Interactions between wild-type and mutant prion proteins modulate neurodegeneration in transgenic mice

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Transgenic mice overexpressing approximately eightfold the mouse (Mo) prion protein (PrP) gene carrying the P102L mutation of GSS developed neurodegeneration between 150 and 300 days of age, while controls expressing the wild-type MoPrP-A transgene at the same level remained healthy. Mice overexpressing the wild-type MoPrP-A transgene were highly susceptible to inoculated mouse prions, exhibiting abbreviated scrapie incubation times of 45 days. After crossing the mutant transgene onto a null (*Prnp*^{0/0}) background, the resulting Tg(MoPrP-P101L)*Prnp*^{0/0} mice displayed a highly synchronous onset of illness at 145 days of age, which was shortened to 85 days upon breeding to homozygosity for the transgene array. Besides occasional PrP plaques and modest spongiform degeneration, Tg(MoPrP-P101L) mice suffered from a myopathy and a peripheral neuropathy. Disruption of the wild-type MoPrP gene increased the number of PrP plaques and the severity of spongiform degeneration. Brain extracts prepared from spontaneously ill transgenic mice transmitted disease to Tg196/*Prnp*^{0/0} mice, expressing low levels of the mutant transgene. Our results demonstrate that the presence of wild-type PrP genes, the level of PrP transgene expression, and the sequence of the transgene can profoundly modify experimental prion disease.

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The prion diseases are unique in that they present as sporadic, inherited, and infectious disorders (Ridley et al. 1986; Prusiner 1989; Richardson and Masters 1995). For many years, they were classified as slow virus infections (Sigurdsson 1954; Gajdusek 1977). Although much emphasis was placed on the transmissible nature of the prion diseases for many decades, studies of the inherited aspects of these disorders are now well appreciated (for review, see Brown et al. 1991; Prusiner 1994; Gambetti et al. 1995).

Nineteen different mutations of the prion protein (PrP) gene that result in non-conservative substitutions or insertions have been identified in families with a history of inherited central nervous system (CNS) disease. Genetic linkage of a P102L mutation to Gerstmann-Sträussler-Scheinker disease (GSS) established that familial prion diseases are hereditary disorders and provided a novel mechanism to explain how the prion diseases could manifest as both genetic and infectious illnesses (Hsiao et al. 1989; Prusiner 1989). The discovery of a mutation at codon 102 in the PrP gene that is responsible for this dominantly inherited disease, permits unambiguous di-

agnosis during life as well as identification of individuals at risk.

In our initial attempt to model GSS in transgenic mice, we constructed a mutated cosmid clone containing the *Prnp*^b gene from the ILn/J mouse (Hsiao et al. 1990). The resulting clone encoded mouse PrP-A (MoPrP-A) with a substitution of leucine for proline at codon 101 designated P101L, which corresponds to codon 102 in human PrP (HuPrP). Two lines of these mice developed CNS dysfunction between 50 and 300 days of age. That overexpression of Syrian hamster (SHa) PrP and wild-type MoPrP-B can produce neurodegeneration of the CNS as well as neuromyopathy (Westaway et al. 1994) forced us to re-examine neurodegeneration in Tg(MoPrP-P101L) mice (Hsiao et al. 1990). We constructed additional lines of Tg(MoPrP-P101L) mice as well as control lines of Tg(MoPrP-A) mice. Mice overexpressing wild-type MoPrP-A remained well for >600 days, in contrast to those overexpressing MoPrP-P101L, all of which become ill between 150 and 300 days of age.

The wide range in the age of onset of spontaneous illness in these Tg(MoPrP-P101L) mice contrasts with experimental scrapie, in which incubation times for a group of animals receiving the same inoculum are

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always similar. We produced high-copy-number Tg(MoPrP-P101L) mice on a background in which the wild-type MoPrP gene had been ablated by gene targeting (Prnp^{0/0}) (Büeler et al. 1992). Prnp^{0/0} mice, which do not express MoPrP, are resistant to mouse prions (Büeler et al. 1993; Prusiner et al. 1993; Manson et al. 1994; Sakaguchi et al. 1995). The resulting Tg(MoPrP-P101L)-Prnp^{0/0} mice exhibited a remarkable uniformity in the timing of their onset of illness, a shorter mean duration of illness and neuropathologic changes characterized by numerous PrP plaques. Brain extracts prepared from spontaneously ill Tg(MoPrP-P101L) mice reproducibly transmitted CNS degeneration to low-copy-number Tg(MoPrP-P101L)196 mice ~350 days after intracerebral inoculation. Crossing of Tg(MoPrP-P101L)196 mice onto the null background shortened these incubation times to ~200 days. Uninoculated Tg196/FVB mice and Tg196/Prnp^{0/0} mice did not develop spontaneous CNS disease nor did Tg196/FVB mice and Tg196/Prnp^{0/0} mice inoculated with brain extracts from healthy nontransgenic control littermates. Comparison of muscle and peripheral nerve from spontaneously ill Tg(MoPrP-P101L) mice, age-matched Tg(MoPrP-P101L)196 and aged Tg(MoPrP-A) mice revealed that overexpression of MoPrP-P101L, but not wild-type MoPrP-A, resulted in pathological abnormalities in muscle and peripheral nerve in addition to neurodegeneration of the CNS. These results demonstrate that disease in Tg(MoPrP-P101L) mice is caused specifically by the adverse effects of mutant PrP rather than overexpression of PrP encoded by the transgene.

Results

Mutant and wild-type MoPrP transgenes

We constructed a series of Tg(MoPrP-P101L) mice using fertilized eggs from FVB mice. The resulting mice express transgene-derived mutant MoPrP-P101L and endogenous wild-type MoPrP. The Tg(MoPrP-P101L)/FVB mice differ from Tg(MoPrP-P101L)87 and Tg(MoPrP-P101L)174 mice produced earlier using fertilized eggs from a (C57BL×SJL)/F2 cross. In addition, the cosShA.Tet vector used to construct the Tg(MoPrP-P101L)/FVB mice differs from the ILn/J cosmid clone used in our initial studies (Hsiao et al. 1990).

Founder transgenic mice were generated by microinjection of linearized DNA from the cosShA. Tet constructs, eight expressing MoPrP(P101L) designated Tg(MoPrP-P101L)/FVB and two expressing wild-type mouse PrP^C-A designated Tg(MoPrP-A)/FVB. Three Tg(MoPrP-P101L)/FVB lines and one Tg(MoPrP-A)/FVB line were established. We estimated that levels of PrP^C in the brains of mice from two lines expressing MoPrP-P101L, designated Tg(MoPrP-P101L)2247/FVB and Tg(MoPrP-P101L)2866/FVB, and one line expressing wild-type mouse PrP^C-A, designated Tg(MoPrP-A)4053/FVB, were each about eightfold higher than PrP^C levels in the brains of nontransgenic mice. The fourth line overexpressing MoPrP-P101L ~32-fold was designated Tg(MoPrP-P101L)2862/FVB.

Expression of MoPrP-P101L transgene produces CNS degeneration

Because wild-type and mutant MoPrP are overexpressed at equivalent levels in the brains of Tg(MoPrP-A)4053/FVB, Tg(MoPrP-P101L)2866/FVB, and Tg(MoPrP-P101L)2247/FVB mice, we were able to assess the relative contributions of the MoPrP(P101L) mutation and PrP overexpression upon the development of spontaneous neurologic disease. In contrast to the Tg(MoPrP-A)4053/FVB mice, which remained free of CNS dysfunction for >600 days of age, the Tg(MoPrP-P101L)2247/FVB and Tg(MoPrP-P101L)2862/FVB lines began to exhibit ataxia, rigidity, and lethargy between 150 and 200 days of age (Fig. 1). By 300 days, 100% of Tg(MoPrP-P101L)2247/FVB mice had developed CNS dysfunction while ~410 days was required for all of the Tg(MoPrP-P101L)2862/FVB mice to exhibit signs of CNS degeneration. The mean age of disease was 227 ± 16 days ($\pm$ S.E.M.) and 321 ± 21 days in 17 Tg(MoPrP-P101L)2247/FVB and 14 Tg(MoPrP-P101L)2862/FVB mice, respectively (Table 1). Nontransgenic littermates of the Tg(MoPrP-P101L)/FVB lines remained asymptomatic for >600 days.

The ages of onset of neurologic dysfunction in Tg(MoPrP-P101L)2866/FVB mice were indistinguishable from those in Tg(MoPrP-P101L)2247/FVB (Fig. 1A, B); both of these Tg(MoPrP-P101L) lines overexpress the transgene about eightfold. The mean age of disease was 220 ± 10 days in 18 Tg(MoPrP-P101L)2866/FVB mice (Table 1). Because Tg(MoPrP-A)4053/FVB, Tg(MoPrP-P101L)2247/FVB, and Tg(MoPrP-P101L)2866/FVB mice all express wild-type or mutant MoPrP^C at the same levels, we conclude that overexpression of PrP cannot account for the spontaneous development of neurologic symptoms in Tg(MoPrP-P101L) mice; rather, expression of MoPrP(P101L) is required for CNS disease in these transgenic mice.

The Tg(MoPrP-P101L)87 and Tg(MoPrP-P101L)174 mice were constructed with a modified ILn/J mouse cosmid (Hsiao et al. 1990; Hsiao et al. 1994), whereas the Tg(MoPrP-P101L)2247/FVB and Tg(MoPrP-P101L)2866/FVB mice were produced using cosShA.Tet vector. An additional difference between the sets of transgenic lines is that the Tg(MoPrP-P101L)87 and Tg(MoPrP-P101L)174 mice were derived from outbred (C57BL/6×SJL)/F2 fertilized oocytes, whereas the Tg(MoPrP-P101L)2247/FVB and Tg(MoPrP-P101L)2866/FVB mice were derived from inbred FVB fertilized oocytes. Because all four transgenic lines display indistinguishable CNS degeneration, it is likely that the substitution of L for P at residue 101 is the only cause of the illness. Additional support comes from transgenic mice expressing chimeric mouse/human MHu2M PrP with the P101L mutation which also spontaneously develop CNS disease. Breeding these Tg(MHu2M-P101L)69 mice to transgene homozygosity shortened the mean age of illness from 362 ± 13 ($\pm$ S.E.M.) to 199 ± 2 days (Telling et al. 1995; G. Telling and S.B. Prusiner, unpubl.).

The Tg(MoPrP-P101L)2862/FVB line that overexpresses mutant PrP ~32-fold is of interest because we

Figure 1. Spontaneous neurologic disease in Tg(MoPrP-P101L) mice as a function of age. (A) Expression of the MoPrP-P102L mutation produced neurologic disease in uninoculated mice. Eighteen Tg(PrP-P101L)2247/FVB mice (■) and 14 Tg(PrP-P101L)2862/FVB mice (●) were observed for the spontaneous development of neurologic disease. (B) Age of onset of CNS dysfunction was modified by the wild-type MoPrP gene and the level of expression of the mutant MoPrP-P101L transgene. Twenty-five Tg(MoPrP-P101L^{+/0})2866/FVB mice (●), 14 Tg(MoPrP-P101L^{+/0})2866/Prnp^{0/0} mice (▲) and 10 Tg(MoPrP-P101L^{+/+})2866/Prnp^{0/0} mice (▼) were observed for the spontaneous development of neurologic disease. The curve for ages of onset of illness of two additional groups of 11 and 22 Tg(MoPrP-P101L)2866/Prnp^{0/0} mice bred >1 year later (◆ and ■) is superimposable on the initial curve. The + superscript denotes the number of copies of the MoPrP-P101L transgene array.

expected these mice to exhibit spontaneous illness at a younger age than the eightfold overexpressors; instead, they remained well up to 250 days of age, and a few survived for more than 400 days (Fig. 1A). It is possible that the ~32-fold overexpression of PrP as measured by dot blot, overestimates the concentration of PrP^C that is correctly folded so as to render it eligible for conversion into PrP^{Sc}.

Although the Tg(MoPrP-A)4053/FVB mice remained free of CNS dysfunction for >600 days of age, 6 of 20 mice developed CNS dysfunction between 605 and 815 days of age (Table 1). At this late time, many animals were also dying of intercurrent illnesses which compli-

cated diagnosis. Apart from minimal spongiform alterations in the white matter normally associated with aging in mice, no neuropathologic evidence for prion disease was evident. In addition, examination of brain extracts by Western blotting failed to detect the presence of protease-resistant PrP^{Sc}. The absence of neuropathologic features of prion disease in the brains of these mice reflects the difficulty of accurately diagnosing clinical prion disease in elderly animals.

Transgenic mice expressing similar levels of MoPrP with an E to K change at residue 199, equivalent to the E200K mutation in humans that produces inherited Creutzfeldt-Jakob disease (CJD), did not develop CNS

Table 1. Age of onset of spontaneous neurologic dysfunction in uninoculated transgenic mice overexpressing mutant or wild-type MoPrP

Transgenic line ^a	Expressed mouse PrP molecules	PrP transgene expression ^b	Age of onset of illness (mean days ± S.E.M.) ^c
Tg(MoPrP-A)4053/FVB	wt	×8	744 ± 31 (6/20)
Tg(MoPrP-E199K)5182/FVB	wt, E199K	×8	704 ± 61 (7/20)
Tg(MoPrP-P101L)2247/FVB	wt, P101L	×8	227 ± 16 (17/17)
Tg(MoPrP-P101L)2866/FVB	wt, P101L	×8	220 ± 10 (18/18)
Tg(MoPrP-P101L)2866/Prnp ^{0/0}	P101L	×8	146 ± 2 (14/14)
Tg(MoPrP-P101L ^{+/+})2866/Prnp ^{0/0}	P101L	N.D.	85 ± 2 (10/10)
Tg(MoPrP-P101L)2862/FVB	wt, P101L	×32	321 ± 21 (14/14)

^aTransgenic mice are designated according to the species of PrP expressed from the integrated transgene array. If mutant PrP constructs are expressed, the location of the mutated codon is indicated preceded by the single letter amino acid code of the wild-type codon followed by the mutant codon. Finally, the founder animal number from which the line was derived is used to distinguish individual lines.

^bProtein expression relative to wild-type, nontransgenic mice.

^cThe number of mice spontaneously developing clinical signs of scrapie divided by the original number of mice is shown in parentheses.

disease (Table 1). A small number of these Tg(MoPrP-E199K)5182/FVB mice were clinically diagnosed with CNS disease at the same time as Tg(MoPrP-A)4053/FVB mice, but the brains of these transgenic mice did not exhibit any of the pathological hallmarks of prion disease, nor did they contain protease-resistant PrP^{Sc}. We conclude that, unlike Tg(MoPrP-P101L) mice, transgenic mice expressing high levels of the mutant MoPrP form of the familial CJD (E200K) mutation do not develop CNS disease.

Interactions between mutant and wild-type MoPrP

Because the Tg(MoPrP-P101L)/FVB mice express two PrP^C molecules differing only at a single amino acid, residue 101, we asked how the disease process might be influenced by eliminating wild-type MoPrP^C. Tg(MoPrP-P101L)2866/Prnp^{0/0} mice were produced by genetic crosses between Prnp^{0/0} mice and Tg(MoPrP-P101L)-2866/FVB mice. Unlike Tg(MoPrP-P101L)2866/FVB mice, the Tg(MoPrP-P101L)2866/Prnp^{0/0} mice all became ill within a narrow interval (Fig. 1B). More than 90% of the Tg(MoPrP-P101L)2866/Prnp^{0/0} mice developed CNS dysfunction and died within a period of ~21 days whereas the Tg(MoPrP-P101L)2866/FVB mice required 161 days. The mean age for development of neurologic disease in the Tg(MoPrP-P101L)2866/Prnp^{0/0} mice was 146 ± 2 days ($\pm$ S.E.M., $n = 14$), in contrast to 220 ± 10 days ($\pm$ S.E.M., $n = 25$) for the Tg(MoPrP-P101L)2866/FVB mice (Table 1). The mean duration of illness, that is, the time from the appearance of clinical signs to death, was reduced by a factor of >5 from 17 ± 3 days in Tg(MoPrP-P101L)2866/FVB mice to 3 ± 1 days in the Tg(MoPrP-P101L)2866/Prnp^{0/0} mice. Thus, expression of wild-type MoPrP^C, albeit at an eight-fold lower level than mutant MoPrP-P101L, profoundly affected the progression of disease in Tg(MoPrP-P101L) mice. When additional groups of Tg(MoPrP-P101L)2866/Prnp^{0/0} mice were observed more than a year after the initial studies described above were performed, the curves for ages of onset of illness in these mice were superimposable on that of the initial study and the mean ages for development of neurologic disease were 149 ± 7 days ($\pm$ S.E.M., $n = 11$) and 137 ± 3 days ($n = 22$) (Fig. 1B).

By mating Tg(MoPrP-P101L)2866/Prnp^{0/0} mice to each other, we produced offspring that were homozygous for the MoPrP-P101L transgene array. These mice, designated Tg(MoPrP-P101L^{+/+})2866/Prnp^{0/0}, had a mean age for the onset of neurologic disease of 85 ± 2 days ($\pm$ S.E.M., $n = 10$) as well as an abbreviated clinical course (Table 1; Fig. 1B).

Transmission of CNS disease: further evidence for interference between mutant and wild-type MoPrP

Brain homogenates prepared from Tg(MoPrP-P101L)2247/FVB and Tg(MoPrP-P101L)2866/FVB mice that had spontaneously developed signs of neurologic disease were inoculated intracerebrally into Tg(MoPrP-P101L)196/FVB and Tg(MoPrP-P101L)196/Prnp^{0/0} mice. Both Tg(Mo-

PrP-P101L)196 lines express low levels of MoPrP^C-(P101L) whereas Tg(MoPrP-P101L)196/Prnp^{0/0} mice do not express wild-type MoPrP^C which is found in Tg(MoPrP-P101L)196/FVB mice. Uninoculated Tg(MoPrP-P101L)196/FVB and Tg(MoPrP-P101L)196/Prnp^{0/0} mice did not spontaneously develop CNS disease [Table 2, (Hsiao et al. 1994)]. Tg(MoPrP-P101L)196/FVB mice inoculated with seven different brain homogenates from spontaneously ill Tg(MoPrP-P101L)2247/FVB or Tg(Mo-

Table 2. Transmission of mutant prions produced in Tg(MoPrP-P101L) mice to inoculated recipients

Inoculum	Incubation time (mean days $\pm$ S.E.M.) ^a	
<i>A. Recipient Tg(MoPrP-P101L)196/FVB mice</i>		
None	>750	(0/9)
Tg2247-1	389 $\pm$ 6	(10/10)
Tg2247-2	304 $\pm$ 11	(10/10)
Tg2247-3	324 $\pm$ 14	(9/10)
Tg2247-4	323 $\pm$ 12	(9/9)
Tg2247-5	384 $\pm$ 37	(5/5)
Tg2866-1	362 $\pm$ 14	(10/10)
Tg2866-2	336 $\pm$ 17	(9/9)
Tg196-1(2247-1) ^b	359 $\pm$ 13	(10/10)
Tg196-2(2247-1)	312 $\pm$ 45	(7/7)
Non-Tg2247-1	>450	(0/8)
Non-Tg2247-2	>450	(0/8)
Non-Tg2247-3	>450	(0/16)
<i>B. Recipient non-Tg196/FVB</i>		
Tg2247-2	>650	(0/5)
Tg2247-3	>600	(0/3)
Tg196-1(2247-1)	>700	(0/5)
Tg196-2(2247-1)	>700	(1/4) ^c
<i>C. Recipient Tg(MoPrP-P101L)196/Prnp^{0/0} mice</i>		
None	>750	(0/8)
Tg2247-1	190 $\pm$ 6	(9/9)
Tg2247-2	205 $\pm$ 6	(8/8)
Tg2247-3	182 $\pm$ 7	(9/9)
Tg2247-5	236 $\pm$ 28	(5/5)
Tg196-1(2247-1)	230 $\pm$ 5	(10/10)
Tg196-2(2247-1)	257 $\pm$ 6	(10/10)
<i>D. Recipient non-Tg196/Prnp^{0/0} mice</i>		
Tg2247-1	>700	(0/4)
Tg2247-3	>700	(0/3)
Tg2247-5	>570	(0/4)
<i>E. Recipient Tg(MoPrP-A)4053/FVB</i>		
Tg196-1(2247-1)	>700	(0/7)
Tg196-2(2247-1)	>350	(0/7)
<i>F. Recipient non-Tg(MoPrP-A)4053/FVB</i>		
Tg196-1(2247-1)	>700	(0/4)
Tg196-2(2247-1)	>650	(1/3) ^d

Tg(MoPrP-P101L)196 mice express the mutant MoPrP-P101L transgene at a low level and do not develop disease spontaneously (Hsiao et al. 1994).

^aNotations in parentheses indicate inoculum used in initial passage.

^bSingle digit following a hyphen distinguishes individual mice from a particular line.

^cOne animal sick at 463 days.

^dOne animal sick at 454 days.

PrP-P101L/2866/FVB mice developed signs of CNS dysfunction with a mean incubation time of ~350 days (Table 2A). In contrast, Tg(MoPrP-P101L)196/FVB mice inoculated with brain homogenates from control non-Tg(MoPrP-P101L)2247/FVB littermates remained free of symptoms. Transmission of neurodegeneration required expression of the MoPrP-P101L transgene because CNS disease was not transmitted to control non-Tg196/FVB or non-Tg196/PrP Prnp^{0/0} mice (Table 2C, D). These control experiments demonstrate that disease in inoculated Tg(MoPrP-P101L)196 mice was not caused by contaminating mouse prions.

Expression of endogenous wild-type MoPrP^C in inoculated Tg(MoPrP-P101L)196 mice had a potent inhibitory effect on the efficiency of prion transmission. Transmission of neurodegeneration from Tg(MoPrP-P101L)2247/FVB mice to recipient Tg(MoPrP-P101L)196/Prnp^{0/0} mice occurred ~200 days after inoculation, a reduction of ~40% compared to Tg(MoPrP-P101L)196/FVB recipients in which disease occurred at ~350 days (Table 2B).

Serial transmission from two ill Tg(MoPrP-P101L)196/FVB mice, designated Tg196-1(2247-1) and Tg196-2(2247-1), was only observed in Tg(MoPrP-P101L)196/FVB and Tg(MoPrP-P101L)196/Prnp^{0/0} mice expressing MoPrP^C(P101L). Transmission of CNS disease did not occur in inoculated nontransgenic 196/FVB mice expressing only endogenous wild-type MoPrP or Tg(MoPrP-A)4053/FVB mice expressing high levels of wild-type MoPrP^C (Table 2C, E), indicating that only MoGSS and not wild-type Mo prions were generated in Tg(MoPrP-P101L)196/FVB mice inoculated with brain homogenates from spontaneously ill Tg(MoPrP-P101L) mice. These findings provide even more persuasive evidence for de novo production of prions in mice expressing a transgene carrying the P102L mutation of GSS than that previously reported by two of us (S.B. Prusiner and S. J. DeArmond) (Hsiao et al. 1994).

Neuropathology of Tg(MoPrP-P101L) mice

Spongiform degeneration and reactive astrocytic gliosis were found in all mice expressing the P101L mutant transgene at the time they exhibited signs of CNS dysfunction (Fig. 2A, B). Like the Tg(MoPrP-P101L)174 and Tg(MoPrP-P101L)87 mice, occasional PrP plaques were detected in the brains of a few ill Tg(MoPrP-P101L)2866/FVB and Tg(MoPrP-P101L)2247/FVB mice (Fig. 2D). Unexpectedly, the brains of all ill Tg(MoPrP-P101L)2866/Prnp^{0/0} mice examined contained abundant PrP plaques that stained with PAS and α -PrP RO73 antiserum (Fig. 2E).

The most severe spongiform change and gliosis was found in the brains of spontaneously ill Tg(MoPrP-P101L)2862/FVB mice which overexpress mutant MoPrP^C(P101L) ~32-fold (Fig. 2C). Vacuolation of the globus pallidus, cerebellum, and substantia nigra was extensive. Severe vacuolation of the neocortex and caudate was also found in these Tg(MoPrP-P101L)2862/FVB mice. The extreme vacuolation of cerebellar white mat-

ter in Tg(MoPrP-P101L)2862/FVB mice is unusual but shares some features with that reported for mice inoculated with an extract prepared from a patient dying of GSS disease (Tateishi et al. 1979). Like Tg(MoPrP-P101L)2866/Prnp^{0/0} mice, Tg(MoPrP-P101L)2862/FVB mice contained abundant PrP plaques, although they were larger and more numerous (Fig. 2F).

The neuropathology in Tg(MoPrP-P101L)196/Prnp^{0/0} mice developing CNS degeneration after inoculation with homogenates prepared from spontaneously ill Tg(MoPrP-P101L)2247/FVB or Tg(MoPrP-P101L)2866/FVB mice consisted of intense reactive astrocytic gliosis, particularly in the cerebellar cortex and along the corticospinal tract (Fig. 2G, H). Spongiform degeneration was most marked in the corticospinal tract reminiscent of the neuropathology found in humans who died of GSS. PrP amyloid plaques were found in the subcallosal region in two of three brains examined from Tg(MoPrP-P101L)196/Prnp^{0/0} mice (Fig. 2I). No PrP plaques were seen in the brains of three ill Tg(MoPrP-P101L)196/FVB mice examined.

Immunoblots of brain extracts from Tg(MoPrP-P101L)Prnp^{0/0} mice

The abundance of PrP amyloid plaques in the brains of ill Tg(MoPrP-P101L)2866/Prnp^{0/0} mice (Fig. 2E) suggested that proteinase K-resistant PrP, that is PrP^{Sc}, was likely to be demonstrable by Western immunoblotting. To the contrary, no PrP resistant to proteinase K digestion was detectable in extracts prepared from the brains of Tg(MoPrP-P101L)2866/Prnp^{0/0} mice (Fig. 3). From these results, we conclude that the PrP deposits in the brains of Tg(MoPrP-P101L)2866/Prnp^{0/0} mice exhibiting clinical signs of CNS dysfunction and the neuropathologic hallmarks of prion disease are not derived from protease-resistant PrP^{Sc} molecules.

Unable to detect proteinase K-resistant PrP by immunoblotting of brain homogenates (Fig. 3), we asked whether detergent-insoluble PrP isolated by ultracentrifugation after limited digestion with proteinase K might reveal protease-resistant PrP molecules. Thus, we examined fractions derived from the brains of spontaneously ill or inoculated Tg(MoPrP-P101L) mice and used as controls the brains of Tg(MoPrP)4053/FVB mice overexpressing wild-type MoPrP and non-Tg(MoPrP-P101L) littermates, both of which were age-matched and well at the time of sacrifice. Both soluble and insoluble fractions were analyzed by immunoblotting after SDS-PAGE (Fig. 4). Protease-resistant PrP was found almost exclusively in the detergent-insoluble fraction of each sample; minor amounts of protease-resistant PrP in the soluble fraction in some samples seem likely to be attributable to mixing during removal of the supernatants. The molecular weight of the detergent-insoluble, protease-resistant PrP was similar to that of PrP^C at 33–35 kD, and thus, different from that of PrP 27–30 (Fig. 3). We found no correlation between the protease-resistant, insoluble PrP and the development of spontaneous CNS disease in Tg(MoPrP-P101L) mice; furthermore, PrP molecules with

Figure 2. PrP amyloid plaques, spongiform degeneration, and reactive astrocytic gliosis in mice expressing the mutant MoPrP-P101L transgene. (A, D) Tg(MoPrP-P101L)2866/FVB mouse. (B, E) Tg(MoPrP-P101L)2866/Prnp^{0/0} mouse. (C, F) Tg(MoPrP-P101L)2862/FVB mouse. (G, I) Tg(MoPrP-P101L)196/Prnp^{0/0} mouse inoculated with brain homogenate from an ill Tg(MoPrP-P101L)2247/FVB mouse. Sections of the globus pallidus stained with hematoxylin and eosin in A–C and with immunoperoxidase using α -PrP R073 antiserum in D–F and I. (G, H) The degree of reactive astrocytic gliosis is shown in sections of the cerebellar cortex (G) and the internal capsule (H) stained for GFAP with immunoperoxidase. Magnification, 400 $\times$.

similar properties were detected in brain extracts from uninoculated Tg(MoPrP-A)4053/FVB mice overexpressing wild-type MoPrP as well as non-Tg(MoPrP-P101L) littermates.

Neuromyopathy in Tg(MoPrP-P101L) mice

Although we found no evidence of CNS degeneration in 6 of 20 Tg(MoPrP-A)4053/FVB mice dying between 605 and 815 days of age (Table 1), we examined quadriceps muscles from these aged mice, as well as clinically ill Tg(MoPrP-P101L)2866/FVB mice and age-matched Tg(MoPrP-P101L)196/FVB mice to ascertain whether overexpression of PrP in these transgenic mice caused peripheral neuropathy and myopathy similar to transgenic mice overexpressing SHa or MoPrP-B (Westaway et al. 1994). Although there was no evidence for neuromyopathic changes in the skeletal muscle of aged Tg(MoPrP-A)4053/FVB mice, the skeletal muscles of Tg(MoPrP-P101L)87, Tg(MoPrP-P101L)174, and Tg(MoPrP-

P101L)2866/FVB mice exhibited neuropathic and myopathic changes concomitantly with the development of CNS disease at $\sim$ 200 days of age (Fig. 5A–C). Only very mild abnormalities of muscle and peripheral nerve were found in Tg(MoPrP-P101L)196/FVB mice sacrificed at the same age.

Skeletal muscle from clinically ill Tg(MoPrP-P101L)2866/FVB mice revealed marked type I fiber predominance and grouping (dark muscle fibers in right two-thirds of the photomicrograph, Fig. 5A) indicative of neurogenic rearrangement of muscle fiber types with a relatively normal distribution of type I and II muscle fibers in the left one-third. Occasional degenerating muscle fibers were observed with rare fibers undergoing phagocytosis (Fig. 5B, arrow). Muscle from age-matched Tg(MoPrP-P101L)196/FVB mice showed normal numbers and distribution of type I and II fibers, whereas the diameter of the type I fibers (darkly stained) appeared to be, on the average, about 10 μ m less than the diameter of type I fibers in normal mice (Fig. 5D). Examination of

Figure 3. Western blot of brain homogenates from transgenic mice expressing MoPrP with the P101L mutation that causes GSS. Tg(MoPrP-P101L) mice developed neurologic dysfunction spontaneously. Aliquots of brain homogenates of ill mice were either treated (odd-numbered lanes) or digested with 20 μ g proteinase K for 60 min at 37°C (even-numbered lanes). Samples were analyzed as described above except α -PrP RO73 antiserum was used. (Lanes 1, 2) Uninoculated CD-1 mouse; (lanes 3, 4) non-transgenic mouse inoculated with RML Mo prions that showed signs of illness after 128 days; (lanes 5, 6) Tg(MoPrP-P101L) 2866/FVB mouse spontaneously ill at 213 days; (lanes 7, 8) Tg(MoPrP-P101L)2862/FVB mouse spontaneously sick at 333 days; (lanes 9, 10) Tg(MoPrP-P101L)2247/FVB mouse spontaneously ill at 160 days; (lanes 11, 12) Tg(MoPrP-P101L) 2866/Prnp^{0/0} mouse spontaneously ill at 138 days. The positions of protein molecular weight markers are shown to the left of the blot and correspond to molecular masses of (from top to bottom) 45, 31, 21, and 14 kD.

muscle from aged Tg(MoPrP-A)4053/FVB mice revealed no abnormalities in type I and II fiber size or distribution (Fig. 5E). The NADH staining (Fig. 5C) revealed subsarcolemmal accumulation of mitochondria, verified by the Gomori trichrome stain (ragged red fibers) and succinic dehydrogenase stain (latter two not shown), and abnormalities in the distribution of the sarcoplasmic reticulum (moth-eaten fibers). Pathological changes in sciatic nerve were found in Tg(MoPrP-P101L)2866/FVB mice and included regional loss of axons and active Wallerian degeneration (Fig. 6). The muscle fiber-type grouping abnormalities shown in Figure 5 is related to the axonal degeneration shown in Figure 6. The degenerating fibers, mitochondrial hyperplasia, and moth-eaten fibers are myopathic features, and suggest that high expression of MoPrP-P101L, but not wild-type MoPrP, causes abnormalities of skeletal muscle both directly and indirectly via peripheral nerve.

Abbreviated scrapie incubation times in Tg(MoPrP-A) mice

Studies with Tg(SHaPrP) mice demonstrated that the level of SHaPrP^C expression was inversely related to the length of the incubation time (Prusiner et al. 1990). As expected, Tg(MoPrP-A)4053/FVB mice expressing high levels of MoPrP^C-A exhibited abbreviated incubation times of ~45 days after inoculation with mouse RML prions (Table 3A). The duration of illness was also shortened. Homogenates from two ill Tg(MoPrP-A)4053/FVB mice, designated RML(4053-1) and RML(4053-2) produced incubation times that were indistinguishable from those of RML prions passaged in CD-1 Swiss mice or Tg(MoPrP-A)4053/FVB mice showing that passage

through Tg(MoPrP-A)4053/FVB mice did not alter the strain characteristics of RML. By mating Tg(MoPrP-A)4053/FVB mice with Prnp^{0/0} mice, we produced a line of mice in which the MoPrP-A transgene was expressed in the absence of endogenous mouse Prnp alleles. Scrapie incubation times in these Tg(MoPrP-A)4053/Prnp^{0/0} mice inoculated with RML prions was prolonged by ~10 days compared to Tg(MoPrP-A)4053/FVB mice reflecting the diminished level of PrP-A expression (Table 3A).

The brains of ill Tg(MoPrP-A)4053/FVB mice inoculated with RML prions showed substantially reduced spongiform degeneration and reactive astrocytic gliosis compared to those of nontransgenic mice (Fig. 7). Whether the shorter incubation time of Tg(MoPrP-

Figure 4. The presence of detergent-insoluble, protease-resistant PrP in mouse brain does not correlate with disease in spontaneously ill or inoculated Tg(MoPrP-P101L) mice. Brain homogenates were adjusted to 100 μ g total protein/ml in TNE (10 mM Tris at pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 0.2% sarkosyl and proteinase K was added at a final concentration of either 10 μ g/ml (A) or 100 μ g/ml (B). Digestion was allowed to proceed for 1 hr at 37°C and was terminated by addition of 100 mM PMSF. Insoluble aggregates were pelleted by centrifugation at 100,000 $\times$ g for 1 hr. Pellets from the high speed centrifugation were resuspended in cell lysis buffer containing 0.5% NP-40, 0.5% deoxycholic acid, 150 mM NaCl and 10 mM Tris-HCl, at pH 8.0 and added to an equal volume of 2 $\times$ SDS-PAGE loading buffer. The supernatants following ultracentrifugation were concentrated by precipitation at -20°C in 5 vol of methanol followed by centrifugation at 3700 $\times$ g for 15 min. Pellets from this low speed centrifugation were also resuspended in cell lysis buffer and added to an equal volume of 2 $\times$ SDS-PAGE loading buffer. The samples were analyzed by SDS-PAGE and Western blotting using the α -PrP RO73 antiserum. Odd numbered lanes are sedimented samples from the high-speed centrifugation, even numbered lanes are supernatants. The lanes are as follows: (Lanes 1, 2) Non-transgenic mouse; (lanes 3, 4) Tg(MoPrP)4053/FVB; (lanes 5, 6) Tg(MoPrP-P101L)2866/FVB; (lanes 7, 8) Tg(MoPrP-P101L)2247/FVB; (lanes 9, 10) Tg(MoPrP-P101L)2862/FVB; (lanes 11, 12) Tg(MoPrP-P101L)196/FVB mice inoculated with MoPrP-P101L prions from a spontaneously sick Tg(MoPrP-P101L)2247/FVB mouse. The sizes of molecular mass markers from top to bottom are 85.0, 50.3, 33.3, and 28.5 kD.

Figure 5. Skeletal muscle in Tg(MoPrP-P101L) mice expressing high levels of the mutant MoPrP transgene develop a neuromyopathy. Only mild abnormalities were found in the muscles or peripheral nerves of well, age-matched Tg(MoPrP-P101L)196 mice expressing low levels of the mutant PrP transgene. No abnormalities were found in the muscles or peripheral nerves of well Tg(MoPrP-A)4053/FVB mice expressing high levels of wild-type MoPrP at 700 days of age. (A) The NADH histochemical stain reveals marked type I fiber predominance and grouping (dark muscle fibers in *right* two-thirds of the photomicrograph) indicative of neurogenic rearrangement of muscle fiber types with a relatively normal distribution of type I and II muscle fibers in the *left* one-third. (B) The hematoxylin and eosin-stained section reveals occasional degenerating muscle fibers with a rare fiber undergoing phagocytosis (arrow). (C) The higher power photomicrograph of the NADH-stained section shows subsarcolemmal accumulation of mitochondria, verified by the Gomori trichrome stain (ragged red fibers) and succinic dehydrogenase stain (latter two not shown), and abnormalities in the distribution of the sarcoplasmic reticulum (moth-eaten fibers). The degenerating fibers, mitochondrial hyperplasia and moth-eaten fibers are myopathic features and suggest that high expression of the MoPrP-P101L transgene causes abnormalities of skeletal muscle directly as well as indirectly via peripheral nerve. (D) The cytochrome-oxidase histochemical stain of a low expressor Tg(MoPrP-P101L)196/FVB mouse muscle shows normal numbers of type I and II fibers and a normal distribution pattern. The diameter of the type I fibers (darkly stained) are, on the average, about 10 μ m less than the diameter of type I fibers in normal mice. (E) The NADH stain of an old Tg(MoPrP-A)4053/FVB shows no abnormalities in type I and II fiber size or distribution. The quadriceps femoris was examined in each case. Bar in E, 100 μ m and also applies to A and D; bars in B and C, 50 μ m.

A)4053/FVB mice compared to nontransgenic controls is responsible, at least in part, for the reduced neuropathology, remains to be determined. Western blot analysis of brain homogenates from ill Tg(MoPrP-A)4053/FVB mice inoculated with RML prions had less PrP^{Sc} than inoculated nontransgenic littermates (Fig. 8). Although the patterns of PrP^{Sc} deposition were similar in scrapied Tg(MoPrP-A)4053/FVB and nontransgenic mice, histoblots showed lower levels of PrP^{Sc} in the brains of ill Tg(MoPrP-A)4053/FVB than in nontransgenic mice (data not shown).

Discussion

The development of a mouse model for human GSS (P102L) has substantially increased our understanding of the inherited prion diseases. Although we were initially concerned about the infrequency of PrP amyloid plaques, the lack of protease-resistant PrP, and transmission of disease to a minority of inoculated recipients with extremely prolonged incubation times (Hsiao et al. 1990, 1994), each of those concerns has been addressed as described here. From our results, we conclude that Tg(MoPrP-P101L) mice faithfully recapitulate virtually all features of human GSS (P102L).

Transgenic mice that overexpress wild-type MoPrP^C at the same level as mutant MoPrP^C(P101L) demonstrate clearly that the P101L mutation is required for CNS degeneration. Our studies also demonstrate a profound suppressive influence of wild-type MoPrP^C on both the clinical and neuropathologic features of the GSS phenotype. The inhibitory effects of wild-type MoPrP^C were manifest in multiple ways: First, ablation of the wild-type MoPrP gene significantly modifies the clinical symptoms of spontaneously sick Tg(MoPrP-P101L) mice, changing the age of onset of CNS disease from a relatively stochastic process in Tg(MoPrP-P101L)FVB mice to a highly uniform process in Tg(MoPrP-P101L)-Prnp^{0/0} mice (Fig. 1). Second, expression of endogenous

Figure 6. Pathological features of sciatic nerves from Tg(MoPrP-P101L) mice include regional loss of axons and active Wallerian degeneration. (A) Cross section of a nerve fascicle showing normal numbers and density of axons to the *left* and reduction in the number of axons with occasional degenerating axons on the *right*. (B) Longitudinal section of the same nerve fascicle shows multiple degenerating axons in the *upper* half of the photomicrograph and normal axons in the *lower* half. Arrows in A and B indicate examples of axonal degeneration. The muscle fiber type grouping shown in Fig. 5 is related to the axonal degeneration demonstrated in this figure. One-micron-thick plastic section of nerve stained with toluidine blue. Bar in A, 50 μ m and also applies to B.

Table 3. Prion incubation times in Tg(MoPrP-A) mice

Recipient mice	Inoculum ^a	Incubation times (mean days ± S.E.M.)	
		Illness ^b	Death ^c
A. Tg(PrP-A) mice			
Tg(MoPrP-A)4053/FVB	RML	48 ± 1 (21/21)	53 ± 2 (12)
Tg(MoPrP-A)4053/FVB	Tg4053-2 (RML)	48 ± 2 (10/10)	58 ± 2 (7)
Non-Tg 4053	RML	127 ± 2 (18/18)	156 ± 2 (5)
Tg(MoPrP-A)4053/Prnp ^{0/0}	RML	59 ± 2 (9/9)	65 ± 2 (6)
B. Swiss CD-1 mice			
Swiss CD-1 ^d	RML	138 ± 1 (24/24)	159 ± 3 (24)
Swiss CD-1	Tg4053-1 (RML)	140 ± 3 (10/10)	163 ± 2 (6)
Swiss CD-1	Tg4053-2 (RML)	138 ± 5 (9/9)	169 ± 2 (6)

^aNotation in parentheses indicates inoculum used in initial passage. Single digit following a hyphen distinguishes individual mice from a particular line.

^bThe number of mice developing clinical signs of scrapie is shown in parentheses.

^cThe number of mice dying of scrapie is shown in parentheses. Mice sacrificed for pathologic examination were excluded from these calculations.

^dData from Butler et al. (1988).

wild-type MoPrP caused the clinical phase of disease to be more protracted in Tg(MoPrP-P101L) mice. Third, elimination of endogenous wild-type MoPrP^C expression modified the neuropathology of Tg(MoPrP-P101L) mice: Whereas relatively few PrP plaques could be detected in Tg(MoPrP-P101L)FVB mice, there was widespread deposition of PrP amyloid plaques in Tg(MoPrP-P101L)-Prnp^{0/0} mice in addition to spongiform degeneration (Fig. 2). Fourth, elimination of wild-type MoPrP^C expression rendered Tg(MoPrP-P101L)196 mice more susceptible to GSS prions from spontaneously sick Tg(MoPrP-P101L) mice, manifested by a ~40% reduction of GSS prion incubation times in inoculated Tg(MoPrP-P101L)-196/Prnp^{0/0} mice compared to Tg(MoPrP-P101L)196/FVB recipients (Table 2).

Transmission of de novo generated prions

The transmission experiments reported here demonstrate unequivocally the formation of infectious prions in the brains of spontaneously ill Tg(MoPrP-P101L) mice and, in conjunction with many other lines of experimental investigation, continue to militate for the absence of a nucleic acid within the prion particle (Prusiner 1991; Prusiner 1982). The reproducible transmission of neurodegeneration from Tg(MoPrP-P101L) mice and not from control nontransgenic mice (Table 2) rules out the trivial possibility of transmission by contamination. Although low level expression of the MoPrP-P101L transgene does not cause spontaneous neurodegeneration in Tg(MoPrP-P101L)196/FVB or Tg(MoPrP-P101L)196/Prnp^{0/0} mice after >750 days (Table 2), efficient transmission of mutant P101L prions requires expression of MoPrP-P101L in these recipient mice. Prions produced de novo in Tg(MoPrP-P101L)2247/FVB mice did not transmit disease to nontransgenic mice which only express wild-type MoPrP or to Tg(MoPrP-A)4053/FVB mice which overexpress wild-type MoPrP and are highly sensitive to wild-type mouse prions.

Transgenic studies argue that PrP^C and PrP^{Sc} form a complex during the conversion of PrP^C into nascent PrP^{Sc} (Prusiner et al. 1990; Scott et al. 1993). The data reported here underscore the results of human CJD and GSS prion transmissions in Tg(MHu2M)5378 and Tg(MHu2M-P102L)69 mice which argue that prion prop-

Figure 7. The intensity of spongiform degeneration and reactive astrocytic gliosis in the brains of Tg(MoPrP-A)4053/FVB mice and non-transgenic littermates inoculated with mouse RML prions. Mice were sacrificed when they showed signs of CNS dysfunction. (A, C, E) Brain sections from inoculated non-transgenic mice. (B, D, F) Brain sections from inoculated Tg(MoPrP-A)4053/FVB mice. A and B are hematoxylin and eosin stained sections showing spongiform degeneration in the cerebellum while panels C and D are hematoxylin and eosin stained sections showing spongiform degeneration in the hippocampus. GFAP immunohistochemistry was performed on sections through the hippocampus and show reactive astrocytic gliosis in E and F. Bar, 100 μ M.

Figure 8. PrP^C and PrP^{Sc} in the brains of ill Tg(MoPrP-A)4053/FVB mice and non-transgenic mice inoculated with mouse RML prions. The total concentrations of protein in 10% brain homogenates were determined by bicinchoninic acid assay. Aliquots of brain homogenates containing equal amounts of total protein in each case were either treated with 20 μ g of proteinase K for 60 min at 37°C (even-numbered lanes) or not treated (odd-numbered lanes). Samples were resolved by SDS-PAGE and analyzed by Western blot. The blot was exposed to the α -PrP RO73 antiserum, developed using enhanced chemiluminescence (ECL) and exposed to X-ray film. (1, 2) Uninoculated CD-1 mouse; (3, 4) uninoculated Tg(MoPrP-A)4053/FVB mouse; (5, 6) Tg(MoPrP-A)4053/FVB mouse inoculated with mouse RML prions sick at 48 days; (7, 8) Tg(MoPrP-A)4053/FVB mouse inoculated with mouse RML prions sick at 56 days; (9, 10) non-transgenic littermate inoculated with mouse RML prions sick at 128 days; (11, 12) non-transgenic littermate inoculated with mouse RML prions sick at 125 days. The positions of protein molecular weight markers are shown to the left of the blot and correspond to molecular masses of (from top to bottom) 31, 21, and 14 kD.

agation is facilitated by homology within the central domains of HuPrP^C and HuPrP^{Sc} (Telling et al. 1995). Since wild-type MoPrP and MoPrP-P101L differ only at a single amino acid position, these results further demonstrate that prion propagation is facilitated by homology between PrP^C and PrP^{Sc} and that mismatch at residue 101 inhibits this process. This conclusion is strengthened by the results of serial passaging experiments which demonstrate that prions produced de novo in the brains of Tg(MoPrP-P101L) mice discriminate between wild-type MoPrP^C and MoPrP^C(P101L) expressed in Tg(MoPrP-P101L)196/FVB mice. In these transgenic mice, MoPrP-P101L prions, but not wild-type mouse prions, were produced as demonstrated by serial passage. Furthermore, expression of endogenous wild-type MoPrP^C in inoculated Tg(MoPrP-P101L)196 mice had a potent inhibitory effect on the efficiency of MoPrP-P101L prion transmission.

Absence of protease-resistant PrP in diseased Tg(MoPrP-P101L) mice

Although earlier studies had shown an excellent correlation between the level of protease-resistant PrP^{Sc} (McKinley et al. 1983) and scrapie prion infectivity, making it a good marker for disease, we were unable to detect protease-resistant PrP^{Sc} in these spontaneously ill mice or in inoculated Tg(MoPrP-P101L)196 mice. Whether additional studies using lower concentrations of proteinase K for very brief periods of time (Lehmann and Harris 1996) will demonstrate a meaningful difference between

mutant PrP from ill Tg(MoPrP-P101L) mice and controls such as uninoculated Tg(MoPrP-A) and Tg(MoPrP-P101L)196 mice remains to be determined. In earlier studies, we observed some protease-resistant PrP in brain extracts prepared from uninoculated Tg(SHaPrP) mice overexpressing SHaPrP when low concentrations of proteinase K were used for digestions (Prusiner et al. 1990). To differentiate what seems to be incompletely digested PrP^C from protease-resistant PrP^{Sc}, we routinely used high concentrations of proteinase K. Since the mutant PrP(P101L) molecules clearly convey prion infectivity (Table 2), we conclude in accord with earlier studies (Hsiao et al. 1994) that the absence of protease-resistant PrP^{Sc} does not rule out prion disease, even though the presence of protease-resistant PrP^{Sc} is considered a diagnostic marker for the prion diseases (Prusiner and Hsiao 1994).

The absence of detectable protease-resistant PrP^{Sc} does not, however, preclude its presence in the infectious prion. The sensitivity of the bioassay for prion detection is several orders of magnitude greater than for the detection of PrP^{Sc}. An alternative explanation for our inability to detect protease-resistant PrP^{Sc} in the brains of Tg(MoPrP-P101L) mice is that the titer of infectious prions in ill Tg(MoPrP-P101L) mice is below the level for detection of protease-resistant PrP^{Sc}. Experiments designed to determine accurately the titer of prions in these mice by endpoint titration are underway and should resolve this issue. Such studies should also help determine whether infectivity generated in Tg(MoPrP-P101L) mice is resistant or sensitive to protease treatment.

Similar to our Tg(MoPrP-P101L)2866/Prnp^{0/0} mice (Fig. 2), the brains of patients who die from GSS (P102L) always contain PrP amyloid plaques, yet detectable protease-resistant PrP is not a constant feature (Telling et al. 1995). Although brain tissue from GSS (P102L) often transmits disease to inoculated recipients, this is also not a constant feature (Masters et al. 1981; Brown et al. 1994; Tateishi and Kitamoto 1995). Whether all cases of GSS (P102L) will transmit disease to Tg(MHu2M-P102L) mice expressing a mutant, chimeric Hu/Mo PrP transgene remains to be established (Telling et al. 1995). It is also of interest that the brains of patients who die of inherited CJD (E200K) have two forms of insoluble PrP; the mutant PrP is protease resistant, and the wild-type is sensitive to protease digestion (Gabizon et al. 1996). Yet, both the mutant and wild-type insoluble PrP can be distinguished from PrP^C which is soluble in non-denaturing detergents.

The P101L mutation causes neurodegeneration and neuromyopathy

The fact that overexpression of wild-type PrP can produce neurodegeneration of the CNS as well as neuromyopathy (Westaway et al. 1994) prompted us to re-examine neurodegeneration in Tg(MoPrP-P101L) mice (Hsiao et al. 1990). The inclusion of additional control transgenic mice which overexpress wild-type MoPrP at levels

equivalent to mutant PrP in Tg(MoPrP-P101L) mice demonstrates that the P101L mutation, not merely overexpression of PrP, causes the clinical and neuropathologic features of GSS. It is noteworthy that in addition to neurodegeneration of the CNS, skeletal muscle in high expressor Tg(MoPrP-P101L) lines developed severe neuropathic and myopathic changes at the time they developed CNS disease (Fig. 5). Features of amyotrophy with muscle fasciculations and fibrillations have been reported in an Italian kindred with GSS (P102L) (Kretzschmar et al. 1992), and the proband from a large English pedigree with GSS, in addition to neurogenic atrophy, showed muscle pathologic changes (Rosenthal et al. 1976). Immunostaining studies have localized PrP at neuromuscular junctions in humans (Askanas et al. 1993) but mice deficient for PrP^C have been shown to exhibit normal electrical transmission at the neuromuscular junction (Brenner et al. 1992). Whether neuromyopathic changes are an integral feature of human GSS (P102L) remains to be established.

Aged mice overexpressing either wild-type SHaPrP or the Prnp^b allele develop hind limb paresis as a result of a necrotizing myopathy and a demyelinating peripheral neuropathy (Westaway et al. 1994). In contrast, aged mice overexpressing the Prnp^a allele showed no evidence of hind limb paresis or pathologic changes in skeletal muscle or peripheral nerves. The mechanism by which overexpression of mutant as well as foreign wild-type transgenes cause neuromyopathies is unknown. Perhaps, heterologous PrPs expressed in Prnp^a FVB mice tend to misfold more frequently than do homologous PrP molecules. SHaPrP differs from MoPrP at 16 of 254 amino acid residues (Basler et al. 1986; Lochter et al. 1986), MoPrP-B and MoPrP-A differ at amino acid residues 108 and 189 (Westaway et al. 1987), and MoPrP-P101L and MoPrP-A differ at a single residue.

Modifying the disease phenotypes of Tg(MoPrP-P101L) mice

Variations in the phenotypes of dominantly inherited neurologic diseases have often been attributed to the interplay of gene products other than that which carries the disease-causing mutation. Two new mechanisms have been discovered recently, and a third is reported here. First, the triplet repeat diseases, such as myotonic dystrophy and Huntington's chorea, exhibit phenotypic variation with respect to the age of onset that depends upon the number of repeats (Fu et al. 1992; Huntington's Disease Collaborative Research Group 1993). In general, there is an inverse relationship between the number of repeats and the age of onset; this finding provided a molecular basis for the phenomenon called anticipation. Second, the inherited prion diseases caused by a D178N mutation of the PrP gene present as dementing illnesses or insomnia, depending upon the polymorphic residue codon 129 (Goldfarb et al. 1992; Monari et al. 1994). When V129 is encoded on the mutant allele, patients present with a dementia; M129 presents as insomnia. Other PrP mutations at codons 102, 105, and 198 often

present with ataxia (Dlouhy et al. 1992; Kitamoto et al. 1993), while the codon 200 mutation seems to present invariably as a dementia (Gabizon et al. 1993). Why a few members of a large pedigree with the codon 102 mutation present with a dementia is unknown (Hsiao et al. 1989).

The studies presented here show that the clinical and neuropathologic phenotypes of transgenic mice can be dramatically altered by ablation of the wild-type MoPrP gene. Tg(MoPrP-P101L) mice that harbor the wild-type MoPrP gene exhibit variable times of onset of neurologic disease ranging from 150 to 300 days followed by a clinical phase of illness lasting almost three weeks (Fig. 1). The neuropathology of these mice shows spongiform degeneration and relatively few PrP plaques. In contrast, >90% of Tg(MoPrP-P101L)/Prnp^{0/0} mice develop clinical signs of CNS dysfunction within a period of ~21 days and have a short duration of illness lasting ~3 days. The neuropathology of these mice not only shows spongiform degeneration but also widespread deposition of PrP amyloid plaques (Fig. 2). Crossing these mice to produce animals homozygous for the mutant transgene array produced spontaneous disease at ~85 days of age (Fig. 1A). Just as expression of wild-type MoPrP dramatically altered the clinical and neuropathologic phenotypes of Tg(MoPrP-P101L) mice, expression of endogenous wild-type MoPrP in recipient Tg(MoPrP-P101L)196 mice had a potent inhibitory effect on the efficiency of Mo prion transmission (Tab. 2).

Our results are consistent with a model in which mutant and wild-type PrP^C bind to an additional component required for prion propagation, which we have designated protein X (Telling et al. 1995). Presumably, protein X facilitates the conversion of MoPrP^C(P101L) into PrP^{Sc}. The elimination of wild-type MoPrP^C expression converts the pathogenesis from what appears to be a stochastic one in Tg(MoPrP-P101L)/FVB mice into a highly regulated process in Tg(MoPrP-P101L)/Prnp^{0/0} mice. This change in prion disease pathogenesis is characterized by a narrowing of the range of ages at which neurologic dysfunction is initially manifest and an abbreviated duration of illness. Mutant and wild-type MoPrP^C differ only at one amino acid residue likely to lie outside the putative binding site for protein X, thought to reside at the carboxy-terminus of PrP^C (Telling et al. 1995). It remains to be determined if the inhibitory effect of relatively low levels of wild-type MoPrP^C on inherited prion disease pathogenesis is a result of competition between mutant and wild-type MoPrP for binding to protein X.

Determinants of CNS pathogenesis in Tg(MoPrP-P101L) mice

The numerous PrP amyloid plaques in the brains of Tg(MoPrP-P101L)2862/FVB and Tg(MoPrP-P101L)2866/Prnp^{0/0} mice (Fig. 2) is particularly important since it clearly documents an abnormality in PrP structure and metabolism, despite the absence of demonstrable protease-resistant PrP^{Sc} on Western blots (Fig. 3). Demon-

strating PrP^{Sc} in the brains of some patients, particularly those dying of inherited prion diseases, has also been problematic (Brown et al. 1986; Medori et al. 1992).

The large numbers of PrP amyloid plaques in the brains of Tg(MoPrP-P101L)2862/FVB and Tg(MoPrP-P101L)2866/Prnp^{0/0} mice suggest that mutant PrP^C is being refolded into molecules with substantial β -sheet content (Huang et al. 1996). Although FTIR and CD spectroscopy have clearly demonstrated that a major conformational change occurs during the conversion of wild-type PrP^C into PrP^{Sc} (Pan et al. 1993), no data on the secondary or tertiary structure of PrP(P101L) is yet available.

From data presented here, it appears that the number of PrP amyloid plaques may be directly proportional to the age of onset of neurologic dysfunction and to the ratio of mutant: wild-type MoPrP^C. Tg(MoPrP-P101L^{+/+})2866/Prnp^{0/0} mice homozygous for the PrP transgene array exhibit a reduced number of PrP amyloid plaques compared to mice that are hemizygous for the transgene array (data not shown) even though they presumably express twice as much MoPrP^C(P101L). These homozygous Tg(MoPrP-P101L^{+/+})2866/Prnp^{0/0} mice develop CNS disease at ~85 days of age compared to hemizygous mice that become ill at ~145 days (Tab. 1). This finding argues that sufficient time must elapse for PrP amyloid deposition to occur. The hypothesis that age of onset of illness is a factor is supported by our finding that Tg(MoPrP-P101L)2862/FVB mice with numerous PrP amyloid plaques (Fig. 2) that express much higher levels of mutant PrP^C and live longer than Tg(MoPrP-P101L)2866/FVB mice. While the Tg(MoPrP-P101L)2862/FVB mice express ~32-fold more mutant MoPrP^C than wild-type MoPrP^C, this high ratio of mutant wild-type MoPrP^C is similar to that in Tg(MoPrP-P101L)2866/Prnp^{0/0} mice.

Whether factors other than the age of onset and the ratio of mutant to wild-type MoPrP^C influence pathogenesis remains to be established. Why the Tg(MoPrP-P101L)2862/FVB mice exhibit such a late onset of disease (Fig. 1) is unclear, as they have the highest levels of expression of MoPrP^C(P101L) and the most severe disease neuropathologically with respect to spongiform degeneration, astrocytic gliosis, and PrP amyloid deposition (Fig. 2). It seems likely that either the measured ~32-fold overexpression of PrP overestimates the concentration of correctly folded PrP^C that is eligible for conversion into PrP^{Sc}, or that insertion of the MoPrP-P101L transgene into the genome of the Tg(MoPrP-P101L)2862/FVB mouse alters the expression of a chromosomal gene that modulates the onset of illness. Because both the Tg(MoPrP-P101L)2247/FVB and Tg(MoPrP-P101L)2286/FVB lines of mice express lower levels of MoPrP^C(P101L) and develop signs of CNS dysfunction more rapidly, additional lines with high levels of MoPrP^C(P101L) need to be constructed in order to study this conundrum.

New approaches arising from these studies

The transmission of CNS degeneration from ill Tg(Mo-

PrP-P101L)2247/FVB and Tg(MoPrP-P101L)2866/FVB mice to Tg(MoPrP-P101L)196/FVB and Tg(MoPrP-P101L)196/Prnp^{0/0} mice and subsequent serial transmissions not only provides convincing evidence for de novo generation of prions but also a new system for studies of genetic and infectious prion disease. The relatively short incubation times of ~200 days in Tg(MoPrP-P101L)196/Prnp^{0/0} mice inoculated with mutant prions (Table 1) make characterization of these prions produced de novo feasible for the first time.

Studies of the kinetics of plaque formation in Tg(MoPrP-P101L)2866/Prnp^{0/0} mice are likely to give important insights into the pathogenesis of the inherited human prion diseases. To our knowledge, this is the first animal model of a dominantly inherited neurologic disease in which the onset of illness is sufficiently uniform that animals sacrificed at different times in the study can be compared, as has been done in studies of rodents inoculated with scrapie prions. Tg(MoPrP-P101L)2866 and Tg(MoPrP-P101L)2247 mice may be useful models for evaluating gene therapy for the inherited human prion diseases. Mating Tg(MoPrP-P101L)2866 or Tg(MoPrP-P101L)2247 with Tg(MoPrP-A)4053 mice that overexpress wild-type PrP-A may prevent neurodegeneration or substantially delay the onset of disease. Alternatively, the uniformity of onset of clinical signs in Tg(MoPrP-P101L)2866/Prnp^{0/0} mice may provide a testable model for evaluating pharmaceuticals that either stabilize PrP^C or inhibit the conversion of PrP^C into PrP^{Sc} (Cohen et al. 1994). Thus, the transgenic mice described here may provide models for developing and evaluating effective treatments to prevent or ameliorate inherited neurodegenerative diseases.

Materials and methods

Production of transgenic mice

The MoPrP-A sequence used in this study was derived from C57BL mice (Locht et al. 1986; Westaway et al. 1987). Construction of the MoPrP ORF cassette has been described previously (Rogers et al. 1991). The construction of the MoPrP-P101L expression cassette containing a substitution of a leucine for proline codon at residue 101 of the MoPrP gene, corresponding to the GSS mutation at codon 102 of the human PrP gene, has been described (Hsiao et al. 1990). ORF cassettes were digested with *Bgl*III which cleaves immediately adjacent to the initiation codon. The 5' protruding termini were filled in because of Klenow, and *Sal*I linkers were added. Recombinant clones were cleaved with *Sal*I and *Xho*I which cleaves immediately past the PrP termination codon, and purified ORF fragments were ligated to the *Sal*I-cut cosShA.Tet cosmid expression vector (Scott et al. 1992). The isolation of recombinant cosmid clones was achieved by methods that have been described previously (Scott et al. 1993). *Not*I fragments, recovered from large-scale DNA cosmid preparations, were used for microinjection into the pronuclei of fertilized FVB/N oocytes as previously described (Scott et al. 1989). Genomic DNA isolated from tail tissue of weanling animals was screened for the presence of incorporated transgenes using a probe that hybridizes to the 3'-untranslated region of the SHaPrP gene contained in the cosShA.Tet vector (Scott et al. 1992).

Preparation of brain homogenates

Ten percent (wt/vol) homogenates of mouse brain were prepared by repeated extrusion through an 18-gauge syringe needle followed by extrusion through a 22-gauge needle in phosphate buffered saline (PBS) lacking calcium and magnesium ions. For immunoblot analysis, samples were cleared of cell debris by a brief low-speed centrifugation.

Sources of prion inocula

The RML isolate from Swiss mice (Chandler 1961) was provided by Dr. William Hadlow (Rocky Mountain Laboratory, Hamilton, Montana) and was passaged in Swiss mice from a closed colony at the Rocky Mountain Laboratory (Hamilton, MT) or in Swiss CD-1 mice obtained from Charles River Laboratories (Wilmington, MA). For transmission of neurodegeneration from spontaneously sick Tg(MoPrP-P101L) mice, brain homogenates were prepared as described above.

Determination of incubation periods

Mice were inoculated intracerebrally with 30 μ l of brain extract using a 27-gauge needle inserted into the right parietal lobe, and observed for signs of disease. Additionally, uninoculated Tg(MoPrP-P101L) and Tg(MoPrP-A) mice were observed for spontaneous CNS disease. The preparation of inocula and criteria for diagnosis of scrapie in mice have been described (Carlson et al. 1986). When clinical signs of CNS dysfunction appeared, the mice were examined daily. To confirm the clinical diagnosis, the brains of some animals whose death was obviously imminent were taken for histopathological studies.

Immunoblotting

Total protein concentrations in brain homogenates were determined by bicinchoninic acid assay. Immuno dot blots for the determination of the relative levels of PrP expression in transgenic mouse brains were performed as described previously (Scott et al. 1993). Samples for Western blot analyses were prepared as described in the figure legends. Western blots were performed as described previously (Towbin et al. 1979; Barry and Prusiner 1986), except that an enhanced chemiluminescent (ECL) detection method (Amersham, Arlington Heights, IL) was used. The blot was exposed to X-ray film. α -PrP rabbit RO73 antiserum was used at a final dilution of 1:5000.

Immunohistochemistry

For immunohistochemistry, endogenous peroxidase activity was blocked with methanol-hydrogen peroxide (2 ml of 30% H_2O_2 in 200 ml methanol). To enhance PrP immunoreactivity, the sections were immersed in 1.3 M HCl and autoclaved at 121°C for 10 min (Muramoto et al. 1992). When the temperature decreased, the slides were placed under running tap water for 10 min followed by rinsing with TB (50 mM Tris-HCl at pH 7.6, with 150 mM NaCl). Nonspecific protein binding was blocked with TBS (25 mM Tris-HCl at pH 7.6, 0.05% Tween 20, 0.5 M NaCl, 5% nonfat milk) for 10 min. The α -PrP polyclonal rabbit RO73 antiserum was diluted 1:250 in TBS. Tissue sections were incubated with the primary antiserum overnight at 4°C. Following two rinses with TB containing 0.1% Triton-X and once in TB, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in TBS, 1:100 dilution, for 30 min at room temperature. After two rinses with TB containing 0.1% Triton-X, the sections were incubated with

an avidin/biotin/peroxidase complex (Vector Laboratories) for 30 min at room temperature. Three rinses in TB were then followed by a 3–5 min reaction with diaminobenzidine solution (5 mg diaminobenzidine tetrahydrochloride, 68 mg imidazole, and 7 mg of NaN_3 in 10 ml of TB), to which 100 μ l of 0.0015% H_2O_2 was added.

Peroxidase immunohistochemistry with antibodies to glial fibrillary acidic protein was used to evaluate reactive astrocytic gliosis.

Neuropathology

CNS, sciatic nerve and skeletal muscle pathologies were assessed by standard procedures (Scott et al. 1989; Westaway et al. 1994).

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