

Research Article

The Lac repressor provides a reversible gene expression system in undifferentiated and differentiated embryonic stem cell

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Abstract. Control of mammalian gene promoters by the bacterial LacI repressor provides reversible regulation and dose-response levels of derepressed expression by the lactose analog isopropyl thiogalactose (IPTG). Here, we show that insertion of LacI-binding sites in the ubiquitous β -actin promoter confers a strong and dose-dependent IPTG-regulatable expression of transiently transfected reporter genes in mouse embryonic stem (ES) cells expressing LacI. We established ES cell lines stably expressing reporter genes under inducible control and found a five- to tenfold IPTG induction of transgene ex-

pression. The kinetics of induction is rapid and stable, and can be rapidly reversed after IPTG removal. Importantly, this regulatable expression was maintained throughout the differentiation process of ES cells, and observed in individual differentiated cardiomyocyte-like cells and neuronal-like cells. This reversible system is the first to function from undifferentiated to individual well-differentiated ES cells, providing a very useful tool to understand molecular mechanisms underlying ES cell self-renewal, commitment and differentiation.

Key words. Mouse embryonic stem cell; LacI/IPTG reversible expression vector; ubiquitous gene promoter; neuron; cardiomyocyte.

Owing to their pluripotency, embryonic stem (ES) cells may be used to generate somatic precursor or differentiated cells in cell and tissue therapy as, for example, in the case of neurodegenerative disorders or myocardial infarction [1, 2]. However, one limitation is the low and variable proportion of mature differentiated cells obtained. Understanding the molecular mechanisms controlling the key steps leading to differentiation is, therefore, crucial for the development of useful ES cell lines. Mouse ES cell lines are maintained, *in vitro*, in an undifferentiated state

in the presence of leukaemia inhibitory factor (LIF) [3] and can be differentiated *in vitro* into various cell lineages, thereby providing a pertinent experimental model. Their differentiation is generally achieved by (i) removing LIF and cultivating the cells in suspension, where they form three-dimensional embryo-like aggregates called embryoid bodies (EBs) and (ii) adding appropriate differentiation agents for defined periods. Cells within the EB undergo specific morphological changes recapitulating the three germ layers. Routinely, after 7 days in suspension, EBs are plated onto tissue culture dishes and differentiated cells develop in the outgrowth area of EBs. Depending on the inducers applied, ES cells differentiate into a variety

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of cell types, including cardiac-like, skeletal muscle-like, or neuronal-like cells [4].

A powerful strategy for studying the role of key genes during early embryonic developmental processes is to genetically engineer ES cells to express foreign genes. Because cell fate-determining factors are usually expressed in a stage-dependent manner, efforts to develop reversible inducible expression systems have been made. The LacI/isopropyl thiogalactose (IPTG) system is a well-known inducible expression system in eucaryotes [5]. This system contains two expression units: one for the bacterial repressor LacI and one for a LacI-response element (operator sequences) promoter to drive expression of the gene of interest. The binding of LacI to operator sequences (repressed state) is relieved by the lactose analog IPTG (activated state). This system has been shown to be functional in various established cell lines and in mice [6, 7].

Here, we demonstrate that the combination of the LacI/IPTG system and the use of β -actin gene promoters constitutes an efficient reversible gene expression induction system in ES cells. The regulatable expression of the reporter gene was maintained from the undifferentiated state through the differentiation process of ES cells and, importantly, induction was observed in individual differentiated cardiomyocyte-like and neuronal-like cells.

Materials and methods

Cell culture and differentiation

Mouse ES cells CGR8 [8] were grown on gelatin-coated plates in DMEM supplemented with non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, 10% (v/v) fetal bovine serum (Life Technologies) and 100 μ M 2-mercaptoethanol (Sigma), maintained undifferentiated by addition of 100 units/ml LIF, and induced to differentiate as described previously [9]. Briefly, ES cells were cultivated for 2 days in hanging drops to form EBs. From day 2 to 7, EBs were maintained in suspension and treated or not, days 2–5, with 0.1 μ M all-trans retinoic acid (RA) (Sigma). From day 7 to 21, EBs were seeded onto gelatin-coated plates and treated for 24 h with or without 1 mM IPTG (Stratagene) as indicated.

Construction of the regulatable promoter CAGop

The synthetic CAG promoter (chicken β -actin promoter plus CMV enhancer) was obtained from Dr Niwa, Kobe, Japan [10], and was modified by insertion of the following sequence:

5'-AGTCAATTGTGAGCGGATAACAATTCCACAG-TCGACCCTAGGTTGTGTCGCGAGTGTTGGATCC-CAGCTGACACCAATTGTGAGCGCTCACAATTG-3'. The two LacI-binding sites O_1 and O_{id} are in bold. This

sequence was inserted into the *HinfI* site located at position +3 from the transcription start of the CAG.

Plasmids

pCMVLacI and pOPI3-Luc are from the LacSwitchII system (Stratagene), except that the CAT gene was replaced by the luciferase gene using *NotI* restriction sites. The human β -actin promoter-driven LacI cDNA plasmid corresponds to the R construct described in Cronin et al. [6] and was a gift of Dr Scrabble, Charlottesville, Va. Construction of the CAGop plasmids starting from pOPI3-Luc was as follows: deletions of *HindIII* and *BstXI-BglII* (replaced by *SpeI* site) fragments and insertion of an *SpeI-HindIII* fragment containing the CAGop promoter, deletion of an *XhoI* fragment, thereby generating the pCAGop-Luc construct. We then inserted a polylinker [5'-*NotI-KpnI-EcoRV-XhoI-XmnI-SmaI/XmaI-ClaI-HpaI-NotI*-3'] at the *NotI* site. The green fluorescent protein (GFP)-encoding plasmid was generated by inserting a *KpnI-SmaI* fragment from pNeoEGFP (BD Bioscience) into the polylinker.

Generation of ES cell lines

ES cell lines were generated sequentially. First, CGR8 ES cells were co-transfected with pLacI + pGK-hygro^R, at a ratio 10 to 1, using the FuGene 6 transfection system (Roche Molecular Biochemicals) according to the manufacturer's protocol, and selected in 100 μ g/ml hygromycin (In Vitrogen) to generate LacI-expressing clones. Then, LacI3 ES cells were co-transfected with pCAGop-Luc or pCAGop-GFP + pGKneo^R, and selected with 200 μ g/ml geneticin (Life Technologies) to generate clones expressing luciferase or GFP proteins in an inducible manner.

RNA, protein analysis and luciferase assay

Total RNA was extracted using Trizol reagent (Life Technologies) and 15 μ g per lane was blotted onto Biotrans nylon membranes (ICN), cross-linked with UV and hybridized with radiolabelled cDNA probes. Hybridization signals were quantified using a Molecular Dynamics Phosphorimager coupled to the Image Quant software.

Fifty micrograms of whole-cell protein extract was resolved by SDS/PAGE on a 10% (w/v) polyacrylamide gel and transferred onto polyvinylidene difluoride membrane. Membranes were blocked in 1% BSA in TBS-0.1% Tween for 1 h at room temperature and blotted for LacI (Clone 9A5; Upstate Biotechnology), luciferase (L2164 antibody; Sigma) or ERK1/2 (Santa Cruz) as internal control.

Luciferase assays were performed as described elsewhere [11].

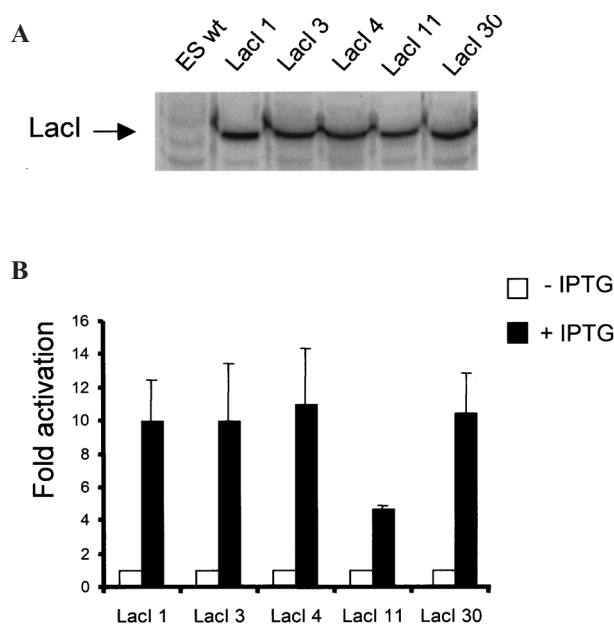


Figure 1. LacI expressing ES cell lines. (A) CGR8 ES cells were co-transfected with pLacI, encoding LacI, and pGK-Hyg, encoding Hygromycin resistance. Cellular lysates of five hygromycin-resistant ES clones stably expressing LacI mRNAs were analysed by Western blotting using anti-LacI antibodies; wild-type CGR8 ES cell extract (ES wt) was used as control. (B) ES clones expressing LacI were transiently transfected with 0.5 μ g pOPI3-Luc for 24 h in the presence or absence of 1 mM IPTG, and luciferase activity was measured on cellular lysates and normalized to 1 without IPTG. Mean values \pm SE of at least three independent experiments are shown.

Immunofluorescence staining

Differentiated EBs were fixed in 4% paraformaldehyde for 20 min at 4°C. Cell membranes were permeabilized in PBS-0.1% triton X-100 for 15 min at 4°C and incubated with either polyclonal anti-GFP antibody (No. 8372; BD Bioscience) or monoclonal anti-troponin T antibody (CT3; Developmental Studies Hybridoma Bank) in PBS-1% BSA overnight at 4°C. After three washes in PBS, cells were incubated with anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (to label the anti-GFP antibody) or anti-mouse Texas red-conjugated antibody (to label the anti-troponin antibody) for 1 h at room temperature. Cell imaging was performed using an Axiovert 200 microscope (Carl Zeiss) equipped with a $\times 20$ Apoplan objective (Carl Zeiss) and a cooled digital CCD CoolSNAP HQ camera (Roper Scientific), using Metamorph image analysis software (Universal Imaging Corporation).

Results

As a first attempt to use the LacI/IPTG system in ES cells, we tested the classical vectors pCMVLacI and pOPI3-Luc, in which viral-derived promoters drive the LacI cDNA and the inducible luciferase reporter construct,

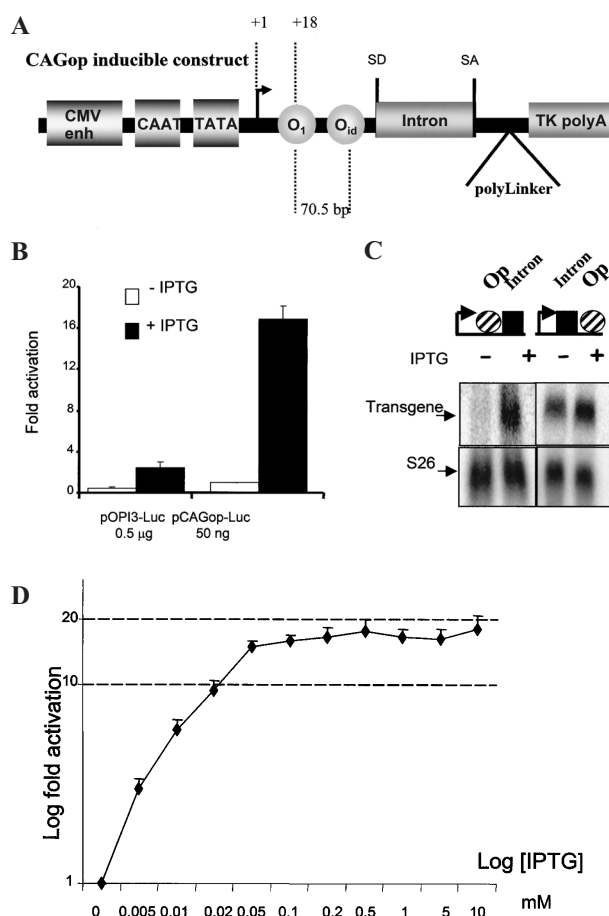


Figure 2. Driven by β -actin promoters, the LacI inducible system is functional in ES cells. (A) Schematic representation of the pCAGop plasmid, including the CAG promoter, composed of the chicken β -actin gene promoter (CAAT and TATA sequences) plus the CMV enhancer (CMV enh), the transcriptional start site (+1), the two LacI-binding sites (O_1 and O_{id}), the first intron of the CAG promoter (intron), the polyadenylation signal (TK polyA) (see Materials and methods for details). (B) ES-LacI3 cells were transiently transfected with 50 ng pCAGop-Luc or 0.5 μ g of pOPI3-Luc for 24 h in the presence or absence of 1 mM IPTG, and luciferase activity was measured on cellular lysates and normalized to 1 for pCAGop-Luc without IPTG. Mean values \pm SE of three independent experiments are shown. (C) ES-LacI3 cells were transiently transfected with 5 μ g of pCAGop constructs for 24 h in the presence or absence of 1 mM IPTG, and analysed by Northern blot with the transgenic probe; the S26 probe was used as internal control. Operator sequences were inserted either between the transcription start site (arrow) and the splice donor, as depicted in A (left panel), or after the first intron (right panel). Migrations of the transgene and S26 mRNAs are indicated. (D) ES-LacI3 cells were transiently transfected with 50 ng pCAGop-Luc for 24 h in the presence of increasing doses of IPTG, and luciferase activity was measured on cellular lysates and normalized to 1 without IPTG. Mean values \pm SE of three independent experiments are shown.

respectively. Although functional in transient transfection experiments, attempts to establish stable cell lines with these vectors were unsuccessful (data not shown). As an alternative to viral promoters, we then transfected ES cells with a human β -actin promoter driving the mam-

malianized LacI cDNA [6]. After co-transfection with a hygromycin gene and hygromycin selection, we found that numerous stable resistant clones (1/3) expressed high levels of LacI mRNA (data not shown) and protein (fig. 1A). Each individual clone expressing LacI was transiently transfected with pOPI3-Luc, and cells were incubated for 24 h with or without 1 mM IPTG. In response to IPTG, all LacI-expressing ES clones induced four to ten fold the expression of the reporter gene (fig. 1B). These results validated the use of this construct to express LacI in ES cells. For the inducible promoter we used the CAG promoter, the chicken β -actin promoter combined with a CMV enhancer, which is strongly and ubiquitously expressed in many cells [10]. To confer LacI responsiveness, the CAG promoter was modified by inserting two operator sequences 7 base pairs downstream of the transcription start site (fig. 2A). The nucleotide sequence of the operators and their positioning relative to the start site were chosen according to Muller et al. [12]. The viral promoter of pOPI3-Luc was then replaced by

the modified CAG promoter, generating pCAGop-Luc (see Materials and methods). Because there was no major difference between the LacI-expressing cell lines, we chose one, clone LacI3, for further studies. LacI3 cells were transiently transfected with either 500 ng pOPI3-Luc or 50 ng pCAGop-Luc and incubated for 24 h with or without 1 mM IPTG. Compared to the viral promoter construct and although the amount of transfected DNA was tenfold lower, the CAGop construct was more efficiently expressed and induced in ES cells, IPTG induction reaching 17-fold (fig. 2B). To further demonstrate operator sequence dependence of the IPTG induction, we also tested a CAG promoter construct where the operators were placed downstream of the intron, spacing them out 1 kb apart. In this case, the LacI binding has no effect on transcription, and this construct was no longer inducible by IPTG, showing constitutive expression (fig. 2C). We then characterized the inducibility of the CAGop promoter by performing transient expression experiments in the presence of increasing doses of IPTG. A dose-response of

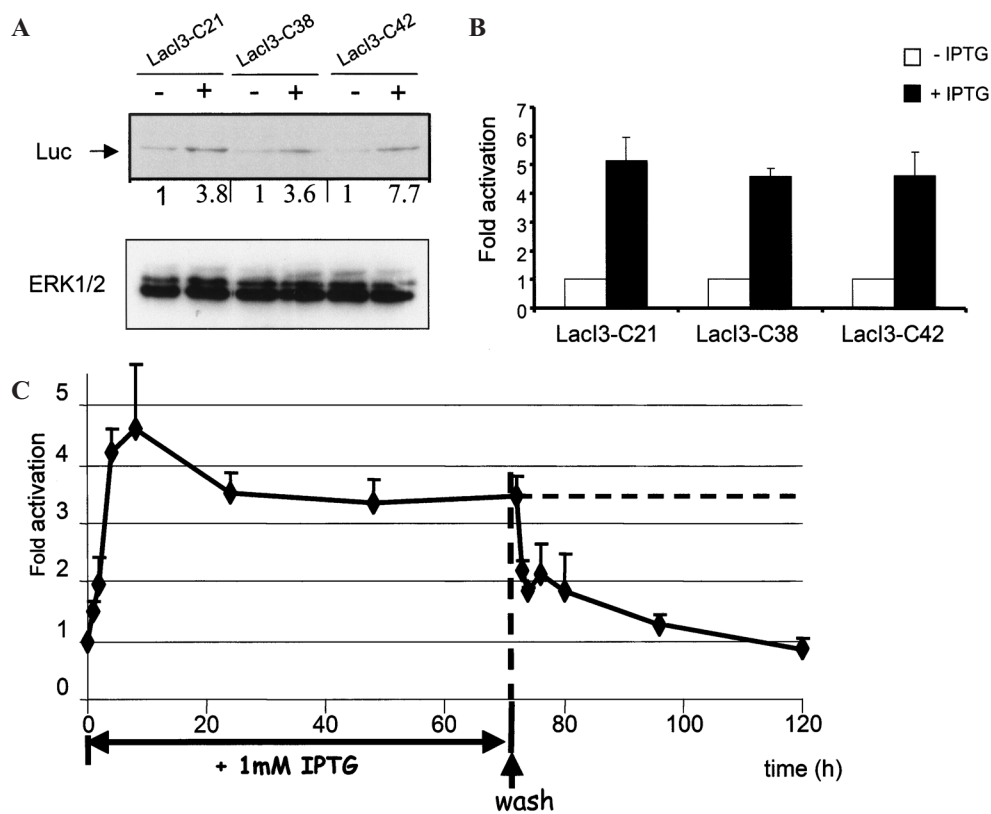


Figure 3. Establishment of ES clones stably expressing the luciferase gene in an IPTG-inducible and reversible manner. (A) ES-LacI3 cells were co-transfected with pCAGop-Luc and pGK-Neo. G418-resistant cells expressing Luc mRNAs in an IPTG inducible manner were cultivated with or without IPTG for 24 h and cellular lysates were analysed by western blotting using anti-Luc antibody. Luc expression signals were quantified, normalized to 1 for each clone without IPTG, and fold induction with IPTG is indicated. (B) The same ES clones were analysed for luciferase activity in the presence or absence of 1 mM IPTG for 24 h. Mean values of three independent experiments are shown. (C) Kinetics and reversibility of the luciferase induction: ES LacI3-C42 cells were cultivated in the presence or absence of 1 mM IPTG and analysed for luciferase activity after 1, 2, 4, 8, 24, 48 and 72 h. After the induction period, IPTG-treated cells were washed (arrow) and incubated with or without IPTG and analysed for luciferase activity after 1, 2, 4, 8, 24, 48 and 72 h. Graph represents luciferase activity normalized to 1 for non-stimulated cells.

derepressed luciferase expression was observed and maximum induction (15- to 20-fold) was reached with 50 μ M IPTG (fig. 2D). To test the system after stable integration into host chromosomes, LacI3-ES cells were co-transfected with pCAGop-Luc and a Neo-resistance selectable marker. G418-resistant colonies were isolated, expanded and individually tested for luciferase expression following a 24-hour IPTG induction. Three out of 30 resistant colonies expressed the luciferase gene and this expression was 3.8- to 7.7-fold inducible by IPTG, as measured either by the amount of luciferase protein or by luciferase activities (fig. 3A, B). The ES clone LacI3-C42 was analysed to test the kinetics and the reversibility of induction. Upon addition of 1 mM IPTG, induction of luciferase activity was readily detected as early as 1 h, reached a maximum by 4–8 h and stayed stable at that level for at least 3 days (fig. 3C). After washing of the cells to remove IPTG, reversibility of the induction was tested. Luciferase activity decreased with a kinetics as rapid as the induction; the diminution was detectable by 1 h and reached the basal level after 24 h (fig. 3C). To test this system in individual cells, we replaced luciferase with the GFP cDNA as a reporter gene in the

inducible construct and established stable transfectants by co-transfection with a Neo^R vector of LacI3-ES cells. Individual G418-resistant colonies were isolated and tested for GFP expression, with or without 24 hours IPTG treatment. A proportion, similar to that obtained with the luciferase construct (1/10), of resistant colonies expressed the GFP gene whose expression was seven- to tenfold IPTG inducible (fig. 4A). IPTG induction of the GFP was readily detected at the protein level by indirect immunofluorescence analysis of fields of ES cell monolayers using anti-GFP antibodies (fig. 4B). Direct fluorescence failed to visualize GFP protein expression, indicating that GFP expression in the presence of IPTG was moderated. Although variable from cell to cell, the IPTG induction of GFP expression occurred in every cell (fig. 4B). These results indicate that this inducible system is functional in all ES cells and is not restricted to a subpopulation of cells. We investigated the possibility of inducing the gene of interest after differentiation. We first tested the stability of LacI expression itself upon differentiation. RNAs from LacI3-ES cells were analysed at various times during the differentiation process from days 0 to 21. The expression of LacI was strong, constant and persisted after 3 weeks

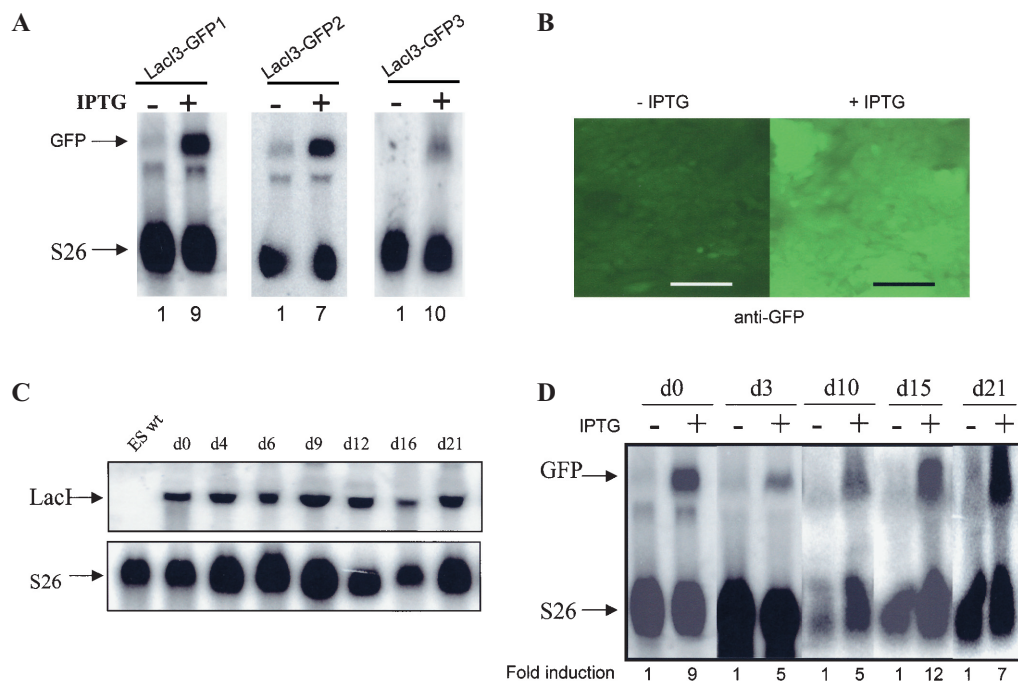


Figure 4. IPTG-inducible expression is conserved throughout the differentiation process. (A) ES-LacI3 cells were co-transfected with pCAGop-GFP and pGK-Neo. mRNAs of G418-resistant cells, cultivated with (+) or without (-) IPTG for 24 h, were analysed by Northern blot with GFP-radiolabelled probe and S26 probe as internal control. GFP expression signals were quantified, normalised to 1 for each clone without IPTG and fold induction with IPTG is indicated. (B) LacI3-GFP1 cells were analysed for GFP protein expression, in the presence or absence of 1 mM IPTG for 24 h, by indirect immunofluorescence using anti-GFP antibody. Acquisition parameters (exposition time) of the images were kept constant in \pm IPTG conditions. Bar, 200 μ m. (C) ES-LacI3 cells were induced to differentiate and mRNAs were extracted at the indicated days of differentiation, d0 corresponding to undifferentiated cells; mRNAs from wild-type (wt) ES cells were used as control. LacI expression was analysed by Northern blot with a LacI probe. (D) ES-LacI3-GFP1 cells were induced to differentiate, cultivated with or without 1 mM IPTG 1 day before the indicated day, and mRNAs were extracted at the indicated days of differentiation. GFP expression was analysed by Northern blot with a GFP probe. GFP expression signals were quantified, normalised to 1 for each day in the condition without IPTG and fold induction with IPTG is indicated.

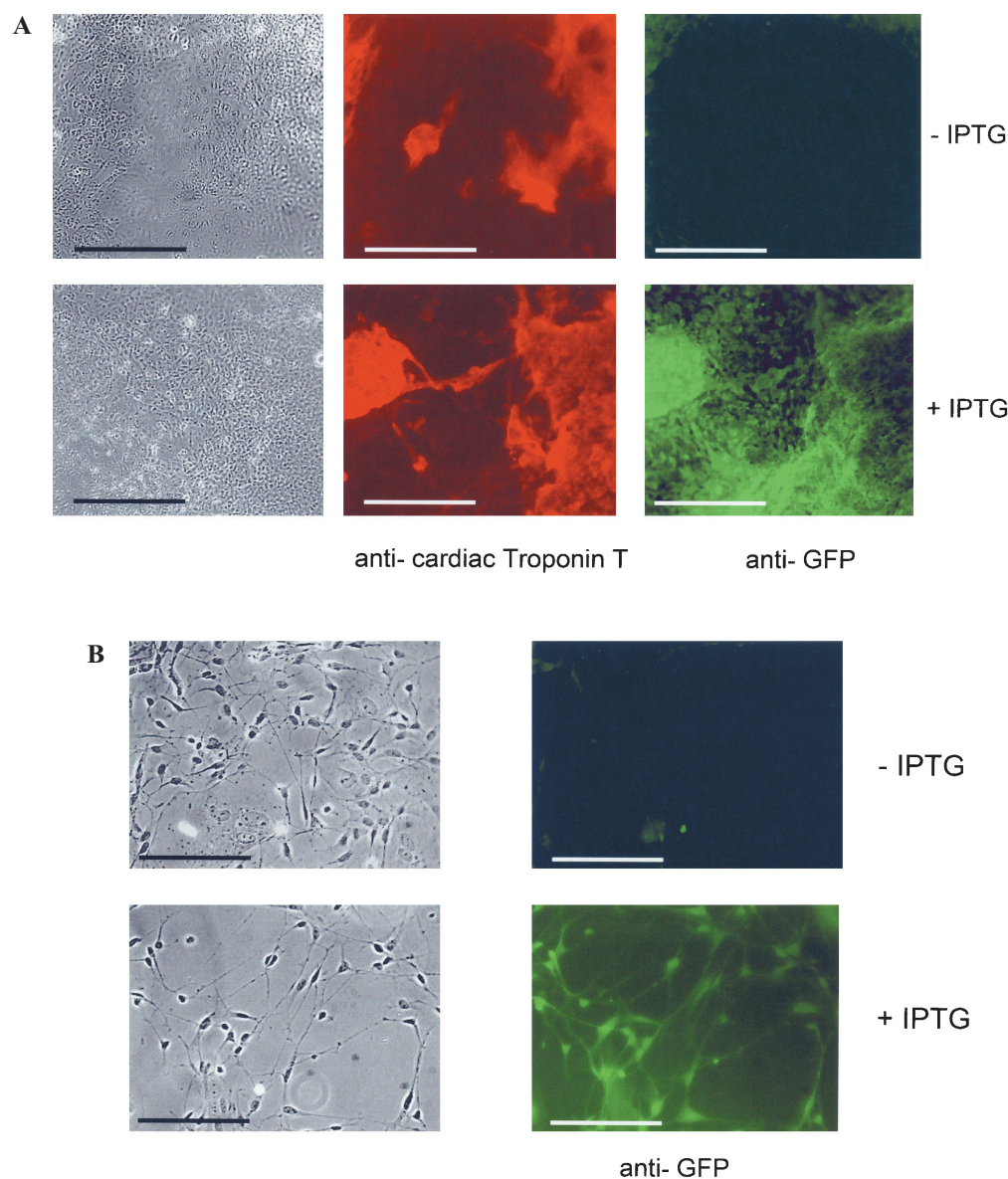


Figure 5. IPTG-inducible expression is maintained in well-differentiated individual cells. ES-LacI3-GFP1 cells were induced to differentiate in suspension to day 7 and EBs were seeded in petri dishes. After the appearance of cardiomyocyte-like and neuronal-like cells, by day 9, cultures were incubated with or without 1 mM IPTG for 24 h, fixed with paraformaldehyde and analysed by indirect immunofluorescence. Cultures were double stained with anti-cardiac troponin-T antibody, specific for cardiomyocytes, visualized in red, and anti-GFP antibody, visualized in green. (A) Representative fields showing cardiomyocyte-like cells (middle panels) are shown. Cardiac-like cells expressed GFP (in green) only in IPTG-treated cells (right panels). Phase contrast images of the same fields (left panels) are given. Bar, 200 μ m. (B) Representative fields showing neuronal-like cells expressing GFP after IPTG treatment (right panels) are shown. Phase contrast images of the same fields (left panels) are given. Acquisition parameters (exposition time) of the images were kept constant in \pm IPTG conditions. Bar, 100 μ m.

of differentiation (fig. 4C). As expected, expression of LacI as well as addition of 1 mM IPTG were well tolerated by ES cells, both in the undifferentiated state and after differentiation, as judged by their generation time and their capacity to form mature differentiated cells similarly to control ES cells (data not shown). We then examined the inducibility of the transgene in differentiated cultures of the LacI3-GFP1 ES cell line. Cells were differentiated without IPTG and GFP expression was monitored at

different times, with or without a 24-hour IPTG induction. Although variable in intensity (5- to 12-fold), sustained IPTG induction was conserved throughout the differentiation process (fig. 4D). These results validated the use of this system in cultures of differentiated ES cells. To investigate whether the induction was present not only in the total population of differentiated cells but also in individual well-differentiated cells, LacI3-GFP1 cells were induced to differentiate, without IPTG, into neuronal-like

or cardiomyocyte-like cells. EBs were treated or not with RA, and thereafter seeded in petri dishes. Cardiac-like cells (in the minus RA condition) and neuronal-like cells (in the RA condition) appeared by day 8 to 10. Cultures were then incubated with or without IPTG for 24 h. Cell cultures presenting contractile cardiomyocyte-like cells were fixed and analysed by immunofluorescence using both anti-cardiac troponin-T, a marker of cardiac cells [4], and anti-GFP antibodies. GFP expression was visualized with fluorescein isothiocyanate (FITC)-conjugated antibody and cardiac troponin-T expression was visualized with Texas red-conjugated antibody. As judged by the red staining, a subpopulation of differentiated ES cells, consisting of dispersed and discrete areas among other differentiated cells, corresponded to differentiated cardiac-like cells (fig. 5A, left and middle panels). In the presence of IPTG, cells presented a uniform induction of GFP expression and, importantly, GFP induction was achieved in all individual cardiac-like cells examined (fig. 5A, compare middle and right panels). In RA-treated cultures, differentiated neuronal-like cells were detected by phase contrast microscopy at the periphery of the outgrowth of the EBs and appeared as dispersed individual cells (fig. 5B, left panels). We found that GFP expression was IPTG induced in all the neuronal-like cells examined (fig. 5B, right panels).

Discussion

The Tet-inducible expression system is probably the most well-known and the most used system. However, attempts to develop this system in ES cells totally or partially failed. One reason is the use of viral promoters to express transgenes. These promoters are weakly expressed and highly methylated in ES cells, leading to transgene silencing [13]. This has been partially overcome by integrating transgenes into host chromosomes via homologous recombination at the ROSA 26 locus [14]. However, established cell lines from this Tet system present profound alteration of the differentiation process, independently of the gene of interest [15]. This adverse effect could be related to the known interference of the VP16 domain of the Tet/VP16 transactivator, which is a fusion between the bacterial Tet protein and the VP16 Herpes viral protein, with cellular functions [16]. We found that the Lac repressor system constitutes an interesting alternative to the Tet system.

Although functional in transient transfection experiments, classical viral-derived promoters, driving LacI cDNA and inducible reporter constructs, did not allow us to establish stable expressing cell lines. Similar to the Tet system, we attributed this problem to the low level of expression of viral promoters after chromosomal integration in ES cells [17]. By contrast, we found that the β -actin promoter,

which is strongly and ubiquitously expressed in many cells, in combination with the mammalianized LacI cDNA developed by Cronin et al. [6] confers a strong IPTG-regulatable expression in ES cells. Furthermore, this modified LacI/IPTG system is functional in both transient experiments and after stable integration into the genome. The non-induced basal levels of expression of both GFP and luciferase reporter genes were low and variable, reflecting a leakiness in LacI repression. This leakiness is higher than in bacterial cells and is detected in both transient experiments and after stable integration into the genome. These results indicate that the leakiness effect is likely due to variable integration sites and to the mammalian chromatin organization itself. Although functional in the present state, this induction system might be improved by reducing the leakiness. Of interest, for example, would be to investigate the effects of insertion of chromatin insulators [18] in the CAG-Op promoter.

Importantly, we systematically found a four- to tenfold induction by IPTG of the expression of each reporter gene in all ES cells analysed. Furthermore, the modified LacI repressor system that we have developed is functional not only before and after differentiation of ES cells but in individual differentiated cells such as cardiomyocyte-like and neuronal-like cells.

Thus we were able to show that the LacI repressor is functional in ES cells and constitutes an efficient IPTG-inducible expression system in both undifferentiated and differentiated ES stable transfectants, with rapid kinetics of induction and reversibility.

Compared to other inducible systems, this system presents several decisive advantages: (i) the use of a β -actin-derived gene promoter instead of viral promoters ensures a ubiquitous robust expression, avoiding gene silencing; (ii) the LacI bacterial protein and IPTG are neutral regarding mammalian cellular physiology, and are thus well tolerated, particularly by ES cells; (iii) no homologous recombination is necessary, standard co-transfections into ES cells are sufficient to produce positive ES clones; (iv) to our knowledge, this inducible system is the first to function from undifferentiated to individual well-differentiated ES cells; (v) the kinetics of induction and reversibility are rapid; (vi) finally, because similar plasmid constructs have been used in transgenic mice [6], construction of chimeric animals from ES cells bearing an inducible gene is conceivable and *in vivo* inducible, reversible expression of the gene is likely to be conserved. This system provides a very useful tool to understand the molecular mechanisms underlying ES cell self-renewal, commitment and differentiation.

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- 1 Lindvall O., Kokaia Z. and Martinez-Serrano A. (2004) Stem cell therapy for human neurodegenerative disorders—how to make it work. *Nat. Med.* **10**: S42–S50
- 2 Lee M. S., Lill M. and Makkar R. R. (2004) Stem cell transplantation in myocardial infarction. *Rev. Cardiovasc. Med.* **5**: 82–98
- 3 Smith A. G., Heath J. K., Donaldson D. D., Wong G. G., Moreau J., Stahl M. et al. (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**: 688–690
- 4 Wobus A. M. (2001) Potential of embryonic stem cells. *Mol Aspects Med* **22**: 149–164
- 5 Hu M. C. and Davidson N. (1987) The inducible lac operator-repressor system is functional in mammalian cells. *Cell* **48**: 555–566
- 6 Cronin C. A., Gluba W. and Scrabble H. (2001) The lac operator-repressor system is functional in the mouse. *Genes Dev.* **15**: 1506–1517
- 7 Scrabble H. (2002) Say when: reversible control of gene expression in the mouse by lac. *Semin. Cell. Dev. Biol.* **13**: 109–119.
- 8 Mountford P., Zevnik B., Duwel A., Nichols J., Li M., Dani C. et al. (1994) Dacistronic targeting constructs: reporters and modifiers of mammalian gene expression. *Proc. Natl. Acad. Sci. USA* **91**: 4303–4307
- 9 Bost F., Caron L., Marchetti I., Dani C., Le Marchand-Brustel Y. and Binetruy B. (2002) Retinoic acid activation of the ERK pathway is required for embryonic stem cell commitment into the adipocyte lineage. *Biochem. J.* **361**: 621–627
- 10 Niwa H., Yamamura K. and Miyazaki J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**: 193–199
- 11 Bost F., Caron L., Vial E., Montreau N., Marchetti I., Dejong V. et al. (2001) The defective transforming phenotype of c-Jun Ala(63/73) is rescued by mutation of the C-terminal phosphorylation site. *Oncogene* **20**: 7425–7429
- 12 Muller J., Oehler S. and Muller-Hill B. (1996) Repression of lac promoter as a function of distance, phase and quality of an auxiliary lac operator. *J. Mol. Biol.* **257**: 21–29
- 13 Chevassut T. and Lim B. (2003) Insights into the role of DNA methylation in murine embryonic stem cells using a modified tetracycline-inducible gene expression system. *Oncol. Res.* **13**: 373–380
- 14 Wutz A. and Jaenisch R. (2000) A shift from reversible to irreversible X inactivation is triggered during ES cell differentiation. *Mol. Cell* **5**: 695–705
- 15 Sonntag K. C., Simantov R., Kim K. S. and Isacson O. (2004) Temporally induced *Nurr1* can induce a non-neuronal dopaminergic cell type in embryonic stem cell differentiation. *Eur. J. Neurosci.* **19**: 1141–1152
- 16 Baron U., Gossen M. and Bujard H. (1997) Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Res.* **25**: 2723–2729
- 17 Chung S., Andersson T., Sonntag K. C., Bjorklund L., Isacson O. and Kim K. S. (2002) Analysis of different promoter systems for efficient transgene expression in mouse embryonic stem cell lines. *Stem Cells* **20**: 139–145
- 18 West A. G. and Fraser P. (2005) Remote control of gene transcription. *Hum. Mol. Genet.* **14**: R101–111



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