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Accumulation of Proteinase K-Resistant Prion Protein (PrP) Is Restricted by the Expression Level of Normal PrP in Mice Inoculated with a Mouse-Adapted Strain of the Creutzfeldt-Jakob Disease Agent

SUEHIRO SAKAGUCHI,[1,2]† SHIGERU KATAMINE,[1] KAZUTO SHIGEMATSU,[3] AKIRA NAKATANI,[1] RYOZO MORIUCHI,[1] NORIYUKI NISHIDA,[1] KENJI KUROKAWA,[1] RYOTA NAKAOKE,[1] HIROSHI SATO,[4] KOUICHI JISHAGE,[2] JUNKO KUNO,[2] TETSUO NODA,[2] AND TSUTOMU MIYAMOTO[1]*

Department of Bacteriology,[1] Second Department of Pathology,[3] and Laboratory Animal Center for Biomedical Research,[4] Nagasaki University School of Medicine, Nagasaki 852, and Department of Cell Biology, Cancer Institute, Toshima-ku, Tokyo 170,[2] Japan

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Creutzfeldt-Jakob disease (CJD) is a transmissible neurodegenerative disease of humans caused by an unidentified infectious agent, the prion. To determine whether there was an involvement of the host-encoded prion protein (PrP^c) in CJD development and prion propagation, mice heterozygous ($PrP^{+/-}$) or homozygous ($PrP^{-/-}$) for a disrupted *PrP* gene were established and inoculated with the mouse-adapted CJD agent. In keeping with findings of previous studies using other lines of PrP-less mice inoculated with scrapie agents, no $PrP^{-/-}$ mice showed any sign of the disease for 460 days after inoculation, while all of the $PrP^{+/-}$ and control $PrP^{+/+}$ mice developed CJD-like symptoms and died. The incubation period for $PrP^{+/-}$ mice, 259 ± 27 days, was much longer than that for $PrP^{+/+}$ mice, 138 ± 12 days. Propagation of the prion was barely detectable in the brains of $PrP^{-/-}$ mice and was estimated to be at a level at least 4 orders of magnitude lower than that in $PrP^{+/+}$ mice. These findings indicate that PrP^c is necessary for both the development of the disease and propagation of the prion in the inoculated mice. The proteinase-resistant PrP (PrP^{res}) was undetectable in the brain tissues of the inoculated $PrP^{-/-}$ mice, while it accumulated in the affected brains of $PrP^{+/+}$ and $PrP^{+/-}$ mice. Interestingly, the maximum level of PrP^{res} in the brains of $PrP^{+/-}$ mice was about half of the level in the similarly affected brains of $PrP^{+/+}$ mice, indicating that PrP^{res} accumulation is restricted by the level of PrP^c.

Creutzfeldt-Jakob disease (CJD), a neurodegenerative spongiform encephalopathy of humans, is transmissible iatrogenically (3, 13) and experimentally (21, 32) to rodent animals. Scrapie is a closely related transmissible disease of sheep and goats (10). The infectious agents, prions, of both CJD and scrapie have yet to be identified, but they share unique biochemical characteristics (2). They are highly resistant to various physical and chemical treatments which inactivate conventional infectious agents such as viruses, bacteria, and fungi. In the infectivity-enriched fractions of CJD- or scrapie-affected brain, the proteinase K-resistant prion protein (PrP), designated PrP^{res}, is detected as a major macromolecule (4, 23, 27). PrP^{res} is thought to be converted from a host-encoded and proteinase-sensitive PrP, PrP^c, which is constitutively expressed in the normal brain tissue. The agents seem to be devoid of a nucleic acid genome because of their resistance to UV irradiation or nuclease treatment (1, 5), and previous intensive studies failed to detect any disease-specific nucleic acid. The prion theory (25) has proposed that PrP^{res} is the agent itself and that the conversion of PrP^c into the protease-resistant isoforms through direct interaction between them is the nature of prion replication. Although accumulating evidence has supported this hypothesis, there is still controversy. For instance, different prion strains distinguishable by the incubation period and the pattern of the brain lesions in inoculated mice can be transmitted serially without changing their properties in the same mouse strain with a single PrP genotype (6, 16), suggesting the presence of a strain-specific replicating component in the prion (34). Moreover, a dissociation between infectivity and PrP accumulation has been observed in the brains of scrapie agent-inoculated mice treated with amphotericin B (37) and in the salivary glands of CJD agent-inoculated mice (30).

Büeler et al. have recently generated mice devoid of the functional *PrP* gene by gene targeting (8). They and another group showed that mice homozygous for the disrupted *PrP* gene were completely resistant to the prion derived from the Chandler scrapie isolate, and heterozygous loss resulted in a prolonged incubation time of scrape in the prion-inoculated mice (7, 9, 26, 29). Manson et al., using another line of PrP-less mice inoculated with the ME7 scrapie strain, later reported a similar finding (19, 20). These studies clearly indicated that PrP^c is necessary for both the disease development and the prion propagation in mice inoculated with the scrapie prions. In the present study, we have independently developed a line of mice with the *PrP* gene disrupted and examined the susceptibility of these mice to a distinct strain of the prion, the mouse-adapted Fukuoka-1 strain of CJD agent (32). In keeping with the previous findings, homozygous loss of the gene resulted in resistance to the infectious agent. Mice heterozygous for the disrupted gene ($PrP^{+/-}$) ultimately developed CJD-like symptoms with pathologic changes similar to those in the wild-type mice ($PrP^{+/+}$), despite the prolonged incubation period. However, the maximum level of PrP^{res} in the brains of $PrP^{+/-}$ mice was about half of the level in the similarly affected

* Corresponding author. Mailing address: Department of Bacteriology, Nagasaki University School of Medicine, 1-12-4 Sakamoto, Nagasaki 852, Japan. Phone: 958-49-7057. Fax: 958-49-7060.

† Present address: Institut für Molekularbiologie I, Universität Zürich, 8093 Zürich, Switzerland.

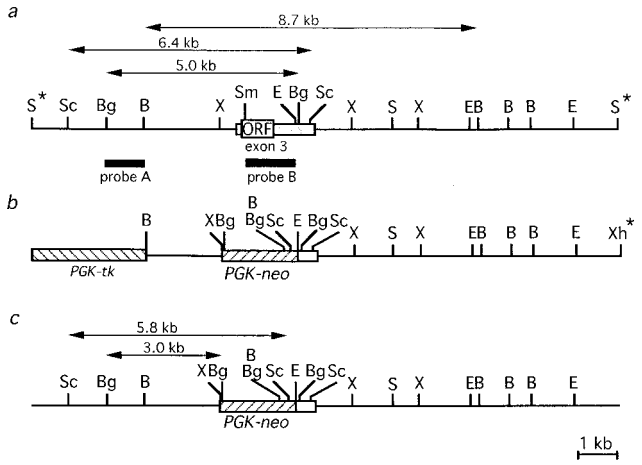

FIG. 1. Maps of the genomic *PrP* locus containing the PrP-coding region, targeting construct (pPrP*neo-tk*), and the mutated *PrP* locus after complete homologous recombination. (a) The cloned 15.5-kb genomic *PrP* DNA derived from 129/Sv mice containing exon 3, encoding PrP, and introns 2 and 3. Locations of the *PrP* open reading frame (ORF) and the probes used in Southern blotting are indicated. (b) Targeting construct which was made by substituting the *Xba*I-*Eco*RI fragment containing the entire PrP open reading frame for *PGK-neo* and adding *PGK-tk* upstream. (c) The mutated *PrP* locus when the homologous recombination occurred in ES cells. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; S, *Sal*I; Sc, *Sca*I; Sm, *Sma*I; X, *Xba*I; S*, *Sal*I site of λ DASH phage; Xh*, *Xho*I site of pBS.

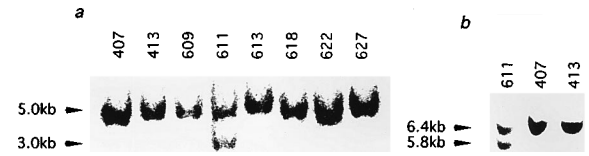

FIG. 2. Southern blotting of ES cell clones. (a) DNAs of eight drug-resistant ES cell clones were digested with *Bgl*II and hybridized with probe A. (b) DNAs of three drug-resistant ES cell clones were digested with *Sca*I and analyzed by Southern blotting using probe A.

brains of $PrP^{+/+}$ mice, indicating that PrP^{res} accumulation is restricted by the level of PrP^{c}.

MATERIALS AND METHODS

Construction of a targeting vector. A genomic DNA clone, 15.5 kb in length, corresponding to the murine *PrP* locus was isolated from a λ library constructed from 129/Sv mouse DNA (28) by screening with a hamster *PrP* cDNA probe (24). The clone consisted of the PrP-encoding exon 3 (2 kb) and the flanking introns 2 and 3, 5.5 and 8 kb, respectively, in length (Fig. 1a). The *Bam*HI-*Xba*I fragment (2 kb) of intron 2 was subcloned into pBluescript II SK+ (pBS). The herpes simplex virus thymidine kinase gene (*tk*) and the neomycin resistance gene (*neo*), both joined to the mouse phosphoglycerate kinase 1 gene (*PGK1*) promoter, were subsequently inserted at the 5′ and 3′ ends, respectively, of the *PrP* fragment in pBS, yielding pPrP5*neo-tk* (28). In *PGK-neo*, a *Bgl*II site was created just downstream of the 5′-end *Xba*I site by linker ligation. The *Eco*RI-*Sal*I fragment (3 kb) containing the 3′ untranslated region of exon 3 plus intron 3 and the *Sal*I-*Sal*I fragment (6 kb) of intron 3 were isolated from the cloned *PrP* genomic DNA and subcloned in order into pBS. The resulting 9-kb insert was cut out at the *Not*I and *Xho*I sites of pBS and was transferred downstream of *PGK-neo* in pPrP5*neo-tk* to yield a targeting vector, pPrP*neo-tk* (Fig. 1b), in which the *Xba*I-*Eco*RI 2.0-kb region of the *PrP* genome including the whole PrP^{c}-coding sequence was replaced by *PGK-neo.*

Generation of PrP-deficient mice. Approximately 2×10^7 J1 embryonic stem (ES) cells (18) were electroporated with 20 μg of the targeting vector linearized with *Xho*I at 400 V and 25 μF in one cuvette (Gene Pulser; Bio-Rad). In total, 8×10^7 ES cells were electroporated. Drug selection was started 36 h after electroporation. The cells were selected by both G418 (200 μg/ml) and FIAU (200 nM) for the first 4 days and by G418 alone thereafter. Drug-resistant colonies were transferred to 96-well dishes with feeder cells (18). Half of the cells grown in the microwell culture were transferred to a 24-well dish, and the remaining cells were stored at −80°C. The expanded cells were subjected to Southern hybridization to screen the cell clone with a disrupted *PrP* allele. The targeted ES cell clone was then thawed, expanded, and injected into blastocysts of C57BL/6J mice, which were then implanted into the uteruses of pseudopregnant ICR female mice. The resulting chimeric mice were mated with C57BL/6J mice, and the germ line transmission of the ES cell genotype in these mice was determined. Resulting F_1 heterozygous ($PrP^{+/-}$) breeding pairs were mated to yield homozygous mice lacking PrP ($PrP^{-/-}$).

Southern hybridization. High-molecular-weight DNA of the mouse tail was isolated as described previously (28). The DNA digested with an appropriate restriction enzyme was separated in a 0.8% agarose gel, denatured in 0.5 N NaOH–1.5 M NaCl, and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) in 1 M ammonium acetate. The membrane was baked at 80°C for 2 h, and prehybridization was performed for 5 h at 42°C in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7])–0.2% sodium dodecyl sulfate (SDS)–5% dextran sulfate–5× Denhardt's solution. Hybridization was performed for 15 h at 42°C in the same buffer with a probe labeled with ^{32}P (10^6 cpm/ml) by the multiprime labeling system (Amersham Japan, Tokyo, Japan) and heat-denatured salmon sperm DNA (0.1 mg/ml). The hybridized membrane was washed four times in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 5 min at room temperature and three times in 0.1× SSC–0.1% SDS for 30 min at 65°C and then exposed to X-ray film with an intensifying screen at −80°C.

In situ hybridization. Fresh mouse brains were fixed with 4% paraformaldehyde and then postfixed in 4% paraformaldehyde with 30% sucrose overnight at 4°C. Frozen 10-μm-thick brain sections were thaw-mounted onto a poly-L-lysine-coated glass slide. After deproteination with 5 μg of proteinase K per ml for 10 min and an acetylation step for 20 min in 0.1 M triethanolamine (pH 8.0)–0.25% acetic anhydride, the slides were incubated with a cRNA probe (2.5 μg/ml) labeled with digoxigenin-UTP (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) by using T7 or T3 RNA polymerase, corresponding to the *Sma*I-*Eco*RI fragment of PrP exon 3, in a solution (2× SSC, 10 mM Tris-HCl [pH 7.5], 50% deionized formamide, 5% skim milk, 500 μg of yeast tRNA per ml, 2× Denhardt's solution) at 60°C for 16 h. Subsequently, hybridized sections were treated with 20 μg of RNase A per ml at 37°C for 30 min and washed finally in 0.1× SSC for 30 min at 60°C. Immunological detection of hybridized digoxigenin-labeled probes was performed with Genius detection kits (Boehringer).

Mice. All mice used in the experiments were fed under specific-pathogen-free conditions. Experiments of prion infection were conducted in the biohazard prevention area (P3) of the Laboratory Animal Center for Biomedical Research of the authors' institution. The $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice were obtained by intercross between F_1 $PrP^{+/-}$ breeding pairs and identified by Southern blotting. C57BL/6J, ICR, and ddY mice were purchased from CLEA Japan, Inc., Tokyo, Japan.

Transmission of prion. The Fukuoka-1 strain of CJD agent (32) (kindly provided by J. Tateishi) was passaged twice in the brains of ddY mice and used for experiments. $PrP^{+/+}$, $PrP^{+/-}$, $PrP^{-/-}$, and C57BL/6J mice, about 4 weeks old, were inoculated intracerebrally with an amount of the agent equivalent to $10^{5.5}$ 50% lethal dose (LD_{50}) units and observed 4 days a week for up to 460 days after inoculation. The onset of the disease was determined as previously described (30), and the diagnosis was confirmed by pathological changes in the brain tissues of all mice.

Infectivity titration of brain tissues of prion-inoculated mice. $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice (35 of each) were inoculated with the agent as described above. Five of each group were sacrificed, and their brain tissues were removed at seven time points after inoculation: 0, 3, 6, 12, 14, 16, and 20 weeks for $PrP^{+/+}$ mice and 0, 3, 6, 12, 20, 25, and 29 weeks for $PrP^{+/-}$ and $PrP^{-/-}$ mice. Week 0 was actually 5 h after inoculation. The infectivity of tissues at each time point was determined by inoculating 0.02 ml of 20% (wt/vol) homogenate in phosphate-buffered saline (PBS) of the pooled replicate tissues into three to six 4-week-old male ddY mice. The same homogenates but heated at 80°C for 20 min were inoculated in the same way. The inoculated mice were observed for up to 260 days, and incubation periods were determined. The titer of infectivity was calculated from the mean value of incubation periods on the basis of a standard curve between titer and incubation period, obtained by inoculating serial 10-fold dilutions of the agent into ddY mice, as described previously (30).

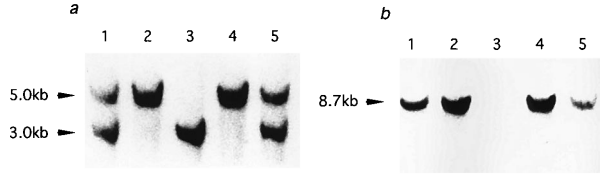

FIG. 3. Southern blotting of the tail DNA of the offspring resulting from the intercross between F_1 $PrP^{+/-}$ mice. (a) DNAs were digested with *Bgl*II and hybridized with probe A. (b) DNAs were digested with *Bam*HI and hybridized with probe B. Lanes: 1 and 5, $PrP^{+/-}$ mice; 2 and 4, $PrP^{+/+}$ mice; 3, $PrP^{-/-}$ mice.

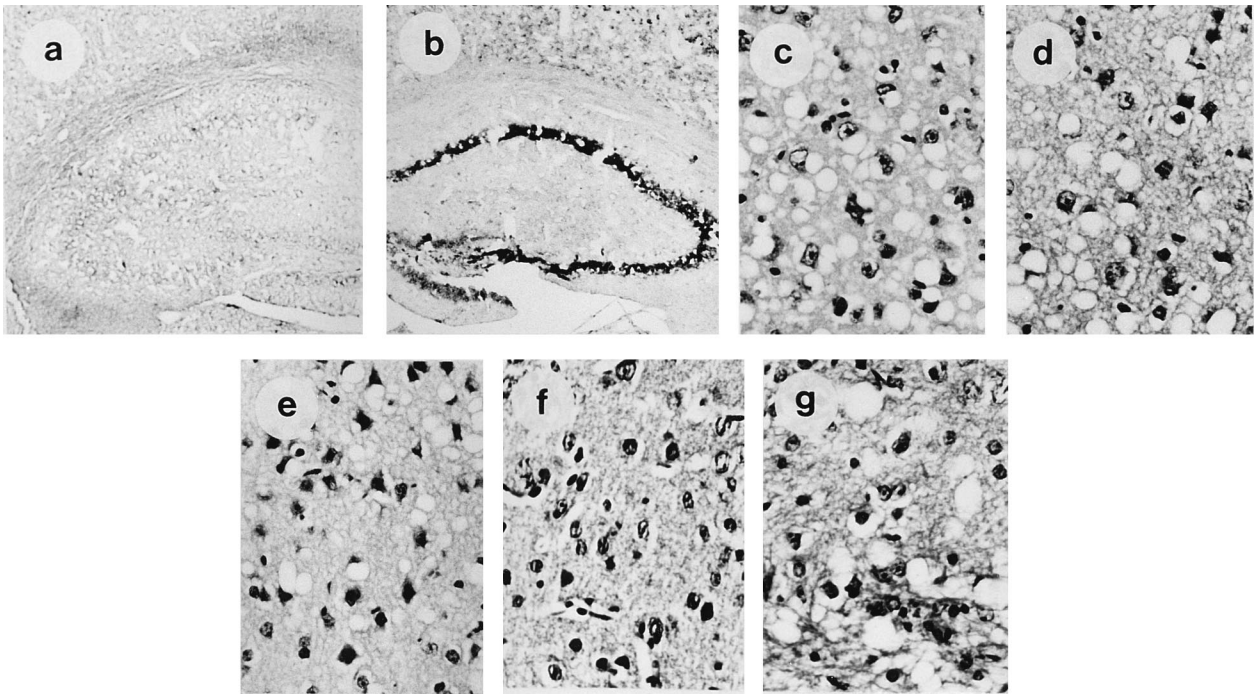

FIG. 4. Expression of PrP mRNA in the brains of $PrP^{+/+}$ and $PrP^{-/-}$ mice and histology of brain tissues from mice inoculated with mouse-adapted CJD agent or brain homogenates. PrP mRNA expression in sections of the hippocampal area from 8-week-old male uninoculated $PrP^{-/-}$ (a) and $PrP^{+/+}$ (b) mice was analyzed by in situ hybridization. PrP mRNA expression is detected in neuronal cells of $PrP^{+/+}$ mice but not of $PrP^{-/-}$ mice. Vacuolization and gliosis were observed in sections of the cortex from C57BL/6J (c), $PrP^{+/+}$ (d), and $PrP^{+/-}$ (e) mice inoculated with the agent and sacrificed when they showed CJD-like symptoms. The cortex of a $PrP^{-/-}$ mouse at 400 days after inoculation shows no pathological change (f). The cortex from a morbid ddY mouse inoculated with the pooled brain homogenate of $PrP^{-/-}$ mice sacrificed 29 weeks after inoculation with the agent reveals vacuolization and gliosis (g). Magnifications: (a and b) ×64; (c to g) ×320.

Immunoblotting. The frozen brain tissues were homogenized in PBS. Homogenate obtained from 5.6 mg (wet weight) of tissue was incubated with 150 μg of DNase I per ml at 37°C for 60 min and then treated with 1% Sarkosyl and 200 μg of proteinase K per ml at 37°C for 1 h. After boiling for 5 min in 0.1% SDS–0.5 M dithiothreitol, the sample was subjected to immunoblotting with antibodies to synthetic peptides of $PrP^{27\text{-}30}$ (31) and then labeled with ^{125}I-protein A as described previously (30).

RESULTS

Establishment of an ES clone with a disrupted *PrP* locus. J1 ES cells were electroporated with a linearized targeting vector, and the resulting drug-resistant colonies were screened for the presence of a disrupted *PrP* allele by Southern hybridization. In the targeting vector, the whole PrP-coding sequence was replaced by the *PGK-neo* gene. The vector also contained the *PGK-tk* gene to allow counterselection against random integration (Fig. 1b). A single targeted ES clone (clone 611) was obtained from among 321 drug-resistant colonies screened. As shown in Fig. 2a, *Bgl*II-digested DNA of clone 611 ES cells gave a 3-kb band derived from a mutated allele in addition to a 5-kb band of a wild-type allele by hybridization with probe A, a *Bgl*II-*Bam*HI 1-kb fragment of the *PrP* intron 2 (Fig. 1a). Homologous recombination in clone 611 was further confirmed on *Sca*I-digested DNA. Probe A hybridized with a mu-

TABLE 1. Infectivity in brains from $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice inoculated with the agent[a]

Sample	0 wk p.i.			3 wk p.i.			6 wk p.i.			12 wk p.i.		
	Incubation time (days)[b]	Survival time (days)[c]	No. dead/ total	Incubation time (days)	Survival time (days)	No. dead/ total	Incubation time (days)	Survival time (days)	No. dead/ total	Incubation time (days)	Survival time (days)	No. dead/ total
$PrP^{+/+}$												
Not heated	144 ± 15	147 ± 17	5/5	144 ± 13	147 ± 14	6/6	145 ± 25	149 ± 23	5/5	125 ± 16	129 ± 14	6/6
Heated[d]	161 ± 18	165 ± 19	5/6	159 ± 23	163 ± 21	6/6	148 ± 8	153 ± 8	6/6	137 ± 3	140 ± 4	5/5
$PrP^{+/-}$												
Not heated	153 ± 19	156 ± 19	5/5	150 ± 22	156 ± 22	4/4	149 ± 4	154 ± 4	4/4	139 ± 11	142 ± 10	5/5
Heated	168 ± 22	174 ± 21	5/6	159 ± 16	163 ± 17	6/6	150 ± 9	154 ± 9	6/6	152 ± 24	159 ± 22	6/6
$PrP^{-/-}$												
Not heated	159 ± 9	164 ± 8	5/5	164 ± 6	167 ± 5	5/6	169 ± 11	172 ± 11	5/6	160	178	1/4
Heated	169 ± 19	176 ± 20	6/6	213	217	1/6	198 ± 18	201 ± 18	3/6	>260	>260	0/6

[a] Infectivity was examined by intracerebral inoculation of the homogenates into ddY mice. p.i., postinfection; ND, not done.
[b] Time to onset of disease. Values are means ± standard deviations.
[c] Time to death. Values are means ± standard deviations.
[d] The homogenates were heated at 80°C for 20 min before inoculation.

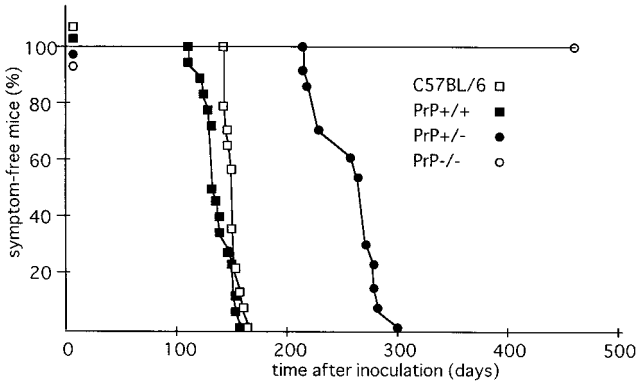

FIG. 5. Percentage of symptom-free mice after inoculation with the Fukuoka-1 strain of CJD agent. Fourteen C57BL/6J, 18 $PrP^{+/+}$, 13 $PrP^{+/-}$, and 11 $PrP^{-/-}$ mice were inoculated with $10^{5.5}$ LD_{50} units of the agent and observed for 460 days.

tated allele-derived 5.8-kb fragment as well as a 6.4-kb fragment from the normal allele, as expected (Fig. 2b).

Generation of mice with homozygously disrupted *PrP* alleles ($PrP^{-/-}$). Four male and five female chimeric mice were generated following injection of clone 611 ES cells into C57BL/6J mouse-derived blastocysts. All of the male chimeras transmitted the genotype of the ES clone to their offspring by breeding with C57BL/6J mice. Of the resulting 172 F_1 offspring with agouti coat color, 80 (47%) had the $PrP^{+/-}$ genotype. All of the F_1 $PrP^{+/-}$ mice developed and grew normally. Subsequently, F_1 $PrP^{+/-}$ breeding pairs were mated to generate $PrP^{-/-}$ mice. Tail DNA of the F_2 offspring was digested with *Bgl*II and hybridized with probe A. A representative result is shown in Fig. 3a. A $PrP^{-/-}$ mouse (lane 3) gave a 3-kb band of two mutated alleles, while $PrP^{+/-}$ (lanes 1 and 5) and $PrP^{+/+}$ (lanes 2 and 4) mice gave bands corresponding to a single copy each of the mutated and normal alleles and two copies of the normal allele, respectively. As shown in Fig. 3b, the loss of the entire PrP-coding sequence in the $PrP^{-/-}$ mice was confirmed by Southern hybridization using a corresponding sequence probe, a *Sma*I-*Eco*RI 1.5-kb fragment of the *PrP* exon 3 which included almost all the PrP-coding region (probe B in Fig. 1a). The 202 F_2 offspring, obtained by the double heterozygous breeding, consisted of 41 (21%) with the $PrP^{+/+}$ genotype, 112 (55%) with the $PrP^{+/-}$ genotype, and 49 (24%) with the $PrP^{-/-}$ genotype. This segregation agreed with Mendelian law, indicating that homozygous disruption of the *PrP* alleles did not cause embryonal death.

To confirm the lack of PrP expression in the $PrP^{-/-}$ mice, Northern (RNA) blotting and in situ hybridization were performed on total cellular RNA and formalin-fixed sections of brain tissue, respectively. Sequences of the probes used corresponded with that of probe B shown in Fig. 1a. Northern hybridization did not detect any PrP mRNA in the brain tissues of $PrP^{-/-}$ mice and revealed that PrP mRNA expression in the tissues of $PrP^{+/-}$ mice was about half of the level in $PrP^{+/+}$ mice (data not shown). By in situ hybridization, no signal was detected in the brain sections of $PrP^{-/-}$ mice (Fig. 4a), while those of $PrP^{+/+}$ mice gave signals of PrP mRNA expression which were especially high in the hippocampus (Fig. 4b).

The $PrP^{-/-}$ mice developed and grew normally. In addition to the normal macroscopic appearance, histological examination of brain, salivary gland, lung, heart, liver, spleen, pancreas, kidney, ovary, and skeletal muscle of these mice sacrificed at 10 weeks after birth revealed no abnormalities.

PrP^c is required for development of CJD-like disease in mice. $PrP^{+/+}$ (n = 18), $PrP^{+/-}$ (n = 13), and $PrP^{-/-}$ (n = 11) mice as well as control C57BL/6J mice (n = 14) were inoculated with the mouse-adapted CJD agent and were observed for up to 460 days. All $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice were derived from the same crosses between F_1 $PrP^{+/-}$ breeding pairs. All of the C57BL/6J, $PrP^{+/+}$, and $PrP^{+/-}$ mice revealed symptoms resembling CJD and subsequently died (Fig. 5). The incubation period in C57BL/6J mice was 151 ± 12 (mean ± standard deviation) days, and they died 161 ± 5 days after inoculation. The incubation and survival periods of $PrP^{+/+}$ mice were 138 ± 12 and 143 ± 14 days, respectively. The onset of the disease in $PrP^{+/-}$ mice was considerably retarded, with disease developing at 259 ± 27 days and death developing at 269 ± 27 days. In contrast, none of the $PrP^{-/-}$ mice has shown any CJD-like symptoms, and all were alive 460 days after inoculation (Fig. 5). We confirmed the characteristic neuropathological changes, including spongiform neurodegeneration and astrogliosis, in all affected brain tissues of C57BL/6J, $PrP^{+/+}$, and $PrP^{+/-}$ mice (Fig. 4c to e), but no pathological change was observed in brain sections of two $PrP^{-/-}$ mice sacrificed 400 days after inoculation (Fig. 4f).

PrP^c is required for propagation of the prion. To confirm the requirement of PrP^c for replication of the agent, the brain tissues of $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice were resected at various times after inoculation of the agent, the homogenate of three pooled replicate tissues was inoculated into three to six male ddY mice, and the incubation period was determined (Table 1). Another aliquot of the homogenate was heated to 80°C and similarly inoculated into ddY mice (Table 1). Infectivity titers of the pooled tissues were calculated from the mean values of incubation periods (Table 2). Infectivity was detectable 5 h (week 0 in Table 2) after inoculation in the nonheated

TABLE 1—*Continued*

Sample	14 wk p.i.			16 wk p.i.			20 wk p.i.			25 wk p.i.			29 wk p.i.		
	Incubation time (days)	Survival time (days)	No. dead/ total	Incubation time (days)	Survival time (days)	No. dead/ total	Incubation time (days)	Survival time (days)	No. dead/ total	Incubation time (days)	Survival time (days)	No. dead/ total	Incubation time (days)	Survival time (days)	No. dead/ total
$PrP^{+/+}$															
Not heated	110 ± 14	117 ± 12	5/5	118 ± 8	120 ± 8	5/5	112 ± 16	115 ± 15	5/5						
Heated[d]	135 ± 19	139 ± 19	5/6	126 ± 12	131 ± 11	6/6	115 ± 12	120 ± 11	3/3						
$PrP^{+/-}$															
Not heated	ND	ND	ND	ND	ND	ND	121 ± 15	127 ± 12	5/5	120 ± 7	124 ± 7	6/6	115 ± 7	118 ± 6	6/6
Heated	ND	ND	ND	ND	ND	ND	133 ± 13	141 ± 17	6/6	124 ± 15	128 ± 14	5/5	137 ± 13	140 ± 13	6/6
$PrP^{-/-}$															
Not heated	ND	ND	ND	ND	ND	ND	225 ± 16	230 ± 17	6/6	>260	>260	0/5	194 ± 14	197 ± 15	5/5
Heated	ND	ND	ND	ND	ND	ND	222 ± 25	227 ± 20	2/6	>260	>260	0/6	>260	>260	0/6

homogenates of all $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice. Titers (5.2, 4.8, and 4.5 log LD_{50} units/g, respectively) of the tissues appeared to correlate with the expression levels of PrP^c. In the $PrP^{+/+}$ mice, an increase in the titer was observed at 12 weeks, and the plateau level, 6.8 log LD_{50} units/g, was reached at 14 weeks. Replication was delayed in the $PrP^{+/-}$ mice, and the maximal level, 6.6 log LD_{50} units/g, was reached at 29 weeks. The infectivity titer in the $PrP^{-/-}$ mice detected soon after inoculation declined gradually and reached a level below the lowest calculable limit, 2.9 log LD_{50} units/g, at 20 weeks. Infectivity was barely detectable thereafter, but at week 29, $PrP^{-/-}$ mice revealed obviously higher infectivity than the mice sacrificed between 12 and 25 weeks. For instance, the 29-week homogenate caused the disease in all of five inoculated mice, with an average incubation period of 194 days, while five mice inoculated with the 25-week homogenate did not show any symptoms of disease for up to 260 days (Table 1). CJD-like pathologic changes in the brains of ddY mice inoculated with the 29-week homogenate prepared from $PrP^{-/-}$ mice were confirmed by histological examination (Fig. 4g). Heat treatment (80°C for 20 min) resulted in a reduction in the infectivity of the homogenates. For instance, 5-h (0-week) homogenates prepared from $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice gave 4.4, 4.1, and 4.0 log LD_{50} units/g, respectively, which again correlated with the PrP^c expression. Heat-treated homogenates from the $PrP^{-/-}$ mice, including that obtained at 29 weeks, barely showed any infectivity thereafter. Titer increases in mice receiving the heat-treated homogenate from $PrP^{+/+}$ and $PrP^{+/-}$ mice were delayed in comparison with those of mice receiving the untreated homogenate, but the titers eventually reached similar levels.

Accumulation of PrP^{res} is restricted by the level of PrP^c expression in the brain. We examined the accumulation of PrP^{res} in the homogenates used for the infectivity titration. Homogenates obtained from 5.6 mg of the pooled brain tissue were treated with proteinase K and subjected to immunoblotting (Fig. 6a). PrP^{res} was not detectable in the brains of $PrP^{-/-}$ mice up to 29 weeks after inoculation, while $PrP^{+/+}$ mice showed a detectable level at 12 weeks, followed by rapid accumulation which reached a plateau at 14 weeks. The accumulation of PrP^{res} in the brains of $PrP^{+/-}$ mice was delayed but became detectable at 20 weeks and reached a plateau at 25 weeks. Interestingly, the maximal level of PrP^{res} reached in the $PrP^{+/-}$ mice was roughly half of that in the $PrP^{+/+}$ mice. To confirm this finding, brain tissues of three $PrP^{+/+}$ and three $PrP^{+/-}$ mice, all of which exhibited clinical symptoms of CJD and showed similar pathological changes, were analyzed individually by immunoblotting. The levels of PrP^{res} in all of the morbid $PrP^{+/-}$ mice were roughly half of those of the similarly affected $PrP^{+/+}$ mice (Fig. 6b), with the intensity of signals from 5.6 mg of tissue of the former roughly equivalent to that from 2.8 mg of tissue of the latter (data not shown).

DISCUSSION

Büeler et al. previously demonstrated the normal phenotype of mice devoid of the functional *PrP* gene (8). The possibility remains, however, that the new mRNA expressed by the disrupted *PrP* gene, which retains the initial 3 codons and final 67 codons of the original gene, may allow the retention of some aspects of normal PrP function (11). In the present study, we developed a line of $PrP^{-/-}$ mice by homologous recombination using a construct in which the whole PrP-coding sequence was replaced by a drug-resistant gene. Homozygous loss of the whole PrP-coding region produced no deleterious effects on the development and morphological appearance of organs of the 10-week-old mice. This finding would appear to suggest that PrP^c is of little use and argues against the loss-of-function theory that the pathological changes in the scrapie- or CJD-affected brain are due to depletion of PrP^c. However, Collinge et al. recently demonstrated by electrophysiological studies that hippocampal slices of another line of $PrP^{-/-}$ mice had weakened γ-aminobutyric acid type A receptor-mediated fast inhibition and impaired long-term potentiation (12), which were rescued by a transgene encoding human PrP^c (36). Longer observation and more detailed evaluation of the neurological features of $PrP^{-/-}$ mice would provide an insight into the function of PrP^c. Indeed, we have observed neurological signs in the uninoculated $PrP^{-/-}$ mice, presumably caused by impaired motor coordination, at some 70 weeks after birth (unpublished observation). Detailed analysis of this interesting phenotype is in progress in our laboratory.

None of the $PrP^{-/-}$ mice inoculated with the mouse-adapted CJD agent developed the disease, and all survived for more than 460 days after inoculation, while all of the $PrP^{+/+}$ and $PrP^{+/-}$ mice developed the disease and died. The survival of the inoculated $PrP^{-/-}$ mice indicates that PrP^c is essential for the agent to exert pathogenicity. PrP^c was also necessary for propagation of the inoculated prion. Even if propagation could occur in the absence of PrP^c, our results estimated its level in the $PrP^{-/-}$ mice to be about 4 orders of magnitude lower than that in the $PrP^{+/+}$ mice. These findings are consistent with those obtained in experiments by others (7, 20, 26) using other lines of $PrP^{-/-}$ mice inoculated with the prions derived from scrapie, indicating that the prion strains share a biological property with respect to the requirement of PrP^c for pathoge-

TABLE 2. Infectivity titers in brains from $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice

Time (wk) after inoculation	Mean infectivity titer (log LD_{50}/g) ± SD[a]					
	$PrP^{+/+}$		$PrP^{+/-}$		$PrP^{-/-}$	
	Not heated	Heated	Not heated	Heated	Not heated	Heated
0	5.2 ± 0.7	4.4 ± 0.9	4.8 ± 0.9	4.1 ± 1.1	4.5 ± 0.4	4.0 ± 0.9
3	5.2 ± 0.6	4.5 ± 1.1	4.9 ± 1.2	4.5 ± 0.8	4.3 ± 0.3	<2.9
6	5.2 ± 1.2	5.0 ± 0.5	5.0 ± 0.2	4.9 ± 0.4	4.0 ± 0.5	<2.9
12	6.1 ± 0.8	5.6 ± 0.1	5.5 ± 0.5	4.8 ± 1.1	4.5	<2.9
14	6.8 ± 0.7	5.6 ± 0.9	ND	ND	ND	ND
16	6.5 ± 0.4	6.1 ± 0.6	ND	ND	ND	ND
20	6.7 ± 0.8	6.6 ± 0.6	6.3 ± 0.7	5.7 ± 0.6	<2.9	<2.9
25			6.4 ± 0.3	6.2 ± 0.7	<2.9	<2.9
29			6.6 ± 0.3	5.6 ± 0.6	<2.9	<2.9

[a] Calculated from the equation log LD_{50}/g = 12.067 − 0.0476 y, $108.5 < y < 192.5$, where y is incubation time (days) (30). ND, not done.

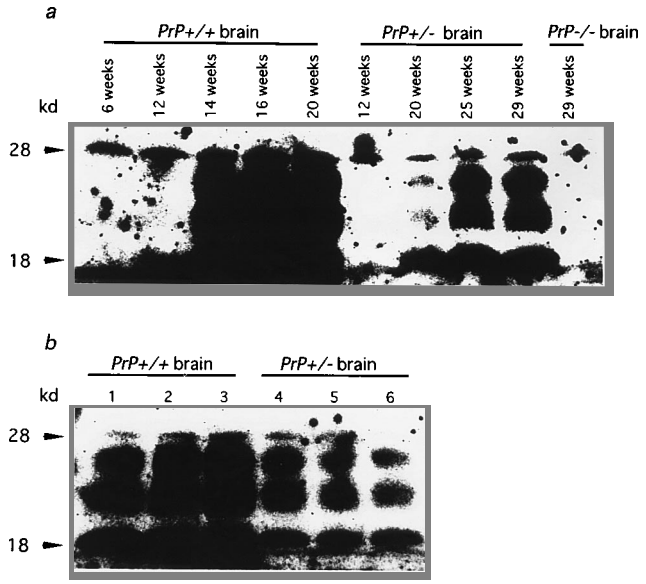

FIG. 6. Accumulation of PrP^{res} in brain homogenates of $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice inoculated with the mouse-adapted CJD agent. (a) PrP^{res} was detected by immunoblotting in proteinase K-treated brain homogenates prepared from $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice sacrificed after inoculation at the indicated time points. (b) PrP^{res} in brain homogenates independently prepared from three $PrP^{+/+}$ and three $PrP^{+/-}$ mice showing CJD-like symptoms. All samples were adjusted to 5.6 mg (wet weight) of brain tissue.

nicity and propagation in mice. The question remains as to how PrP^{c} is involved in prion propagation. We observed an interesting correlation between the levels of PrP^{c} expression and the different infectivity titers detected in the brains of $PrP^{+/+}$, $PrP^{+/-}$, and $PrP^{-/-}$ mice 5 h after inoculation. Since it is well known that the CJD or scrapie agent spreads rapidly throughout the body following intracerebral inoculation, the infectivity titers in brains 5 h after inoculation were thought to represent the residual inoculum. Furthermore, titers at 5 h in the $PrP^{-/-}$ mice declined thereafter, while those in the $PrP^{+/+}$ or $PrP^{+/-}$ mice remained unchanged by 6 weeks. These findings suggest that PrP^{c} interacts with the infectious agent and plays a role in retaining infectivity in the tissue. This interaction may be between PrP^{c} and PrP^{res} and subsequently result in conversion of the former to the latter. However, it is still possible that PrP^{c} functions only at an initial step of propagation, for instance as a cell surface receptor for an unidentified agent as suggested by others (11). An experimental system capable of manipulating PrP^{c} expression in mice after inoculation with the prion will be required to definitively answer this question.

Infectivity titers in the brains of the $PrP^{-/-}$ mice declined continuously after inoculation of the nonheated agent and reached an undetectable level by 25 weeks. However, the pooled 29-week brains gave an obvious elevation in infectivity despite the low titer, which was completely abrogated by the heat treatment. Büeler et al. found a similar increase in the infectivity titer in the $PrP^{-/-}$ mice at a single time point (20 weeks) after inoculation with the scrapie prion (7). Their subsequent study showed that the increase was transient: no infectivity was found in the samples of 25, 33, and 48 weeks in the same experiment, and it was not reproducible in an independent experiment (29). The most likely explanation for this result is persistence of the inoculum in the brain or contamination in the sample homogenate. However, an intriguing possibility remaining to be evaluated is that an unknown heat-labile agent, unrelated to PrP^{res} and present in the inoculum as an extremely minor component, was able to propagate in the absence of PrP^{c} after a long incubation period. This putative agent might have the ability to convert PrP^{c} to PrP^{res} in the brain tissues of mice harboring PrP^{c}.

Interestingly, the maximum levels of PrP^{res} in the brains of $PrP^{+/-}$ mice were about half of those in the brains of $PrP^{+/+}$ mice. This finding indicated that PrP^{res} accumulation is restricted by the PrP^{c} concentration. It is possible that PrP^{c} is a rate-limiting factor in the catalytic interaction between a converting factor, probably PrP^{res} itself, and PrP^{c}. Alternatively, this may be a consequence of the exhaustion of PrP^{c} in the affected region of the brain. If the conversion of PrP^{c} to PrP^{res} occurs only in a limited range of cell types or in a limited area of the organs, neuronal cell death would cause the exhaustion of PrP^{c} and subsequent discontinuation of PrP^{res} synthesis. It would be of interest to know whether the infectivity titers in the affected brains of the $PrP^{+/+}$ and $PrP^{+/-}$ mice correlate with the levels of PrP^{res}. Unfortunately, the twofold difference in infectivity was too subtle to be distinguished in the bioassay. A more detailed comparison between the kinetics of PrP^{res} accumulation and prion propagation in $PrP^{+/+}$ and $PrP^{+/-}$ mice will provide new insight into the nature of the prion.

A causal relationship between the PrP^{res} accumulation and pathological change in the brain tissue has been postulated. The absence of PrP^{res} accumulation in the $PrP^{-/-}$ mice would appear to support the argument that PrP^{res} accumulates through the conversion from PrP^{c} and that this accumulation is associated with the pathological changes (14). However, the higher amounts of PrP^{res} would be expected to cause more serious pathological changes, and our findings of different levels of PrP^{res} accumulation in the $PrP^{+/+}$ and $PrP^{+/-}$ mice even at similar clinical and pathological stages are inconsistent with this. Rather, we suspect the presence of a factor(s) other than PrP^{res} involved in the pathogenesis, with or without coordination with PrP^{res}. This notion is more consistent with the previous findings that transgenic mice overexpressing a normal or mutant PrP gene developed pathological changes characteristic of scrapie in the brain despite undetectable or low levels of PrP^{res} (15, 35). Alternatively, the accumulated PrP^{res} is directly involved in the pathological changes in the brain, and progression of the disease may be rate limited by the PrP^{res} concentration. If this is the case, the prolonged incubation period in $PrP^{+/-}$ mice is reasonably explained by the lower level of accumulated PrP^{res}.

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REFERENCES

1. **Alper, T.** 1985. Scrapie agent unlike viruses in size and susceptibility to inactivation by ionizing or ultraviolet radiation. Nature (London) **317:**750.
2. **Bendheim, P., J. M. Bockman, M. P. McKinley, D. T. Kingsbury, and S. B. Prusiner.** 1985. Scrapie and Creutzfeldt-Jakob disease prion proteins share physical properties and antigenic determinants. Proc. Natl. Acad. Sci. USA **82:**997–1001.
3. **Bernoulli, C., J. Siegfried, G. Baumgartner, F. Regli, T. Rabinowicz, D. C. Gajdusek, and C. J. Gibbs, Jr.** 1977. Danger of accidental person-to-person transmission of Creutzfeldt-Jakob disease by surgery. Lancet **i:**478–479.
4. **Bolton, D. C., M. P. McKinley, and S. B. Prusiner.** 1982. Identification of a protein that purifies with the scrapie prion. Science **218:**1309–1311.
5. **Brown, P., P. P. Liberski, A. Wolff, and D. C. Gajdusek.** 1990. Conservation of infectivity in purified fibrillary extracts of scrapie-infected hamster brain after sequential enzymatic digestion or polyacrylamide gel electrophoresis. Proc. Natl. Acad. Sci. USA **87:**7240–7244.

6. **Bruce, M. E., and A. G. Dickinson.** 1987. Biological evidence that scrapie agent has an independent genome. J. Gen. Virol. **68:**79–89.
7. **Büeler, H., A. Aguzzi, A. Sailer, R.-A. Greiner, P. Autenried, M. Aguet, and C. Weissmann.** 1993. Mice devoid of PrP are resistant to scrapie. Cell **73:**1339–1347.
8. **Büeler, H., M. Fischer, Y. Lang, H. Bluethmann, H.-P. Lipp, S. J. DeArmond, S. B. Prusiner, M. Aguet, and C. Weissmann.** 1992. Normal development and behaviour of mice lacking the neuronal cell-surface PrP protein. Nature (London) **356:**577–582.
9. **Büeler, H., A. Raeber, A. Sailer, M. Fischer, A. Aguzzi, and C. Weissmann.** 1994. High prion and PrP^{Sc} levels but delayed onset of disease in scrapie-inoculated mice heterozygous for a disrupted PrP gene. Mol. Med. **1:**19–30.
10. **Chandler, R. L.** 1961. Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet **i:**1378–1379.
11. **Chesebro, B., and B. Caughey.** 1993. Scrapie agent replication without the prion protein? Curr. Biol. **3:**696–698.
12. **Collinge, J., M. A. Whittington, K. C. L. Sidle, C. J. Smith, M. S. Palmer, A. R. Clarke, and J. G. R. Jefferys.** 1994. Prion protein is necessary for normal synaptic function. Nature (London) **370:**295–297.
13. **Duffy, P., J. Wolf, G. Collins, A. G. DeVoe, B. Streeten, and D. Cowen.** 1974. Possible person-to-person transmission of Creutzfeldt-Jakob disease. N. Engl. J. Med. **290:**692.
14. **Forloni, G., N. Angeretti, R. Chiesa, E. Monzani, M. Salmona, O. Bugiani, and F. Tagliavini.** 1993. Neurotoxicity of a prion protein fragment. Nature (London) **362:**543–546.
15. **Hsiao, K. K., M. Scott, D. Foster, D. F. Groth, S. J. DeArmond, and S. B. Prusiner.** 1990. Spontaneous neurodegeneration in transgenic mice with mutant prion protein. Science **250:**1587–1590.
16. **Kimberlin, R. H., and C. A. Walker.** 1978. Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture. J. Gen. Virol. **39:**487–496.
17. **Kitamoto, T., T. Muramoto, S. Mohri, K. Doh-ura, and J. Tateishi.** 1991. Abnormal isoform of prion protein accumulates in follicular dendritic cells in mice with Creutzfeldt-Jakob disease. J. Virol. **65:**6292–6295.
18. **Li, E., T. H. Bestor, and R. Jaenisch.** 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell **69:**915–926.
19. **Manson, J. C., A. R. Clarke, M. L. Hooper, L. Aitchison, I. McConnell, and J. Hope.** 1994. 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. Mol. Neurobiol. **8:**121–127.
20. **Manson, J. C., A. R. Clarke, P. A. McBride, I. McConnell, and J. Hope.** 1994. PrP gene dosage determines the timing but not the final intensity or distribution of lesions in scrapie pathology. Neurodegeneration **3:**331–340.
21. **Manuelidis, E. E., J. N. Angelo, E. J. Gorgacz, and L. Manuelidis.** 1977. Transmission of Creutzfeldt-Jakob disease to Syrian hamster. Lancet **i:**479.
22. **Medori, R., P. Montagna, H. J. Tritschler, A. LeBlanc, P. Cortelli, P. Tinuper, E. Lugaresi, and P. Gambetti.** 1992. Fatal familial insomnia: a second kindred with mutation of prion protein gene at codon 178. Neurology **42:** 669–670.
23. **McKinley, M. P., D. C. Bolton, and S. B. Prusiner.** 1983. A protease-resistant protein is a structural component of the scrapie prion. Cell **35:**57–62.
24. **Oesch, B., D. Westaway, M. Wälchli, M. P. McKinley, S. B. H. Kent, R. Aebersold, R. A. Barry, P. Tempst, D. B. Teplow, L. E. Hood, S. B. Prusiner, and C. Weissmann.** 1985. A cellular gene encodes scrapie PrP 27-30 protein. Cell **40:**735–746.
25. **Prusiner, S. B.** 1982. Novel proteinaceous infectious particles cause scrapie. Science **216:**136–144.
26. **Prusiner, S. B., D. Groth, A. Serban, R. Koehler, D. Foster, M. Torchia, D. Burton, S.-L. Yang, and S. J. DeArmond.** 1993. Ablation of the prion protein (PrP) gene in mice prevents scrapie and facilitates production of anti-PrP antibodies. Proc. Natl. Acad. Sci. USA **90:**10608–10612.
27. **Prusiner, S. B., D. F. Groth, D. C. Bolton, S. B. Kent, and L. E. Hood.** 1984. Purification and structural studies of a major scrapie prion protein. Cell **38:**127–134.
28. **Rudnicki, M. A., T. Braun, S. Hinuma, and R. Jeanisch.** 1992. Inactivation of MyoD in mice leads to up-regulation of myogenic HLA gene Myf-5 and results in apparently normal muscle development. Cell **71:**383–390.
29. **Sailer, A., H. Büeler, M. Fischer, A. Aguzzi, and C. Weissmann.** 1994. No propagation of prions in mice devoid of PrP. Cell **77:**967–968.
30. **Sakaguchi, S., S. Katamine, K. Yamanouchi, M. Kishikawa, R. Moriuchi, N. Yasukawa, T. Doi, and T. Miyamoto.** 1993. Kinetics of infectivity are dissociated from PrP accumulation in salivary glands of Creutzfeldt-Jakob disease agent-inoculated mice. J. Gen. Virol. **74:**2117–2123.
31. **Shinagawa, M., E. Munekata, S. Doi, K. Takahashi, H. Goto, and G. Sato.** 1986. Immunoreactivity of synthetic pentapeptide corresponding to the N-terminal region of the scrapie prion protein. J. Gen. Virol. **67:**1745–1750.
32. **Tateishi, J., M. Ohta, M. Koga, Y. Sato, and Y. Kuroiwa.** 1979. Transmission of chronic spongiform encephalopathy with kuru plaques from humans to rodents. Ann. Neurol. **5:**581–584.
33. **Weber, T., H. Tümani, B. Holdorff, J. Collinge, M. Palmer, H. A. Kretzschmar, and K. Felgenhauer.** 1993. Transmission of Creutzfeldt-Jakob disease by handling of dura mater. Lancet **341:**123–124.
34. **Weissmann, C.** 1991. A 'unified theory' of prion propagation. Nature (London) **352:**679–683.
35. **Westaway, D., S. J. DeArmond, J. Cayetano-Canlas, D. Groth, D. Foster, S.-L. Yang, M. Torchia, G. A. Carlson, and S. B. Prusiner.** 1994. Degeneration of skeletal muscle, peripheral nerves, and the central nervous system in transgenic mice overexpressing wild-type prion proteins. Cell **76:**117–129.
36. **Whittington, M. A., K. C. L. Sidle, I. Gowland, J. Meads, A. F. Hill, M. S. Palmer, J. G. R. Jefferys, and J. Collinge.** 1995. Rescue of neurophysiological phenotype seen in PrP null mice by transgene encoding human prion protein. Nat. Genet. **9:**197–201.
37. **Xi, Y. G., L. Ingrosso, A. Ladogana, C. Masullo, and M. Pocchiari.** 1992. Amphotericin B treatment dissociates in vivo replication of the scrapie agent from PrP accumulation. Nature (London) **356:**598–601.