BMC Molecular Biology

Methodology article

Retroviral vectors for establishing tetracycline-regulated gene expression in an otherwise recalcitrant cell line

Paraic A Kenny*[1,3], Tariq Enver[1] and Alan Ashworth[2]

Address: [1]Section of Gene Function and Regulation, Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, United Kingdom, [2]The Breakthrough Breast Cancer Research Centre, Institute of Cancer Research, Chester Beatty Laboratories, 237 Fulham Road, London SW3 6JB, United Kingdom and [3]Current address: Life Sciences Division, Lawrence Berkeley National Laboratory, 1 Cyclotron Road, Berkeley, CA 94720 USA

E-mail: Paraic A Kenny* - pakenny@lbl.gov; Tariq Enver - tariq@icr.ac.uk; Alan Ashworth - alana@icr.ac.uk

*Corresponding author

Abstract

Background: Tetracycline-regulated systems have been used to control the expression of heterologous genes in such diverse organisms as yeast, plants, flies and mice. Adaptation of this prokaryotic regulatory system avoids many of the problems inherent in other inducible systems. There have, however, been many reports of difficulties in establishing functioning stable cell lines due to the cytotoxic effects of expressing high levels of the tetracycline transactivator, tTA, from a strong viral promoter.

Results: Here we report the successful incorporation of tetracycline-mediated gene expression in a mouse mammary epithelial cell line, HC11, in which conventional approaches failed. We generated retroviruses in which tTA expression was controlled by one of three promoters: a synthetic tetracycline responsive promoter (TRE), the elongation factor 1-alpha promoter (EF1α) or the phosphoglycerate kinase-1 promoter (PGK), and compared the resulting cell lines to one generated using a cytomegalovirus immediate early gene promoter (CMV). In contrast to cells produced using the CMV and PGK promoters, those produced using the EF1α and TRE promoters expressed high levels of β-galactosidase in a tetracycline-dependent manner.

Conclusions: These novel retroviral vectors performed better than the commercially available system and may have a more general utility in similarly recalcitrant cell lines.

Background

The ability to control the spatial and temporal expression of a transgene, either in cell culture or in transgenic animals, is a valuable tool in gene function studies. Constitutive overexpression of a transgene over long periods in culture may have several undesired effects that are inimical to its functional characterisation. This is particularly true in the case of gene products with growth inhibitory or toxic effects. Temporal and spatial control of transgene expression is conferred by the choice of heterologous promoter. Many methods have been described to achieve this aim in mammalian cells. Some of these methods involve the use of endogenous mammalian proteins. These include the use of heat shock proteins [1–3], metal-

lothionein [4], the glucocorticoid receptor [5], the estrogen receptor [6], the progesterone receptor [7] and the aryl hydrocarbon receptor [8]. The ecdysone receptor of *Drosophila melanogaster* has also been used to confer ligand-dependent activation on heterologous transgenes *in vivo* and *in vitro*[9]. Other approaches have harnessed components from two well-understood paradigms of prokaryotic gene regulation – the Lac repressor [10,11] and the Tet repressor [12].

While all of the methods listed have been used in transgenic animals, the tetracycline-mediated method is one of the most commonly used. It has a number of significant advantages over the other systems. Mammals do not express an endogenous tetracycline repressor and systemic administration of this antibiotic to animals over long periods is, in itself, innocuous. The method exploits the properties of the *E. coli* Tn10-encoded tetracycline repressor protein, TetR. TetR binds Tet operator (TetO) elements in the Tn10 transposon and represses the transcription of tetracycline resistance genes. The binding of tetracycline to TetR results in the derepression of these genes [13]. Analysis of the structure of the TetR:tetracycline complex revealed that binding of tetracycline alters the relative orientation of the helix-turn-helix motifs thereby abolishing the DNA binding of the repressor [14].

This system has been adapted to control the expression of heterologous genes in mammalian cells by fusion of the Tet Repressor to the VP16 transcriptional activation domain of *Herpes simplex* virus to produce the tetracycline transactivator, tTA. Expression of this protein in mammalian cells was shown to regulate the expression of a luciferase transgene downstream of a tetracycline responsive promoter (a minimal CMV promoter containing tandem tetO sites) in a tetracycline-dependent manner [12]. The utility of the system in *in vivo* transgenic models has been demonstrated in yeast [15], plants [16], flies [17] and mice [18]. The system was further improved by the identification in a random mutagenesis screen of a TetR variant with reversed DNA-binding properties. This reverse tetracycline transactivator, rtTA, activates transcription upon ligand-binding [19].

Some attempts have been made to address problems of cytotoxicity of the tTA protein as these can have a negative impact on the successful generation of stable tetracycline-regulated cell lines. The initial system used the strong viral CMV promoter to drive tTA expression [12]. Plasmid vectors in which the CMV promoter was replaced with weaker promoters were shown to significantly increase the probability of establishing a functional cell line [20,21]. Generation of stable cell lines using plasmid vectors is typically inefficient and time-consuming, requiring the integration of the plasmid at a random chromosomal

breakpoint, often in resulting in multiple copies of the plasmid at this locus. In contrast, the retroviral life cycle includes a genomically integrated proviral stage so that the use of recombinant retroviruses can lead to the rapid and efficient generation of stable cell lines [22].

In this study, we compare the effect of the choice of promoter used to drive tTA expression on the successful generation of stable β-galactosidase-inducing cells using the mouse mammary epithelial cell line, HC11 [23]. Attempts to establish tetracycline-regulated gene expression in this cell line using conventional approaches were unsuccessful. The promoters evaluated are the cytomegalovirus immediate early promoter (CMV), the mammalian elongation factor 1-alpha (EF1α) and phosphoglycerate kinase-1 (PGK) promoters, and a tetracycline responsive element consisting of seven 42 bp TetO repeats upstream of a minimal CMV promoter (TRE). Of the four promoters tested, both the EF1α and the TRE yielded cell lines in which a β-galactosidase transgene was regulated in a tetracycline-dependent manner, while no induction was detected in the case of the CMV and PGK promoters. These novel retroviral vectors may have a general applicability in cell lines in which commercially available tetracycline are unsuccessful.

Results

HC11 cells stably infected with RevTetOFF(CMV-tTA) and TRE-lacZ fail to express *β*-galactosidase in a tetracycline-dependent manner

The RevTetOFF system (Clontech) was chosen to establish tetracycline-regulated gene expression in a mouse mammary epithelial cell line, HC11. This system consists of two retroviral constructs – RevTetOFF in which tTA expression is under the control of the CMV promoter (CMV-*tTA*), and RevTRE, containing a tetracycline responsive promoter, into which the cDNA of interest can be cloned. As this paper describes a number of variants of the commercially available RevTetOFF vector, this original vector will be henceforth referred to as CMV-*tTA*. As a preliminary experiment, HC11 cells were transiently transfected with CMV-*tTA* and TRE-*lacZ* to investigate whether tetracycline-mediated regulation of gene expression in this cell line was a viable prospect. These cells expressed β-galactosidase in a tetracycline-dependent manner (data not shown and Fig. 2B-II). Culture of transiently transfected cells in either tetracycline (2 µg/ml) or doxycycline (1 µg/ml) was sufficient to repress transgene expression in these experiments.

A "double-stable" cell line, HC11(CMV-*tTA*/TRE-*lacZ*), was generated using these retroviruses in serial infection and antibiotic selection as described in Materials and Methods. When these cells were induced for 48 hours by doxycycline withdrawal, X-gal staining revealed no detect-
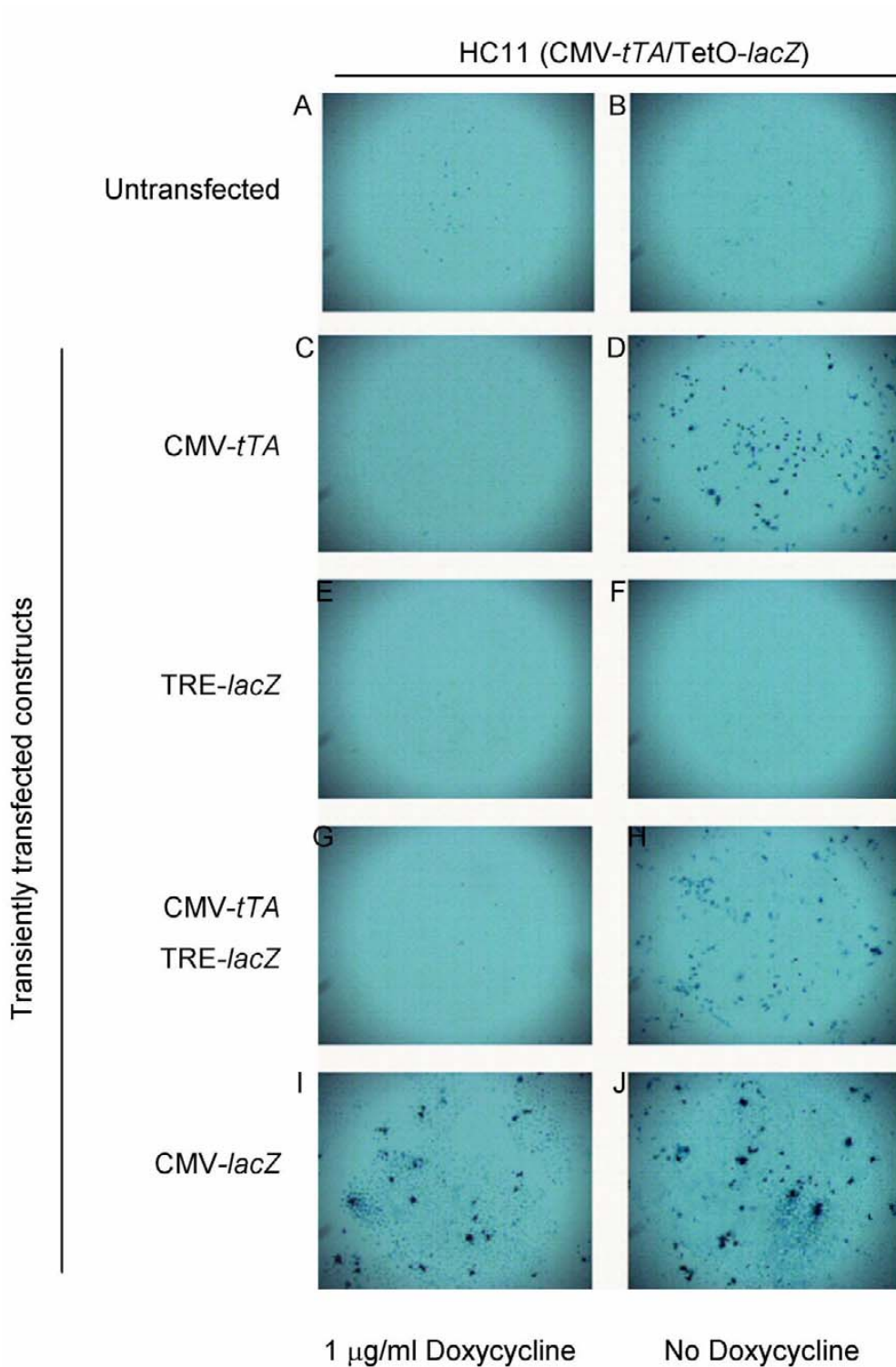
Figure 1
Lack of tTA expression prevents β-galactosidase induction in HC11(CMV-*tTA*/TRE-*lacZ*) cells. The cells were transiently transfected with the indicated constructs and were cultured either in the presence (**A,C,E,G,H,I**) or absence (**B,D,F,H,J**) of 1 μg/ml doxycycline for 48 hours. The CMV-*lacZ* expression vector was used as a positive control for β-galactosidase expression. The cells were fixed and stained for β-galactosidase expression using X-gal 48 hours after transfection.
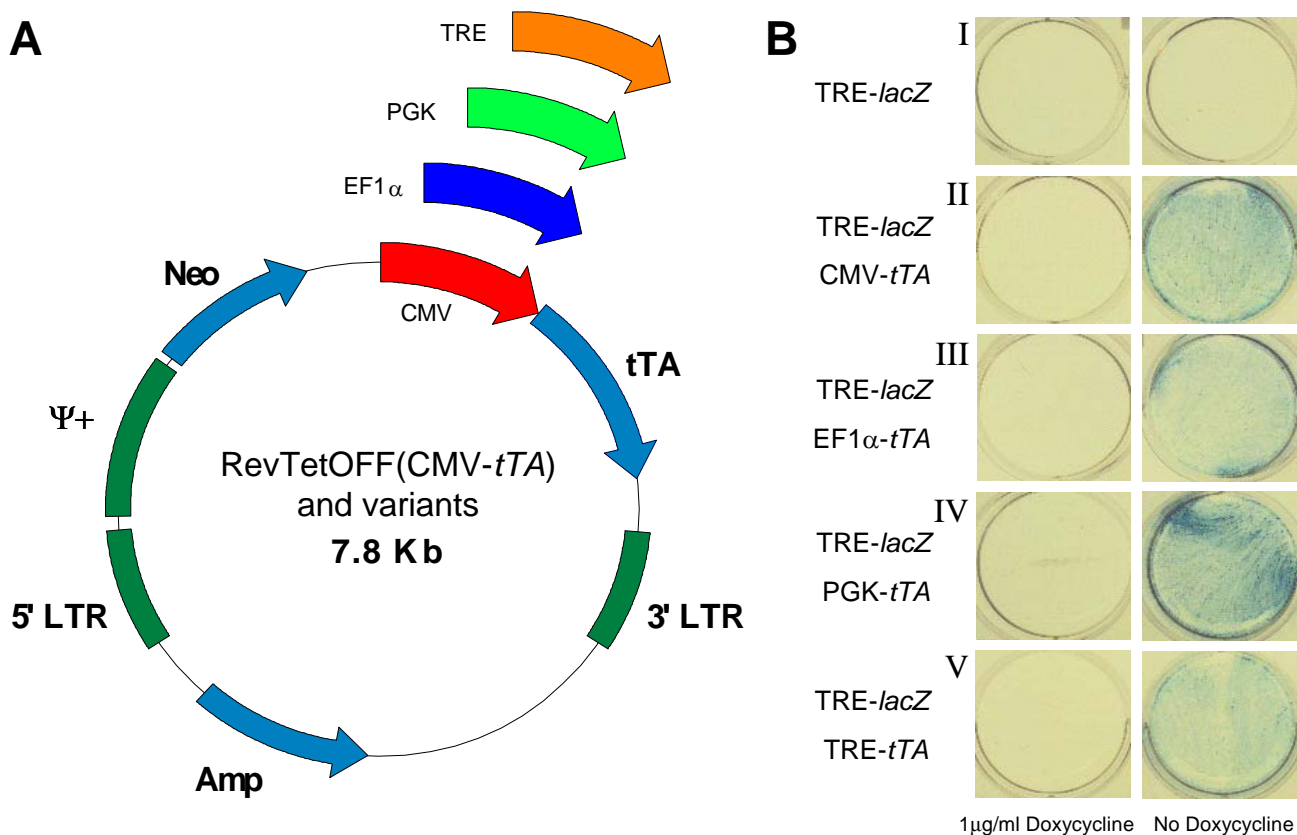
Figure 2
Generation and analysis of modified RevTetOFF constructs. (**A**) Three additional constructs were generated in which the CMV promoter was replaced by either the elongation factor 1-alpha or phosphoglycerate kinase-1 promoters, or a tetracycline responsive element. (**B**) Parental HC11 cells were transiently co-transfected with the indicated constructs and maintained for 48 hours in the presence of absence of 1 μg/ml doxycycline. The cells were fixed and stained for β-galactosidase expression using X-gal 48 hours after transfection.

able increase in β-galactosidase activity (Fig. 1A and 1B). This induction time was adequate for HC11 cells transiently transfected with these constructs. Increasing the induction time did not lead to any increase in β-galactosidase activity.

Addition of tTA is necessary and sufficient to rescue the induction in HC11 (CMV-tTA/TRE-lacZ) cells
As these cell pools were resistant to both G418 and hygromycin B it was assumed that the retroviruses had become stably integrated. To investigate the failure to obtain inducible expression, these "double-stable" cells were transiently transfected with the constructs used to generate the retroviruses (Fig. 1). Co-transfection of both CMV-*tTA* and TRE-lacZ led to a strong induction of β-galactosidase upon withdrawal of tetracycline, indicating that there are

no factors intrinsic to these cells which prevent tetracycline-mediated gene regulation (Fig. 1G and 1H). Transfection of TRE-*lacZ* alone into this pool led to no increase in expression (Fig. 1E and 1F). Surprisingly, transfection of CMV-*tTA* alone led to a strong induction of β-galactosidase activity in the pool (Fig. 1C and 1D). This strongly suggested that the expression level of tTA was the limiting factor in the pool and that its absence was preventing the induction of the *lacZ* transgene.

Effect of promoter choice on the generation of functional inducible cell lines
The results above suggested that tTA was limiting and previous reports indicated that the choice of promoter used to drive tTA or rtTA expression affected the probability of successfully generating a functional inducible cell line
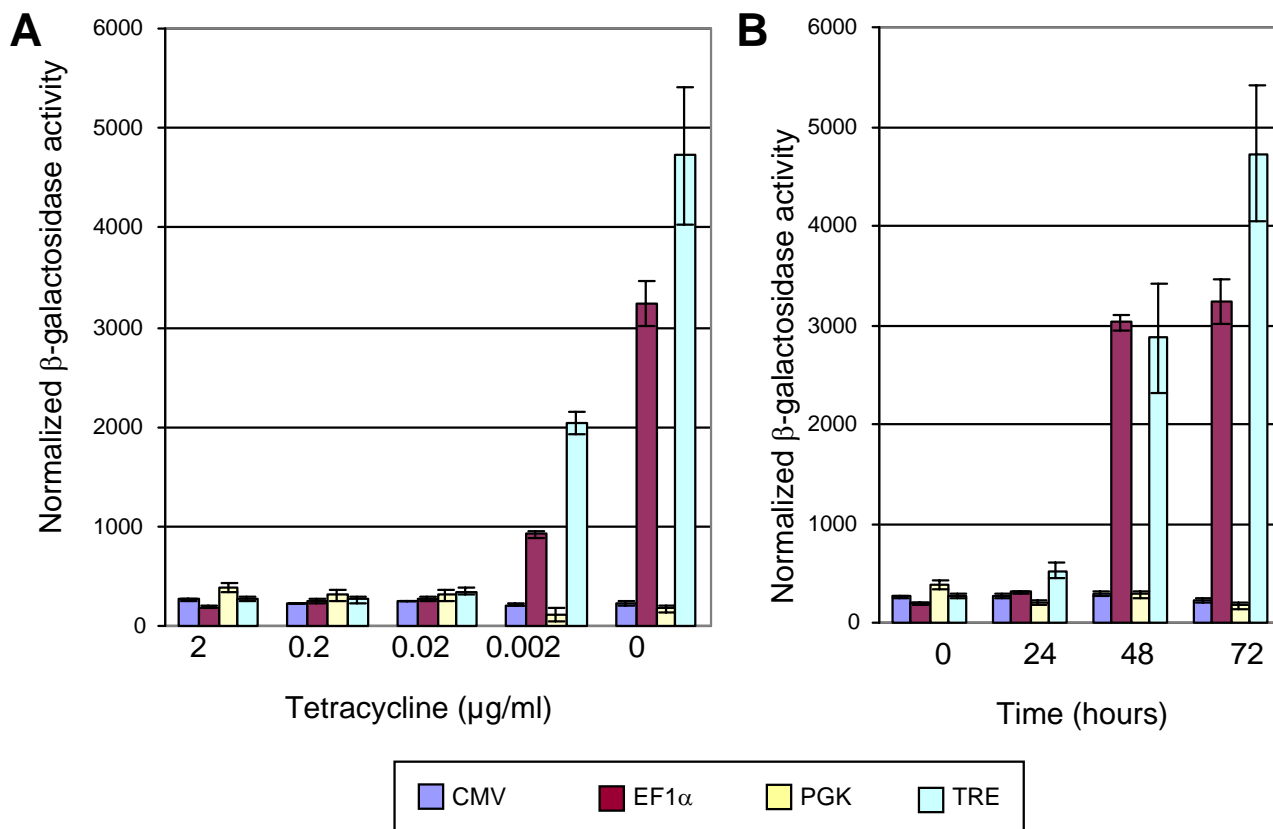
Figure 3
Analysis of the kinetics of β-galactosidase induction in the HC11 cell pools with tTA under the control of the CMV, EF1α, PGK or TRE promoters. (**A**) Dose-response curve. 5000 cells were plated per well for each condition and β-galactosidase activity was determined after 72 hours of induction. (**B**) Time course of induction. 5000 cells were plated per well for each condition and β-galactosidase activity was measured after the indicated periods of induction. In both cases, the β-galactosidase activity has been normalised to the protein concentration of the sample. Results are presented as mean normalised β-galactosidase activity of three replicates ± standard error.

[20,21]. To address whether this was significant in the cell line of interest, we generated three additional retroviral constructs based on CMV-*tTA* (Fig. 2A). In these variants, the CMV promoter was replaced with either the elongation factor 1-alpha (EF1α) or phosphoglycerate kinase-1 (PGK) promoters or the same Tetracycline Responsive Element (TRE) that confers inducibility on the transgenes in this system. By using the TRE, it was hoped that an autoregulatory system would be created in which tTA would be expressed at minimal levels in the presence of tetracycline and, upon tetracycline withdrawal, would activate its own transcription and the transcription of the TRE-*lacZ* transgene. Transient co-transfection of TRE-*lacZ* with each of these constructs led to the expression of β-galactosidase in a tetracycline-dependent manner, indicating that these constructs were functioning as expected (Fig. 2B).

Each of these tTA-expressing retroviruses was used to generate a pool of stable cells, as previously described. Each pool was then infected with the TRE-*lacZ* retrovirus. Following selection, the induction kinetics of each of these "double-stable" pools was investigated using a CPRG-based β-galactosidase activity assay (Fig. 3). In agreement with the previous results, the cells infected with the CMV-*tTA* retrovirus yielded no detectable induction in this sensitive assay. Similarly the PGK-*tTA* cells could not be induced to express β-galactosidase. Interestingly, a strong induction was observed in the cell pools in which tTA expression was controlled by either the EF1α promoter or the Tetracycline Responsive Element. This induction was effectively repressed by culturing the cells in the presence of 20 ng/ml tetracycline. When tetracycline was withdrawn from cells cultured in 2 μg/ml of the antibiotic, a
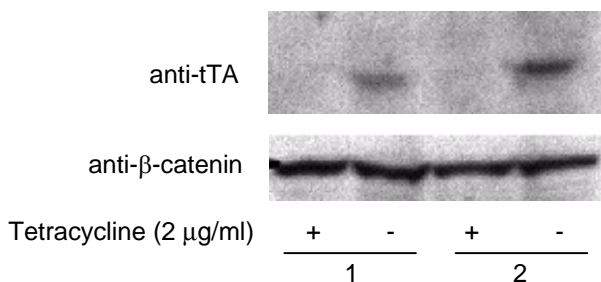
Figure 4
Western Blot analysis of tTA expression in two independent pools of TRE-*tTA* infected cells. The cells were induced for 72 hours. In pools 1 and 2, tTA is expressed in a tetracycline-dependent manner, demonstrating that the system functions in an autoregulatory manner. β-catenin is used as a loading control.

time course analysis showed a strong induction of β-galactosidase activity occurred between 24 and 48 hours (Fig. 3B). The expression of tTA in two independently generated TRE-*tTA* pools was examined by western blotting (Fig. 4). tTA was expressed in these lines in a tetracycline-dependent manner, indicating that this system was functioning in an autoregulatory manner as designed.

Discussion
In this study we have analysed the effect of the promoter chosen to drive tTA expression on establishing tetracycline-mediated transcriptional regulation in a mouse mammary epithelial cell line, HC11. Although this was feasible by transient transfection, this cell line was recalcitrant to the generation of tetracycline-regulated stable cells in our hands using the retrovirally-mediated RevTet-OFF system (Clontech). We evaluated the effect of four promoters in parallel in the context of the RevTetOFF vector – cytomegalovirus immediate early (CMV), elongation factor 1-alpha (EF1α), phosphoglycerate kinase-1 (PGK) and Tetracycline Responsive Element (TRE). Of these, only those cells in which either EF1α or TRE were used to drive tTA expression yielded functional β-galactosidase-inducing cell lines. Use of the TRE resulted in an autoregulatory system in which tTA was not expressed at detectable levels prior to induction. Upon tetracycline withdrawal, tTA activated both its own transcription and that of the *lacZ* transgene.

While it is clearly advantageous to use either the EF1α or TRE promoters to drive tTA expression in this system, the

reasons for the failure of the CMV and PGK promoters are unclear. Overexpression of tTA has been shown to have a growth inhibitory effect (Gallia 1998). It is possible that the expression of this transgene at high levels from the CMV or PGK promoters is detrimental to the cell. The lack of inducibility in the cell pools obtained using these promoters could be explained by either the death of tTA expressing cells or the rapid overgrowth of the culture by non-tTA expressing cells. The mechanism of tTA toxicity has been proposed to be squelching – the inhibition of cell function by the sequestration of endogenous proteins – first described in the case of GAL4 overexpression in yeast [24]. The tTA protein is a fusion of the transcriptional activation domain of VP16 and the DNA-binding domain of the tet Repressor. VP16 is a potent transcriptional activator and binds to a large number of proteins involved in the regulation of transcription – Host Cell Factor, Oct-1, TBP, hTAFII31, TFIIA, TFIIB, TFIIH, PC4 and the RNA polymerase II holoenzyme [25]. Expression of high levels of a VP16 fusion protein is likely to reduce the available cellular pool of these factors and consequently inhibit the process of RNA transcription.

No tTA expression was detected by immunofluorescence or western blotting in the cell lines in which the CMV promoter was used to drive tTA expression in this study (data not shown). The efforts of others to reduce the toxicity of tTA initially focussed on the VP16 domain. Elimination of the binding sites for other proteins by using only the amino acid sequence required for transcriptional activation produced a modified tTA that was tolerated at higher intracellular concentrations while still retaining the ability to modulate transgene expression in a ligand-dependent manner [26]. The issue of VP16 toxicity has also been addressed by replacing this domain with a number of non-viral transcriptional activation domains. Fusion of the acidic p65 domain of NF-kB to the tet-repressor yielded a functional tetracycline dependent transactivator [27]. A more sensitive and potent transactivator has been produced by replacing the VP16 domain with a hybrid trans-activation domain containing sequences from NF-kB p65 and Heat Shock Factor 1 [28]. The transcriptional activation domain of the ubiquitously expressed E2F4 protein has also proved useful. In a parallel comparison with tTA transfected cells, the E2F4 variant yielded a higher percentage of clones expressing the transactivator, and these cells were not subject to the growth retarding effects of the VP16 fusion protein [29]. Some success has been reported using a plasmid-based autoregulatory system similar to that described here [20], however the use of retroviruses as described here should facilitate the more rapid generation of functional cell lines.

Conclusions

There have been many anecdotal reports of difficulties in establishing tetracycline-mediated gene expression in a variety of cell lines. We believe that the EF1α– and TRE-*tTA* retroviral constructs described here may have a general applicability for the introduction of tetracycline-mediated gene expression in otherwise recalcitrant systems.

Methods

Cell culture

All reagents were purchased from Sigma unless otherwise noted. HC11 mouse mammary epithelial cells were cultured in 5% CO_2 at 37°C in RPMI 1640, supplemented with 10% Foetal Bovine Serum (PAA Laboratories, Linz, Austria), 2 mM L-glutamine, 2.5 µg/ml insulin, 5 ng/ml epidermal growth factor and 50 µg/ml gentamycin (Invitrogen) [23]. Cells infected with retroviruses encoding tTA were routinely cultured in either 2 µg/ml tetracycline or 1 µg/ml doxycycline to repress transcription of the tetracycline-regulated transgene.

Bosc23 cells were used to produce ecotropic retroviruses [30]. Cells were transiently transfected with the appropriate retroviral construct and the supernatant was collected 48 hours post-transfection. Polybrene was added to a final concentration of 5 µg/ml and the supernatant was added to HC11 cells for 24 hours. HC11 cells were then subjected to antibiotic selection using 250 µg/ml G418 (Invitrogen) when selecting for RevTetOFF based constructs. These pools were then infected with TRE-*lacZ* retroviruses and subjected to antibiotic selection with 200 µg/ml hygromycin B (Invitrogen). These were the empirically determined lowest concentrations of antibiotics required to kill untransfected HC11 cells within seven days.

Generation of constructs

The RevTetOFF construct containing the CMV promoter driving tTA expression and the empty RevTRE construct were purchased from Clontech.

A BglII fragment containing the *lacZ* cDNA was excised from the CMV-*lacZ* construct and sub-cloned into BamHI digested RevTRE to make TRE-*lacZ*. To make the EF1α-, PGK- and TRE-*tTA* retroviral constructs, the *tTA* cDNA was excised from RevTetOFF using HindIII and BamHI and subcloned into the HindIII and BamHI sites of pBluescript (Stratagene) to make pSK-*tTA*. The EF1α promoter from pEF6V5HisC (Invitrogen) was excised using HindIII and subcloned into the HindIII site of pSK-*tTA* to make pSK-EF1α-*tTA*. The PGK promoter was excised from a pPGK-Neo derived vector using XhoI and HindIII and subcloned into the XhoI and HindIII sites of pSK-*tTA* to make pSK-PGK-*tTA*. The Tetracycline Responsive Element was excised from RevTRE using XhoI and HindIII and subcloned into the XhoI and HindIII sites of pSK-*tTA* to make pSK-TRE-*tTA*. The CMV-*tTA* cassette was excised from RevTetOFF by BamHI digestion and the ends were filled in with Klenow polymerase. The promoter-*tTA* cassettes from each pSK-Promoter-*tTA* construct were excised with XhoI and BamHI, the ends were filled in with Klenow polymerase and they were subcloned into the blunt-ended BamHI digested RevTetOFF construct to make the EF1α-*tTA*, PGK-*tTA* and TRE-*tTA* retroviral constructs.

Transient transfection

Transient transfections were performed using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Briefly, 1.5×10^5 cells were plated in 3.5 cm wells on the day prior to transfection. Each well was transfected with a total of 1 µg DNA under serum-free conditions for six hours, after which the cells were washed and incubated for a further 48 hours before assaying expression.

Western Blotting

Cell monolayers were rinsed twice with ice-cold Phosphate Buffered Saline and total cell lysates were prepared by scraping cells into a minimal volume of 50 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.5% NP40 and Complete™ protease inhibitor cocktail (Roche). The protein content of the clarified total cell lysates was measured using the Biorad Protein Assay. Aliquots containing 80 µg protein from each sample were analysed by SDS-PAGE [31], and transferred electrophoretically to a PVDF membrane. Mouse monoclonal antibodies were used to detect tTA (Clontech) and β-catenin (Transduction Laboratories). A HRP-conjugated anti-mouse antibody (Amersham Pharmacia Biotech) was used as a secondary antibody in both cases. Detection was by Enhanced Chemiluminescent Detection according to the manufacturer's instructions (Amersham Pharmacia Biotech).

β-galactosidase activity assays

5000 cells from each cell line, for each condition, were cultured in triplicate in 96 well plates for 72 hours, and were lysed *in situ* by addition of 40 µl 1 × Reporter Lysis Buffer (Promega) followed by two freeze-thaw cycles. The protein concentration of 5 µl of lysate was determined and the β-galactosidase activity of the remaining 35 µl of lysate was measured using a spectrophotometric chlorophenol red β-D-galactopyranoside (CPRG) assay. 175 µl of a solution containing 5 mM CPRG, 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.14% (v/v) 2-mercaptoethanol was added to each well. The rate of change of absorbance (at 575 nm) was measured using an MRX-TC spectrophotometer (Dynex Technologies) and this β-galactosidase activity was normalised to the protein concentration of each sample to control for any variation in cell number.

Cell monolayers were stained for β-galactosidase expression using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The cells were fixed for 5 minutes in 4% (w/v) paraformaldehyde and incubated at 37°C in a solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% (v/v) sodium deoxycholate, 0.02% NP40, 2.5 mM X-gal.

Authors' contributions

All experimental procedures were carried out by PK. PK, TE and AA contributed to the design of the study. All authors read and approved the final manuscript.

Acknowledgements

References

1. Kay RJ, Boissy RJ, Russnak RH, Candido EP: **Efficient transcription of a Caenorhabditis elegans heat shock gene pair in mouse fibroblasts is dependent on multiple promoter elements which can function bidirectionally.** *Mol Cell Biol* 1986, **6:**3134-3143
2. Kothary R, Clapoff S, Darling S, Perry MD, Moran LA, Rossant J: **Inducible expression of an hsp68-lacZ hybrid gene in transgenic mice.** *Development* 1989, **105:**707-714
3. Schweinfest CW, Jorcyk CL, Fujiwara S, Papas TS: **A heat-shock-inducible eukaryotic expression vector.** *Gene* 1988, **71:**207-210
4. Mayo KE, Warren R, Palmiter RD: **The mouse metallothionein-I gene is transcriptionally regulated by cadmium following transfection into human or mouse cells.** *Cell* 1982, **29:**99-108
5. Huang AL, Ostrowski MC, Berard D, Hager GL: **Glucocorticoid regulation of the Ha-MuSV p21 gene conferred by sequences from mouse mammary tumor virus.** *Cell* 1981, **27:**245-255
6. Webster N, Jin JR, Green S, Hollis M, Chambon P: **The yeast UASG is a transcriptional enhancer in human HeLa cells in the presence of the GAL4 trans-activator.** *Cell* 1988, **52:**169-178
7. Wang Y, O'Malley BW Jr, Tsai SY, O'Malley BW: **A regulatory system for use in gene transfer.** *Proc Natl Acad Sci U S A* 1994, **91:**8180-8184
8. Campbell SJ, Carlotti F, Hall PA, Clark AJ, Wolf CR: **Regulation of the CYP1A1 promoter in transgenic mice: an exquisitely sensitive on-off system for cell specific gene regulation.** *J Cell Sci* 1996, **109:**2619-2625
9. No D, Yao TP, Evans RM: **Ecdysone-inducible gene expression in mammalian cells and transgenic mice.** *Proc Natl Acad Sci U S A* 1996, **93:**3346-3351
10. Hu MC, Davidson N: **The inducible lac operator-repressor system is functional in mammalian cells.** *Cell* 1987, **48:**555-566
11. Labow MA, Baim SB, Shenk T, Levine AJ: **Conversion of the lac repressor into an allosterically regulated transcriptional activator for mammalian cells.** *Mol Cell Biol* 1990, **10:**3343-3356
12. Gossen M, Bujard H: **Tight control of gene expression in mammalian cells by tetracycline-responsive promoters.** *Proc Natl Acad Sci U S A* 1992, **89:**5547-5551
13. Bertrand KP, Postle K, Wray LV Jr, Reznikoff WS: **Overlapping divergent promoters control expression of Tn10 tetracycline resistance.** *Gene* 1983, **23:**149-156
14. Hinrichs W, Kisker C, Duvel M, Muller A, Tovar K, Hillen W, Saenger W: **Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance.** *Science* 1994, **264:**418-420
15. Gari E, Piedrafita L, Aldea M, Herrero E: **A set of vectors with a tetracycline-regulatable promoter system for modulated gene expression in Saccharomyces cerevisiae.** *Yeast* 1997, **13:**837-848
16. Weinmann P, Gossen M, Hillen W, Bujard H, Gatz C: **A chimeric transactivator allows tetracycline-responsive gene expression in whole plants.** *Plant J* 1994, **5:**559-569
17. Bello B, Resendez-Perez D, Gehring WJ: **Spatial and temporal targeting of gene expression in Drosophila by means of a tetra-cycline-dependent transactivator system.** *Development* 1998, **125:**2193-2202
18. Furth PA, St Onge L, Boger H, Gruss P, Gossen M, Kistner A, Bujard H, Hennighausen L: **Temporal control of gene expression in transgenic mice by a tetracycline-responsive promoter.** *Proc Natl Acad Sci U S A* 1994, **91:**9302-9306
19. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H: **Transcriptional activation by tetracyclines in mammalian cells.** *Science* 1995, **268:**1766-1769
20. Gallia GL, Khalili K: **Evaluation of an autoregulatory tetracycline regulated system.** *Oncogene* 1998, **16:**1879-1884
21. Gopalkrishnan RV, Christiansen KA, Goldstein NI, DePinho RA, Fisher PB: **Use of the human EF-1alpha promoter for expression can significantly increase success in establishing stable cell lines with consistent expression: a study using the tetracycline-inducible system in human cancer cells.** *Nucleic Acids Res* 1999, **27:**4775-4782
22. Colosimo A, Goncz KK, Holmes AR, Kunzelmann K, Novelli G, Malone RW, Bennett MJ, Gruenert DC: **Transfer and expression of foreign genes in mammalian cells.** *Biotechniques* 2000, **29:**314-324
23. Ball RK, Friis RR, Schoenenberger CA, Doppler W, Groner B: **Prolactin regulation of beta-casein gene expression and of a cytosolic 120-kd protein in a cloned mouse mammary epithelial cell line.** *EMBO J* 1988, **7:**2089-2095
24. Gill G, Ptashne M: **Negative effect of the transcriptional activator GAL4.** *Nature* 1988, **334:**721-724
25. Flint J, Shenk T: **Viral transactivating proteins.** *Annu Rev Genet* 1997, **31:**177-212
26. Baron U, Gossen M, Bujard H: **Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential.** *Nucleic Acids Res* 1997, **25:**2723-2729
27. Urlinger S, Helbl V, Guthmann J, Pook E, Grimm S, Hillen W: **The p65 domain from NF-kappaB is an efficient human activator in the tetracycline-regulatable gene expression system.** *Gene* 2000, **247:**103-110
28. Go WY, Ho SN: **Optimization and direct comparison of the dimerizer and reverse tet transcriptional control systems.** *J Gene Med* 2002, **4:**258-270
29. Akagi K, Kanai M, Saya H, Kozu T, Berns A: **A novel tetracycline-dependent transactivator with E2F4 transcriptional activation domain.** *Nucleic Acids Res* 2001, **29:**E23
30. Pear WS, Nolan GP, Scott ML, Baltimore D: **Production of high-titer helper-free retroviruses by transient transfection.** *Proc Natl Acad Sci U S A* 1993, **90:**8392-8396
31. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970, **227:**680-685