

# Homogeneous Tetracycline-Regulatable Gene Expression in Mammalian Fibroblasts

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**Abstract** The expression of transfected genes in mammalian cells is rapidly repressed by epigenetic mechanisms such that, within a matter of weeks, only a fraction of the cells in most clonal populations still exhibit detectable expression. This problem can become prohibitive when one wants to express two ectopically introduced genes, as is necessary to establish cell lines that harbor genes regulated by the tetracycline-controlled transactivators. We describe an approach to establish Chinese hamster ovary (CHO) cell lines that stably induce a tet-responsive reporter gene in all cells of a transfected clonal population. Screening of more than 100 colonies resulting from a standard co-transfection of the tetracycline transactivator (tTA) with a green fluorescent protein (GFP) reporter plasmid failed to identify a single colony that could induce GFP in more than 20% of cells. The presence of chromatin insulator sequences, previously shown to protect some transfected genes from epigenetic silencing, moderately improved stability but was not sufficient to produce homogeneous transformants. However, when cell lines were first established in which selection could be maintained either for the expression of tTA activity (co-transfection with a tTA-responsive selectable marker) or the presence of tTA mRNA (bicistronic message encoding a selectable marker), these cell lines could be subsequently transfected with the GFP reporter construct, and nearly 10% of the resulting colonies exhibited stable homogeneous tet-responsive GFP expression in 100% of the expanded clonal cell population. *J. Cell. Biochem.* 76:280–289, 1999. © 1999 Wiley-Liss, Inc.

**Key words:** GFP; tetracycline; CHO cells; transfection; gene expression

The tetracycline (tet)-regulatable gene expression systems have become extremely popular for the inducible expression of reporter genes in mammalian cells [Shockett and Schatz, 1996; Blau and Rossi, 1999]. In this system, a fusion protein (tTA) consisting of the tetracycline repressor (tetR) linked to the herpes simplex virus (HSV) transactivator VP16 regulates the expression of a promoter (Ptet) linked to seven tandemly repeated binding sites for tetR [Resnitsky and Reed, 1995]. tTA can only bind tetR and activate transcription in the absence of tet. Unfortunately, the construction of stable transformants that allow for tet-regulatable

gene expression has been a challenge because of the epigenetic instability of transfected genes [Gilbert, 1989; Pikaart et al., 1998] and the potential growth toxicity of the tTA gene product [Gossen and Bujard, 1992]. Typically, extensive screening is required to find a cell line that can express tTA for extended periods [Yin and Zhu, 1996; van Steensel and de Lange, 1997]. Expression of tTA is usually evaluated by transfection with a reporter gene, such as luciferase, by means of an enzymatic assay. Such assays measure the total amount of tTA activity in a cell population but are unable to assess the percentage of cells that express tTA. Once a tTA-expressing cell line has been established, a second transfection is required to introduce the tet-responsive reporter gene, which will again be subject to the same epigenetic instability. In most cases, the resulting cell lines express the tet-responsive gene product in only a fraction of cells in the population. This heterogeneity goes unnoticed by investigators who evaluate the level of protein expression in cell populations.

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However, for experiments in which similar levels of transgene expression are required in most cells in a clonal population, the difficulties are often insurmountable.

We have investigated several strategies to construct stable homogeneous tet-regulatable populations of Chinese hamster ovary (CHO) cells, using standard transfection techniques. We demonstrate that flanking the tTA expression cassette with chromatin insulator sequences [Pikaart et al., 1998] to protect against epigenetic silencing is helpful but is not sufficient for stability. Our results indicate that growth inhibition by tTA is the major obstacle to stable tet-responsive gene expression. The ability to select directly for the presence of tTA-expressing cells had a profound effect on the stability of reporter gene expression. Using this approach, clonal cell populations could be established in which 100% of transfected cells expressed tet responsive GFP.

## MATERIALS AND METHODS

### Construction of Plasmids

The *XhoI* site of pTRE (Clontech) was converted to an *AseI* site by linker substitution, resulting in pTRE-A. The *AseI-NheI* fragment of pEGFP-C1 (Clontech) containing the CMV promoter was then replaced by the *AseI-SacII* fragment of pTRE-A (containing Ptet), using an *NheI-SacII* adapter. The resulting plasmid, ptetGFP, encodes the GFP sequence under the control of Ptet. A 1.2-kb *XbaI* fragment of pJC13-1 containing a chicken insulator sequence [Chung et al., 1993] was isolated; the ends were filled in with Klenow, and one copy each was inserted first into the *AseI* site (also filled in with Klenow) and then into the *DraIII* site (chewed back with T4 polymerase) of ptetGFP to construct ptetGFPins.

To construct ptet*bsr*, the *HindIII* fragment of pSV2*bsr* [Izumi, Miyazawa et al., 1991] was inserted into the *EcoRI* site of pTRE by blunt end-ligation after restriction sites were filled in with Klenow. ptTA-IRES-*hyg* was made by insertion of the *EcoRI-BamHI* fragment of pUHD15.1 [Gossen and Bujard, 1992] containing the tTA coding sequences into the *BamHI* site of pIRES*hyg* (Clontech) after filling in restriction sites with Klenow.

To flank the tTA-expressing genes with insulator sequences, pJC13-1 [Chung et al., 1993], containing a multiple cloning sequence and a locus control region (LCR) flanked on each side

by two copies of a chromatin insulator, was digested with *EcoRI* and self-ligated, deleting the 1.1-kb LCR fragment and creating pJC13-1 $\Delta$ LCR (not shown). The 2.3-kb *XhoI-HindIII* fragment from pUHD15.1, containing the cytomegalovirus (CMV) promoter and tTA coding sequences, and the 4.3-kb *MluI-XhoI* fragment from ptTA-IRES*hyg* were filled with Klenow and blunt end-ligated to the 7.5-kb *BamHI* fragment of pJC13-1 $\Delta$ LCR (also filled with Klenow) to construct ptTAins2 and ptTA-IRES-*hyg*ins, respectively.

### Cell Culture and Transfection

CHOC 400 cells, a derivative of CHO in which the dihydrofolate reductase gene has been amplified ~500-fold [Hamlin et al., 1994], were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and nonessential amino acids. All plasmids were linearized before stable transfection. For the standard co-transfection approach,  $3 \times 10^5$  cells were grown in 35-mm dishes for 24 h and transfected with 1.0  $\mu$ g of the expression vector for tTA (pUHD15.1 or ptTAins2) and 0.1  $\mu$ g of the expression vector for green fluorescent protein (GFP) (ptetGFP or ptetGFPins), using Lipofectoamine reagent (Gibco). After exposure to the DNA-liposome complex for 2 h in the presence of 2  $\mu$ g/ml of tet, cells were refed with fresh medium and cultured for 48 h in the presence of 2  $\mu$ g/ml of tet. After culturing with 0.7 mg/ml of G418 (Gibco) and 2  $\mu$ g/ml of tet for 2 weeks, cells were washed three times with tet-free medium and cultured for 24 h without tet. Cells were then fixed at 4°C for 20 min in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) and stained with 0.1  $\mu$ g/ml 4',6-diamidino-2-phenylindole (DAPI); GFP-positive colonies were counted using an inverted fluorescence microscope. Although it was not necessary to fix cells for GFP observation, it was more convenient during the lengthy periods of time necessary for scoring; fixation allowed for the staining of cells with DAPI, which aided in the identification of nuclei.

To select for cells expressing tTA activity,  $7 \times 10^5$  cells were grown in 60-mm dishes for 24 h, and 0.1  $\mu$ g of ptet*bsr* was co-transfected with 2.4  $\mu$ g of the expression vector for tTA (pUHD15.1 or ptTAins2) for 5 h, using Lipofectoamine. After culturing with fresh medium for 48 h, cells were selected with 10  $\mu$ g/ml of blastidicin S (Bs) for 2 weeks. Bs-resistant (Bsr)

colonies were isolated and maintained in the presence of 10  $\mu\text{g/ml}$  of Bs until the second transfection. A similar protocol was employed to transfect bicistronic constructs ptTA-IRES-*hyg* or ptTA-IRES-*hyg/ins*, except that cells were selected with various concentrations of hygromycin B (*hyg*) for 2 weeks. Hyg-resistant colonies were isolated and maintained in the presence of 0.5 mg/ml of *hyg*.

For transfection of Bsr26 with GFP reporter plasmids,  $3 \times 10^5$  cells were transfected with 1.0  $\mu\text{g}$  of ptetGFP or ptetGFPins, using Lipofectamine. After exposure to the DNA-liposome complex for 2 h in the presence of 2  $\mu\text{g/ml}$  of tet, the cells were refed with fresh medium and cultured for 48 h in the presence of 2  $\mu\text{g/ml}$  of tet. After culturing with 0.7 mg/ml of G418 (Gibco) and 2  $\mu\text{g/ml}$  of tet for 2 weeks, colonies of the GFP100 series (transfected with ptetGFP) and GFP200 series (transfected with ptetGFPins) were isolated from some of the dishes. GFP100 and GFP200 series clones were maintained in the presence of 0.5 mg/ml of G418 and 2  $\mu\text{g/ml}$  of tet. Colonies from the remaining dishes were cultured for 24 h without tet, and GFP-positive colonies were scored as before.

Hyg<sup>r</sup>25 and Hyg<sup>r</sup>16 were similarly transfected with GFP reporter plasmids, except that one-half of the dishes were selected for the 2-week period in the presence of 0.5 mg/ml *hyg*. Colonies of the GFP300 series, isolated from some of the dishes of Hyg<sup>r</sup>25 transfections, were expanded and maintained in the presence of 0.5 mg/ml of G418 (Gibco), 2  $\mu\text{g/ml}$  tet, and 0.5 mg/ml *hyg*. Colonies from the remaining dishes were cultured for 24 h without tet, and GFP-positive colonies were scored as before.

To evaluate the percentage of tTA-expressing cells in the various clonal populations,  $3 \times 10^4$  cells were grown on 24-well plate for 24 h and transfected with 0.15  $\mu\text{g}$  of ptetGFP and 0.15  $\mu\text{g}$  of pSV-beta-gal (Promega, Madison, WI) using Lipofectamine and supercoiled plasmids. After exposure to the DNA-liposome complex for 5 h, the cells were refed with fresh medium and cultured for 24 h both in the presence and in the absence of tet. The cells were then fixed with 3.7% formaldehyde at 4°C for 20 min and stained with X-gal for  $\beta$ -galactosidase activity as described by MacGregor and Mogg et al., 1987. The percentage of  $\beta$ -galactosidase-positive cells that were also GFP-positive was scored.

## Flow Cytometry Analysis and Fluorescence Microscopy

For the induction of GFP expression, cells were washed three times with tet-free medium and cultured for 24 h without tet. Cells were collected by trypsinization, washed twice with in PBS, and then fixed with 4% paraformaldehyde/PBS at 4°C for 30 min. Ten thousand cells were analyzed for GFP expression level on a FACScan (Becton Dickinson). Consistent with a previous report [Jiang and Hunter, 1998], we found that ethanol fixation resulted in a significant loss of GFP signal per cell, whereas fluorescence after fixation with paraformaldehyde was indistinguishable with unfixed cells. Fixation was performed for convenience of handling large numbers of samples and was unnecessary for flow cytometry. For microscopy, cells were grown on sterile dishes or coverslips, rinsed with PBS, and fixed at 4°C for 30 min in 4% paraformaldehyde/PBS.

## RESULTS

In designing a strategy to construct homogeneously inducible CHO cell lines, we considered several criteria that were addressed with the plasmids described in Figure 1. First, we wanted to employ a reporter gene that would allow us to rapidly evaluate both the percentage of posi-

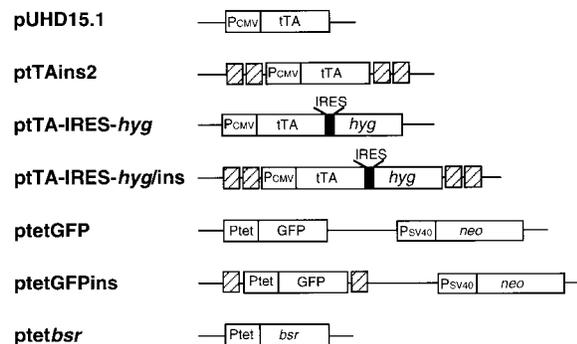
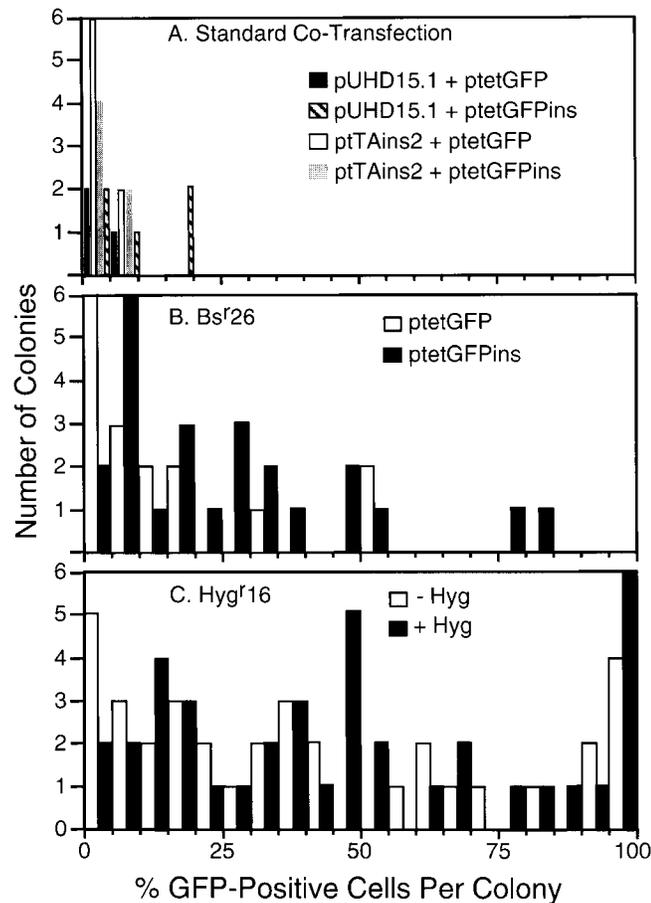


Fig. 1. Schematic diagram of the expression vectors. pUHD15.1 [Gossen and Bujard, 1992], the original vector expressing tetracycline transactivator (tTA) from the cytomegalovirus (CMV) promoter ( $P_{CMV}$ ), was modified to construct three expression vectors: ptTAins2, ptTA-IRES-*hyg*, and ptTA-IRES-*hyg/ins*. ptTAins2 is flanked on both sides with two copies of the chicken  $\beta$ -globin insulator sequence (hatched box). ptTA-IRES-*hyg* (without insulators) and ptTA-IRES-*hyg/ins* (with insulators) co-express tTA and *hyg*, from a bicistronic message (using encepharovirus IRES), permitting direct selection for tTA expression. Reporter plasmids expressing green fluorescent protein (GFP) from the tet-responsive promoter (Ptet) were constructed both with (ptetGFPins) and without (ptetGFP) flanking insulator sequences. Reporter plasmid ptetbsr was constructed to select cell lines expressing tTA activity, using blasticidin S.



**Fig. 2.** Frequency of green fluorescent protein (GFP)-positive cells in the individual colonies. **A:** CHO 400 cells were co-transfected with expression vectors for tetracycline transactivator (tTA) (pUHD15.1 or ptTAins2) and GFP (ptetGFP or ptetGFPins), as indicated. Stable colonies were selected in G418 and tet; tet was removed from the medium to induce GFP expression, and the percentage of GFP-positive cells in each colony was scored (more than 100 colonies were obtained—the vast majority of colonies did not express GFP). Data are presented as

a histogram of the number of colonies showing various percentages of GFP-positive cells, in increments of five percentage points. **B:** Bs'26 (which harbors ptTAins2) was transfected with ptetGFP (white) or ptetGFPins (black), selected in G418 and tet, and GFP-positive colonies were scored as in A. **C:** Hygf16 (which harbors ptTAins2) was transfected with ptetGFPins and selected in G418 and tet either in the presence (black) or absence (white) of hyg, and GFP-positive colonies were scored as in A.

tive cells and the level of expression per cell. Using GFP as a reporter, colonies of cells can be directly monitored during their selection with an inverted fluorescence microscope and candidates demonstrating a high percentage of positive cells can subsequently be analyzed for the amount of expression per cell, using the fluorescence-activated cell sorter (FACS). Second, we wanted to evaluate the effectiveness of insulator sequences to stabilize expression from the viral CMV promoter (driving expression of tTA) and the Ptet promoter. In particular, an insulator sequence derived from the chicken  $\beta$ -globin locus has been shown to protect certain promoters against position effects, stabilizing the expression of transfected genes [Pikaart et al., 1998]. Thus, plasmids were constructed both

with and without flanking insulator elements (Fig. 1). Finally, we considered the possibility, suggested by others [Gossen and Bujard, 1992], that the tTA gene product itself is toxic to cell growth, probably due to the presence of the VP16 transactivation domain [Gilbert et al., 1992]. For this reason, we designed strategies to select either for activity of tTA or for expression of the messenger RNA (mRNA) encoding tTA.

#### Standard Co-transfection

We employed three strategies to construct stable tetracycline regulatable cell lines. In the first strategy (Fig. 2A), cells were co-transfected with a tTA-expressing plasmid (both with

and without insulator sequences) and a reporter GFP plasmid (both with and without insulator sequences) in all four combinations. Selection was made for the neomycin gene present on the reporter plasmid. Stable transfection efficiencies (~2% of transfected cells were resistant to G418), as well as the percentage of G418-resistant colonies that exhibited at least some GFP-positive cells (~7%) were the same in all four transfections in two independent experiments. Of more than 100 colonies obtained from these selections, we were not successful in identifying a single colony in which more than 20% of cells expressed GFP, regardless of the presence of flanking insulator sequences and the continued selection for G418 resistance (Fig. 2A). However, it was evident from these experiments that the presence of insulator sequences flanking both the tTA plasmid and the reporter plasmid moderately increased the percentage of GFP-positive cells. These experiments confirmed what we expected from previous experience [Gilbert, 1989]; that is, standard transfection does not provide a practical means to achieve homogeneous expression of ectopic genes in a variety of mammalian cell types.

#### Selection for Cells Expressing tTA Activity

In a second approach, the insulator-flanked tTA expressing plasmid (ptTAins) was co-transfected with a reporter construct in which the blasticidin S-resistance (*bsr*) gene was placed downstream of Ptet (Fig. 1; *ptetbsr*). Blasticidin S (Bs)-resistant colonies were obtained at a frequency of 0.01%, whereas no colonies (<0.0003%) were obtained in control transfections in which *ptetbsr* was transfected without tTA, demonstrating that transfected clones must express a functionally active tTA to acquire Bs resistance. These colonies were expanded and then cultured in the absence of Bs for 2 weeks, allowing those colonies that harbor epigenetically unstable tTA integrations to accumulate tTA-negative cells. To evaluate the stability of tTA within these clonal populations, aliquots of these cells were transiently (48 h) co-transfected with *ptetGFP* and *pSV-β-gal* and the percentage of β-gal-positive cells that were also GFP positive was evaluated. Figure 3 plots the doubling times of 38 Bs<sup>r</sup> colonies against the percentage of cells positive for tTA activity. These results demonstrate an inverse correlation between the level of tTA activity and the

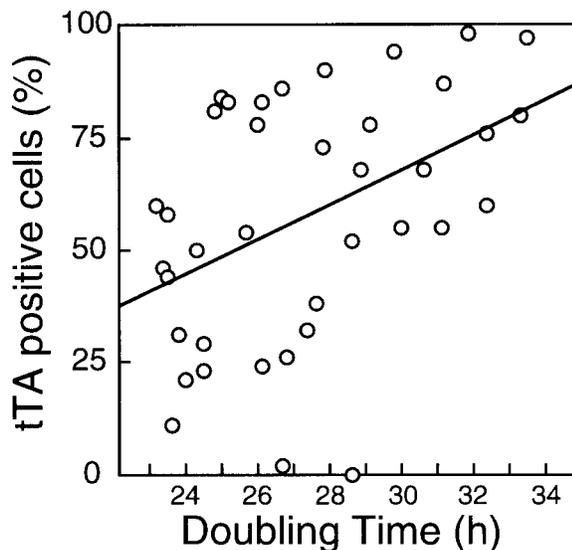


Fig. 3. Toxicity of tetracycline transactivator (tTA) for cell growth. Thirty-eight blasticidin S-resistant clones were isolated from the co-transfection of *ptetbsr* and *ptTAins2* into CHO400 cells and then grown for 2 weeks in the absence of blasticidin S. Each clonal population was transiently co-transfected with *pSV-β-gal* and *ptetGFP* and the percentage of β-galactosidase positive cells that were also green fluorescent protein (GFP) positive was scored to give a measure of the percentage of cells in each population that expressed tTA. The doubling times in the absence of blasticidin S were directly measured and plotted against the percentage of tTA-positive cells.

growth rate of cells and support a growth inhibitory effect of the tTA. We conclude from this experiment that continuous selection for tTA-negative cells can be expected within tTA-transfected cell populations.

One of these clones (Bs<sup>r</sup>26) was chosen for stable transfection with *ptetGFP* and *ptetGF-Pins*, selecting for a linked G418 resistance marker. We obtained the same stable transfection efficiency for G418 resistance (~2%) as with the standard co-transfection strategy. However, colonies containing at least some GFP-positive cells were observed at a much higher frequency (~20%); several of these colonies harbored more than 50% GFP-positive cells (Fig. 2). Consistent with the results from the standard co-transfection approach, the presence of the insulator sequences did not alleviate epigenetic instability but did result in a moderate increase in the stability of GFP expression (Fig. 2B).

To investigate the level of uninduced and induced expression of GFP in these individual colonies, 10 G418-resistant colonies were expanded in the presence of tet; aliquots of these

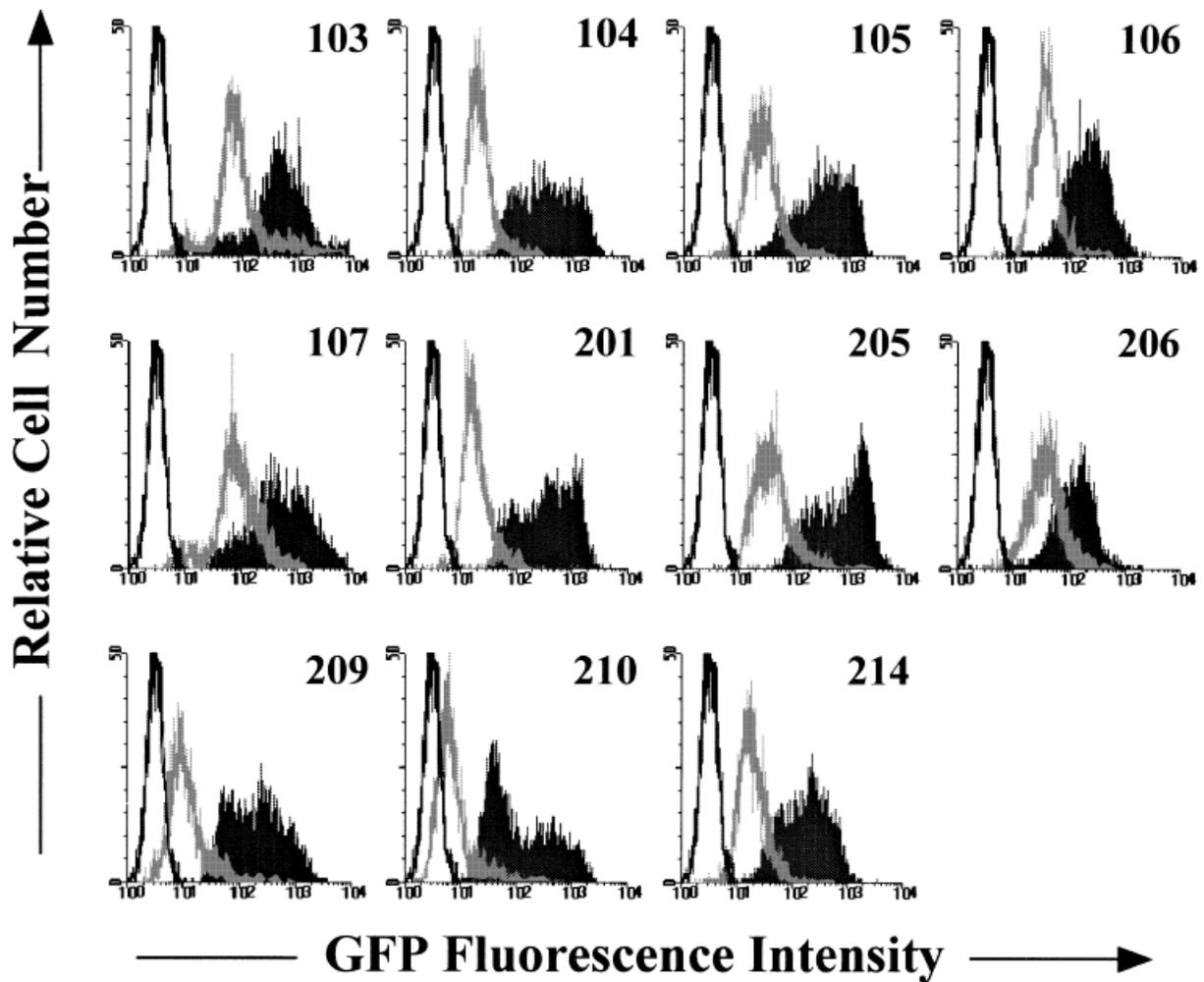


Fig. 4. FACS analysis of green fluorescent protein (GFP)-expressing cell lines from Bs'26. Bs'26 cells were transfected with ptetGFP and ptetGFPins to establish the GFP100 and GFP200 series of clones, respectively. After culturing in the

absence (filled black) or presence (open gray) of tet for 24 h, cells were analyzed by flow cytometry. The open black histogram represents untransfected Bs'26 cells.

cells were transferred to tet-free medium for 24 h, and the expression of GFP was analyzed by flow cytometry. As shown in Figure 4, a significant increase in GFP expression was observed upon removal of tet from the medium. For most clones harboring ptetGFP, and nearly all clones with ptetGFPins, close to 100% of cells expressed tet-responsive GFP. Notably, a higher percentage of GFP-positive cells was detected by flow cytometry versus direct microscopic observation (Fig. 2B), demonstrating the higher sensitivity of the FACS in detecting GFP fluorescence. Both the uninduced and induced levels of GFP expression were highly variable from cell line to cell line. As expected [Forster et al., 1999], all colonies exhibited a basal level of GFP expression in the continuous presence of

tet, which for most clones was not observable by microscopic examination. However, one clone (#210) displayed an uninduced level of GFP expression close to that of untransfected cells (Fig. 4). In general, cells harboring GFP constructs flanked by insulator sequences had a higher percentage of positive cells, as predicted from Figure 2.

#### Selection for Cells Expressing tTA mRNA

The approach described above selects for the maintenance of tTA activity. However, if the tet-regulatable system is to be employed for the conditional expression of a gene that will arrest cell growth, it will not be possible to maintain selection for the active conformation of tTA after introduction of a reporter construct. In a

**TABLE I. Transfection Efficiencies With Bicistronic Plasmids**

Plasmid	Concn of Hygromycin B (mg/ml)	Efficiency of stable transformation
ptTA-IRES-hyg	0.5	$5.6 \times 10^{-4}$
	1.0	$7.0 \times 10^{-5}$
	3.0	$<1.3 \times 10^{-6}$
	10	$<1.3 \times 10^{-6}$
ptTA-IRES-hyg/ins	0.5	$7.9 \times 10^{-4}$
	1.0	$3.7 \times 10^{-4}$
	3.0	$5.3 \times 10^{-6}$
	10	$<1.3 \times 10^{-6}$

third approach, tTA was co-expressed with the *hyg* gene from a bicistronic message in which the two coding sequences are separated by an internal ribosome entry site (IRES). In this strategy, culturing in the presence of hyg maintains selection for tTA mRNA in the presence or absence of tet, as there is no requirement for tTA activity. We also reasoned that increasing the concentration of hyg could select for cells that express higher levels of tTA. Hence, CHO 400 cells were transfected with the tTA-IRES*hyg* vector with and without flanking insulator sequences (Fig. 1) and selected in the presence of 0.5, 1.0, and 3.0 mg/ml hyg (Table 1). Stable transfection efficiencies with this vector were relatively low, presumably due to inefficient *hyg* expression from the IRES. As expected, transfection efficiencies were reduced at higher concentrations of hyg. Interestingly, the presence of flanking insulator sequences increased the frequency of stable colony formation and permitted the growth of colonies at higher concentrations of hyg. Twenty individual colonies from transfections with insulated constructs selected at 1 mg/ml Hyg were expanded in the presence of Hyg and analyzed for the percentage of tTA-positive cells by transient co-transfection with ptet-GFP and pSV- $\beta$ -gal (as in Fig. 3). In this case, all clones displayed some expression of GFP, and more than one-half displayed GFP expression in most cells (>60%) in the population. One of these clones (Hyg<sup>r</sup>16) was chosen for selection of stable transformants with the GFP reporter genes.

Hyg<sup>r</sup>16 was transfected with ptetGFPins and selection for G418 resistance was carried out both in the presence and in the absence of hyg (to evaluate the stability of tTA in these clonal populations without hyg selection; Fig. 2C).

Stable transfection efficiencies (~1.3%) were similar for all four transfections and in the same range as previous selections for G418 resistance. After removal of tet for 24 h, approximately 30% of the colonies arising from all four of these transfections displayed at least some GFP-positive cells. Maintaining hyg selection had only a minimal effect on the percentage of GFP-positive cells within each colony (Fig. 2C), indicating that, after initial selection for high levels of hyg expression from the IRES, expression of tTA is relatively stable. Most importantly, several clones were found to express GFP in 100% of the cells (Fig. 2C). By fluorescence microscopy, induced GFP expression appeared to be higher and more homogeneous than in the Bs<sup>r</sup>26 background (Fig. 5). To evaluate further the heterogeneity of GFP expression within these clones, 13 colonies from the Hyg<sup>r</sup>16 transfection were expanded in the presence of tet and hyg, aliquots of these cells were transferred to tet-free medium for 1 day, and the expression of GFP was analyzed by flow cytometry (Fig. 6). As can be seen, several of these clones expressed homogeneous amounts of GFP. This finding suggests that the heterogeneity seen in the Bs<sup>r</sup>26 background may be due to heterogeneous or lower levels of tTA in Bs<sup>r</sup>26. Selection for high levels of tTA message in Hyg<sup>r</sup>16 may stabilize the induced level of expression from Ptet. Uninduced levels of expression were also quite high, suggesting that higher levels of tTA protein in Hyg<sup>r</sup>16 contribute to a background interaction of tTA with Ptet even in the presence of tet. However, one clone (#312) demonstrated a low background level of expression by FACS analysis (Fig. 6) that was undetectable by fluorescence microscopy (Fig. 5). We conclude that the selection for tTA message, using a bicistronic gene construction, permits the establishment of homogeneous tet-responsive gene expression within CHO cells, using standard transfection methodology.

## DISCUSSION

Achieving regulatable ectopic gene expression after the transfection of mammalian cells has proven a difficult task. Usually, investigators are satisfied with cell lines that induce variable levels of a gene product in a fraction of cells within a population. We demonstrate that homogeneous tet-responsive gene expression can be obtained in CHO cells, using standard transfection techniques but, only after careful

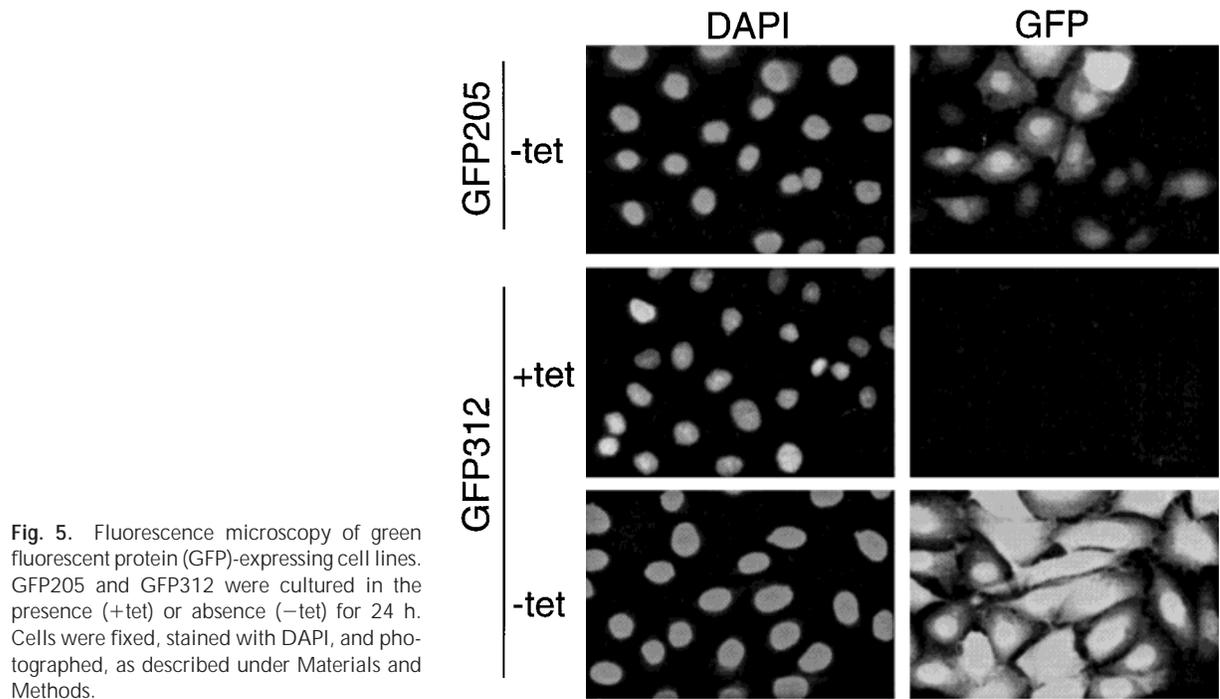


Fig. 5. Fluorescence microscopy of green fluorescent protein (GFP)-expressing cell lines. GFP205 and GFP312 were cultured in the presence (+tet) or absence (-tet) for 24 h. Cells were fixed, stained with DAPI, and photographed, as described under Materials and Methods.

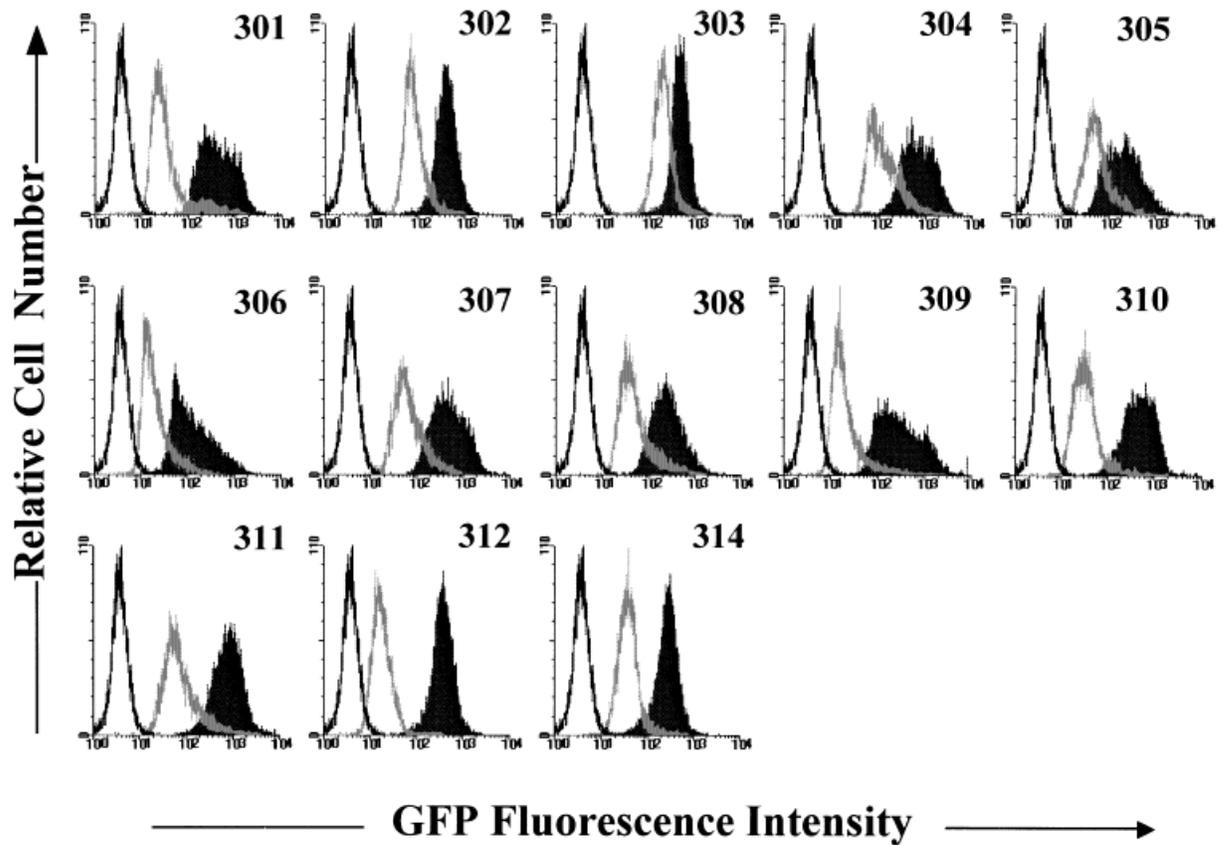


Fig. 6. FACS analysis of green fluorescent protein (GFP)-expressing cell lines from Hyg<sup>r</sup>16. Hyg<sup>r</sup>16 cells were transfected with ptetGFPins to establish the GFP300 series. After culturing in the absence (filled gray) or presence (open gray) of tet for 24 h, cells were analyzed by flow cytometry. The open black histogram represents untransfected Hyg<sup>r</sup>16 cells.

selection and screening procedures. Our experiments define several important considerations when establishing such cell lines.

First and foremost of these considerations is the tendency of mammalian fibroblasts to extinguish gene expression epigenetically from ectopically introduced promoters. Since some chromosomal integration sites extinguish the ectopic promoter less rapidly than others, one approach to achieve stable gene expression has been to screen many transfected colonies for one that may have integrated the transfected gene(s) at a favorable chromosomal site. However, using this approach, we were unsuccessful in finding a homogeneously positive clone after screening more than 100 colonies. Another approach is to flank the gene of interest with chromatin insulator sequences to protect the transfected promoter from epigenetic silencing. The most well-defined vertebrate insulator sequence is derived from the chicken  $\beta$ -globin locus. This insulator sequence was very effective at stabilizing gene expression from a chicken gene promoter introduced into a chicken fibroblast cell line [Pikaart et al., 1998] as well as *Drosophila* genes in transgenic flies [Chung et al., 1993]. Our results indicate that this sequence is only partially effective at insulating the CMV promoter from position effects in CHO cells. This could be attributable to species specificity of factors that interact with the chicken insulator sequence. Alternatively, the insulator may function more effectively with some promoters than with others. Clarification of the mechanism of insulator function and the identification of more examples of insulator sequences will be important in resolving these issues.

A second consideration is the toxicity of the tTA gene product. This toxicity was suggested by the inability to detect the apparently very low levels of tTA gene product within transfected cells [Gossen and Bujard, 1992]. We have directly demonstrated an inverse relationship between the level of tTA expression and the growth rates of 38 clonal cell populations. This underscores the need to maintain some degree of selective advantage for those cells that express tTA. We have exploited two different strategies to select for tTA expression. One method has the advantage of selecting directly for tTA activity. The obvious disadvantage to this approach is that selective pressure must be removed whenever one wishes to culture cells under conditions that maintain Ptet in an inac-

tive state (i.e., presence of tet). The second strategy selects for the expression of tTA mRNA so that selection can be maintained even in the presence of tet. A potential disadvantage to this strategy is that selection is not for tTA activity directly, so that mutations in the tTA coding sequences, including frameshift mutations that would eliminate tTA protein completely, could accumulate even in the presence of selection. However, we found that within the course of the experiments described in this report, no such loss of tTA protein was detected. We conclude that the latter strategy is superior. In any case, should such mutations prove a significant problem in certain applications, a combination of the two approaches, in which p $tet$ *bsr* is introduced along with p $tTA$ -IRES-hyg/ins, would allow for the periodic elimination of tTA-negative cells by passage of cells in the presence of Bs.

A final consideration is the level of uninduced expression from Ptet in the presence of tet. As described previously [Forster et al., 1999], the basal level of expression can be quite high. Furthermore, our results demonstrate that when GFP is used as a reporter, this background expression may be underestimated when observed by microscopic examination. Uninduced levels of expression were more readily detected by FACS analysis. We found that, although uninduced levels of expression were never equivalent to cells lacking the reporter gene construct, low levels of uninduced expression were achievable in a reasonable percentage of colonies. Nonetheless, under certain circumstances, it may be necessary to reduce the background expression to near-untransfected levels (e.g., to achieve the conditional expression of extremely toxic gene products). Recently, modified transactivators that repress transcription from Ptet have been described; it has been shown that regulators that activate Ptet in the presence of tet and repress Ptet in the absence of tet can be simultaneously expressed [Blau and Rossi, 1999; Forster et al., 1999]. These new regulators may provide a means of eliminating uninduced levels of expression. In combination with the approaches described in this report, it should be possible to construct homogeneous populations of cells that exhibit tight regulation of conditional gene expression from Ptet, using conventional transfection techniques.

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