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Self-Contained, Tetracycline-Regulated Retroviral Vector System for Gene Delivery to Mammalian Cells

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Retroviral vectors that contain the tetracycline-inducible (Tet) system were developed. The two components of the Tet system were organized within the vectors in a manner that stringently maintains tetracycline-dependent regulation. Regulated expression of an indicator gene inserted into the retroviral vectors was examined in several different cell types. In infected NIH 3T3 cells, levels of induction in the absence of tetracycline were observed to be as much as 336-fold higher than levels in the presence of tetracycline, which were extremely low. Tetracycline-dependent regulation was observed in all other transduced cell types and ranged from 24- to 127-fold. The generation of retroviral vectors containing regulatory elements that allow for the regulated expression of heterologous genes and that have the ability to infect virtually all dividing target cells should greatly facilitate the biochemical and genetic examination of a broad range of genes. Moreover, these inducible retroviral vectors should prove useful in gene therapy applications.

The introduction of heterologous genes into cultured mammalian cells or tissues is fundamental for understanding the biochemistry, genetics, and functions of genes and gene products. Much of the understanding of biological processes has been determined by transfection, electroporation, or viral delivery of genes into cultured cells or tissues. More recently, gene therapy approaches have emphasized the need for gene delivery vectors that can efficiently introduce, and control the expression of, heterologous genes in mammalian cells.

An important feature of any gene delivery system is the ability to regulate the expression of the delivered gene. This is important when the translation product of the delivered gene is being examined for its functional role in cell biology and in therapeutic situations in which the gene product is toxic or must be maintained at appropriate levels. Ideally, regulation of expression of the delivered gene should be in an on or off manner. When turned on, gene expression should be highly induced, and when turned off, gene expression should be silenced.

The tetracycline-controlled transactivator (tTA)-responsive promoter (Tet system) is a prokaryotic inducible promoter system which has been adapted for use in mammalian cells (7, 8). In the previously developed Tet system, there are two components, each of which is carried on a separate plasmid. One component, the response unit, is composed of the *Escherichia coli*-derived tetracycline resistance operon regulatory elements (*tet*O) embedded within a minimal cytomegalovirus (CMV) promoter. The second component, the regulator unit, encodes a hybrid tTA protein composed of the tetracycline repressor (*tet*R) fused to the herpes simplex virus (HSV) transactivator protein, VP16. Expression of a gene inserted downstream of the *tet*O-minimal CMV promoter in the response unit is highly dependent on tTA, which binds *tet*O sequences through its *tet*R domain and recruits positively acting cellular transcription factors through its VP16 domain. Gene expression is inhibited by the addition of tetracycline, which binds the transactivator protein, causing it to dissociate from the *tet*O-minimal CMV promoter and leading to cessation of gene transcription.

Although the Tet system has proven to be an important tool for examining the effects of genes on cells, it is limited in its applications for the following reasons. (i) For the Tet system to regulate the expression of a heterologous gene, two separate plasmids must be introduced into the cells of interest. The introduction of the two plasmids into cells is performed by using chemical or electrochemical procedures that are inherently inefficient in terms of the number of cells that take up the plasmid DNAs. The limitations of these DNA transfection procedures restrict the use of the two-plasmid-based Tet system to cultured cells, and it cannot be effectively used in vivo except in the case of transgenic animals (5, 6). (ii) The original two-plasmid-based Tet system developed by Gossen and Bujard (8) can also demonstrate some gene expression when the system is switched off (1, 15a). This means that the current Tet system can allow low-level uninduced gene expression, which can be a significant problem in some applications.

We have developed a modified version of the Tet system for use within a retroviral vector. The two components of the Tet system have been organized within the same vector so that high levels of constitutively produced tTA transcripts function not only for production of tTA protein in the on state but also to decrease basal expression of the response unit by apparent antisense inhibition. Levels of induction when these vectors were used in the absence of tetracycline were observed to be as much as 340-fold higher than levels in the presence of tetracycline, which were extremely low.

MATERIALS AND METHODS

Construction of retroviral plasmids. The retroviral constructs described here are derived from a modified version of pBABEpuro, which is a Moloney murine leukemia virus-based vector containing a puromycin resistance gene under the control of an internal simian virus 40 (SV40) promoter (14, 15). First, the *Not*I site was removed from the plasmid backbone by cleavage with *Not*I and fill-in with T4 DNA polymerase in the presence of deoxynucleoside triphosphates (dNTPs). A new *Not*I site was then added to the polylinker by cleavage with *Sna*BI and addition of a *Not*I linker. Next, the puromycin resistance gene in

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pBABEpuro was excised with *Hin*dIII and *Cla*I and replaced by the tTA gene. To do this, the entire 1,017-bp coding sequence of tTA was generated by PCR amplification using plasmid pUHD15-1 (kindly provided by H. Bujard) (1) as a template and primers corresponding to bp 774 to 790 (including a *Hin*dIII restriction site and the optimal Kozak sequence CCACCATG) and bp 1790 to 1774 (including a *Cla*I site). This pBABE-based plasmid coding for tTA under the control of the internal SV40 promoter was designated pBST. To obtain the pBJT plasmid, in which the tTA gene is under the control of the JC promoter, the SV40 promoter in pBST was removed by excision with *Not*I and *Hin*dIII and replaced by a 299-bp *Hin*dIII-*Not*I fragment from pCR1000-Mad-1 corresponding to the JC virus Mad-1 strain early promoter (11). The puromycin resistance gene, isolated as a 660-bp *Hin*dIII-*Cla*I fragment from pBabePuro, was then reinserted into the *Bam*HI-*Not*I polylinker site of pBST and pBJT (located 5′ to the SV40 and JC promoters) by blunt-end ligation after fill-in with Klenow enzyme, thereby destroying the *Not*I and *Bam*HI sites. The resulting plasmids were designated pBPST and pBPJT, respectively.

The response plasmid pUHD10-3 (provided by H. Bujard) contains the heptamerized *tet*O sequences, a minimal CMV promoter, a cloning site for insertion of the gene of interest, and an SV40 polyadenylation signal (8). To facilitate cloning of genes into the response cassette within the retroviral plasmids, the cloning site in pUHD10-3 was replaced with a polylinker containing restriction sites unique to the retroviral constructs; to this end, a *Sac*II-*Not*I-*Pac*I-*Pme*I-*Bam*HI adaptor (5′-GGCCGCTTAATTAAGTTTAAACG-3′ and 5′-GATC CGTTTAAACTTAATTAAGCGGCCGC-3′) was ligated into the *Sac*II-*Bam*HI cloning site of pUHD10-3. The 857-bp *Hin*PI fragment of this modified response cassette (including *tet*O sequences, minimal CMV promoter, polylinker, and SV40 polyadenylation signal) was then inserted into pBPST or pBPJT in an antisense orientation (relative to the tTA transcription unit) into either the *Cla*I site (located immediately downstream of the tTA gene; −1 series of plasmids) or, using Klenow fill-in and blunt-end ligation, into the *Bgl*II site which was introduced into the unique *Nhe*I site in the U3 region of the 3′ long terminal repeat (LTR) by digestion with *Nhe*I, fill-in with T4 DNA polymerase in the presence of dNTPs, and addition of a *Bgl*II linker (−2 series of plasmids). The SV40 polyadenylation signal, derived from plasmid pUHD10-3 and included in the *Hin*PI fragment that had been inserted into the retroviral plasmids, was removed by excision of a 135-bp fragment containing the relevant polyadenylation sequences with *Bam*HI and *Hpa*I and replaced by a *Bam*HI-*Sma*I adaptor (5′-GATC CCCCGGG-3′ and 5′-CCCGGGG-3′). Depending on the promoter responsible for transcription of the tTA gene (SV40 or JC) and the location of the response unit, the resulting retroviral cloning vectors were designated pBPSTR1, pBPJTR1, pBPSTR2, and pBPJTR2.

To construct the retroviral reporter plasmids pBPSTluc1, pBPJTluc1, pBPSTluc2, and pBPJTluc2, the 1,700-bp gene for firefly luciferase was excised from pGEM-luc (Promega, Madison, Wis.) with *Not*I and *Stu*I and inserted into the *Not*I-*Pme*I polylinker site of the four retroviral cloning vectors.

Cell culture. The following cell lines were used: rat C6 glioma cells (American Type Culture Collection, Rockville, Md.), mouse NIH 3T3 fibroblasts (American Type Culture Collection), rat H56 hepatoma cells (2), mouse Pam212 squamous carcinoma cells (21), and the ecotropic packaging cell line BOSC23 (16). C6 glioma cells were grown in Ham's F10 medium (Mediatech, Washington, D.C.) containing 15% horse serum (Sigma, St. Louis, Mo.), 2.5% fetal calf serum (Sigma), penicillin G (100 U/ml; Sigma), and streptomycin sulfate (100 μg/ml) (Sigma). The other cell lines were maintained in Dulbecco's modified Eagle's minimal essential medium (Mediatech) supplemented with 10% fetal calf serum and penicillin G-streptomycin sulfate.

Transfections, infections, and determination of viral titer. Transfections of BOSC23 cells were performed essentially as recommended elsewhere (16). Briefly, 5×10^6 BOSC23 cells were plated in 10 ml of medium per 100-mm-diameter dish. After 24 h, the medium was replaced with 5 ml of fresh medium. After an additional 3 h, cells were transfected with 15 μg of retroviral plasmid DNA, using the calcium phosphate precipitation technique. Just prior to transfection, chloroquine was added to a final concentration of 25 μM. Six hours posttransfection, the cells were washed twice and 10 ml of medium was added. Twenty-four hours posttransfection, the medium was removed and 5 ml of fresh medium was added. The medium containing the virus was removed 48 h posttransfection, filtered through a 0.45-μm-pore-size filter, and either used directly or stored frozen until use. Media from different plates containing BOSC23 cells transfected with the same retroviral construct were pooled to provide a common stock of viral titer for each set of experiments. BOSC23 cells were grown in either the presence (1 μg/ml) or absence of tetracycline (Sigma) during packaging of virus.

Cells which had been plated at a density of 5×10^5/100-mm-diameter dish the day before infection were infected in the presence of Polybrene (4 μg/ml; Sigma) with 5 ml of filtered medium containing virus. After 4 h, the medium was aspirated and fresh medium was added. Cells were washed twice 20 h postinfection, and luciferase assays were performed 48 h postinfection. To study tetracycline-dependent regulation of gene expression, cells were grown in the presence (1 μg/ml) or absence of tetracycline from 3 h before infection until harvesting. All experiments were performed at least twice in duplicate.

For determination of viral titer, NIH 3T3 cells were infected with serial dilutions of filtered BOSC23 supernatant. Cells were split 1:10 into selection medium containing puromycin (1.5 μg/ml; Sigma) 48 h postinfection. Fresh medium was added every 3 days, and colonies were counted 12 days later after fixation and Giemsa staining.

Luciferase assay. Cells were washed three times in phosphate-buffered saline and then lysed in 1 mM dithiothreitol–1% Triton X-100–luciferase buffer (25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA [pH 7.8]) at 4°C. Cell lysates were collected from plates with a cell scraper and centrifuged for 4 min at 7,800 $\times g$. Aliquots (100 μl) of the supernatant were mixed with 370 μl of luciferase buffer containing 2 mM ATP, 1 mM dithiothreitol, and 15 mM potassium phosphate (pH 7.8) and assayed for luciferase activity in a model 1251 luminometer (Wallac, Gaithersburg, Md.), using the integral mode. D-Luciferin (Sigma) was used at a saturating substrate concentration (0.2 mM in luciferase buffer). Luciferase activity was adjusted to protein content of the lysates by using a Bradford assay (Bio-Rad).

RESULTS

Construction of retroviral vectors. To incorporate the Tet system into a retroviral vector, we first constructed retroviral vectors containing the tTA gene under the control of either the broadly active SV40 promoter or the glial cell-specific JC virus promoter (4, 17) (regulator unit). An important consideration for efficient regulation of a gene inserted into the response unit is where to insert this unit relative to the promoters within the regulator unit and the 5′ LTR of the virus. For the Tet system to function efficiently, the response unit must operate independently of other promoters within the virus. We therefore used two different cloning strategies to determine the optimal placement of the response unit within the retroviral vectors. In all of these constructs, the luciferase gene was inserted into the response unit to allow quantitation of tetracycline-dependent regulation.

In one strategy, the response unit was cloned into a *Cla*I site, located immediately downstream of the 3′ end of the regulator unit, in the opposite orientation to the tTA-containing regulator unit (Fig. 1a and b, pBPSTR1 and pBPJTR1). In this orientation, expression of the coding strand of a gene within the response unit can be directed only from the response unit promoter (*tet*O-minimal CMV), not from the promoters in the regulator unit or viral 5′ LTR. Another advantage of inserting the response unit in this antisense orientation is that, in principal, a small amount of transcript produced as a result of potential low-level leakiness of the response unit promoter should form RNA duplexes with the vast excess of transcripts originating from the constitutively active promoters within the regulator unit and 5′ LTR.

A second strategy was to clone the response unit into a *Bgl*II site within the U3 region of the 3′ LTR (Fig. 1c and d, pBPSTR2 and pBPJTR2), again in the antisense orientation relative to the regulator unit. These vectors are considered double-copy vectors (9) because during replication and integration of the retrovirus, the viral 3′ LTR is duplicated and replaces the viral 5′ LTR that is present in the plasmid form of the vector. Thus, after replication and integration, any gene cloned into the U3 region of the 3′ LTR will ultimately be located 5′ to the promoter within the U5 region of the LTRs. The logic for this cloning strategy is that a gene regulated by the *tet*O-minimal CMV promoter within the 5′ LTR unit will not be transcribed by the promoters within the viral 5′ LTR or regulator unit (Fig. 1c and d).

Regulation of luciferase activity with single- and double-copy vectors and different packaging conditions. Using NIH 3T3 cells as target cells, we observed tetracycline-dependent regulation of luciferase activity for all retroviral constructs (Table 1). However, variability in the regulation of luciferase activity was observed, depending on (i) whether tetracycline was present during packaging of the retrovirus in BOSC23 cells and (ii) the organization of the components of the Tet system within the vectors. When pBPSTluc1 virus are packaged in

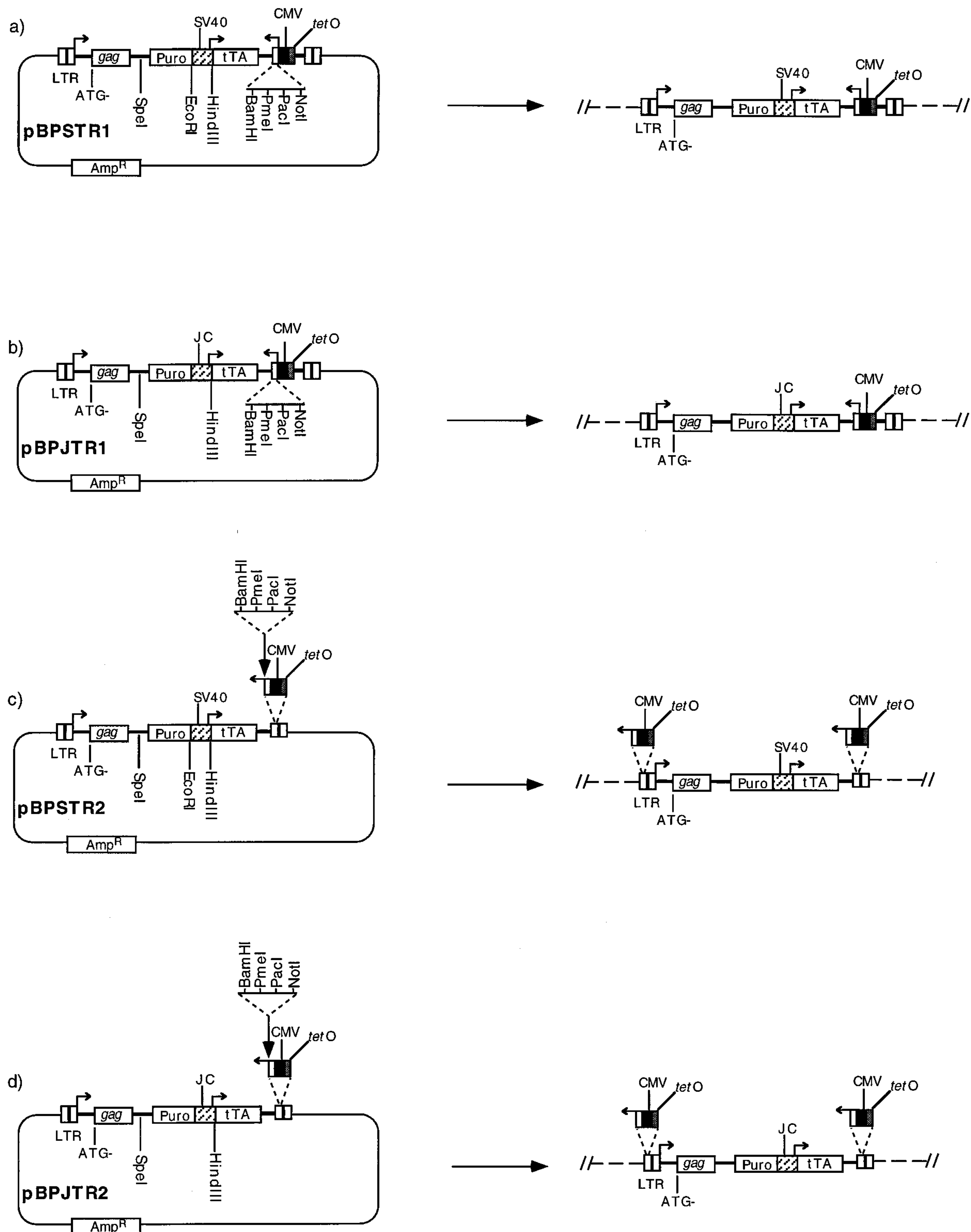

FIG. 1. Inducible and cell-specific retroviral cloning vectors. All retroviral vectors contain both components of the Tet system, including the regulator unit (containing the tTA gene), which is under the transcriptional control of internal SV40 or JC virus promoter, and the response unit, which is under the transcriptional control of a minimal CMV promoter (lacking enhancers) containing seven *tet* operators (*tet*O). Note that the response unit is inserted into the retroviral vectors in an antisense orientation relative to the regulator unit. A multiple cloning site containing four unique restriction sites (*Not*I, *Pac*I, *Pme*I, and *Bam*HI) has been inserted downstream of the response unit to facilitate the insertion of heterologous genes into the vectors. The puromycin resistance gene (Puro) is present in all constructs and is under the transcriptional control of the 5′ retroviral LTR. The retroviral vectors are modular in that both the SV40 promoter and puromycin resistance gene can be removed and replaced by different promoters or selectable marker genes. Transcription units and direction of transcription are indicated by arrows. The vectors carrying the response gene within the 3′ LTR (pBPSTR2 and pBPJTR2; c and d) are considered double-copy vectors (14). The vectors carrying the response unit between the 5′ and 3′ LTRs (pBPSTR1 and pBPJTR1; a and b) are single-copy vectors. B, pBabe family-based retroviral vector; P, puromycin resistance gene; S, SV40 early promoter; J, JC virus early promoter; T, tTA; R, response unit. The predicted structure of the retrovirus after integration into host cell DNA (provirus) is shown at the right.

TABLE 1. Regulated expression of luciferase in NIH 3T3 cells, using retroviral vectors with self-contained tetracycline-dependent inducible elements[a]

Vector	Tet during packaging	Tet during infection	Luciferase activity (relative light units/mg of total protein)	Fold induction (+Tet/−Tet)	Titer (CFU/ml)
pBPSTluc1	+	−	91,082		
	+	+	284	321	2.1×10^5
	−	−	83,671		
	−	+	467	179	1.3×10^5
pBPSTluc2	+	−	21,412		
	+	+	434	49	2.7×10^4
	−	−	21,530		
	−	+	1,174	18	2.4×10^4

[a] Retroviral vectors pBPSTluc1 and pBPSTluc2, containing the luciferase gene under the transcriptional control of the tetracycline-responsive tTA, were transiently transfected into BOSC23 packaging cells (2 days). Tetracycline (Tet) was present (+) or absent (−) during packaging. Packaged retroviral particles were used to infect target NIH 3T3 cells. NIH 3T3 cells were plated in the presence or absence of tetracycline for measurement of inducible luciferase activity. Serial dilutions of media from packaging cells were used for determining virus titers on NIH 3T3 cells. Infected cells were placed under puromycin selection, and resistant colonies were counted. Experiments were performed in duplicate and confirmed in at least two additional experiments performed in the same manner.

BOSC23 cells either in the presence (1 μg/ml) or absence of tetracycline and then used to infect NIH 3T3 cells plated either in the presence (1 μg/ml) or absence of tetracycline, luciferase activity was 321- or 179-fold lower than in the absence of tetracycline. This vector thus allows efficient transfer of the components of the Tet system into target cells, including stringent repression of gene expression by tetracycline and up to 300-fold differences between on and off states (Table 1).

To examine whether the presence of one or two copies of the response gene within the provirus affects regulation, luciferase activities were compared in NIH 3T3 cells infected with either the single-copy vector pBPSTluc1 or the double-copy vector pBPSTluc2. When tetracycline was present during the packaging of retroviral constructs in BOSC23 cells but absent during infection of NIH 3T3 cells, luciferase activity with the single-copy vector was about fourfold higher than that obtained with the double-copy vector (Table 1). When tetracycline was present during infection of NIH 3T3 cells, repression of luciferase activity appeared similar for both the single-copy and double-copy vectors; however, taking into account that the induced activity in the double-copy vector is about fourfold lower than that of the single-copy vector and that there is a corresponding increase in repressed activity as induced activity increases (for example, compare values for NIH 3T3 cells infected with pBPSTluc1 in Tables 1 and 2), the true magnitude of repressed luciferase activity in the double-copy vector should also be correspondingly fourfold higher.

Tetracycline-dependent regulation of luciferase activity after packaging in the presence of tetracycline was 321-fold with the single-copy vector but only 49-fold with the double-copy vector. When tetracycline was absent during packaging of retrovirus, regulation appeared to be lower, but single-copy vectors still showed better regulation than double-copy vectors (179-fold versus 18-fold; Table 1). Because of the better regulation of the Tet system observed with virus that had been packaged in BOSC23 cells in the presence of tetracycline, in all subsequent experiments we used virus packaged in this manner.

Regulation of luciferase activity by different internal promoters controlling tTA expression. To examine whether cell-type-specific expression could be introduced into the Tet system-containing retroviral vectors, the glial cell-specific JC virus early promoter was used to control expression of the tTA gene in order to evaluate whether luciferase activity could be restricted to cells of glial origin. pBPSTluc1 or pBPJTluc1 retrovirus was used to infect cell lines derived from different tissue origins: NIH 3T3 (mouse fibroblasts), C6 (rat glioblastoma), H56 (rat hepatoma), and Pam212 (mouse squamous carcinoma). In glial cell-derived C6 cells infected with the pBPJTluc1 retrovirus, induced luciferase activity was marginally higher than the activity observed with the pBPSTluc1 retrovirus (Fig. 2; Table 2). In contrast, in all nonglial cell types, induced luciferase activities were significantly higher when the pBPSTluc1 virus was used than when the pBPJTluc1 virus was used (Fig. 2; Table 2).

Regulation and induced luciferase activities were generally higher in the mouse cell lines (NIH 3T3 and Pam212) than in the rat cell lines (C6 and H56), probably because of higher infectibility of the mouse cells with the ecotropic virus used (Fig. 2; Table 2). The highest tetracycline-dependent regulation was observed in NIH 3T3 cells, with an induction of 336-fold (regulation of the other cell types varied from 11- to 127-fold).

Viral titers. In transient packaging reactions using BOSC23 cells, the single-copy vector pBPSTluc1 produced about 10-fold-higher viral titers than the double-copy vector pBPSTluc2 (Table 1). To examine whether transcriptional interference occurs in the retroviral vectors as a result of the placement of transcriptional units in opposing orientations, BOSC23 cells were grown in the presence or absence of tetracycline during

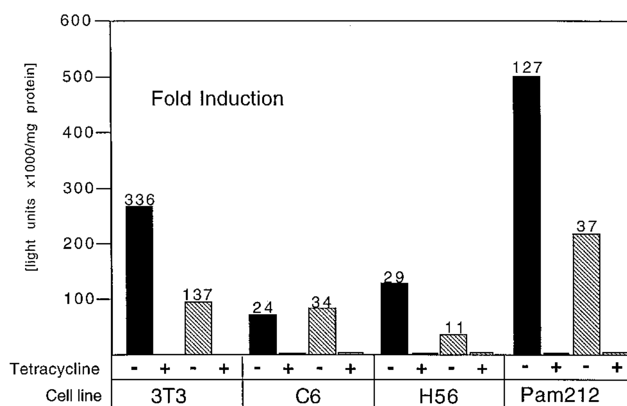

FIG. 2. Effects of different internal promoters on gene expression and regulation. NIH 3T3, C6, H56, and Pam212 cells were infected with either pBPSTluc1 (SV40 promoter; ■) or pBPJTluc1 (JC promoter; ▨) retrovirus. Ratios of luciferase activity in the absence (−) and presence (+) of tetracycline during and after infection reflect inducible regulation (shown above boxes). Values were obtained from Table 2.

TABLE 2. Regulated expression of luciferase in different cell types, using retroviral vectors with self-contained tetracycline-dependent inducible elements[a]

Cell type	Vector	Tet during infection	Mean luciferase activity (relative light units/mg of protein) ± SE	Fold induction (+Tet/−Tet)
NIH 3T3	pBPSTluc1	−	263,190 ± 24,739	
	pBPSTluc1	+	784 ± 32	336
	pBPJTluc1	−	91,714 ± 7,979	
	pBPJTluc1	+	669 ± 15	137
C6	pBPSTluc1	−	66,347 ± 3,317	
	pBPSTluc1	+	2,787 ± 167	24
	pBPJTluc1	−	76,713 ± 4,603	
	pBPJTluc1	+	2,271 ± 50	34
H56	pBPSTluc1	−	86,970 ± 8,175	
	pBPSTluc1	+	3,014 ± 184	29
	pBPJTluc1	−	25,783 ± 2,759	
	pBPJTluc1	+	2,267 ± 95	11
Pam212	pBPSTluc1	−	512,346 ± 49,185	
	pBPSTluc1	+	4,039 ± 185	127
	pBPJTluc1	−	201,830 ± 20,788	
	pBPJTluc1	+	5,490 ± 203	37

[a] Retroviral vectors containing the luciferase gene under the transcriptional control of the tetracycline-responsive tTA were transiently transfected into BOSC23 cells and packaged in the presence of tetracycline. tTA is under the control of the SV40 promoter in pBPSTluc1 or the JC promoter in pBPJTluc1. Packaged retroviral particles were used to infect NIH 3T3 fibroblasts, C6 glioma cells, H56 hepatoma cells, and Pam212 squamous carcinoma cells. During and after infection, NIH 3T3 cells were plated in the presence (+) or absence (−) of tetracycline (Tet) for measurement of luciferase activity. Experiments were performed in duplicate and confirmed in at least two additional experiments performed in the same manner.

packaging of pBPSTluc1 and pBPSTluc2 constructs. Because tetracycline causes tTA to dissociate from *tet*O sequences, thereby inhibiting transcription of the response unit, the presence of tetracycline may facilitate the production of genomic transcripts and increase the number of viral particles. However, the titer did not significantly increase after packaging in the presence of tetracycline (Table 1). No differences in the viral titer were observed between pBPSTluc1 and pBPJTluc1 or pBPSTluc2 and pBPJTluc2 (data not shown).

DISCUSSION

We have constructed retroviral vectors that contain a modified version of the original Tet system in order to adapt the system for use in a broad spectrum of applications. The essential features of these retroviral vectors are as follows. The Tet system was organized within a retroviral vector so that high levels of constitutively produced tTA mRNA function not only for production of tTA protein but also to decrease basal expression of the response unit by apparent antisense inhibition. This antisense inhibition is evident when one compares tetracycline-dependent regulation of the single- and double-copy vectors. For example, in one set of experiments using NIH 3T3 cells as target cells, a 321-fold induction of luciferase activity was observed in the single-copy vector pBPSTluc1, whereas a 49-fold induction was observed in the double-copy vector pBP STluc2. The lower induction value observed for the double-copy vector is due to higher basal expression in the off state (plus tetracycline) and lower induced expression in the on state (minus tetracycline) compared with activities observed for the single-copy vector. Although basal expression of the response unit within the 3′ LTR of the double-copy vector is subject to antisense inhibition, the response unit within the 5′ LTR is not. Thus, the higher basal expression observed in the double-copy vector is from the response unit within the 5′ LTR. The true magnitude of the antisense effect in the single-copy vector is probably higher than shown in Table 1 because of the lower titers of the double-copy vector. The basal luciferase activity of the single-copy vector is therefore much lower than that expected on the basis of gene dosage, which is probably the consequence of antisense inhibition. Interestingly, although it would be expected that antisense inhibition would also occur when transcription in the response unit is activated with tTA binding, this apparently is not a major problem, since relatively high levels of luciferase activity are detectable in the on state.

Presently, it is unclear why better induction ratios are obtained when the vectors are packaged in the presence of tetracycline. However, this phenomenon is observed regardless of whether we used single- or double-copy vectors and was observed in all experiments when we generated virus in the presence of tetracycline. Further experiments are needed to understand this phenomenon.

Tetracycline-dependent regulation of the retroviral vectors was measured in several different cell types. The best regulation was observed in NIH 3T3 cells; however, regulation was still relatively high in the other cell types examined. Variation in the efficiency of tetracycline-controlled gene expression in different cell types using the two-plasmid-based Tet system has been observed and discussed previously (1). In that study, a complete lack of regulation was observed in some cell lines. In our studies, we observed regulation in all cell lines examined. A possible explanation for this difference is that in the two-plasmid-based system, stable tTA-expressing cell lines need to be established. Because the VP16 motif of tTA functions in mammalian cells as a transcription factor, some cell types may not be able to tolerate high-level expression of the tTA protein and may not grow well. It is also likely that the promoter responsible for expression of the regulator (encoding tTA) demonstrates variable activity depending on the cell type and site of integration in the host cell genome. Further, basal expression of the response unit (containing the *tet*O-minimal CMV promoter) may vary depending on cell type. In this regard, the incorporation of the Tet system into a retroviral vector alleviates the need to preestablish cell lines with stable expression of tTA, because both the regulator and response units are transduced in parallel and integrated adjacent to each other in infected cells.

It is important to note that because the site of integration of the virus into the host cell genome is random, rare integrations that place the inducible system adjacent to an active cellular gene likely occur (for a review see reference 18). This situation could occur in a host cell containing a single site of integration or in a host cell containing multiple sites of integration. In either of these situations, inducible regulation would be bypassed; however, this would affect only the regulation within individual cells, not the overall regulation in a population of target cells.

We also examined the feasibility of using tissue-specific promoters (glial cell-specific JC virus promoter) to add the feature of cell-specific expression to the retroviral Tet system. When either the JC virus or SV40 promoter was used to direct expression of the tTA protein to cells of glial origin, marginally higher levels of tTA-dependent expression could be directed to C6 glioma cells with the JC virus promoter. However, in nonglial cell types, tTA-dependent expression was decreased but not abolished with the JC virus promoter. Reasons for this lack of stringent cell-specific regulation by the JC virus promoter in

nonglial cell types are currently unclear, but the internal JC promoter might be influenced by the enhancer within the viral 5′ LTR. In this regard, previous studies have shown that the 3′ LTR enhancer and promoter can be deleted from the plasmid form of a retroviral vector without affecting virus titers or the ability of the virus to integrate into the host cell genome (3, 10, 13, 19, 20). In the context of a retroviral vector, the JC virus promoter may not be absolutely specific for glial cells; however, it remains to be determined whether the incorporation of other cell-type-specific promoters into the retroviral Tet system results in a more selective pattern of gene expression in the on state.

The retroviral vectors described here are modular in that components can be easily replaced. We incorporated a multiple cloning site immediately downstream of the *tet*O-minimal CMV sequences in the response unit for the insertion of heterologous genes. In addition, the puromycin resistance gene was included upstream of the regulator unit and under the control of the 5′ LTR promoter. Including this selectable marker into the vectors is important for determining the virus titer and for the selection and cloning of high-level producer cell lines.

Potential applications of the inducible retrovirus. Coupling the inducible retroviral vectors with the extremely efficient ecotropic BOSC23 packaging cells (16) allows production of virus after a 2-day transient transfection and packaging reaction. Because we have included the puromycin resistance gene into the inducible retroviral vectors, it may be possible to increase the titer of virus produced from the BOSC23 cells by introducing a brief selection period in order to establish puromycin-resistant producer pools. Target cells can then be infected with retrovirus carrying a gene of interest, and the effect on target cells can be measured quickly. Furthermore, the relatively high titer of the virus produced in the transient packaging reactions is in general sufficient to infect the majority of target cells, bypassing the need to generate stable cell lines and decreasing the potential negative selection if target cells poorly tolerate the tTA protein. The ability to efficiently "ping-pong" ecotropic to amphotropic retrovirus provides a means of generating retrovirus that will infect nonrodent species (12). Thus, these inducible retroviral vectors should facilitate the cloning or expression of genes with negative growth influences on cells such as those encoding cell cycle inhibitors or dominant negative mutants. Lastly, in gene therapy research, these inducible retrovirus vectors will potentially be helpful for applications requiring regulated expression of a therapeutic gene.

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