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Design and In Vitro Characterization of a Single Regulatory Module for Efficient Control of Gene Expression in Both Plasmid DNA and a Self-Inactivating Lentiviral Vector

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Abstract

Background: Regulation of transgene expression in target cells represents a critical and challenging aspect of gene therapy. Recently, a two-plasmid tetracycline-inducible system was developed in which the tetracycline repressor (tetR) alone, rather than the tetR-VP16 fusion derivative, was shown to function as a potent trans-modulator of a second plasmid that contains two tandem repeats of the tetracycline operator (tetO) inserted between the TATA box and the transcription start site of the hCMV major immediate-early promoter. A technological advance in this area would be the development of a single autoregulatory cassette that incorporates both of these components into nonviral and viral gene transfer vectors. For the latter, an inducible lentiviral vector that is capable of temporal and quantitative control of gene expression in either dividing or nondividing cells is highly desirable.

Materials and Methods: A one-piece inducible (1Pi) autoregulatory cassette was constructed to provide IRES-mediated translation of the tetR as well as tight control over the tetO unit preventing transcription initiation of the first cistron in the absence of the tetracycline. To increase efficiency of tetR-mediated repression, a nuclear localization signal was incorporated at the 3′ end of the tetR gene. Regulation of gene expression at the transcriptional and protein level was analyzed in transient transfection experiments using plasmid DNA. Construction of a self-inactivating lentiviral vector containing this 1Pi cassette allowed the study of its long-term effectiveness in primary human cells.

Results: The 1Pi autoregulatory cassette when incorporated into plasmid DNA allows efficient control of the secretable hEGF as well as eGFP expression in a variety of cell types. Transient transfection studies demonstrated that the time course of repression is different for the 1Pi and two-plasmid system (2Pi). In the 2Pi system, greater repression is seen with the first 24–48 hr; however, by 72 hr, similar levels of repression with the 1Pi and 2Pi systems are obtained. This regulation is reached three times faster when the tetR is modified with a nuclear localization signal to direct nascent proteins into the nuclear compartment. In addition, stable transduction of human umbilical vein endothelial cells (HUVEC) with a self-inactivating lentiviral vector incorporating this single regulator cassette provided tetracycline-inducible control of gene expression that is not diminished over time and is completely reversible upon removal of tetracycline.

Conclusions: These results suggest a model in which the 1Pi autoregulatory system reaches a steady state over time, the minimal amount of tetR produced by the basal activity of the CMV promoter and accumulated is adequate to replace the tetR that is lost over time. These studies also show that the inducible self-inactivating lentiviral vector can temporally and reversibly regulate transgene expression in HUVECs. The use of this transcriptional control unit in both nonviral and viral vector delivery systems will constitute an attractive technological advance for many gene therapy applications where temporal and quantitative control of gene expression is desired. The strengths and limitations of the 1Pi system are discussed.

Introduction

Inducible expression systems provide a valuable tool for the study of gene functions in prokaryotic and eukaryotic cells and are particularly useful for investigations of developmental genes. The ability to tightly control the level and timing of gene expression with regulatable gene transfer vectors would have clear advantages in many gene therapy applications. A number of controllable systems have been developed; however, many of these systems suffer from significantly high basal levels of gene expression under noninduced conditions and/or exhibit cellular toxicity from the inducer/repressor (1,2). The ideal inducible system would allow modulation of expression levels of the candidate gene within single cells in a homogeneous manner and

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exhibit at a minimum three desirable characteristics, namely, specificity, efficiency, and dose dependence (3). The four main systems that fulfill these ideal requirements for an inducible system are the tetracycline, FK506/rapamycin, RU486/mifepristone, and ecdysone systems (3).

Recently, Yao et al. (4) demonstrated that the tetracycline repressor (tetR) itself, rather than tetR-mammalian cell transcription factor fusion derivatives described previously, can function as a powerful transcriptional modulator to regulate gene expression from the tetracycline operator (tetO)-bearing hCMV major immediate-early promoter. Because this system employs tetR itself as transcriptional modulator, it avoids some of the potential pleiotropic and toxic effects that are sometimes seen with the chimeric transactivator used in the model system initially described by Gossen and Bujard (5), where regulation of transgene expression is achieved by an heptamerized tetO sequence fused to the hCMV minimal promoter and the hybrid transactivator between tetR and the transcription activation domain of herpes simplex virus protein, VP16 (5–8).

In the present study, a modified version of this tetracycline-inducible system was used to create a single inducible cassette that provides simultaneous expression of both the target transgene and its modulator tetR under the tetO-containing hCMV major immediate-early promoter. To achieve autoregulatable activity in the single inducible system, an internal ribosomal entry site (IRES) from the encephalomyocarditis virus (EMCV) was used to direct cap-independent translation of tetR from the second cistron of a bicistronic transcription unit (9–12). In this format, the initial transcripts are not under tetracycline (Tc) regulation until sufficient tetR is produced and then the de novo synthesized tetR binds to the tetO, blocking further transcription. Rapid activation of the system with the consequent expression of the target gene can be achieved by the addition of tetracycline. Remarkably, within about 24 hr after DNA transfection at a time when sufficient tetR has accumulated, inhibition of basal transcription has occurred and the system displays markedly tightened Tc regulation over time. This accumulation is reached faster when the tetR has been modified with a nuclear localization signal because all of the tetR proteins are present in the nuclear compartment. In addition, following transduction with a self-inactivating, Tc-regulatable lentiviral vector, longer term regulation of gene expression is maintained. Thus, this autoregulatory unit should have broad applications in a number of gene therapy and gene targeting studies where temporal and quantitative control of gene expression in either dividing or nondividing cells is desired. However, given that the single inducible cassette must be switched "on" before sufficient tetR accumulation has occurred to completely "shut off" further RNA transcription, the system may not be suitable in all cases, particularly if the co-regulated gene of interest is detrimental to the cell or important in cell development.

Materials and Methods

Construction of a Tetracycline-Inducible Single Regulatory Cassette

Basic One-Plasmid System The single inducible cassette [one-plasmid system (1Pi)] outlined in Figure 1A was constructed by three-piece ligation of an IRES from the EMCV and the tetR fragment removed from pcDNA3tetR (4), into a NotI/ClaI sites of pCMVtetOhEGF (4). The plasmid pCMVtetOhEGF, used as the parental vector for all our constructs, contains the human epidermal growth factor (hEGF) gene driven by a chimeric promoter composed by ~650 bp of the immediate early cytomegalovirus enhancer/promoter (CMVie) and two tandem repeats of the tetO positioned 10 bp downstream of the TATA box. A NotI-NheI fragment encoding the IRES sequence was removed from the vector, pCMV-Fab 105/21H previously described by Levin et al. (13). An intermediate cloning vector pGEM7Zf(+) vector (Promega, Madison, WI, USA) was modified by incorporating a synthetic linker containing a HindIII-NheI-Kozak (CCACC)-ATG-XbaI-EcoRI-Stop (TATTAA)-SpeI-ClaI-SphI restriction sites. A pair of oligonucleotides carrying the corresponding sequence was synthesized and equivalent amounts of each (10 μg) were hybridized prior to the final ligation into the HindIII-SphI sites of pGEM7Zf(+) vector. A subcloning step, using the pGem7Zf(+) vector, was required to clone the XbaI-EcoRI tetR-containing fragment from pcDNA3tetR, allowing the introduction of the flanking restriction sites (NheI-ClaI) necessary for the final cloning step as well as the insertion of a Kozak sequence preceding the first ATG (14). The resulting 0.65-Kb NheI-ClaItetR fragment was inserted downstream from the IRES sequence and prior to the polyadenylation site of the pCMVtetOEGF vector. This position allows cap-independent translation of tetR from the single mRNA transcript. The final three-piece ligation step was performed using a DNA ligation kit from TaKaRa (PanVera Corp., Madison, WI, USA) according to manufacturer procedures. Similarly, a pCMVhEGF plasmid lacking the tetO was modified to incorporate the IRES sequence and the tetR gene for its use as nonregulatable control (1Pc) plasmid.

Introduction of a Carboxy Terminal Nuclear Localization Signal on tetR

A three tandem repeat sequence corresponding to the nuclear localization signal (NLS) from simian virus large T-antigen (GATCCAAAAAAGAAGAGAAAGGTA) was incorporated at the 3′ end of tetR preceding the stop codon. A pair of complementary oligonucleotides containing the NLS sequence were synthetically prepared and, after hybridization, cloned in frame between the EcoRI and SpeI sites of

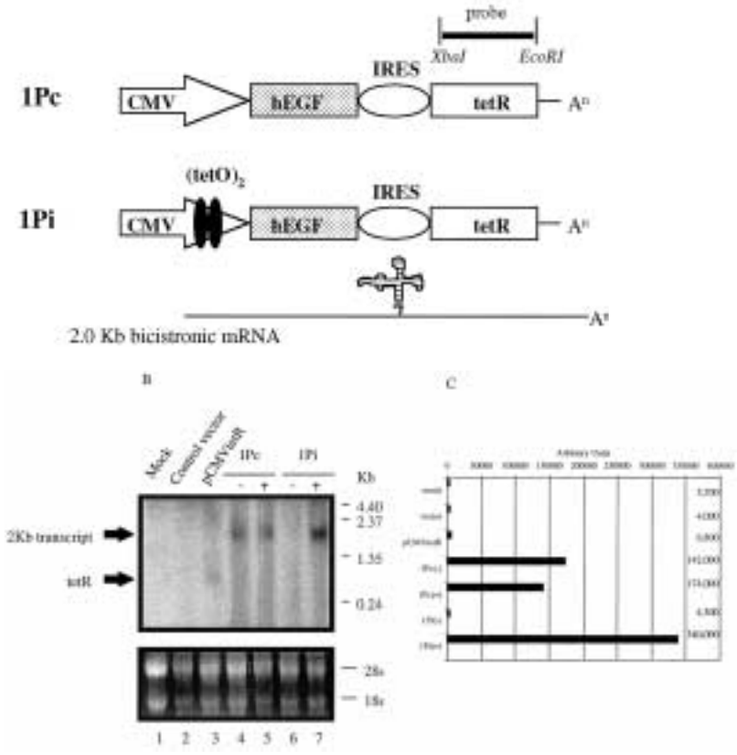

Fig. 1. TetR-mediated repression of transcription initiation. (A) Schematic diagram of the tetracycline-regulatable single expression cassette and the expected bicistronic mRNA. The tetR probe for Northern blot analysis is underlined. **(B)** Northern Blot analysis. Mock-treated Vero cells and cells transfected independently with the empty vector, the pcDNAtetR plasmid, the 1Pc, and the 1Pi were harvested 2 days posttransfection. Total RNA was separated using the TRIzol reagent, followed by chloroform extraction and precipitation with isopropanol. Total RNA (20 μg) was run in denaturing conditions and blotted on Hybond-N membranes to detect the presence of specific mRNAs that hybridize with a ^{32}P-labelled tetR probe (XbaI-EcoRI DNA fragment indicated in **A**). A transcript of about 0.6 Kb corresponding to the tetR mRNA is shown. Tc regulation of the 2.0 Kb bicistronic mRNA expression from the 1Pi is observed. **(C)** Densitometric analysis of the radiolabeled 2-Kb bands is represented. Results obtained with the different constructs are expressed in arbitrary units.

pGEM7Zf(+)-tetR. Plasmids previously described were modified by replacing the tetR gene for the tetRNLS fragment.

Replacement of the hEGF Reporter Gene by eGFP Gene

The BamHI/NotI fragment containing the hEGF gene was excised from the basic inducible system and replaced by the enhanced green fluorescent protein (eGFP) gene. The 700-bp fragment encoding for eGFP gene was removed from peGFP-IRES-neo vector (Clontech, Palo Alto, CA, USA) and directly ligated into the parental constructs.

Cell Culture and Transient Transfection

African green monkey kidney cells, Vero, COS-1, COS-7 cell lines, and human kidney 293-T cells were grown and maintained in Dulbecco's modified Eagle's medium (D-MEM) (GIBCO-BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Tissue Culture Biologicals, Tulare, CA, USA) and antibiotics. D-MEM media containing 10% of the Tet System approved FBS (Clontech) was used for functional testing of the inducible system. The day before transfection, cells were subcultured into six-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) at densities of 2×10^5 cells/well. Transient transfection assays were performed using the Superfect reagent (Qiagen, Valencia, CA, USA) as described by the manufacturer. DNA complexes were prepared using 2.5 μg of plasmid DNA and Superfect reagent at a 1:2 ratio of DNA to condensing agent, followed by incubation at room temperature for 10 min and finally, addition of the complexes to the cells. Comparison with the two-plasmid system (2Pi) was carried out using 0.5 μg of pCMVtetOhEGF or pCMVhEGF and in each case alone or in combination with 2 μg pcDNA3tetR or to normalize the amount of transfected DNA, 2 μg empty vector DNA, pcDNA3.1(−). After 18 hr incubation at 37°C in a humidified atmosphere of 5% CO_2, cells were washed with PBS and refed with fresh media in the presence or absence of tetracycline (1 μg/ml). Reporter gene expression was measured either at a fixed time or as a function of time after transduction as described in the results.

Primary human umbilical vein cord endothelial cells (HUVEC) were used to test the regulatable lentiviral vectors. Second passage cells were cultured on gelatin-coated plates using Kaighn's F12K media (GIBCO-BRL) supplemented with 15% of FBS, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.1 mg/ml heparin and 0.03–0.05 mg/ml of endothelial cell growth supplement (Sigma, St. Louis, MO, USA).

Evaluation of Reporter Gene Expression

Expression of hEGF in cultured media was performed by enzyme-linked imunnosorbent assay (ELISA). Briefly, 96-well plates were coated with an

anti-hEGF monoclonal antibody (MAB236; R&D Systems, Minneapolis, MN, USA) (100 ng/well) at room temperature (RT) for 5 hr, and then blocked using 3% nonfat milk in phosphate-buffered saline (PBS). Recombinant hEGF standards, prepared in a 2-fold dilution series ranging from 9.7–5000 pg/ml (234-EG; R&D Systems), as well as test samples harvested from extracellular medium, were incubated at 4°C overnight. A secondary polyclonal antibody specific to hEGF (sc275; Santa Cruz Biotechnologies, Santa Cruz, CA, USA) was then added (100 ng/well) and incubated for 2 hr at RT. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit polyclonal antibody (sc2004; Santa Cruz) was the tertiary antibody (3.33 ng/well). Finally, the peroxidase assay was performed (Bio-RAD, Hercules, CA, USA), according to manufacturer's procedures and the reactions analyzed on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Production of the green fluorescent protein from plasmids encoding the eGFP gene was detected by flow cytometric analysis (FACS) and fluorescence microscopy (see below).

RNA Extraction and Northern Blot Analysis

Total cytoplasmic RNA was extracted from transfected cells using the TRIzol Reagent (GIBCO-BRL) and according to manufacturer's procedures. RNA (20 μg) was separated on 1.2% formaldehyde/agarose gels and transferred to nylon Hybond-N filter membranes (Amersham, Arlington Heights, IL, USA) by pressure blotting. Blots were probed with a XbaI-EcoRI-tetR DNA fragment (25 ng) labeled using the Megaprime DNA labeling system (Amersham) [α^{32}P]-dCTP (NEN, Boston, MA, USA). Overnight hybridization was performed using 4×10^7 cpm of labeled probe in a solution containing 0.5% (w/v) SDS, 5× Denhardt's solution (0.1% BSA, 0.1% Ficoll, 0.1% PVP), and 5× SSPE (0.9M NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.7) at 42°C. Blots were washed at a final stringency of 0.1% SDS, 0.1% SSPE at 60°C, and then visualized by autoradiography after exposure at −80°C. Intensity of the radiolabeled products was quantified using ImageQuant v1.11 Software from Molecular Dynamics (Sunnyvale, CA, USA).

Immunolocalization of tetR in Transfected Cells

Vero cells (5×10^4/well) were plated the day before transfection on chamber glass slides. Transfection of the constructs was performed as described. Forty-eight hours after treatment (minus or plus 1 μg/ml Tc), cells were fixed with 4% formaldehyde in PBS for 20 min at RT. Upon fixation, cells were permeabilized with 0.2% Triton X-100 for 5 min at RT and blocked with 10% normal goat serum, 5% BSA in PBS for 30 min. A monoclonal antibody raised against tetR (Clontech) was added in a 1:100 dilution and incubated for 1–2 hr. A goat anti-mouse IgG coupled to FITC (Sigma) or alternatively labeled with PE (Boehringer Mannheim, Indianapolis, IN, USA) at 1:250 dilution was added to the cells and incubation continue for 1 hr. After washing with PBS, coverslips were mounted in Sigma medium and examined under the UV light using a fluorescence microscope (Nikon Diaphot 300) with a FITC and Rhodamine exchangeable filters. Images recorded in a spot cooled color digital camera were analyzed using the Oncor Image software and printed from Adobe Photoshop, V3.0 for Macintosh.

Generation of Tetracycline-Inducible Lentiviral Vectors

Self-inactivated (SIN) lentiviral vectors based on the HIV-1 proviral clone HXB2 were genetically modified to incorporate the one-piece cassette for the evaluation of long-term transgene regulation. Three deletions, one (nts. 1441–4550) within the *gag-pol* region and two additional (nts. 6094–7654 and 8475–9028) within *env*, vpu, and nef open-reading frames, generated a replication-deficient HIV-1 vector. Inactivation of the viral promoter was achieved by introducing an additional 120-bp deletion (nts. 8897–9616) spanning the U3 region of the 3′ LTR. The DNA fragments containing the bicistronic cassettes were excised from the original vectors and reinserted into the BamHI/XbaI sites of the SIN backbone (see scheme in Figure 9A).

Pseudotyped single round viruses were generated by transient cotransfection of 293T human kidney cells using three-plasmid combination. The transfer vectors carrying the control or inducible constructs (15 μg), the packaging construct pCMVΔR8.2 (10 μg) encoding HIV-1 gag and pol genes under control of the CMV immediate-early promoter was a kind gift from Didier Trono. The envelope-expressor plasmid, pCMV VSV-G (4.5 μg), contains the vesicular stomatitis virus glycoprotein (VSV-G) gene under regulation of the CMV promoter. Transient cotransfection of 293T was carried out by the conventional calcium phosphate technique. Supernatants harvested 48 hr posttransfection were cleared by passing the cultured media through a 0.45-μm filter and then kept in aliquots at −80°C. Reverse transcriptase (RT) levels were used to determine the total particle number in the preparation. Infection of HUVEC (2×10^5 cells/well, six-well plates) was carried out using equal number of RT units for each construct and in the presence of 20 μg/ml of DEAE-dextran. Four hours postinfection, cells were washed with 1× PBS and refed with fresh media in the presence or absence of antibiotic. Regulation of eGFP expression was studied at 16 days postinfection by FACS analysis.

Results

Design of a Tetracycline-Regulatable Single Expression Cassette

A single regulatory module was designed that provides coexpression of the repressor protein, tetR, as

well as the target gene, in a controllable fashion (Figure 1A). Production of tetR by cap-independent translation is mediated through an IRES sequence. Concomitantly with tetR production and intracellular accumulation, transcriptional shut-off occurs in the absence of Tc through a high-affinity and effective interaction between dimers of tetR and two tandem tetO sequences located between the TATA box and transcription start site of the CMV promoter. When Tc is added to the system, tetR releases binding to the tetO because of a higher association constant between the repressor and the antibiotic (15). As a result, high levels of expression can be achieved through activation of the chimeric CMV promoter. A bicistronic mRNA of about 2 Kb, encoding the reporter gene as well as the tetR, is the final gene transcription product from both inducible and control plasmids.

Transcriptional Control of mRNA Expression by TetR

The transcription levels of the one-piece control (1Pc) and one-piece inducible (1Pi) plasmids described in Figure 1A were analyzed by Northern blotting in Vero cells 48 hr posttransfection. A radiolabeled tetR probe was used to visualize mRNAs produced from the control and inducible plasmids (Figure 1A, DNA probe is underlined). Total RNA from nontransfected cells and from cells transfected with an empty control plasmid served as negative controls (Fig. 1B, lanes 1 and 2). In parallel, cells were transduced with pcDNAtetR plasmid and its RNA was used as positive control (lane 3). The probe was able to detect a transcript of approximately 0.6 Kb, corresponding to the mRNA size of the tetR gene. Cells transduced with the 1Pc plasmid showed expression of the ~2 Kb mRNA corresponding to the expected size of the full-length transcript. No differences in expression could be detected in the absence or presence of 1 μg/ml Tc (Fig. 1B, lanes 4 and 5 and Fig. 1C). However, the level of 2-Kb transcripts in cells transduced with the 1Pi plasmid showed regulation of expression by Tc (Fig. 1B, lanes 6 and 7). Densitometric analysis demonstrated an approximately 1000-fold repression of the 1Pi mRNA expression in the absence of Tc (Fig. 1C). Total RNA used in this experiment is shown in the bottom panel of Figure 1B, which demonstrates that comparable amounts of RNA were loaded in each of the lanes.

Regulation of hEGF Expression From the Tetracycline-Regulatable Single Expression Cassette

Tight control of gene expression requires a regulatable system that provides high inducibility, and specific and dose-dependent response to the inducer, as well as the capability to return to basal levels after the inducer is removed. We have tested these three properties using the 1Pi plasmid by in vitro transfection experiments.

Efficiency Functional studies evaluating the efficiency of both one-plasmid (1Pi) and 2Pi systems were performed in parallel by measuring the amount of hEGF secreted to the culture media of transfected Vero cells (Fig. 2). Every 24 hr, the extracellular medium was harvested and the amount of secreted hEGF quantified by ELISA. The data obtained using the 2Pc and 2Pi plasmids are consistent with the results reported previously by Yao et al. (4). Expression of hEGF from the 2Pc system did not exhibit any variation with antibiotic administration. For the 2Pi system, expression of hEGF from pCMVtetOhEGF was unaffected unless tetR is cotransfected and in the absence of Tc, achieved about 340-fold repression during the first 24 hr, increasing to 600-fold and 950-fold during the two consecutive time points, respectively. Similarly, using the 1Pc cassette, no difference in hEGF expression levels was observed in the presence or absence of Tc. However, a significant time-dependent delay in tetR repression, owing to initially higher basal levels of hEGF, is observed using the 1Pi system resulting in a 55-fold, 100-fold, and 900-fold repression at 0–24 hr, 24–48, and 48–72 hr posttransfection, respectively, compared to the 2Pi system. These data imply that, as a consequence of simultaneous expression of both genes from the bicistronic mRNA occurring during the first rounds of transcription, cap-mediated translation of the first cistron occurs in the absence of Tc, contributing to higher levels of hEGF production from the 1Pi system compared with the 2Pi system, until sufficient IRES-mediated

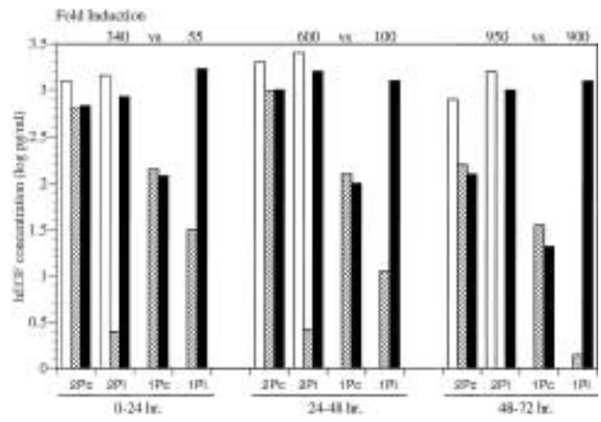

Fig. 2. Comparison of the regulation of hEGF expression using 2P and 1P plasmid systems. Vero cells (duplicate wells or $n = 2$) were transfected with 0.5 μg of pCMVtetOhEGF (2Pi), or the nonregulated version pCMVhEGF (2Pc) plus 2 μg of pcDNA 3.1(−) control plasmid (white bars), or in combination with 2 μg of pcDNAtetR and then, cells were incubated in the absence (striped bars) or presence (black bars) of 1 μg/ml of Tc. To test the 1Pc and 1Pi plasmids, cells in triplicate were independently transfected with 2.5 μg of the corresponding DNA in the absence (striped bars) or presence (black bars) of the antibiotic. Extracellular medium was collected from the transfected cells at the indicated times and the expression of hEGF was measured by ELISA.

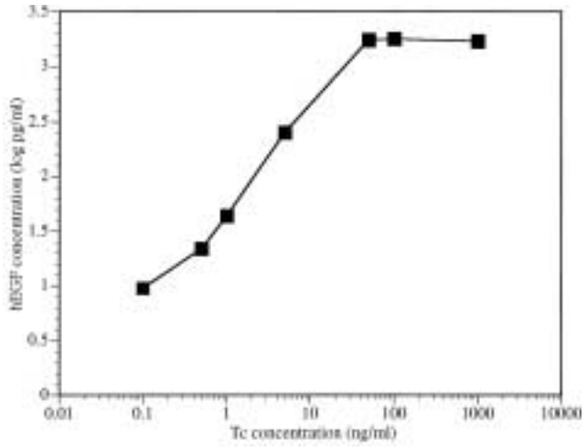

Fig. 3. Dose–response effects to tetracycline. Vero cells transfected with the 1Pi cassette were treated with increasing concentrations of Tc in the culture media ($n = 2$). After 48 hr, the quantity of hEGF released to the medium was analyzed by ELISA.

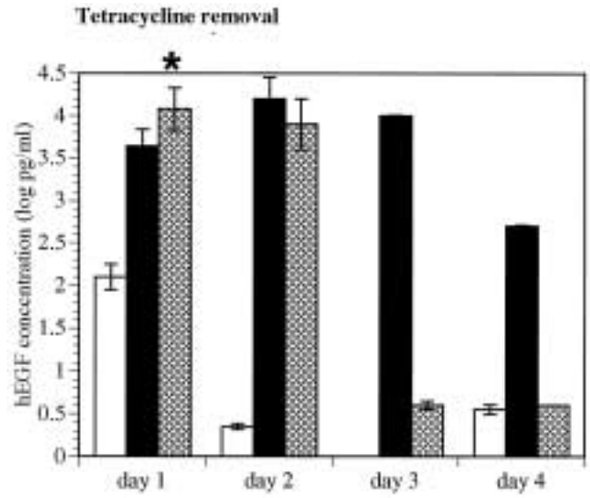

Fig. 4. Reversible effects of our single cassette in Vero cells. Transfected cells were cultured in the absence (white bars) or presence (black bars) of Tc during the entire experiment or alternatively, after 24 hr of Tc treatment (*), the cells were maintained in media without Tc (shaded bars). Culture media (triplicate samples) was analyzed for hEGF production at the indicated time points. The results are means ± SD of triplicate samples (error bars are too small to see).

tetR synthesis is achieved to block gene expression. Thus for the 1Pi system, the levels of tetR that are required to achieve saturable binding to tetO may not be reached until 48 hr posttransfection. However after 48 hr, comparable levels of repression are obtained from both systems.

Dose Response Release of tetR-mediated repression was observed after addition of increasing concentration of Tc to the culture media of transfected Vero cells with the 1Pi system (Fig. 3). Full activation of the system is achieved using 50 ng/ml of Tc.

Reversibility The capability of the 1Pi system to respond to Tc removal after activation was tested (Fig. 4). Vero cells transfected with the 1Pi plasmid were incubated in the absence or presence of 1 μg/ml of Tc. After 24 hr, a set of cells previously exposed to Tc were refed with fresh medium without antibiotic and the amount of hEGF released to the culture media was determined. As shown in Figure 4, hEGF secretion continued almost unaffected for the next 24 hr, but dramatically returned to basal levels after 48 hr in absence of the Tc. Upon removal of Tc, transcription initiation of the hEGF gene in cells previously undergoing gene expression ceases and a 2500-fold repression of hEGF expression is achieved for at least 2 days. In addition, cells that were kept in the uninduced state exhibited a maximum of 10,000-fold repression 3 days posttransfection.

Control of eGFP Expression in Different Cell Lines

An important aspect of the 1Pi system to explore was the ability of the tetO-bearing CMV promoter to control expression of the reporter gene in cell lines other than Vero cells. For that purpose, expression of the eGFP gene was used to screen diverse cells lines for transgene expression by detecting the intensity of fluorescence using FACS analysis (Fig. 5). In all the cases, mock-transfected cells or cells transfected with an empty vector did not show significant background fluorescence. The monkey-derived cell lines,

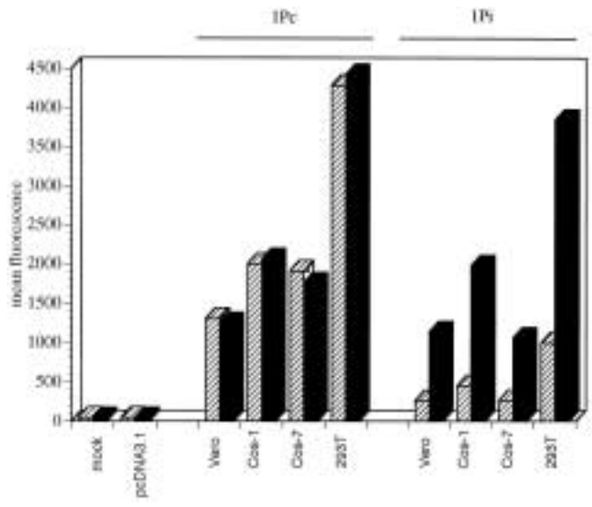

Fig. 5. Regulation of eGFP expression in different cell lines. Nontransfected cells and cells transfected either with an empty vector, pcDNA3.1(−) (only background data from Vero cells is exemplified) or with the 1Pc or 1Pi plasmids were analyzed by FACS analysis 48 hr posttransfection to determine eGFP expression in different cell lines in the absence (striped bars) or presence (black bars) of 1 μg/ml Tc. Differences in mean fluorescence between samples ($n = 3$) were less than 5%.

Vero, COS-1 and COS-7 transfected with the 1Pc eGFP plasmid, all exhibited similar degrees of fluorescence intensity. In contrast, eGFP expression in the human cell line 293T displayed two times higher fluorescence intensity than did the monkey cell lines. Overall, no variation in terms of mean fluorescence was observed in the absence or presence of the Tc. Performance of the 1Pi chimeric promoter was again similar between the monkey-derived cell lines reaching 5-fold repression of eGFP intensity in the absence of the antibiotic. However, the activity of tetO-bearing CMV promoter differed between cell lines, and in particular for the human 293T cell line, higher background and expression levels were observed in absence of Tc, probably due to the presence of E1A/B gene products from adenoviruses that have been shown to promote activity of the viral CMV promoter. Similar results were obtained after analyzing cells harvested 24, 48, and 72 hr posttransfection (data not shown).

A general observation from the previous experiments is that the cell lines studied for expression of eGFP exhibited significant background levels in the absence of Tc. The two most likely explanations for this observation are that the high basal levels of eGFP expression were a consequence of leakage of the system and/or were merely caused by slow turnover of the eGFP protein. To examine the latter hypothesis in greater detail, immunocytochemical staining was performed to simultaneously analyze the production of eGFP (FITC filter) and tetR (PE filter) in transduced Vero cells without or with the addition of Tc. As can be seen in Figure 6, cap-mediated eGFP (panel 1a and c) and IRES-mediated tetR (panel 1b and d) production from the 1Pc plasmid remained unaffected in the absence or presence of Tc. Cytoplasmic and nuclear distribution of the both proteins is observed in different cells, being mostly nuclear for eGFP and mostly cytoplasmic for tetR. Cells transfected with the 1Pi construct exhibited a different behavior. Although in the absence of Tc eGFP protein could be visualized (panel 2a), tetR expression is only faintly seen (panel 2b). However, when the system is released from repression by adding Tc, both eGFP and tetR expression was detected (panel 2c and d). Therefore, tetR-mediated repression works efficiently in these cells, but the long life and stability of eGFP (>120 hr) does not allow us to determine precisely the level of activation or repression of the system using this marker gene. A similar observation was recently reported by Kafri et al. (16).

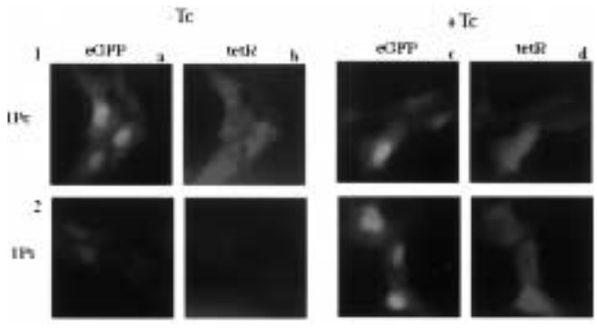

Fig. 6. Coexpression of eGFP and tetR in transfected Vero cells. Vero cells transfected with the 1Pc eGFP (panel 1, a–d) and with the 1Pi eGFP (panel 2, a–d) plasmids were grown for 2 days in the absence (panels 1 and 2, a, b) or presence (panels 1 and 2, c, d) of Tc prior to analysis. Simultaneous detection of eGFP (panels 1 and 2, a, c) and tetR (panels 1 and 2, b, d) expression was performed by immunostaining of the tetR protein using a primary antibody against tetR and a secondary goat anti-mouse IgG coupled to PE that allow detection of the immune complexes under a different wavelength.

Introduction of a NLS Sequence Accelerates TetR-Mediated Repression

Having demonstrated that tetR distribution is mostly cytoplasmic, a NLS was introduced at the 3′ end of the tetR gene to facilitate nuclear import of tetR in an attempt to increase tetR-mediated repression of transcription (17–20). Transient transfection experiments were performed on Vero cells using the 1Pi system with the modified tetRNLS (Fig. 7). Measurement of hEGF secretion demonstrated that no significant difference between both plasmids was seen during the initial 24 hr posttransfection ($p < 0.05$). However, a more rapid tetR-mediated repression was observed at 24–48 hr with the NLS construct obtaining 300-fold repression or three times higher efficiency of the tetRNLS protein than the untargeted tetR, in the absence of the antibiotic. After 48 hr,

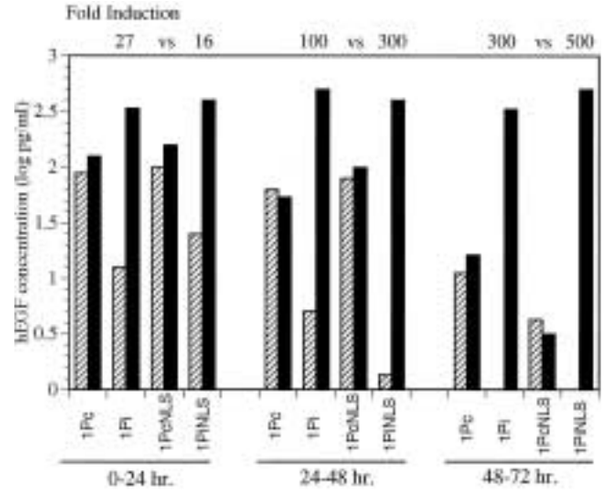

Fig. 7. TetR-mediated repression is enhanced by inserting a NLS sequence. Vero cells ($n = 3$) transfected either with the control (1Pc or 1PcNLS) or the inducible (1Pi or 1PiNLS) plasmids were grown in the absence (striped bars) or presence (black bars) of Tc. Aliquots of harvested supernatants ($n = 3$) were analyzed to determine the amount of hEGF secreted to the culture media. Values represent mean ± SD of triplicate samples (error bars are too small to see).

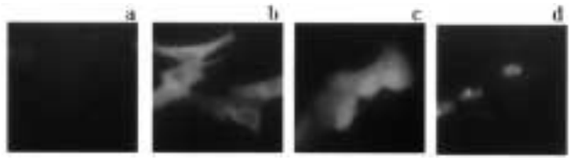

Fig. 8. Immunolocalization of tetR after addition of the NLS sequence. Localization of tetR protein after transfection of Vero cells with different plasmid constructs was performed by immunofluorescence staining. Cells transfected with a control plasmid (panel a), the pcDNAtetR plasmid (panel b), and the 1PihEGF plasmid (panel c) or 1PihEGFNLS (panel d) in the presence of Tc were fixed with 4% formaldehyde/PBS and permeabilized with a detergent before incubation with an anti-tetR mAb. After 2 hr incubation with the primary antibody, a goat anti-mouse IgG coupled to FITC allowed visualization under a fluorescence microscope (final magnification 400×).

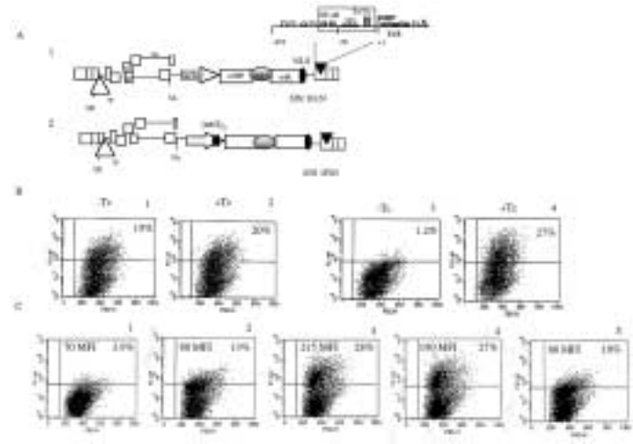

Fig. 9. Regulatable expression of eGFP in primary HUVEC cells transduced with a SIN lentiviral vector. (A) Structure of SIN HIV vector constructs. The 1PcN and 1PiN cassettes were cloned into a SIN HIV-1 vector within the third deletion in the envelope region. The viral backbone contains a truncated gag/pol region and, vif and vpr open reading frames. The triangle in the U3 region of the 3′ LTR indicates the SIN deletion. **(B)** Efficiency of eGFP expression in HUVEC cells by FACS analysis. Cells (2×10^5 cells/well into six-well plates) were infected with 1PcN (panels 1 and 2) or 1PiN (panels 3 and 4) VSV-G pseudotyped SIN vectors using equal numbers of RT units (50,000 cpm) and in the presence of 20 μg/ml of DEAE-dextran. Four hours postinfection, cell monolayers were washed and fed with fresh media without (panels 1 and 3) or with (panels 2 and 4) 1 μg/ml of Tc. Sixteen days postinfection, cells were tested for expression of the reporter gene by FACS analysis. **(C)** Effects of delay in Tc treatment on eGFP induction and of removal of Tc on reversibility of eGFP induction. Mock-transduced cells (panel 1) and cells transduced with the 1PiN vector (panels 2–5) were maintained in culture for 16 days. Expression of eGFP in the absence (panel 2) or presence (panel 3) of Tc was analyzed by FACS analysis. At day 7, a portion of transduced HUVECs was harvested (panels 4 and 5). Cells that were maintained in the absence of Tc were fed with media containing the antibiotic (panel 4); withdrawal of Tc occurred to those that have been previously treated with Tc (panel 5). Incubation continued for another 9 days.

300-fold and 500-fold repression was achieved from the 1Pi and 1PiNLS plasmids, respectively. No significant differences in terms of fully activation of the system were observed between these plasmids. All plasmids containing the wild-type CMV promoter did not show any regulatory effects by Tc throughout the experiment.

The distribution of tetR in the different constructs was analyzed by immunofluorescence staining using anti-tetR mAb (Fig. 8). In order to detect tetR production, the cells were treated with Tc for 2 days prior to fixation. As can be seen, cells transfected with an empty vector stained poorly (panel a), whereas cells transduced with pcDNAtetR as a positive control showed cytoplasmic and to a lesser extent nuclear staining (panel b). Likewise, tetR expression from 1Pi is present in both cytoplasmic and nuclear compartments (panel c), while in contrast tetRNLS protein is found mostly in the nucleus (panel d).

Long-Term Regulatable Transgene Expression Using a SIN Lentiviral Vector

A SIN lentiviral vector was constructed to determine if incorporation of the 1PiNLS cassette would allow long-term regulatable transgene expression. This vector backbone contains a 120-bp deletion (bp −136 to −16 relative to the transcription start site) encompassing the NF-κB, SP1, and TATA elements of the U3 region of the HIV-1 LTR. In control experiments with lentiviral vectors without an internal CMVie promoter where the 5′ HIV-1 LTR drives eGFP expression, SIN resulted in a 77% reduction in eGFP MFI in transduced 293T cells (data not shown). Both the 1PcNLS and the 1PiNLS cassettes were cloned into the SIN vector to produce SIN 1PcN and SIN 1PiN, respectively (Fig. 9A).

Equal numbers of RT units from the SIN 1PcN and SIN 1PiN vector preparations were used to infect primary HUVEC cells. After infection, cells were maintained in the absence or presence of 1 μg/ml Tc. At 16 days postinfection, eGFP expression was analyzed by FACS analysis (Fig. 9B). Mock-infected cells, either in the absence or presence of the antibiotic did not exhibit any significant background (data not shown). Stable long-term expression was detected for at least 2 weeks postinfection, the longest time point that was examined. Cells (~20%) infected with virus containing the 1PcN construct produced green fluorescence protein in a noncontrolled fashion. However, regulated eGFP expression was obtained in cells containing the 1PiN unit. Although the number of eGFP-positive cells found in the absence of Tc was slightly above the background level (1.2% versus 0.15%), the mean fluorescence in both populations was equivalent (134 versus 115, respectively). In the presence of Tc, 27% of SIN 1PiN transduced cells exhibited green fluorescence, reaching the overall intensity observed in the SIN 1PcN transduced cells expressing eGFP in a constitutive manner (270 versus 250, respectively).

Separate experiments were conducted on transduced HUVEC to evaluate whether a delay in Tc treatment would diminish the induction of eGFP expression and whether the induction of eGFP by Tc was reversible on its removal. As shown in Figure 9C, the number of eGFP-positive cells in the absence of Tc was again above background (13% versus 3.9%, absolute values being greater in this experiment due to lower placement of the threshold bar compared to Figure 9B). In the presence of Tc for 16 days (panel 3) or in the absence for 7 days followed by the presence of Tc for 9 days (panel 4), equivalent levels of gene expression were seen (28% verses 27%, respectively). Furthermore, when cells were treated with Tc for 7 days followed by removal of Tc for 9 days (panel 5), eGFP expression returned to background (panel 2) levels (10% verses 13%, respectively). The mean fluorescent intensity (MFI) was also close to the background levels (80 MFI units verses 70 MFI units, respectively). The low basal levels of eGFP expression detected in the absence of Tc may be attributed to the leakiness of the inducible promoter and/or the partial SIN of the HIV LTR. Further experiments will be required to determine whether one or both processes are involved. Thus, the 1Pi system allows Tc-regulatable long-term gene expression within the SIN 1PiN lentiviral vector and this regulation is reversible upon removal of Tc.

Finally, an analysis was performed to determine if the repression levels would be different or whether the ratio between repressed and induced states would be maintained by varying the amount of lentiviral vector used for the transductions. HUVEC were infected with SIN 1PiN at three different RT values (25K, 50K, and 75K) and eGFP expression as detected by FACS 9 days postinfection. As expected under these experimental conditions, the percentage of eGFP-positive cells increased as the amount of vector used for infection increased. However, neither the repression levels in the absence (4, 5, and 9%) or presence (13, 19, and 22%) nor the MFI in the absence or presence of Tc were significantly effected by increasing the amount of vector for infection (data not shown). These results suggest that a fixed ratio exists between the bicistronic mRNAs and their gene products so that any additional tetR that is expressed as a result of multiple integration events is balanced by its binding to the additional tetO-bearing hCMV immediate-early promoter elements.

Discussion

In the present study, the design of a single regulatory module is described that maintains the three essential features of an inducible system: specificity, reversibility, and controllable levels of transgene expression. Specific activation by Tc is achieved through a chimeric promoter containing two tandem copies of the tetO positioned 10 bp downstream of the last nucleotide of the TATA box of the CMV immediate-early promoter. To achieve autoregulatable activity in the 1Pi system, an IRES was used to direct cap-independent translation of tetR from the second cistron of a bicistronic transcription unit. In this single vector system, a minimal level of TetR is necessary to shut off the tetO-regulatable CMVIE promoter and this, in the absence of Tc, is reached only after an accumulation of the TetR itself. This accumulation is reached approximately three times faster when the tetR was modified with a nuclear localization signal because all the tetR proteins are present in the nuclear compartment (Figs. 7 and 8). Presumably, in time, the system reaches a steady state in which the minimal amount of tetR produced by the basal activity of the CMV promoter is adequate to replace the tetR that is lost over time. Furthermore, the 1Pi cassette functions well in both a plasmid construction and in the context of a SIN lentiviral vector.

One of the differences observed between the 1Pi and 2Pi systems is in the time course of repression. In the 2Pi system, greater repression is seen within the first 24–48 hr; however by 72 hr, similar levels of repression with the 1Pi and 2Pi systems are obtained (Fig. 2). One likely explanation for this observation is that cap-independent translation of tetR in the 1Pi system may be less efficient that cap-dependent transcription and translation of tetR in the 2Pi system. As a result, less tetR is produced from the initially limited number of bicistronic mRNAs to allow comparable levels of tetR production for the first 24–48 hr and only after 48 hr are adequate levels of tetR accumulated in the 1Pi system to achieve full repression (Fig. 1B). Remarkably, after 48–72 hrs, comparable levels of hEGF and eGFP are inducible in the 1Pi and 2Pi systems. In addition, the 1Pi system still provides precise external control of transgene expression by varying the dose of the antibiotic administered to the cells (Fig. 3). Moreover, the 1Pi system efficiently turns “off” expression of the reporter gene between a 24-hr period of adjustment (Fig. 4).

Whether the 1Pi system represents the “ideal” inducible system will depend on its intended use. The strengths of the 1Pi system are several. First, its limited size may be ideal for vectors such as AAV, where severe size limitations have required the incorporation of tetO and tetR sequences in different vectors to obtain regulatable gene expression (21). Second, coexpression of tetR from the same controllable promoter provides adequate functional levels of tetR and avoids the potential deleterious cellular effects that could be caused by higher constitutive production of the repressor when using two separate plasmids for production of tetR and the transgene of interest. Third, although several laboratories have in the past constructed single plasmid systems suitable for gene delivery using adenoviral (22), retroviral

(23–27), and lentiviral (16) vectors, these regulated viral vectors retain the basic principle of the model system initially described by Gossen and Bujard (5), where regulation of transgene expression is achieved by an heptamerized tetO sequence fused to the CMV promoter and the transactivator, tetR fused to the transcriptional activation domain of the herpes simplex virus VP16 protein to produce tTA (28–30). Thus, in certain experimental settings, an additional advantage of the 1Pi system relates to the use of tetR as a repressor instead of tTa because even low levels of expression of the chimeric tTA protein have been reported to be associated with cellular toxicity (5,8,31) and transcriptional squelching of cellular genes by the fusion tetR-VP-16 protein has been reported to occur (32). Finally, in the 1Pi system presented here, Tc is used to induce transgene expression rather than repress transgene expression, a relative advantage for in vivo clinical use and for basic research with transgenic animals where continuous addition of Tc to the drinking water will not be required to repress expression of the transgene of interest.

Likewise, there are theoretical limitations of the 1Pi system that under certain experimental settings may affect its use. First, the efficiency of IRES-mediated translation is highly cell-type specific, which in turn may alter the optimal IRES activity among different cell types (33,34). However, in the studies presented here, high enough levels of tetR expression were obtained in the diverse cell lines tested to allow regulatable transgene expression (Fig. 5). Second, the 1Pi cassette must be switched "on" before sufficient tetR accumulation has occurred to completely "shut off" further RNA transcription, and as a result the system may not be suitable in all cases, particularly if the coregulated gene of interest is detrimental to the cell (e.g., suicide gene) or important in cell development. Thus, the 1Pi autoregulatory system may be limited to those cases were constitutive expression of the gene of interest is tolerated, at least initially, and "shut off" could occur after a critical cellular concentration of tetR has accumulated that is sufficient to prevent mass production of both genes.

The lentiviral vector system described in this paper proposes an attractive addition to a limited number of single viral vectors that have been reported to be both regulatable and capable of integrating into nondividing cells. One such system recently reported by Kafri et al. (16) that incorporates the Tet system developed by Gossen and Bujard deserves comment. These authors constructed a first-generation lentiviral vector (containing intact 3′ LTR) by inserting an inducible cassette between the constitutively active CMV promoter and the GFP reporter gene. The inducible cassette contained the Tc-dependent transactivator (tTA) at its 5′ end and the inducible promoter [minimal CMV promoter and seven copies of the Tet operon (tetO)] at its 3′ end that drives GFP expression. Their results demonstrated that although the expression of GFP was not completely repressed by the presence of doxycycline following transduction of 293 cells, it was 500- to 1000-fold lower than its expression in the absence of doxycycline. In addition, induction of GFP mRNA was observed 3 days post-doxycycline withdrawal and reached a peak by day 8. Thus, despite the theoretical constraints discussed above regarding "leakage" of the 1Pi system, it functions well within the lentiviral vector backbone and comparable to the more traditional "Tet system" developed by Gossen and Bujard, which, in the report by Kafri et al. (16), was not designed to be autoregulatable.

In summary, the 1Pi system described in this paper can be used to control expression of candidate gene(s) in both nonviral and viral transfer systems. The cycling of the 1Pi system depends on three critical parameters: the half-life of tetR, the strength of the CMV promoter in the given cell type, and the efficiency of the IRES-mediated translation of tetR. The autoregulatory design of the 1Pi system facilitates expression of candidate genes in a spatial, temporal, and quantitative fashion, and should constitute an attractive strategy for a number of applications in developmental biology and human gene therapy studies if used with attention to its limitations discussed above. Furthermore, the recent report by Ohkawa and Taira that a similar 2Pi system, one containing the chimeric tetO/human U6snRNA promoter and the second plasmid providing the tetR alone, was capable of regulating expression of an antisense RNA (35) suggests that the 1Pi system can be modified to allow regulatable transgene expression from both cell-specific and tissue-specific promoters. Indeed, our current efforts are to examine this type of transcriptional regulation with the latest generation of lentiviral vectors.

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