from agarose gel. The VP16E2 sequence was amplified from the CMV-VP16 plasmid by PCR using primers generating XbaI and HindIII sites at the 5′ and 3′ ends of the resultant PCR products. The PCR products were digested with XbaI and HindIII and then ligated into the cut pHlTERT 19 and pHl2023 using the XbaI and HindIII sites to create pTERT-VP16E2 and pTR-VP16E2, respectively. The hTERT-Gal4VP16 and hTR-Gal4VP16 were cloned in a similar fashion using the pBind-Gal4VP16 plasmid as a template for the Gal4VP16 insert. pBind-Gal4VP16 is based on the pBind vector containing the Gal4 DNA binding domain (Promega). The VP16 sequence was inserted downstream from the Gal4 DNA binding encoding sequence using a BamHI- and KpnI-digested PCR product prepared from CMV-VP16.

Incorporation of the TSTA System into a Single Plasmid. The p6xE2-3bp-TERT-VP16E2 forward and p6xE2-3bp-TERT-VP16E2 reverse plasmids were prepared as follows. The p6xE2-3bp-Luc vector was cut with BamHI to linearize the vector and then phosphatased. A hTERT-VP16E2 PCR product with 5′ and 3′ ends containing BglII restriction sites was generated and digested with BglII and then ligated into the BamHI-digested p6xE2-3bp-Luc, creating p6xE2-3bp-TERT-VP16E2 forward and p6xE2-3bp-TERT-VP16E2 reverse.

Construction of TRAIL Expression Plasmids. The TERT-containing vectors pCMV-TRAIL and p6xE2-3bp-TRAIL were generated as follows. pcDNA 3.1 (Invitrogen) and p6xE2-3bp-Luc vectors were digested with XbaI and HindIII (which removes the luciferase coding sequence from p6xE2-3bp-Luc), and both vectors were purified by agarose gel electrophoresis. The full-length TRAIL coding sequence was PCR amplified from the pORF-hTRAIL vector (Invitrogen) with XbaI and HindIII ends, digested with the appropriate enzymes, and then ligated into the cut pcDNA 3.1 and p6xE2-3bp-Luc, creating pCMV-TRAIL and p6xE2-3bp-TRAIL, respectively. The p6xE2-3bp-TERT-VP16E2 forward was generated by linearizing p6xE2-3bpTRAIL with BamHI followed by ligation of the TERT-VP16E2 sequence using BglII digestion of a TERT-VP16E2-encoding PCR product generated with BglII restriction sites.

All PCRs were done using a Kod Hot Start proofreading polymerase (Merck Biosciences). Correct clones were determined by either sequencing or restriction digest or both. Ligations were done using the TAKARA 2.1 ligation kit.
(Cambrex Bioscience) according to the manufacturer’s instructions. Transformations were all done using JM-109 chemical competent cells.

Cell Growth

All cell lines were grown in DMEM/10% FCS, 1% penicillin, and streptomycin solution at 37°C with 5% CO2. Cells were trypsinized and split 1 in 10 when they reached 90% confluency. The telomerase status of all cells was investigated and cells were as predicted from many previous studies (data not shown).

Transfection of Cells

Cells (2 x 10^5; 3 x 10^5 for MRC-5 and C33a cells) were plated in a 60-mm tissue culture dish with growth medium and incubated for 24 h to allow cells to adhere and grow. 293-T, C33a, MRC-5, and U2OS (for transcription assays) cells were transfected using calcium phosphate (22, 23). GM847, MCF7, and U2OS [for terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)] were transfected with TransIT LT1 Transfection Reagent according to the manufacturer (Cambridge Bioscience Ltd.).

Western Blot

Transfected cells were harvested 40 h after transfection using trypsin followed by two washes in PBS. Cell lysates were prepared by resuspending cells pellets in 100 μL lysis buffer [0.5% NP40, 150 mmol/L NaCl, 50 mmol/L Tris (pH 8.0) containing 1:25 volume dissolved protease inhibitor cocktail tablet from Roche Molecular Biochemicals]. Protein levels in lysates were determined using a bicinchoninic acid/CuSO4 assay (Promega). Sample volumes containing equal amounts of protein were separated by SDS-PAGE gel electrophoresis and transferred to a nitrocellulose membrane using a semidry blotter at 10 V for 1 h. The membrane was placed in blocking solution [PBS with 0.1% Tween 20 and 5% (w/v) skimmed milk powder] for 1 h. The membrane was incubated with primary antibody [rabbit polyclonal IgG anti-TRAIL (ab2435, diluted 1:200; Abcam) and rabbit polyclonal IgG anti-VP16 tag (ab4808, diluted

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Figure 1. VP16-E2 is a more potent transcriptional activator than Gal4-VP16. A, a representation of the sequences used to drive VP16-E2 and Gal4-VP16 expression and the reporter gene used to monitor the transcriptional activation properties of the two proteins. B, a titration of the TERT-driven VP16-E2 and Gal4-VP16 plasmids was assayed for transcriptional activation of the luciferase reporter shown in A in 293T cells. C, a titration of the TERT-driven VP16-E2 and Gal4-VP16 plasmids was assayed for transcriptional activation of the luciferase reporter shown in A in MCF7 cells. D, a titration of the TERT-driven VP16-E2 and Gal4-VP16 plasmids was assayed for transcriptional activation of the luciferase reporter shown in A in C33a cells. All transcription results are presented as fold activation over the activity generated by the luciferase reporter by itself and are normalized to protein concentration. All experiments were carried out at least three times in duplicate. Bars, SE. A numerical summary of the transcription results is shown in Table 1A. The P values shown represent the result of t test analysis on at least three independent experiments.
1:1,500; Abcam]) diluted in blocking buffer for 1 h at room temperature. The membrane was washed with PBS with 0.1% Tween 20 and incubated with secondary antibody (goat anti-rabbit IgG, peroxidase conjugated, A6154, diluted 1:5,000; Sigma) for 1 h. The membrane was then washed with PBS with 0.1% Tween 20. The proteins were detected using ECL-Plus (Amersham Biosciences) followed by exposure of the membrane to film.

**TUNEL Staining**

U2OS and 293-T cells (2 x 10^5) were plated in six-well plates, in which a glass coverslip was placed in each well. For the 293-T cells, the coverslips were treated with poly-L-lysine. Twenty-four hours after plating, U2OS and 293-T cells were transfected using TransIT and calcium phosphate, respectively. Twenty-four hours later, cells on coverslips were fixed in 4% formaldehyde in PBS. TUNEL staining of the cells was done using the DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's protocol for adherent cell lines. After staining, coverslips were mounted and counterstained using Vectashield mounting medium with 4',6-diamidino-2-phenylindole stain (Vector Laboratories). Stained coverslips were visualized using a fluorescent microscope, and five random pictures were taken of cells. The number of 4',6-diamidino-2-phenylindole–stained and fluorescein-stained cells for each exposure was quantified and the percentage of 4',6-diamidino-2-phenylindole–stained cells, which were also fluorescein stained, was determined. The statistical significance of the difference in fluorescein staining between groups in each experiment was determined using Yates corrected χ^2 analysis.

**Transcription Assays**

Transfected cells were harvested ∼40 h after transfection using Reporter lysis buffer (Promega); 300 μL was added directly onto the adherent cells that were washed twice with PBS. Luciferase activity of the cell lysates was determined after transferring 80 μL of each sample to an opaque microtiter plate, and luciferase activity was monitored using the Luciferase Assay System substrate (Promega) by reading the plate in a Luminoskan Ascent Luminometer and Ascent

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**Figure 2.** A comparison of the potency of the TERT and TR promoters for driving transcriptional activation by VP16-E2 and Gal4-VP16 in telomerase-positive cells. A, a titration of the TERT- and TR-driven VP16-E2 and Gal4-VP16 plasmids was assayed for transcriptional activation of the luciferase reporter shown in Fig. 1A in 293T cells. B, a titration of the TERT- and TR-driven VP16-E2 and Gal4-VP16 plasmids was assayed for transcriptional activation of the luciferase reporter shown in Fig. 1A in MCF7 cells. C, a titration of the TERT- and TR-driven VP16-E2 and Gal4-VP16 plasmids was assayed for transcriptional activation of the luciferase reporter shown in Fig. 1A in C33a cells. All transcription results are presented as fold activation over the activity generated by the luciferase reporter by itself and are normalized to protein concentration. All experiments were carried out at least three times in duplicate. Bars, SE. A numerical summary of the transcription results is shown in Table 1A. The P values shown represent the result of t test analysis on at least three independent experiments; only those values < 0.05 are shown with TR versus TERT for Gal4 and E2. D, a Western blot detecting VP16-E2 and Gal4-VP16 following transfection into 293T cells. Bottom, results obtained with the VP16 antibody; top, γ-tubulin, which serves as a loading control. Lane 1, pCG-VP16E2 (1,000 ng); lane 2, pCG-VP16E2 (100 ng); lane 3, pTERT-VP16E2 (1,000 ng); lane 4, pTERT-VP16E2 (100 ng); lane 5, pTR-VP16E2 (1,000 ng); lane 6, pTR-VP16E2 (100 ng); lane 7, pTERT-Gal4VP16 (1,000 ng); lane 8, pTERT-Gal4VP16 (100 ng); lane 9, pTR-Gal4VP16 (1,000 ng); lane 10, pTR-Gal4VP16 (100 ng); lane 11, nontransfected control.
software (Thermo Labsystems Corp.). The luciferase expression readings were adjusted by accounting for protein content in each sample by doing a bicinchoninic acid/CuSO4 assay and dividing the luciferase activity by the protein concentration. All transcription assays were done a minimum of three times in duplicate. As a positive control in luciferase assays, cells transfected with 1 μg GP G LV3 - RV - RVNCConnection were always included.

Results

VP16-E2 Is a Better Transcriptional Activator than Gal4-VP16 in the TSTA System

To compare the abilities of the VP16-E2 and Gal4-VP16 proteins in a TSTA, they were placed under transcriptional control of the potentially cancer-specific TERT promoter. A luciferase reporter gene was used for these assays driven by the thymidine kinase promoter from herpes simplex virus 1 with six E2 and five Gal4 DNA binding sequences inserted upstream in the same plasmid to allow comparison (Fig. 1A). A constant amount of the reporter plasmid (1 μg) was cotransfected with increasing amounts of the TERT-VP16 fusion protein expressing plasmids into several telomerase-positive cell lines: 293T (Fig. 1B), the breast cancer line MCF7 (Fig. 1C), and the cervical cancer line C33a (Fig. 1D). All of the results from Fig. 1 and those from Fig. 2 are given numerical value in Table 1. In 293T cells at 1 and 10 ng of transfected activator plasmid, VP16-E2 is a better activator than Gal4VP16, becoming statistically significant at 10 ng (from now on the VP16-E2 fusion will be referred to as E2 and the Gal4VP16 as Gal4, referring to the different DNA binding domains used in the two fusion proteins). This benefit is lost at higher activator concentrations, with both proteins activating transcription to a similar degree. However, in the two carcinoma cell lines, E2 is better than Gal4 at all plasmid concentrations, being 8- and 10-fold better at the highest concentrations in MCF7 and C33a, respectively. In these latter lines, the E2 protein increases the activation levels with increasing plasmid concentrations, whereas the Gal4 activation plateaus. This suggests that increasing the amount of expression plasmid could increase the differential between the two fusion proteins even further.

P values show that E2 is statistically better than Gal4 at activation in these two carcinoma cell lines. Throughout these experiments, a CMV-driven VP16-E2 fusion has been used as a control; this is a strong promoter and is highly active at low plasmid concentrations. However, at higher TERT E2 plasmid levels, the transcriptional activation became comparable with the CMV promoter-driven protein. The TERT promoter is used in most gene therapy protocols to drive expression in telomerase-positive cells (24, 25), although the TR promoter has also been used (26). The preference for TERT relates to the suggested more promiscuous expression of the TR gene, although the TR promoter has been proposed as the stronger of the two (24). To compare both of these promoters, identical experiments to those shown in Fig. 1 were carried out with TR-driven expression of the E2 and Gal4 fusion proteins. Figure 2 shows the results in comparison with the TERT results in Fig. 1, whereas

Table 1. A numerical summary of transcription results shown graphically in Figs. 1, 2, and 3

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<td>1,000 ng</td>
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<td>VP16-E2</td>
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<td>129.3 ± 59.1</td>
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NOTE: (A) Numerical summary of the transcriptional activation by VP16-E2 and Gal4-VP16 in telomerase-positive cells shown graphically in Figs. 1 and 2. (B) Numerical summary of transcriptional activation mediated by VP16-E2 driven by the TERT and TR promoters in telomerase-negative cells shown graphically in Fig. 3.
Table 1A summarizes the results numerically. In Fig. 2, only significant differences between the TR and TERT promoters with Gal4 and E2 are highlighted for the sake of clarity. In 293T cells at the lowest plasmid concentration, the TR promoter is statistically better than TERT with E2 (Fig. 2A). In MCF7 cells at a variety of concentrations, the TR promoter is statistically better at activation than TR and this is true for E2 and Gal4 (Fig. 2B), whereas in C33a cells there is no statistical difference between TR and TERT at any plasmid concentrations with E2 or Gal4 (Fig. 2C).

To investigate protein levels produced from the TERT- and TR-driven plasmids, 1 μg and 100 ng of the plasmids were transfected into 293T cells and harvested 48 hours later. Cell lysates were prepared and Western blotted using a VP16 antibody and the results are shown in Fig. 2D. In lanes 1 and 2, pCMV-VP16-E2 was used and, as expected due to the strong promoter, high levels of protein expression are detected. It is also clear, whether using the TERT or TR promoter, that the E2 fusion protein is better expressed than the Gal4 fusion. This is a possible explanation for the differences in transcriptional activity that are summarized in Table 1 and shown in Figs. 1 and 2. To control for protein loading, expression of γ-tubulin was also detected and also shown in Fig. 2D.

Comparing the TERT and TR Promoters in Telomerase-Negative Cells

The results in Figs. 1 and 2 suggest that the TR promoter would be the better promoter to choose for the TSTA, as it shows increased levels of activation at low plasmid concentrations. However, this does not take account specificity, and therefore, the ability of TR- and TERT-driven E2 to activate transcription in a TSTA system in telomerase-negative cell lines was tested. In these experiments, only the E2 fusion protein was used, as Figs. 1 and 2 show the superiority of this protein over Gal4. A further modification was the introduction of another promoter-driven expression of the luciferase gene. The thymidine kinase promoter was ideal for making comparisons between the E2 and Gal4 fusions but is less ideal for gene therapy as it has low to medium transcriptional activity in a lot of cell types. In the experiments described in Fig. 3, the thymidine kinase promoter was replaced by a minimal promoter from BPV4 that we have previously shown to have no activity in the absence of E2 (23). This promoter has only 3 bp upstream from the TATA box, and the lack of activity in the absence of E2 is advantageous in gene therapy. For example, if the TSTA was being used to deliver a suicide gene to a telomerase-positive cancer cell, the lack of expression from the 3-bp promoter would ensure no suicide gene expression in telomerase-negative cells. Therefore, this promoter adds another level of selectivity to the TSTA. In addition, the BPV4 promoter is preferentially responsive to transcriptional activators in epithelial cells (22, 23) and therefore, in combination with the epithelial-derived E2 transcription factor, will provide optimum activity in epithelial cells.

In the GM847 and U2OS cells, the TR promoter gives higher activity at all levels of input plasmid, whereas in MRC-5 cells there is very little difference between the TR and TERT

Figure 3. A comparison of the potency of the TERT and TR promoters for driving transcriptional activation by VP16-E2 in telomerase-negative cells. A, a titration of the TERT- and TR-driven VP16-E2 plasmids was assayed for transcriptional activation of the p6xE2-3bp-luciferase reporter (23) in GM847 cells. B, a titration of the TERT- and TR-driven VP16-E2 plasmids was assayed for transcriptional activation of the p6xE2-3bp-luciferase reporter (23) in U2OS cells. C, a titration of the TERT- and TR-driven VP16-E2 plasmids was assayed for transcriptional activation of the p6xE2-3bp-luciferase reporter (23) in MRC-5 cells. All transcription results are presented as fold activation over the activity generated by the luciferase reporter by itself and are normalized to protein concentration. All experiments were carried out at least three times in duplicate. Bars, SE. A numerical summary of the transcription results is shown in Table 1B. The P values shown represent the result of t test analysis on at least three independent experiments.

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promoters; none of the differences were statistically significant (Fig. 3). Taken together with the results in Figs. 1 and 2, it was decided that the TERT promoter would be taken forward for further development of the TSTA system as it shows robust activity in telomerase-positive cells while having a more restricted expression than the TR promoter in telomerase-negative cells, although statistical analysis suggests there is no significant difference between the TR and TERT promoters in the telomerase-negative cell lines. These properties allow selective levels of reporter/therapeutic gene expression to be delivered to telomerase-positive cells. The results in the telomerase-negative cells are summarized in Table 1B.

Incorporation of the TSTA System into a One Plasmid

To further develop the TSTA system, the 6E2-3bp-Luc unit was combined with the TERT-VP16-E2 unit in one plasmid. They were joined together “head to tail” (p6xE2-3bp-TERT-VP16E2 forward) or “head to head” (p6xE2-3bp-TERT-VP16E2 reverse) as shown in Fig. 4A. The activities of these two plasmids were assayed in 293T cells and the results are shown in Fig. 4B. The reverse version gave higher activity than the forward one, but these differences were not statistically significant. At the highest level of input plasmid (800 ng), the fold induction in reporter gene activity generated is similar to the highest plasmid levels in the two-plasmid system (Fig. 1) and similar to the levels induced by VP16-E2 driven by the CMV promoter. To confirm that this system generates increased reporter activity when compared with the TERT promoter by itself, p6xE2-3bp-TERT-VP16E2 forward was compared with pTERT-Luc where the TERT promoter is driving expression of luciferase. The results of this experiment are shown in Fig. 4C using 1 μg of input plasmid, and as predicted, there is increased activity of p6xE2-3bp-TERT-VP16E2 forward; it generates a 34-fold increase in activity when compared with pTERT-Luc, giving a statistically significant difference. These results show successful incorporation of the TSTA system into one plasmid and that this system is significantly better at inducing reporter gene expression than the TERT promoter by itself. To reconfirm the superiority of E2 over Gal4, an identically designed Gal4 one-plasmid system incorporating Gal4 sites
upstream from the 3-bp promoter was compared with p6xE2-3bp-TERT-VP16E2 reverse and the results are shown in Fig. 4D. Clearly, the E2-containing vector is a better activator than Gal4 in this system.

**Cell Killing Induced by the Novel TSTA System**

The intention of most gene therapy for cancer is to kill the transformed cells while not harming nontransformed cells. This is generated using selective promoters such as TERT linked to genes that directly kill cells and suicide genes or by the production of oncolytic viruses. To test the cell killing potential of our new system, we incorporated the full-length TRAIL gene into our TSTA plasmid, generating p6xE2-3bp-TERT-VP16E2-TRAIL as shown in Fig. 5A. This plasmid was transiently transfected into telomerase-positive (293T) and telomerase-negative (U2OS) cell lines, and apoptosis was measured using TUNEL assays 24 hours later. These cell lines were chosen as they were readily transfectable, and the TUNEL assay was chosen as it is quantitative. Several controls were included in these experiments: pCMV-TRAIL uses the strong CMV promoter to drive TRAIL expression, pGL3 contains no TRAIL gene but simply the luciferase gene, and p6E2-3bp-TRAIL contains the TRAIL gene driven by the 3-bp promoter but no TERT-VP16-E2. The results of these experiments are shown in Fig. 5B and summarized in Table 2, with the numbers from the individual experiments shown and the results of Yates corrected $\chi^2$ for each experiment shown. In 293T cells, pCMV-TRAIL and p6xE2-3bp-TERT-VP16E2-TRAIL induce statistically significant increases in apoptosis when compared with p6E2-3bp-TRAIL and pGL3, showing that the TSTA system
is functional at inducing apoptosis and indeed does so to levels similar to that induced by a CMV-driven TRAIL. In telomerase-negative U2OS cells, pCMV-TRAIL is again efficient at inducing apoptosis, but p6xE2-3bp-TERT-VP16E2-TRAIL does not induce cell death above that of background controls pGL3 and p6E2-3bp-TRAIL. This is due to the low TERT promoter activity in this cell type–driven expression of the TRAIL gene (see Fig. 3 for reporter gene activity). The conclusions from these experiments are that both 293T and U2OS are sensitive to cell death following TRAIL expression and that the TSTA system can induce cell death in telomerase-positive cells while having no effect in telomerase-negative cells. Therefore, this TSTA system tightly restricts cell death to the target telomerase-positive cell type. Of note is the observation that in U2OS cells with 1,000 ng of pTERT-VP16 (Fig. 3B), there is significant luciferase activity detected, but here, we detect no cell killing with 1,000 ng of p6xE2-3bp-TERT-VP16E2-TRAIL. This suggests that there may be a threshold of TRAIL expression required for induction of cell killing.

To evaluate the TRAIL expression in the one vector system, a Western blot was carried out. 293-T cells were transfected with 1 μg of CMV-TRAIL, p6E2-3bp-TRAIL, p6xE2-3bp-TERT-VP16E2-TRAIL, or pGL3 and a Western blot was carried out using a primary antibody against TRAIL. As shown in Fig. 5C, TRAIL is expressed in cells transfected with CMV-TRAIL and p6xE2-3bp-TERT-VP16E2-TRAIL, but no expression is detected in the pGL3-transfected or p6E2-3bp-TRAIL–transfected cells, confirming the tight regulation of this system.

Discussion

This report describes a new TSTA system using a transcriptional activator, VP16-E2, demonstrably better than the standard used in these systems, Gal4-VP16. Both fusion proteins have their transcriptional activation domain replaced with VP16 in the same part of the protein as their natural activation domains: NH2 terminus to the DNA binding domain for E2 and COOH terminus for Gal4. TSTA systems have been developed for use in gene therapy to amplify transcription from cell type–specific promoters, and in this report, we have used the TERT and TR promoters, both of which show increased activity in cancer cells due to the telomerase activity in these cells and have been used extensively in gene therapy protocols. The amplification of the TERT promoter activity using the TSTA system described is evident from Fig. 4C; the activity with the TERT promoter by itself is amplified ~30-fold when incorporated into the TSTA system, showing the potential of the system for gene therapy delivery. Clearly, the E2 fusion with VP16 is better than the Gal4 fusion as shown in Figs. 1, 2, and 4D. One of the reasons for this may be related to the increased expression of the E2 fusion in the cells under study (Fig. 2D); this is perhaps not surprising as the E2 protein must operate in a mammalian cell type, whereas Gal4 is an intracellular yeast protein not optimized for function in mammalian cells. Figure 1 also suggests that the E2 fusion is much better than Gal4 in epithelial cells; in the nonepithelial 293T cells, E2 and Gal4 both reach similar levels of transcriptional activation when high levels of expression plasmid are used in the transcription assays. However, in the epithelial MCF7 and C33a cells, the E2 protein is better at all plasmid levels tested, and noticeably, the Gal4 activation plateaus, whereas the E2 results show an increasing activation with increasing levels of expression vector. Although this has only been done in one nonepithelial cell line, the results do suggest preferential activity in epithelial cells. Three important conclusions can be drawn from these experiments: first, at low levels of activator (represented by low levels of transfected plasmid), E2 is much better than Gal4 in all cell types; second, in the 293T cells, Gal4 matches E2 at high plasmid levels, showing that the failure of Gal4 to match E2

Table 2. Statistical analysis of the TUNEL assays

<table>
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<tr>
<th></th>
<th>pGL3-Basic</th>
<th>pCMV-TRAIL</th>
<th>p6xE2-3bp-TRAIL</th>
<th>p6xE2-3bp-TRAIL-TERT-VP16E2</th>
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<tbody>
<tr>
<td></td>
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<td>U2OS 293-T</td>
<td>U2OS 293-T</td>
<td>U2OS 293-T</td>
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<td>Experiment 1</td>
<td></td>
<td></td>
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<tr>
<td>Yates P</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Live/apoptotic</td>
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<td>5,792/24</td>
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<td>0.000</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>4,207/7</td>
<td>1,012/71</td>
<td>4,979/84</td>
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<tr>
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<td>4,301/10</td>
<td>2,451/322</td>
<td>4,009/4</td>
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<td>ND 6,655/170</td>
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</table>

NOTE: This table shows the results from each individual experiment and the P value generated in each experiment was determined using the Yates corrected χ² test. This test is ideal for data sets when there are a few changes in large populations as observed in the TUNEL assays. Abbreviation: ND, not determined.
transcriptional activity in the epithelial cells is not simply due to a failure of the Gal4 to activate the promoter due to the different location of the Gal4 DNA binding sites in the promoter; and third, the E2 protein shows enhanced activity in epithelial cells when compared with Gal4. The natural target cell type for E2 function is epithelial, and therefore, this protein will be optimal in these cells, making it an ideal protein for TSTA systems targeting epithelial-diseased tissue such as carcinomas.

The expression of telomerase in cancer cells has made the telomerase promoters obvious tools for targeting gene therapy to cancers. The promoters for the two components required for telomerase activity, the TERT enzyme and the TR, have therefore been used in cancer-directed gene therapy. Both of these promoters were tested in the TSTA system in telomerase-positive cells as shown in Fig. 2. The TR promoter is better than TERT in these assays, particularly at low levels of transfected plasmid.

To decide whether to develop the TSTA using the TERT or TR promoter, the activity of these promoters with E2 was tested in telomerase-negative cell lines. An alternative promoter-driven luciferase was used in these assays; this promoter contains only 3 bp of the BPV4 promoter upstream from the TATA box with six E2 DNA binding sites upstream from this (23). This promoter has minimal activity in the absence of E2 activator proteins, putting another level of control into the TSTA system; if the TERT/TR promoter is not activated, no E2 will be expressed, and therefore, no transcription will occur from the 3-bp promoter. This is important for restricting transgene expression in nontarget cells (in this case, those with no or low TR or TERT promoter activity). In two of three telomerase-negative cells, the TR promoter gave higher levels of activity than TERT (Fig. 3) and this is perhaps indicative of the TR promoter being more promiscuous than TERT in telomerase-negative cells. The RNA component of telomerase seems to be more widely expressed than the TERT enzyme, and therefore, the failure of a cell to express telomerase activity may be controlled more by the level of TERT than the TR component, and this is perhaps reflected in the assays reported here. Due to the TR promoter activity in the telomerase-negative cells, and the fact there was not a huge difference between the two promoters in telomerase-positive cells, we elected to develop the TSTA system based on the TERT promoter.

Figure 4 shows the incorporation of the TERT-driven TSTA system into one plasmid. In Fig. 5, the reporter luciferase gene is replaced by TRAIL and the ability to kill telomerase-positive cells is measured. When TRAIL is driven by the CMV promoter, there are high levels of apoptosis in both telomerase-positive 293T cells and telomerase-negative U2OS. The 3-bp-driven TRAIL gene does not induce an increase in cell death over background in either cell line, and this shows that the low level of transcriptional activity from this promoter is ideal for selective delivery of gene therapy. When the TSTA plasmid is used, there is clearly killing of the telomerase-positive 293T cells but no increase in killing of the telomerase-negative U2OS cells. This shows that our TSTA system has no background killing activity when TERT promoter activity is relatively low. The TRAIL protein we have used in our TSTA system consists of the full-length coding region of the TRAIL gene. This full-length TRAIL construct can induce apoptosis in a broader cell population than the very commonly used recombinant soluble TRAIL. Western blot of our TRAIL-transfected cells also confirmed apoptosis, as there was cleavage of caspase-3 (data not shown) backing up the TUNEL assays.

This report describes a new TSTA system that should be adapted for use in gene therapy, particularly when targeting epithelial cells. Much can be done to improve this system, such as increase the number of E2 binding sequences in the promoter, increase the stability of the E2 protein (25), and increase the number of VP16 domains in the E2 fusion, which has been shown to enhance the activity of the Gal4-VP16 fusion (6). We propose that this new system should be adopted in future TSTA experiments to improve gene therapy both for the delivery of therapeutic genes and also to assist with in vivo imaging in gene therapy trials.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References
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A novel two-step transcriptional activation system for gene therapy directed toward epithelial cells

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