

ACCELERATED COMMUNICATION

Circumventing Recombination Events Encountered with Production of a Clinical-Grade Adenoviral Vector with a Double-Expression Cassette

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ABSTRACT

Delivery of multiple exogenous genes into target cells is important for a broad range of gene therapy applications, including combined therapeutic gene expression and noninvasive imaging. Previous studies (*Mol Ther* 4:223-231, 2001) have described the adenoviral vector RGDTKSSTR with a double-expression cassette that encodes herpes simplex virus thymidine kinase (HSVtk) for molecular chemotherapy and human somatostatin receptor subtype-2 (hSSTR2) for indirect imaging. In this vector, both genes are inserted in place of the E1 region of the adenoviral genome and expressed independently from two cytomegalovirus (CMV) promoters. During production of clinical-grade RGDTKSSTR, we found that the CMV promoters and simian virus 40 (SV40) poly(A) regions located in both expres-

sion cassettes provoked homologous recombination and deletion of one of the cassettes. To resolve this problem, we designed a strategy for substituting the duplicate promoters and poly(A) regions. We placed the hSSTR2 gene in the new Ad5.SSTR/TK.RGD vector under the control of a CMV promoter with a bovine growth hormone poly(A) region, whereas the SV40 promoter, enhancer, and poly(A) signal controlled HSVtk expression. This use of different regulatory sequences allowed independent expression of both transgenes from a single adenoviral vector and circumvented the recombination problem. Reconstruction of the vector with a double-expression cassette enables its use in human clinical trials.

Adenovirus-mediated gene therapy is promising for advanced cancers refractory to other treatment modalities because it enables the delivery of therapeutic genes directly into the target cells. So far, however, clinical trials of

adenovirus-mediated gene therapy have had limited success. One of the primary obstacles to the realization of the full potential of this promising modality is the inability of currently used adenoviral vectors to efficiently transduce target cancer cells. Inefficient transduction is principally caused by inconsistent expression of coxsackie-adenovirus receptor (CAR), which is often low or absent in primary cancer cells (Li et al., 1999a,b). Investigators have performed extensive research in an effort to enhance the transduction capacity of adenoviral vectors (Dmitriev et al., 1998; Belousova et al., 2003). An important corollary to

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ABBREVIATIONS: CAR, coxsackie-adenovirus receptor; HSVtk, herpes simplex virus thymidine kinase; hSSTR2, human somatostatin receptor subtype-2; CMV, cytomegalovirus; SV40, simian virus 40; RGD-4C, arginine-glycine-aspartic motif; GCV, ganciclovir; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction; MOI, multiplicity of infection; pfu, plaque-forming units; bp, base pair(s).

this line of research is development of the means to monitor gene transfer in vivo.

Several studies have suggested the use of a noninvasive imaging approach to observe the efficiency of gene transfer and expression over time. This approach is based on the coupled expression of therapeutic and reporter genes; the level of expression of the reporter gene predicts the level of expression of the therapeutic gene. In general, researchers have used four approaches to link the expression of two genes in the context of an adenoviral vector. One approach is based on the use of two adenoviral vectors (Kwong et al., 1997; Yaghoubi et al., 2001). However, this approach cannot guarantee uniform infection of all cells with both vectors because of the relative inefficiency of gene transfer procedures. Another approach involves the use of bicistronic vectors coexpressing the genes linked by an internal ribosomal entry site (Wen et al., 2001; Wang et al., 2005). Use of this method results in valid correlations in expression, but the absolute level of expression of the second gene is generally reduced. The third approach is expression of multiple gene products from a single cistron through a fusion protein that can be postsynthetically cleaved and processed into individual biologically active proteins (Gaken et al., 2000) or that can itself have two or more functions (Ray et al., 2004). The fourth approach involves the insertion of multiple expression cassettes into a single vector, with each cassette having its own independent promoter and poly(A) signal. Different groups have employed this approach, including ours (Hemminki et al., 2001; Buchsbaum, 2004). To be specific, we developed the infectivity-enhanced adenoviral vector RGDTKSSTR, which incorporates a genetically modified fiber with an arginine-glycine-aspartate (RGD-4C) motif in the HI loop of the knob (Hemminki et al., 2001). The RGD-4C modification allows the virus to use cellular integrins, which are frequently overexpressed in ovarian tumors (Cannistra et al., 1995) and other types of tumors (Mortarini and Anichini, 1993; Westlin, 2001; Cooper et al., 2002), for binding and internalization. Thus, the vector can circumvent dependence on CAR. For a therapeutic effect, HSVtk/ganciclovir (GCV) suicide system (Driesse et al., 1998) was applied. A reporter gene, hSSTR2, was used to provide high-sensitivity imaging with positron emission tomography and single-photon emission computed tomography. Both hSSTR2 and HSVtk expression cassettes in RGDTKSSTR were driven by CMV promoters and included SV40 poly(A) regions. This gene therapy approach had cytotoxic effects on both ovarian cell lines and primary cancer cells. Moreover, in a subsequent study, we were able to detect the virus for more than 2 weeks in an ovarian cancer animal model, and we showed that the RGD-4C modification can enhance the in vivo effects of the HSVtk/GCV approach (Hemminki et al., 2002). Based on the promising in vitro and in vivo data, we suggested that the RGDTKSSTR vector could be a useful agent in the treatment of ovarian cancer and could set the stage for clinical testing.

In the study described herein, we found, while upscaling RGDTKSSTR for a clinical trial in patients with ovarian cancer, that the presence of homologous promoters and poly(A) regions in expression cassettes induced recombination and led to the deletion of one of two transgenes. This

defect rendered the use of this vector in its original configuration impossible in human clinical trials. We hypothesized that to resolve this problem, we had to reconstruct the vector by replacing the duplicate promoters and poly(A) signals with nonhomologous ones.

Materials and Methods

Cells. The human embryonic kidney (HEK) 293 cell line used for propagation of recombinant adenoviruses was obtained from Magenta Corporation (Rockville, MD). The lung cancer cell line A549 was purchased from the American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 medium (50:50 mix) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. FBS was purchased from Invitrogen (Carlsbad, CA), and media and supplements were purchased from Mediatech (Herndon, VA). Cells were propagated at 37°C in a 5% CO₂ atmosphere.

Production of Clinical-Grade RGDTKSSTR. Production of the RGDTKSSTR vector, in which expression of the *hSSTR2* and *HSVtk* genes is driven by CMV promoters, was described previously (Hemminki et al., 2001). Small-scale virus propagation was performed in 175-cm² flasks. Further cell expansion and upscaled adenovirus production in 850-cm² roller bottles (Corning Life Sciences, Acton, MA) were performed as described previously. In all, three rounds of virus amplification were performed. Adenoviral vectors were isolated from infected cells and purified by equilibrium centrifugation in CsCl gradients.

Recombination Assay. Viral particles (10⁷) were used for polymerase chain reaction (PCR) analysis with pairs of primers located in the region, including hSSTR2 and HSVtk expression cassettes: P1-Ad5.F277 (CGCGGGAAACTGAATAAGA) and P2-pShuttle.R (GCGCGTTGTCAAATATGAG), P3-SSTR.F1014 (GGAGCGGAGTGACAGTAAGC) and P4-TK.R11129 (AGCCTCCCCCATCTCCC), P1 and P6-TK.R150 (CAGTAGCGTGGGCATTTTCT), and P2 and P5-SSTR.F79 (TCAACCAACACCTCAAACCA). The primers were designed to amplify both full-size DNA fragments corresponding to double-expression cassettes and potentially truncated fragments corresponding to recombinant genomes with only one expression cassette. Localization of the primers is shown in Fig. 1A.

Genetic Re-engineering of Ad5.SSTR/TK.RGD. The HSVtk gene was amplified from pORF-HSVtk (InvivoGen, San Diego, CA) by using sticky-end PCR (44). This was accomplished using two pairs of primers: HindIII.F.L (AGCTTGCCACCATGGCTTCGTACCCC) and XbaI.R.S (ATCATTTCACTCAGTTAGCCTCCCCCAT), and HindIII.F.S (TGCCACCATGGCTTCGTACCCC) and XbaI.R.L (CTAGATCATTTCACTCAGTTAGCCTCCCCCAT). The PCR products were mixed in equimolar amounts, denatured, annealed, and ligated with a HindIII-XbaI-digested pGL3-control vector (Promega, Madison, WI). The resultant plasmid was designated pGL-TK.

Sequences corresponding to the CMV promoter and hSSTR2 gene were amplified from pDC.SSTR2 plasmid (Hemminki et al., 2001), and the BGHpA region was amplified from pcDNA3.1 (Invitrogen, Carlsbad, CA). In all cases, the sticky-end PCR technique was employed. Next, pairs of primers were used for amplification: for the CMV promoter, NotI.F.L (GGCCGCGATCTAATTCCTGGCAT) and AgeI.R.S (TCGGTCCCGGTGTCTTCTAT), and NotI.F.S (GCGATCTAATTCCTGGCAT) and AgeI.R.L (CCGGTCCGGTCCCGGTGTCTTCTAT); for the hSSTR2 gene, AgeI.F.L (CCGGTGAACCATGGACATGGCGGA) and BstBI.R.S (TCATTTCACTCAGATACTGGTTTGGAGGTC), and AgeI.F.S (TGCAACCATGGACATGGCGGAT) and BstBI.R.L (CGAATCATTTCACTCAGATACTGGTTTGGAGGTC); and for the BGHpA signal, BstBI.F.L (TTCAAGACTGTGCCTTCTAGTTGC) and NheI.R.S (CGGTGGGCTCTATGGG), and BstBI.F.S (AAGACTGTGCCTTCTAGTTGC) and NheI.R.L (CTAGCCCATAGAGCCCACCGC). The PCR products for each structure were mixed in equimolar amounts,

denatured, and annealed. Then, they were combined and ligated with an NheI-SalI fragment from pGL-TK containing the HSVtk expression cassette and a NotI-SalI-digested pShuttle vector (Quantum Biotechnologies, Montreal, QC, Canada). The final plasmid was designated pShuttle.SSTR/TK.

To generate recombinant adenoviral genomes containing hSSTR2/HSVtk-expressing cassettes in place of the E1 region and a fiber gene incorporating an RGD-4C-encoding peptide, homologous DNA recombination with PmeI-digested pShuttle.SSTR/TK and an E1, E3-deleted pVK554 vector was performed (Hemminki et al., 2001). The resultant plasmid was designated pNB557.

Ad5.SSTR/TK.RGD Vector. The Ad5.SSTR/TK.RGD vector containing hSSTR2 and HSVtk expression cassettes (driven by CMV and SV40 promoters, respectively) and an RGD-4C-incorporating fiber gene was rescued after transfection of HEK 293 cells with PacI-digested pNB557. The virus was amplified three times consecutively in the HEK 293 cells and purified by equilibrium centrifugation in CsCl gradients with the use of a standard protocol. Determination of the virus particle titer was accomplished spectrophotometrically using a conversion factor of 1.1×10^{12} viral particles per absorbance unit at 260 nm. The infectious titer of the vector in HEK 293 cells was determined using a standard 50% tissue culture infective dose assay (Quantum Biotechnologies, Montreal, QC, Canada).

Cytotoxicity Assay. A549 cells were plated in 96-well plates (3×10^3 cells per well). The next day, cells were infected with an adenovirus diluted in 5% FBS-DMEM/F-12 medium at a multiplicity of infection (MOI) of 5 pfu/cell. Medium without a virus was added to the control wells. After 4 h, GCV was added to the cells to a final concentration of 0.25, 0.50, and 0.75 mM. Plates were incubated in a CO₂ incubator for 4 more days, and the medium was replaced with 100 μ l of phosphate-buffered saline buffer. Cytotoxicity was analyzed by using a colorimetric method with the CellTiter 96 AQueous nonradioactive cell proliferation assay (Promega, Madison, WI) as described by the manufacturer. Absorbance was measured at a wavelength of 490 nm by using an EMax microplate reader (Molecular

Devices, Sunnyvale, CA). Each data point was set in triplicate and calculated as the mean of three determinations.

hSSTR2 Expression. Experiments were performed with adherent cells in 96-well plates; each treatment was evaluated in five separate wells. A total of 5×10^3 A549 cells per well were infected with an adenovirus diluted in 5% FBS-DMEM/F-12 medium at an MOI of 5 pfu/cell. After 96 h, the medium was removed, and cells were incubated with an internalization medium alone. Detection and measurement of in vitro gene transfer was performed by using γ camera imaging. Two groups were set with an internalization medium or with internalization medium containing 2 μ g of unlabeled P2045 peptide (Diatide Research Laboratories, Londonderry, NH) (Fig. 2) per well. Unlabeled P2045 was used as a competitor for blocking labeled P2045 to bind to the expressed hSSTR2. After blocking for 5 min, 99m Tc-P2045 (specific activity, approximately 1000 μ Ci/ μ g) was added to all of the wells on the plate (15 μ Ci/well; final concentration, 11.5 nM) and incubated for 2 h. After incubation, the cells were washed twice with ice-cold Hanks' balanced salt solution, pH 7.2, and lysed with 1 M NaOH. The radioactivity in the lysed cells was measured by using a γ -ray counter (MINAXI 5000 series gamma counter; PerkinElmer Life and Analytical Sciences, Boston, MA). All of the data were normalized for protein concentration measured in the samples by Lowry assay (Bio-Rad, Hercules, CA).

Results

The Homologous Regions in RGDTKSSTR Provoke Recombination and Internal Deletions. We upscaled RGDTKSSTR (Hemminki et al., 2001) for use in a phase I clinical trial in patients with ovarian cancer, and we examined the stability of the adenoviral genome, particularly the E1-modified region, using PCR analysis with primers located in that region (Fig. 1A). We clearly detected truncated PCR products corresponding to the genome but only one expression cassette (hSSTR2 or HSVtk; Fig. 1B). Thus, because of

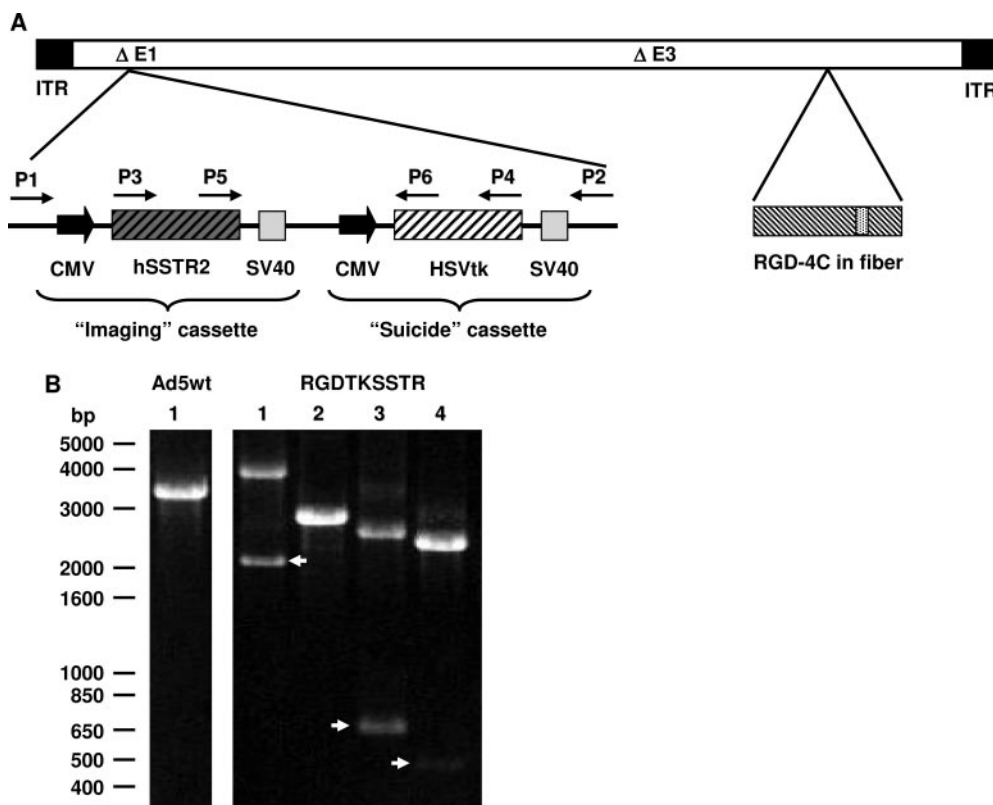


Fig. 1. Analysis of the RGDTKSSTR genome structure. A, schema of the RGDTKSSTR genome organization. The E1 and E3 regions were deleted and replaced with two expression cassettes: an "imaging" cassette encoding the hSSTR2 receptor and a "suicide" cassette encoding HSVtk. Both cassettes included CMV promoters and SV40 poly(A) regions as regulatory sequences. A fiber gene was modified to incorporate an RGD-4C-encoding motif to increase the viral infectivity to CAR-negative, integrin-positive cancer cells. Localization of primers P1–P5 is indicated by the arrows. B, PCR analysis of the CsCl-purified virions after the third round of upscaling. Pairs of primers were used for PCR: lane 1, P1 and P2; lane 2, P3 and P4; lane 3, P1 and P6; lane 4, P2 and P5. The arrows point to truncated PCR products. Wild-type Ad5 was used as a control.

the presence of the homologous regions corresponding to the CMV promoter (414 bp) and SV40 poly(A) (165 bp) sequences in both the hSSTR2 and HSVtk cassette, homologous recombination occurred and induced internal deletion. Therefore, the viral population was heterogeneous and included viral particles with genomes encoding both transgenes as well as particles with only one expression cassette. This defect hindered our ability to use the original configuration of RGDTKSSTR in human clinical trials.

Reconstruction of Ad5.SSTR/TK.RGD. To resolve the problem of recombination, we designed a strategy to replace duplicate promoters and poly(A) regions in the RGDTKSSTR vector with nonhomologous ones. To that end, we placed the hSSTR2 gene in the first expression cassette under the control of the CMV promoter and BGHpA, and we placed the HSVtk gene in the second expression cassette under the control of the SV40 promoter with the SV40 enhancer and SV40 poly(A) signal (Fig. 3A). We combined the two cassettes in a pShuttle vector and then transferred them by homologous recombination into an E1, E3-deleted pVK555 backbone. We rescued the new vector, named Ad5.SSTR/TK.RGD, after transfection of HEK 293 cells with the resultant adenoviral genome. We consecutively amplified Ad5.SSTR/TK.RGD three times on HEK 293 cells and used CsCl-puri-

fied virions for PCR analysis. The analysis of Ad5.SSTR/TK.RGD revealed no truncated products (Fig. 3B). Thus, use of the different regulatory sequences circumvented the problem of recombination experienced with the vector containing two expression cassettes under the control of identical regulatory regions.

Validation of Ad5.SSTR/TK.RGD Transgene Expression. We confirmed the cytotoxic effect of HSVtk by using a cytotoxicity assay (Fig. 4A). This assay showed that Ad5.SSTR/TK.RGD effectively killed A549 cells. The percentage of surviving cells depended on the GCV concentration and varied from 39% to 55%. The number of viable cells was higher with Ad5.SSTR/TK.RGD compared with the positive control vector, Ad5.CMV-TK, in which expression of the HSVtk gene was driven by the CMV promoter, much lower than two negative control groups, Ad5Luc1 and no virus.

The γ camera imaging assay with the hSTTr2-avid Tc 99m-P2045 peptide confirmed that the hSSTR2 gene was also biologically active (Fig. 4B). Only A549 cells infected with Ad5.SSTR/TK.RGD bound the ^{99m}Tc -P2045 peptide; unlabeled P2045 specifically blocked this binding. This meant that the functional hSSTR2 receptor was expressed on the surface of infected cells. Noninfected cells and cells infected with the control Ad5Luc1 virus, which expressed the luciferase gene, did not demonstrate significant binding. In addition, planar gamma camera imaging detected binding of ^{99m}Tc -P2045 to the Ad5.SSTR/TK.RGD-infected cells (data not shown).

Discussion

In this study, we upscaled the adenoviral vector RGDTKSSTR for a clinical trial involving patients with ovarian cancer and tested the stability of the adenoviral genome. PCR analysis showed that the genome was unsta-

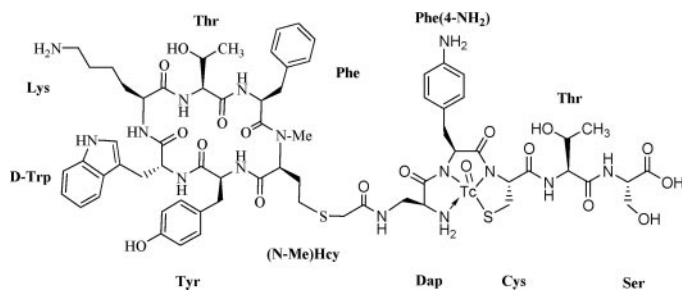


Fig. 2. Structure of P2045.

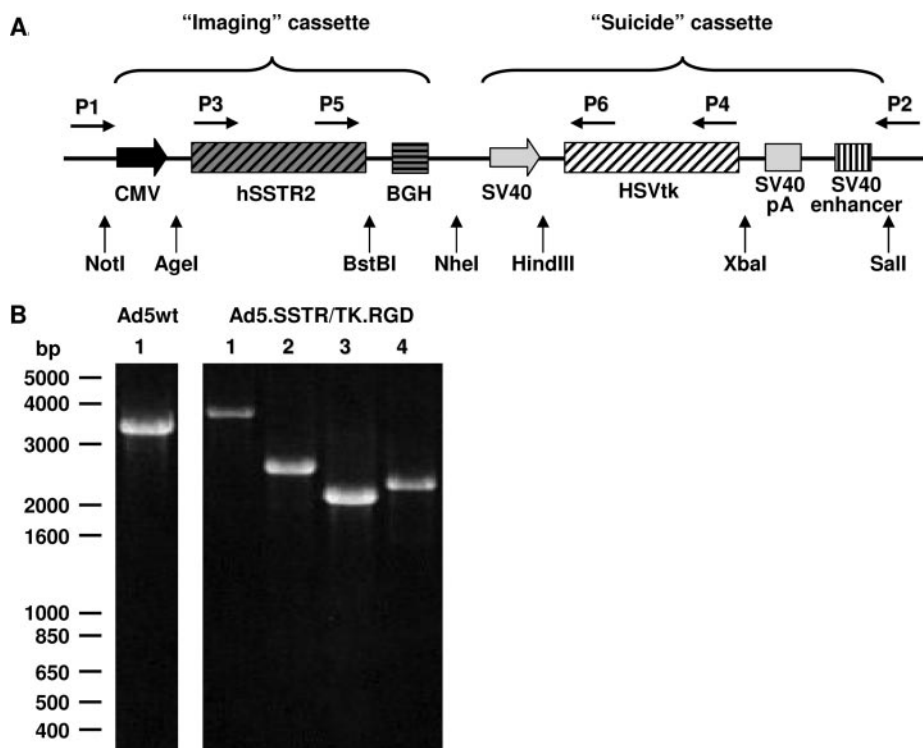


Fig. 3. Reconstruction and analysis of the double-expression cassette in the Ad5.SSTR/TK.RGD vector. A, schema of the double-expression cassette in Ad5.SSTR/TK.RGD. Expression of the hSSTR2 gene in the first expression cassette was placed under the control of a CMV promoter and BGHpA sequence. Expression of the HSVtk gene in the second expression cassette was regulated by an SV40 promoter, SV40 enhancer, and SV40 late poly(A) signal. The arrows indicate unique restriction endonucleases in pShuttle.SSTR/TK. B, diagnostic PCR. To imitate the conditions of RGDTKSSTR analysis, the Ad5.SSTR/TK.RGD vector was subjected to three rounds of amplification and used for PCR with the primers employed for RGDTKSSTR: lane 1, P1 and P2; lane 2, P3 and P4; lane 3, P1 and P6; lane 4, P2 and P5. Ad5Luc1 was used as a control.

ble, and recombination events occurred in the homologous regions of the expression cassettes. We reconstructed the vector by replacing the duplicate promoters and poly(A) regions with nonhomologous ones. We detected no recombinant events, and both hSSTR2 and HSVtk were functional in the new Ad5.SSTR/TK.RGD construct. Thus, we achieved independent expression of two transgenes and genetic stability in the dual-expression cassette region of a single adenoviral vector with different regulatory sequences, allowing the use of this new vector in human clinical trials.

Investigators have developed a number of approaches to link the expression of two genes in a single adenoviral vector. One approach that has been employed by several groups is based on the independent expression of two transgenes from two CMV promoters (Hemminki et al., 2001). In the present study, we found that the adenoviral genome carrying the double-expression cassette was unstable and that one of the cassettes was deleted by homologous recombination during vector upscaling. This finding led us to conclude that even relatively small repeated sequences (approximately 150 bp) in the viral genome can participate in recombination and induce deletion of the sequence located between the repetitive elements.

Researchers observed a similar phenomenon when two CMV promoters were located in two different vectors (helper-dependent and helper vectors) (Jozkowicz and Dulak, 2005). The recombination occurred when the CMV promoters were positioned close to one end of the vector, and the promoters from both vectors were oriented in the same direction, resulting in the appearance of dominating new vector species. The emergence of replication-competent adenoviruses during large-scale production of replication-deficient adenoviral vec-

tors is another example of homologous recombination, which in this case occurs between vector DNA and a homologous sequence in the genome of E1-complementing HEK 293 cells (Schiedner et al., 2000). Therefore, to create a stable genome, one should avoid homologous sequences within one adenoviral vector or between the vector and the cell genome as well as coreplicating adenoviral vectors. If these sequences are located too close to each other within one genome, adenoviral genomes with a deletion should be properly packed into adenoviral particles so that the final viral population will be heterogeneous.

The combined delivery of two transgenes is important for a number of biomedical applications. Use of cooperatively interacting immunomodulatory genes or the combination of an immunomodulatory gene and a tumor antigen is a promising approach to immunotherapy for cancer in vivo (Antony et al., 2006). In addition, linkage of angiogenic, preapoptotic, and chemotherapeutic genes with reporter gene expression may enable monitoring of the magnitude, location, and duration of therapeutic gene expression with the use of indirect imaging in vivo. Our finding that an adenoviral vector with a dual-expression cassette has a stable genome and functional transgenes when they are placed under the control of different regulatory sequences can be used for generation of adenoviral vectors for any of these applications.

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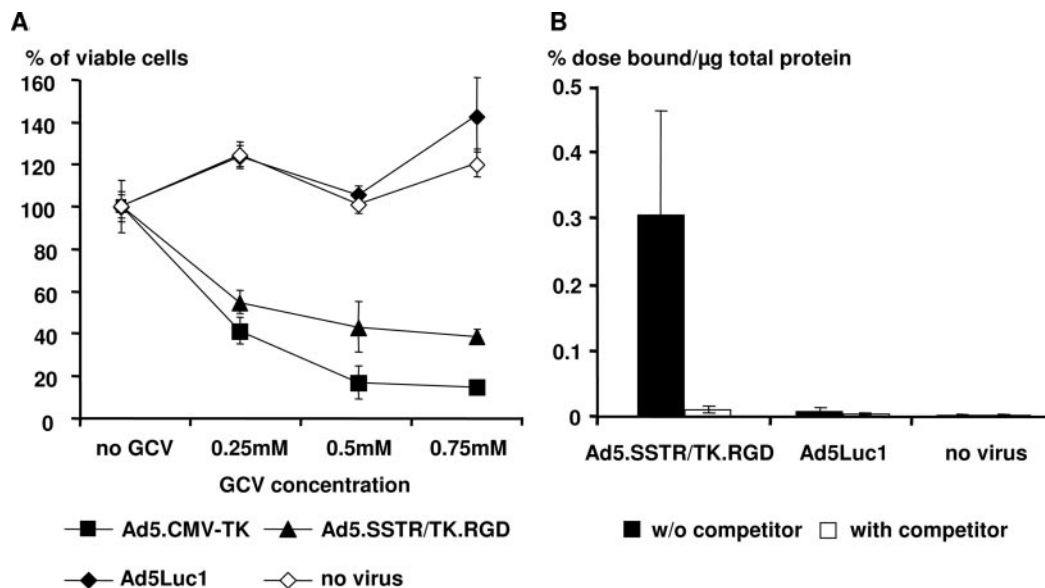


Fig. 4. Analysis of TRANSGENE activity in A549 cells infected with Ad5.SSTR/TK.RGD. **A**, validation of HSVtk activity by cytotoxicity assay. A549 cells grown in 96-well plates were infected with viruses at an MOI of 5 pfu/cell. After 4 h, GCV was added to the cells to a final concentration of 0.25, 0.50, and 0.75 mM. After 4 days, the medium was replaced with a phosphate-buffered saline buffer, and an MTS/PMS solution was added to each well. The absorbance was measured 1 h later at a wavelength of 490 nm. One hundred percent of viable cells points to absorbance in the absence of GCV. Each data point is the mean of three determinations. **B**, imaging of hSSTR2. A549 cells grown in 96-well plates were infected with viruses at an MOI of 5 pfu/cell. After 96 h, unlabeled P2045 peptide as a competitor for blocking labeled P2045 peptide to bind to expressed hSSTR or an internalization medium was added to the plates. Five minutes later, labeled ^{99m}Tc -P2045 (11.5 nM) was applied. Five minutes after that, the control wells were washed and subjected to the first round of imaging. After 2 h, all cells were washed, lysed with 1 M NaOH, and imaged. The data were normalized on protein concentrations measured in the samples by Lowry assay.

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