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# Novel Retroviral Vector Transferring a Suicide Gene and a Selectable Marker Gene with Enhanced Gene Expression by Using a Tetracycline-Responsive Expression System

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**A retroviral vector for the enhanced expression of the herpes simplex virus thymidine kinase (HSV *tk*) gene was developed by using a tetracycline-responsive expression system (TRES). The two components of the TRES, the chimeric transactivator (tTA) and the corresponding tTA-binding *cis* element (*tetO*), were both incorporated into a retroviral vector and resulted in high levels of *tk* gene expression from *tetO* in target cells. Amphotropic virus supernatants from stable producer cells, generated by the retroviral vector containing the TRES, gave titers of $10^4$ to $10^5$ G418-resistant CFU/ml on murine NIH 3T3 cells. The retroviral vector (G1tTA-[tetOTkINa]$_R$), in which *tetO* was used in the opposite orientation relative to viral transcription, was capable of transducing *tk* and *neo* genes into murine NIH 3T3 cells to yield a high level of *tk* gene expression. TK enzyme activity in NIH 3T3 cells transduced by this vector was 417-fold higher than in control cells. This increased TK activity was returned to basal levels in the presence of tetracycline. The level of *tk* gene expression driven by *tetO* from G1tTA-[tetOTkINa]$_R$ vector in NIH 3T3 cells was fourfold higher at both the mRNA level and the TK enzyme level than that produced by the long terminal repeat of G1Tk1SvNa, the vector being used in the ongoing brain tumor gene therapy trial. Retroviral vectors containing the TRES may be useful therefore in achieving higher levels of *tk* gene expression, which should facilitate gene therapy approaches in the treatment of cancer.**

Recombinant retroviruses are widely used as an efficient gene delivery system into eukaryotic cells (for reviews, see references 1, 5, 11, 19, 23, 26, and 29 and references therein). The availability of safety-improved packaging cell lines and retroviral vectors to generate replication-incompetent retroviruses (20) expands this system as a tool for human gene therapy. The ultimate goal in clinical applications of retroviral vector-mediated gene delivery is not only highly efficient gene transfer but also appropriately regulated and stable gene expression from a safely integrated provirus. In an effort to maximize expression of the clinically relevant herpes simplex virus thymidine kinase (HSV *tk*) gene in target cells, we have constructed a retroviral vector containing a tetracycline-responsive expression system (TRES) and exploited the high levels of *tk* gene expression from the provirus.

Gossen and Bujard (12) have previously described a regulatable transcriptional system based on the bacterial tetracycline resistance operator/repressor. The system consists of two components, the transcriptional activator (tTA) and its corresponding *cis* element (*tetO*). A chimeric protein (tTA) composed of the tetracycline repressor fused to the activation domain of the HSV transcriptional activator, VP16, serves as the transactivator. The target of tTA is a synthetic promoter (*tetO*) consisting of tandem repeats of the tetracycline operator and a minimal promoter sequence from the cytomegalovirus immediate-early promoter. The TRES has been originally shown to function as a regulatable transcriptional system, with up to $10^5$-fold induction in the absence of tetracycline compared with the presence of tetracycline, in mammalian cells both in cell lines and in vivo in the skeletal muscle of mice and in transgenic mice (3, 9, 12, 28, 31). In addition, the use of the TRES is attractive as it potentially allows an additional safety feature to be incorporated for in vivo gene therapy, namely an ability to shut off gene expression in the presence of tetracycline.

In this study we have incorporated into a single retroviral vector the *tk* gene under control of *tetO* and also provided the tTA transactivator in the same retroviral vector. We have tested whether this arrangement allows a high level of constitutive *tk* gene expression in the absence of tetracycline and examined the repressive effect of tetracycline on the expression of the *tk* gene under *tetO* control. This present study reports that the retroviral vector (G1tTA-[tetOTkINa]$_R$), in which the *tetO* transcriptional unit was positioned in the opposite orientation relative to viral transcription, conferred a greater than 400-fold higher TK enzymatic activity on transduced NIH 3T3 cells than on nontransduced control cells. This increased *tk* gene expression, which could be repressed by the addition of tetracycline, was significantly higher than *tk* expression obtained from the retroviral long terminal repeat (LTR).

**Retroviral vectors containing the TRES.** Various recombinant retroviral vectors, including the control G1Tk1SvNa vector (17), are schematically illustrated in Fig. 1. We first constructed G1TkINa, in which the sequences encoding the clinically useful HSV *tk* gene and the selectable marker (neomycin phosphotransferase gene) are connected by internal ribosome entry site (IRES) sequences (10, 22), to allow these two gene products to be expressed from the same promoter. Retroviral vectors encoding a dicistronic transcription unit permit both genes to be expressed more predictably by avoiding promoter interference or epigenetic gene suppression after selection pressure (6, 7, 16, 22). The G1TkINa vector was made by inserting the HSV *tk* gene from the plasmid pG1NaSvTk1

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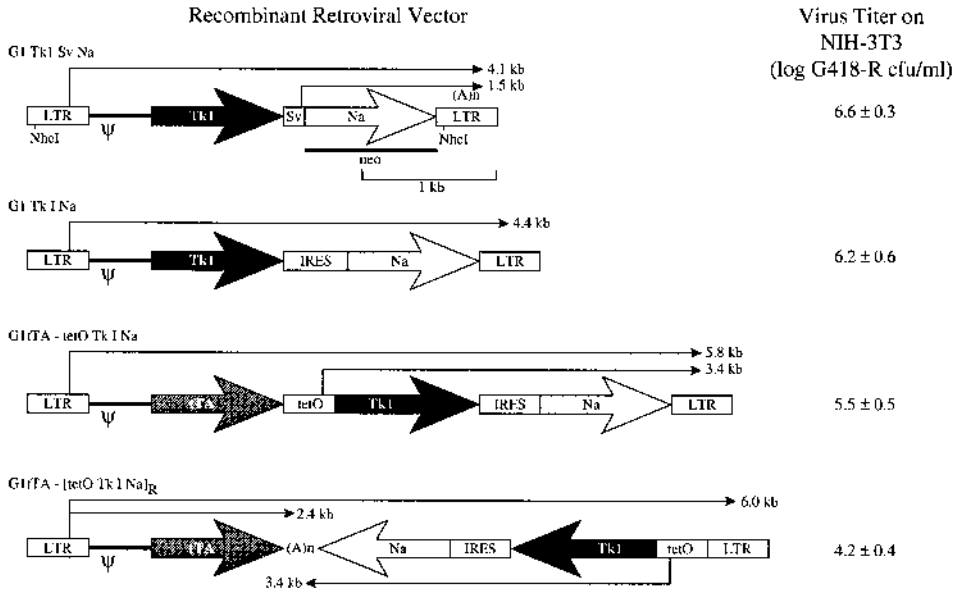


FIG. 1. Schematic representation of the recombinant retroviral vectors containing various promoters/enhancers and the *tk* and *neo* genes. (A)n, polyadenylation signal; ψ, packaging signal sequence; Sv, simian virus 40 enhancer/promoter; Tk1, HSV type 1 *tk* gene; IRES, encephalomyocarditis virus internal ribosome entry site sequence; Na, neomycin phosphotransferase (*neo*) gene; tTA, chimeric transactivator; *tetO*, tTA-binding synthetic promoter. The arrows indicate the approximate location of promoters and the direction of transcription. Sizes of the predicted transcripts are indicated at the ends of the arrows. The thick line under Na indicates a *neo* cDNA probe used in Northern analysis. The G418-resistant titers on NIH 3T3 cells of the virus supernatants from producer cells containing the diagramed vectors are shown on the right side (mean ± standard deviation).

and the encephalomyocarditis virus IRES sequences (10) from the plasmid pEMC-F into a modified pG1XSvNa plasmid which contains a linker sequence (5′-GTCGACGGATCCCT CGAGAAGCTTCCTAGG-3′).

The retroviral vectors G1tTA-tetOTkINa and G1tTA-$[tetOTkINa]_R$ were designed to carry both the tTA and *tetO* components of the TRES within the same vector and therefore contain two independent promoters, the LTR and *tetO*. To construct the G1tTA-tetOTkINa vector, tTA from the plasmid pUHD15-1 (12), *tetO* from the plasmid pUHD10-3, and TkINa sequences from the vector G1TkINa were inserted into the G1 vector (18). The G1tTA-$[tetOTkINa]_R$ vector was constructed by inserting tTA from the plasmid pUHD15-1, a simian virus 40 poly(A) signal from the plasmid pSG5 (Stratagene, La Jolla, Calif.), *tetO* from the plasmid pUHD10-3, and TkINa sequences from the vector G1TkINa into the G1 vector.

**Producer cell lines and virus titer.** PE501 cells (murine ecotropic retroviral packaging line) ($6 \times 10^5$) (21) were plated per 100-mm tissue culture dish in Dulbecco's modified Eagle medium with high glucose (4.5 g/liter) and supplemented with 2 mM glutamine and 10% fetal bovine serum (BioWhittaker, Walkersville, Md.). After 6 h, cells were transfected with 20 μg per plate of the indicated retroviral vector, using calcium phosphate precipitation (5 Prime-3 Prime, Inc., Boulder, Colo.). Cells were washed, replaced in fresh medium after 16 h of transfection, and incubated for an additional 48 h after which time the supernatant was collected. Ecotropic replication-defective virus supernatants from the transfected PE501 cells were filtered through a 0.45-μm-pore-size Millex-GS filter (Millipore, Bedford, Mass.) and used to transinfect the amphotropic packaging cell line, PA317 (murine amphotropic retroviral packaging line; American Type Culture Collection, Rockville, Md.). These transinfected PA317 cells were selected for 10 days with 0.6 mg of G418 (Geneticin; Gibco, Grand Island, N.Y.) per ml until individual resistant cells formed colonies. Colonies were pooled and used as producer cells. To produce amphotropic replication-defective viral supernatants, $5 \times 10^6$ producer cells were plated into a roller bottle (Corning, Corning, N.Y.) and supernatants were collected after the cells reached 90% confluence.

To demonstrate the feasibility of producing functional viral particles carrying the TRES, virus supernatants collected from various stable producer cells were compared for their ability to transfer resistance to G418. Replication-defective virus particles that were produced by the stable producer cells generated with all of these vectors produced functional particles with viral titers of $10^4$ to $10^6$ CFU/ml in NIH 3T3 cells (Fig. 1). Recombinant G1tTA-tetOTkINa and G1tTA-$[tetOTkINa]_R$ retroviruses have two promoters, the LTR and *tetO*, carry exogenous inserts of approximately 4.5 kb, and produce G418-resistant viral titers that are one log ($3 \times 10^5$ CFU/ml) and two logs ($2 \times 10^4$ CFU/ml) lower, respectively, when compared with the titer of G1Tk1SvNa recombinant virus. These results suggest that the orientation of the inserted gene, besides its length and content, affects the titer of vector supernatant. It appears that the reduced titer of G1tTA-$[tetOTkINa]_R$ virus is due to the production of antisense RNA from *tetO* (RNA complementary to that synthesized from the 5′ LTR) (14), as its titer is increased one log by addition of 1 μg of tetracycline per ml to repress *tetO*-driven transcription (data not shown).

**Analysis of transcripts from the provirus in the transduced NIH 3T3 cells.** A total of $3 \times 10^5$ NIH 3T3 $TK^-$ cells (ATCC, Rockville, Md.) was plated in a 100-mm tissue culture dish and transduced with various amphotropic vector supernatants supplemented with 8 μg of Polybrene per ml at a multiplicity of infection of 1. Cells were selected with 0.6 mg of G418 per ml for 10 days and used for RNA extraction. The expression of proviral transcripts was analyzed by Northern hybridization (27) with a *neo* cDNA probe in parallel with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe using 20 μg of total RNA. NIH 3T3 cells transduced with the G1Tk1SvNa vector produced two *neo* transcripts (4.1 and 1.5 kb), generated

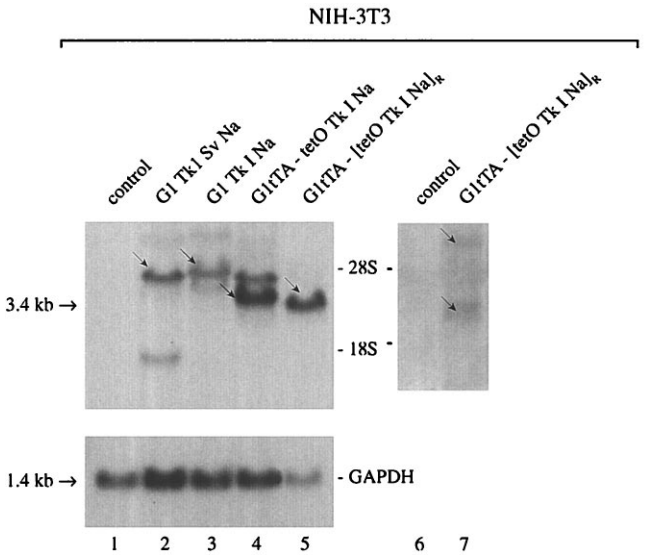


FIG. 2. Northern blot analysis of transcripts in retrovirally transduced NIH 3T3 cells and control cells. To identify transcripts expressed in different NIH 3T3 cells after transduction with the indicated retroviral particles, aliquots containing 20 μg of total RNA from the transduced NIH 3T3 cells and control cells were characterized by Northern blot analysis. The RNA blots were independently hybridized with a $^{32}$P-labeled *neo* probe (top left panel), a $^{32}$P-labeled tTA probe (top right panel), or a $^{32}$P-labeled human GAPDH probe (bottom panel). The characteristic predicted transcripts (4.1, 4.4, and 3.4 kb) either from the LTR or *tetO* are indicated by arrows (see text and Fig. 1). The positions of 18S and 28S rRNA species are indicated.

from the LTR and the simian virus 40 promoter (Fig. 1 and Fig. 2, lane 2). NIH 3T3/G1TkINa cells showed a 4.4-kb transcript initiated from the LTR promoter (Fig. 1 and Fig. 2, lane 3). For the *tetO*-tTA-containing vectors, a transcript of 3.4 kb initiated from the internal *tetO* promoter was expected. This product was seen in both NIH 3T3/G1tTA-tetOTkINa and NIH 3T3/G1tTA-[tetOTkINa]$_R$ cells (Fig. 1 and Fig. 2, lanes 4 and 5). However, for GltTA-tetOTkINa the predicted 5.8-kb transcript originating from the LTR was not observed and instead a short 4.1-kb transcript was seen (Fig. 2, lane 4).

For G1tTA-[tetOTkINa]$_R$, we were surprised to find only a major transcript of 3.4 kb and not the expected 6-kb transcript (Fig. 2, lane 5). However, when tTA cDNA was used as a probe, the expected 6- and 2.8-kb transcripts were observed in NIH 3T3/G1tTA-[tetOTkINa]$_R$ cells after longer exposure of the hybridized membrane (Fig. 2, lane 7). After treatment with 0.1 to 10 μg of tetracycline (Sigma, St. Louis, Mo.) per ml for 24 h, the level of 3.4-kb transcript in target NIH 3T3/G1tTA-[tetOTkINa]$_R$ cells was reduced to levels comparable to those found in untransduced NIH 3T3 cells, whereas the 3.4-kb transcript in NIH 3T3/G1tTA-tetOTkINa cells was not sensitive to tetracycline treatment (data not shown). Furthermore, Southern analysis with the *neo* probe of PA317/G1tTA-tetOTkINa producer cells indicated significant deletion of the proviral genome (data not shown), suggesting the TRES is unstable in this gene configuration. Although Hoshimaru et al. have achieved regulatable c-*myc* gene expression from a tetracycline-responsive promoter in the same orientation as the LTR (13), our present observation suggests that the G1tTA-tetOTkINa vector has undergone rearrangements or deletions and that the 3.4-kb transcripts do not originate from the intact *tetO*. A tetracycline-regulated retroviral vector recently reported by Paulus et al. also had a *tetO*-driven luciferase gene in the opposite orientation to the LTR (25).

TABLE 1. Comparison of HSV TK enzymatic activity in retrovirally transduced NIH 3T3 cells and untransduced control cells[a]: Tetracycline-dependent TK activity in NIH 3T3/GltTA-[tetOTkINa]$_R$ cells

| Retroviral vector | TK activity[b] | Tetracycline (1 μg/ml) | Fold induction (−Tet/+Tet) |
|---|---|---|---|
| None | 0.11 ± 0.06 | − | |
| G1Tk1SvNa | 10.49 ± 0.53 | − | |
| G1TkINa | 16.51 ± 3.74 | − | |
| G1tTA-[tetOTkINa]$_R$ | 45.90 ± 1.80 | − | 353 |
| | 0.13 ± 0.06 | + | |

[a] Retroviral vectors containing the HSV *tk* gene under the transcriptional control of either the LTR or *tetO* were transduced into NIH 3T3 cells.

[b] TK activity values are mean ± standard deviation from triplicate assays. One TK enzyme activity unit is defined as one picomole of phosphorylated GCV production min$^{-1}$ mg$^{-1}$. Enzymatic TK activity in the transduced NIH 3T3/G1tTA-[tetOTkINa]$_R$ cells was quantified in the presence and absence of tetracycline.

After quantification by Phosphorimager (Molecular Dynamics, Sunnyvale, Calif.) and standardization against constitutive levels of GAPDH mRNA, the relative level of the *tetO*-originated *tk-neo* fusion transcripts from G1tTA-[tetOTkINa]$_R$ was shown to be fourfold higher than that of the transcripts generated from the 5′ LTR promoter in both G1Tk1SvNa and G1TkINa (Fig. 2, compare lanes 2 and 3 with lane 5). Furthermore, vector G1tTA-[tetOTkINa]$_R$ could be transduced into human cancer cells and resulted in different levels of gene expression in these cell lines. Both human hepatocellular carcinoma (Hep 3B) and human epithelioid carcinoma (HeLa) cells were efficiently transduced with amphotropic G1tTA-[tetOTkINa]$_R$ retrovirus and expressed 15- and 2-fold higher levels of *tk-neo* fusion transcripts from *tetO* than from the LTR (data not shown).

**A fully functional TK protein which is regulatable by tetracycline is produced in NIH 3T3 cells transduced with the G1tTA-[tetOTkINa]$_R$ retrovirus.** The expressed protein in retrovirally transduced cells was examined by HSV TK enzymatic activity assay, using aliquots (60 μg) of crude lysate protein and 1 μCi of [8-$^3$H]ganciclovir (GCV) (Moravic Biochemicals, Inc., Brea, Calif.) (2). The filter-bound phosphorylated GCV resulting from the HSV TK enzymatic reaction was determined by scintillation counting. The TK enzyme activities in transduced NIH 3T3/G1Tk1SvNa and NIH 3T3/G1TkINa cells were 95- and 150-fold higher than the activity observed in untransduced cells (Table 1). Of particular note, the TK enzyme activity in NIH 3T3/G1tTA-[tetOTkINa]$_R$ cells was 417-fold higher than the background observed in control cells and was 4-fold higher than the TK activity achieved from the LTR in NIH 3T3/G1Tk1SvNa cells (Table 1).

Similar to vector G1TkINa, which contains a dicistronic transcription unit, a single transcription event from *tetO* in the G1tTA-[tetOTkINa]$_R$ retroviral vector generates a dicistronic mRNA that can be translated to yield both functional TK and Neo proteins (Fig. 1). To demonstrate that TK expression was from *tetO*, we added tetracycline to NIH 3T3/G1tTA-[tetOTkINa]$_R$ cells. Addition of 1 μg of tetracycline per ml to these transduced cells for 24 h was sufficient to block the increase in TK enzymatic activity (Table 1). This observation indicates that *tk* gene expression in these cells solely comes from *tetO*. In agreement with our results, Paulus et al. have recently reported that luciferase expression levels in NIH 3T3 cells transduced with an ecotropic retroviral vector containing the TRES were 300-fold higher in the absence of tetracycline than in the presence of tetracycline (25).

While the structure and expression of retroviruses generally has been considered to be stable, both genetic and epigenetic events have been shown to result in a high-frequency loss or shutdown of viral gene expression in vitro and in vivo (4, 8, 15, 24, 30). These reports caution that the results from in vitro tests may not always directly predict expression in vivo. We have now analyzed the vector contributing to stable vector design for a retroviral vector containing a tetracycline-regulatory transcriptional system. The pattern of in vitro gene expression of retroviral vectors used in this study was stable for the tested period for up to 8 months (data not shown). We are now examining the expression of the G1tTA-$[tetOTkINa]_R$ vector in vivo.

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