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Mice Devoid of PrP Are Resistant to Scrapie

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Summary

S. B. Prusiner proposed that the infectious agent of scrapie, the prion, is PrP^{Sc}, a modified form of the normal host protein PrP^{C}. *Prn-p*$^{0/0}$ mice devoid of PrP^{C} showed normal development and behavior. When inoculated with mouse scrapie prions, they remained free of scrapie symptoms for at least 13 months while wild-type controls all died within 6 months. Surprisingly, heterozygous *Prn-p*$^{0/+}$ mice also showed enhanced resistance to scrapie. After introduction of Syrian hamster PrP transgenes, *Prn-p*$^{0/0}$ mice became highly susceptible to hamster but not to mouse prions. These experiments show that PrP^{C}, possibly at close to normal levels, is required for the usual susceptibility to scrapie and that lack of homology between incoming prions and the host's PrP genes retards disease.

Introduction

The nature of the prion, the agent causing transmissible spongiform encephalopathies such as scrapie in animals or Creutzfeld–Jakob disease in humans, has been a long-standing enigma. Considerable evidence has accumulated in support of Prusiner's proposal (Prusiner, 1982), subsequently espoused by others (Bolton, 1988; Bolton and Bendheim, 1988; Gajdusek, 1991), that the prion consists of PrP^{Sc}, a modified form of the normal host protein PrP^{C}, and is devoid of nucleic acid (the "protein only" model; for reviews see Prusiner, 1991; Weissmann, 1991). The propagation of the prion is thought to come about by the conversion of endogenous PrP^{C} to PrP^{Sc} catalyzed by PrP^{Sc} (Prusiner, 1991; Prusiner et al., 1990).

There are two major lines of evidence in favor of the protein only hypothesis. First, PrP^{Sc} and scrapie infectivity copurify by several procedures (Bolton et al., 1982; Diringer et al., 1983; Prusiner et al., 1982, 1983), including affinity chromatography on an anti-PrP monoclonal antibody column (Gabizon et al., 1988), and no scrapie-specific nucleic acid has been detected in highly purified prion preparations, despite long-standing efforts (Oesch et al., 1988; Meyer et al., 1991; Kellings et al., 1992). Second, genetic evidence points to an intimate linkage between prion disease and *Prn-p*, the gene for PrP. Thus, *Prn-i*, a host gene codetermining incubation time for scrapie (most likely identical to *Sinc*) (Carlson et al., 1986; Hunter et al., 1987), is closely linked if not congruent to *Prn-p* (Carlson et al., 1986; Hunter et al., 1987; Carlson et al., 1988, 1989; Race et al., 1990). Furthermore, a seminal experiment by Prusiner and his colleagues showed that the species barrier (Pattison, 1966) to prion transmission from hamster to mouse could be overcome by introducing a Syrian hamster PrP transgene into the recipient mouse line (Scott et al., 1989). Even more significantly, the prions generated in such transgenic mice were hamster specific when the prion inoculum was hamster derived and mouse specific when it was mouse derived (Prusiner et al., 1990). Another strong argument in favor of the protein only hypothesis was the seminal discovery of Hsiao et al. (1989), confirmed and extended by others (reviewed by Baker and Ridley, 1992), which led to the realization that in most, if not all, cases of the human familial spongiform encephalopathies occurrence of the disease is linked to mutations in the PrP gene.

Nonetheless, some workers believe that the scrapie agent is but an unconventional, nucleic acid–containing virus and that the accumulation of PrP^{Sc} is secondary, reflecting a consequence of infection and cell damage (Diringer et al., 1988; Manuelidis et al., 1988; Rohwer, 1991; Kimberlin, 1990).

If indeed PrP^{Sc} is an essential component of the scrapie agent, then mice devoid of PrP should be resistant to infection, developing neither symptoms of scrapie nor allowing propagation of the infectious agent. Conversely, if the animals succumb to the disease or propagate infectivity, albeit without showing symptoms of neurological disease, the protein only hypothesis would be falsified.

We have reported earlier on the generation of mice homozygous for disrupted *Prn-p* genes (*Prn-p*$^{0/0}$ mice) and have shown that although no PrP is detectable in the brains of these animals, they develop and reproduce normally and show no detectable physical or behavioral defect (Büeler et al., 1992). It thus became possible to study the response of *Prn-p*$^{0/0}$ mice to inoculation with scrapie prions, as well as of animals carrying a single *Prn-p* allele (*Prn-p*$^{0/+}$ mice) and of *Prn-p*$^{0/0}$ mice reconstituted with Syrian hamster PrP genes.

Results

Challenge of *Prn-p*$^{0/0}$ Mice with Scrapie Prions

Both the mice homozygous for the wild-type and the disrupted *Prn-p* genes had a genetic background derived from 129/Sv and C57BL/6J animals. Fifty-seven each of *Prn-p*$^{0/0}$ and *Prn-p*$^{+/+}$ mice were inoculated intracerebrally with a high dose (about 10^7 LD_{50} units) of the Chandler isolate of mouse-adapted prions (Chandler, 1961). As a further control, 13 Swiss CD-1 mice, a strain commonly used for the titration of mouse prions (Eklund et al., 1967), were inoculated in the same fashion. Four animals of each group (one animal only in the case of CD-1 mice) were

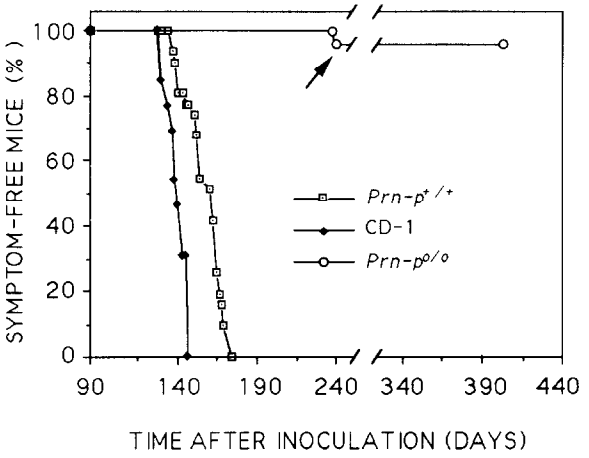


Figure 1. *Prn-p*$^{0/0}$ and *Prn-p*$^{+/+}$ Mice Remaining Symptom Free at Different Times after Inoculation with Mouse Scrapie Prions

Prn-p$^{0/0}$ and *Prn-p*$^{+/+}$ mice were offspring of F1 *Prn-p*$^{0/+}$ breeding pairs and therefore had a similar, heterogeneous genetic background. Swiss CD-1 mice were obtained from Charles River WIGA (Sulzfeld, Germany). Inoculations were as described in Experimental Procedures. Thirty-one *Prn-p*$^{+/+}$ and 25 *Prn-p*$^{0/0}$ animals were kept under observation. In addition, 26 *Prn-p*$^{+/+}$ and 30 *Prn-p*$^{0/0}$ animals were sacrificed at various times to determine infectivity titers and to perform histology. One *Prn-p*$^{0/0}$ mouse (arrow) showed ataxic gait and was sacrificed at 240 days: it showed no histopathological changes typical for scrapie, and brain homogenate from this mouse did not transmit scrapie to CD-1 indicator mice after more than 170 days. Another *Prn-p*$^{0/0}$ mouse died of intercurrent disease without scrapie symptoms.

sacrificed after 4 days, 2, 8, 12, and 20 weeks, and in the cases of *Prn-p*$^{0/0}$ and mock-inoculated mice, they were also sacrificed after 33 and 57 weeks; brain and spleen were recovered for infectivity determination, and brain was recovered for histological examination. A pool of four brains from *Prn-p*$^{+/+}$ mice that died of scrapie between 23 and 25 weeks after inoculation was also assayed for infectivity.

CD-1 mice showed typical neurological symptoms at 140 ± 6 days (Figure 1) and died at 153 ± 7 days. All of 31 *Prn-p*$^{+/+}$ mice with the C57BL-129/Sv background showed symptoms at 158 ± 11 days (Figure 1) and died at 171 ± 11 days. The slightly different course of the disease in CD-1 mice and the mice with mixed genetic background may have been due to secondary genes such as *Pid-1*, which influences incubation times for scrapie along with *Prn-i* (Carlson et al., 1986, 1988; Kingsbury, 1990). In stark contrast, 23 of 25 *Prn-p*$^{0/0}$ mice were alive and free of symptoms at the time of writing, more than 13 months after inoculation and 7 months after the last *Prn-p*$^{+/+}$ controls died (Figure 1). One *Prn-p*$^{0/0}$ mouse died of intercurrent disease without scrapie symptoms. Another presented ataxic gait and was sacrificed; it showed no scrapie pathology in the brain, that is, vacuolization, loss of neurons or astrogliosis. Amyloid plaques, which consist mainly of PrP^{Sc} (DeArmond et al., 1985; Kitamoto et al., 1986), were not looked for.

In a further experiment heterozygous *Prn-p*$^{0/+}$ mice were inoculated intracerebrally with about 10^7 infectious units of the Chandler isolate of mouse-adapted prions. Nine of 10 animals showed early signs of scrapie 253–322 days after inoculation, but were still alive after 322 days, so that the average incubation time to onset of disease was about 125 days longer than in the case of the wild-type controls.

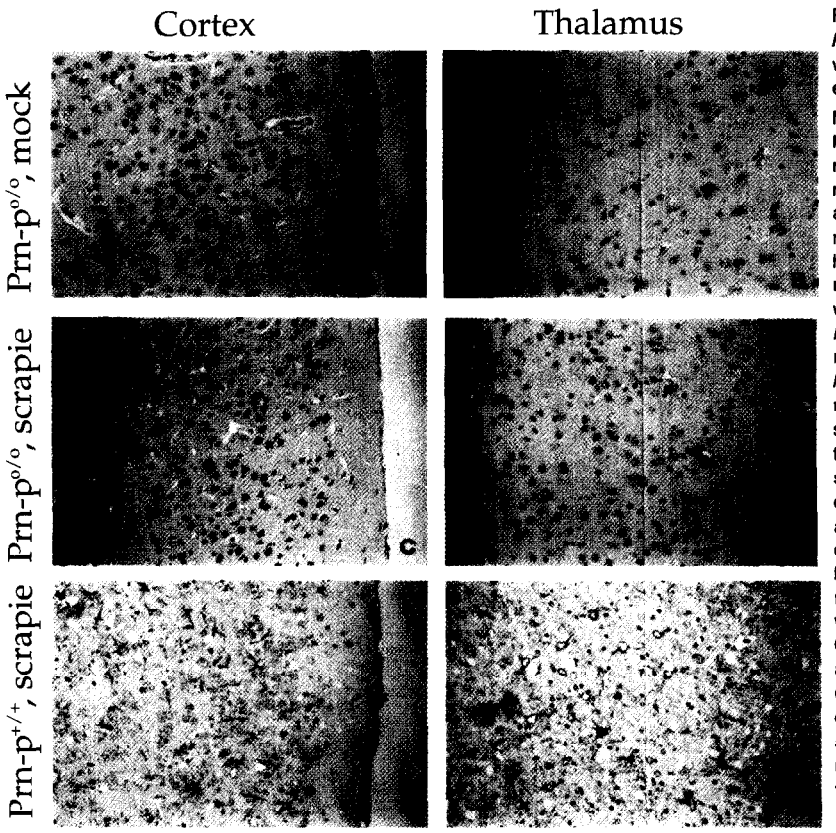
Lack of Neuropathology in Scrapie-Infected *Prn-p*$^{0/0}$ Mice

Brain sections of *Prn-p*$^{0/0}$ and *Prn-p*$^{+/+}$ mice inoculated with normal or mouse prion-containing brain homogenate were stained with anti–glial fibrillary acidic protein (GFAP) antibodies (Figures 2A–2F) or hematoxylin and eosin (Figures 2G–2L). *Prn-p*$^{+/+}$ mice 23–25 weeks after inoculation with mouse prions showed pronounced astrogliosis and vacuolation mainly in the cortex (Figures 2E and 2K), thalamus (Figures 2F and 2L), and hippocampus (not shown). In addition, some of these mice displayed neuronal loss in the hippocampus and thalamus. In contrast, brains from *Prn-p*$^{0/0}$ animals 33 or 57 weeks after inoculation with mouse scrapie prions (Figures 2C, 2D, 2I, and 2J) showed no scrapie-specific pathology and were indistinguishable from those 56 weeks after inoculation with normal brain homogenate (Figures 2A, 2B, 2G, and 2H). A few nonreactive astrocytes were observed to the same extent in mock-infected and scrapie-inoculated animals. Additional stains, Luxol–Nissl and Bodian histochemistry, as well as immunohistochemistry for synaptophysin, neuron-specific enolase, neurofilament proteins, and myelin basic protein, revealed no differences between scrapie-inoculated *Prn-p*$^{0/0}$ and mock-inoculated mice (data not shown).

Titration of Scrapie Infectivity in Brains and Spleens of *Prn-p*$^{+/+}$ and *Prn-p*$^{0/0}$ Mice

At different times after inoculation, brains and spleens from four replicate mice were pooled and homogenized (10% w/v), and aliquots were heated at 80°C for 20 min to inactivate conventional infectious agents. The samples were serially diluted from 10^{-1} to 10^{-8}, and each dilution was inoculated intracerebrally into 6 indicator CD-1 mice. In some cases aliquots of the 10^{-1} dilutions were also inoculated without the heating step. The appearance of scrapie symptoms and death is recorded in Tables 1 and 2. The prion titers of the wild-type heated brain samples were calculated by endpoint dilution (Table 3). The titers of the spleen samples and the nonheated brain samples were calculated from the incubation times to death of 5 to 8 inoculated mice, using standard curves derived from the endpoint dilution experiment (see legend to Table 3).

Spleen homogenates from inoculated *Prn-p*$^{+/+}$ mice gave titers of about 5.7 log LD_{50} units/ml at 4 days after infection and increased slightly thereafter, whereas in the case of *Prn-p*$^{0/0}$ mice the titer was around 2.3 log LD_{50} units/ml at 4 days after inoculation, and no infectivity (<1.5 log LD_{50} units/ml) was detected at later times (Table 3). The low titer at day 4 is presumably due to residual inoculum. It is well documented that viruses as well as scrapie agent spread rapidly throughout the body after intracerebral inoculation (Cairns, 1950; Millson et al., 1993; Hotchin et al., 1983). The fact that after 4 days the infectivity titer in spleen was barely detectable in *Prn-p*$^{0/0}$ animals while it was high in *Prn-p*$^{+/+}$ mice suggests that in wild-type animals substantial prion propagation had occurred within this or-



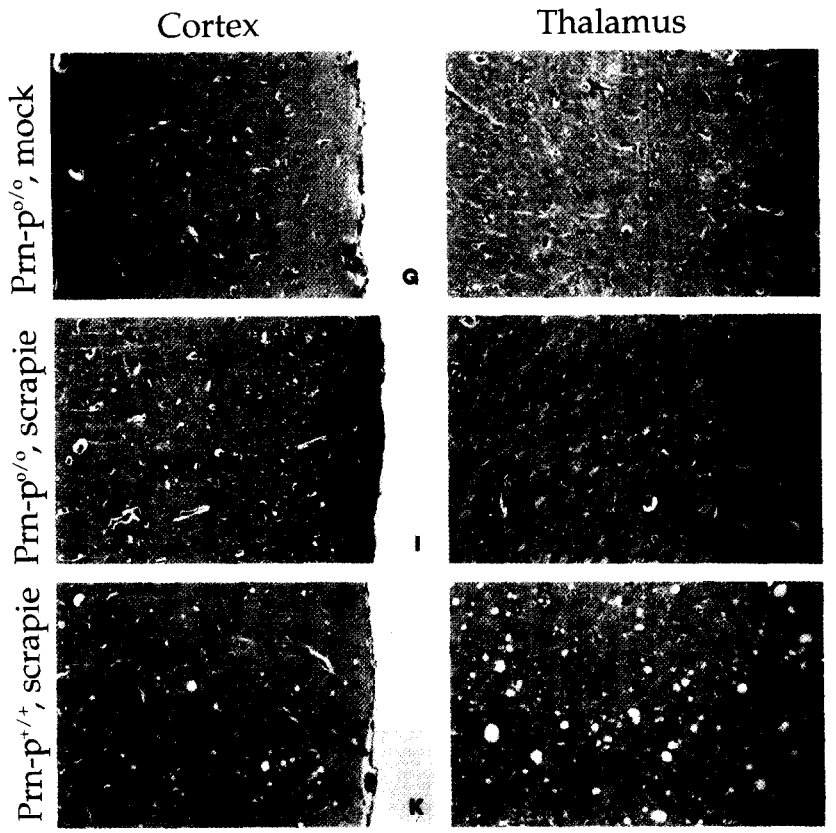


Figure 2. Histology of Brains from *Prn-p*$^{+/+}$ and *Prn-p*$^{o/o}$ Mice at Various Times after Inoculation with Scrapie-Infected or Normal Brain Homogenates

Micrographs of the telencephalic cortex (molecular and pyramidal layers) and of the ventromedial thalamic nucleus were taken from coronal sections at the level of the chiasma opticum at a nominal magnification of 100×. (A–F) Immunocytochemical stain for GFAP with light hematoxylin counterstain. Immunoreactive material stains brown. (G–L) Sections stained with hematoxylin and eosin. Prn-p$^{o/o}$, mock: *Prn-p*$^{o/o}$ mice 56 weeks after inoculation with normal brain homogenate. Prn-p$^{o/o}$, scrapie: *Prn-p*$^{o/o}$ mice 57 weeks after inoculation with mouse scrapie prions (inoculum RML). Prn-p$^{+/+}$, scrapie: *Prn-p*$^{+/+}$ mice 24 weeks after inoculation with mouse scrapie prions. In the GFAP-stained sections, occasional scattered astrocytes in *Prn-p*$^{o/o}$ mock and *Prn-p*$^{o/o}$ scrapie mice are marginally immunoreactive; the processes of these cells are long and delicate. In contrast, both cortex and thalamus of *Prn-p*$^{+/+}$ scrapie mice contain numerous reactive astrocytes with abundant cytoplasm and thick, strongly GFAP-positive processes, indicative of glial reaction to tissue damage. The hematoxylin- and eosin-stained sections show that the neuropil of *Prn-p*$^{o/o}$ mock and *Prn-p*$^{o/o}$ scrapie mice has a normal texture, whereas vacuolization is readily discernible in cortex and thalamus of the *Prn-p*$^{+/+}$ scrapie animals.

Table 1. Titration of Scrapie Infectivity in *Prn-p*$^{+/+}$ Mice

Tissue/Log Dilution	Time after Inoculation						
	4 Days	2 Weeks	8 Weeks	12 Weeks	20 Weeks	23/25 Weeks	Inoculum[a]
Spleen/−1							
Disease	153 ± 14	147 ± 6	144 ± 4	155 ± 2	143 ± 4	ND	–
Death	177 ± 9	171 ± 7	163 ± 10	174 ± 3	163 ± 3	ND	–
Dead/total	6/6	6/6	6/6	5/5	7/7	ND	–
Brain/−1							
Disease	–	–	149 ± 5	144 ± 8	130 ± 11	135 ± 8	137 ± 2
Death	–	–	166 ± 5	158 ± 12	149 ± 8	149 ± 7	149 ± 12
Dead/total	0/6	0/6	5/5	6/6	6/6	8/8	5/5
Brain/−2							
Disease	–	–	151 ± 11	150 ± 10	138 ± 14	ND	151 ± 6
Death	–	–	166 ± 6	166 ± 10	163 ± 12	ND	168 ± 11
Dead/total	0/6	0/6	6/6	6/6	6/6	ND	6/6
Brain/−3							
Disease	–	–	155 ± 9	159 ± 13	148 ± 3	ND	157 ± 10
Death	–	–	174 ± 10	177 ± 11	166 ± 11	ND	172 ± 11
Dead/total	0/6	0/6	6/6	6/6	6/6	ND	6/6
Brain/−4							
Disease	–	–	173 ± 19	178 ± 4	158 ± 3	ND	160 ± 4
Death	–	–	190 ± 6	190 ± 10	178 ± 6	ND	176 ± 10
Dead/total	0/6	0/6	2/5	6/6	6/6	ND	6/6
Brain/−5							
Disease	–	–	–	211 ± 3	179 ± 13	ND	183 ± 14
Death	–	–	–	225 ± 6	191 ± 15	ND	198 ± 18
Dead/total	0/6	0/6	0/6	4/6	6/6	ND	5/5
Brain/−6							
Disease	–	–	–	214	183 ± 9	ND	201 ± 11
Death	–	–	–	230	196 ± 15	ND	215 ± 12
Dead/total	0/6	0/6	0/6	1/6	5/6	ND	5/5
Brain/−7							
Disease	–	–	–	–	211 ± 9	ND	199 ± 10
Death	–	–	–	–	225 ± 7	ND	211 ± 10
Dead/total	0/6	0/6	0/6	0/6	4/6	ND	3/6
Brain/−1 not heated							
Disease	ND	ND	147 ± 1	137 ± 5	127 ± 8	ND	ND
Death	ND	ND	154 ± 3	161 ± 18	154 ± 12	ND	ND
Dead/total	ND	ND	6/6	5/5	6/6	ND	ND

Titration of infectivity and monitoring the mice for scrapie symptoms was as described in Experimental Procedures. "Disease" indicates time of onset of disease (in days); "Death" indicates time to death (in days); ND, not done.

[a] The Chandler-derived isolate of mouse-adapted prions (Chandler, 1961).

gan; alternatively, in *Prn-p*$^{0/0}$ animals transport of prions from brain to spleen may be severely impaired or degradation in the spleen may be accelerated.

As shown in Table 3, brain homogenates prepared from *Prn-p*$^{+/+}$ mice showed an increase in titer from <1.5 log LD_{50} units/ml at day 4 to 5.4 log LD_{50} units/ml at 8 weeks and 8.1 log LD_{50} units/ml at 23–25 weeks. In the case of *Prn-p*$^{0/0}$ mice, borderline infectivity was noted at day 4 (presumably due to residual inoculum), and no transmission was noted for 1:10 diluted brain homogenates at 2, 8, 12, and 25 weeks after inoculation. Only the unheated sample from *Prn-p*$^{0/0}$ mice 20 weeks after inoculation, injected at a 1:10 dilution into CD-1 mice, caused typical scrapie symptoms and death in all 6 animals after 204 ± 14 days, equivalent to a titer of about 3.2 log LD_{50} units/ml. One of 6 indicator mice injected with the heated 1:100 diluted 20 week sample died after 253 days.

From our results we conclude that if infectious agent is propagated in brain of *Prn-p*$^{0/0}$ mice, this occurs at a level 5 orders of magnitude lower than in the wild-type controls. However, it is more likely that this one instance of transmission was due to either adventitious contamination, despite all precautions taken to avoid such an event, or residual infectivity from the inoculum.

Complementation of *Prn-p*$^{0/0}$ Mice with Syrian Hamster PrP Genes

Prusiner and his colleagues have generated mice containing an autosomal chromosome with 30–50 copies of the Syrian hamster PrP gene, believed to be present in a tandem array (Scott et al., 1989). These Tg(SHaPrP)81 mice were shown to be very susceptible to hamster prions and somewhat less susceptible to mouse prions than their wild-type counterparts (Scott et al., 1989). We mated

Table 2. Titration of Scrapie Infectivity in *Prn-p*$^{0/0}$ Mice

Tissue/Log Dilution	Time after Inoculation					
	4 Days	2 Weeks	8 Weeks	12 Weeks	20 Weeks	23/25 Weeks
Spleen/−1						
Disease	191 ± 6	–	–	–	–	–
Death	213 ± 4	–	–	–	–	–
Dead/total	2/6	0/6	0/6	0/5	0/6	0/8
Brain/−1						
Disease	180	–	–	–	–	–
Death	190	–	–	–	–	–
Dead/total	1/6	0/6	0/6	0/6	0/3	0/8
Brain/−2						
Disease	–	–	–	–	230	ND
Death	–	–	–	–	253	ND
Dead/total	0/6	0/6	0/5	0/6	1/6	ND
Brain/−3						
Disease	–	–	–	–	–	ND
Death	–	–	–	–	–	ND
Dead/total	0/6	0/6	0/6	0/6	0/6	ND
Brain/−4						
Disease	–	–	–	–	–	ND
Death	–	–	–	–	–	ND
Dead/total	0/4	0/6	0/6	0/6	0/6	ND
Brain/−5						
Disease	–	–	–	–	–	ND
Death	–	–	–	–	–	ND
Dead/total	0/6	0/6	0/6	0/6	0/6	ND
Brain/−6						
Disease	–	–	–	–	–	ND
Death	–	–	–	–	–	ND
Dead/total	0/6	0/6	0/6	0/6	0/6	ND
Brain/−7						
Disease	–	–	–	–	–	ND
Death	–	–	–	–	–	ND
Dead/total	0/6	0/6	0/5	0/6	0/5	ND
Brain/−1 not heated						
Disease	ND	ND	–	–	188 ± 7	ND
Death	ND	ND	–	–	204 ± 14	ND
Dead/total	ND	ND	0/6	0/4	6/6	ND

Titration of infectivity and monitoring the mice for scrapie symptoms was as described in Experimental Procedures. "Disease" indicates time of onset of disease (in days); "Death" indicates time to death (in days); ND, not done.

Table 3. Prion Titers in Brain and Spleen of *Prn-p*$^{+/+}$ and *Prn-p*$^{0/0}$ Mice

Time after Inoculation	Log LD_{50} units/ml					
	Brain (Heated)		Brain (Not Heated)		Spleen (Heated)	
	$PrP^{+/+}$	$PrP^{0/0}$	$PrP^{+/+}$	$PrP^{0/0}$	$PrP^{+/+}$	$PrP^{0/0}$
4 days	<1.5	2.0[a]	ND	ND	5.7 ± 0.9	2.3[b]
2 weeks	<1.5	<1.5	ND	ND	6.2 ± 0.8	<1.5
8 weeks	5.4	<1.5	7.7 ± 0.6	<1.5	6.9 ± 1.0	<1.5
12 weeks	6.8	<1.5	7.1 ± 1.6	<1.5	5.9 ± 0.6	<1.5
20 weeks	8.6	<1.5	7.7 ± 1.1	3.2 ± 1.4[c]	6.9 ± 0.6	<1.5
23/25 weeks	8.1 ± 0.8	<1.5	ND	ND	ND	<1.5

The titers in 10% (w/v) homogenates of brains recovered at 8, 12, and 20 weeks after inoculation and the titer of the Chandler-derived mouse prion inoculum RML (8.5 log LD_{50} units/ml) were calculated by multiplying the LD_{50} units at the endpoint dilution (Table 1) with (33 × dilution factor). For the brain samples recovered at 12 weeks and 20 weeks and the mouse prion inoculum RML log LD_{50} units/ml of serially diluted homogenates (1%, 0.1%, etc. down to the endpoint dilution) were plotted against average survival times of inoculated mice. Linear regression curves were calculated with the linear regression function of the HEWLETT PACKARD 15C calculator. The curves were described by the equations $y = 20.344 - 0.093\,x$, $y = 22.826 - 0.102\,x$; and $y = 18.851 - 0.077\,x$, with correlation coefficients of −0.992, −0.988, and −0.971, respectively, where y is the titer (log LD_{50} units/ml) and x is the survival time (days). For 1% homogenates not analyzed by endpoint titration, survival times of five to eight inoculated mice were inserted into the three equations described above, resulting in 15 to 24 titers. The final titer given in the table refers to 10% homogenates and is expressed as 10 times the mean of these titers ± SD.

[a] One of six mice died after inoculation with 10^{-1} diluted homogenate (Table 2).

[b] Two of six mice died after inoculation with 10^{-1} diluted homogenate (Table 2).

[c] Six of six mice died after inoculation with 10^{-1} diluted homogenate (Table 2).

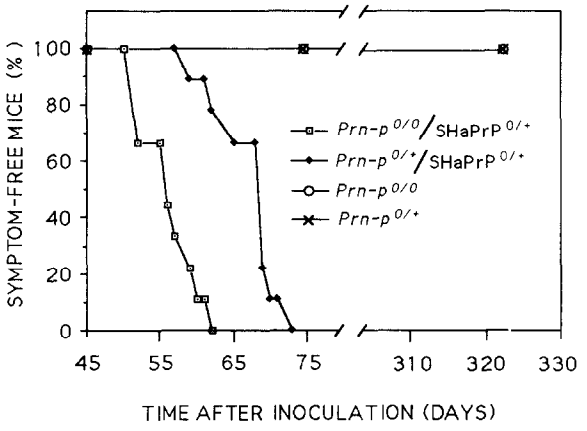


Figure 3. *Prn-p*$^{0/0}$ and *Prn-p*$^{0/+}$ Mice with Hamster PrP Transgenes Remaining Symptom Free at Different Times after Inoculation with Hamster Scrapie Prions

Prn-p$^{0/0}$/SHaPrP$^{0/+}$, *Prn-p*$^{0/+}$/SHaPrP$^{0/+}$, *Prn-p*$^{0/0}$, and *Prn-p*$^{0/+}$ mice were obtained from crosses between *Prn-p*$^{0/0}$ and *Prn-p*$^{0/+}$/SHaPrP$^{0/+}$ mice (see Experimental Procedures). Groups of 9 to 11 mice of each genotype were inoculated with the Sc237 isolate of hamster prions.

Prn-p$^{0/0}$ mice with Tg(SHaPrP)81 mice homozygous for the transgene-containing locus SHaPrP and obtained *Prn-p*$^{0/+}$/SHaPrP$^{0/+}$ progeny. Such progeny were backcrossed to *Prn-p*$^{0/0}$ mice, and the *Prn-p* and SHaPrP alleles of the progeny were determined by Southern blot analysis (see Experimental Procedures). Groups of 9 to 11 *Prn-p*$^{0/0}$/SHaPrP$^{0/+}$ and *Prn-p*$^{0/+}$/SHaPrP$^{0/+}$ mice were inoculated with the Sc237 isolate of Syrian hamster prions or with the Chandler isolate of mouse scrapie prions. The *Prn-p*$^{0/0}$/SHaPrP$^{0/+}$ mice inoculated with hamster prions showed neurological symptoms after 56 ± 3 days and died after 59 ± 5 days, while the values for the *Prn-p*$^{0/+}$/SHaPrP$^{0/+}$ mice were 67 ± 4 and 71 ± 6 days, respectively (Figure 3). In contrast, all the nontransgenic *Prn-p*$^{0/0}$ and *Prn-p*$^{0/+}$ mice were still healthy 322 days after inoculation with hamster prions.

At the time of writing, 322 days into the experiment, 8 of 9 *Prn-p*$^{0/0}$/SHaPrP$^{0/+}$ mice inoculated with mouse prions showed scrapie symptoms between 271 and 318 days and all 7 of the *Prn-p*$^{0/+}$/SHaPrP$^{0/+}$ mice inoculated with mouse prions fell ill between 223 and 300 days after inoculation (data not shown). It would seem that hamster PrP^{C} is able to interact with mouse prions, albeit with low efficiency, as might be expected from the fact that hamsters inoculated with mouse prions ultimately also succumb to scrapie disease (Scott et al., 1989).

Search for Antibodies against PrP in *Prn-p*$^{0/0}$ Mice Inoculated with Brain Homogenate from Scrapie-Infected *Prn-p*$^{+/+}$ Mice

After inoculation with the mouse prion Chandler inoculum (RML), a scrapie-infected brain homogenate derived from *Prn-p*$^{+/+}$ mice, antibodies against PrP might be formed in *Prn-p*$^{0/0}$, but not in *Prn-p*$^{+/+}$ and *Prn-p*$^{0/+}$ mice. Such antibodies might affect the susceptibility of *Prn-p*$^{0/0}$ mice to scrapie infection. Western blots of *Prn-p*$^{+/+}$ and *Prn-p*$^{0/0}$ brain homogenates (Serban et al., 1990), as well as of partially purified (Hilmert and Diringer, 1984) mouse PrP27-30 (protease-treated PrP^{Sc}), were incubated with sera obtained from *Prn-p*$^{0/0}$ mice 6 weeks (serum A) and 20 weeks (sera B–D) after inoculation with the Chandler inoculum or from noninoculated *Prn-p*$^{0/0}$ controls (serum M). The blots were treated with goat anti-mouse immunoglobulin G/immunoglobulin M labeled with horseradish peroxidase and developed with chemiluminescence reagents.

Multiple bands were detected when blots of brain homogenate were used as targets for sera derived from scrapie-inoculated *Prn-p*$^{0/0}$ mice; however, no band clearly corresponded to PrP^{C} (33–35 kd). In particular, there was no difference in the band patterns of the blotted brain homogenate between *Prn-p*$^{+/+}$ and *Prn-p*$^{0/0}$ mice (Figure 4, sera A–D, lanes + and 0). A control serum from a noninoculated *Prn-p*$^{0/0}$ mouse gave only a few bands (Figure 4, serum M, lanes + and 0). No bands were detected with any of the mouse sera when partially purified murine PrP27-30 was used as target (Figure 4, lanes P). An affinity-purified polyclonal anti-PrP antiserum was used as a positive control to reveal PrP^{C} in *Prn-p*$^{+/+}$ brain homogenates (Figure 4, antiserum R073, lane +) or PrP27-30 (Figure 4, antiserum R073, lane P). Thus, antibodies against (undefined) mouse brain proteins could be easily detected after intracerebral injection of *Prn-p*$^{+/+}$ brain homogenate into *Prn-p*$^{0/0}$ mice, but no antibodies against mouse PrP27-30 were observed.

Discussion

Our results show that mice devoid of PrP are completely protected against scrapie disease, at least up to 13 months after inoculation. Moreover, even heterozygous *Prn-p*$^{0/+}$ mice are partially protected, inasmuch as 9 of 10 scrapie-inoculated animals showed signs of scrapie only 253–322 days after inoculation but are still alive after 322 days, while all *Prn-p*$^{+/+}$ controls died within about 180 days. Moreover, disease progression in *Prn-p*$^{0/+}$ mice is distinctly slower than in *Prn-p*$^{+/+}$ mice: the interval between first symptoms and death was 13 days in the case of *Prn-p*$^{+/+}$ mice, while no *Prn-p*$^{0/+}$ mice have died to date, about 2 months after the appearance of scrapie symptoms.

We conclude that development of scrapie symptoms and pathology is strictly dependent on the presence of PrP and that incubation time and disease progression are inversely related to the level of PrP. It has previously been found that the length of scrapie incubation time for hamster-derived prions in mice expressing SHaPrP genes was inversely related to the level of SHaPrP (Prusiner et al., 1990). The finding that *Prn-p*$^{0/0}$ mice carrying hamster PrP genes become very susceptible to hamster-derived prions (incubation time of 56 days), but show long incubation times (>271 days) with mouse-derived prions, demonstrates the requirement of a homotypic relationship between incoming prion and resident PrP protein for prion propagation and development of pathology, as foreshadowed by the results of Prusiner et al. (1990).

It is also clear that if infectious agent is propagated in

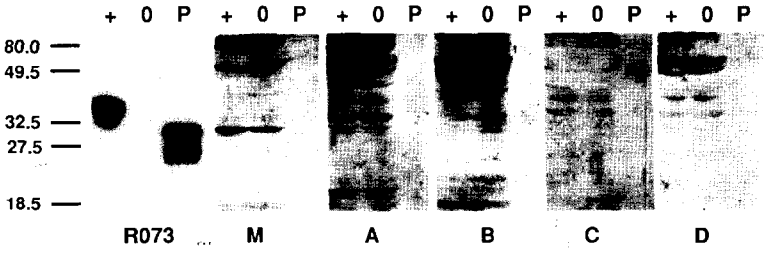


Figure 4. Search for Antibodies in Sera from Scrapie-Inoculated *Prn-p*$^{0/0}$ Mice

Western blot analysis of 10% mouse brain homogenates (Serban et al., 1990) and partially purified murine PrP27-30 (Hilmert and Diringer, 1984) using various sera is described in Experimental Procedures. Plus sign, 10 μl of *Prn-p*$^{+/+}$ brain homogenate; zero, 10 μl of *Prn-p*$^{0/0}$ brain homogenate; P, 2.4 μg of partially purified mouse PrP27-30; R073, polyclonal affinity-purified antibody against hamster PrP27-30 that cross-reacts with mouse PrP (Serban et al., 1990); M, serum from a noninoculated *Prn-p*$^{0/0}$ mouse; A, serum obtained from a *Prn-p*$^{0/0}$ mouse 6 weeks after intracerebral inoculation with scrapie-infected *Prn-p*$^{+/+}$ brain homogenate (Chandler-derived mouse prion inoculum RML); B–D, sera obtained from three *Prn-p*$^{0/0}$ mice 20 weeks after intracerebral inoculation with scrapie-infected brain homogenate from *Prn-p*$^{+/+}$ mice (Chandler-derived mouse prion RML). The size markers (in kilodaltons) were from Bio-Rad.

Prn-p$^{0/0}$ mice, this would be at a level about 5 orders of magnitude lower than that in *Prn-p*$^{+/+}$ animals. In scrapie-inoculated *Prn-p*$^{+/+}$ animals, infectious agent was detected in the brain at 8 weeks and increased to about 8.6 log LD_{50} units/ml by 20 weeks after inoculation. No infectious agent was detected in *Prn-p*$^{0/0}$ animals 25 weeks after inoculation, the latest time point for which data are presently available. However, a low level of infectivity, about 3.2 log LD_{50} units/ml, was found in the nonheated 20 week sample. Tissues from the 4 animals from which the pooled brain homogenate had been prepared were retyped by Southern blotting and polymerase chain reaction and confirmed to be *Prn-p*$^{0/0}$. Because only one sample in the entire experiment was positive, we cannot exclude accidental contamination of the homogenate. Another possibility is that the infectivity is due to residual traces of inoculum. However, the fact that all samples taken between 2 and 12 weeks after inoculation were negative is evidence against this explanation. Finally, we must consider the possibility that the infectious agent is something other than PrP^{Sc}, and that in the absence of PrP^{Sc} in the host it is much less infectious than in its presence. This question can only be resolved by further experiments, which are now underway.

We considered the possibility that antibodies against PrP might be formed in *Prn-p*$^{0/0}$ mice (but not in *Prn-p*$^{+/+}$ mice) after exposure to the Chandler mouse prion inoculum, a scrapie-infected brain homogenate from *Prn-p*$^{+/+}$ mice, and that such antibodies could affect susceptibility to scrapie infection. We failed to detect anti-PrP antibodies in inoculated *Prn-p*$^{0/0}$ mice. Moreover, *Prn-p*$^{0/+}$ mice, which should be as tolerant toward PrP as *Prn-p*$^{+/+}$ mice, show prolonged incubation times, suggesting that resistance to scrapie of *Prn-p*$^{0/0}$ mice is not due to an immune response.

Our results show that it is possible to generate normal mice that are resistant to scrapie by knocking out their PrP genes; in principle, it should thus be possible to breed sheep or cattle resistant to this disease, either by PrP gene disruption or by the introduction of transgenes expressing PrP antisense RNA. Moreover, the fact that *Prn-p*$^{0/+}$ heterozygous mice show much longer scrapie incubation times than their wild-type counterparts argues that disease progression may be rate limited by the PrP^{C} concentration. This conclusion is consistent with the observation that in several mouse lines containing hamster PrP transgenes the incubation time for hamster prion-induced scrapie is a function of the hamster PrP^{C} expression level (Prusiner et al., 1990). A practical implication of this conclusion is that a moderate reduction of PrP^{C} synthesis, such as might eventually be achieved by antisense oligonucleotide therapy, could substantially mitigate disease progression in incipient cases of spongiform encephalopathies.

Experimental Procedures

Animals

Female mice were kept in groups of 3 to 8 animals. Males were kept alone or together with a maximum of 3 other males. *Prn-p*$^{+/+}$ and *Prn-p*$^{0/0}$ mice were obtained from F1 *Prn-p*$^{0/+}$ breeding pairs and identified by the polymerase chain reaction as described (Büeler et al., 1992). To introduce hamster PrP genes into *Prn-p*$^{0/0}$ mice, *Prn-p*$^{0/0}$ mice were mated with Tg(SHaPrP)81 mice homozygous for the transgene-containing locus SHaPrP to obtain *Prn-p*$^{0/+}$/SHaPrP$^{0/+}$ progeny. Such progeny were backcrossed to *Prn-p*$^{0/0}$ mice, and the *Prn-p* and SHaPrP alleles of the offspring were determined by Southern blot analysis of EcoRI-digested tail DNA using probe A described by Büeler et al. (1992). *Prn-p*$^{0/+}$ mice were obtained from the same crosses between *Prn-p*$^{0/0}$ and *Prn-p*$^{0/+}$/SHaPrP$^{0/+}$ mice.

Inoculations and Scrapie Diagnosis

The Chandler-derived mouse prion preparation RML (obtained from S. B. Prusiner) was heated at 80°C for 20 min in the presence of 0.1% sarkosyl (except where stated otherwise) to inactivate possible viral or bacterial contaminants, adjusted to 0.5% sarkosyl, and diluted 10-fold in phosphate-buffered saline–5% bovine serum albumin to yield the inoculum. The hamster prion Sc237, also provided by S. B. Prusiner, was diluted and inoculated without prior incubation at 80°C. Mice were intracerebrally inoculated with 30 μl of inoculum using a 26-gauge hypodermic needle that was inserted into the right parietal lobe. Beginning 13 weeks after inoculation, mice were examined every 2 days for the following scrapie-associated symptoms: ataxia, mincing gait, "plastic tail," disorientation, feet clasping when lifted, and depression. Monitoring of the mice reconstituted with the SHaPrP transgene was started 7 weeks after infection.

Preparation of Tissue Homogenates

Brain homogenates (10% w/v) in 0.32 M sucrose were prepared by slowly passing the brains back and forth first through an 18-gauge and then a 22-gauge needle. Once the solution appeared homogeneous, it was spun for 10 min at 1500 rpm (about 500 × g) and the supernatant was frozen at −25°C. Spleen homogenates (10%) were prepared using a Dounce tissue homogenizer. For decontamination, the homogenizer was submerged in 3 N NaOH for at least 30 min and washed with water and ethanol before it was used to process a new spleen.

Titration of Infectivity

Homogenates (10% w/v in 0.32 M sucrose) of brains and spleens

from *Prn-p*$^{+/+}$ and *Prn-p*$^{0/0}$ mice were heated at 80°C for 20 min in the presence of 0.1% sarkosyl (except where stated otherwise) and adjusted to 0.5% sarkosyl. Brain homogenates were diluted serially in phosphate-buffered saline–5% bovine serum albumin from 10^{-1} to 10^{-8}. Aliquots (30 μl) of each dilution were injected intracerebrally into 6 Swiss CD-1 mice. In some cases 1% brain homogenates were also inoculated without prior heating. Spleen homogenates were inoculated as 1% dilutions only. Mice were monitored for scrapie symptoms as described above.

Detection of Antibodies in Mouse Serum

Aliquots (10 μl) of 10% brain homogenate (Serban et al., 1990) from *Prn-p*$^{+/+}$ and *Prn-p*$^{0/0}$ mice or 2.4 μg of partially purified mouse PrP27-30 (a gift from H. Diringer) were electrophoresed through a 1.5 mm SDS–polyacrylamide (12.5%) gel (acrylamide:bisacrylamide, 20:1) (Laemmli, 1970). Proteins were transferred to nitrocellulose (Schleicher & Schuell, BA 85, 0.45 mm pore size) by semidry electroblotting (IKA Biotech, Denmark). Membrane strips comprising three lanes were preincubated for 1 hr in TBS (0.01 M Tris–HCl [pH 7.2], 0.15 M NaCl) containing 5% nonfat dry milk and incubated overnight at 4°C in TBS, 1% nonfat dry milk, and 1:100 diluted serum from scrapie-inoculated or uninoculated *Prn-p*$^{0/0}$ mice. The positive control antibody was R073 (1:2000) (Serban et al., 1990). After washing four times for 5 min in TBS, 0.1% Tween 20, the strips were incubated for 1 hr in TBS, 1% nonfat dry milk, 0.1% Tween 20, and 1:1000 diluted goat anti-mouse immunoglobulin G and immunoglobulin M coupled to horseradish peroxidase (Milan Analytica, catalog number 115-036-068) or 1:1000 diluted protein A–horseradish peroxidase (Sigma), washed once for 10 min and three times for 5 min in TBS, 0.1% Tween 20, developed using the enhanced chemiluminescence detection kit from Amersham, and exposed to Kodak X-ray film for 15 s to 1 min.

Histology

Mouse brains were fixed in phosphate-buffered saline containing 4% formaldehyde for at least 24 hr, incubated for 60 min in concentrated formic acid (Brown et al., 1990), and washed with phosphate-buffered saline. Coronal slices of approximately 2 mm were dehydrated through graded alcohols and embedded in paraffin. Sections of 3 μm nominal thickness were stained with hematoxylin and eosin. Immunostains for GFAP were performed using a rabbit anti-GFAP antiserum (DAKO) at a dilution of 1:300. The secondary antibody was a biotinylated swine anti-rabbit immunoglobulin serum at a working dilution of 1:250. Visualization was achieved using avidin–peroxidase (DAKO, Copenhagen) and diaminobenzidine (Sigma). Slices were mounted in Entellan (Merck).

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References

Baker, H. F., and Ridley, R. M. (1992). The genetics and transmissibility of human spongiform encephalopathy. Neurodegeneration *1*, 3–16.

Bolton, D. C., and Bendheim, P. E. (1988). A modified host protein model of scrapie. In Novel Infectious Agents and the Central Nervous System, G. Bock and J. Marsh, eds. (Chichester, England: John Wiley & Sons), pp. 164–177.

Bolton, D. C., McKinley, M. P., and Prusiner, S. B. (1982). Identification of a protein that purifies with the scrapie prion. Science *218*, 1309–1311.

Brown, P., Wolff, A., and Gajdusek, D. C. (1990). A simple and effective method for inactivating virus infectivity in formalin-fixed tissue samples from patients with Creutzfeldt–Jakob disease. Neurology *40*, 887–890.

Büeler, H., Fischer, M., Lang, Y., Bluethmann, H., Lipp, H. P., DeArmond, S. J., Prusiner, S. B., Aguet, M., and Weissmann, C. (1992). Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature *356*, 577–582.

Cairns, J. (1950). Intracerebral inoculation of mice: fate of the inoculum. Nature *166*, 910–911.

Carlson, G. A., Kingsbury, D. T., Goodman, P. A., Coleman, S., Marshall, S. T., DeArmond, S., Westaway, D., and Prusiner, S. B. (1986). Linkage of prion protein and scrapie incubation time genes. Cell *46*, 503–511.

Carlson, G. A., Goodman, P. A., Lovett, M., Taylor, B. A., Marshall, S. T., Peterson, T. M., Westaway, D., and Prusiner, S. B. (1988). Genetics and polymorphism of the mouse prion gene complex control of scrapie incubation time. Mol. Cell. Biol. *8*, 5528–5540.

Carlson, G. A., Westaway, D., DeArmond, S. J., Peterson Torchia, M., and Prusiner, S. B. (1989). Primary structure of prion protein may modify scrapie isolate properties. Proc. Natl. Acad. Sci. USA *86*, 7475–7479.

Chandler, R. L. (1961). Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet *1*, 1378–1379.

DeArmond, S. J., McKinley, M. P., Barry, R. A., Braunfeld, M. B., McColloch, J. R., and Prusiner, S. B. (1985). Identification of prion amyloid filaments in scrapie-infected brain. Cell *41*, 221–235.

Diringer, H., Gelderblom, H., Hilmert, H., Ozel, M., Edelbluth, C., and Kimberlin, R. H. (1983). Scrapie infectivity, fibrils and low molecular weight protein. Nature *306*, 476–478.

Diringer, H., Braig, H. R., and Czub, M. (1988). Scrapie: a virus-induced amyloidosis of the brain. Ciba Found. Symp. *135*, 135–145.

Eklund, C. M., Kennedy, R. C., and Hadlow, W. J. (1967). Pathogenesis of scrapie virus infection in the mouse. J. Infect. Disease *117*, 15–22.

Gabizon, R., McKinley, M. P., Groth, D., and Prusiner, S. B. (1988). Immunoaffinity purification and neutralization of scrapie prion infectivity. Proc. Natl. Acad. Sci. USA *85*, 6617–6621.

Gajdusek, D. C. (1991). The transmissible amyloidoses—genetical control of spontaneous generation of infectious amyloid proteins by nucleation of configurational change in host precursors—Kuru-CJD-GSS-Scrapie-BSE. Eur. J. Epidemiol. *7*, 567–577.

Hilmert, H., and Diringer, H. (1984). A rapid and efficient method to enrich SAF-protein from scrapie brains of hamsters. Biosci. Reports *4*, 165–170.

Hotchin, J., Sikora, E., and Baker, F. (1983). Disappearance of scrapie virus from tissues of the mouse. Intervirology *19*, 205–212.

Hsiao, K., Baker, H. F., Crow, T. J., Poulter, M., Owen, F., Terwilliger, J. D., Westaway, D., Ott, J., and Prusiner, S. B. (1989). Linkage of a prion protein missense variant to Gerstmann-Sträussler syndrome. Nature *338*, 342–345.

Hunter, N., Hope, J., McConnell, I., and Dickinson, A. G. (1987). Linkage of the scrapie-associated fibril protein (PrP) gene and Sinc using congenic mice and restriction fragment length polymorphism analysis. J. Gen. Virol. *68*, 2711–2716.

Kellings, K., Meyer, N., Mirenda, C., Prusiner, S. B., and Riesner, D. (1992). Further analysis of nucleic acids in purified scrapie prion preparations by improved return refocusing gel electrophoresis. J. Gen. Virol. *73*, 1025–1029.

Kimberlin, R. H. (1990). Scrapie and possible relationship with viroids. Sem. Virol. *1*, 153–162.

Kingsbury, D. T. (1990). Genetics of response to slow virus (prion) infection. Annu. Rev. Genet. *24*, 115–132.

Kitamoto, T., Tateishi, J., Tashima, T., Takeshita, I., Barry, R. A., DeArmond, S. J., and Prusiner, S. B. (1986). Amyloid plaques in Creutzfeldt-Jakob disease stain with prion protein antibodies. Ann. Neurol. *20*, 204–208.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Manuelidis, L., Murdoch, G., and Manuelidis, E. E. (1988). Potential

involvement of retroviral elements in human dementias. Ciba Found. Symp. *135*, 117–134.

Meyer, N., Rosenbaum, V., Schmidt, B., Gilles, K., Mirenda, C., Groth, D., Prusiner, S. B., and Riesner, D. (1991). Search for a putative scrapie genome in purified prion fractions reveals a paucity of nucleic acids. J. Gen. Virol. *72*, 37–49.

Millson, G. C., Kimberlin, R. H., Manning, E. J., and Collis, S. C. (1993). Early distribution of radioactive liposomes and scrapie infectivity in mouse tissues following administration by different routes. Vet. Microbiol. *1979*, 89–99.

Oesch, B., Groth, D. F., Prusiner, S. B., and Weissmann, C. (1988). Search for a scrapie-specific nucleic acid: a progress report. Ciba Found. Symp. *135*, 209–223.

Pattison, I. H. (1966). The relative susceptibility of sheep, goats and mice to two types of the goat scrapie agent. Res. Vet. Sci. *7*, 207–212.

Prusiner, S. B. (1982). Novel proteinaceous infectious particles cause scrapie. Science *216*, 136–144.

Prusiner, S. B. (1991). Molecular biology of prion diseases. Science *252*, 1515–1522.

Prusiner, S. B., Bolton, D. C., Groth, D. F., Bowman, K. A., Cochran, S. P., and McKinley, M. P. (1982). Further purification and characterization of scrapie prions. Biochemistry *21*, 6942–6950.

Prusiner, S. B., McKinley, M. P., Bowman, K. A., Bolton, D. C., Bendheim, P. E., Groth, D. F., and Glenner, G. G. (1983). Scrapie prions aggregate to form amyloid-like birefringent rods. Cell *35*, 349–358.

Prusiner, S. B., Scott, M., Foster, D., Pan, K.-M., Groth, D., Mirenda, C., Torchia, M., Yang, S.-L., Serban, D., Carlson, G. A., Hoppe, P. C., Westaway, D., and DeArmond, S. J. (1990). Transgenetic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. Cell *63*, 673–686.

Race, R. E., Graham, K., Ernst, D., Caughey, B., and Chesebro, B. (1990). Analysis of linkage between scrapie incubation period and the prion protein gene in mice. J. Gen. Virol. *71*, 493–497.

Rohwer, R. G. (1991). The scrapie agent: "a virus by any other name." Curr. Top. Microbiol. Immunol. *172*, 195–232.

Scott, M., Foster, D., Mirenda, C., Serban, D., Coufal, F., Wälchli, M., Torchia, M., Groth, D., Carlson, G., DeArmond, S. J., Westaway, D., and Prusiner, S. B. (1989). Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. Cell *59*, 847–857.

Serban, D., Taraboulos, A., DeArmond, S. J., and Prusiner, S. B. (1990). Rapid detection of Creutzfeldt-Jakob disease and scrapie prion proteins. Neurology *40*, 110–117.

Weissmann, C. (1991). A "unified theory" of prion propagation. Nature *352*, 679–683.