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Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie

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The 'protein only' hypothesis postulates that the prion, the agent causing transmissible spongiform encephalopathies, is PrP^{Sc}, an isoform of the host protein PrP^{C}. Protease treatment of prion preparations cleaves off ~60 N-terminal residues of PrP^{Sc} but does not abrogate infectivity. Disruption of the PrP gene in the mouse abolishes susceptibility to scrapie and prion replication. We have introduced into PrP knockout mice transgenes encoding wild-type PrP or PrP lacking 26 or 49 amino-proximal amino acids which are protease susceptible in PrP^{Sc}. Inoculation with prions led to fatal disease, prion propagation and accumulation of PrP^{Sc} in mice expressing both wild-type and truncated PrPs. Within the framework of the 'protein only' hypothesis, this means that the amino-proximal segment of PrP^{C} is not required either for its susceptibility to conversion into the pathogenic, infectious form of PrP or for the generation of PrP^{Sc}.

Keywords: PrP complementation/PrP expression vectors/PrP knockout mice/PrP mutations/reverse genetics

Introduction

A wealth of data supports the 'protein only' hypothesis (Griffith, 1967) which proposes that the prion, the agent responsible for transmissible spongiform encephalopathies (TSEs), is a modified, pathogenic form of a constitutive host protein and that it multiplies by converting the normal form into a likeness of itself (Prusiner, 1991). The host protein, PrP^{C}, and its modified form, PrP^{Sc}, have been isolated; both are encoded by the same gene (Basler *et al.*, 1986) and are believed to be conformational isomers. Co-purification experiments demonstrate physical linkage of infectivity with PrP^{Sc} (Bolton *et al.*, 1982; Gabizon *et al.*, 1988). A scrapie-specific nucleic acid has never been detected (Kellings *et al.*, 1993), arguing against the virus (Diringer *et al.*, 1994) and virino (Dickinson and Outram, 1988) hypotheses. Because the ratio of infectious units to PrP^{Sc} molecules is only ~1:100 000 (Bolton *et al.*, 1991), the structure of the prion protein (PrP) molecule actually associated with infectivity cannot be inferred definitively. For this reason, and because specific infectivity can vary considerably, the PrP species responsible for infectivity is presently better designated as PrP* (Weissmann, 1991); it may or may not be identical with PrP^{Sc}, the major species that has been characterized chemically and physico-chemically. If it is identical, the low specific activity could be due to low efficiency of infection or to the infectious unit being an aggregate of a large number of PrP^{Sc} molecules.

Genetic evidence linking PrP to prion diseases is compelling. Ablation of the PrP gene renders mice resistant to experimental scrapie and precludes replication of the infectious agent; reintroduction of PrP transgenes restores susceptibility to scrapie (Büeler *et al.*, 1993; Sailer *et al.*, 1994). The so-called species barrier, which hinders prion transmission from one host species to another, is overcome by introducing PrP transgenes from the prion donor to the recipient (Prusiner *et al.*, 1990; Büeler *et al.*, 1993). Remarkably, all familial cases of TSE in man are probably linked to one of many possible mutations in the PrP gene (Baker and Ridley, 1992); these mutations are thought to facilitate occasional spontaneous conversion of PrP^{C} to PrP^{Sc} (or PrP*), which is then followed by the autocatalytic process.

PrP^{C} and PrP^{Sc} are chemically indistinguishable (Stahl *et al.*, 1993) but differ in their secondary structure and physico-chemical properties. PrP^{C} has a very low and PrP^{Sc} a high content of β-pleated sheet structure (Caughey *et al.*, 1991; Pan *et al.*, 1993). Under conditions where PrP^{C} is readily degraded by proteinase K, PrP^{Sc} is partially resistant. About 60 N-terminal residues are cleaved off and the remainder of the molecule is quite stable. Under these conditions of protease treatment, prion preparations retain full infectivity. It has been shown earlier, by introducing a truncated version of the PrP gene into scrapie-infected N2A neuroblastoma cells, that PrP^{C} devoid of 66 N-terminal amino acids can still give rise to PrP^{Sc}; however, it was not determined whether infectivity was associated with the truncated PrP^{Sc} molecule (Rogers *et al.*, 1993).

We now show that introduction of truncated PrP transgenes encoding PrP^{C} devoid of as many as 49 amino-proximal amino acids into PrP knockout mice restores susceptibility to scrapie, allows prion propagation and leads to accumulation of PrP^{Sc}. Within the framework of the 'protein only' hypothesis, this means that the amino-proximal segment of PrP^{C} is required neither for conversion into the pathogenic, infectious form of PrP nor for the generation of PrP^{Sc}.

Results

Introduction of transgenes encoding wild-type murine PrP into $Prnp^{0/0}$ mice restores susceptibility to mouse prions

We have shown earlier that *$Prnp^{0/0}$* mice are resistant to experimental scrapie and do not propagate the infectious

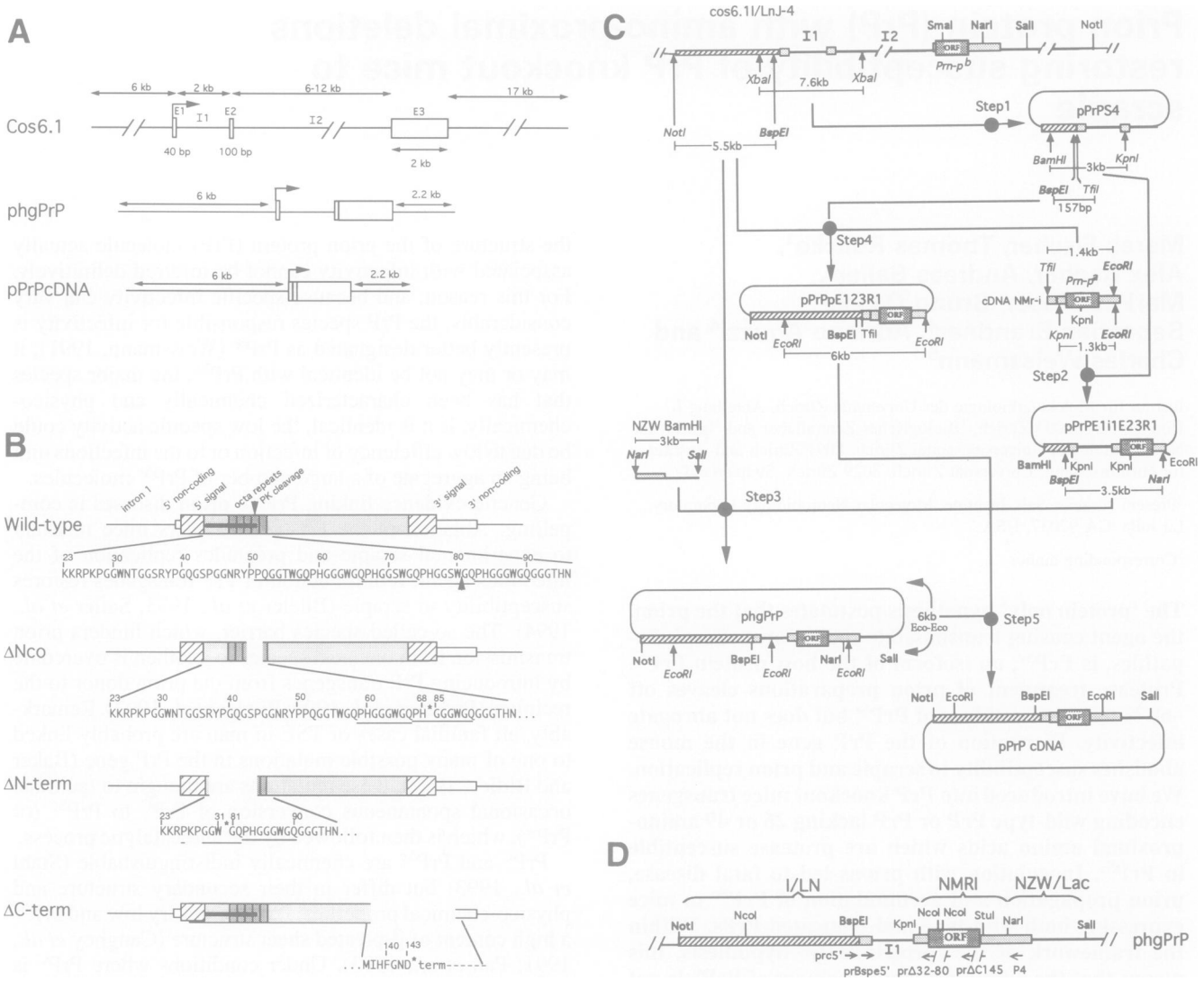

Fig. 1. Wild-type and mutant PrP-encoding genes. (**A**) Maps of constructs encoding wild-type PrP. Cos6.11, a cosmid-derived clone comprising three exons (E1, E2 and E3), two introns (I1 and I2), 6 kb of 5′- and 17 kb of 3′-flanking regions; phgPrP, as before, but lacking intron 2 and with only 2.2 kb of 3′-flanking region; pPrPcDNA, as phgPrP, but lacking both introns. (**B**) Wild-type and mutant constructs derived from phgPrP. The boxes represent the part of the transcription unit comprised of exons 2 and 3. Open boxes, 5′- and 3′-non-coding; hatched, 5′ and 3′ signal sequences; lightly stippled, coding sequence of mature PrP; dark stippled boxes and underlined sequence, octa repeats; horizontal black bar, protease-sensitive in PrP^{Sc}; arrowhead, beginning of the sequence resistant to protease in PrP^{Sc}; *, deletion breakpoints. (**C**) Flowsheet for the construction phgPrP and pPrPcDNA; details are given in Materials and methods. (**D**) Detailed view of phgPrP showing the origin of the various segments and the primers (arrows) used in various PCRs. Hatched box, 5′-flanking region; light stippled, non-coding regions; heavy stippled, ORF; heavy lines, intron or 3′-flanking regions.

agent (Büeler *et al.*, 1993; Sailer *et al.*, 1994). Moreover, breeding multiple hamster *Prnp* transgenes into $Prnp^{0/0}$ mice rendered the latter highly susceptible to hamster prions but much less so to mouse prions.

We now undertook to introduce genes encoding wild-type and mutated murine PrP into $Prnp^{0/0}$ mice by transgenesis. For this purpose, we examined several PrP-encoding constructs for their efficacy in restoring susceptibility to scrapie. Wild-type mice containing multiple *Prnp* transgenes have been generated previously by injecting 40 kb cosmid DNA containing genomic *Prnp* into one-cell embryos of wild-type mice, and efficient expression of PrP was achieved (Scott *et al.*, 1989; Hsiao *et al.*, 1990).

The mouse PrP gene contains an upstream intron of 2 kb and a downstream intron of 6–12 kb (Westaway *et al.*, 1994a). In order to facilitate mutagenesis, we generated a PrP-encoding construct from which the large intron ('half-genomic construct' or phgPrP) or both introns ('cDNA construct' or pPrPcDNA) were deleted and which contained 5.5 kb of 5′- and 2 kb of 3′-flanking sequence (Figure 1A). These constructs, as well as the *Prnp*-containing cosmid DNA (cosmid cos6.I/lnJ4; Westaway *et al.*, 1991) were introduced into $Prnp^{0/0}$ or $Prnp^{0/+}$ mice by nuclear injection. Founders were identified and bred to yield $Prnp^{0/0}$ lines homozygous or heterozygous for the PrP-encoding transgene array.

PrP expression in the brain was monitored by Northern analysis (Table I). In all but one of the six lines carrying half-genomic *Prnp* transgenes tested, PrP was expressed. In contrast, the cDNA construct, in all eight lines tested, even in those (*tg*b9/+, *tg*b24/+) with very high transgene copy numbers (>150 copies), showed no detectable levels

Table I. Characteristics of mouse lines with various PrP genotypes[a]

Line	PrP-encoding gene	Gene copy No.[b]	PrP RNA[c]	PrP protein[d]	Inoculum	Days to symptoms	Days to terminal disease	No. of animals inoculated
Prnp$^{+/+}$	*Prnp*^{a}	2	1	1	RML	131 ± 9	166 ± 8	9
Prnp$^{0/+}$	*Prnp*^{a}	1	0.5	0.5	RML	290 ± 33	415 ± 30	19/15
tga19/+	*hgPrnp*^{a}	30	no data	3–4	RML	87 ± 13	100 ± 17	13
tga20/+	*hgPrnp*^{a}	30	1.2	6–7	RML	64 ± 9	68 ± 10	15
tga20/tga20	*hgPrnp*^{a}	60	no data	10	RML	60 ± 4	62 ± 4	6
tgc35/+	*Prnp*^{b}*(cosmid)*	40	no data	4–5	RML	121 ± 10	130 ± 7	8
tgb24/+	*PrPcDNA*	150	<0.5	not detected	none	–	–	none
tge19h/+	*PrPΔNco*	150	1.8	3–4	RML	67 ± 17	72 ± 16	7
tge19l/+	*PrPΔNco*	15	1	1.5–2.5	RML	101 ± 5	104 ± 9	4
tgd11/+	*PrPΔN-term*	30	2.7	6	RML	97 ± 9	103 ± 11	6
tgd12/+	*PrPΔN-term*	40	3.9	10–12	RML	86 ± 11	89 ± 12	8

[a]*Prnp*$^{0/+}$, from Büeler *et al.* (1994); *tga20/tga20*, partly from Brandner *et al.* (1995).
[b]Relative to wild-type; determined by quantitative PCR.
[c]Relative to wild-type; determined by quantitative Northern analysis.
[d]Relative to wild-type; determined by densitometric analysis of Western blots.

of PrP protein in the brain and only very weak (*tgb24/+*) or no expression of PrP RNA (data not shown). The one line of cosmid transgenic mice examined showed efficient PrP expression, as expected from previous work (Westaway *et al.*, 1991). These results, in agreement with earlier data (Scott *et al.*, 1989), show that the presence of at least one intron is essential for the efficient expression of a PrP-encoding transgene under the control of its own promoter. A requirement of introns for efficient (Brinster *et al.*, 1988) and/or tissue-specific (Lozano and Levine, 1991) expression of some transgenes has been described previously.

Two half-genomic (*tga19/+; tga20/+*) mouse lines and one cosmid mouse line (*tgc35/+*), all with similar gene copy numbers of ~30–40, were analyzed in more detail. As shown in Figure 2 and Table I, all three lines overexpressed PrP in the brain at levels ranging from ~3- to 4-fold and 4- to 5-fold (*tga19/+* and *tgc35/+* respectively) to 6- to 7-fold (*tga20/+*) of those observed in wild-type *Prnp*$^{+/+}$ animals. The distribution of PrP transcripts in the brains of the two half-genomic and the cosmid lines was analyzed by *in situ* hybridization of brain sections with a PrP antisense riboprobe (Figure 3). Overall levels of PrP RNA were higher in the brains of transgenic than in those of wild-type animals. The expression patterns were similar in transgenic and wild-type mice, with highest PrP RNA levels in the hippocampus (Figure 3A, C and E) and the cerebellum (Figure 3B, D and F). In some areas, like the molecular layer of the cerebellum, PrP RNA-positive cells were clearly visible in transgenic but only very faintly in wild-type mice, supporting the notion that expression of PrP RNA was generally increased. Interestingly, however, PrP RNA was not detected in the Purkinje cells of animals carrying half-genomic transgenes (*tga19*, Figure 3F; *tga20*, not shown), while it was abundant in Purkinje cells of wild-type and cosmid transgenic mice (Figure 3B and D). This suggests that one or more control elements responsible for the specific expression of PrP in Purkinje cells are absent from the half-genomic construct.

Some animals containing the cosmid transgene (*tgc35/+*; *tgc35/c35*, *Prnp* allele b) gradually developed paresis of their hind-limbs as early as 4–6 months after birth. Similar symptoms in mice overexpressing this as well as other types of *Prnp* cosmid transgenes have been described earlier (Westaway *et al.*, 1994b). Animals expressing half-genomic PrP-A, at even higher levels in the brain (*tga20/+*, *tga20/tga20),* revealed no obvious pathological phenotype. This difference could be due to the different allelic nature of the two transgenes (Carlson *et al.*, 1988), to differences in expression patterns or to overexpression of an unidentified gene product encoded by sequences present in the cosmid but not in the half-genomic construct. Because these symptoms were found in several independent PrP cosmid transgenic mouse lines, it seems unlikely that they were due to position effects of the transgene integration.

To determine whether the half-genomic construct could restore susceptibility to scrapie, mice hemizygous for the half-genomic *tga20/+* and *tga19/+* transgene insertion were inoculated with mouse prions. As positive controls, *tgc35/+* animals hemizygous for the cosmid transgene array and *Prnp*$^{+/+}$ mice of comparable genetic background to the *Prnp*$^{0/0}$ mice used for the generation of the transgenic lines (offspring of C57/Bl6×129/SV crosses; Büeler *et al.*, 1992) were inoculated similarly. Negative controls were *Prnp*$^{0/0}$ mice inoculated with prions and mock-inoculated transgenic animals. Results are compiled in Figure 4. The negative control groups remained free of scrapie during the time of observation, for up to 500–600 days post-inoculation (data not shown). One mock-inoculated *tga20/+* animal died 538 days p.i., after showing kyphosis and tremor, symptoms which occur in scrapie-infected but occasionally also in uninoculated aging mice; it was not analyzed further.

Scrapie-inoculated mice expressing the half-genomic construct or the cosmid displayed typical scrapie symptoms, such as hind-limb paresis, foot-clasp reflex, kyphosis, ataxia, disorientation and minced gait. Incubation times to the occurrence of first symptoms as well as to the terminal state (Table I) were shorter than in the wild-type control group, namely 68 ± 10 days for *tga20/+* and 100 ± 17 days for *tga19/+* (the two lines expressing the half-genomic transgene) and 130 ± 7 days for *tgc35/+* (the line carrying the cosmid), as compared with 166 ± 8 days for wild-type mice. In addition, the duration of clinical disease was greatly decreased in the transgenic animals: in the *tga20/+* line, terminal disease occurred as early as 4 ± 2 days after the observation of the first symptoms, as compared with 35 ± 12 days in

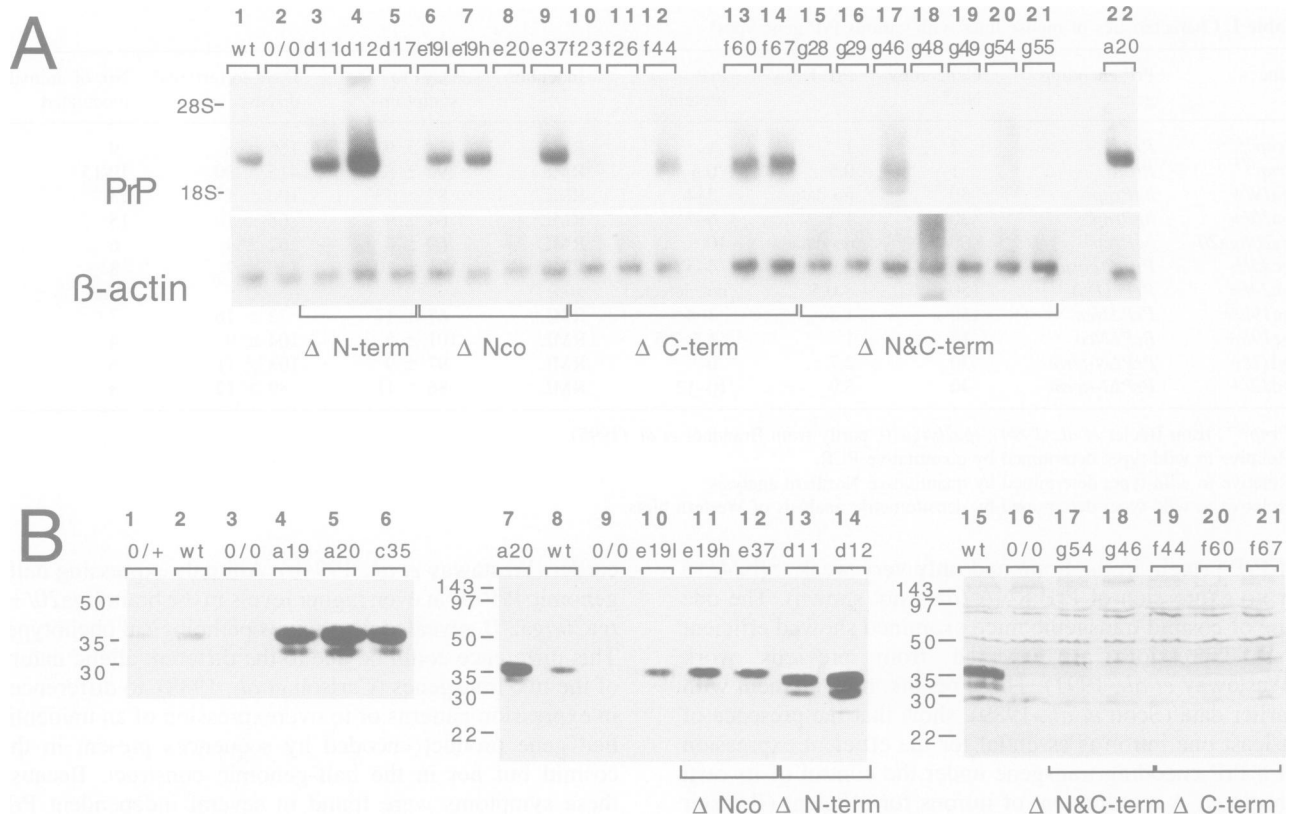

Fig. 2. PrP mRNA and protein levels in brains of mice transgenic for wild-type and mutant PrP-encoding genes. (**A**) Northern blots were performed on 10 µg of total RNA as described in Materials and methods. PrP: the labeled probe corresponded to a region of the *Prnp* gene deleted in $Prnp^{0/0}$ mice. Actin: the filter was stripped and rehybridized with a chicken β-actin cDNA probe. (**B**) Western blots of total mouse brain homogenates. One half mouse brain was homogenized in 1.35 ml of 0.5% SDS, 50 mM Tris–HCl (pH 6.8), 5 mM EDTA, 0.25 M PMSF, and 200 µl of lysate was mixed with 500 µl of SDS loading buffer (Laemmli, 1970) without reducing agent. Samples (20–40 µl) were electrophoresed through a 12.5% polyacrylamide gel. Western blotting was carried out as described (Büeler *et al.*, 1994) using antiserum RO73 (lanes 1–6), 1B3 (lanes 7–14) or R3430 (lanes 15–21) and developed with the ECL system. All mice were hemizygous for the mutant alleles and $Prnp^{0/0}$, except for the wild-type (wt).

the wild-type group. Furthermore, shorter incubation times correlated with higher expression levels of PrP^C when lines *tga19/+* and *tga20/+* were compared, consistent with an inverse correlation between these two parameters, as suggested earlier (Scott *et al.*, 1989; Büeler *et al.*, 1994).

Because *tga20* mice showed such a short incubation time compared with the usual wild-type indicator mice, we explored the possibility of using them for prion titration by the incubation time assay (Prusiner *et al.*, 1982). The titer of a prion preparation was determined by end point titration in *tga20* mice (Reed and Muench, 1938). Table II shows the relationship between incubation period and inoculated dose; the data were used to determine the parameters of the equation: log10(titer×dilution) = *a–b* (incubation time).

Generation and characterization of mice transgenic for mutant PrP genes

The observation that moderate treatment of preparations of scrapie agent with proteinase K removes amino acids 23–80 of mouse PrP^{Sc} (Hope *et al.*, 1988) without abrogating infectivity (Neary *et al.*, 1991) suggested that, within the framework of the 'protein only' hypothesis, the amino-proximal deletion mutants described above may be functional, provided that the truncated protein is susceptible to conversion. Moreover, a human PrP allele with a nonsense mutation in codon 145 has been found in a patient who suffered from neurodegenerative disease and had amyloid plaques containing PrP truncated at the cognate position (Kitamoto *et al.*, 1993). This suggested that such truncated PrP might also be pathogenic.

Deletion of codons 23–88 of the hamster PrP gene as well as truncation of the C-terminal codons downstream of the attachment site for the GPI-anchor did not preclude conversion of PrP^C to PrP^{Sc} in scrapie-infected neuroblastoma cells (Rogers *et al.*, 1993). However, protease resistance of PrP is not necessarily linked to scrapie infectivity (Manuelidis *et al.*, 1987; Xi *et al.*, 1992; Pocchiari, 1994) and is therefore not a reliable marker for this property.

In order to define the requirements of PrP for conferring susceptibility to scrapie and promotion of prion replication, N- and C-terminal deletion mutants of the PrP gene were constructed and mice transgenic for these genes were assessed for susceptibility to scrapie, propagation of prions and, where appropriate, formation of PrP^{Sc}.

Four constructs, based on the half-genomic vector (Figure 1), were prepared and introduced as transgenes into $Prnp^{0/0}$ mice. The founders were mated to $Prnp^{0/0}$ partners and the hemizygous offspring were tested for the presence and the expression of the PrP transgenes (Table I, Figure 2). (i) Construct d (pPrPΔN-term): the sequence

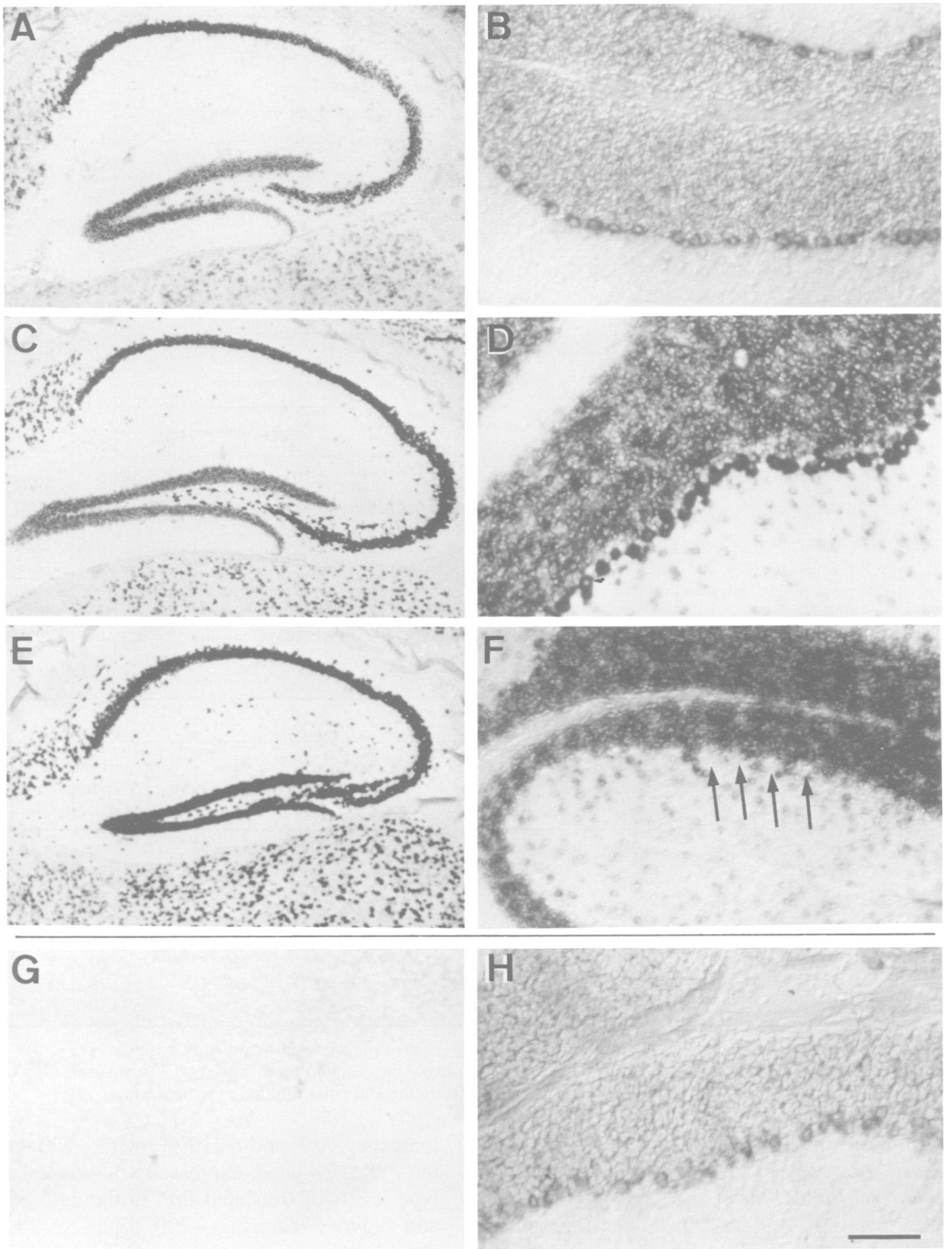

Fig. 3. Expression pattern of PrP mRNA in wild-type mice and mice expressing PrP transgenes. Sagittal brain sections of 129/SV(ev)×C57BL/6-derived wild-type (**A** and **B**), *tgc35/+* (*Prnp* cosmid; **C** and **D**), *tga19/+* (*hgPrnp*; **E**, **F** and **H**) or $Prnp^{0/0}$ mice (**G**) were hybridized *in situ* with digoxigenin-labeled riboprobes detecting PrP (A–G) or neuron-specific enolase (NSE) RNA (H) as described (Moser *et al.*, 1995). Regions from the hippocampus (A, C and E) and the cerebellum (B, D, F, G and H) are shown. Note the absence of a PrP RNA-specific signal in Purkinje cells of *tga19/+* mice (F) even though NSE mRNA can be detected in them in a parallel section (H). Arrows in (F) indicate the positions of Purkinje cell bodies. Hybridization of PrP probe to $Prnp^{0/0}$ (G) mice served as a control for non-specific hybridization signals. Bar, 310 μm in (A), (C) and (E).

encoding amino acids 32–80 (inclusive) was deleted but the codons for the signal sequence and nine additional amino acids were retained to ensure correct processing and localization in the cell. (ii) Construct e (pPrPΔNco): the sequence encoding two of the five copies of the octa repeat PxGGxWGQ (from amino acid 68 to 83 inclusive) was deleted. (iii) Construct f (pPrP–C-term): the sequence encoding amino acids 144 (inclusive) to the end was ablated by fusing codon 143 to the termination codon of the PrP reading frame. (iv) Construct g (pPrPΔN&C-term): the deletions of constructs d and f were combined, to give a sequence encoding a mature protein of 73 amino acids.

Most animals carrying the *PrPΔN-term* and *PrPΔNco* transgenes expressed them at both the RNA (Figure 2A) and protein level (Figure 2B). As expected, the protein encoded by the *PrPΔN-term* gene showed distinctly higher mobility than wild-type PrP (Figures 2B and 4), while the

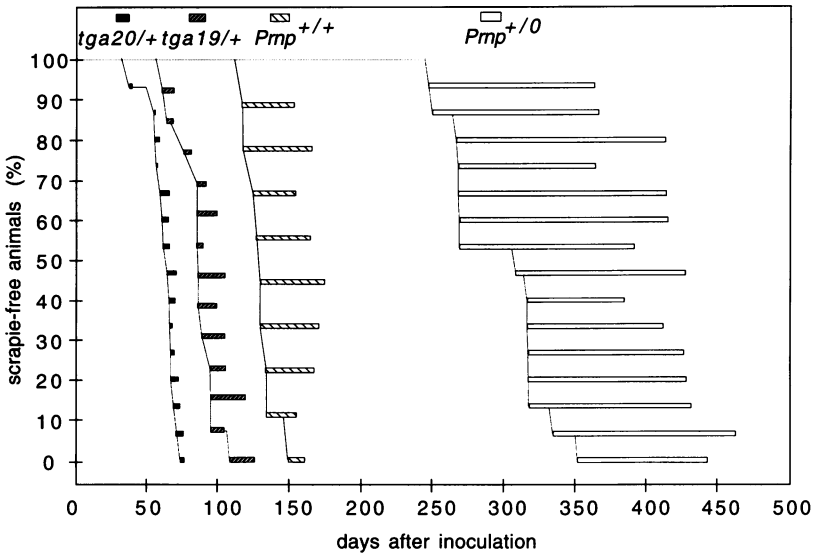

Fig. 4. Scrapie incubation time of mice expressing different levels of PrP. All mice were derived from 129×C57BL crosses. $Prnp^{0/+}$ mice express about half the wild-type PrP level. *tga19/+* and *tga20/+* are $Prnp^{0/0}$ mice containing ~30 copies of the hgPrP construct and expressing ~3–4 and 6–7 the wild-type level of PrP, respectively. Intracerebral inoculation with mouse scrapie prions was as described (Büeler *et al.*, 1993). Scrapie was diagnosed if mice showed three or more scrapie-specific symptoms (decreased motor activity, hind-limb paresis, foot-clasp reflex, kyphosis, ataxia, disorientation, minced gait). The horizontal bars indicate for each mouse the time elapsed from diagnosis until the terminal stage of disease (immobility or inability to procure food or drink).

Table II. Determination of prion titers in *Tga20* mice

Expt	Genotype of donor	PrP-encoding gene	Time after inoculation of donor (days)	Dilution of sample	Genotype of indicator	Mice succumbing to scrapie	Time to terminal disease	Titer[a] (log LD_{50} unit/ml 10% homogenate)
1	*tga20/+*	*hgPrnp*	68	10^{-1}	*tga20/tga20*	1/1	69	7.9
	tga20/+	*hgPrnp*	68	10^{-2}	*tga20/tga20*	3/3	69 ± 2	8.9 ± 0.2
	tga20/+	*hgPrnp*	68	10^{-4}	*tga20/tga20*	3/3	87 ± 5	8.9 ± 0.5
	tga20/+	*hgPrnp*	68	10^{-6}	*tga20/tga20*	4/4	111 ± 1	8.3 ± 0.1
	tga20/+	*hgPrnp*	68	10^{-8}	*tga20/tga20*	0/4	>274	–
2	$Prnp^{+/+}$ (CD-1)	*Prnp*	~150	10^{-1}	*tga20/tga20*	6/6	61 ± 3	7.1 ± 0.5
3	*tgd12/+*	*PrPΔN-term*	98	10^{-1}	*tga20/tga20*	3/3	78 ± 0	6.9
4	*tgd12/+*	*PrPΔN-term*	73	10^{-1}	*tga20/tga20*	3/3	70 ± 1	7.8 ± 0.1

In all cases, 30 μl of the indicated dilution of a 10% brain homogenate were injected intracerebrally into *tga20/tga20* mice.
[a]Titers were determined by the incubation time assay (Prusiner *et al.*, 1982), using the relationship $y = 14.37–1.1x$, where y is log LD_{50} and x is incubation time (days) to terminal disease; this relationship was determined from the end point titration of experiment 1.

PrPΔNco product showed only a slight decrease in mobility (not detectable in the analyses shown). In contrast, the mice transgenic for *PrPΔC-term* and *PrPΔN&C-term* expressed lower levels of PrP transcripts per transgene copy than wild-type animals and no PrP was detected in their brains. In some lines carrying 200 or more transgene copies (*tgf60/+*, *tgf67/+*, *tgg46/+*), mRNA was present at levels similar to wild-type animals, and yet it was not possible to detect PrP in brain extracts using polyclonal antibodies directed against full-length murine PrP (data not shown) or a polyclonal antiserum directed against a peptide present within the expected mutant proteins (Figure 2B, lanes 15–21).

Inoculation of mice carrying mutant PrP transgenes with prions

Lines of $Prnp^{0/0}$ mice carrying a high copy number of mutated transgenes were chosen for inoculation experiments (Table I). In the case of *PrPΔN-term*, *tgd12/+* mice with copy numbers of 30–40 in the hemizygous state expressed truncated PrP at ~10–12 times wild-type. The lines *tge19h/+* and *tge19l/+*, with ~150 and 15 copies of the *PrPΔNco* gene, expressed ~3–4 times and twice wild-type levels of truncated PrP. In the case of lines *tgf67/+* and *tgg46/+* carrying ~200 copies of *PrPΔC-term* and *PrPΔN&C-term* respectively, PrP RNA was expressed at high levels but PrP could not be detected.

Inoculation was with mouse prions as above; the end-point of the experiment was the appearance of terminal scrapie symptoms, at which time the animals were sacrificed. As shown in Table I, both N-proximally truncated transgenes were capable of restoring susceptibility to scrapie in $Prnp^{0/0}$ mice. The two lines expressing PrPΔNco, as well as those expressing wild-type PrP, showed incubation times that appeared to be dependent on PrP levels: 104 ± 9 days and 72 ± 16 days for *tge19l/+* and *tge19h/+* animals, respectively, values very close to those of *tga19/+* and *tga20/+* animals (100 ± 17 days and 68 ± 10 days respectively (Table I). The two lines expressing *PrPΔN-term* transgenes, *tgd11/+* and *tgd12/+*, displayed incubation times of 103 ± 11 days and 89 ± 12 days, respectively. Both the determination of PrP levels

Table III. Determination of prion titers in CD-1 indicator mice

Expt	Genotype of donor	PrP-encoding gene	Time after inoculation of donor (days)	Dilution of sample	Mice succumbing to scrapie	Time to terminal disease	Estimated titer[a] (log LD_{50} unit/ml 10% homogenate)
1	*Prnp*$^{+/+}$ (CD-1)	*Prnp*	~150	10^{-1}	6/6	154 ± 4	7.7 ± 0.6
2	*tga20/+*	*hgPrnp*	68	10^{-1}	4/4	172 ± 4	6.1 ± 0.6
	tga20/+	*hgPrnp*	68	10^{-2}	3/3	165 ± 6	7.7 ± 0.7
3	*tge19h/+*	*PrPΔNco*	56	10^{-1}	4/4	166 ± 11	6.6 ± 1
	tge19h/+	*PrPΔNco*	56	10^{-2}	4/4	180 ± 10	6.4 ± 1
4	*tgd12/+*	*PrPΔN-term*	98	10^{-1}	4/4	173 ± 0	6.0 ± 0.6
	tgd12/+	*PrPΔN-term*	98	10^{-2}	3/3	170 ± 7	7.3 ± 0.8

In all cases, 30 µl of the the indicated dilution of a 10% brain homogenate were injected intracerebrally in CD-1 mice.
[a]Titers were determined by the incubation time assay as described previously (Büeler *et al.*, 1993; see Table II).

and the measurement of incubation times, due to the small numbers of samples tested in both cases, are only approximate.

In contrast to the results outlined above, no scrapie symptoms were observed in scrapie-inoculated animals transgenic for either *ΔC-term* or *ΔN&C-term* transgenes (>270 days p.i. at the time of writing). This finding is not unexpected in view of the lack of detectable PrP^C in these lines. Transmission experiments to determine whether prion replication has occurred in the brains of these animals have been initiated and have failed to demonstrate the presence of scrapie infectivity up to 270 days p.i.

Mock-inoculated N-terminal mutant mice remained free of scrapie symptoms for the entire observation period (>290 days p.i. at the time of writing) (Table I), except for one mock-inoculated *tgd12/+* animal which was found dead at 101 days p.i. after showing kyphosis and footclasp reflex. Histopathology of the poorly preserved brain was inconclusive.

Determination of scrapie infectivity in brains of transgenic mice expressing truncated and normal PrP

We assayed scrapie infectivity on CD-1 wild-type mice, because the *tga20* assay system had not been available initially. As shown in Table III, titers of brain homogenates from scrapie-infected, terminal or close to terminal mice carrying the wild-type *hgPrnp* transgenes (*tga20),* or the transgenes with the large (*tgd12/+*) or small (*tge19h/+*) deletion were all in the range 6–8 log LD_{50} units/ml of 10% homogenate. These results are supported by similar data in Table II (the 10^{-1} dilutions used for inoculation are, however, outside the linear range of the assay and thus do not yield absolute values).

From these data, it is clear that PrP devoid of amino acids 32–80 is able to restore the capacity of propagating mouse scrapie prions. Because of the imprecision of the assay and because incubation times could be prolonged when the PrP sequence in prion donor and recipient is different (Scott *et al.*, 1989; Prusiner *et al.*, 1990; Büeler *et al.*, 1993), it is not possible to conclude whether or not the truncation of PrP diminished its efficacy to some degree.

Determination of protease-resistant PrP in mice overexpressing PrP

A pathognomonic feature of prion diseases is the accumulation of PrP^{Sc}, defined by its partial resistance to limited protease digestion, in scrapie-diseased brains.

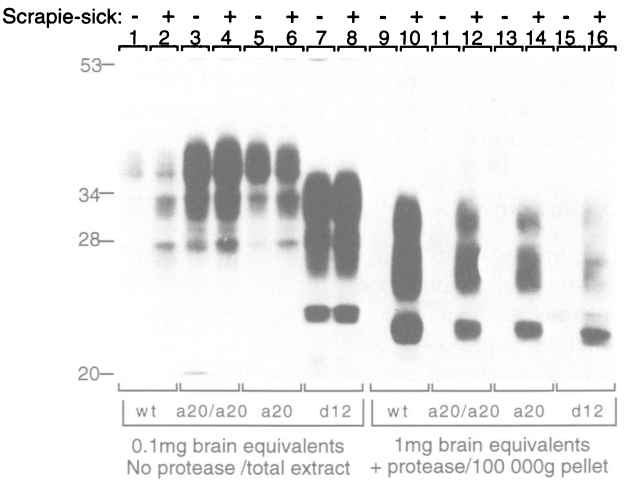

Fig. 5. Western blot analysis for total PrP and PrP^{Sc} in brains of mice transgenic for wild-type and mutant PrP-encoding genes. Samples from healthy or terminally sick animals were analyzed by Western blotting as described in Materials and methods using antiserum IB3. (**A**) Mouse brain homogenates were analyzed directly, applying the equivalent of 0.1 mg of brain tissue. (**B**) Homogenates were treated with proteinase K and the solubilized 100 000 *g* pellet analyzed, applying the equivalent of 1 mg of brain tissue.

Protease treatment of mouse PrP^{Sc} gives rise to PrP27–30, a truncated form of the mature protein, lacking (in addition to the signal sequence) 58 N-terminal amino acids, from Lys23 to Trp80 (Hope *et al.*, 1988). The large N-terminal mutant gene *PrPΔN-term* was designed to yield a protein with a similar primary structure to PrP27–30, except for the retention of the first nine residues of the mature sequence, Lys23–Trp31 (Figure 1). Therefore, it was of interest to determine whether scrapie infection of mice carrying the *PrPΔN-term* transgene would lead to accumulation of protease-resistant mutant PrP and whether such molecules would be similarly truncated by protease treatment.

As shown earlier, PrP^{Sc} can be pelleted from detergent-treated brain homogenates by centrifugation at 100 000 *g*, while PrP^C remains in the supernatant (Meyer *et al.*, 1986). Brain homogenates were prepared from healthy and scrapie-sick animals, and samples were subjected to proteinase K digestion and centrifuged at 100 000 *g*. Aliquots of the untreated, total homogenates equivalent to 0.1 mg of brain tissue and pellet fractions equivalent to 1 mg of brain tissue were analyzed by immunoblotting (Figure 5). As reported earlier, the total PrP level of wild-

type brain increased ~5-fold in scrapie-inoculated brains of terminal animals (Büeler *et al.*, 1994); however, no such increase was found in the brains of PrP-overexpressing transgenic animals, either in the case of full-length or of the ΔN-term truncated PrP (Figure 5). Unexpectedly, in PrP-overexpressing scrapie-infected brains, the level of PrP^{Sc} was only about half the wild-type level, in the case of full-length and only ~25% in the case of truncated PrP. Thus, in brains of terminally scrapie-sick wild-type animals, PrP^{Sc} was ~50% of total PrP, while in animals overexpressing PrP this value ranged from 3 to 10%.

The electrophoretic mobility of the amino-proximally truncated PrP lacking amino acids 32–80 from a *tgd12/+* animal (Figure 5, lanes 7 and 8) is, as expected, greater than that of wild-type PrP (lanes 1–6); however, proteinase K-treated PrP^{Sc} from wild-type (lanes 10, 12 and 14) and mutant (lanes 16) have a similar mobility. This suggests that the primary structure of truncated PrP^{Sc} encoded by *PrPΔN-term* is similar to that of the protease-resistant core of normal PrP^{Sc}.

Discussion

The finding that mice devoid of PrP were viable and resistant to scrapie opened up the possibility of carrying out reverse genetics on the *Prnp* gene with regard to its function in prion diseases.

To facilitate the introduction of mutations, we prepared a mini-*Prnp* gene ('half genomic *Prnp*') lacking the large intron, and determined its capacity to restore expression of PrP and susceptibility to mouse scrapie. The expression pattern generated by the mini-*Prnp* gene in brain differed slightly from that in wild-type mice or mice transgenic for the complete *Prnp* gene introduced as a cosmid clone, in that PrP mRNA in Purkinje cells was undetectable. This suggests that the mini-gene lacks a cell-specific enhancer which could be located in the large intron or in 3′-flanking regions present in the cosmid clone but not in the truncated construct. Removal of both introns precluded expression altogether.

Mice carrying ~ 60 copies of the half-genomic sequence *hgPrnp*, such as *tga20/tga20*, expressed ~10 times the normal PrP level and were very susceptible to scrapie: the incubation time to terminal disease after inoculation with ~10^6 LD_{50} units of mouse prions was only 61 ± 3 days, as compared with 149 ± 12 days for a standard wild-type mouse such as CD-1. Because of the short and reproducible incubation times, we have been using these mice routinely for titering mouse prion levels (Brandner *et al.*, 1996).

We obtained transgenic mice which overexpressed PrP with amino-proximal deletions at levels commensurate with those of *tga20/+* mice; however, no PrP expression was found in lines of mice carrying up to ~200 genes encoding PrP truncated at residue 144, even when the cognate mRNA was expressed at wild-type level. Because the C-terminal truncation removed the domain responsible for anchoring PrP to the cell surface, the protein may have been secreted and degraded rapidly. It is not clear why in the case of a patient heterozygous for a *Prnp* gene encoding PrP with the analogous deletion (Kitamoto *et al.*, 1993) the amyloid plaques contained exclusively a truncated form of PrP. Possibly, the truncated PrP was not the product of the mutated allele, but a proteolytic fragment derived from the product of the normal allele. Similarly truncated fragments have been found in amyloid of Gerstmann–Sträussler–Scheinker disease (GSS) cases and shown to be derived from full-length PrP, albeit with missense mutations (Tagliavini *et al.*, 1994).

Curiously, all PrP-overexpressing mice showed lower absolute levels of PrP^{Sc} at the final stages of disease than wild-type mice, resulting in a ratio of PrP^{Sc} to total PrP of ~0.1 or less in overexpressing animals and ~0.5 in wild-type mice. This may come about because accumulation of PrP^{Sc} is a time-dependent process and PrP-overexpressing mice die early on. In view of our earlier finding that mice containing a single copy of the *Prnp* gene have incubation times of ~41 weeks and already have high levels of PrP^{Sc} after 24 weeks, when they are still months away from showing scrapie symptoms, and of reports that mice (Hsiao *et al.*, 1994) and humans (Collinge *et al.*, 1995) succumbing to prion disease may be devoid of detectable PrP^{Sc}, it seems likely that accumulation of high levels of PrP^{Sc} is neither necessary nor sufficient for disease and death. It should, however, be noted that because the detection of infectivity is orders of magnitude more sensitive than that of PrP^{Sc}, it is possible that the prion is associated with a subset of protease-resistant PrP which we have previously dubbed PrP* (Weissmann, 1991).

The hypothesis that scrapie pathology is due to depletion of PrP^{C} is not supported by the finding that PrP^{C}-overproducing mice succumb to disease rapidly, although relatively little of it is converted to PrP^{Sc}.

It has been reported that moderate protease treatment of scrapie-infected hamster brain homogenates resulted in little if any decrease of infectivity (McKinley *et al.*, 1983), and that ~66 amino acids were cleaved off hamster PrP^{Sc} under such conditions. Similar conclusions can be drawn for mouse PrP^{Sc} (Hope *et al.*, 1988; Neary *et al.*, 1991). If the 'protein only' hypothesis is correct, this means that the protease-susceptible residues are not required for the conversion of mature full-length PrP^{C} into PrP^{Sc}. Our experiments comparing mice expressing high levels of wild-type PrP or PrP lacking residues 32–80 show that both are susceptible to scrapie and capable of allowing efficient propagation of the infectious agent. Our data do not allow us to conclude whether the somewhat longer incubation times in the case of the shortened PrP molecules are due to experimental variation or to a moderately lower efficiency of the shortened molecule in mediating disease. In order to clarify this point, it will be necessary to replace the wild-type *Prnp* gene by the mutated version using homologous recombination, so as to ensure identical gene copy numbers and genetic environment. Such experiments are currently underway.

In any event, it is clear that, by and large, the course of the disease and the levels of PrP^{Sc} attained are very similar in mice carrying similar copy numbers of truncated or wild-type PrP^{C}. Within the framework of the 'protein only' hypothesis, this means that not only is protease-truncated PrP^{Sc} capable of converting PrP^{C} into PrP^{Sc}, but that PrP^{C} devoid of 49 amino-proximal residues is subject to conversion by full-length PrP^{Sc} into a shortened version of PrP^{Sc} with the same susceptibility to protease as its normal counterpart.

Materials and methods

DNA constructions

DNA constructions (Figure 1) were carried out according to standard cloning protocols (Ausubel *et al.*, 1987; Sambrook *et al.*, 1989) using the following starting DNAs: cos6.I/LNJ-4 (Westaway *et al.*, 1994a); pNZWBamHI (containing an 8.9 kb segment of the mouse *Prnp*^{a} gene; Büeler *et al.*, 1992); pPrPS4 (provided by D.Westaway) consisted of the 7.6 kb *Xba*I fragment from cos6.I/LNJ-4 comprising *Prnp* exons 1 and 2 in the *Xba*I site of pBluescribe; cDNA-NMRI was amplified from total brain cDNA from NMRI mice using Vent DNA polymerase (Biolabs), the previously described primer P4 (Büeler *et al.*, 1992) and primer prc5′ (5′GTCGGATCAGCAGACCGATTCTGGGCGCT3′). Plasmids encoding wild-type and mutant PrPs were constructed as follows.

pPrPE1i1E23R1. The 3 kb *Kpn*I–*Bam*HI fragment from pPrPS4 (comprising exons 1 and 2, intron 1 and part of the promoter) was isolated. cDNA-NMRI was digested with *Eco*RI and partially with *Kpn*I to yield a 1.3 kb fragment. The two fragments were joined to dephosphorylated *Bam*HI- and *Eco*RI-cleaved pBluescript (Stratagene).

pPRPHG. The *Not*I–*Bsp*EI 5.5 kb fragment from cos6.I/LNJ-4 (comprising part or all of the *Prnp* promoter), the 3.5 kb *Nar*I–*Bsp*EI fragment from pPrPE1i1E23R1 (containing exons 1 and 2, the coding region of exon 3 as well as intron 1) and the 3 kb *Nar*I–*Sal*I fragment of pNZW *Bam*HI (comprising the 3′ end of *Prnp*) were joined to *Not*I- and *Sal*I-cleaved, dephosphorylated pBluescript in a four-way ligation.

pPrPPE123R1. The 1.4 kb *Tfi*I–*Eco*RI fragment of NMRI-cDNA (comprising exons 1, 2 and 3 up to the *Eco*RI site in the 3′ non-coding region), the 157 bp *Tfi*1–*Bsp*EI fragment from pPrPS4 (comprising the promoter sequences proximal to exon 1) and the 5.5 kb *Not*I–*Bsp*EI fragment from cos6.I/LNJ-4 (containing the promoter) were joined to *Not*I- and *Eco*RI-digested, dephosphorylated pBluescript in a four-way ligation.

pPrPcDNA. The 6 kb *Eco*RI fragment from pPrPPE123R1 (part of the promoter, exons 1 and 2, the coding region of exon 3 as well as intron 1) was ligated to a dephosphorylated 6 kb *Eco*RI fragment from PrPHG (comprising the plasmid backbone as well as promoter and 3′-untranslated sequences).

pPrPΔNco. pPrPHG was digested with *Nco*I and religated. Resulting clones were screened for the presence and correct orientation of the fragment comprising exon 1, and the absence of the small *Nco*I fragment derived from exon 3.

pPrPΔN-term. A PCR product was prepared using pPrPHG as template, primer prBspE5′ (5′caagcattaagccagtccggagcggtga3′), the mutation primer prΔ32–80 (5′tccaccgccatggggttgccccccaccctccaggct3′) and cloned *Pfu* DNA polymerase (Stratagene). The 2 kb *Bsp*EI–*Nco*I fragment of this PCR product (comprising exon 1, intron 1 and exon 3 up to the deletion breakpoint) was joined to two fragments derived from pPrPHG, the 9 kb *Bsp*EI–*Sal*I fragment (comprising the vector backbone and the PrP promoter) and the 3 kb *Nco*I–*Sal*I fragment (comprising the 3′ end of the half-genomic gene).

pPrPΔC-term. A PCR product was prepared using pPrPHG as template, primer prBspe5′ (5′caagcattaagccagtccggagcggtga3′) and the mutation primer prΔC145 (5′cctccctcagtcgttgccaaaatggatcat3′). The 2.1 kb *Bsp*EI fragment of this product (containing exon 1, intron 1, exon 2 and the ORF of the mutated gene), the 9 kb *Bsp*EI–*Sal*I (comprising the vector backbone and the PrP promoter) and the 2.7 kb *Stu*I–*Sal*I fragment (containing the 3′ end of pPrPHG) of pPrPHG were joined in a three-way ligation.

pPrPΔN&C-term. All steps were carried out as for pPrPΔC-term, except that the template for PCR was pPrPΔN-term rather than PrPHG.

Plasmid DNA from *E.coli* XL1-blue (Stratagene) was purified using the Qiagen purification system. Linear amplification DNA sequencing (PCR sequencing) was carried out essentially according to Adams and Blakesly (1991).

Generation of transgenic mice

Plasmid DNA was digested with *Not*I and *Sal*I (only with *Not*I in the case of the cosmid), heated for 5 min at 65°C and electrophoresed through a 1% agarose gel (Seakem GTG; FMC BioProducts, Denmark). The PrP insert was purified using the Prep-A-Gene DNA extraction kit (Bio-Rad, USA), diluted to 1–2 ng/μl with TE buffer (10 mM Tris–HCl pH 7.5, 0.5 mM EDTA), filtered through a Micropore filter (Millipore Ultrafree MC 0.45 μm) pre-rinsed twice with TE buffer. Microinjection into oocytes and re-implantation were as previously described (Brinster *et al.*, 1985; Wilmut *et al.*, 1991).

PCR for the detection and quantitation of transgenes

Prnp^{0} and *Prnp*$^{+}$ alleles, both transgenic and resident, were detected and quantified by a modification of a previous protocol (Büeler *et al.*, 1992). One microlitre of either purified genomic DNA or crude tail lysate (Laird *et al.*, 1991) was added to 25 μl of PCR mix [50 mM KCl, 10 mM Tris–HCl (pH 9), 1.5 mM $MgCl_2$, 2% deionized formamide, 0.1% bovine serum albumin (BSA), 0.1% Triton X-100, 0.5 μCi [α-^{32}P]dATP, 0.2 mM each of dATP, dCTP, dGTP, dTTP, 0.7 μM of each primer and 0.6 units of *Taq* DNA polymerase]. Three primers were used to detect transgenes simultaneously and, as internal standard, the two *Prnp*^{0} alleles P3 (specific for *Prnp*^{0}) and P10 (specific for *Prnp*$^{+}$) (Büeler *et al.*, 1992) and prPrP3′nc (5′ataacccctcccccagcct3′) (complementary to both *Prnp*$^{+}$ and *Prnp*^{0}). The primer prPrP3′nc is proximal to the coding region of PrP, resulting in products of 350 bp for the *Prnp*^{0}-specific signal and 550 bp for the wild-type and N-terminal mutant *Prnp*-specific signals. After 21 cycles in a thermal cycler (Perkin Elmer/Cetus, USA; 45 s at 95°C, 45 s at 62°C and 1 min at 72°C) followed by 7 min at 72°C and subsequent cooling to 4°C, 5 μl samples were electrophoresed through agarose gels. After drying, the gels were analyzed using a PhosphorImager (Molecular Dynamics, USA). Copy numbers were estimated by comparison with control reactions containing appropriate cloned standards run in parallel.

Northern blots

Total brain RNA was prepared essentially as described (Chomczynski and Sacchi, 1987) and electrophoresed through a 1% agarose gel. Prehybridization and hybridization were according the Quikhyb protocol (Stratagene). A 140 nucleotide single-stranded DNA probe was prepared by linear amplification for 10 cycles of a *Nco*I–*Ava*II murine PrP cDNA fragment comprising the sequence from codon 84 (His) to codon 145 (Glu), using a primer hybridizing to the DNA plus strand (pr del-: 5′tccccagcatgtagccaccaagg3′). The β-actin probe, an *Eco*RI–*Hin*dIII fragment of chicken β-actin cDNA (Cleveland *et al.*, 1980), was ^{32}P-labeled by the random primer method (Prime-It, Stratagene).

Western blot analysis

Electrophoresis of brain homogenates and blotting were carried out essentially as described (Büeler *et al.*, 1994). The antibodies used were R073, a rabbit polyclonal antiserum raised against PrP27–30 from hamster (Serban *et al.*, 1990), 1B3, a rabbit polyclonal serum directed against SAF (aggregated PrP) from mouse (Farquhar *et al.*, 1989) and R3430, a rabbit polyclonal serum directed against the N-terminal peptide P1 from hamster PrP27–30 (GQGGGTHNQWNKP, identical to the murine sequence comprising amino acids 89–102) (Barry *et al.*, 1986), at a 1:2000 dilution.

Determination of PrP^{Sc}

Brain homogenates (10%) in 0.32 M sucrose were adjusted to 0.5% in SST buffer (20 mM Tris–HCl pH 7.5, 2% *N*-lauryl sodium sarcosine, 0.32 M sucrose) and further homogenized by drawing through a 26 gauge needle. Of this, 900 μl were digested with 5 μg/ml proteinase K for 15 min at 37°C. After adding 10 μl of 0.1 M phenylmethylsulfonyl fluoride, 800 μl were centrifuged at 100 000 *g* for 2 h at 10°C. The final pellet was resuspended in 80 μl of SST and 40 μl of 5× SDS loading buffer containing 1% mercaptoethanol and heated for 5 min in a boiling waterbath.

Inoculations and scrapie diagnosis

Inocula were prepared by incubating cleared 10% brain homogenates for 20 min at 80°C and subsequently diluting in phosphate-buffered saline (PBS)–5% BSA. Mice were inoculated intracerebrally with 30 μl of 1% brain extract, which was prepared from terminally scrapie-sick mice (CD-1) inoculated with mouse-adapted (RML) prions as described previously (Büeler *et al.*, 1993). The animals were monitored at weekly intervals for the first 40 days p.i. and at 2-day intervals thereafter.

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