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Rapid retroviral delivery of tetracycline-inducible genes in a single autoregulatory cassette

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ABSTRACT **We describe a single autoregulatory cassette that allows reversible induction of transgene expression in response to tetracycline (tet). This cassette contains all of the necessary components previously described by others on two separate plasmids that are introduced sequentially over a period of months [Gossen, M. & Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5547–5551]. The cassette is introduced using a retrovirus, allowing transfer into cell types that are difficult to transfect. Thus, populations of thousands of cells, rather than a few clones, can be isolated and characterized within weeks. To avoid potential interference of the strong retroviral long terminal repeat enhancer and promoter elements with the function of the tet-regulated cytomegalovirus minimal promoter, the vector is self-inactivating, eliminating transcription from the long terminal repeat after infection of target cells. Tandem tet operator sequences and the cytomegalovirus minimal promoter drive expression of a bicistronic mRNA, leading to transcription of the gene of interest (*lacZ*) and the internal ribosome entry site controlled transactivator (Tet repressor–VP16 fusion protein). In the absence of tet, there is a progressive increase in transactivator by means of an autoregulatory loop, whereas in the presence of tet, gene expression is prevented. Northern blot, biochemical, and single cell analyses have all shown that the construct yields low basal levels of gene expression and induction of one to two orders of magnitude. Thus, the current cassette of the retroviral construct (SIN-RetroTet vector) allows rapid delivery of inducible genes and should have broad applications to cultured cells, transgenic animals, and gene therapy.**

Studies of gene function in cultured cells, analyses of gene expression during mammalian development, and delivery of proteins via gene therapy would all greatly benefit from the ability to regulate gene expression in a temporal and quantitative manner. In cultured cells, a frequent approach to the study of genes is stable transfection of plasmids and isolation of constitutively expressing cells using a drug selectable marker, a procedure that is time consuming, labor intensive, and may result in loss of function of the gene of interest over time. In mouse development, the role of genes is typically studied by irreversible changes: for example, gene inactivation or gene augmentation in transgenic animals from the onset of embryogenesis or use of *flp* or *cre* recombinases later in development (1, 2). In gene therapy, only constitutive delivery of proteins has been achieved, yet constant expression levels are often not physiological and can even be life threatening. In each of these three cases, it would be highly advantageous if the gene of interest could be expressed during a particular window of time in a dose-dependent and reversible manner.

Several inducible gene expression systems have been developed over the past decade in an attempt to meet the need for regulated gene expression. Most of the commonly used methods for inducing gene expression such as heat shock, steroids, or metallothionein, suffer from either high basal levels of gene expression under noninduced conditions, pleiotropic effects on host cell genes, or both (reviewed in refs. 3 and 4). Although usefully applied in a multitude of studies over the past decade, these inducible systems are frought with problems.

Recently two systems have been developed that appear to overcome many of the problems associated with the first generation of inducible vectors in that the inducers are specific to the gene of interest and lead to low basal and high inducible levels of gene expression: the tetracycline (tet) and progesterone antagonist (RU486) regulatable systems (5, 6). Both systems use microbial proteins and microbial DNA response elements to drive the expression of a desired gene in heterologous cells. As a result, regulation is restricted to the gene of interest, host genes are not affected, and there appears to be no associated toxicity. In addition, gene expression can frequently be modulated over a broad range of levels during a defined temporal window.

The two-plasmid systems described above (5, 6), represent theoretical milestones in the development of inducible systems, yet widespread application is severely hindered for several reasons. First, two sequential transfection steps are necessary to introduce each of the two plasmids stably, requiring several months in order that individual clones be tested at each step. Second, use in poorly transfectable cell types such as primary myoblasts or lymphocytes remains difficult. Thus, in practice, obtaining well-regulated clones using the two-plasmid systems is not only limited to certain cell types, but is also cumbersome, labor intensive, and low in yield of inducible clones.

To overcome these problems, we developed a single cassette that contains all of the components required for tet regulation. In this report, the cassette is contained in a retrovirus, which can be produced at high titer and infects dividing cells with high efficiency, allowing use in a large number of cell types. The retrovirus requires minimal effort to prepare, is stable, and can yield populations of cells with regulatable gene expression within weeks, instead of months. The potential problem of cis-regulatory effects due to the close proximity of the tet-regulatable cytomegalovirus (CMV) minimal promoter elements to strong viral regulatory elements has been circumvented by utilizing a self-inactivating (SIN) retroviral vector (7). Following infection of the target cell, the SIN vector, which contains a deletion in the enhancer and promoter sequences of the 3′ long terminal repeat (LTR), transfers this deletion to the 5′ LTR, resulting in transcriptional inactivation of the provirus. As a result, the tet-regulatable minimal promoter drives expression of a bicistronic mRNA encoding the gene of interest and an internal ribosome entry site (IRES) (8) controlled transactivator. In the presence of tet, the low levels of transactivator transcribed are effectively inhibited from binding the tet operator sequences due to allosteric changes, whereas in the absence of tet, the transactivator promotes its

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Abbreviations: tet, tetracycline; Dox, doxycycline; CMV, cytomegalovirus; SIN, self-inactivating; IRES, internal ribosome entry site; LTR, long terminal repeat; β-gal, β-galactosidase; FACS, fluorescence-activated cell sorter.

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own transcription in an autoregulatory loop, reaching a plateau when all operator sequences are presumably occupied. Due to its rapid introduction into large numbers of primary cells, potential for analyses of gene expression shortly after introduction, and broad host cell range, the retroviral vector has all of the positive attributes of the original two-plasmid tet regulatable system, while being much more amenable to use in a variety of contexts.

MATERIALS AND METHODS

Vector Construction. All cloning steps were performed as described (9). For the vector backbone pBABE M *lacZ*, a pBABE based retroviral vector (10) bearing the MFG packaging signal Moloney murine leukemia virus was used (S. Kinoshita and G.P.N., unpublished data). Vectors bearing the elements of the tet-regulatable system including the minimal human CMV promoter, tet repressor VP16 fusion protein (transactivator), and tet operator sequences were gifts from H. Bujard (5). The IRES was derived from Mengo virus (gift of J. P. Morgenstern, Mellenium Corporation). The final vector construct was named pBABE SIN CMV 15–1 (SIN-RetroTet vector). To construct a SIN vector, the 3′ LTR of pBABE M *lacZ* was exchanged with the mutant 3′ LTR of pJrPro$^-$ (gift of R. Mulligan, Whitehead Institute, Massachusetts Institute of Technology), which lacks the promoter and enhancer sequences in the 3′ LTR.

Cell Culture. Primary mouse myoblasts (C57BL/6) were purified earlier (11). Cells were grown in myoblast growth medium consisting of 50% F10 medium (GIBCO/BRL) and 50% DMEM (GIBCO/BRL), supplemented with 20% fetal bovine serum (HyClone), basic fibroblast growth factor (2.5 ng/ml; Promega), penicillin G (200 units/ml), and streptomycin (200 μg/ml). Cells were grown on collagen-coated dishes (Sigma) in a humidified 5% CO_2 incubator at 37°C. tet (Sigma) and doxycycline (Dox) (Sigma) were stored at −20°C in the dark as 1 mg/ml stock solutions in 30% ethanol and 50% ethanol, respectively, and added to the medium as needed.

β-Galactosidase (β-gal) Assays. *Cell lysates.* β-gal activity was measured with 1 μg of protein using the Galacto-Light Plus kit (Tropix, Bedford, MA) according to the specifications of the manufacturer. For these chemiluminescence assays, cells were grown on 60-mm tissue culture plates for 2–3 days until they reached 70–80% confluency. At that time, cells were harvested and assayed by integration for 10 sec with a Monolight 2001 luminometer. All samples were measured twice and the mean value was corrected for the background activity of the assay (10 μl of lysis buffer), which varied in different experiments between 900 and 3000 relative light units.

Fixed cells. Staining of cells for β-gal expression on culture dishes was performed as described (12).

Live cells. β-gal expression in living cells was determined by fluorescence-activated cell sorting (FACS) as described (13) except that the fluorescein di-β-D-galactopyranoside (FDG) concentration was reduced to 0.5 mM to reduce the background signal seen with 2 mM (unpublished observations).

Transfection of BOSC23 Cells and Transduction of C57BL/6 Primary Myoblasts. Retroviral particles were obtained after transient transfection of BOSC23 cells as described (14). The viral supernatant was collected and either was used immediately for transductions or was frozen on dry ice, stored at −80°C, and used in later transductions. Primary myoblasts (5×10^5) (C57BL/6) were plated 1 day prior to transduction, and just before transduction, myoblast growth medium was removed and replaced with undiluted viral supernatant supplemented with 8 μg/ml Polybrene. In general, cells were incubated overnight at 32°C in a humidified 5% CO_2 incubator. The viral supernatant was then aspirated and cells were transferred to myoblast growth medium and growth conditions. Cells were then either expanded or assayed for β-gal activity 48 hr posttransduction.

Northern Blot Analysis. Northern blotting was performed as described (15) using a digoxigenin-labeled probe (approximately 2 kb), complementary to the *lacZ* gene. The bound probe was visualized by chemiluminescence using CSPD substrate (Tropix) at a final concentration of 0.1 mg/ml.

RESULTS

Design of the tet-Regulatable Retroviral Vector. In the tet system of Gossen and Bujard (5), a first plasmid constitutively expresses a transactivator, a fusion protein between the tet repressor (TetR) and the transcriptional activation domain of the herpes simplex virus VP16 protein. On a second plasmid is the heptamerized tet operator (TetO) sequence followed by a human CMV immediate early minimal promoter (P_{hCMV^*-1}) and the gene of interest.

The retroviral construct shown in Fig. 1 was designed to contain all of these components as follows. To achieve low basal levels of expression of the gene of interest and of the transactivator, the relatively weak P_{hCMV^*-1} promoter was used. Low promoter activity leads to the transcription of an mRNA that is initiated upstream of the gene of interest (in this case, the *lacZ* gene), progresses through the transactivator sequence, and terminates at the polyadenylylation site located in the R region of the viral 3′ LTR. Thus, a bicistronic mRNA is produced coding for β-gal and an IRES controlled transactivator. As a result, translation ceases downstream of the stop codon of the gene of interest (*lacZ*) in the IRES-

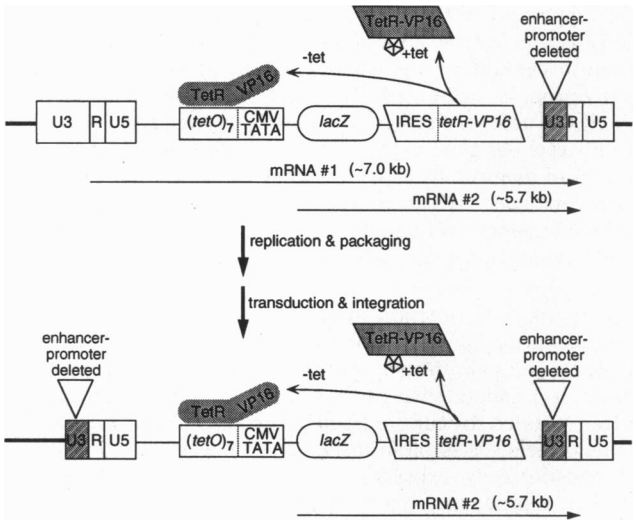

FIG. 1. Schematic representation of the tet-regulatable retroviral vector (SIN-RetroTet vector). An autoregulatory cassette was cloned into a retroviral SIN vector backbone, which consists of a heptamerized tet operator sequence $(TetO)_7$ fused to the human CMV immediate early minimal promoter (CMV TATA) designated P_{hCMV^*-1}. Upon infection of target cells (myoblasts), the basal activity of the P_{hCMV^*-1} promoter drives the expression of a bicistronic mRNA (mRNA 2) with a calculated length of approximately 5.7 kb, coding for β-gal (*lacZ* gene) and the IRES-controlled tet repressor-VP16 fusion protein (TetR-VP16). In the presence of tet, the TetR-VP16 does not bind the TetO sequences, and expression levels are reduced to those of the basal promoter. In the absence of tet, the binding of the TetR-VP16 to the TetO sequences results in increased expression levels from the P_{hCMV^*-1} promoter due to the recruitment of transcription factors by the VP16 transcriptional activation domain. In packaging cells in which the virus is produced, mRNA 1 (≈7.0 kb) and mRNA 2 (≈5.7 kb) are transcribed (*Upper*). Upon infection of the myoblast target cell (*Lower*), the promoter and enhancer elements in the viral 5′ LTR are deleted upon integration into the host genome resulting in transcription of mRNA 2 (≈5.7 kb).

sequence, but is reinitiated at the first ATG at the very 3′ end of the IRES-sequence which mediates translation of a second protein, the transactivator. Like the two-plasmid system of Gossen and Bujard (5), in the presence of tet the transactivator undergoes an allosteric change and does not bind the tet operator sequences leading to a lack of gene expression, whereas in its absence the transactivator binds tightly and gene expression is induced. However, basal transcription is essential for some transactivator molecules to be produced. In the absence of tet, as the concentration of transactivator reaches a necessary threshold, the heptamerized operator sequences are bound, further increasing transcription levels of the bicistronic mRNA until they reach a maximum, at which point gene expression plateaus. Thus, a major difference between the two-plasmid system and the retroviral vector is that induction using the single vector results from an autoregulatory feedback loop.

Other features critical to the design of the retroviral vector are the use of the MFG packaging sequence of pBABE M (S. Kinoshita and G.P.N., unpublished data) and the construction of a SIN retroviral vector (7). The MFG packaging sequence leads to high efficiency incorporation of the vector RNA into the maturing virions (G.P.N., unpublished observation). Due to the high titer of virus produced, it is possible to transduce cells that are difficult to transfect, such as primary myoblasts (Fig. 2) and lymphocytes (unpublished observation). Potential cis-regulatory problems in the tet regulation of the $P_{hCMV^{*}-1}$ promoter due to proximity to potent Moloney viral enhancer and promoter elements are eliminated by the use of a SIN vector, which lacks these elements.

FACS-Enrichment for a Population of tet-Regulatable Transduced Primary Myoblasts. The high transduction efficiency characteristic of retroviral vectors allows stable introduction of the inducible gene of interest into a population of cells within a few days. A caveat of all protocols that involve integration of DNA in the genome is that the expression of the gene of interest may be influenced by its proximity to endogenous promoters, enhancers, or silencers, leading to constitutive gene expression or lack of expression. Because the likelihood of a vector integrating next to such a region within the host genome increases with the number of integrations per cell, transductions were performed under conditions that resulted in approximately 10% efficiency, a frequency that should statistically yield, at most, one viral integration per genome. The actual transduction efficiency was determined as β-gal expression in the absence of tet by X-gal staining assessed at the single-cell level by microscopy. For this purpose, three randomly selected fields were scored containing approximately 2000 cells per field and an average of 240 cells per field stained blue in the absence of tet in accordance with the predicted frequency. Under these conditions, the viral titer was approximately 1.6×10^4 infectious particles per ml. There was a range of β-gal expression. Some of the cells exhibited basal levels of expression that could be detected by X-gal staining even in the presence of tet, whereas others did not. Upon tet removal, the majority of the transduced cells could be induced to express at least one to two orders of magnitude greater levels of β-gal (see below).

To isolate a more uniform population of cells with particularly low basal and high inducible levels of gene expression, the FACS was used, which allows isolation of live transduced cells en masse (Fig. 2*A*). After transduction, primary myoblasts were first grown in a medium that contained the tet analogue Dox (1 μg/ml) for 5 days and cells that exhibited levels of β-gal indistinguishable from untransduced control cells were collected. After expansion and growth without Dox for one week, the myoblasts were reanalyzed by FACS. Cells with approximately 1 order of magnitude higher levels of β-gal activity than untransduced control cells were collected. Following two rounds of FACS sorting after growth without Dox and collection of β-gal expressing cells using similar fluores-

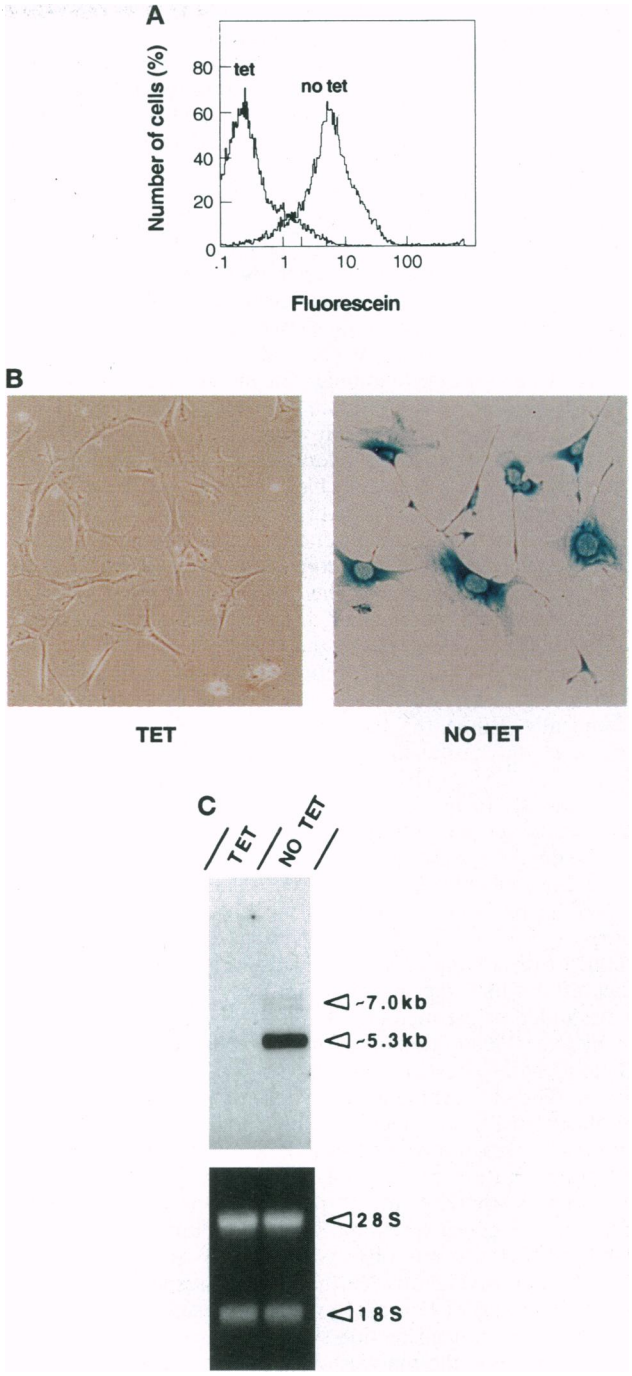

FIG. 2. Inducible β-gal activity in primary myoblasts transduced with the SIN-RetroTet vector. (*A*) FACS-enriched C57 mouse primary myoblasts transduced with the RetroTet vector and designated C57 BABE SIN CMV 15-1 were grown either in the presence (tet) or absence (no tet) of 0.1 μg/ml tet for 4 days and analyzed by FACS. As shown in the histogram of number of cells (%) (ordinate) and log fluorescein (abscissa) gene expression is induced in the majority of cells. (*B*) At the single-cell level, X-gal staining and microscopic analysis of this population revealed no blue cells under noninduced conditions (tet) but almost 100% blue cells upon induction (no tet). (*C*) Northern blot analyses were carried out for C57 BABE SIN CMV 15-1 cells grown either in the presence (tet) or absence (no tet) of 1 μg/ml tet for 2 days. Total RNA (10 μg) was separated on a 1% agarose gel and probed with a digoxigenin-labeled riboprobe complementary to the *lacZ* DNA (*Upper*). Only under induced conditions (no tet), a band of ≈5.3 kb (calculated based on the migration of the 18S and 28S rRNA) was visible approximating the expected size of mRNA 2. The ethidium bromide staining of the same gel (*Lower*) shows that equal amounts of RNA were loaded per lane.

cence gates, more than 90% of the isolated cell population exhibited regulatable β-gal expression (Fig. 2*A*). This population of regulatable transduced myoblasts, referred to as C57 BABE SIN CMV 15-1, remained stable in its inducible properties for 2 months as determined by FACS and by X-gal staining (Fig. 2 *A* and *B*) and was used in all subsequent experiments described below.

To test whether the retroviral vector acts as a SIN vector upon myoblast infection, Northern blot analysis (Fig. 2*C*) of C57 BABE SIN CMV 15–1 myoblasts was carried out. In RNA from uninduced cells (+tet) no transcript was evident. After long exposures a transcript was barely detectable at ≈5.3 kb, but never at ≈7.0 kb (data not shown), providing evidence that transcription was driven by the CMV promoter and that the viral 5′ LTR was nonfunctional. The major band evident in the absence of tet (−tet) approximates the expected size of ≈5.7 kb. The faint higher molecular weight transcripts are likely to be read-through products or result from activation of a cryptic promoter(s). Thus, Northern blot analysis confirmed that the vector results in a low basal level of mRNA expression, and that the fold induction in the absence of tet is relatively high.

Regulation of *lacZ* Gene Expression Assessed by Chemiluminescence. We compared the properties of the new single-vector retroviral system with the previously described two-vector plasmid system (5) in the C57 BABE SIN CMV 15-1 myoblast population. In a preliminary experiment a chemiluminescence assay was used to determine β-gal activity biochemically. When the background levels for four replicate plates of untransduced myoblasts were subtracted from the values for C57 BABE SIN CMV 15-1 cells grown either continuously in tet for 2 days or grown continuously in the absence of tet, a 600-fold induction was observed.

The dose-response curve for β-gal activity in the presence of different concentrations of tet and its analog Dox was determined using a chemiluminescence assay (Fig. 3). For this purpose C57 BABE SIN CMV 15-1 cells were plated in replicate dishes and grown in the absence of tet or Dox for one day, after which the indicated amounts of either tet or Dox were added to the medium. At 70 to 80% cell confluency, or 60 hr after the addition of tet or Dox, cells were harvested and β-gal activities measured in cell lysates by chemiluminescence assays. The mean value for each dose of antibiotic obtained in duplicate dishes was determined as indicated in Fig. 3, which shows a representative of two independent experiments. At a tet concentration of 5 ng/ml, β-gal activity was reduced to basal levels whereas only 50 pg/ml of Dox was sufficient to prevent *lacZ* gene expression. At the antibiotic concentrations used, no toxicity was observed, assayed as changes in cell morphology or viability. Both the dose response curve and greater potency of Dox agree well with results reported using the two-plasmid transfection system (5, 16).

To determine the kinetics of inactivation and activation of gene expression, β-gal activity was assessed in replicate dishes after addition or withdrawal of tet, respectively (Fig. 3 *lower*). Addition of tet led to an average reduction of β-gal activity to 50% of levels of maximally expressing control cells within 8 hr, and basal levels after 48 hr. The kinetics of induction were slower. Twenty-four hours after tet removal only a slight increase in β-gal activity was detectable, but that level approximated maximal levels by 48 hr. Clearly with this vector the loss of *lacZ* gene expression is faster than the induction of gene expression.

Single-Cell Analysis of Kinetics of Induction of *lacZ* Gene Expression by FACS. To determine whether the increase in β-gal activity seen between 24 and 48 hr was due to a few cells rapidly reaching maximal β-gal activities or to the concerted action of the entire population, C57 BABE SIN CMV 15-1 cells were analyzed by FACS at various time points after tet removal (Fig. 4). The histograms obtained for β-gal activity in this population 24 hr after removal of tet were largely coincident with the histograms obtained for cells that were not

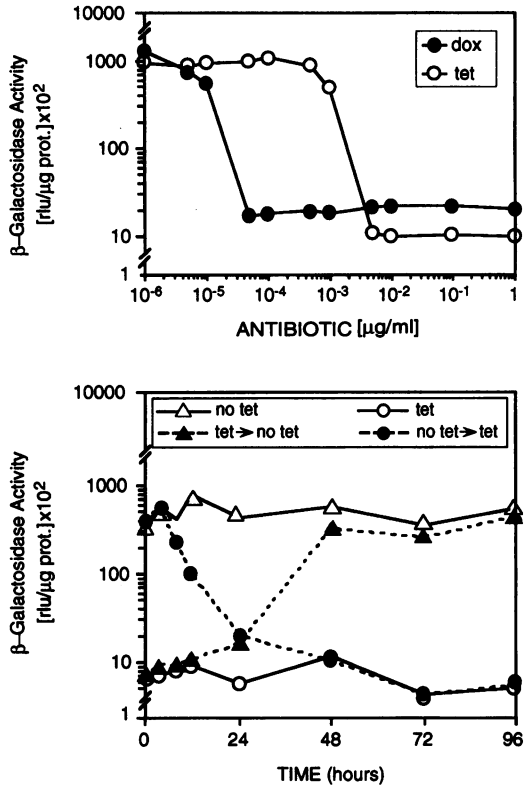

FIG. 3. Pharmacologic properties of regulation of β-gal expression in primary mouse myoblasts transduced with the SIN-RetroTet vector. (*Upper*) Dose-response to tet and Dox. C57 BABE SIN CMV 15-1 cells were grown in the presence of different tet and Dox concentrations, respectively, and β-gal activities in cell lysates were determined after 60 hr by chemiluminescence. A representative of two independent experiments, each performed in replicate, is shown with a maximal difference among measurements of 15%. Low concentrations of 50 pg/ml of Dox and 5 ng/ml of tet were sufficient to reduce β-gal activities to basal levels. (*Lower*) Reversibility of gene expression: kinetics of induction and de-induction. C57 BABE SIN CMV 15–1 cells were grown either in the presence or absence of 0.1 μg/ml of tet for 4 days. At time 0, cells which were grown in the presence of tet were washed with PBS and grown in tet-free medium (▲ tet → no tet). Cells grown in the absence of tet were also washed with PBS and then grown in medium containing tet (● no tet → tet). Control cells were grown either continuously in the absence of tet (Δ no tet) or continuously in the presence of tet (○ tet). β-gal activity in cell lysates was measured for duplicate dishes by chemiluminescence (rlu, relative light units) at the time points indicated and the mean values were plotted. Eight hours after tet addition (●) β-gal activities were reduced to approximately 50% of minimal levels (○). Cells in which gene expression was induced (▲) approached β-gal activities of control cells (Δ) after 48 hr and were equivalent to control levels within 96 hr.

transduced with the retroviral vector (*Top*). A significant shift toward higher β-gal activity had occurred by 48 hr and this increase was characteristic of the entire FACS sorted population (*Middle*). By 72 hr, maximal induction of gene expression was observed in that the histogram for the induced population was coincident with that of positive controls never exposed to tet. These properties were characteristic of the population even after a period of 2 months. Thus, with this particular gene, by two independent quantitative assays (chemiluminescence and FACS), basal levels of the entire cell population were low and then increased one to two orders of magnitude when the inhibitor was removed, an induction which should suffice for most biological applications.

DISCUSSION

The single retroviral vector described in this report extends the use of the elegant tet system developed by Gossen and Bujard

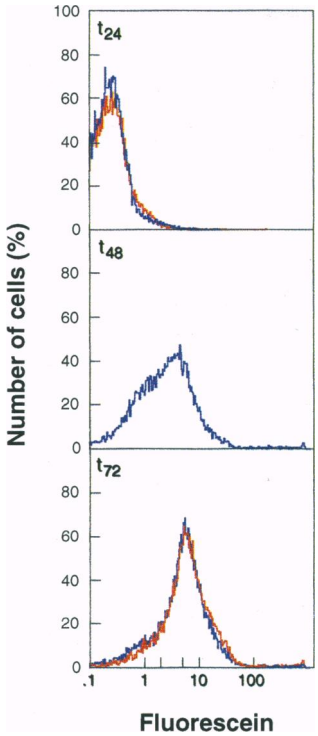

FIG. 4. Reversibility of gene expression by FACS analyses of the population of primary myoblasts expressing the SIN-RetroTet vector. C57 BABE SIN CMV 15-1 cells were grown in the presence of 0.1 μg/ml of tet, and at time 0 hr, cells were washed with PBS and grown in tet-free medium. At different time points the population was analyzed by FACS and plotted as a histogram as the percent of cells (ordinate) and log fluorescein (abscissa). Whereas 24 hr after induction (t_{24}) the induced population (blue) exhibited background activity and was essentially indistinguishable from untransduced C57 primary myoblasts (red), after 48 hr (t_{48}) the entire population had shifted to express β-gal as reflected by higher fluorescein values. By 72 hr after induction (t_{72}), the induced population (blue) was indistinguishable from constitutively induced C57 BABE SIN CMV 15-1 cells (red) and expressed maximal β-gal levels.

(5) to allow widespread regulatable transgene expression *in vitro* and *in vivo* in a manner not possible using that original system (5). The Gossen and Bujard two-vector system requires the stable transfection of first one and then another plasmid, a procedure that is both cumbersome and time consuming, requiring months for the selection and testing of individual clones at each step. By contrast, use of the tet-regulatable retroviral vector described here allows rapid (within weeks) isolation and characterization of large numbers of cells ($>10^4$) that exhibit regulated gene expression. This is advantageous since a population of cells is more representative than a few carefully selected clones for analysis of gene expression. Individual clones may differ from one another and may fluctuate after long-term propagation. Indeed, one or both of the two tet-regulated plasmids ceased to be expressed or was eliminated from myogenic cells over time (S. E. Elson, M. Conboy, H.M.B., unpublished). An added advantage of the retroviral vector described here for regulated gene expression is that retroviruses generally integrate as a single copy into the genome of the target cell, whereas sequential plasmid transfection can be problematic because the integration of multiple plasmids in tandem can raise basal levels of gene expression. A further advantage is that the virus is easily prepared at high titers and is stable after long-term storage. Due to the MFG packaging sequence of this retroviral vector, it can infect a broad range of primary cells that are not easily transfected, including primary muscle cells and lymphocytes at efficiencies as high as 95 and 40%, respectively (unpublished observations).

To avoid potential effects on tet regulation by the weak minimal CMV promoter due to close proximity to the strong Moloney viral 5′ LTR enhancer and promoter control elements, these retroviral sequences were deleted postinfection resulting in a self-inactivating vector. That the vector was indeed a SIN vector (7) was evident from the size of the mRNA observed on Northern blots, which was the reduced size expected if transcription was driven by the CMV minimal promoter, not the retroviral regulatory sequences in the 5′ LTR. Due to the P_{hCMV^*-1} promoter, transactivator production was constitutive but low, leading to low basal levels of gene expression. However, in the absence of tet, this finite amount of transactivator was sufficient to act in an autoregulatory loop, binding the heptameric tet operator sequences upstream of the CMV promoter. The progressive accumulation of transactivator presumably resulted in the observed plateau of gene expression, between one to two orders of magnitude above basal levels.

By contrast with previously used inducers of mammalian gene expression, modulation of transgene expression by tet is easily achieved over several orders of magnitude, is specific to the gene of interest, and is not toxic, because of the nonmammalian origin of the control elements and the low doses of regulator used. Other recently developed two-plasmid inducible systems that use microbial regulatory components (6, 17) suffer from the same problems as the original tet system (5), as they have not yet been combined into a single cassette. To capitalize on the power of the FACS to isolate populations of cells that regulate a gene of interest that encodes a gene product that cannot itself be detected by the FACS, further modifications are now being introduced into the vector such as a second IRES followed by a small gene encoding a sortable marker such as the green fluorescent protein (18).

The time course for the decline in β-gal activity with the retroviral vector was significantly shorter than the time course for induction. The kinetics of de-induction depend on the turnover rate of the transcript and the protein it encodes. In this case, the t_{50} was 8 hr, which approximates the half-life of the regulated gene product, β-gal (G.P.N., unpublished observation). For a gene that has a shorter half-life of either its encoded mRNA or protein, loss of gene expression may be more rapid. By contrast with the kinetics of de-induction, the kinetics of induction of gene expression were relatively slow. The kinetics of induction of the current retroviral vector are presumably due to the autoregulatory nature of the vector that requires multiple rounds of transcription to reach a maximum level of transcription. Other inducible retroviral constructs are being developed that may overcome this limitation and activate gene expression more rapidly (A.H. and H.M.B., unpublished).

In cultured cells, there are numerous potential applications of inducible retroviral vectors. Studies of gene products deleterious to the growth of cells such as cell cycle regulators or tumor suppressors have largely been restricted to analysis by transient transfection or inducible regulation in yeast. The retroviral vector described here may advance these studies by allowing induction and de-induction of expression of such regulators in the same stably infected populations of mammalian cells in a manner previously not possible.

In mice, the ability to induce gene expression reversibly and to modulate the levels of the gene product of interest over time is not currently possible. Regulated gene expression should be of great utility, especially when overexpression or inactivation of the gene of interest results in embryonic lethality. Although site-specific recombinases can target activation or inactivation of gene expression at later times in development (1, 2), they are not dose-dependent or reversible. Since the two-plasmid system functions when naked DNA is directly injected into the

muscles of mice (19), the cassette present in the retroviral vector described here is likely also to be regulated in a dose-dependent manner *in vivo*. Transgenic animals that express tet-inducible reporter genes have been successfully produced by mating transgenic animals into which one or the other of the two plasmids was introduced in one-cell embryos (20, 21). The tet-regulatable cassette described here could be introduced into fertilized cells producing transgenic animals in a single step thereby precluding the need for raising and breeding mice containing single plasmids in order to obtain progeny in which both plasmids are present and the gene of interest is regulated, a time-consuming and expensive undertaking. Furthermore, tet-regulatable cassettes could be used to evade embryonic lethality allowing the generation of mouse models of adult onset human genetic diseases in a manner previously not possible.

For gene therapy, gene delivery that leads to constitutive expression of products such as hormones, neurotrophins, or cytokines is often not physiological and may decrease efficacy or lead to deleterious effects. Typically endogenous expression is regulated or pulsatile. tet-regulated retroviral vectors could be readily delivered to a range of primary cells *ex vivo*, which following implantation *in vivo* could exhibit regulated gene expression. This possibility is presently precluded by the need for successive transfection of plasmids. Thus, both efficacy and safety should be increased by the facility of delivering tet-regulated retroviral vectors that allow modulation of gene expression in gene therapy.

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