Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 12580–12585, October 1998
Medical Sciences

Doxycycline control of prion protein transgene expression modulates prion disease in mice

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Contributed by Stanley B. Prusiner, August 20, 1998

ABSTRACT **Conversion of the cellular prion protein (PrP^C) into the pathogenic isoform (PrP^{Sc}) is the fundamental event underlying transmission and pathogenesis of prion diseases. To control the expression of PrP^C in transgenic (Tg) mice, we used a tetracycline controlled transactivator (tTA) driven by the PrP gene control elements and a tTA-responsive promoter linked to a PrP gene [Gossen, M. and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5547–5551]. Adult Tg mice showed no deleterious effects upon repression of PrP^C expression (>90%) by oral doxycycline, but the mice developed progressive ataxia at ≈50 days after inoculation with prions unless maintained on doxycycline. Although Tg mice on doxycycline accumulated low levels of PrP^{Sc}, they showed no neurologic dysfunction, indicating that low levels of PrP^{Sc} can be tolerated. Use of the tTA system to control PrP expression allowed production of Tg mice with high levels of PrP that otherwise cause many embryonic and neonatal deaths. Measurement of PrP^{Sc} clearance in Tg mice should be possible, facilitating the development of pharmacotherapeutics.**

Prion diseases are fatal neurodegenerative illnesses that can present as genetic, sporadic, or infectious disorders (1). In these diseases, the normal, cellular prion protein (PrP^C), which is encoded by a chromosomal gene, undergoes a posttranslational modification to generate the pathogenic isoform PrP^{Sc}). Prion diseases include scrapie of sheep and goats, bovine spongiform encephalopathy, and Creutzfeldt–Jakob disease of humans.

The function of PrP^C is unknown. In two lines of PrP-deficient ($Prnp^{0/0}$) mice, no clinical signs of illness have been reported, and the animals seem to develop normally (2, 3). Brain slices from these two $Prnp^{0/0}$ lines have been reported to show defective neurotransmission at GABAergic synapses and diminished long-term potentiation (4, 5). In two other studies, no detectable electrophysiological defects could be identified in one of the $Prnp^{0/0}$ lines (6, 7). However, defective sleep–wake cycles and altered circadian rhythms have been reported for both of these $Prnp^{0/0}$ lines (8). Subsequently, an additional line of $Prnp^{0/0}$ mice was reported to develop ataxia due to Purkinje cell degeneration at about 70 weeks of age (9). Whether PrP has a role in Cu^{2+} metabolism is unclear, but studies of Cu^{2+} in $Prnp^{0/0}$ mice support such a hypothesis (10). Several studies have suggested that PrP may bind Cu^{2+} in the octarepeat region (11). Two Cu^{2+} ions appear to be bound per PrP molecule through a square planar geometry (12).

To challenge the hypothesis that PrP^{Sc} is required for transmission and pathogenesis of prion disease (13), $Prnp^{0/0}$ mice were inoculated with prions. These PrP-deficient mice neither developed disease (14) nor replicated prions (15, 16). Moreover, mice hemizygous for PrP gene ablation showed prolonged incubation times (15, 17, 18).

On this background, we undertook development of a system where the level of PrP expression could be regulated to modulate the rate of prion formation. We chose the tetracycline-responsive gene system that was developed by using the *Escherichia coli* tetracycline resistance Tn10 operon (19). It makes use of a transactivator (tTA) obtained by fusing the tetracycline repressor with the transactivation domain of the herpes simplex virus VP16 transcription factor. The tTA binds specifically with high affinity to the tetracycline operator (tetO) and activates transcription from a minimal promoter linked to the target gene. Binding of doxycycline, a tetracycline analog, to tTA prevents the protein from binding to the tetO region, thereby preventing target gene expression.

MATERIALS AND METHODS

Constructs. Target constructs containing the MoPrP-A ORF were obtained by cloning a 5-kbp promoterless $Prnp^a$ genomic fragment (*Xba*I) excised from pPrP-5′HG (20) downstream from the tTA responsive promoter at the *Xba*I site of the pUHD10-3 vector. This $Prnp^a$ minigene contains the first, second, and third exons of MoPrP-A together with the 3′ untranslated region. The transactivator construct was generated with the *Eco*RI–*Bam*HI tTA fragment from pUHD15-1 (19). Fragments were rendered blunt by using Klenow enzyme and introduced into the *Sal*I-digested blunt-ended CosSHa.tet vector as described (21, 22).

Tg Mice. Tg mice were obtained by microinjection of FVB. $Prnp^{0/0}$ N4F1 obtained by crossing $Prnp^{+/0}$ with FVB animals for four generations before interbreeding to homozygosity. Breeding and screening of Tg mice was performed as described (21). Antibiotics were administered as follows. Doxycycline (2 mg/ml) was added to the drinking water with 5% sucrose to mask the bitter taste. Alternatively, a 21-day-release doxycycline or minocycline pellet of 50 or 200 mg (Innovative Research of America, Sarasota, FL) was surgically implanted subcutaneously. Intravenous injection of doxycycline at 25 mg/kg was carried out in the tail vein once a day for 3 days before animals were terminated on the fourth day.

Immunodetection. Protein concentration was assessed for cell lysates and brain homogenates by bicinchoninic acid assay

Abbreviations: PrP, prion protein; PrP^C, cellular isoform of PrP; PrP^{Sc}, pathogenic isoform of PrP; Tg, transgenic; tTA, tetracycline transactivator; $Prnp^{0/0}$, PrP-deficient mice; tetO, tetracycline operator; CNS, central nervous system; GFAP, glial fibrillary acidic protein.

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(Pierce). Western blot analyses were performed as already described (23) by using an enhanced chemiluminescent detection method (Amersham) with the RO73 polyclonal α-PrP antiserum at 1:5000 dilution. Histoblots were performed on coronal brain sections (10 mm), transferred onto a nitrocellulose membrane, and processed for immunohistochemistry by using the α-PrP polyclonal RO73 antiserum as described (24).

Pathology. Central nervous system (CNS), sciatic nerve, and muscle histology were assessed by standard procedures as described (21, 25). Brains were taken and immediately immersion-fixed in 10% buffered formalin. Specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin for neuropathological evaluation. Evaluation of reactive astrocytic gliosis was performed by immunostaining of glial fibrillary acidic protein (GFAP) by using an α-bovine-GFAP rabbit antiserum (Dako) as described (26).

RESULTS

Establishment of Tg(Prnp-tTA) and Tg(tetO-PrP) Lines. The tTA gene (19) was introduced into the CosSHa.tet cosmid expression vector (22) and microinjected into *Prnp*$^{0/0}$ zygotes to establish three Tg(Prnp-tTA)FVB/*Prnp*$^{0/0}$ lines (Table 1). Individual lines were characterized by assessing the level and distribution of mRNA by *in situ* hybridization by using antisense tTA as well as Prnp probes for wild-type FVB mice. The Tg(Prnp-tTA/F973) mice exhibited the highest expression levels followed by the Tg(Prnp-tTA/F959) and Tg(Prnp-tTA/F966) lines, respectively (Table 1). Expression was detected throughout the brain with the strongest signals in the cortex and hippocampus and lower levels in the thalamic, hypothalamic, and brainstem regions. Interestingly, the cerebellar granular layer of Tg(Prnp-tTA) mice presented a stronger expression signal relative to that of Prnp signals observed in FVB and Tg(MoPrP-A)4053/*Prnp*$^{0/0}$ mice (data not shown).

Target MoPrP-A transgenes were constructed by introducing a promoterless MoPrP-A minigene downstream from the tetO-heptamer linked to a CMV IE minimal promoter (designated tetO-PrP). The construct was introduced into *Prnp*$^{0/0}$ zygotes to establish five Tg lines referred to as Tg(tetO-PrP) (Table 2). Brains from Tg(tetO-PrP) lines displayed basal expression, ranging from less than 1% to 50% of that found in FVB mice as determined by immunoblot by using α-PrP RO73 rabbit antiserum (27).

Production of Tg(tTA:PrP) Mice. The Tg(tetO-PrP) target lines were selected for inducibility prior to breeding with the tTA lines. After primary fibroblast cultures were established and transfected with CMV-tTA, MoPrP expression was measured. The Tg(tetO-PrP/E6740) and Tg(tetO-PrP/E6550) lines exhibited high inducibility, whereas Tg(tetO-PrP/E7655) mice showed moderate induction (Table 2). These mice were bred to the transactivator Tg(Prnp-tTA/F959) and Tg(Prnp-tTA/F966) lines (Table 3). For convenience, animals expressing both the Prnp-tTA and tetO-PrP transgenes are designated Tg(tTA:PrP) mice. Tg(tTA:PrP) mice untreated or treated up to 4 weeks with doxycycline administered orally (2 mg/ml doxycycline) were tested for PrP^{C} expression. Brains processed for Western blots (Fig. 1) displayed high levels of PrP expression that could be repressed by treatment with tetracycline analogs.

Table 1. Characteristics of tTA lines

Line	Expression level, mRNA	Ability to induce target line
Prnp-tTA/F959	+++	++
Prnp-tTA/F966	++	++
Prnp-tTA/F973	+++	ND

Table 2. Characteristics of target lines

Line	Basal PrP-A expression, %*	Induction by tTA	
		Fibroblasts	Tg mice
tetO-PrP/E6539	<1	++	−
tetO-PrP/E6550	25–50	++++	+++
tetO-PrP/E6725	10	++	ND
tetO-PrP/E6740	10	++++	++
tetO-PrP/E7655	2	++	+

ND, not determined.
*Expression levels reported were standardized by using FVB PrP^{C} levels as 100%

The genotypic distribution of Tg(Prnp-tTA × tetO-PrP)F_1 mice obtained by crossing Tg(Prnp-tTA) with Tg(tetO-PrP) was non-Mendelian. For example, from the 460 pups obtained by using Tg(Prnp-tTA/F959) crossed with Tg(tetO-PrP/E6740), 92 (20.0%) died during the first 2 weeks of life and out of the 368 remaining, only 28 (7.6%) were found to harbor both transgenes (Table 4). Out of 4 randomly selected litters ranging from 2 to 14 days of age ($n = 33$), 7 (21%) severely runted pups were sacrificed and found to be the only double Tg neonates among these litters. The proportion of double Tg mice (24.0%) that survived through weaning was restored to the expected Mendelian ratio when mating pairs were kept on doxycycline during the entire pregnancy and through weaning of the litters (Table 4). A similar phenomenon was observed when Tg(Prnp-tTA/F966) mice were crossed with Tg(tetO-PrP/E6740) (Table 4). Doxycycline treatment initiated during the last third of the pregnancy appeared sufficient to rescue Tg pups harboring both transgenes (Table 4). These observations implicate the overexpression of wt MoPrP^{C} during the last third of the gestation or during the neonatal period as a cause of neonatal death of Tg(tTA:PrP) mice.

Regulation of PrP^{C} Expression *in Vivo*. We examined the ability of tetracycline analogs to repress the expression of MoPrP^{C} mediated by tTA in Tg mice. Crosses of Tg(Prnp-tTA/F959) mice with Tg(tetO-PrP/E7655), Tg(tetO-PrP/E6740), or Tg(tetO-PrP/E6550) mice produced Tg(tTA:PrP)2, Tg(tTA:PrP)3, and Tg(tTA:PrP)4 mice, respectively (Table 3). The levels of inducible MoPrP^{C} expression in these three lines of Tg(tTA:PrP) mice were 0.5- to 8-fold that of wild-type FVB mice (Table 3; Fig. 1 *A* and *B*). Although induction of MoPrP^{C} expression was also observed when Tg(Prnp-tTA/F966) mice were crossed with Tg(tetO-PrP/E6740) mice to produce Tg(tTA:PrP)1 mice, crosses using Tg(Prnp-tTA/F959) mice were superior with respect to the higher levels of expression obtained (Fig. 1 *A* and *B*).

Of all the Tg mice studied, the best regulated expression of MoPrP^{C} by doxycycline was found in Tg(tTA:PrP)3 mice. Basal expression of MoPrP^{C} after 7 days of doxycycline treatment was ≈15% of that found in FVB mice, whereas expression in the absence of doxycycline was ≈2-fold that of FVB (Table 3, Fig. 1 *A* and *C*).

MoPrP^{C} expression was nearly fully repressed by various treatments with tetracycline analogs as demonstrated by using Tg(tTA:PrP)1 mice: 7 days of doxycycline in the drinking water (2 mg/ml), 7 days of a 50 or a 200 mg subcutaneous doxycycline pellet (21-day release period), 7 days of a 200 mg minocycline subcutaneous pellet (21-day release), or 3 days of a daily intravenous injection of doxycycline (25 mg/kg) (Fig. 1*B*). The residual expression is unlikely because of an insufficient dose of antibiotic because it was observed in all animals treated, irrespective of the route or dose administered. A 30-day oral doxycycline treatment of Tg(tTA:PrP)1 mice was effective in repressing expression to levels equivalent to those seen in target Tg(tetO-PrP/E6740) mice (Fig. 1*B*).

Repression of MoPrP^{C} Expression in Adult Mice. A time course of repression and induction was performed by using

Table 3. Characteristics of regulated Tg lines

Regulated Tg line	Genotype	Residual expression, %*†			Induction level, %
		2 days	7 days	30 days	
Tg(tTA:PrP)1	tTA/F966:PrP/E6740	50	15	10	100–200
Tg(tTA:PrP)2	tTA/F959:PrP/E7655	ND	5	ND	50–100
Tg(tTA:PrP)3	tTA/F959:PrP/E6740	ND	15	ND	200–400
Tg(tTA:PrP)4	tTA/F959:PrP/E6550	ND	50–100	ND	400–800

*Expression levels reported were standardized by using FVB PrP^c levels as 100%. †Measurements were made after the mice were orally administered doxycycline (2 mg/ml) for 2, 7, or 30 days, as indicated. ND, not determined.

doxycycline administered orally (2 mg/ml) to Tg(tTA:PrP)1 mice (Fig. 1*B*). PrP^C expression was repressed ≈50% after 2 days of treatment and nearly fully repressed after 7 days; baseline levels were found in Tg(tTA:PrP)1 mice sacrificed after 30 days of treatment. Stopping doxycycline after 7 days produced an ≈50% reinduction of PrP^C expression within 2 days, and complete reactivation was observed by 7 days.

Two-month-old adult Tg(tTA:PrP)1 and 3 mice remained well for 30 days with no signs of systemic or CNS dysfunction when PrP expression was repressed ≈90% to basal levels by oral doxycycline. The Tg(tTA:PrP) mice were either treated with doxycycline in the drinking water for 1 month ($n = 8$) or untreated ($n = 7$). Groups of age-matched controls consisting of $Prnp^{0/0}$ ($n = 8$), FVB ($n = 8$), Tg(MoPrP-A)4053/$Prnp^{0/0}$ ($n = 10$), Tg(Prnp-tTA/F959) ($n = 4$), and Tg(tetO-PrP/6740) ($n = 4$) mice were treated similarly or kept untreated. All of the treated and untreated mice remained well over the entire duration of the experiment. At the end of the observation period, animals were sacrificed and their brains taken for measurement of PrP^C levels and histologic examination (Figs. 1*B* and 2).

In related experiments described below, 8- to 10-week-old adult Tg(tTA:PrP)3 mice were given oral doxycycline for over 380 days and have displayed no signs of illness (Table 5). These findings extend those described above for adult Tg(tTA:PrP)1 mice that were studied after 30 days of oral doxycycline treatment.

Immunoblot analysis showed that Tg(tTA:PrP)1 and Tg(tTA:PrP)3 mice treated for 30 days with doxycycline exhibited levels of PrP^C equivalent to those of target Tg(tetO-PrP) mice alone (Fig. 1 and data not shown). Histoblots (24) revealed high expression of PrP^C in the hippocampus, cingulate gyrus, neocortex, entorhinal cortex, thalamus, substantia nigra, molecular, and granular layers of the cerebellum in untreated Tg(tTA:PrP)3 mice (Fig. 2 *A–C*).

The expression of PrP^C was more restricted in the grey matter of Tg(tTA:PrP)3 mice than in FVB mice (Fig. 2 *J–L*). For example, PrP^C was not detected in a portion of the CA3 region of the hippocampus (Fig. 2*A*), the parietal neocortex (Fig. 2 *A* and *B*), or the brainstem tegmentum of Tg(tTA:PrP)3 mice (Fig. 2 *B* and *C*). In contrast, the expression of PrP^C was higher in the substantia nigra (Fig. 2 *B* and *K*), the entorhinal

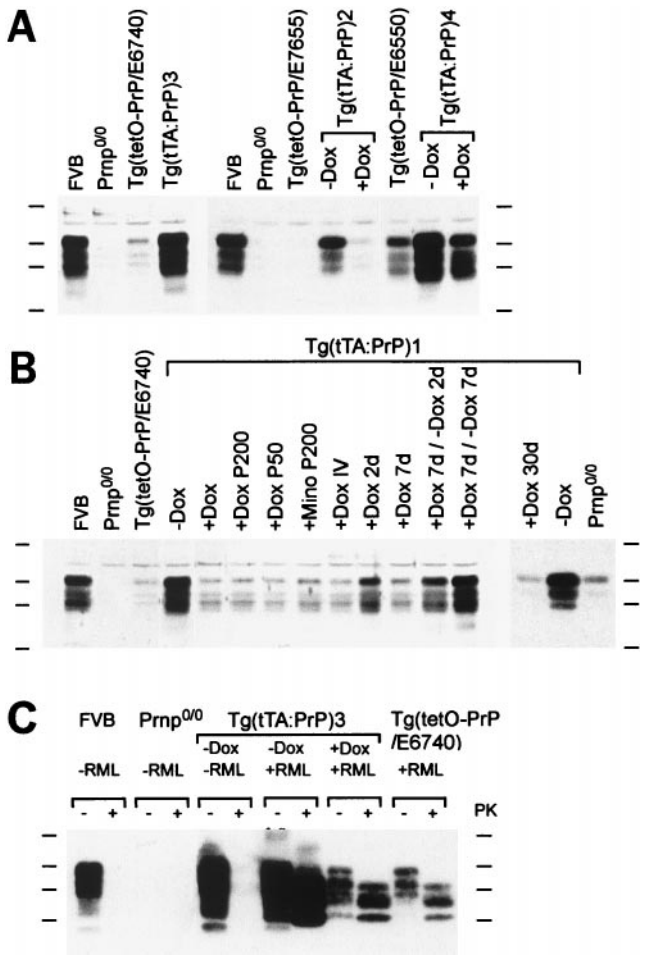

FIG. 1. PrP^C and PrP^{Sc} in the brains of Tg(tTA:PrP) mice. Four lines of Tg(tTA:PrP) mice (Tables 1–3) were tested for PrP expression by Western blot analysis by using α-PrP polyclonal RO73 antiserum. Levels of expression in FVB, $Prnp^{0/0}$, and Tg(tetO-PrP) lines are also shown. (*A* and *B*) Experimental conditions were as follows: "− Dox" denotes no treatment; "+ Dox" corresponds to doxycycline administered for 7 days in the drinking water at 2 mg/ml unless otherwise specified. "P50" corresponds to a 50 mg pellet (21-day release) of doxycycline placed subcutaneously for 7 days, and "P200" is a 200 mg pellet (21-day release). "IV" corresponds to a daily intravenous dose of 25 mg/kg of doxycycline for 3 days, and "d" is the abbreviation used for day. Induction of PrP^C expression was observed in Tg(tTA:PrP) mice. Repression was obtained upon administration of doxycycline or minocycline (Mino) by using various routes and could be reversed upon ceasing antibiotic treatment. (*C*) Tg(tTA:PrP)3 animals left untreated (− Dox) and inoculated with RML prions (+ RML) were sacrificed at the time they presented neurological deficits consistent with development of scrapie (69 days postinoculation). Tg(tTA:PrP)3 mice treated with doxycycline (+ Dox) 1 week before inoculation as well as uninoculated Tg(tTA:PrP)3 and inoculated Tg(tetO-PrP/E6740) mice remained clinically healthy and were sacrificed at 200 days postinoculation. Brain homogenate consisting of 40 μg (*A* and *B*) or 60 μg (*C*) of protein was loaded per lane. Proteinase K digestion (20 mg/ml) was performed for 60 min at 37°C. Protein molecular weight markers from top to bottom correspond to 48, 35, 28, and 19 kDa.

Table 4. Genotypic frequency distribution of transgenic mice obtained from tetO-MoPrP × tTA crosses

Crosses				No. neonatal	Deaths,		Genotypes, %			
tTA	PrP	Treatment*	No. born	deaths	%†	No. screened	TetO-MoPrP	tTA	Non-Tg	tTA:PrP
F959	E6740	− Dox	460	92	20.0	368	30.7	32.3	29.3	7.6
		+ Dox	187	14	7.5	175	22.9	25.1	28.0	24.0
		+ Dox E13 or E14‡	24	4	16.7	20	30.0	25.0	25.0	20.0
		+ Dox E17 or E18‡	45	3	6.7	42	21.0	31.0	43.0	5.0
		+ Dox P2, P4, or P6§	39	8	20.5	31	21.0	31.0	43.0	0.0
F966	E6740	− Dox	188	22	11.7	166	42.2	23.5	24.1	10.2
		+ Dox	216	8	3.7	208	29.8	24.5	24.5	21.2

*Treatment refers to 2 mg/ml of doxycycline administered orally; − Dox, no treatment; + Dox, doxycyline during embryonic and postnatal development.
†Neonatal deaths were observed during the first 3 postnatal weeks.
‡E refers to the embryonic days at which doxycycline treatment was initiated.
§P refers to the postnatal days at which treatments were initiated.

cortex (Fig. 2 *B* and *K*), and the molecular and granule cell layers of the cerebellar cortex (Fig. 2 *C* and *L*) of Tg(tTA:PrP)3 mice than that seen in FVB mice. Similar patterns of PrP^C expression were found in Tg(tTA:PrP)2 and Tg(tTA:PrP)4 mice (data not shown).

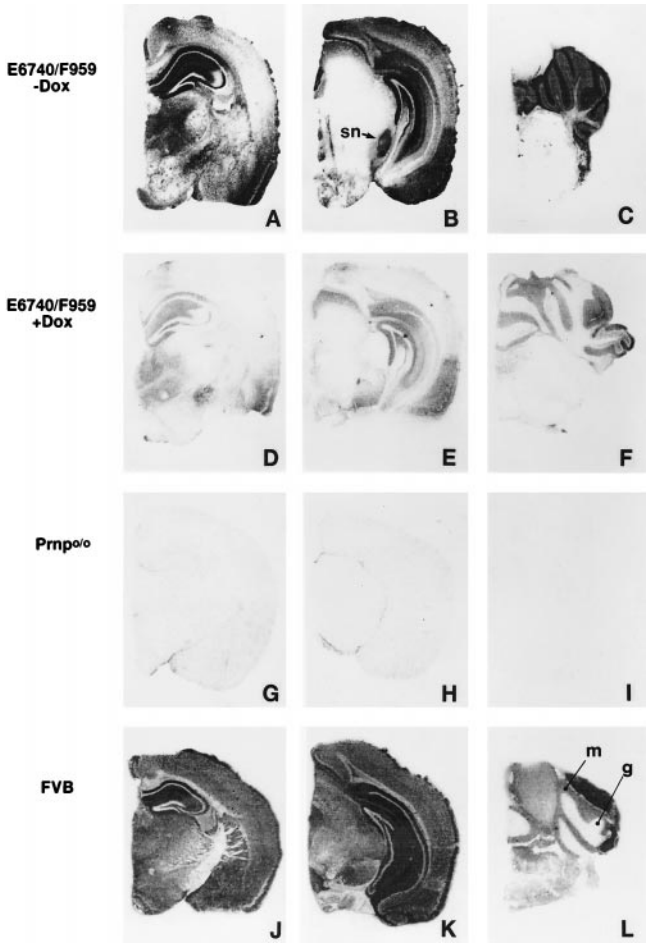

FIG. 2. Distribution of PrP^C expression in the brain of Tg(tTA:PrP)3 mice. Distribution of $MoPrP^C$ is revealed by histoblots by using α-PrP polyclonal RO73 antiserum on half coronal brain sections of Tg(tTA:PrP)3 mice (*A–C*) untreated or (*D–F*) treated with oral doxycycline (2 mg/ml) for 30 days. Control brain sections from untreated (*G–I*) $Prnp^{0/0}$ and (*J–L*) FVB mice. Tg(tTA:PrP)3 mice express PrP^C at high levels in the hippocampus, neocortex, entorhinal cortex, substantia nigra (sn), and thalamus, as well as cerebellar granular (g) and molecular (m) layers. Although doxycycline treatment repressed PrP^C expression, low levels of residual expression were still detected in the hippocampus, neocortex, entorhinal cortex, and granular layer of the cerebellum.

After 30 days of doxycycline treatment, PrP^C expression was substantially repressed (Fig. 1*B*), with residual expression still detectable within the cingulate gyrus, caudate nucleus, hippocampus, thalamus, entorhinal cortex, and cerebellum (Fig. 2 *D–F*). Microscopic examination revealed no specific pathological feature associated with acute repression of PrP^C after 30 days (data not shown).

High PrP^C Expression Induces Reactive Astrocytic Gliosis. A moderate degree of reactive astrocytic gliosis was detected in the thalamus, substantia nigra, and cerebellum granular and molecular layers of Tg(tTA:PrP)3 mice ($n = 5$) and Tg(tTA:PrP)4 mice ($n = 2$) expressing high levels of $MoPrP^C$ (data not shown). However, the neocortex, entorhinal cortex, hippocampus, and caudate nucleus, which also expressed high levels of PrP^C, were normal. Repression of PrP^C for 1 month had no effect on the extent of astrocytic gliosis observed, suggesting that this phenomenon may have resulted from the early overexpression of PrP^C. Astrocytic gliosis was not observed in doxycycline-treated or untreated age-matched controls consisting of $Prnp^{0/0}$ ($n = 2$), FVB ($n = 4$), Tg(tetO-PrP/E6740) ($n = 3$), Tg(Prnp-tTA/F959) ($n = 4$), and Tg(MoPrP-A)4053/$Prnp^{0/0}$ ($n = 6$) mice or in randomly selected 10-month-old $Prnp^{0/0}$ ($n = 4$), FVB ($n = 5$), and Tg(MoPrP-A)4053/$Prnp^{0/0}$ ($n = 4$) animals.

Oral Doxycycline Prevents Prion Disease in Tg(tTA:PrP) Mice. When doxycycline was administered orally to adult Tg(tTA:PrP)3 mice, beginning 7 days before inoculation with RML prions, the mice remained free of any signs of CNS dysfunction for more than 380 days. In contrast, mice from the same line not given doxycycline developed a progressive ataxia starting ≈50 days after inoculation with prions (Fig. 3; Table 5). Wild-type FVB mice developed signs of prion disease at ≈120 days after inoculation, whereas $Prnp^{0/0}$ mice were not susceptible to prions, as previously reported (15, 17, 18).

Tg(tTA:PrP)3 mice not treated with doxycycline were sacrificed after developing progressive CNS dysfunction. Levels and distribution of PrP^C and PrP^{Sc} in Tg(tTA:PrP)3 mice were assessed by Western blot analysis (Fig. 1*C*) and histoblot analysis (data not shown). Both PrP^C and PrP^{Sc} levels were high in the brains of untreated Tg(tTA:PrP)3 mice that developed scrapie. PrP^{Sc} accumulated in the neocortex, hippocampus, corpus callosum, and white matter tracts of the cerebellum and pons. Doxycycline treatment repressed PrP^C to low levels similar to those observed in target Tg(tetO-PrP/E6740) (Fig. 1*C*). This residual expression was nonetheless sufficient to permit the conversion of low levels of PrP^{Sc} (Fig. 1*C*), which accumulated mostly within the forebrain and in particular in the corpus callosum and caudate nucleus (data not shown).

The brains from untreated Tg(tTA:PrP)3 mice exhibited extensive neuronal loss in the hippocampal pyramidal cell layer (Fig. 3*G*) and dentate gyrus and focal loss of Purkinje cells and

Table 5. Inhibition of scrapie disease development by repression of *Prnp* gene expression in transgenic mice

Recipient	Treatment	Inoculum	CNS dysfunction	Incubation time (mean days ± SEM)
FVB	—*	RML	11/11	122 ± 3
Tg(tTA:PrP)3	—*	RML	4/4†	51 ± 0
Tg(tTA:PrP)3	− Dox‡	—	0/6	>200
Tg(tTA:PrP)3	+ Dox§	RML	0/7	>380 (n = 5)¶
Non Tg(tTA:PrP)3‖	—*	RML	0/10	>380 (n = 8)¶
Tg(tetO-PrP/E6740)	—*	RML	0/8	>380 (n = 6)¶
$Prnp^{0/0}$	—*	—	0/10	>380

*Animals from these groups were kept at all times without doxycycline.
†Animals in this category first presented with ataxia at 51 days postinoculation and with additional signs of neurologic dysfunction at 69 days. Traditionally, the diagnosis of experimental scrapie in mice requires two signs of neurologic dysfunction as described (32).
‡Animals were born from parents maintained on doxycycline in the drinking water. Treatment was ceased at 3 weeks of age.
§Doxycycline was administered in the drinking water (2 mg/ml) 1 week before inoculation.
¶Two animals from each of these groups were sacrificed for histopathology at 200 days postinoculation.
‖Animals in this group did not harbor any transgene.

granular cells in the cerebellum (Fig. 3*C*). These changes were accompanied by moderate to severe astrocytic gliosis in all regions examined, including the neocortex, hippocampus, entorhinal cortex, thalamus, caudate nucleus, and substantia nigra, as well as cerebellar granular and molecular layers. As noted above, the doxycycline-treated Tg(tTA:PrP)3 mice did not show signs of CNS dysfunction. When these mice were sacrificed ≈200 days after inoculation with prions, their brains showed no signs of neurodegeneration.

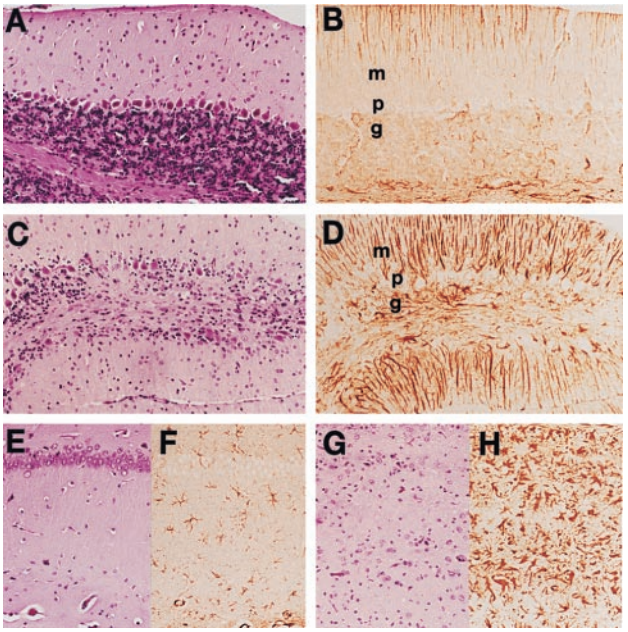

FIG. 3. Doxycycline prevented neuronal loss, vacuolation, and gliosis in Tg(tTA:PrP)3 mice inoculated with RML prions. (*A*, *B*, *E*, and *F*) Tg(tTA:PrP)3 mice treated with oral doxycycline (2 mg/ml) and sacrificed at 200 days postintracerebral inoculation with RML prions. (*C*, *D*, *G*, and *H*) Tg(tTA:PrP)3 mice untreated with doxycycline and sacrificed at 70 days postintracerebral inoculation with RML prions when they showed multiple signs of CNS dysfunction; the first sign of neurologic disease was ataxia at 50 days after inoculation. (*A*, *C*, *E*, and *G*) Hematoxylin and eosin-stained brain sections. (*B*, *D*, *F*, and *H*) α-GFAP-immunostained brain sections. (*A–D*) Views of cerebellar molecular (m), Purkinje cell (p), and granular (g) layers. (*E–H*) Views of the hippocampus CA1 pyramidal cell layer (aligned in the upper part of *Insets*) with underlying stratum radiatum. Focal loss of Purkinje cells and granule cells was observed in the cerebellum of the ill Tg(tTA:PrP)3 mice and was accompanied by low grade vacuolation. Reactive astrocytic gliosis was demonstrated by the α-GFAP immunostaining that revealed moderate to severe gliosis in the granular and molecular layers (*D*) (Bergmann's gliosis). In the hippocampal CA1 region most neuronal cell bodies were lost, and low grade vacuolation was apparent together with severe astrocytic gliosis (*H*).

DISCUSSION

To test the hypothesis that developmental compensation in PrP-deficient mice prevented any recognizable dysfunction in adult mice, we used the tTA system in Tg mice to regulate PrP expression. Doxycycline administered to adult Tg(tTA:PrP) mice acutely repressed the expression of PrP^C but did not produce any recognizable adverse effects in the mice over a 30-day period. Neither the viability nor the neurological status of the mice was compromised, and histological examination of the brains did not reveal any abnormalities. These results indicate that high levels of PrP^C are not essential for short-term neuronal survival, as its expression can be repressed over 20-fold without adverse effects. It is noteworthy that adult Tg(tTA:PrP)3 mice were placed on oral doxycycline to repress their PrP^C expression and have remained well for >380 days with continual administration of doxycycline (Table 5).

PrP^{Sc} Accumulation Causes Prion Disease. The accumulation of PrP^{Sc} in the brains of animals and humans is a specific hallmark of prion disease. Often, but not always, proteolytic fragments of PrP^{Sc} coalesce in the extracellular space to form amyloid plaques. Such extracellular accumulations of PrP^{Sc} were thought to be of little consequence in the pathogenesis of prion disease because they are a nonobligatory feature of the disease (28). Moreover, PrP^{Sc} either within caveolae or within an intracellular compartment has been implicated in the pathogenesis of prion disease (29, 30), a conclusion supported by neuronal cell grafts producing PrP^{Sc} in the CNS of $Prnp^{0/0}$ mice (31).

With the production of Tg(tTA:PrP) mice, it is possible to examine the effects of low or intermediate levels of PrP^{Sc} in the CNS. We found that low levels of PrP^{Sc} did not produce any deleterious clinical or histological effects up to 380 days after inoculation of RML prions in Tg(tTA:PrP)3 mice (Fig. 3*C*). Studies of $Prnp^{+/0}$ mice with one functional PrP allele show greatly prolonged incubation times (15) but at a higher accumulation of PrP^{Sc} than was anticipated (17). Studies with Tg(tTA:PrP) mice where the levels of PrP^C expression are held at different levels throughout the incubation time should help to clarify this issue.

The findings reported here clearly show that repression of PrP^C expression in young adult Tg(tTA:PrP) mice is not

deleterious, whereas accumulation of PrP^{Sc} in the same line of animals is lethal (Table 5). Even though Purkinje cell degeneration in 70-week-old *Prnp*$^{0/0}$ mice has been found (9), our data continue to argue that the accumulation of PrP^{Sc} and not a loss of PrP^{C} function is responsible for the pathogenesis of prion disease.

New Approaches Arising from These Studies. The use of the tTA-regulated PrP transgene expression revealed that high levels of even wild-type PrP^{C} are often incompatible with neonatal development, as most Tg(tTA:PrP) mice died within the first 3 weeks of life (Table 4). Repression of PrP^{C} expression by beginning doxycycline treatment during embryonic development was sufficient to prevent this mortality. The ability to control PrP^{C} expression should allow the establishment of Tg mice expressing higher levels of wild-type or mutant PrP than was previously possible. Such mice may demonstrate unique sensitivities to prion infection and provide the basis for a truly rapid bioassay.

Reversing the course of prion diseases by blocking the production of PrP^{Sc} through repression of PrP^{C} expression will allow us to measure the removal of PrP^{Sc}. Such clearance studies, which were not previously possible, are a prelude to the development of effective therapies where drugs that block PrP^{Sc} formation are administered at the earliest onset of symptoms to patients with sporadic Creutzfeldt–Jakob disease. At present, we have no understanding of how much PrP^{Sc} can be tolerated by the CNS and how rapidly it will disappear once synthesis of its precursor, PrP^{C}, is repressed.

We thank F. E. Cohen, D. Groth, A. Serban, S. Freundlieb, and U. Baron for many helpful discussions during the course of these studies. This work was supported by grants from the National Institutes of Health and the American Health Assistance Foundation, as well as by a gift from the Bernard Osher Foundation. Z.M. was supported by a Brody Grant from the Alzheimer's Association. M.G. is supported by a Human Frontiers of Science Fellowship.

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