Benchmarks

Plasmid Harboring *lac* Repressor and *tTA* Activator Genes Can Regulate Two Inducible Genes in Mammalian Cells

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This study demonstrates that the plasmid pTet-Lac-Hyg containing a lac repressor gene (*lacI*) and a tetracycline activator gene (*tTA*) could regulate two inducible genes in either transiently or stably transfected mammalian cells. The *Escherichia coli* tetracycline responsive promoter (Tet system) and lactose responsive promoter (Lac system) have been used in combination (designated Lac/Tet dual-inducible sys-

tem) to regulate two inducible genes simultaneously in a single cell, which were demonstrated by either transient or stable transfection study (4). The reporter gene of the Lac system can be specifically activated by addition of the lactose analogue, isopropyl- β -D-thiogalactoside (IPTG), which binds to the repressor and disables it from binding to the operator sequence by conformational change (3,5). Tetracycline activator (tTA) of the Tet system is encoded by the plasmid pTet-off (CLONTECH Laboratories, Palo Alto, CA, USA) and is a fusion of the wild-type Tet repressor to the VP16 activation domain of herpes simplex virus (HSV). The tTA binds and thereby activates transcription in the absence of tetracycline (Tc). Thus, as Tc is added to the culture medium, transcription is turned off (1,2). IPTG and Tc (used for the operation of the Lac and the Tet systems, re-

spectively) were non-cytotoxic to the cells while added together into the cells around the optimal concentrations (IPTG, ≤ 5 mM and Tc, < 1.5 μ g) (4).

Traditionally, four plasmids are required to regulate two inducible genes in a cell. Therefore, the Lac system needs a lac repressor plasmid (pH-lacINLSneo) and an IPTG inducible target gene plasmid (5), and the Tet system needs a tTA plasmid (pTet-off) and a Tc inducible target gene plasmid. To cut down the number of plasmids for transfection and subsequently reduce the variation caused by delivering multiple plasmid DNA, we put *lacI*, *tTA* and a drug resistant gene (*hygromycine*) into a single plasmid pBSSK⁺ (Stratagene, La Jolla, Ca, USA) and named it pTet-Lac-Hyg (Figure 1). We demonstrated that the mRNA levels of lac repressor and tTA proteins expressed from pTet-Lac-Hyg are similar to the levels of *lacI*

Table 1. Plasmid pTet-Lac-Hyg Functions in Three Cell Lines Demonstrated by Transient Transfection Analysis

Cell Line ^a	Tet (1 μg/mL)	IPTG (5 mM)	Luciferase Activity			β-Galactosidase Activity		
			Repressed (RLU/μg) ^c	Induced	Fold ^b Increase	Repressed (RLU/μg) ^c	Induced	Fold ^b Increase
T2								
1-1-4 ^d	+	-	1.4 × 10 ⁵					
	-	-		5.0 × 10 ⁶	35			
	+	-				3.0 × 10 ⁴		
	+	+					4.6 × 10 ⁵	15
HepG2								
1-1-3 ^d	+	-	5.0 × 10 ⁴					
	-	-		1.5 × 10 ⁶	31			
	+	-				1.5 × 10 ⁴		
	+	+					9.0 × 10 ⁴	6
NIH/3T3								
1-1-3 ^d	+	-	4.6 × 10 ⁵					
	-	-		5.5 × 10 ⁶	12			
	+	-				3.7 × 10 ⁴		
	+	+					5.8 × 10 ⁵	15.7

^aT2, bladder carcinoma cell line; HepG2, hepatoma; NIH/3T3, mouse fibroblast.

^bThe fold increase is a comparison between repressed and induced levels of expression.

^cRelative luciferase unit per microgram cell extract.

^dpTet-Lac-Hyg (1 μg): pTREluc (1 μg): pSVlacOZ (3 or 4 μg) were co-transfected into the tumor cells by LIPOFECTIN® method (Life Technologies). The cell extracts were analyzed at 48 h post-transfection. The luciferase and β-galactosidase activities were determined by a Dual-Light™ Luciferase and β-Galactosidase Reporter Gene Assay System (Tropix, Bedford, MA, USA) (6). These data represent the results of three independent experiments.

Figure 1. The construct of plasmid pTet-Lac-Hyg. Hygromycin resistant gene (Hygromycine) driven by a RSV promoter, Tc activation gene (*tTA*) driven by a cytomegalovirus (CMV) promoter and lac repressor gene (*lacI*) driven by a HSV thymidine kinase promoter were cloned into the vector pBSSK⁺.

and *tTA* expressed from their individual plasmids pH β lacINLSneo and pTet-off, respectively (Figure 2). Moreover, pTet-Lac-Hyg could regulate the expression of luciferase and β -galactosidase, two inducible reporter genes in bladder car-

cinoma (T2) (7), hepatoma (HepG2) and NIH/3T3 cell lines by the transient transfection analysis. The induction folds for luciferase activity are 35-, 31- and 12-fold, respectively, and the induction folds for β -galactosidase ac-

Figure 2. The expression of *lacI* and *tTA* genes from pTet-Lac-Hyg is equivalent to *lacI* gene expression from pH β lacINLSneo and *tTA* gene expression from pTet-off by northern blot analysis. Total RNA (30 μ g) was extracted from 293T cells transiently transfected with pH β lacINLSneo, pTet-off or pTet-Lac-Hyg (20 μ g) and analyzed by northern blotting using [α -³²P]dCTP labeled *lacI* and *tTA* probes. Lanes 1 and 2, *tTA* expression; lanes 3 and 4, *lacI* expression.

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Table 2. The Induction Capability of the Stable Cell Lines Containing pTet-Lac-Hyg

Cell Line ^a	Tet (1μg/mL)	IPTG (5 mM)	Luciferase Activity		Fold ^b Increase	β-Galactosidase Activity		Fold ^b Increase						
			Repressed (RLU/μg) ^c	Induced		Repressed (RLU/μg) ^c	Induced							
HA-22T														
2-4	+	-	5.8 × 10 ⁵	9.3 × 10 ⁶	16	3.0 × 10 ⁴	8.9 × 10 ⁴	3						
	-	-												
	+	-	4.9 × 10 ⁵	5.4 × 10 ⁶	11	2.5 × 10 ⁴	1.1 × 10 ⁵	4.5						
	+	+												
3-4	+	-							4.1 × 10 ⁵	4.9 × 10 ⁶	12	3.1 × 10 ⁴	1.2 × 10 ⁵	4
	-	-												
	+	-												
	+	+												
Huh-7														
2-7	+	-	4.1 × 10 ⁵	4.9 × 10 ⁶	12	3.1 × 10 ⁴	1.2 × 10 ⁵	4						
	-	-												
	+	-												
	+	+												

pTREluc (2.5 μg) and pSVlacOZ (2.5 μg) were co-transfected into the tumor cells by LIPOFECTIN method. The same procedures as described in Table 1 were used to process the cell extracts. This data represent the results of three independent experiments.

^aHA22T and Huh-7, hepatoma cell lines.

^bThe fold increase is a comparison between repressed and induced levels of expression.

^cRelative luciferase unit per microgram cell extract.

tivity are 15-, 6- and 15.7-fold, respectively (Table 1). No difference was detected while comparing the induction fold and the suppression stringency controlled by the regulatory proteins expressed from pTet-Lac-Hyg, pHβlac-INLSneo and pTet-off (data not shown).

In the stable cell line study, we demonstrated that the lac repressor and tTA expressed from pTet-Lac-Hyg could induce the expression of signal transducer and activator of transcription (*Stat-3*) and *Ha-ras* transgenes in a human kidney cell line 2-4 (a derivative of 293T). Figure 3 shows that *ras* and *Stat-3* expression could be induced individually in the presence of both IPTG (5 mM) and Tc (1 μg/mL) and in the absence of both IPTG and Tc, respectively (Figure 3, lanes 2 and 4). Both inducible gene products were overexpressed simultaneously in the presence of IPTG but in the absence of Tc (Figure 3, lane 3). A very low amount of inducible gene expression was induced in the absence of IPTG but in the presence of Tc (Figure 3, lane 1). The maximal induction fold for *Stat-3* and *Ha-ras* are 3× and 11×, respectively (Figure 3). Collectively, the regulatory proteins

Figure 3. Both *Ha-ras* and *Stat-3* transgenes are inducible in the stable cell line 2-4 harboring plasmid pTet-Lac-Hyg. The stable cell line 2-4, a derivative of 293T, was established by co-transfection with pSVlacOraz (5), pTetStat-3 and pTet-Lac-Hyg, followed by hygromycin selection (0.15 mg/mL). Cells (1×10^6 /plate) were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (both from Life Technologies, Gaithersburg, MD, USA) in the presence or absence of IPTG and/or Tc for 24 h. Cells were then harvested, the protein was extracted and analyzed by western blotting using anti-Ras (Oncogene Science, San Diego, CA, USA) and anti-STAT-3 Monoclonal Antibody (PharMingen, San Diego, CA, USA) to detect Ras and STAT-3. Lane 5, negative control, 293T cells; lane 6, NIH/3T3 cells express STAT-3 and Ras, respectively.

produced by pTet-Lac-Hyg showed sufficient induction-fold and tight repression.

Finally, three stable hepatoma cell lines (HA22T-2-4, 3-4 and Huh7-2-7) containing pTet-Lac-Hyg have been established after hygromycine selection. Western blot analysis confirmed the expression of lac repressor and tTA in the cells (data not shown). The induction efficiency of pTet-Lac-Hyg in the above cells was evaluated by the reporter gene luciferase (Tet system) β -galactosidase (Lac system), which were transiently delivered into the cells. Our results show that the induction for luciferase activity is 16-, 11- and 12-fold, respectively, and induction for β -galactosidase activity is 3-, 4.5- and 4-fold, respectively, in HA22T and Huh7 cell lines (Table 2). The possible reason for the low induction fold of luciferase and β -galactosidase reporter genes in our study is that the molar ratio between the reporter gene and pTet-Lac-Hyg is not optimized. Nevertheless, we demonstrate that the lac repressor and tTA were expressed efficiently from pTet-Lac-Hyg and could regulate their target genes in various mammalian cell lines, which were either transiently or stably transfected. Most importantly, this unique plasmid is very useful for studying the transient effects of two genes on the cell either individually or simultaneously, as well as the relationship between these two inducible genes. In conclusion, pTet-Lac-Hyg simplifies the transfection procedure and extends the application of prokaryotic inducible systems from the regulation of a single gene to two genes. It could be valuable for the functional study of multiple novel genes discovered from the human genomic projects.

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