



# Efficient transgene regulation from a single tetracycline-controlled positive feedback regulatory system

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Control of gene expression in eukaryotic cells is clearly important in many applications including modifications of the level of a therapeutic gene product. For effective gene delivery and regulation, the regulatory system must be contained on a single vector and it must exhibit high transgene expression on induction and low basal expression on repression. Here, we have investigated several self-contained vectors carrying both the tetracycline-controlled transactivator (tTA) and a potentially therapeutic gene in transient studies. An enhancerless positive feedback regulatory vector (pSialV) transcribing both tTA and mGM-CSF from a modified tTA-responsive bidirectional promoter

demonstrated over 200-fold gene regulation in HeLa cells. This was comparable to the degree of regulation obtained on cotransfection of vectors expressing tTA and tTA-responsive mGM-CSF. The maximal transcriptional activity of pSialV was comparable to that of CMV IE promoter and its basal activity as low as the leakiness of the tetracycline-responsive promoter (tRP) in several commonly used cell lines, resulting in 47- to 328-fold regulation. Furthermore, pSialV also showed efficient regulation in stable cells. Overall, the positive feedback regulatory system (PFRS) offers efficient gene regulation which is suitable for most applications, especially gene therapy.

**Keywords:** bidirectional promoter; positive feedback regulatory system; gene therapy; mGM-CSF; tetracycline-controlled transactivator; tTA-responsive promoter

## Introduction

The basic principle of gene-based therapies is efficient delivery of therapeutic genes to mammalian cells and their adequate expression. Advances in the field of gene therapy have mainly focused on the development and employment of viral and nonviral systems for delivery of foreign genes into target cells. However, recent work has shown that a specific dose of the therapeutic gene product is required for successful treatment of certain diseases<sup>1,2</sup> and excessive production of the gene product may be toxic.<sup>3,4</sup> Thus, a regulatable system that can offer tight control of gene expression in response to inducible agents that can be safely administered is of value for modifying specific therapies. Although a variety of regulatable promoters have been described, most suffer from high promoter leakiness, poor regulation of gene expression and employment of inducible agents that are associated with adverse effects to mammalian cells.<sup>5–10</sup> The tetracycline-regulatable system (TRS)<sup>11</sup> avoids the problems related to many of the other systems by offering substantial regulation of transgene expression in response to concentrations of tetracycline (Tc) that can be repetitively administered. Indeed, the low cytotoxicity of Tc and its high affinity for the tetR,  $10^9 \text{ M}^{-1}$ ,<sup>12</sup> has enabled

the use of this antibiotic at concentrations that cause no appreciable adverse effects in transgenic animals expressing the TRS.<sup>13–16</sup> The TRS is founded on the efficiency of Tc resistance operon of *E. coli*<sup>17</sup> and the general functionality of the herpes simplex virus transactivator (VP16)<sup>18</sup> in eukaryotic cells.

The TRS has obvious applications in gene therapy where the level and timing of gene expression can be modified to meet specific therapeutic regimens. However, for this purpose, the original system is not ideal as it requires the co-delivery of a plasmid containing the tTA expression cassette and another containing a foreign gene under the control of tRP to the same cells. Nevertheless, this approach has demonstrated two orders of magnitude of gene regulation on co-injection of plasmids into rat heart<sup>19</sup> and into mouse muscle,<sup>20</sup> and on co-infection of cells with two recombinant adenoviruses.<sup>21</sup> Although, for efficient *in vivo* gene delivery a single construct is clearly preferable.

Our initial studies of a single plasmid, containing the two essential elements of the TRS (pSialII), showed significant expression of mGM-CSF in the absence of Tc and a time-dependent reduction of mGM-CSF level in the presence of Tc. The maximal degree of repression of mGM-CSF was about 15-fold when the Tc-repressed tRP expression from pSialII was about five-fold higher than the basal expression from the minimal promoter. The lack of total repression from pSialII is presumably due to the presence of enhancer elements on this plasmid (unpublished data). Similarly, others have described sin-

gle Tc-controlled constructs<sup>22-27</sup> that show modest regulation in transient studies, a maximum of 40-fold,<sup>22,23</sup> despite significant control in some stable cell lines.<sup>22-27</sup> With the increasing interest in the use of episomal plasmids, herpes viruses, autonomous parvoviruses and adenoviruses, the benefits of a single regulatable unit with high induction and low basal expression in a nonintegrated form are clear.

Here, we investigated control of gene expression using single Tc-regulatable vectors in transient transfection assays. We describe a positive feedback regulatory vector with high maximal expression and low Tc-repressed basal expression. This vector was characterised in several different cell lines to establish its general application. The superior efficiency of the system in regulating gene expression in a nonintegrated form was shown to apply to the integrated form of the vector. Thus, we demonstrate a novel PFRS that displays substantial expression and tight regulation of a transgene.

## Results

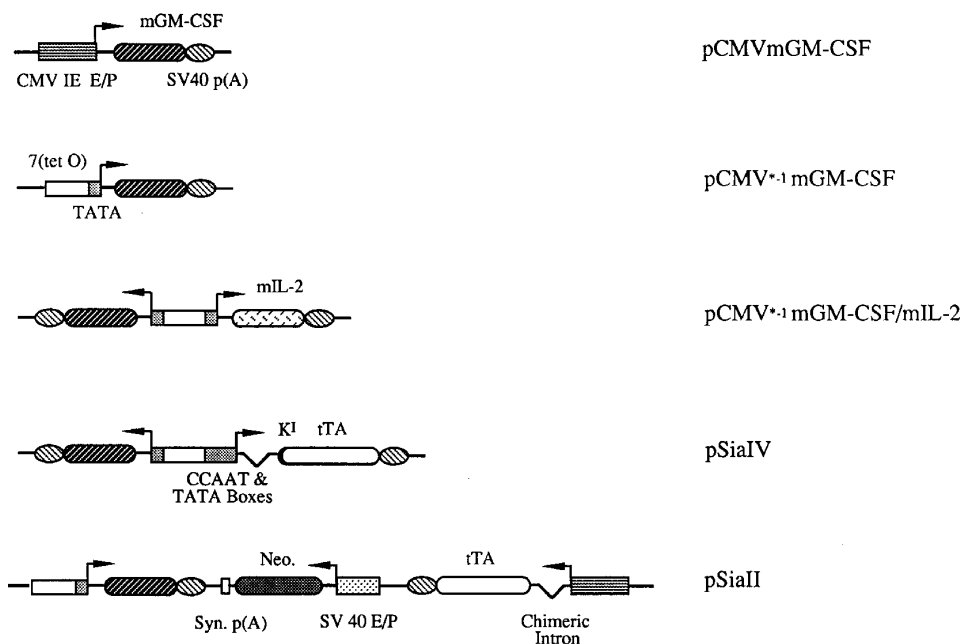
### Characterisation of a bidirectional promoter based on the tTA-responsive element

In order to improve the degree of regulation from a self-contained plasmid, we aimed at reducing the basal expression of the system without compromising its high level of expression by employing an enhancerless bidirectional promoter.<sup>28</sup> The degrees of expression and regulation of mIL-2 and mGM-CSF from the bidirectional promoter of pCMV<sup>\*-1</sup>mGM-CSF/mIL-2 (Figure 1) were quantitatively compared with their parental controls in a tTA-expressing 293 cell line (293-pCEP.tTA). The detected level of mIL-2 (Figure 2) is approximately twice that of mGM-CSF. This is not only seen with pCMV<sup>\*-1</sup>mGM-CSF/mIL-2 but also with the parental pCMV<sup>\*-1</sup>

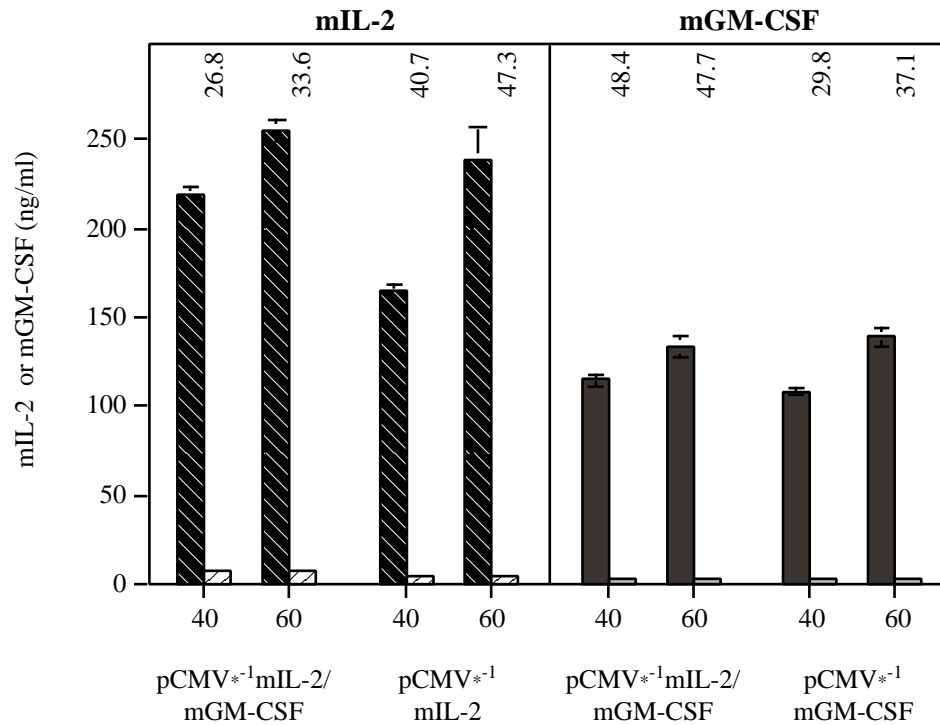
mGM-CSF and pCMV<sup>\*-1</sup>mIL-2 vectors and may reflect more efficient detection, secretion or longer half-life of mIL-2 in these cells. At 60 h, the degree of repression of mIL-2 was about 47.3-fold for pCMV<sup>\*-1</sup>mIL-2 and about 33.6-fold for pCMV<sup>\*-1</sup>mGM-CSF/mIL-2, and that of mGM-CSF was about 37.8-fold for pCMV<sup>\*-1</sup>mGM-CSF and about 47.7-fold for pCMV<sup>\*-1</sup>mGM-CSF/mIL-2. The degrees of induction and repression of mIL-2 and mGM-CSF for pCMV<sup>\*-1</sup>mGM-CSF/mIL-2 were comparable with those of the corresponding parental controls and therefore with one another. This indicates that the bidirectional promoter is quantitatively regulated by tTA in a direction-independent manner. We also obtained comparable repression for luciferase indicating that our studies with mIL-2 and mGM-CSF can be compared with those of other investigators using luciferase as a reporter gene (data not shown).

### Construction of a tetracycline-controlled, positive feedback regulatory system

Having established that the constructed promoter is bidirectionally regulated by tTA, the capability of the promoter to positively up-regulate itself was tested using pCMV<sup>\*-1</sup>tTA/mGM-CSF. This plasmid is identical to pCMV<sup>\*-1</sup>mGM-CSF/mIL-2 apart from having the tTA sequence replacing mIL-2 cDNA. In theory, both tTA and mGM-CSF will be produced at basal levels on transfection of eukaryotic cells. The tTA will interact with its promoter, increasing the expression of its own gene and mGM-CSF. This will result in increased concentration of tTA which will perpetually continue to up-regulate its promoter bidirectionally. The system should therefore behave as a positive feedback mechanism and should also offer increased sensitivity to Tc repression (discussed below). The positive feedback regulatory vectors function



**Figure 1** Schematic representation of the major plasmids used in the study. CMV IE E/P, CVM immediate-early enhancer/promoter; mGM-CSF, mouse granulocyte-macrophage colony-stimulating factor; Neo, neomycin phosphotransferase; SV40 E/P, SV40 enhancer/promoter; SV40 p(A), SV40 poly(A); Syn p(A), synthetic poly(A); tetO, 7, tet operator; tTA, Tc-controlled transactivator, K<sup>I</sup>, modified translational initiation sequence.



**Figure 2** Characterisation of the bidirectional promoter. 293-pCEP.tTA cells were transfected with 0.75 pmoles of pCMV<sup>\*-1</sup>mGM-CSF/mIL-2, pCMV<sup>\*-1</sup> mIL-2, or pCMV<sup>\*-1</sup>mGM-CSF per well in six-well plates. In addition, 0.2 pmoles of pCH110 per well was included in the transfection mixtures. Cells were either maintained in culture medium or in medium containing 1 µg/ml of Tc at the time of transfection. Samples of supernatant were taken at 20, 40 and 60 h after transfection for mIL-2 and mGM-CSF ELISA analysis when the medium was replaced with fresh culture medium with or without 1 µg/ml of Tc. Each experiment was repeated more than once. The results of a typical experiment are shown above. The value represents the mean of six samples in duplicate at 40 and 60 h and the bar indicates the standard error of the mean. The degree of repression relative to that of control with no Tc, is shown at the top of each bar. β-Galactosidase expression was used as an internal control to monitor the efficiencies of the transfections. Numbers of cells expressing β-galactosidase were within 1.0- to 1.12-fold of each other between the wells. For mIL-2 assay, pCMV<sup>\*-1</sup>mGM-CSF/mIL-2 and pCMV<sup>\*-1</sup>mIL-2 - Tc (▨) and + Tc (▩). For mGM-CSF assay, pCMV<sup>\*-1</sup>mGM-CSF/mIL-2 and pCMV<sup>\*-1</sup>mGM-CSF - Tc (▧) and + Tc (▫).

on a similar basis to the autoregulatory inducible vectors.<sup>29,30</sup>

To characterise pCMV<sup>\*-1</sup>tTA/mGM-CSF, HeLa cells were transfected with pSiaII, pCMV<sup>\*-1</sup>tTA/mGM-CSF, or pCMV<sup>\*-1</sup>mGM-CSF. As can be seen from Figure 3, at 60 h, the mGM-CSF expression from pCMV<sup>\*-1</sup>tTA/mGM-CSF was about three to four times lower than that of pSiaII and about eight to nine times higher than the basal expression of pCMV<sup>\*-1</sup>mGM-CSF. In the presence of Tc, pCMV<sup>\*-1</sup>tTA/mGM-CSF was repressed to the basal expression of pCMV<sup>\*-1</sup>mGM-CSF. The degree of regulation from pCMV<sup>\*-1</sup>tTA/mGM-CSF was about 35-fold, relative to 15-fold of pSiaII. In repeat experiments, generally, maximal expression from pCMV<sup>\*-1</sup>tTA/mGM-CSF was lower than seen here. With higher intracellular concentration of tTA, expression and therefore regulation of the system should be enhanced. This can be achieved either by increasing tTA expression selectively or lengthening the duration of its expression.

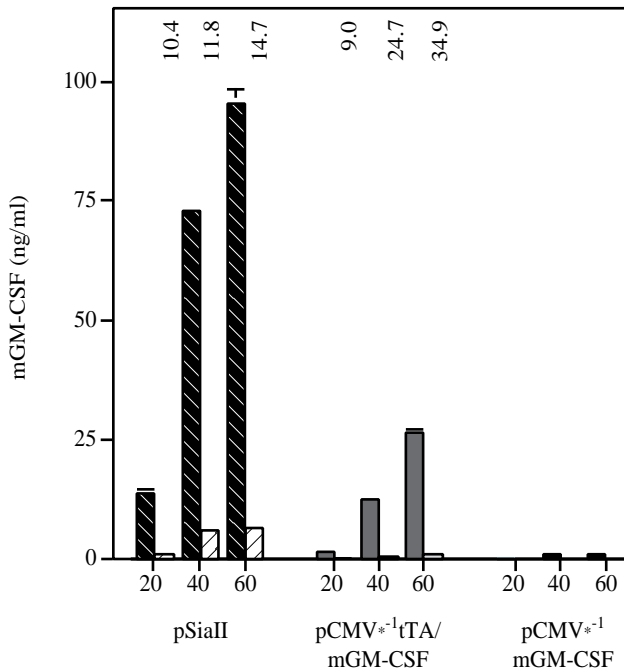
#### Selective modifications of the tTA-half of the bidirectional promoter

To increase the expression of mGM-CSF from the bidirectional promoter and therefore to enhance the degree of regulation, we attempted modifications aimed at increasing tTA expression without compromising the basal expression of mGM-CSF expression. To that end, the

pCMV<sup>\*-1</sup>tTA/mGM-CSF was reconstructed in stages to make pSiaIV (Figure 1). This vector contains the upstream region of CMV IE promoter including the CCAAT and TATA boxes. It also contains the chimeric intron of pCI.neo and a modified sequence for translational initiation of tTA. The vectors were studied in conjunction with pSiaII, pCMV<sup>\*-1</sup>mGM-CSF and cotransfected pUHD 15-1/pCMV<sup>\*-1</sup>mGM-CSF. The results for pSiaIV are shown in Figure 4. As seen from the results, the maximal expression from pSiaIV was comparable with that of pSiaII. However, the degree of mGM-CSF regulation from pSiaIV, about 186-fold, was far greater than that of pSiaII, about 19-fold. pSiaIV displayed a degree of regulation comparable with cotransfected pUHD 15-1 and pCMV<sup>\*-1</sup>mGM-CSF, about 201-fold. Thus, modifications of the tTA-half of the bidirectional promoter designed to increase expression of tTA led to enhanced gene regulation, without compromising the basal expression of mGM-CSF.

#### Applicability of the positive feedback regulatory system in other cell lines

The plasmid pSiaIV was tested and compared with pSiaII, pCMV<sup>\*-1</sup>mGM-CSF and pCMVmGM-CSF in several commonly used cell lines, including HeLa, A431, 293 and NIH3T3. The results at the time of maximal expression of pCMVmGM-CSF, pSiaII and pSiaIV are



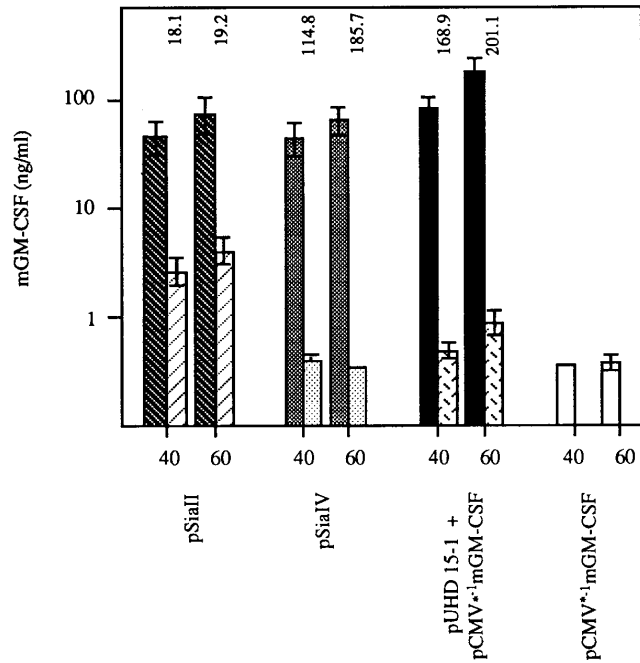
**Figure 3** Characterisation of the Tc-controlled PFRS. HeLa cells were transfected with 0.75 pmoles of pSiaII, pCMV\*1tTA/mGM-CSF, or pCMV\*1mGM-CSF per well in six-well plates. In addition, 0.2 pmoles of pCH110 per well was included in the transfection mixtures. Cells were treated with medium containing 1 µg/ml of Tc or no Tc at the time of transfection. Samples of supernatant were taken at 20, 40, 60 and 80 h after transfection for mGM-CSF ELISA analysis when the medium was replaced for fresh culture medium with or without 1 µg/ml of Tc. Each experiment was repeated more than once. The results at the time of maximal expression of all vectors are shown above. Each value represents the mean of five samples in duplicate at 40 and 60 h and the bar indicates the standard error of the mean. The degree of repression relative to that of control with no Tc, is shown at the top of each bar. β-Galactosidase expression was used as an internal control to monitor the efficiencies of the transfections. Numbers of cells expressing β-galactosidase were within 1.0- to 1.32-fold of each other between the wells. pSiaII - Tc (▨) and + Tc (▩), pCMV\*1tTA/mGM-CSF - Tc (▧) and + Tc (▦), pCMV\*1mGM-CSF (■).

shown in Figure 5. In most of the lines tested, mGM-CSF expression from pSiaII, pSiaIV and pCMVmGM-CSF were relatively comparable and within a two-fold difference. At 60 h, depending on the cell lines used, the degree of regulation achieved with pSiaIV was from six- to 40-fold better than that seen with pSiaII. The difference in the levels of regulation of the two plasmids is dictated by the difference in the basal activity of the Tc-repressed tRPs. Whereas activity of the Tc-repressed tRP of pSiaIV reaches that of the leakiness of pCMV\*1mGM-CSF in all the cells tested, that of pSiaII remains at a significantly higher level.

Finally, consistent with the observations of Ackland-Berglund and Leib,<sup>31</sup> we confirm that the degree of leakiness varies in different cell lines and is dependent on other cellular factors characteristic of a particular cell type.

#### Comparison of pSiaII and pSiaIV in stable forms

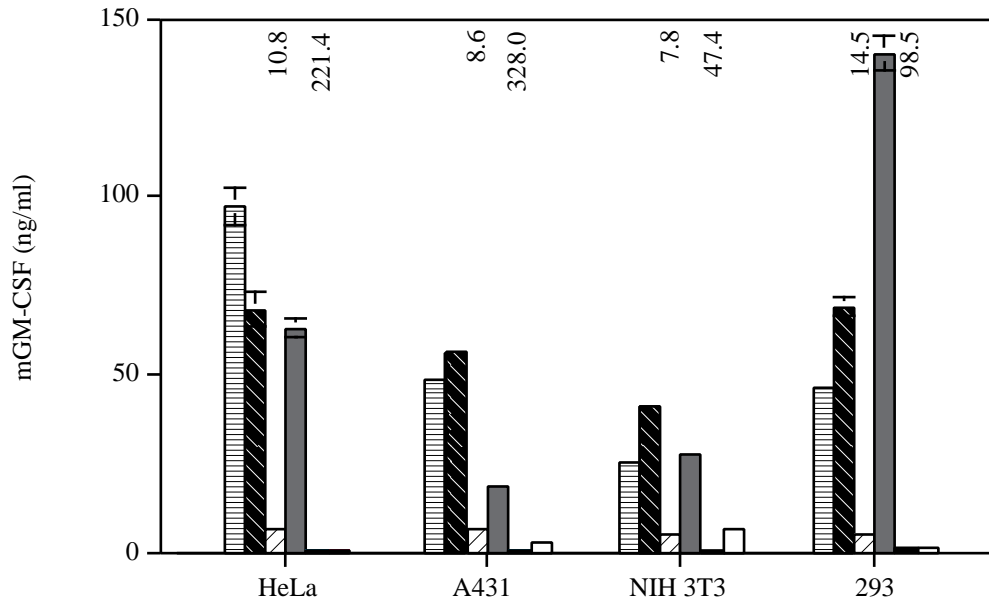
To explore the potential use of the PFRS in stably transfected cells, the efficiency of pSiaIV in regulating transgene expression was compared with that of pSiaII in inte-



**Figure 4** Characterisation of pSiaIV. HeLa cells were transfected with 0.75 pmoles of pSiaII, pSiaIV, pCMV\*1mGM-CSF, or cotransfected with 0.75 pmoles of pUHD 15-1 and 0.75 pmoles of pCMV\*1mGM-CSF per well. To achieve equal molar concentrations in each transfection mixture, 0.75 pmoles of pC.neo was included with pSiaIV, pSiaII, and pCMV\*1mGM-CSF, per well. Also, 0.2 pmoles of pCH110 per well was included in all the transfection mixtures. Cells were treated as described in Figure 3. Numbers of cells expressing β-galactosidase were within 1.0- to 1.27-fold of each other between the wells. pSiaII-Tc (▨) and + Tc (▩), pSiaIV-Tc (▧) and + Tc (▦), pUHD 15-1/pCMV\*1mGM-CSF - Tc (■) and + Tc (▦), pCMV\*1mGM-CSF (□).

grated forms in 293, A431, AM12 and NIH3T3 cells. HeLa cells were not included, since we have never been able to establish stable mGM-CSF expressing HeLa clones, presumably due to the sensitivity of this cell line to high concentrations of mGM-CSF. Stable cells expressing pSiaII and pSiaIV were established and grown in the presence of 1 µg/ml of doxycycline (Dox) for several weeks. Dox was used instead of Tc since this analogue is more effective in inactivating tTA and maintains repression of tRP for a longer period following initial treatment.<sup>32</sup> Despite several attempts, we failed to establish stable 293 cells with pSiaIV. With this cell line, growing clones died following Dox removal from the culture medium. This may be a result of tTA cytotoxicity,<sup>33,34</sup> since the activity of the positive feedback regulator vectors are remarkably high in this cell type. Shockett *et al*<sup>30</sup> have also observed greater than 50% cell death, upon the removal of Tc from the culture medium, in NIH3T3 clones transfected stably with a plasmid transcribing tTA under the control of tRP.

As seen in transient studies, the degree of transgene regulation with pSiaIV was again superior to that of pSiaII (Table 1). Thus pSiaIV offers a convenient approach for generating stable cells with a significant degree of transgene regulation. The results reported above show the degree of regulation in a pool of cells. In selected clones, a higher degree of control may be expected. pSiaII showed a low degree of gene regulation



**Figure 5** Applicability of the PFRS in HeLa, A431, 293 and NIH3T3 cells. These cells in five-well plates were transfected with 0.75 pmoles of pSiaII, pSiaIV, pCMV\*1 mGM-CSF or pCMVmGM-CSF per well. Also, 0.2 pmoles of pCH110 per well was included in all the transfection mixtures. Cells were treated as described in Figure 3. Each value represents the mean of five samples in duplicate at 60 h and the bar indicates the standard error of the mean. Numbers of cells expressing  $\beta$ -galactosidase were within 1.0- to 1.34-fold of each other between the wells. pCMVmGM-CSF (□), pSiaII - Tc (▨) and + Tc (▧), pSiaIV - Tc (▩) and + Tc (■), pCMV\*1 mGM-CSF (□).

**Table 1** Comparison of pSiaII and pSiaIV in stable forms in 293, A431, AM12 and NIH3T3 cells

Cell line	Tetracycline	Mean	s.e.	Degree of regulation
pSiaII A431	-	52.50	3.30	13.40
	+	3.90	0.37	
NIH3T3	-	17.90	0.48	21.20
	+	0.85	0.03	
AM12	-	115.3	5.06	10.34
	+	11.5	0.85	
293	-	147.60	3.38	185.43
	+	0.79	0.03	
pSiaIV A431	-	53.40	2.02	161.80
	+	0.33	0.01	
NIH3T3	-	281.90	12.32	320.30
	+	0.88	0.01	
AM12	-	213.60	3.82	187.10
	+	1.14	0.02	
293	-	-	-	-

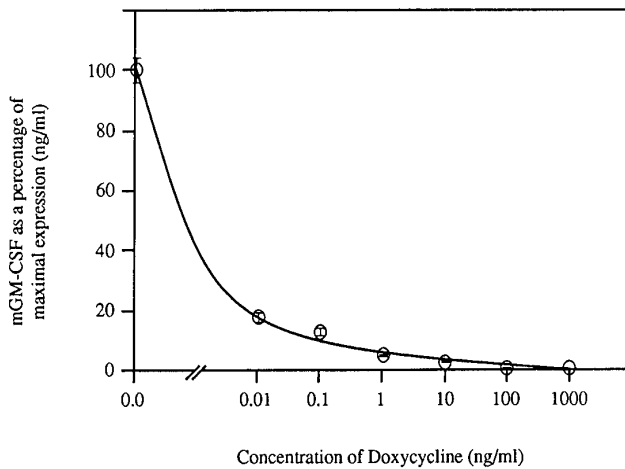
293 Cells transfected with pSiaIV died upon the removal of Dox from the medium. Cells were grown in the absence of Tcs for 15 days and then seeded as a pool of  $2 \times 10^5$  cells per well of six-well plates in culture medium with or without  $1 \mu\text{g/ml}$  of Tc. The medium was replaced with fresh culture medium with or without  $1 \mu\text{g/ml}$  of Tc at 80 h. Samples of the supernatants were taken at 100 h following seeding. Mean shows the average of mGM-CSF level for five wells in duplicate and s.e. indicates the standard error of the mean.

in most cells apart from 293 where it produced over 150-fold of control.

*Characterisation of the positive feedback regulatory system in response to different concentrations of doxycycline*

To establish the sensitivity of the PFRS, the effect of different Dox doses was studied on AM12-pSiaIV. Dox has

been shown to be more efficient than Tc,<sup>26,32</sup> anhydrotetracycline, minocycline and oxytetracycline in inactivating tTA.<sup>32</sup> At 60 h, the PFRS was completely repressed at Dox concentration range of 1000 ng/ml to 10 ng/ml, with minimal expression at 1 ng/ml. Partial expression from the system occurred in the range of 1 ng/ml to 0.0 ng/ml of Dox (Figure 6).



**Figure 6** Dose-response curve of the PFRS in response to Dox. AM12-pSiaIV cells were seeded as a pool of  $2 \times 10^5$  cells per well of five-well plates in culture medium containing no Dox or this antibiotic at final concentrations of 0.01, 1, 10, 100 and 1000 ng/ml. Samples of supernatant were taken at 20, 40 and 60 h for mGM-CSF ELISA analysis when the medium was replaced with culture medium with or without Dox as above. Each value shows the mean of five samples in duplicate at 60 h and the bar indicates the standard error of the mean. Both values are represented as a percentage of the mean of maximal expression in the absence of Tc.

## Discussion

The general use of the Tc-regulatable system for gene therapy depends on the delivery of both the tTA and the tRP on a single vector. Here, we describe the construction and analysis of several suitable self-contained vectors. The constructs were tested in transient transfections as a model for nonintegrating gene delivery systems and their functionality was further confirmed in stable transfectants. As a reporter gene, mGM-CSF, a cytotoxic protein in excess<sup>3,4</sup> with potential application for gene therapy<sup>1,2,4,35</sup> was used. The lack of regulation of pSiaII in our initial studies is mainly due to the high basal activity of its Tc-repressed tRP, presumably due to the presence of enhancer elements on the plasmid. Wasylyk *et al*<sup>36</sup> have in fact shown that for the SV40 promoter, the transcriptional activity of the promoter is 10-fold lower in enhancerless constructs. To address this issue, we characterised a bidirectional promoter totally lacking enhancer elements and employed it in the construction of a PFRS. The bidirectional promoter expresses mIL-2 and mGM-CSF, with the same efficiency as the corresponding parental vectors. Also, the repression of the bidirectional promoter in response to Tc treatment is comparable for both genes and with those of the parental vectors. This indicates that quantitative regulation from the bidirectional promoter is comparable for the two genes. Obviously, this control could either be down-regulation or up-regulation in the presence of Tc depending on the use of tTA or rtTA,<sup>37</sup> respectively.

Having demonstrated that tRP of the bidirectional system can function efficiently and comparably in either direction, a regulatable, positive feedback system on the basis of the bidirectional promoter was constructed. Despite a reasonable level of regulation, the plasmid exhibited a low level of reporter gene expression. To improve both the expression of the gene and its regulation, the bidirectional promoter was redesigned to

increase the expression of tTA without compromising the basal activity of the promoter. We show that a modified bidirectional vector, pSiaIV, containing the CCAAT and TATA boxes, a chimeric intron and a modified translational initiation sequence exhibited over 200-fold regulation in HeLa cells after 60 h of treatment with 1  $\mu$ g/ml of Tc. This plasmid also demonstrated superior regulation relative to pSiaII in Vero, NIH3T3, 293 and A431 cells. Moreover, the expression level of pSiaIV is comparable within two-fold to that of the intact CMV IE promoter and pSiaII in most of the cells studied. In all the cell lines tested, the activity of the Tc-repressed pSiaIV is comparable to the basal expression of pCMV<sup>\*</sup>-1mGM-CSF. Since the expression from pSiaIV is comparable to that from CMV IE promoter, the degree of regulation from pSiaIV is predominantly dependent on the leakiness of tRP in a given cell line. The maximal efficiency of pSiaIV may not have been demonstrated in these experiments. A higher degree of regulation may be seen if longer duration of pSiaIV expression could be followed.

The positive feedback bidirectional promoter system offers several advantages compared with other existing approaches. (1) The system offers enhanced gene expression and Tc sensitivity by offering two levels of regulation, namely tTA inactivation and termination of tTA transcription. With the conventional system, Tc abolishes the activity of the tTA, but its production continues driven from the CMV IE promoter. At steady level, an equilibrium may be reached between the production of tTA and its inactivation by Tc, whereby some active tTA may remain in the cell. (2) Attempts to obtain stable tTA-expressing clones have been unsuccessful in certain cell types,<sup>38</sup> suggesting possible cytotoxicity of tTA in high concentrations. It has been suggested that high-level expression of VP16 in animal cells inhibits normal transcription by RNA polymerase II and thus results in arrest of cell growth<sup>34</sup> by sequestering transcription factors in a nonproductive initiation complex.<sup>33</sup> Having the production of tTA controlled effectively may thus be advantageous in those cells sensitive to high concentrations of tTA. Moreover, using this system, establishment of cell lines expressing tTA and a toxic gene should become more feasible due to the inactivation of tTA and increased repression of both tTA and the toxic gene expression in response to Tc. (3) As with other single plasmids, the approach prevents segregation of the expression and regulation units, ensuring equal copy number of these units at the same chromosomal locus. This avoids unbalanced copy numbers, which may result in poor regulation due to excess background reporter gene or limited tTA supply. (4) The PFRS shows enhanced sensitivity to Dox in comparison to the dose-response studies of Dox in 293-pSiaII.<sup>32</sup> The enhanced sensitivity of the PFRS to Dox is not only exhibited by its repressibility at lower Dox concentrations but also by a more prolonged delay in resumption of tRP activity. Following the removal of 1000 ng/ml of Dox from AM12-pSiaIV cells, the system takes over 9 days to reach about 30% of its maximal expression (data not shown). This is in contrast to our observation on integrated pSiaII in 293 cells (293-pSiaII) which reaches its maximal expression within 9 days.<sup>32</sup>

Recently, several single plasmids have been constructed and delivered to cells directly or via recombinant viruses. In contrast to our results, similar constructs to

pSiaII have been reported to attain higher levels of regulation.<sup>23–25,27,39</sup> Others have studied single autoregulatory vectors which function similarly to pSiaIV. Recently, Liang *et al*<sup>22</sup> investigated the potential use of a bicistronic vector, expressing a reporter gene from a tRP followed by tTA expression from a CAP-independent translational enhancer sequence distal to the reporter gene. They reported a 40-fold regulation of gene expression in mice 7 days after intramuscular injection of a bicistronic vector. A similar bicistronic construct on a retrovirus demonstrated one or two orders of magnitude gene regulation after two rounds of enrichment by FACS sorting for cells expressing high reporter gene activity.<sup>26</sup> Compared to the bicistronic vectors, the level of regulation from pSiaIV is higher in both transient and stable forms. In fact, the level of control achieved with pSiaIV is the highest regulation that has been reported from a single construct in transient studies. Furthermore, pSiaIV in the integrated form displays a similar or superior degree of gene regulation to most of the approaches mentioned above despite using pooled stable cells rather than selected clones.

Unlike bicistronic vectors, it is possible to selectively enhance the expression of tTA in positive feedback regulatory vectors without compromising the expression of the reporter gene. Our data suggest that, by modifying the intrinsic properties of a bidirectional promoter, it is feasible to obtain different levels of expression from each half of the promoter. Excising the sequence between the tet operators and the TATA box of the mGM-CSF-half of the bidirectional promoter decreases the level of basal and maximal expression of mGM-CSF by five-fold relative to the unmodified bidirectional promoter (data not shown). Also as our data indicate, inclusion of a CCAAT box upstream of tTA had little or no effect on the basal expression of mGM-CSF from the other half of the bidirectional promoter. Expression and regulation from pSiaIV could potentially be further enhanced by inclusion of a suitable transcription factor binding site<sup>40</sup> or a terminal deoxynucleotidyl transferase initiator element<sup>41</sup> within the tTA-half of the promoter, by including a nuclear localisation signal within tTA, and/or by using an alternative poly (A) sequence.

The PFRS could prove to be a powerful approach for targeting specific tissues or cells. A number of *cis*-acting control sequences conferring tissue specificity have been identified.<sup>42–46</sup> By inclusion of a *cis*-acting control sequence on the tTA-half of the promoter, it is possible to regulate expression of tTA in a tissue-specific manner. Tissue-specific expression can be further restricted by inclusion of a second *cis*-acting control sequence to the other half of the bidirectional promoter.

In conclusion, we show that it is possible to modify the intrinsic properties of a bidirectional promoter so as to construct a novel single Tc-controlled positive feedback regulatory vector with a high expression upon induction and low basal expression upon repression. This vector transcribes a reporter gene as efficiently as the intact CMV IE promoter and is repressed to the leakiness of the tRP in response to Tc. The system functions well in an intra- and extra-chromosomal form and could be applied in many gene therapy applications.

## Materials and methods

### Plasmids and DNA manipulation

All nucleic acid manipulations were carried out according to established protocols.<sup>47</sup> Large-scale plasmid preparations were carried out by anion exchange chromatography (Qiagen, Surrey, UK), according to the manufacturer's protocol. All the restriction endonucleases were purchased from New England Biolabs (Hertfordshire, UK).

Complementary DNA was prepared from concanavalin A-stimulated<sup>48</sup> spleen cells of a Balb/c mouse using a standard method.<sup>49</sup> The mouse GM-CSF and mouse interleukin-2 (mIL-2) cDNAs were generated by the polymerase chain reaction and were subsequently verified by sequencing (USB, Ohio, USA).

The plasmid pUHD 15-1 contains the tTA transactivator gene and pUHD 10-3 carries a tRP followed by a polylinker.<sup>11</sup> The 530 bp *Xba*I/*Bam*HI mIL-2 cDNA and the 462 bp *Xba*I/*Bam*HI mGM-CSF cDNA were inserted into the *Xba*I site of pUHD 10-3 to construct pCMV\*<sup>-1</sup> mIL-2 and pCMV\*<sup>-1</sup> mGM-CSF, respectively. pCMV mGM-CSF was constructed by cloning the *Nhe*I-*Bam*HI fragment of pCMV\*<sup>-1</sup> mGM-CSF, containing mGM-CSF, into the *Eco*RI-*Bam*HI site of pUHD 15-1. To construct pCEP4.tTA, the hygromycin expression cassette together with the chloramphenicol resistance gene, *OriP* and the *EBNA1* gene of pCEP4.Cm<sup>R</sup> were digested by *Clal*-*Nru*I and inserted into the *Xho*I site of pUHD 15-1. pCEP4.Cm<sup>R</sup> is an EBV-based vector whereby the CMV promoter and SV40 poly(A) sequences of parental pCEP4 are replaced with the chloramphenicol resistance gene from pACYC184 (New England Biolabs). For construction of pSiaII, the 1 kb *Eco*RI-*Bam*HI tTA coding sequence of pUHD 15-1 was subcloned into pCI.neo (plasmid containing chimeric intron; Promega, Southampton, UK) at *Not*I-*Eco*RI site to construct pCI.tTA.neo. The 1.35 kb *Pvu*II-*Ssp*I mGM-CSF expression cassette of pCMV\*<sup>-1</sup> mGM-CSF was then cloned at the *Bam*HI site of pCI.tTA.neo in the opposite direction to the tTA and neomycin phosphotransferase expression cassettes (being in sense). To construct, pCMV\*<sup>-1</sup> mGM-CSF/mIL-2, the *Pvu*II-*Sma*I mIL-2 expression cassette of pCMV\*<sup>-1</sup> mIL-2 was inserted at the *Xho*I site of pCMV\*<sup>-1</sup> mGM-CSF in the opposite direction to the mGM-CSF expression. The upstream regions of the mIL-2 and the mGM-CSF genes, up to -33 bp from the TATA box, are therefore separated by seven copies of the tet operator. For construction of pCMV\*<sup>-1</sup> tTA/mGM-CSF, the *Eco*RI-*Bam*HI tTA sequence of pUHD 15-1 was subcloned into pUHD 10-3 at *Eco*RI-*Bam*HI site to construct pCMV\*<sup>-1</sup> tTA. The 1.8 kb *Pvu*II-*Sma*I fragment of pCMV\*<sup>-1</sup> tTA, containing the tTA expression cassette, was cloned at the *Xho*I site of pCMV\*<sup>-1</sup> mGM-CSF in the opposite direction to the mGM-CSF expression. The plasmid pCI\*<sup>-1</sup> tTA/mGM-CSF was constructed by removing the tTA expression cassette from pCI.tTA.neo with *Sgf*I-*Dra*III. This fragment includes the CCAAT and TATA boxes of the CMV IE promoter, the chimeric intron, the tTA sequence and the SV40 poly (A). It was cloned into the *Xho*I site of pCMV\*<sup>-1</sup> mGM-CSF in the opposite direction to the mGM-CSF expression. To construct pSiaIV, the oligonucleotides 5' TCGAGGCGGCCGACCATGT 3' and 5' CTAGAC-ATGGTGC GGCCGCC 3' (translational initiation codon underlined)<sup>50</sup> were annealed and ligated at the *Xho*I-*Xba*I

site to replace the translational initiation sequence of tTA. This will replace the sequence immediately upstream to the translational initiation codon by CGCACC.

### Transfections

Transfection of purified plasmid DNA into the cell lines was carried out using calcium phosphate precipitation. The molecular weight of each plasmid was taken into consideration so approximately equal molar concentration of each vector was used in transfections ( $2 \mu\text{g} = 0.375 \text{ pmol}$  for pSiaII, 8200 bp). To ensure similar transfection efficiencies among wells transfected with the same plasmid, a single precipitate was prepared and divided among the wells. Plasmid pCH110 (Pharmacia) encoding for  $\beta$ -galactosidase was included in the transfections as an internal control for comparing the efficiencies of transfections.  $\beta$ -Galactosidase expression was detected by standard methods.<sup>47</sup>

For establishment of stable cell lines with pCEP4.tTA, 293 cells were transfected with  $10 \mu\text{g}$  of pCEP4.tTA using calcium phosphate precipitation. This plasmid carries the *EBNA1* gene and the *OriP* of the EBV virus and can replicate in eukaryotic cells. Clones were selected in the presence of  $0.2 \text{ mg/ml}$  of hygromycin (Calbiochem, La Jolla, CA, USA) 36 h after transfection. At 1 week after transfection, growing clones of 293 cells were noted and a stable cell line readily established. For establishment of stable cell lines with pSiaII or pSiaIV, cells were transfected with either  $10 \mu\text{g}$  of pSiaII, or  $9.5 \mu\text{g}$  of pSiaIV plus  $0.5 \mu\text{g}$  of pCI.neo and treated with  $1 \mu\text{g/ml}$  of Dox (Sigma, Poole, UK) and G418 (Gibco, Dorset, UK) 36 h later. G418 was used at concentrations of  $300 \mu\text{g/ml}$  for the A431 cell line,  $750 \mu\text{g/ml}$  for Vero, HeLa, 293 and AM12 cell lines, and  $1000 \mu\text{g/ml}$  for the NIH3T3 cell line.

### Assays for reporter genes

Mouse IL-2 and mGM-CSF assays were done using mIL-2 and mGM-CSF ELISA kits (Endogen, Leicestershire, UK) according to the manufacturer's protocol.

### Cell culturing

HeLa (ECACC, 85060701), 293 (ECACC, 85120602), AM12,<sup>51</sup> A431 (ECACC, 85090402), Vero (84113001) and NIH3T3 cell lines were maintained in DMEM supplemented with 10% FBS, 100 units/ml of penicillin and 100 mg/ml of streptomycin. For transfections of monolayer cultures, cells were subcultured into six-well plates (Corning, New York, USA) a day before transfection at densities of about  $5 \times 10^5$  cells for 293 and  $5 \times 10^4$  cells for all the other cell lines. Tetracycline and doxycycline were prepared as described previously.<sup>32</sup>

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