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Note

Unexpected Expression Pattern of Tetracycline-Regulated Transgenes 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 *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.

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 (Figure 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.

Restricted cell-type expression *in vivo* for both enhanced green fluorescent protein and human SOD1 products: We observed a powerful induction of human SOD1 and enhanced green fluorescent protein (EGFP) expression in the transiently transfected NSC-34 motor-neuron-like cell line (Figure 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 EGFP and human SOD1 products were restricted to some incoming projections toward the main and accessory olfactory bulbs in the double-transgenic mice (MOB and AOB in Figure 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) (Figure 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 on a single occasion (data not shown), and it was confirmed by Western blotting (Figure 2B). Analysis of total RNA samples revealed that the human SOD1 and EGFP mRNAs were present exclusively in extracts from the olfactory epithelium (OE; Figure 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 behavior in terms of doxycycline response in the expressing cells (Figure 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 efficiency of transgene expression in olfactory receptor neurons is 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 (Figure 3A), which could be related to the levels of the transgenic proteins in OE homogenates across the transgenic lines (Figure 3B). The levels of production of both the human SOD1 and EGFP were inversely proportional to the number of transgene responder

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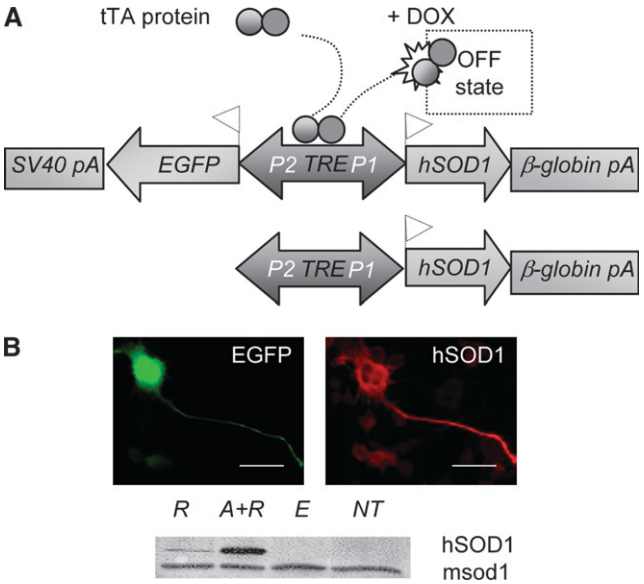

Figure 1.—Experimental settings. (A) The tTA binds the core promoter (*TRE*; *tetracycline-responder element*) and activates bidirectional expression (*P1*, *P2*; minimal promoters of *cytomegalovirus*). Antibiotic administration (DOX; the tetracycline derivative doxycycline) abolishes transcriptional 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) and two linear constructs—4.4 kb (top: *EGFP-TRE-hG93ASOD1cDNA*; *997*, *995*, *497* transgenic lines) and 2.4 kb (bottom: *TRE-hG93ASOD1cDNA*; *450*, *446*, *428*, *419*, *413* transgenic lines)—were generated and micro-injected into BDF1 (C57BL/6xDBA/2) fertilized 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 cells (green, EGFP; red, monoclonal anti-human SOD1 antibody; MBL International, Woburn, MA). A representative immunoblot is shown. Bar, 50 μm. *R*, responder plasmid alone; *A+R*, activator and responder plasmid; *E*, empty responder vector; *NT*, nontransfected cells; msod1, murine superoxide dismutase type I.

copies (Figure 3C), irrespective of the linear construct tested (Figure 3B). This trend is consistent with the repeat-induced transgene-silencing theory (Garrick *et al.* 1998) observed in constitutively expressing transgenes. The presence of a major number of transgene copies has been associated with a major chance of epigenetic repression (Manuelidis 1991; Jones and Takai 2001). Thus, in our mice, the responder sequences might favor 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 (Opsahl *et al.* 2002; Padjen *et al.* 2005) and number (Saveliev *et al.* 2003) and orientation (Stam *et al.* 1998) or sequence composition (Ramírez *et al.* 2001) 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 either to 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 (supplemental 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 (Figure 2B and data not shown).

The lack of EGFP and/or human SOD1 mRNAs can be interpreted as a consequence of the absence of transcriptional activation of the responder promoter in almost all cell types. The proportional trend between human SOD1 and EGFP production (Figure 3, B and D) suggests that the system works bidirectionally (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 (Figure 3D). Otherwise, it must be assumed that post-transcriptional regulation affected each independent element separately and along all of the transgenic lines. The expression variegation observed at the ORNs, and especially the inverse dependence of expression levels on 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 having obtained the same outcome in all of the responder transgenic lines that were generated. In conclusion, we raise concerns about the composition of the responder transgene sequences because the aberrant patterns of transgene expression can be dramatic when these murine models are used to study human diseases, a concern that can be

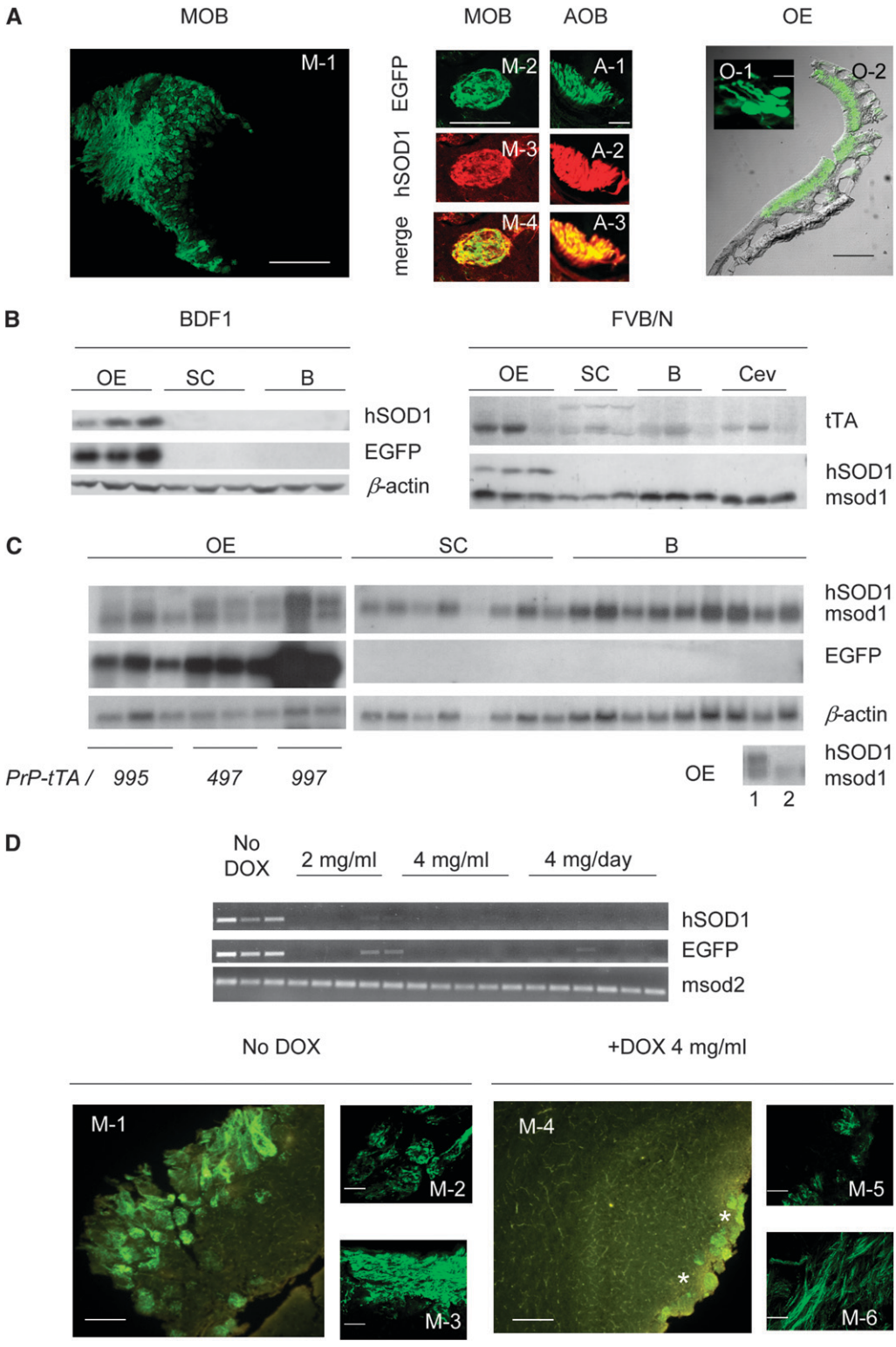

FIGURE 2.—Restricted patterns of expression and doxycycline dependence. (A) Representative pattern of expression in tissue sections obtained by transcardial perfusion of double-transgenic mice (two to five animals/line). Main olfactory bulbs (MOB, M-1: bar, 800 μm; M-2–M-4: bar, 100 μm) and accessory olfactory bulbs (AOB, A-1–A-3: bar, 25 μm). Olfactory receptor neurons (O-1: bar, 10 μm) and olfactory epithelium (OE, O-2: bar, 500 μm). (B) Representative immunoblots [anti-EGFP monoclonal (Roche, Manheim, Germany); anti-SOD1 (Upstate, Lake Placid, NY); and monoclonal anti-β-actin (Immunological Sciences, Rome)]. (Left) Homogenates from double-transgenic mice from a representative *EGFP-TRE-hG93ASOD1cDNA* responder line in the BDF1 mouse strain background (tTA immunoblot in supplemental information). (Right) The same situation after changing the background of the same line to FVB/N (up to the eighth generation). (C) Northern blots (two to three double-transgenic mice from the three *EGFP-TRE-hG93A-SOD1cDNA* lines). (Inset bottom right) Controls: 1, double-transgenic mouse; 2, nontransgenic mouse. (D) RT–PCR and representative tissue sections of doxycycline-treated mice dosed as indicated in drinking water with 5% sucrose over 1 week, compared to nontreated (No DOX) counterparts (three to six mice/group). The glomerular layer in the main olfactory bulb (M-1 and M-4: bar, 200 μm; M-2 and M-5: bar, 100 μm) and axonal projections (M-3 and M-6: bar, 50 μm) are shown. Stars indicate weak and low numbers of autofluorescent glomeruli. OE, olfactory epithelium; SC, spinal cord; B, brain; Cev, cerebellum; msod1 and msod2, murine superoxide dismutase types I and II.

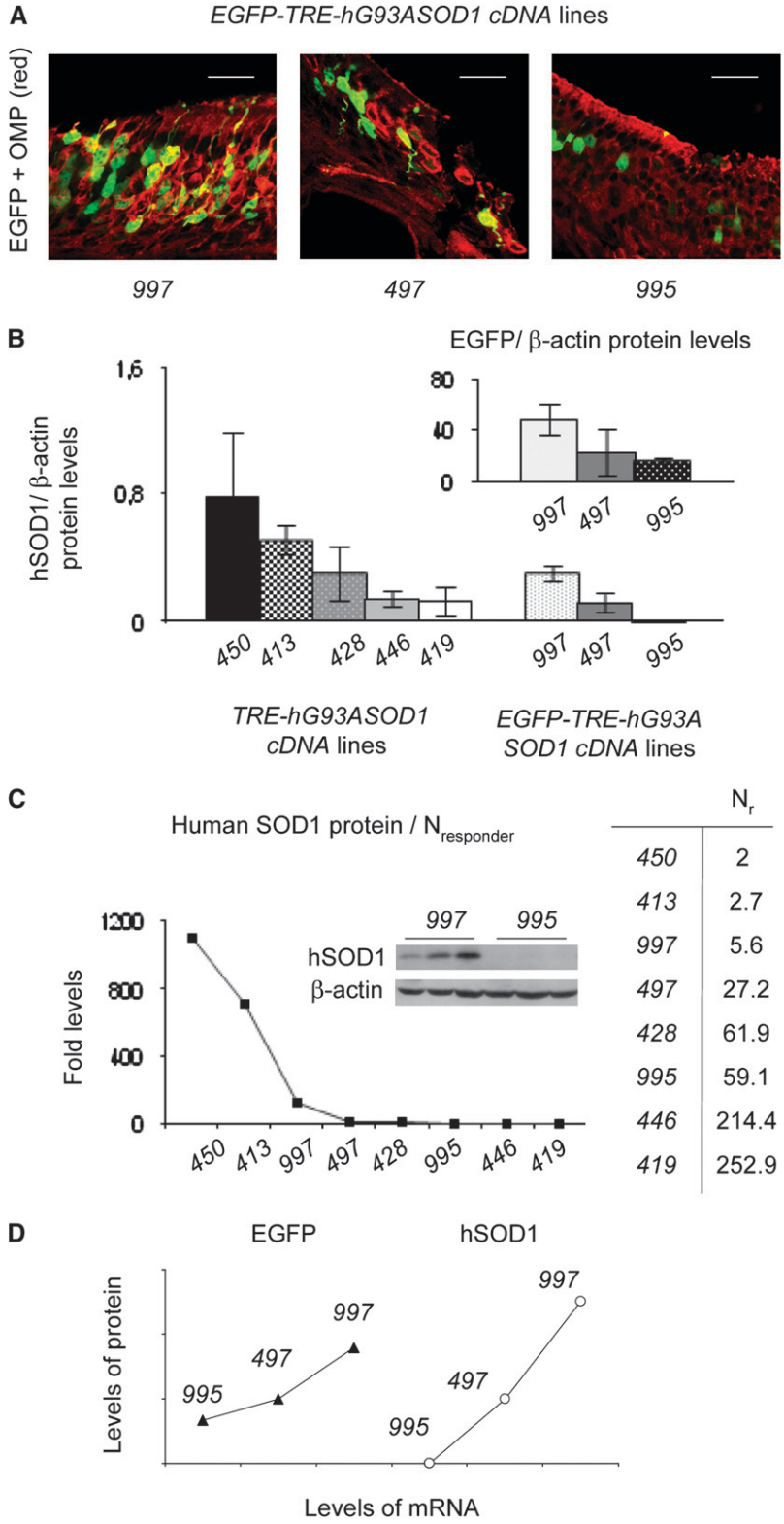

FIGURE 3.—Degree of copy-number-dependent expression variegation in the olfactory epithelium. (A) EGFP (green) signal in OMP-stained (red) sections of olfactory epithelium of *EGFP-TRE-hG93ASOD1cDNA* double-transgenic mice. Bars: 20 μm (997 and 497) and 30 μm (995). (B) Quantification of protein immunoblots from olfactory epithelium extracts (three to five mice/transgenic line), normalized to β-actin. (C) Efficiency of human SOD1 expression (fold levels). Protein levels normalized to β-actin and the number of responder transgene copies [$N_{responder}$ (N_r), inset right] estimated by real-time PCR and Southern blotting (data not shown). Inset center: immunoblot showing decrease of hSOD1 production when responder copies increase only 10-fold. (D) Correlation between mean mRNA and protein levels based on Western and Northern blots (Figure 1C and data not shown).

extended to *in vivo* conditional RNA-interference-related technologies.

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