

Retroviral Vectors Encoding a Reverse Transcription-activated Transgene Efficiently Limit Expression of the Gene to Target Cells

John Douglas Burke¹ and John C Morris¹

¹Metabolism Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland, USA

Recombinant retroviral vectors are indispensable tools for the study of gene function and for therapeutic gene transfer owing to their ability to transfer and stably express foreign genes in target cells. A limitation of these vectors, however, is the difficulty in generating stable vector producer cell (VPC) lines when the vectors encode cytotoxic proteins. We developed a series of Moloney murine leukemia virus-based vectors encoding a reverse transcription-activated transgene. These vectors preclude gene expression in the producer cells, yet allow lines for transgene expression in target cells. The vectors were generated by cloning the gene of interest in reverse orientation either just upstream of the viral 3' long terminal repeat (LTR) or in the U3 region of the 3'LTR. An exogenous promoter was inserted, also in reverse orientation, at the R-U5 border of the viral 5'LTR. Upon transduction of target cells, the inserted promoter is copied to the 3'LTR during reverse transcription of the vector genomic RNA, where it then drives transgene expression. We tested this system using a green fluorescent protein (GFP) gene and the SV40 promoter. Reverse transcription-activated retroviral vectors may allow for the generation of stable retroviral VPC lines encoding cytotoxic or inhibitory genes.

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INTRODUCTION

The utility of viral gene transfer vectors for the study of protein function and for gene therapy is well established.^{1,2} Retroviral vectors, especially, have been extensively used owing to their ability to stably express proteins in target cells. (reviewed in refs. 3–5) As a result, retroviruses have been used to generate stable cell lines expressing exogenous genes,⁶ and for therapeutic gene transfer for the treatment of cancer, genetic conditions, and autoimmune disease.^{3,6–10} In the former application, the ability of retroviruses to integrate into the host cell genome allows for the generation of stable cell lines expressing the gene of interest. In therapeutic approaches, these vectors are used to replace

mutated or deleted genes to correct a disease phenotype or, in the case of tumors, to deliver potentially toxic genes into cancer cells. In this latter case, often termed “suicide” or enzyme-prodrug gene therapy, the transferred gene usually encodes a toxin, or a viral or prokaryotic enzyme that converts an ordinarily innocuous prodrug into a cytotoxic metabolite.^{9,11} The efficacy of this approach correlates with the fraction of cells that ultimately express the transferred gene, which is often quite limited. To increase the efficiency of gene transfer, direct intratumoral injection of retroviral vector producer cell (VPC) lines has been clinically used to increase the delivery of viral particles expressing the suicide gene.^{8,12–14}

A significant limitation of recombinant retroviral vectors has been the difficulty in developing stable cell-based systems capable of producing vectors encoding cytotoxic or growth inhibitory genes. When the encoded protein is toxic, constitutive expression by prototypical retroviral vectors precludes the generation of stable producer cells because of the adverse effect of the toxic protein in the VPC themselves. In the case of enzyme-prodrug therapy, as the VPC also express the suicide gene, they too are subject to toxicity in the presence of the prodrug. This requires re-administration of the VPC for repeated treatment cycles, something that is not always practical or safe.

The inability to generate stable cell lines producing retroviral vectors that express toxic genes is principally due to the design of these vectors, where the spatial arrangement of the promoter, whether it be it the retroviral long terminal repeat (LTR) or an exogenous promoter, constitutively drives gene expression in both the VPC and target cells. A number of systems have been developed in attempts to overcome this problem. One approach uses transient transfection for the production of retroviral vectors.¹⁵ As the producer cells in these systems are only briefly exposed to the gene product, short-term vector production is possible. Although useful, these systems can be inconvenient, prone to recombination, generally produce low titers, and are not ideal for standardized mass production of vectors for clinical use.

Another approach is to place transgene expression under the control of an inducible promoter, or have its expression driven

Correspondence: John C Morris, Metabolism Branch, Center for Cancer Research, National Cancer Institute, Mark O. Hatfield Clinical Research Center, Rm. 4-5330, Bethesda, Maryland 20892-1457, USA. E-mail: jmorris@mail.nih.gov.

by a tissue-specific promoter. Inducible promoters are usually derived from prokaryotic antibiotic resistance gene promoters and require the addition or removal of an exogenous agent such as tetracycline for their activity.^{16–20} Alternatively, tissue-specific promoters, exemplified by the tyrosinase and α -fetoprotein promoters, are activated in the presence of endogenous transcription factors specific to a particular cell or tissue type.^{20–23} Gene expression using inducible promoters can be accomplished by the appropriate manipulation of the inducer or repressor agent, and in those utilizing tissue-specific promoters by targeting a cell or tissue that permits promoter activity. The major difficulty with these systems is that they may suffer from some level of promoter “leakiness” as well as a degree of non-specificity, and their use may be limited to specific tissues or cells. Additionally, in those situations where the VPCs are administered *in vivo*, an inducible system would not necessarily selectively preclude transgene expression in the producer cells, as both vector producer and target cells are simultaneously exposed to the inducer molecule.

We report the generation of a series of retroviral vectors that utilize the natural retroviral life cycle to regulate transgene expression. We exploited the LTR “copying” mechanism peculiar to retroviral reverse transcription, used to generate self-inactivating and “double copy” vectors,^{24,25} to bring an initially promoterless transgene under the control of a strong exogenous promoter. In so doing, we developed a gene transfer system that limits the expression of a transgene to the target cells and may allow for the efficient generation of stable retroviral VPC producing retroviruses encoding cytotoxic genes.

RESULTS

Vectors and producer cells

To preclude transgene expression in the VPC, a promoterless green fluorescent protein (GFP) gene was cloned in reverse orientation relative to the viral LTR (**Figure 1c–e**). To achieve activation upon transduction, gene expression was made dependent upon reverse transcription by cloning the SV40 promoter in reverse orientation into the R-U5 border of the 5′LTR (**Figure 1d and e**). The R-U5 region of the 5′LTR is copied to the corresponding locus of the 3′LTR during reverse transcription. The resulting constructs were transfected into PG13 retroviral vector packaging cells because the GaLV-pseudotyped murine cell line is relatively refractory to autotransduction.³⁹

To determine whether insertion of the inverted SV40 promoter in proximity to the 5′LTR affected the activity of the latter, we measured viral titers and analyzed expression of the forward-oriented transgene *cd* by determining the sensitivity (IC_{50}) of the VPC to the prodrug 5-fluorocytosine.⁹ Insertion of the promoter resulted in a modest decrease in viral titers. Titers of the SV40(+) vectors, RET (1.5×10^4 colony-forming units(CFU) mL^{-1}) and RETdc (3.6×10^4 CFU mL^{-1}), were reduced compared with those of the SV40(–) control vectors, retG (2×10^5 CFU mL^{-1}) and GLEN (8×10^5 CFU mL^{-1}) (**Figure 1**). In contrast to the effect on viral titers, the insertion of the SV40 promoter did not appear to adversely affect

functional expression of *cd*. Untransfected PG13 cells, PG13/RET producer cells, and PG13 cells stably transfected with a standard retroviral *cd* expression construct (*cd* cloned in forward orientation into the multiple cloning site of GCsamEN, such that its expression is constitutively driven by the vector LTR) treated with 5FC (0.001 μM to 10 mM) were similarly sensitized to the drug (IC_{50} 100 μM). This concentration was 2 logs below that required to inhibit growth of unmodified PG13 cells (10 mM).

Molecular analysis

To demonstrate the approximation of the SV40 promoter to the inserted GFP gene that occurs on target cell transduction and reverse transcription, we analyzed genomic DNA from both the VPC and the transduced TE671 cells by Southern blot and polymerase chain reaction (PCR). For the Southern analysis, DNA was digested with *StuI* and *HindIII*, which cut the integrated vector provirus once in the SV40 promoter and the internal ribosomal entry site (IRES) sequence, respectively. Reverse transcription of the vector genomic RNA copies the SV40 promoter with the *StuI* site into the 3′LTR. Therefore, hybridization of the genomic DNA with a GFP-specific probe should exhibit a band only from the vector-transduced target cells. Bands of 2.9 and 2.8 kb were observed only in DNA digests from TE671 cells transduced with RET and RETdc, respectively (**Figure 2a**). Longer exposures of the blots did not reveal additional hybridization bands.

For the PCR analysis, we used a sense primer (with respect to the viral LTR) specific for the inverted GFP gene and an antisense primer specific for the inverted SV40 promoter. This arrangement allows amplification only if the SV40 promoter and the GFP gene have been approximated to each other in the correct orientation by reverse transcription. Thus, PCR using a genomic DNA template should only generate products in vector-transduced target cells. Products of 886 and 777 bp in the RET and RETdc lanes, respectively, were the only bands detected (**Figure 2b**). The greater intensity of the RETdc band resulted primarily from the presence of two SV40-GFP cassette templates compared with the single copy in the RET-transduced cells. Sequencing confirmed the identity of the PCR products. A control PCR for 18S ribosomal RNA demonstrated that similar amounts of DNA template were used for all reactions (**Figure 2c**).

To demonstrate restriction of GFP expression at the transcriptional level, we analyzed total RNA from the producer and target cells by Northern blot. Copying of the SV40 promoter into the 3′LTR by reverse transcription is expected to generate GFP-encoding transcripts only in the vector-transduced target cells. Further, as we cloned the SV40 early small *t* antigen splice donor and splice acceptor immediately downstream of the SV40 promoter and just upstream of the GFP ATG-site, respectively, we expect to detect both spliced and unspliced GFP transcripts. Using a GFP complementary DNA (cDNA) probe, unique mRNA transcripts were detected in the RET- and RETdc-transduced TE671 cells (**Figure 3**). These transcripts were not found in the corresponding PG13 producer cells, or in TE671 cells transduced with the GLEN or the retG vectors, even on

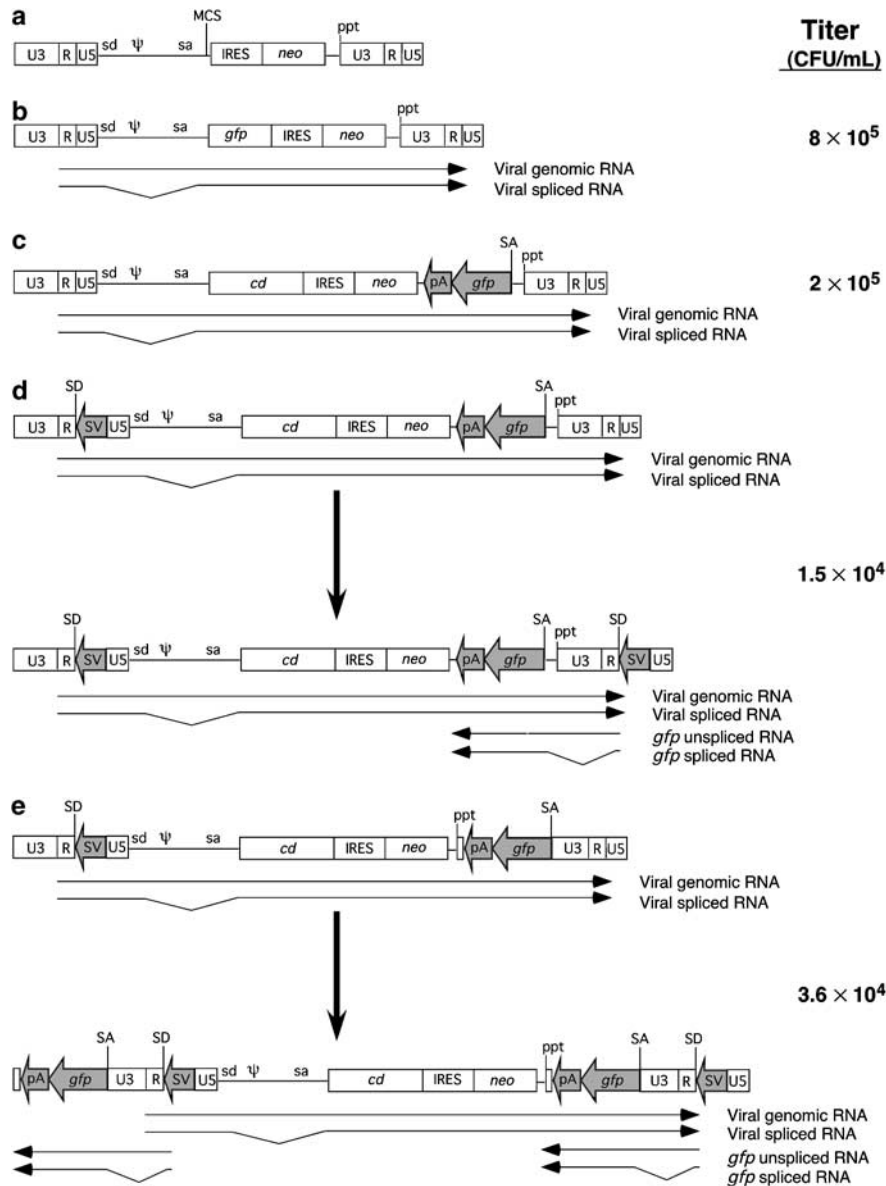


Figure 1 Schematic representation of retroviral vectors and their corresponding proviruses. The plasmid constructs and only those corresponding proviruses that differ structurally from the parental plasmids are shown. **(a)** GCsamEN parental vector. **(b)** GLEN constitutive GFP-expressing control vector. **(c)** retG SV40(-) control vector. **(d)** RET vector, plasmid, and corresponding provirus. **(e)** RETdc "double copy" vector, plasmid, and corresponding provirus. Shaded arrows indicate elements cloned in reverse orientation. Expected transcripts and splicing patterns are shown beneath each vector. Where available, the average titer for each vector is shown to the right of the vector's diagram. Abbreviations: sd, vector splice donor; sa, vector splice acceptor; ψ , encapsidation signal; ppt, polypurine tract; SD, splice donor of the SV40 early small t antigen intron (reverse orientation); SA, splice acceptor of the SV40 early small t antigen intron (reverse orientation); SV, SV40 early promoter; pA, BGH polyadenylation signal.

prolonged exposures of the blots. The greater intensity of the RETdc band again reflected the presence of two SV40-GFP cassettes. Hybridization of the blots with an oligonucleotide probe specific for 18S rRNA revealed that similar amounts of total RNA were loaded in each lane (**Figure 3**). The size of the transcripts suggested that splicing occurred at the expected junctions. RT-PCR of the RET and RETdc RNA samples and sequencing of the cDNA products demonstrated that while most of the RNA was indeed spliced, a small percentage was not (**Figure 4**).

Analysis of gene expression

To assess vector-mediated GFP expression, we analyzed both the producer and the target cells by flow cytometry (**Figure 5**). Expression of GFP in both GLEN-transfected PG13 cells and GLEN-transduced TE671 cells, wherein the GFP is cloned in forward orientation, was more than 1.5-logs greater than the control cells. Analysis of RET and RETdc producer cells and their TE671 target cells demonstrated GFP fluorescence only in the TE671 cells. The fluorescence of the RETdc-transduced TE671 cells was twice as intense as that of the RET target cells, but was

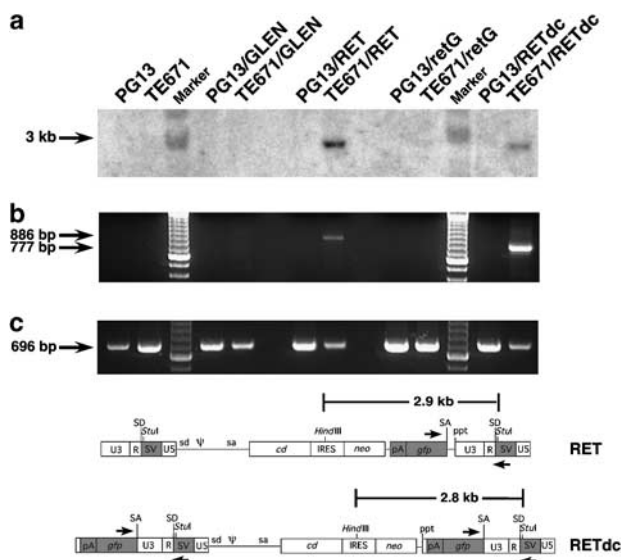


Figure 2 Physical approximation of the reverse-oriented SV40-promoter and GFP gene in integrated proviruses. **(a)** Southern blot of genomic DNA from G418-selected PG13 producer and TE671 target cells, digested with *Stul* and *HindIII*, and hybridized with a ³²P-labeled GFP-specific cDNA probe. Sample/lane identities are indicated along the top. Bands are observed where the SV40 promoter (*Stul* site) has been copied via reverse transcription to the 3'LTR, i.e., in the TE671 cells transduced with RET and RETdc vectors. Bands are not detected in the corresponding PG13 producer cells or TE671 target cells transduced with retG or GLEN. Proviral structures with relevant restriction sites and expected band sizes are indicated at the bottom. **(b)** PCR of genomic DNA from the same samples used for Southern analysis using oligonucleotides specific for the GFP gene and the SV40 promoter. Reactions were run on a 1.5% agarose gel stained with ethidium bromide (EtBr). Sample/lane identities are as in **a**. Successful amplification requires the copying of the reverse-oriented SV40 promoter to the 3'LTR. Appropriately sized PCR bands were obtained from TE671 cells transduced with the RET and RETdc vectors, but from no other samples. The greater intensity of the bands for the RETdc compared with the RET-transduced cells is likely the result of the second copy of the SV40-GFP cassette. Arrows in the vector diagrams indicate the location and orientation of the PCR primers. **(c)** Control PCR performed on the same genomic DNA samples using primers specific for 18S ribosomal RNA.

lower than that observed in TE671 cells transduced with GLEN. GFP fluorescence was not observed in either the producer or the target cells of the retG control vector, demonstrating the dependence of the expression on the presence of the SV40 promoter and its approximation to the GFP gene on reverse transcription. The magnitude of this expression was also, at least in part, dependent on the functioning of the synthetic intron. Analysis of GFP expression in TE671 cells transduced with a vector identical to RETdc, except that it lacked the introduced splice donor, demonstrated 1/2-log lower expression relative to that observed in target cells transduced with the original vector (data not shown).

Finally, we assessed the stability of GFP expression in RET-transduced TE671 cells cultured continuously in the absence of G418 selection by periodic flow cytometry. Under these conditions, GFP expression was maintained for more than 6 months (data not shown).

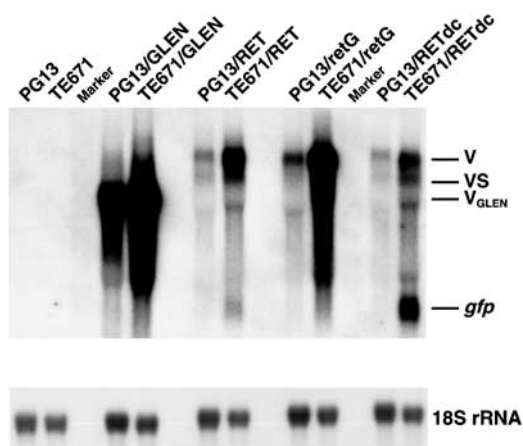


Figure 3 Analysis of GFP mRNA expression in producer and target cells. The upper panel shows a Northern blot of total RNA from G418-selected PG13 producer and TE671 target cells hybridized with a ³²P-labeled GFP-specific cDNA probe. Sample/lane identities are indicated along the top. In TE671 cells transduced with the RET and RETdc vectors, copying of the SV40 promoter to the 3'LTR by reverse transcription resulted in the generation of a small (~1 kb) GFP-encoding transcript not seen in the corresponding PG13 producer cells or in TE671s transduced with retG or GLEN. Vector diagrams and expected transcripts are shown in **Figure 1**. Abbreviations: V: RET, retG, and RETdc genomic RNAs; VS: RET, retG, and RETdc spliced vector RNAs; VGLEN: GLEN genomic and spliced vector RNAs (indistinguishable); GFP: GFP spliced RNAs. Lower panel shows a control Northern blot of the same RNA samples using a ³²P-labeled oligonucleotide probe specific for 18S ribosomal RNA.

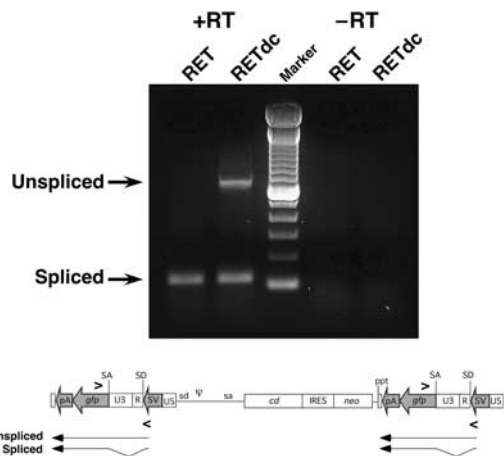


Figure 4 Analysis of the splicing efficiency of the GFP mRNA synthetic intron. RT-PCR of total RNA from G418-selected RET- and RETdc-transduced TE671 target cells. The oligonucleotides used for cDNA synthesis are specific for the GFP gene and for the 5' untranslated region of the SV40 promoter-driven transcript. Only the GFP-specific oligo was used for minus-strand synthesis to ensure that no products were derived from reverse transcription and amplification of the vector genomic RNA. Reactions were run on a 1.5% agarose gel stained with EtBr. Sample/lane identities are indicated along the top. The two samples on the right are reverse transcriptase (RT) minus controls. Successful amplification requires the copying of the reverse-oriented SV40 promoter to the 3'LTR and its subsequent transcription of the GFP gene. Products from both spliced (~100 bp) and unspliced (~700 to 800 bp) transcripts were observed for the samples due to differential splicing of the inserted intron. The RETdc proviral structure and expected transcripts are shown at the bottom. Small arrows indicate the location and orientation of the PCR primers.

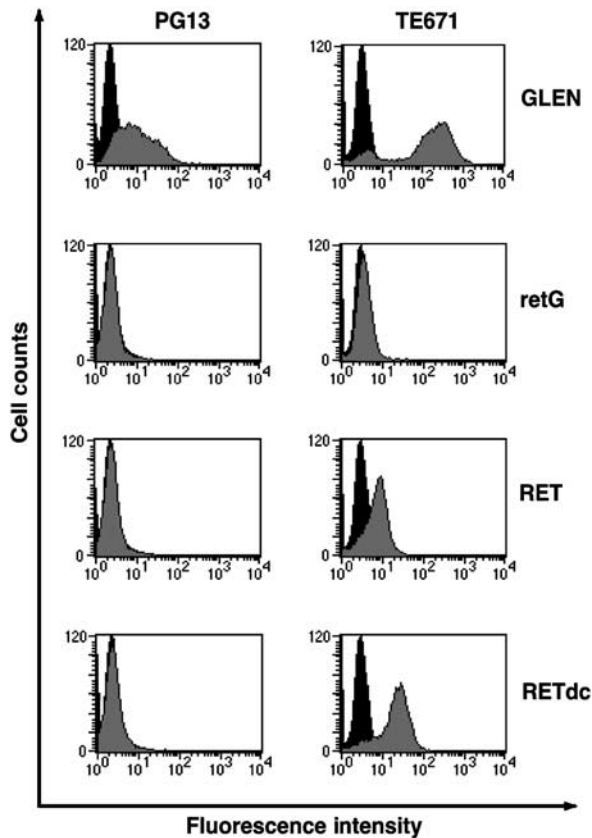


Figure 5 Flow cytometry analysis of GFP expression in producer and target cells. Dark shading shows the baseline fluorescence in untransduced cells. Light shading indicates expression in transfected producer or transduced target cells. PG13 cells were transfected with the indicated vectors, selected in G418, and the surviving colonies pooled. Supernatants were harvested and used to transduce TE671 cells, which were then selected in G418 and pooled. Analysis of the PG13 cells was performed at the time of supernatant harvesting from the G418-selected populations, and on TE671 cells after selection and pooling.

DISCUSSION

We generated prototypes of a retroviral vector that restricted expression of their encoded transgenes to target cells without the use of an inducible promoter or tissue-specific promoter. Gene expression was efficiently restricted by tying it to retroviral reverse transcription, part of the natural viral life cycle that occurs after budding of the virus from its producer cell.³⁻⁵ These vectors appear to be highly efficient at restricting gene expression to the transduced target cells and preventing expression in the producer cells. This does not imply, however, that no circumstances exist under which expression might be observed in VPC. Theoretically, “head-to-tail” tandem integration of transfected vector plasmids or autotransduction could allow for aberrant gene expression in the VPC. This was not observed and, given the architecture of our vectors and the use of the PG13 packaging cell line that precluded autotransduction, this possibility is not likely to be realized.

The design of our vectors raised a number of concerns regarding potential deleterious effects of the requisite manipulations of the vector backbone on viral titers and gene expression. A chief concern was the effect of the inverted SV40 promoter

placed in the 5'LTR of the vector. The proximity of a transcriptionally active, reverse-oriented element to the viral LTR-promoter might interfere with the latter's activity, perhaps owing to steric interference between two oppositely oriented RNA polymerases, reducing both vector titer and LTR-mediated expression of the forward-oriented transgene. Further, given the well-documented sensitivity of the viral genomic region proximal to the viral primer binding site to manipulation,²⁶⁻³⁰ there was concern that a promoter insertion at or near the U5 region of the 5'LTR would result in a drop in titer by adversely affecting reverse transcription, perhaps because of the disruption or displacement of a required element of the vector genome at the primer binding site. Joshi *et al.*^{31,32} demonstrated, however, that insertions of up to 829 bp at the border of R and U5 have little deleterious effect on titers. Similarly, we found that transcriptionally inert insertions as large as 2.4 kb at this position had no significant effects on vector titers and gene expression (data not shown). Indeed, the titers of the RET and RETdc vectors containing the SV40 promoter were only modestly reduced (≤ 1 log) relative to those of the retG promoterless control vector, and the functional expression of the *cd* and *neo* genes were unaffected. The reduction in titer was likely the result of the SV40 promoter interfering with transcription from the viral LTR promoter, rather than from any effect on reverse transcription. It should be remembered, however, that these lower titers were obtained from polyclonal VPC populations. Subcloning should allow for the selection of producing higher titers VPC clones.

Another concern with the design of the vectors was the possibility that the viral LTR might adversely affect or completely dampen expression of the transgene from the copied SV40 promoter in target cells. Interference by the viral LTR on exogenous promoters by an as yet unidentified mechanism is well documented.^{3,6,7,33} Furthermore, there was a concern that an antisense effect between the LTR-driven transcript and the SV40-driven transcript might reduce the efficiency of translation of the latter.⁶ Indeed, although the magnitude of GFP expression in TE671 cells transduced with RET and RETdc vectors was significant, it was still lower by about 1 log that of TE671 target cells transduced with the control GLEN vector. One or both mechanisms may have contributed to the drop in gene expression.

There are means by which gene expression might be increased in this system. We explored one possibility with the construction of the “double copy” or RETdc version of the vector.²⁴ The presence of two proviral GFP-encoding minigenes had the effect of increasing GFP expression by greater than two fold. Also, the generation of a self-inactivating or “SIN” version of the vectors might have the desired effect by eliminating promoter interference and antisense effects.²⁵ Additionally, insertion into the vectors of an intron-disrupted polyadenylation signal sequence,³⁴ cloned in forward orientation just upstream of each vector's polypurine tract, might also eliminate these adverse effects by leaving the reverse-oriented minigene essentially outside of the transcriptional unit of the 5'LTR in transduced cells.²⁴ Finally, incorporation of stronger promoters such as the cytomegalovirus (CMV) immediate-early promoter or a second

Moloney murine leukemia virus (MoMLV) LTR, in lieu of the SV40 promoter, may also increase the level of gene expression.

Julias *et al.*³⁵ and Delviks *et al.*^{36,37} used the phenomenon of tandem repeat deletion peculiar to retroviral reverse transcription to develop the “E”- and “ ψ ”- vectors, respectively, that effectively limit expression of a transgene to either the VPC or target cells, depending upon the architecture of the vector. These vectors can be produced at reasonable titers; however, we believe that our vectors offer ease of cloning compared with these other systems, in that an intact transgene can be readily inserted into a multiple cloning site in our vector, whereas cloning into these other vectors requires splitting of the transgene. Tabotta *et al.*³⁸ reported the ReCon vector, which bears resemblance to the current work. The high vector titers reported with this system are somewhat surprising, given that in their system the SV40 polyadenylation signal is cloned into the 5' most portion of the U3 region of the vector's 3'LTR. It would be expected that the bi-directionality of this powerful signal, consisting of the early mRNA polyadenylation signal in one orientation and the late mRNA signal in the other, would truncate the mRNAs generated in both orientations, including premature truncation of the vector LTR-driven genomic transcript, but this was apparently not observed. Although successful at achieving high titers and expression, the ReCon system appears less efficient at limiting expression of the transgene to target cells. Aberrant expression in the VPC was attributed to read-through from adjacently integrated vector plasmids, although autotransduction resulting from the use of an amphotropic retroviral packaging cell line may contribute. We believe the careful exclusion from our vectors of elements containing putative cryptic splice sites and polyadenylation signals in the forward orientation, as well as the use of a packaging cell line incapable of autotransduction represent an improvement.

Others sought to improve the efficacy of retrovirus-mediated suicide gene therapy of tumors by enhancing vector spread through stereotactic intratumoral injection of VPC producing vectors encoding suicide genes.^{8,12-14} However, as the VPCs also express the suicide gene, exposure to the prodrug also results in killing of the VPCs, limiting vector production and transduction, and negating much of the advantage of direct VPC administration. Use of inducible or tissue-specific promoters to overcome this difficulty is of limited efficacy for a number of reasons, including the problems with promoter “leakiness.” The approach described generated Moloney-based retroviral gene transfer vectors encoding reverse transcription-activated transgenes that can be produced in stable VPC lines at reasonable titers. These vectors may allow the generation of stable VPC for retroviruses encoding cytotoxic genes. Our vector system effectively precludes transgene expression in VPC and may thus prevent their destruction when exposed to the prodrug, potentially allowing for repeated rounds of prodrug administration after a single VPC inoculation.

MATERIALS AND METHODS

Cell lines. PG13 gibbon ape leukemia virus (GaLV)-pseudotyped retroviral vector packaging cells³⁹ were obtained from ATCC (Manassas, VA) and TE671 human rhabdomyosarcoma cells⁴⁰ were a kind gift of

Dr BA Bunnell (NHGRI, Bethesda, MD). They were grown in Dulbecco's modified Eagle's medium (4.5 g/L glucose) supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bio-Products, Calabasas, CA), 2 mM glutamine, and gentamicin at 37°C and 5% CO₂.

Retroviral constructs and transductions. The GCsamCDENretSG (RET), GCsamCDENretSGdc (RETdc), and GCsamCDENretG (retG) retroviral constructs were derived from pGCsamEN¹⁰ (generous gift of RA Morgan, NCI, Bethesda, MD). Briefly, pGCsamEN is a bicistronic vector consisting of 5' Moloney murine sarcoma virus and 3' Moloney murine leukemia virus LTRs, a multiple cloning site (MCS), and a picornaviral internal ribosomal entry site (IRES) that serves as an additional translation initiation site for the neomycin phosphotransferase II (*neo*) gene (**Figure 1a**). The RET construct consists of GCsamEN modified with: (1) the simian virus 40 (SV40) early promoter and a synthetic splice donor cloned in reverse orientation into the R-U5 border of the vector 5' LTR, (2) a GFP gene and a synthetic splice acceptor assembly cloned in reverse orientation just upstream of the vector polypurine tract; and (3) an *Escherichia coli* cytosine deaminase (*cd*) gene inserted into the multiple cloning site (MCS) in forward orientation (**Figure 1d**). The plasmid was generated by inserting the synthetic, double-stranded fragment, 5'-CCTCTCTAAGGTAAATTCG CGGAAT-3', which carries the SV40 small *t* antigen splice donor and possesses 5' *StuI* and 3' *Clal* compatible ends, into the *StuI*-*Clal* site of pEGFP-N1 (Clontech, Palo Alto, CA). This arrangement places the splice donor immediately downstream of the SV40 early promoter present in pEGFP-N1. The 385 bp *SspI*-*Clal* fragment of the resulting construct, containing the SV40 promoter/splice donor assembly, was inserted in reverse orientation by blunt-end ligation into a *Clal* site introduced at the R-U5 border of the 5' LTR of GCsamEN. The *Clal* site was introduced by PCR mutagenesis of GCsamEN using the primers 5'-TTT CTAGTGAATCATCAGATGTTTCCAGGGTGCC-3' and 5'-CCCTCCCA AGGAACAGCGAGACCACGATTCGATCGATGCAAACAG-3'. The 256 bp PCR product was digested with *StyI* and ligated into the *StyI*-*StyI* site in the 5' LTR of GCsamEN. Because of the multiple *StyI* sites in GCsamEN, the 5' LTR was first isolated by subcloning into pBluescriptKS(+) (Stratagene, La Jolla, CA). Next, the 1.5 kb *EcoRI*-*BamHI* fragment of pCD2²⁴ encoding a mutated *E. coli cd* gene was inserted in forward orientation into the *SnaBI*-*XhoI* site of the modified GCsamEN by blunt-end ligation. This places the *cd* gene under the constitutive transcriptional control of the viral 5' LTR. The small 146 bp *StuI*-*XhoI* fragment generated between the *cd* gene and IRES was excised and the plasmid blunt-ended and religated to reduce the vector's size and, to remove a *BamHI* site, the *cd* gene was inserted into the vectors as a negative selection marker. Its expression was also used to assess the effect of the inverted SV40 promoter on LTR-driven gene expression. The final step was to clone by blunt-end ligation an enhanced GFP (the 732 bp *NotI* fragment of pGreenLantern-1 (Invitrogen, Carlsbad, CA)), with upstream synthetic splice acceptor derived from the SV40 small *t* antigen and downstream bovine growth hormone (BGH) polyadenylation signal, in reverse orientation into the remaining *BamHI* site of the modified GCsamEN. The RETdc construct is similar to RET, except that the GFP/splice acceptor assembly is cloned in reverse orientation into the U3 region of the viral 3' LTR in a “double copy” configuration¹³ (**Figure 1e**). This was performed by inserting the GFP assembly into the *AflIII* site of the 3' LTR via blunt-end ligation. RetG is similar to RET, except that it has an unmodified 5' LTR lacking the SV40 promoter/splice donor assembly. In addition, the 1.5 kb fragment encoding *cd* was cloned into the *Sall*-*XhoI* site of GCsamEN, rather than into the *SnaBI*-*XhoI* site (**Figure 1c**). The GCsamGLEN control vector (GLEN) consists of GCsamEN, modified with the GFP gene, inserted into the MCS in forward orientation (**Figure 1b**). It was generated by blunt-end insertion of the 732 bp *NotI* fragment of pGreenLantern-1 into the

SnaBI-XhoI site of GCsamEN. This places the GFP under the constitutive transcriptional control of the viral 5' LTR.

Vectors were generated by liposomal transfection of the above constructs into PG13 cells using Lipofectamine Reagent (Invitrogen). Transfected cells were selected in 1 mg/mL G418 and the survivors were pooled to generate a polyclonal vector producer cell population. Supernatants were harvested from populations controlled for cell number, supplemented with 8 µg/mL polybrene, passed through a 0.45-µm filter, and titered by serial dilution on TE671 cells with G418 (1 mg/mL) selection. Experimental transductions using supernatants supplemented with polybrene were carried out on TE671 cells overnight at 37°C, 10% CO₂, at multiplicities of infection ranging from 0.0001 to 1 colony-forming unit (CFU) per cell. Transduced cells were selected in 1 mg/mL G418 and the surviving cells was pooled for analysis.

Southern analysis. Genomic DNA was isolated using the Nucleon BACC 3 kit (Scotlab Bioscience, Lanarkshire, Scotland) from PG13 producer cells at the time (t_0) that supernatant was harvested (after transfection, G418 selection, pooling, and replating) and from TE671 target cells after transduction and G418 selection. DNA was digested overnight at 37°C with *HindIII* and *StuI*. Digests were run on 1.5% agarose gels and blotted onto nylon membranes. Blots were probed overnight at 68°C with the 732 bp *NotI* fragment of pGreenLantern-1 random-labeled with [α -³²P]dCTP.

PCR analysis. Genomic DNA from the same samples used for Southern analysis was analyzed by PCR using the following oligonucleotides: 5'-CGCCATCCAGTTCCACGAGAAT-3' and 5'-CTCCGCCAGTTCCGCCATTCTC-3'. The former is specific for a region of the GFP gene and the latter for a region of the SV40 promoter. A control PCR for 18S ribosomal RNA was also performed on the DNA using the following oligonucleotides: 5'-AACGCTGCCCCTATCAACTTTCG-3' and 5'-CCTCCGACTTTCGTTCTTGATTA-3'. These primers are specific for both human and mouse 18S rRNA. Platinum Taq DNA Polymerase High Fidelity (Invitrogen) was used for all amplifications. Reactions were run on a 1.5% agarose gel stained with ethidium bromide (EtBr) and photographed.

Northern analysis. Total RNA was isolated from PG13 producer cells at t_0 and from TE671 target cells after transduction and G418 selection using Trizol (Invitrogen). The RNA was denatured for 15 min at 65°C and then run on 1.1% agarose-formaldehyde gels and blotted onto nylon membranes. Blots were hybridized overnight at 42°C with either the random-labeled *NotI* fragment of pGreenLantern-1 or the oligonucleotide, 5'-TGCCCCCGGCCGTCCCT-3', specific for human and mouse 18S ribosomal RNA, end-labeled with ³²P.

RT-PCR analysis. Total RNA isolated from TE671 target cells transduced with either the RET or RETdc vectors was analyzed by RT-PCR using the Access RT-PCR kit (Promega, Madison, WI), with some modification of the manufacturer's instructions. Specifically, the minus-strand synthesis was performed using the oligonucleotide 5'-CGCCATCCAGTTCCACGAGAAT-3' alone, whereas the oligonucleotide 5'-TAGTGAGGAGGCTTTTTGGAG-3' was added for the amplification only after heat inactivation of the reverse transcriptase, but before initiation of cycling. The former oligo is specific for a region of the GFP gene and the latter for the 5' untranslated region of the SV40 promoter-driven transcript, upstream of the introduced splice donor. Reactions were run on a 1.5% agarose gel stained with EtBr and photographed. Automated dideoxy cycle sequencing of the PCR products was performed (SeqWright, Houston, TX) on an ABI sequencing system (PE Corporation, Norwalk, CT) using the GFP-specific oligo as the primer.

Flow cytometry. Cells were trypsinized, washed with PBS, and analyzed on a Becton Dickinson FACSCalibur. PG13 producer cells were analyzed by flow cytometry for GFP expression at t_0 . TE671 target cells were analyzed for GFP expression after transduction and selection.

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