An Adenovirus Vector Containing the Suicide Gene Thymidine Kinase for a Broad Application in Cancer Gene Therapy

GS Magalhães, AR Muotri, MCN Marchetto, CFM Menck, AM Ventura+

Departamento de Microbiologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes 1374, 05508-900 São Paulo, SP, Brasil

Treatment of cancer using gene therapy is based on adding a property to the cell leading to its elimination. One possibility is the use of suicide genes that code for enzymes that transform a pro-drug into a cytotoxic product. The most extensively used is the herpes simplex virus thymidine kinase (TK) gene, followed by administration of the antiviral drug ganciclovir (GCV). The choice of the promoter to drive the transcription of a transgene is one of the determinants of a given transfer vector usefulness, as different promoters show different efficiencies depending on the target cell type. In the experiments presented here, we report the construction of a recombinant adenovirus carrying TK gene (Ad-TK) driven by three strong promoters ($P_{CMV\ IE}$, SV40 and EN1) and its effectiveness in two cell types. Human HeLa and mouse CCR2 tumor cells were transduced with Ad-TK and efficiently killed after addition of GCV. We could detect two sizes of transcripts of TK gene, one derived from the close together $P_{CMV\ IE}$/SV40 promoters and the other from the 1.5 Kb downstream EN1 promoter. The relative amounts of these transcripts were different in each cell type thus indicating a higher flexibility of this system.

Key words: ganciclovir - adenovirus - thymidine kinase - cancer - gene therapy

Insertion of genes that activate pro-drugs to produce cytotoxity in tumor cells, is considered a potential therapeutic strategy for cancer treatment. One specific cytotoxic gene therapy approach is viral transduction of the herpes simplex virus (HSV)-thymidine kinase (TK) gene, followed by treatment with the antiviral drug, ganciclovir (GCV) (Moolten 1986). GCV is a good substrate for viral TK, but is not recognized by the mammalian analogous enzyme (Ellion et al. 1977). Viral TK phosphorylates GCV to the monophosphate form, which is then converted to the triphosphate form by cytoplasmic enzymes. GCV triphosphate, when incorporated into replicating DNA, stops chain elongation and results in cell death. In addition, GCV treatment exerts a bystander effect, as it causes also the death of the neighboring untransduced cells (Ezzeddine et al. 1991, Freeman et al. 1993, Samejima & Meruelo 1995).

For several reasons such as stability of the particle and broad range of target cells, adenoviruses are attracting increasing attention as expression vectors, especially for human gene therapy (Berkner 1992). The adenovirus vectors that have been used for this purpose carry the transgenes replacing the E1 region from their genome, since its deletion renders the viruses defective for replication and incapable of producing infectious viral particles in target cells. These vectors are able to grow only in HEK 293 cells, which supply E1 products *in trans* (Graham et al. 1977). Adenovirus vectors with the TK gene have been used extensively in a variety of tumor cells in vitro and in vivo (Chen et al. 1994, Su et al. 1997, Sterman et al. 1998, Herman et al. 1999, Trask et al. 2000). Since it has been reported that the cell type affects the transcription from a given promoter (Everett 1988, Shillitoe & Noonan 2000), different promoters should be considered when an efficient expression has to be achieved.

Differently from the previous adenovirus vectors that have only one promoter (Chen et al. 1994, Rosenfeld et al. 1995, Park et al. 1997, Anderson et al. 1999), we report here the construction of an adenoviral vector harboring three different strong promoters to drive TK gene expression: the human cytomegalovirus immediate early promoter ($P_{CMV\ IE}$), the SV40 early promoter and the EN1 promoter (Ventura et al. 1990, Ventura & Villa 1993). This Ad-TK was tested in two distinct tumor cell lines, human HeLa and the mouse CCR2. This is an effort to improve the efficiency of TK gene expression regardless of the tumor cell target since when one promoter is weak, one of the others could properly drive the transcription of TK gene to achieve a better level of expression.

MATERIALS AND METHODS

Cell lines, medium, and viruses - HeLa and HEK 293 cells (Graham et al. 1977) were purchased from the American Type Culture Collection, and CCR2 (NIH3T3 transformed by *EJras*) was described previously (Jasiulionis et al. 1996). HEK 293 cells were grown in Minimum Essential Medium (MEM, Life Technologies, Inc., USA), HeLa and CCR2 cells in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies, Inc., USA), both supplemented with 10% fetal calf serum (Cultilab, Brazil) and gentamicin (400 µg/ml). The cells were maintained at 37°C in a 5% CO_2 atmosphere. As a control, a recombinant adenoviral vector encoding the *Escherichia coli* β-galactosidase gene under the control of cytomegalovirus promoter/enhancer (Ad-βgal) was utilized (obtained from Généthon III – Service Assurance Qualité, France).

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+Corresponding author. Fax: +55-11-3091.7354. E-mail: amventur@icb.usp.br
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Enzymes and primers - Restriction endonucleases (except *PI-SceI* and *I-CeuI* which were provided together with Adeno-X^{TM} Expression System-Clontech, USA), T4 DNA ligase, Taq DNA polymerase, and proteinase K were from either New England Biolabs or Boehringer Mannheim (USA). The TK gene forward (F), reverse (R) and internal primers (synthesized by Life Technologies, Brazil) were drawn based on sequence data obtained by McKnight (1980). Their sequences and respective positions according to that data are presented below.

TKF - 5'TATGGCTTCGTACCCCGGCC3' (308-328)
TKR - 5'CTCCTTCCGTGTTTCAGTTAGC3' (1432-1453)
TKR1 - 5'CCATTGTTATCTGGGCGC3' (676-694)
TKR2 - 5'CAGGTCCAGCCGCTCG3' (980-996)
TKR3 - 5'TGCATGGAACGGAGGCG3' (1261-1287)
TKF1 - 5'TGGGGGCTTCCGAGAC3' (580-596)
TKF2 - 5'TGGCCCTCATCCCGCC3' (880-896)

Plasmids construction - TK gene was amplified via PCR from the plasmid pMC19TK kindly provided by Dr Mario Capechi (Mansour et al. 1988) with the primers TKF and TKR. Amplification was performed during 30 cycles of 94^oC (1 min), 57^oC (1 min) and 72^oC (1 min 30 sec), after an initial 5 min at 94^oC to denature input DNA, with a final 5 min at 72^oC for strand extension. The 1200 bp product was purified and cloned into the *EcoRV* site of pSH plasmid (Ventura & Villa 1993) that contains SV40 and EN1 promoters, generating pSHTK. TK gene sequence was confirmed, after cloning, by automatic sequencing with the ABI prism® Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems, USA) using the primers described above (TKF, TKR, TKR1, TKR2, TKR3, TKF1, TKF2 and TKF3) and ABI PRISM^{TM} 310 Genetic Analyzer (Applied Biosystems, USA). No mismatch by the PCR cloning was observed in the TK gene.

The insertion of the TK gene, SV40 and EN1 promoters in pShuttle (Clontech Inc., USA) was done with an intermediate vector, pBI-TK, constructed by sub-cloning TK gene and the two promoters (*XhoI-NotI* fragment) into pBI-L (Clontech Inc., USA). In this construct, *XbaI* sites flank the cassette. *XbaI* digested pBI-TK was used to transfer TK and promoters to pShuttle, resulting in the vector pShuttleTK. In this construct, TK gene is under the control of three promoters ($P_{CMV\ IE}$, SV40 early and EN1).

Characterization of pSHTK clones - CCR2 cells were co-transfected with both pSHTK (10 µg) and pSV40-neo (1 µg) using Lipofectin (Life Technologies, Inc., USA) according to manufacturer's instructions. After 48 h the cells were treated with 400 µg/ml of Geneticin (Life Technologies, Inc., USA) in growth media to select the clones. The clones obtained were incubated in growth media containing different concentrations (12.5, 25 and 50 µg/ml) of GCV. DNA from the sensitive clones was extracted and submitted to PCR as described previously. RNA was extracted according to the acid guanidinium thiocyanate-phenol-cloroform method (Chomczynski & Sacchi 1987) and Northern blot (Sambrook et al. 1989) was performed using Hybond N$^+$ nylon membranes (Amersham, USA), and hybridized with a [^{32}P] labeled probe of 1200 bp generated by PCR of pSHTK using the TKF and TKR primers.

Generation of recombinant adenoviral plasmid - Recombinant adenovirus vector expressing TK downstream of three promoters (SV40, EN1 and $P_{CMV\ IE}$) was constructed using the Adeno-X^{TM} Expression System (Clontech Inc., USA), following manufacturer's instructions, which exploits the ligation method (Mizuguchi & Kay 1998). Briefly, the pShuttle-TK was digested with *PI-SceI* and *I-CeuI* to release the expression cassette containing the three promoters (SV40, EN1 and $P_{CMV\ IE}$), TK gene and the bovine growth hormone polyadenylation signal (BGA poly A). This DNA fragment was subsequently ligated to the pAdeno-X, digested with the same enzymes to generate pAdeno-XTK. After ligation it was digested with *Swa I* restriction enzyme to avoid the occurrence of non-recombinant plasmids and used to transform *E. coli* DH5-α, clones bearing ampicilin resistance were selected. Plasmids obtained were screened by digestion with the restriction enzymes *Xho I*, *EcoR I* and *Xba* I.

Ad-TK production - Approximately 10^6 HEK 293 cells were plated in 25 cm^2 flasks 24 h before transfection, by which time they reached 60-70% confluency. After that, 2.5 µg of pAdeno-XTK, digested with *PacI*, were transfected using the calcium phosphate co-precipitation technique (CalPhos^{TM} Mammalian Transfection kit, Clontech Inc., USA) according to the manufacturer's instructions. Upon evidence of extensive cytopathic effect the cells were harvested, submitted to three freeze and thawing cycles and centrifuged at 2,000Xg for 10 min. The supernatant was collected and viral titer determined by plaque assay in HEK 293 cells. The presence of TK gene was accessed by Southern blot (Sambrook et al. 1989). Viral DNA was isolated (Hitt et al. 1998) and digested with *XbaI*, separated on a 0.8% agarose gel, transferred onto a Hybond N$^+$ nylon membrane (Amersham, USA) and hybridized with a [^{32}P] labeled probe of 680 bp generated by PCR from pSHTK using the TKF1 and TKR3 primers.

Adenovirus infection and ganciclovir treatment - HeLa and CCR2 cells were plated 24 h prior to infection, with all experiments being performed when cells reached a confluency of 70-80%. The infection procedures were carried out with the recombinant adenoviruses (Ad-TK and Ad-βgal) diluted in DMEM without serum and antibiotics for 1 h at 37^oC. Complete growth media containing GCV (25 µg/ml) was then added to the cell culture. After 4 days the cells were fixed and stained.

Analysis of TK gene transcripts after tranduction with Ad-TK - Hela and CCR2 cells (10^6 cells in 75cm^2 flasks) were infected with 2 pfu/cell of Ad-TK. After 48 h RNA was extracted and a Northern blot performed as described above, with a [^{32}P] labeled probe of 680 bp generated by PCR from pSHTK using the TKF1 and TKR3 primers. Quantification of the probe radioactivity hybridized in the TK transcripts was done by exposition of the bloted Nylon membrane in a PhosphorImager Storm 840 (Molecular Dynamics, USA).

RESULTS

TK gene expression from pSHTK - Prior to the construction of Ad-TK it was necessary to verify if the subcloned TK gene sequence was correct and the expressed

gene working properly. The vector pSHTK, constructed as described in Materials and Methods, has the TK gene driven by the EN1 and SV40 promoters. pSHTK and pSV40-neo (which carries a eucariotic resistance marker) were co-transfected into CCR2 and clones were selected with geneticin (see Materials and Methods). All clones obtained were submitted to ganciclovir treatment and among them two showed sensitivity. The best GCV concentration was 25 µg/ml, which did not show any apparent toxic effect to the control cells and was effective to kill cells harboring TK gene (data not shown). The presence and expression of TK gene in the GCV sensitive clones was also assessed by PCR and northern blot respectively (data not shown).

Construction of pAdeno-XTK vector - The overall strategy developed here is diagrammed in Fig. 1 and involves three steps. To begin with, the TK gene and promoters SV40 and EN1 were transferred from pSHTK to pBI-TK, and then to pShuttle vector, which contains the human cytomegalovirus immediate early promoter ($P_{CMV\ IE}$), in such a way that the TK gene was placed downstream of the three promoters (see Materials and Methods). Next, the entire expression cassette, which in pShuttleTK is flanked by unique *PI-SceI* and *I-CeuI* sites, was excised using these enzymes and ligated into the pAdeno-X vector digested with the same enzymes. The new vector contains a different resistance marker (ampicilin) from pShuttleTK (kanamycin), preventing pShuttleTK recovery. The candidate clones were screened by digestion with appropriate restriction endonucleases. In our experience over 70% of the ampicilin resistant clones obtained carried the sought-after recombinant adenoviral plasmid.

Obtaining Ad-TK adenovirus in HEK293 cells - The plasmid pAdeno-XTK contains two *PacI* restriction sites, which are located at both ends of the viral genome, 3' and 5' of the inverted terminal repeats (ITRs). Thus, to ensure efficient replication and packaging of recombinant adenovirus carrying TK gene (Ad-TK), the plasmid was digested with *PacI* and used to transfect HEK 293 cells. When cells grew to confluence before viral plaques appeared, they were trypsinized and transferred to a larger flask, and after 21 days plaques appeared turbid due to light scattering by dead cells indicating the adenovirus presence. To confirm the presence of the TK gene in the recombinant adenovirus, the viral DNA was extracted and a Southern blot was carried out, using the DNA of recombinant adenovirus with the β-galactosidase gene (Ad-βgal) as negative control and pShuttleTK as positive control. Fig. 2 shows the probe hybridized with viral DNA extracted from HEK 293 cells transfected with the Ad-TK construct.

Cytotoxicity of adenoviral vectors containing TK gene in vitro - To determine whether recombinant adenovirus containing the three promoters ($P_{CMV\ IE}$, SV40 and EN1) upstream of TK gene would render tumor cells sensitive to ganciclovir-mediated cell killing, 40,000 HeLa cells were infected with either Ad-βgal at 80,000 pfus or Ad-TK at different titers. The transduced cells were then supplied or not with GCV at 25 µg/ml in the culture medium. Four days later the cells infected with Ad-βgal remained attached while the cells infected with Ad-TK detached completely from the plate at 60,000, 30,000 or 15,000 pfus. Cells infected with Ad-TK cultivated in the absence of GCV did not show evidence of cell killing (Fig. 3A). In a similar experiment, 40,000 CCR2 cells infected with Ad-TK at 60,000 or 30,000 pfus and treated with GCV were completely killed. Lower virus titers (15,000, 7,500 or 3,750 pfus) were not totally efficient. No toxic effect to the CCR2 cells was observed after infection with Ad-TK without GCV treatment (Fig. 3B).

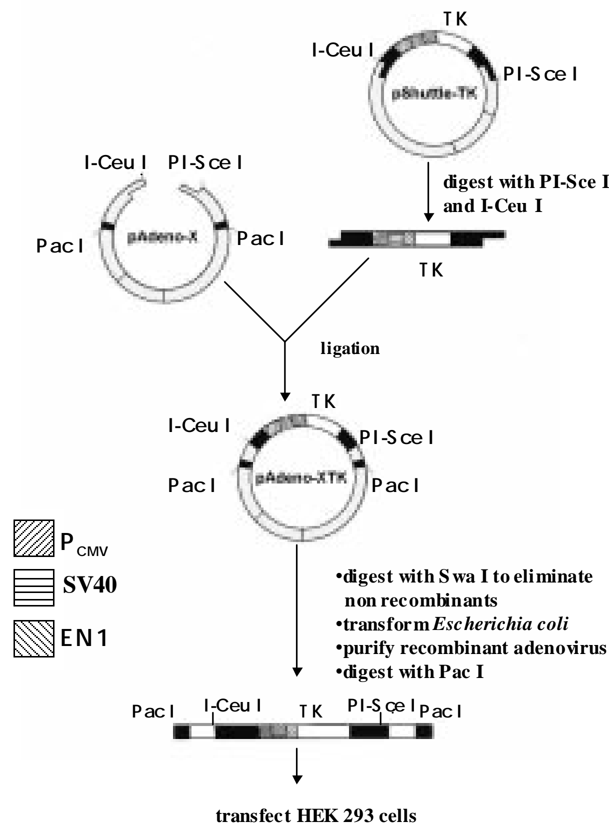

Fig. 1: schematic view of the construction of adenovirus carrying thymidine kinase gene using Adeno-X^{TM} Expression System.

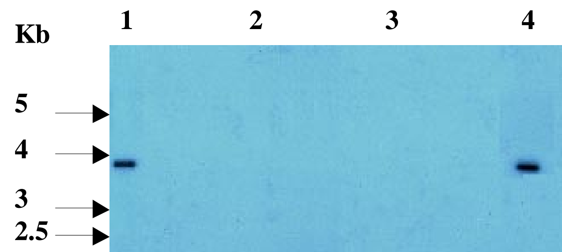

Fig. 2: Southern blot characterization of adenovirus carrying thymidine kinase gene (Ad-TK). Recombinant viral vectors were grown in HEK293 cells, purified, digested with Xba I and run in agarose gel. The resulting blot probed to detect TK gene is presented. Lanes - 1: 0.01 ng of Ad-TK DNA; 2: 2 ng of adenovirus carrying β-galactosidase gene (Ad-βgal) DNA; 3: 4 ng of Ad-βgal DNA and 4: 0.001 ng of pShuttle-TK plasmid digested with XbaI. The positions of molecular weight markers are indicated on the left.

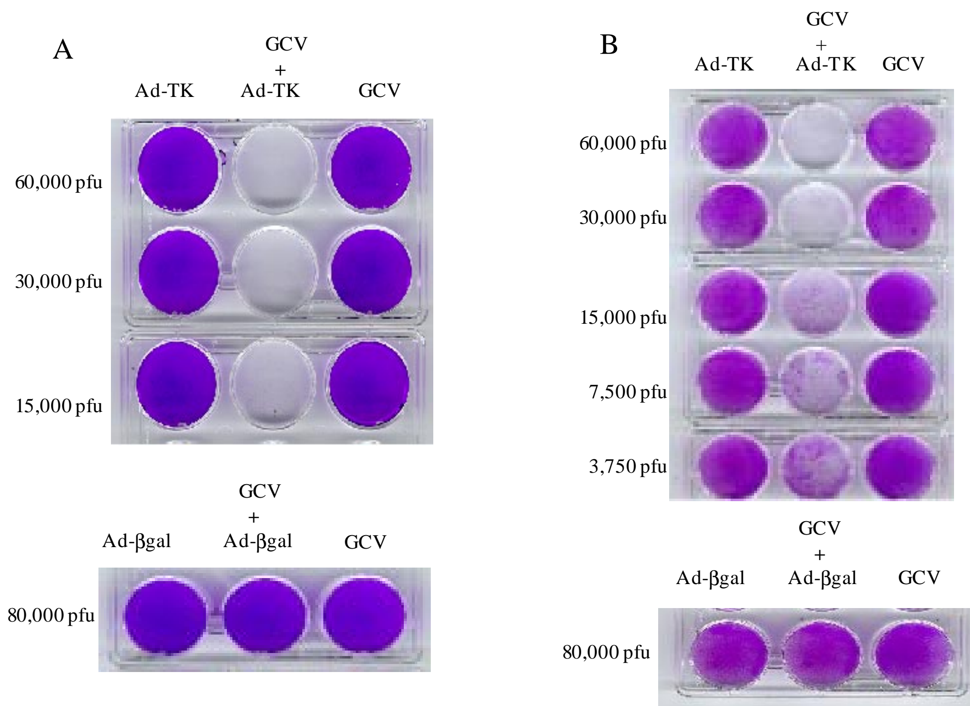

Fig. 3: infection of HeLa or CCR2 cells with adenovirus carrying thymidine kinase gene (Ad-TK). HeLa (A) or CCR2 (B) cells (40,000/ well), when indicated, were infected with Ad-TK (upper panels) or adenovirus carrying β-galactosidase gene (lower panels) with the indicated pfu number. Infected and uninfected (control) cells when indicated were treated with glanciclovir (GCV) (25 μg/ml). GCV treatment started 1h after infection and the cells were fixed and stained 4 days later.

Analysis of TK gene expression in Ad-TK transduced cells - After transduction with Ad-TK, a northern blot analysis was done in RNA extracted from HeLa or CCR2 cells 48 h after transduction, to verify the size of the transcripts starting from any of the three promoters. We could detect two sizes of transcripts of TK gene, one derived from the close together $P_{CMV\ IE}$/SV40 promoters and the other from the 1.5 Kb downstream EN1 promoter (Fig. 4). The relative amount of these transcripts was measured for each cell type in a PhosphorImager, and the ratio $P_{CMV\ IE}$/SV40:EN1 was 3:2 for CCR2 and 4:1 for HeLa cells.

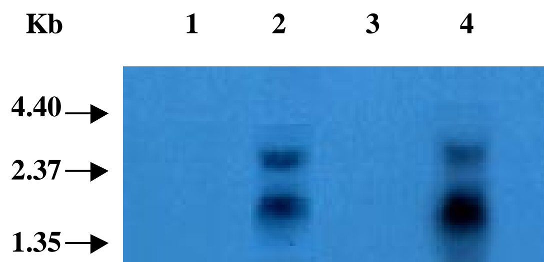

Fig. 4: Northern analysis of thymidine kinase (TK) gene expression in adenovirus carrying thymidine kinase gene (Ad-TK) transduced cells. RNA was extracted from HeLa or CCR2 cells 48 h after transduction with Ad-TK. The resulting blot probed to detect TK gene transcripts is presented. Lanes - 1: untransduced CCR2 cells; 2: transduced CCR2 cells; 3: untransduced HeLa cells; 4: transduced HeLa cells. The positions of molecular weight markers are indicated on the left.

DISCUSSION

Conventional methods for creating recombinant adenovirus vectors rely on in vitro homologous recombination in HEK 293 cells or *E. coli*. We did experiments with pJM17 and pΔE1sp1A (Bett et al. 1994) and the pAdEasy system (He et al. 1998) but in our hands they were inefficient. The protocol originally developed by Mizuguchi and Kay (1998, 1999) gave us straightforward results. The procedure is based on the application of an in vitro ligation to incorporate the gene of interest into a plasmid containing replication-incompetent human adenoviral type 5 genome (pAdeno-X). This system provides a mean to obtain a homogeneous population of recombinant adenovirus, which avoids the need of plaque purification procedures.

In the experiments reported here, we first tested if the TK gene sub-cloned in the vector pSHTK was functional. That was accomplished by the transfection of CCR2 cells that showed sensitivity to GCV treatment. Using the TK gene expression cassete from pSHTK, Ad-TK was constructed and $P_{CMV\ IE}$ added upstream of the two previous promoters (Fig. 1). From the results shown in Fig. 3, we conclude that the presence of the three promoters ($P_{CMV\ IE}$, SV40 and EN1) resulted in efficient expression of TK gene from the Ad-TK virus. Furthermore, in spite of using a number of pfus smaller (30,000) than the number of cells (40,000), there was complete elimination of CCR2 cells (Fig. 3B). In the experiment using the same number of HeLa cells, complete elimination occurred at 30,000 and

15,000 pfus (Fig. 3A). This phenomenon has been termed the bystander effect (Culver et al. 1992, Freeman et al. 1993), where GCV kills cells expressing TK gene and nearby cells that are not expressing it. Previous studies (Fick et al. 1995, Mesnil et al. 1996) have shown that gap junctions play a role in the bystander effect of the TK/GCV system, by allowing the passage of phosphorylated GCV metabolites from TK^+ to TK^- cells. This is of key importance in the treatment of solid tumors in vivo, were an injection of Ad-TK into the center of the cell mass resulted in the regression of the whole tumor (Eastham et al. 1996).

In Fig. 4 is shown that the Ad-TK transduced cells have two sizes of transcripts for TK gene, one derived from the close together $P_{CMV\ IE}$/SV40 promoters and the other from the 1.5 Kb downstream EN1 promoter. The ratios of transcription indicate a differential expression in the target cells since the ratio $P_{CMV\ IE}$/SV40:EN1 was 3:2 for CCR2 and 4:1 for HeLa cells. Suicide gene employed in cancer gene therapy requires expression from promoters that can be efficient in different cell types, and as it has been reported different cell types affect the efficiency of a given promoter activity (Everett 1988, Shillitoe & Noonan 2000). This may cause important differences in the levels and duration of the suicide gene expression and lead to a failure of efficiency of one promoter in a given tumor cell. This failure is less likely to occur with the Ad-TK described here due to the presence of three strong promoters, in such a way that when one promoter is weak the others may be more effective, thus optimizing this system and having the potential to be effective in a large variety of tumor cells.

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