Unexpected expression pattern of tetracycline-regulated transgene in mice

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ABSTRACT

In generating a conditional transgenic murine model based on a tetracycline-regulated system, we obtained unexpected patterns of expression due to the transcriptional inactivity of the \textit{tet}-responder promoter. Here we show strong cell-type-restricted expression that was variegated to an extent determined by the number of responder transgene copies integrated into the host genome.

\textbf{Sequence data: None needed}

\textbf{Running head: Transgene silencing in a tet-off model}

\textbf{Key words:} transgenesis, \textit{tet-off} system, olfactory receptor neurons (ORNs).
The tetracycline-based *tet-off* system (Gossen and Bujard 1992) has emerged as a powerful tool for consistent and conditional induction of transcription of transgenic sequences (Bockamp *et al.* 2002). This has been exploited to generate transgenic organisms, including models for the study of pathological mechanisms involved in human diseases, such as nervous system targets (Mayford *et al.* 1996; Tremblay *et al.* 1998; Yamamoto *et al.* 2000; Lucas *et al.* 2001; SantaCruz *et al.* 2005). In this context, we planned a *tet*-conditional murine model (Fig. 1A) to dissect out the chain of events related to the G93A amino-acid substitution in the human superoxide dismutase type 1 (SOD1) protein sequence (Gurney *et al.* 1994), which leads to disease in a subset of familial amyotrophic lateral sclerosis (FALS).

**Restricted cell-type expression in vivo for both the EGFP and human SOD1 products:** We observed powerful induction of human SOD1 and EGFP (enhanced green fluorescent protein) expression in the transiently transfected NSC-34 motor-neuron-like cell line (Fig. 1B). This confirmed that our *tet-off* based system was working in a cell-culture context (Babetto *et al.* 2005). However, when eight independent responder transgenic mouse lines were generated, both the EGFP and human SOD1 products were restricted to some incoming projections towards the main and accessory olfactory bulbs in the double transgenic mice (MOB and AOB; Fig. 2A). An extended analysis of the olfactory mucosa revealed that the reporter EGFP signal could be ascribed exclusively to the mature olfactory receptor neurons (ORNs) (Fig. 2A). In contrast, there was no evidence of exogenous protein expression in the remaining cells of the olfactory epithelium or in the central nervous system (data not shown). This restricted pattern was observed in all of the scored animals (38 double-transgenic mice) along all of the transgenic lines, except for on a single occasion (data not shown), and it was confirmed by Western blotting (Fig. 2B). Analysis of total RNA samples revealed that the human SOD1 and EGFP mRNAs were present exclusively in extracts from the olfactory epithelium (OE; Fig. 2C). This observation strongly indicated that the responder promoter could be inactive at the transcriptional level, therefore leading to a lack of...
protein synthesis. However, the system showed fully functional behaviour in terms of doxycycline response in the expressing cells (Fig. 2D). Therefore, the ability to turn off expression of both EGFP and human SOD1 in the ORNs after administration of doxycycline demonstrated that in vivo the expressed tetracycline-controlled transactivator (tTA) protein retained all of its functional features.

The levels of expression in olfactory receptor neurons are inversely correlated with the number of responder transgene copies inserted: Immunohistochemical analysis of the OE showed that expression of EGFP in the ORNs was variegated (Fig. 3A), which could be related to the levels of the transgenic proteins in OE homogenates across the transgenic lines (Fig. 3B). The levels of both the human SOD1 and EGFP products were inversely proportional to the number of transgene responder copies (Fig. 3C), irrespective of the linear construct tested (Fig. 3B). This trend is consistent with the repeat-induced transgene silencing theory (Garrick et al. 1998) observed in constitutive-expressing transgenes. The presence of a major number of transgene copies has been associated to a major chance of epigenetic repression (Manuelidis 1991; Jones and Takai 2001). Thus, in our mice, the responder sequences might favour the attraction of repressing states in concomitance with an increase in copy numbers, leading to a minor number of expressing ORNs.

The responder transgene sequences as the cause of the unexpected expression pattern: There is documented evidence that random integration of transgenes can fail to drive consistent exogenous expression due to several factors, such as genome position influences (Sabl and Henikoff 1996), mouse genetic background (Robertson et al. 2002; Opsahl et al. 2002; Padjen et al. 2005), number (Saveliev et al. 2003) and orientation (Stam et al. 1998), or sequence composition (Ramírez et al. 2001; Lotti et al. 2002) of transgene-item repeats, especially if viral sequences are included (Schumacher et al. 2000). Failure of tet-based strategies in vivo has also been reported. This has been related to either defective tTA expression (Böger and Gruss 1999; Fedorov et al. 2001; Lee et
al. 2006) or to epigenetic repression on the tet-promoter activity (Janicki et al. 2004; Pankiewicz et al. 2005; Kues et al. 2006), in addition to undesired interactions of eukaryotic cell factors with the tet-promoter sequences (Rang and Will 2000; Gould and Chernajovsky 2004).

In our mice, the genomic insertion context had little influence on the pattern of distribution observed for EGFP/human SOD1 expression, since the same distribution patterns occurred in eight different responder transgenic lines (data not shown). Neither qualitative nor quantitative tTA defects can explain the expression patterns displayed by our transgenic lines (Supplementary Information, and data not shown). Therefore, consistent data support the ubiquitous production of tTA and the effective transcriptional activation of tet-promoters (Boy et al. 2006) when other responder mouse lines are crossed to the same PrP-tTA activator line used in this study. Moreover, the unexpected pattern observed in our mice persisted after changing some responder lines to the FVB/N strain background (Fig. 2B, and data not shown).

The lack of EGFP and/or human SOD1 mRNAs can be interpreted as a consequence of absence of transcriptional activation of the responder promoter in almost all cell types. The proportional trend between human SOD1 and EGFP production (Fig. 3B, D) suggests that the system works bidirectionally (Baron et al. 1995; Krestel et al. 2001), and reinforces the hypothesis of transcriptional inactivity since the EGFP and human SOD1 coding sequences share the same transcriptional link. In addition, the striking correspondence between mRNA and protein levels supports this impression (Fig. 3D). Otherwise, it must be assumed that posttranscriptional regulation affected each independent element separately and along all of the transgenic lines. The expression variegation observed at the ORNs, and specially the inverse dependence of expression levels to responder transgene copy numbers, could be indirectly indicative of repressing states affecting the responder sequences and might also be influenced by the composition of our transgene sequences. This is dramatically underlined by the same outcome obtained in all of the responder
transgenic lines generated. In conclusion, we raise concerns about the composition of the responder transgene sequences, as the aberrant patterns of transgene expression can be dramatic with the use of these murine models to study human diseases, which can be extended to \textit{in vivo} conditional RNA-interference-related technologies.

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LITERATURE CITED


FIGURE LEGENDS

Figure 1. Experimental settings

A. The tetracycline-controlled transactivator (tTA) binds the core promoter (TRE; tetracycline-responder element) and activates bidirectional expression (P1, P2; minimal promoters of cytomegalovirus). Antibiotic administration (DOX; the tetracycline derivative doxycycline) abolishes transcriptional activation (OFF state). The cDNA coding for a mutated version of human SOD1 (hSOD1) was cloned into the pBI-EGFP vector (Clontech, Palo Alto, CA, USA) and two linear constructs were generated: 4.4 kb (top; EGFP-TRE-hG93ASOD1cDNA; 997, 995, 497 transgenic lines); 2.4 kb (bottom; TRE-hG93ASOD1cDNA; 450, 446, 428, 419, 413 transgenic lines) and microinjected into BDF1 (C57BL/6xDBA/2) oocytes. The activator mouse line PrP-tTA F959 in the FVB/N strain (Tremblay et al. 1998) was crossed to each responder transgenic line to obtain double-transgenic mice. B. Transiently transfected NS34 (green, EGFP; red, monoclonal anti-human SOD1 antibody; MBL International, Woburn, MA, USA). Scale bar, 50 μm. A representative immunoblot is shown. R: responder plasmid alone; A+R: activator and responder plasmid; E: empty responder vector; NT: non-transfected cells; msod1, murine superoxide dismutase type I.

Figure 2. Restricted patterns of expression and doxycycline dependence

A. Representative pattern of expression in tissue sections obtained by transcardial perfusion of double-transgenic mice (2-5 animals per line). Main (MOB; M-1, scale bar 800 μm, M-2 to M-4, scale bar 100 μm) and accessory (AOB; A-1 to A-3, scale bar 25 μm) olfactory bulbs. Olfactory receptor neurons (O-1, scale bar 10 μm) and olfactory epithelium (OE; O-2, 500 μm). B. Representative immunoblots (anti-EGFP monoclonal; Roche, Manheim, Germany; anti-SOD1; Upstate, Lake Placid, NY, USA; monoclonal anti-β-actin; Immunological Sciences, Roma, Italy). Left panel, homogenates from double-transgenic mice from a representative EGFP-TRE-
hG93ASOD1cDNA responder line in the BDF1 mouse strain background (tTA immunoblot in Supplementary Information). Right panel, the same situation after changing the background of the same line to FVB/N (up to 8th generation). C. Northern blots (2-3 double transgenic mice from the three EGFP-TRE-hG93ASOD1cDNA lines). Insert below right: controls; 1, double-transgenic mouse; 2, non-transgenic mouse. D. RT-PCR and representative tissue sections of doxycycline-treated mice dosed as indicated in drinking water with 5% sucrose over one week, compared to non-treated (No-dox) counterparts (3-6 mice/group). Glomerular layer in the main olfactory bulb (M-1 and M-4, 200 µm; M-2 and M5, 100 µm) and axonal projections (M-3 and M-6, 50 µm) are shown. Stars indicate weak and low numbers of autofluorescent glomeruli. OE, olfactory epithelium; SC, spinal cord; B, brain; Cev, cerebellum; msod1, msod2, murine superoxide dismutase types I, II.

Figure 3. Degree of copy-number-dependent expression variegation in the olfactory epithelium

A. EGFP (green) signal in olfactory marker protein (OMP)-stained (red) sections of olfactory epithelium of EGFP-TRE-hG93ASOD1cDNA double-transgenic mice. Scale bars: 20 µm (997, 497), 30 µm (995). B. Quantification of protein immunoblots from olfactory epithelium extracts (3-5 mice/ transgenic line), normalized to β-actin. C. Human SOD1 protein levels (fold levels) normalized to β-actin and number of responder transgene copies (Nresponder or N, insert right) estimated by real-time PCR and Southern blotting (data not shown). Insert centre: immunoblot showing decrease of hSOD1 levels when responder copies increase only ten-fold. D. Correlation between mean mRNA and protein levels based on Western and Northern blots (Fig. 1C, and data not shown).
A

iTA protein

SV40 pA

EGFP

TRE

hSOD1

β-globin pA

+/− DOX

OFF state

B

EGFP

hSOD1

R A+R E NT

hSOD1

mSOD1
### A 

MOB  

**M-1**  

**MOB**  

**AOB**  

**OE**  

![Images of MOB, AOB, and OE with merged images of hSOD1, EGFP, and β-actin](image)

### B 

#### BDF1  

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### C 

**OE**  

![Images of hSOD1, EGFP, and β-actin](image)

**SC**  

![Images of hSOD1, EGFP, and β-actin](image)

**B**  

![Images of hSOD1, EGFP, and β-actin](image)

*PrP-tTA*  

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### D 

**No DOX**  

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**+DOX 4 mg/ml**  

![Images of MOB with merged images of hSOD1, EGFP, and msod2](image)
A

EGFP-TRE-hG93ASOD1 cDNA lines

B

EGFP/β-actin protein levels

C

Human SOD1 protein / N_{responder} 

D

Levels of protein

Levels of mRNA