

# BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein

Emmanuel A.Asante, Jacqueline M.Linehan, Melanie Desbruslais, Susan Joiner, Ian Gowland, Andrew L.Wood, Julie Welch, Andrew F.Hill, Sarah E.Lloyd, Jonathan D.F.Wadsworth and John Collinge<sup>1</sup>

MRC Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College, Queen Square, London WC1N 3BG, UK

<sup>1</sup>Corresponding author  
e-mail: j.collinge@prion.ucl.ac.uk

**Variant Creutzfeldt–Jakob disease (vCJD) has been recognized to date only in individuals homozygous for methionine at *PRNP* codon 129. Here we show that transgenic mice expressing human PrP methionine 129, inoculated with either bovine spongiform encephalopathy (BSE) or variant CJD prions, may develop the neuropathological and molecular phenotype of vCJD, consistent with these diseases being caused by the same prion strain. Surprisingly, however, BSE transmission to these transgenic mice, in addition to producing a vCJD-like phenotype, can also result in a distinct molecular phenotype that is indistinguishable from that of sporadic CJD with PrP<sup>Sc</sup> type 2. These data suggest that more than one BSE-derived prion strain might infect humans; it is therefore possible that some patients with a phenotype consistent with sporadic CJD may have a disease arising from BSE exposure.**

**Keywords:** BSE/Creutzfeldt–Jakob disease/prion/transgenic

## Introduction

Prion diseases, such as Creutzfeldt–Jakob disease (CJD) in humans, and scrapie and bovine spongiform encephalopathy (BSE) in animals, are transmissible neurodegenerative diseases associated with accumulation of a disease-associated isoform of host-encoded cellular prion protein (PrP<sup>C</sup>), designated PrP<sup>Sc</sup>. PrP<sup>Sc</sup> is thought to comprise an aggregated form of a conformational isomer of PrP<sup>C</sup>. According to the protein-only hypothesis, infectious prions are composed principally, if not entirely, of an abnormal isoform of PrP. Distinctive isolates or strains of prions can be propagated in the same type of host and may be encoded by differences in PrP<sup>Sc</sup> conformation (Bessen and Marsh, 1992, 1994; Collinge *et al.*, 1996b; Telling *et al.*, 1996) and glycosylation (Collinge *et al.*, 1996b). Variant CJD (vCJD), recognized in 1996, is thought to be caused by exposure to BSE-like prions (Collinge *et al.*, 1996b; Lasmézas *et al.*, 1996; Bruce *et al.*, 1997; Hill *et al.*, 1997). We have previously described four human PrP<sup>Sc</sup>

types in brain tissue from patients with CJD: types 1–3 are seen in classical (sporadic or iatrogenic) CJD, while type 4 is seen in vCJD (Collinge *et al.*, 1996b).

A common polymorphism at codon 129 of the human PrP gene (*PRNP*), where either methionine (M) or valine (V) can be encoded, is a key determinant of susceptibility to sporadic and acquired prion diseases, and may affect age at onset in inherited prion disease (Baker *et al.*, 1991; Collinge *et al.*, 1991; Palmer *et al.*, 1991). To date, all patients recognized with vCJD have been of the *PRNP* 129MM genotype (Collinge *et al.*, 1996a; Zeidler *et al.*, 1997; our unpublished data). PrP polymorphisms are known to affect prion strain propagation in mice and sheep (Bruce, 1993). Similarly, codon 129 genotype may play a role in human prion strain propagation, since certain PrP<sup>Sc</sup> types are closely associated with codon 129 genotypes. To date, we have found types 1 and 4 PrP<sup>Sc</sup> only in individuals of the *PRNP* 129MM genotype and type 3 PrP<sup>Sc</sup> only in genotypes MV or VV, while type 2 PrP<sup>Sc</sup> is seen in association with all three genotypes (Collinge *et al.*, 1996b; Wadsworth *et al.*, 1999; our unpublished data). We have previously reported that Tg(HuPrP129V<sup>+/+</sup> Prnp<sup>0/0</sup>)-152 mice, which express only human PrP V129 (129VV Tg152 mice), are highly susceptible to infection with human prions from patients with sporadic and iatrogenic forms of CJD, regardless of patient genotype at polymorphic codon 129 (Collinge *et al.*, 1995; Hill *et al.*, 1997). However, these mice are much less susceptible to prions from patients with vCJD. Indeed, the transmission properties of vCJD closely resembled those of BSE, and these experiments form part of the extensive data arguing that vCJD is caused by a BSE-like prion strain (Collinge *et al.*, 1996b; Bruce *et al.*, 1997; Hill *et al.*, 1997). These mice lacked a species or transmission barrier to classical CJD prions and were also used to model the transmission barrier between cattle and humans (Collinge *et al.*, 1995; Hill *et al.*, 1997). These data were relatively reassuring, in that transmission of BSE to transgenic mice expressing only human PrP was inefficient, with <40% of intracerebrally inoculated mice succumbing to prion disease after prolonged incubation periods, consistent with the presence of a substantial transmission barrier. However, an important caveat with respect to public health considerations was that vCJD was occurring in humans of the *PRNP* 129MM genotype, while these mice expressed human PrP 129V (Collinge *et al.*, 1995; Hill *et al.*, 1997). Although classical CJD from patients with all three *PRNP* codon 129 genotypes (MM, VV and MV) transmitted efficiently to these mice, it is possible that part of the transmission barrier to vCJD infection of these mice resided in the mismatch at codon 129 between inoculum and host (Hill *et al.*, 1997). Using the same inocula, we have now extended these studies to mice expressing human PrP M129 to further study both the bovine-to-human species

**Table I.** vCJD, BSE and sporadic CJD transmissions to transgenic mice expressing human PrP 129M

	Inoculum			Tg(HuPrP129M <sup>+/+</sup> Prnp <sup>0/0</sup> )-35		
	Code	PRNP129 genotype	Human PrP <sup>Sc</sup> type	Clinical signs	Incubation period (days ± SEM)	Total affected <sup>a</sup>
Sporadic CJD	I1199	MM	T1	3/3	237 ± 10	3/3
	I1202	MM	T1	8/8	229 ± 5	8/8
	I1196	MM	T1	8/8	225 ± 7	8/8
	I026	MM	T2	7/7	223 ± 1	7/7
	I024	MV	T2	4/4	241 ± 1	4/4
	I022	VV	T2	2/6	700, 708	4/6
	I020	MV	T3	6/7	437 ± 31	7/7
	I021	VV	T3	2/7	354	3/7
vCJD	I336	MM	T4	0/2	>600	2/2
	I342	MM	T4	1/5	690	5/5
	I344	MM	T4	0/7	>340–720	7/7
BSE	I038	MM <sup>b</sup>		2/20	344, 468	8/20
	I060	MM <sup>b</sup>		0/6	>570	1/6
	I062	MM <sup>b</sup>		2/7	338, 340	3/7
	I064	MM <sup>b</sup>		2/10 <sup>c</sup>	344, 492	2/10
	I066	MM <sup>b</sup>		0/6	>500	0/6

<sup>a</sup>Positive either by clinical signs, western blotting and/or immunohistochemistry.

<sup>b</sup>Genotype at corresponding bovine PrP gene codon.

<sup>c</sup>One brain not available for either western blotting or immunohistochemistry.

barrier and the propagation of human and BSE prion strains. Detailed study of the relative transmission barriers to BSE in transgenic mice expressing human PrP M129 and V129 will be published elsewhere. Here we report the unexpected finding that BSE prion inoculation can induce replication of two distinct prion strains in mice expressing human prion protein.

## Results

### **Susceptibility of transgenic mice expressing human PrP M129 to human and bovine prions**

We produced transgenic mice homozygous for a human PrP M129 transgene array and murine PrP null (Bueler *et al.*, 1992) alleles (*Prnp*<sup>0/0</sup>), designated Tg(HuPrP129M<sup>+/+</sup> Prnp<sup>0/0</sup>)-35 (129MM Tg35), with expression levels of human PrP two times that of pooled normal human brain (data not shown). These mice were challenged with prions from cases of sporadic CJD, vCJD and BSE. 129MM Tg35 mice were highly susceptible to prions from patients with sporadic CJD of the *PRNP* 129MM genotype, but were less susceptible to classical CJD prions from individuals of the *PRNP* 129VV genotype (Table I). Transmission of sporadic CJD of the *PRNP* 129MV genotype was associated with either consistent short-duration characteristics as with MM cases (I024) or long and variable incubation periods (I020). This may reflect stochastic propagation of either 129M or 129V PrP<sup>Sc</sup> in these patients. This was in contrast to Tg(HuPrP129V<sup>+/+</sup> Prnp<sup>0/0</sup>)-152 mice, expressing human PrP V129 (129VV Tg152), which, as we have reported previously (using the same inocula), are highly susceptible to classical CJD prions from all three *PRNP* genotypes (Collinge *et al.*, 1995; Hill *et al.*, 1997). The presence of a transmission barrier can be estimated by measuring the fall in mean incubation period on primary and second passage

in the same host. Second passage of prions from sporadic CJD (I1202)-inoculated 129MM Tg35 mice resulted in an incubation period of 249 ± 3 days (4/4 mice), which was not lower than primary passage [229 ± 5 days (8/8 mice)]. It is possible that the small increase in incubation period reflects a lower prion titre in mouse than human brain since affected mice are culled at an early clinical stage. Consistent short incubation periods on primary passage with 100% attack rate and no fall in incubation period on second passage of CJD in these mice, as with our earlier studies with Tg152 mice (Collinge *et al.*, 1995), are consistent with lack of a transmission barrier to classical CJD 129MM prions. However, as with 129VV Tg152 mice (Hill *et al.*, 1997), 129MM Tg35 mice were much more resistant to vCJD 129MM prions, with only 1/14 mice succumbing to clinical prion disease at a prolonged incubation period (690 days) (Tables I and II). Indeed, as judged by development of clinical disease, 129MM Tg35 mice, expressing human PrP 129M, appeared less susceptible to vCJD than 129VV Tg152 mice, expressing human PrP 129V (Hill *et al.*, 1997). Similarly, 129MM Tg35 mice appeared highly resistant to BSE prions, with 6/49 clinically scored transmissions at variable and prolonged incubation periods (338–492 days) (Tables I and II).

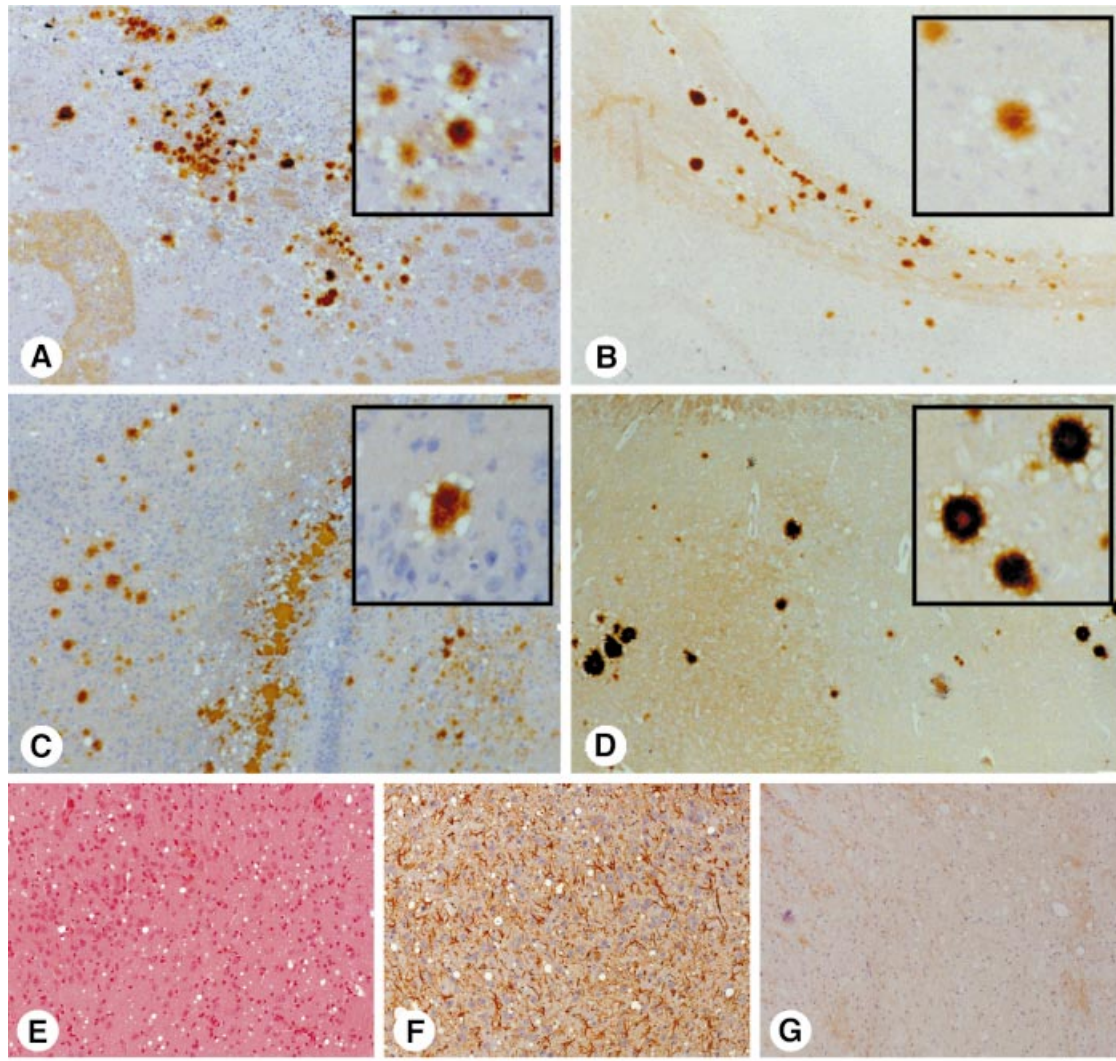
### **Sub-clinical infection in mice expressing human PrP M129**

While by clinical criteria these data might be interpreted as consistent with the existence of a substantial species barrier between cattle BSE and transgenic mice expressing 129M human PrP, we investigated all these mice for evidence of sub-clinical infection. We and others have previously demonstrated extensive sub-clinical prion infection in mice inoculated with a strain of hamster prions (Sc237 or 263K) thought to be non-pathogenic to wild-type mice (Hill *et al.*, 2000; Race *et al.*, 2001),

**Table II.** Summary of BSE and vCJD transmission to Tg35 and Tg45

Transgenic line	BSE					vCJD				
	Total attack rate	Clinical disease	Sub-clinical infection	Type 2 PrP <sup>Sc</sup>	Type 4 PrP <sup>Sc</sup>	Total attack rate	Clinical disease	Sub-clinical infection	Type 2 PrP <sup>Sc</sup>	Type 4 PrP <sup>Sc</sup>
Tg35	14/49	6/49	8/49	10/11 <sup>a</sup>	1/11 <sup>a</sup>	14/14	1/14	13/14	0/14	14/14
Tg45	9/12	0/12	9/12	0/9	9/9	4/4	1/4	3/4	0/4	4/4

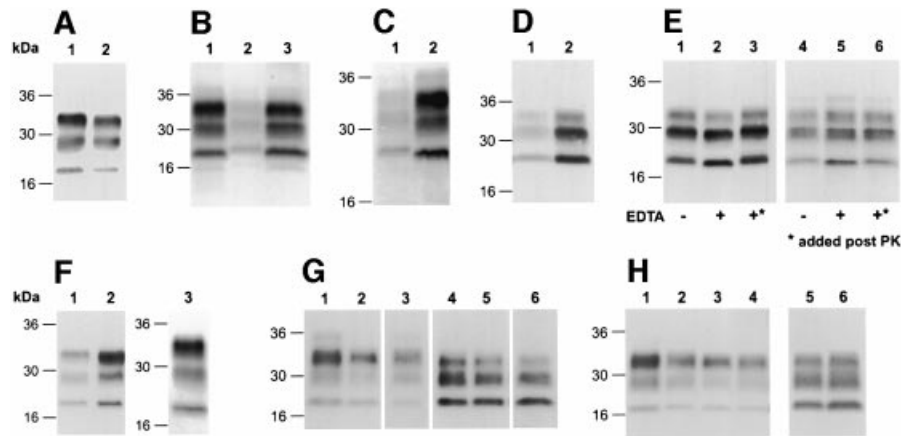
<sup>a</sup>Three brains not analysed by western blotting (one brain from a clinically infected animal was unavailable for either western blotting or immunohistochemistry; single brains from clinically affected and sub-clinically affected animals were scored positive by immunohistochemistry).



**Fig. 1.** Immunohistochemistry of cerebral cortex and hippocampal regions of transgenic mouse brain showing abnormal PrP immunoreactivity, including PrP-positive florid plaques (enlarged in insets). (A) vCJD-inoculated 129MM Tg35 mouse. (B) BSE-inoculated 129MM Tg35 mouse. (C) vCJD-inoculated 129MM Tg45 mouse. (D) BSE-inoculated 129MM Tg45 mouse. (E–G) Histological analysis showing the thalamus of a BSE-inoculated 129MM Tg35 mouse propagating type 2 human PrP<sup>Sc</sup> with widespread vacuolation (E; H&E), extensive gliosis (F; GFAP), but no specific PrP immunoreactive deposits (G; ICSM 35). Scale bar: (A–D) = 100  $\mu$ m; (E), (F) and (G) = 50  $\mu$ m.

questioning current definitions of transmission barriers, which have been conventionally assessed on the basis of occurrence of clinical disease in inoculated animals. Surprisingly, as assessed by histology, immunohistochemistry and/or the presence of human PrP<sup>Sc</sup> on western blotting of brain tissue, we found that all (13/13) vCJD-inoculated 129MM Tg35 mice, which had died apparently

of age-related causes without clinical signs of prion disease at ages typical for uninoculated or mock-inoculated mice, had pathological (Figure 1A) and/or biochemical (Figure 2A) evidence of prion infection. Only a single (1/14) (Table II) 129MM Tg35 mouse challenged with vCJD developed clinical prion disease. Excluding those animals that died soon after inoculation, which were



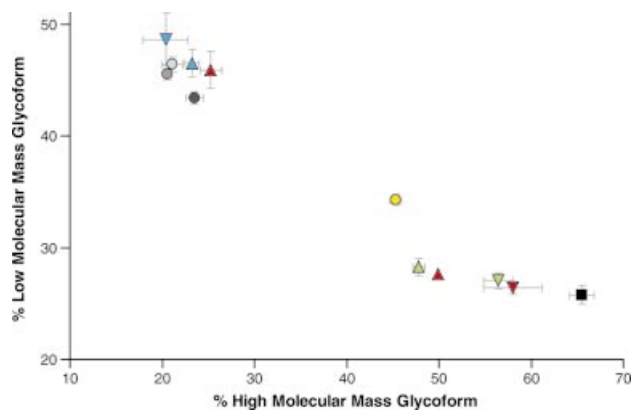
**Fig. 2.** Western blots of proteinase K (PK)-treated brain homogenates from transgenic mice, human cases of variant and sporadic CJD, and lines of wild-type mice. (A) Lane 1, vCJD; lane 2, vCJD-inoculated 129MM Tg35 mouse. (B) Lane 1, vCJD-inoculated 129MM Tg35 mouse; lane 2, BSE-inoculated 129MM Tg35 mouse propagating type 2 PrP<sup>Sc</sup>; lane 3, vCJD. (C) Lanes 1 and 2, BSE-inoculated 129MM Tg35 mouse propagating either type 2 PrP<sup>Sc</sup> (lane 1) or type 4 PrP<sup>Sc</sup> (lane 2). (D) Lane 1, BSE-inoculated 129MM Tg35 mouse propagating type 2 PrP<sup>Sc</sup>; lane 2, human sporadic CJD type 2 PrP<sup>Sc</sup> (*PRNP* genotype 129MM). (E) Lanes 1–3, human sporadic CJD type 2 PrP<sup>Sc</sup> (*PRNP* genotype 129MM); lanes 4–6, BSE-inoculated 129MM Tg35 mouse propagating type 2 PrP<sup>Sc</sup>. Samples were PK digested in the absence (lanes 1, 3, 4 and 6) or presence (lanes 2 and 5) of 25 mM EDTA. \*Following proteolysis, samples in lanes 3 and 6 were boiled in SDS sample buffer and subsequently adjusted to 25 mM EDTA before electrophoresis. (F) Transmission of vCJD and BSE to 129MM Tg45 mice. Lane 1, vCJD; lane 2, vCJD-inoculated 129MM Tg45 mouse; lane 3, BSE-inoculated 129MM Tg45 mouse. (G) Primary transmission of vCJD and BSE to wild-type inbred mice. Lane 1, BSE-inoculated FVB mouse; lane 2, vCJD-inoculated FVB mouse; lane 3, BSE-inoculated C57BL/6 mouse; lane 4, BSE-inoculated SJL mouse; lane 5, vCJD-inoculated SJL mouse; lane 6, BSE-inoculated RIIS mouse. (H) Secondary transmission of vCJD and BSE in wild-type inbred mice. Lanes 1–4, BSE was passed twice in C57BL/6 mice and then passaged in different wild-type mice: lane 1, C57BL/6 mouse; lane 2, FVB mouse; lane 3, SJL mouse; lane 4, RIIS mouse. Lanes 5 and 6, second passage of SJL-passaged BSE in further SJL mice (lane 5) or FVB mice (lane 6). Western blots were analysed by high-sensitivity ECL using biotinylated anti-PrP monoclonal antibody ICSM 35 (A–D, F–H) or 3F4 (E).

not examined further, all other mice (13/14), which died of intercurrent or age-related illness between 342 and 726 days post-inoculation without showing clinical signs of prion disease, were positive for type 4 PrP<sup>Sc</sup> in their brains. There appeared, therefore, to be a 100% infection rate of vCJD-inoculated 129MM Tg35 mice (Table I). A smaller proportion (8/49) of BSE-inoculated 129MM Tg35 mice also developed sub-clinical disease (Table I; Figure 2D). Widespread sub-clinical disease was not seen, however, in vCJD- or BSE-inoculated 129VV Tg152 mice (Hill *et al.*, 1997). Since the methods used for PrP<sup>Sc</sup> detection in the current study are more sensitive than those used in our earlier study (Hill *et al.*, 1997), we re-analysed the 129VV Tg152 mouse brains (16) for which frozen tissue remained for study, using the same methods used here, and found no PrP<sup>Sc</sup>. Sub-passage of infectivity from both 129V and 129M human PrP-expressing lines of transgenic mice will be necessary to further characterize and quantitate these transmission barriers.

#### **Transgenic mice expressing human PrP M129 develop the neuropathological features and PrP<sup>Sc</sup> type of vCJD following inoculation with BSE or vCJD prions**

Inoculation of vCJD prions into 129MM Tg35 mice resulted in clinical disease in only a single mouse, but widespread sub-clinical disease with human PrP<sup>Sc</sup> readily detectable in brain by western blot analysis. In previous transmission studies of vCJD prions to 129VV Tg152 mice, a novel type 5 PrP<sup>Sc</sup> pattern was obtained, and thought to represent a prion strain switch resulting from mismatch of the codon 129 polymorphism in inoculum and host human PrP (Hill *et al.*, 1997). A prediction of the

hypothesis that prion strain type is encoded by PrP<sup>Sc</sup> structural properties, and that the *PRNP* codon 129 polymorphism plays a key role in human prion strain propagation, is that transmission of vCJD prions (containing human PrP<sup>Sc</sup> type 4) to 129MM Tg35 mice would result in faithful propagation of type 4 PrP<sup>Sc</sup>. This was indeed what we observed: the PrP<sup>Sc</sup> type seen, as judged by PrP<sup>Sc</sup> fragment sizes (Figure 2A, compare lanes 1 and 2), was the type 4 pattern characteristic of vCJD prions in human brain. The glycoform ratio also closely resembled that of type 4 PrP<sup>Sc</sup> in human brain (Figure 3). As we have reported previously, a small difference is seen on glycoform ratios of the same prion strain propagated in mice and human brain, presumably reflecting the superimposition of species-specific glycosylation effects on the prion strain-specific pattern (Hill *et al.*, 1997). Furthermore, the neuropathological features in the vCJD-inoculated 129MM Tg35 mice were quite different from those of 129VV Tg152 mice propagating type 5 human PrP<sup>Sc</sup>, where no PrP immunoreactive plaques were seen (Hill *et al.*, 1997). Remarkably, the vCJD-inoculated 129MM Tg35 mice not only developed abundant PrP plaques, an uncommon feature of prion disease in mice, but many of these were of the 'florid' type (a central plaque core surrounded by a ring of spongiform vacuoles), which are characteristic of vCJD in humans (Will *et al.*, 1996) (Figure 1A) but rarely seen in mice. Florid plaques were first described in Icelandic scrapie and have also been described in mice infected with the 111A scrapie strain (McBride *et al.*, 1988). More recently, florid plaques have been reported in BSE-inoculated primates (Lasmézas *et al.*, 1996) and in transgenic mice expressing ovine PrP infected with sheep-passaged BSE prions (Crozet *et al.*, 2001).



**Fig. 3.** Scattergraph of proportions of protease-resistant PrP in higher molecular mass (diglycosylated) and low molecular mass (monoglycosylated) glycoforms seen in brain tissue from sporadic CJD, vCJD, BSE and in transgenic mice following challenge with CJD, vCJD and BSE. Data points are plotted as mean  $\pm$  SEM. Human cases, indicated as circles: sporadic CJD type 1 PrP<sup>Sc</sup>, light grey ( $n = 12$ ); sporadic CJD type 2 PrP<sup>Sc</sup>, mid-grey ( $n = 49$ ); sporadic CJD type 3 PrP<sup>Sc</sup>, dark grey ( $n = 22$ ); vCJD type 4 PrP<sup>Sc</sup>, yellow ( $n = 16$ ). Cattle BSE, black square ( $n = 3$ ). Transmissions to 129MM Tg35 mice, upward triangles: sporadic CJD type 1 PrP<sup>Sc</sup>-inoculated mice, blue ( $n = 7$ ); vCJD type 4 PrP<sup>Sc</sup>-inoculated mice, green ( $n = 10$ ); BSE-inoculated mice, red ( $n = 9$ ;  $n = 1$ ). Transmissions to 129MM Tg45 mice, inverted triangles: sporadic CJD type 2 PrP<sup>Sc</sup>-inoculated mice, blue ( $n = 3$ ); vCJD type 4 PrP<sup>Sc</sup>-inoculated mice, green ( $n = 4$ ); BSE-inoculated mice, red ( $n = 4$ ).

BSE prion inoculation of 129MM Tg35 mice also resulted in both clinical disease and sub-clinical infection (Tables I and II). In sharp contrast to BSE transmission to 129VV Tg152 mice, where we were unable to detect PrP<sup>Sc</sup> in brain (Hill *et al.*, 1997), PrP<sup>Sc</sup> was readily detectable in brains of clinically sick 129MM Tg35 mice and in mice not showing clinical signs of prion disease when they died at advanced age (Figures 2C and 3). In one of the eight sub-clinically affected mice, type 4 human PrP<sup>Sc</sup> was seen (Figure 2C, lane 2), indistinguishable from that seen in vCJD-inoculated 129MM Tg35 mice and in human vCJD itself. In this mouse, neuropathological features were identical to those of vCJD-inoculated mice, with abundant florid plaques as in human vCJD (Figure 1B). These data further supported the conclusion that vCJD is caused by a BSE-like prion strain. However, in all other sub-clinically affected BSE-inoculated 129MM Tg35 mice (7/8), an alternate phenotype was observed (Table II). This was also seen in all clinically affected BSE-inoculated 129MM Tg35 mice where brain was available for analysis (5/6) (Table II).

#### **Some Tg(HuPrPM129) mice develop a distinct phenotype following inoculation with BSE prions**

In 4/6 clinically affected and 6/8 sub-clinically affected BSE-inoculated 129MM Tg35 mice, a distinctive human PrP<sup>Sc</sup> type was seen with a quite different fragment size of unglycosylated PrP<sup>Sc</sup> following proteinase K digestion and a different ratio of the three glycoforms, monoglycosylated PrP<sup>Sc</sup> being most abundant (in marked contrast to type 4 PrP<sup>Sc</sup>, where diglycosylated PrP<sup>Sc</sup> predominates) (Figure 2B, C and E, compare lanes 1 and 2). Comparison with known human PrP<sup>Sc</sup> types in CJD indicated that this type corresponded, both with respect to fragment sizes and

glycoform ratio, to the type 2 PrP<sup>Sc</sup> seen in sporadic and iatrogenic CJD (Figure 2D, compare lanes 1 and 2, and Figure 3). Human PrP<sup>Sc</sup> types can also be distinguished by their metal-binding properties. Both type 1 and type 2 human PrP<sup>Sc</sup> undergo a shift in fragment size following proteinase K treatment if treated with the metal chelator EDTA (Wadsworth *et al.*, 1999). Type 3 (also seen in classical CJD) and type 4 PrP<sup>Sc</sup> do not undergo a metal-dependent shift in proteinase K cleavage site on treatment with EDTA (Wadsworth *et al.*, 1999). Treatment of BSE-inoculated 129MM Tg35 mouse brain homogenates with EDTA prior to proteinase K cleavage demonstrated that while that showing a type 4 pattern was unaltered by this treatment (data not shown), those showing a type 2 pattern underwent the expected band shift indistinguishable from type 2 PrP<sup>Sc</sup> from CJD-affected human brain (Figure 2E, compare lanes 1 and 2 with 4 and 5).

Routinely, in our transmission studies, individual mouse brains from a group are either frozen for biochemical studies or fixed for histology; in some mice, one hemisphere is frozen and the other fixed to allow both techniques on an individual mouse. Histopathological analysis on fixed tissue and biochemical analysis on frozen tissue was only available on a single animal showing type 2 PrP<sup>Sc</sup>. However, neuropathological features of this sub-clinically affected, BSE-inoculated 129MM Tg35 mouse, showing a type 2 PrP<sup>Sc</sup> pattern in the brain, were quite distinct from those with type 4 PrP<sup>Sc</sup>. There was no specific PrP immunoreactivity; in particular, there were no florid or other plaques (Figure 1G). However, widespread neuronal vacuolation (Figure 1E) and extensive gliosis (Figure 1F), consistent with spongiform encephalopathy, clearly confirm sub-clinical disease in this mouse.

While vCJD prions produce a neuropathological pattern in 129MM Tg35 mice similar to that seen in human vCJD, and the characteristic PrP<sup>Sc</sup> type of vCJD is maintained in all mice, BSE inoculation results in two distinct but highly consistent phenotypes: one indistinguishable from the vCJD transmissions, and associated with the characteristic molecular 'signature' of BSE; and a second that resembles transmission of the commonest molecular sub-type of classical CJD.

#### **vCJD and BSE transmission to a further HuPrP129M-expressing transgenic line**

We also inoculated a second transgenic line expressing HuPrPM129, generated as described for Tg35, with vCJD and BSE prions. Tg(HuPrP129M<sup>+/+</sup> Prnp<sup>0/0</sup>)-45 (129MM Tg45) mice were produced similarly to 129MM Tg35 mice, but have a level of expression of human PrP 4-fold higher than a pooled normal human brain standard (data not shown). These mice were also highly susceptible to sporadic CJD, with a 100% attack rate, extremely short and consistent incubation periods (I024: 7/7 mice developed disease with an incubation time of  $155 \pm 5$  days), and no fall in incubation period on second passage, consistent with lack of a transmission barrier to classical CJD prions. Again, as judged by clinical disease, we found that these animals were much less susceptible to vCJD and BSE. However, as seen with BSE- or vCJD-inoculated 129MM Tg35 mice, evidence of sub-clinical prion infection was seen in most clinically unaffected mice (Table II). While only 1/4 vCJD-inoculated 129MM Tg45

**Table III.** BSE and vCJD transmissions to inbred lines of mice

Inoculum	SJL/OlaHsd		RIIS/J		FVB/NHsd		C57BL/6/OlaHsd	
	Incubation time (days $\pm$ SEM)	Clinical signs	Incubation time (days $\pm$ SEM)	Clinical signs	Incubation time (days $\pm$ SEM)	Clinical signs	Incubation time (days $\pm$ SEM)	Clinical signs
BSE (I783)	196 $\pm$ 13	25/40	241 $\pm$ 15	20/29	589 $\pm$ 21	22/31	710 $\pm$ 15	6/25
vCJD (I336)	139 $\pm$ 17	6/10			342 $\pm$ 31	8/8		
vCJD (I344)	256 $\pm$ 46	5/7			402 $\pm$ 34	7/8		
vCJD (I342)	169, 169	2/11			475 $\pm$ 68	3/10		

mice developed clinical disease (at 580 days), the remaining 3/4 mice had neuropathological and biochemical evidence of prion infection. Again, in close agreement with the results from 129MM Tg35 mice, analysis of brains of vCJD-inoculated 129MM Tg45 mice consistently revealed widespread florid plaque deposition (Figure 1C) and type 4 PrP<sup>Sc</sup> (Figure 2F, lane 2 and Figure 3). Similarly, none of the BSE-inoculated 129MM Tg45 mice developed clinical signs of prion disease for >700 days, but 9/12 had sub-clinical prion infection (Table II). Neuropathological examination of BSE-inoculated 129MM Tg45 mice revealed closely similar pathological findings to that of vCJD-inoculated 129MM Tg45 mice with florid plaques (Figure 1D) and western blot analysis of brain tissue revealed type 4 PrP<sup>Sc</sup> (Figure 2F, lane 3 and Figure 3). To date, the alternate neuropathological pattern associated with type 2 PrP<sup>Sc</sup> has not been detected in BSE-inoculated 129MM Tg45 mice.

#### **vCJD and BSE transmission to various inbred lines of non-transgenic mice**

BSE prions transmit readily to wild-type mice but with prolonged and variable incubation periods. We have previously reported the PrP<sup>Sc</sup> type of both FVB and C57BL/6 mice when inoculated with BSE (Collinge *et al.*, 1996b; Hill *et al.*, 1997). As with other species naturally or experimentally infected with BSE that we have reported, a BSE-like pattern is produced with a characteristic PrP<sup>Sc</sup> fragment size and glycoform ratio. However, these transmissions involve PrP from another mammalian species of different molecular mass, such that the proteins are not directly comparable, as with transmissions of human prion disease to transgenic mice expressing only human PrP. This mouse PrP<sup>Sc</sup> pattern is, therefore, referred to as ‘diglycosylated dominant’.

In independent experiments to determine the range of incubation periods of BSE in many inbred mouse lines, as part of studies to map prion incubation time genes (Lloyd *et al.*, 2001), we have identified two inbred lines of mice in which BSE transmission is associated with the production of a distinctive PrP<sup>Sc</sup> type, with PrP<sup>Sc</sup> glycoform ratios closely similar to that of human sporadic CJD and referred to here as a ‘monoglycosylated dominant’ PrP<sup>Sc</sup> pattern. Interestingly, these lines are also associated with unusually short incubation periods for BSE (Table III). All four inbred mouse lines have the same *Prnp* coding sequence (*Prnp-a*; data not shown) and are homozygous for methionine at codon 128, the corresponding murine codon to *PRNP* codon 129.

Following inoculation with BSE prions, both FVB and C57BL/6 mice show the characteristic diglycosylated dominant PrP<sup>Sc</sup> pattern in the brain (Figures 2G, lanes 1 and 3, and 4A). However, when inoculated with the same BSE inoculum, SJL and RIIS mice exhibit a monoglycosylated dominant PrP<sup>Sc</sup> pattern (Figures 2G, lanes 4 and 6, and 4A). This PrP<sup>Sc</sup> type is stable on further passage to both SJL and FVB mice (Figures 2H, lanes 5 and 6, and 4C) and is unaffected by EDTA treatment (data not shown).

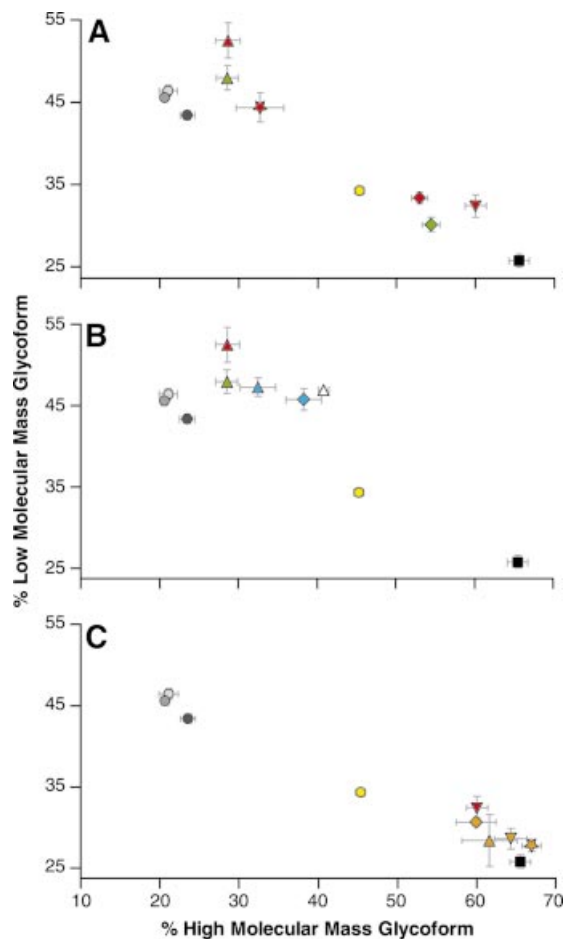
The propagation of the monoglycosylated PrP<sup>Sc</sup> glycoform pattern is established by the host in which primary passage is carried out, as both SJL and RIIS mice are capable of propagating the diglycosylated dominant PrP<sup>Sc</sup> pattern when challenged with BSE passaged twice in a C57BL/6 mouse (Figures 2H, lanes 3 and 4, and 4C).

vCJD prions behave in the same way as BSE prions in FVB mice, producing a prolonged and variable incubation period and a diglycosylated dominant PrP<sup>Sc</sup> type (Hill *et al.*, 1997) (Figures 2G, lane 2, and 4A; Table III). vCJD transmissions to SJL mice also resemble BSE transmissions to these mice. Inoculation with vCJD gives unusually short incubation times (Table III) and produces a monoglycosylated dominant PrP<sup>Sc</sup> pattern which is closely similar to that produced by BSE transmission (Figures 2G, lane 5, and 4A) and is unaffected by EDTA treatment (data not shown). To our knowledge, the PrP<sup>Sc</sup> pattern seen on BSE or vCJD transmissions to RIIS and SJL mice used in our study, or to the RIIS strain of mice routinely used for biological strain typing experiments, has not been reported previously (Bruce *et al.*, 1997).

The neuropathological features seen in inbred lines of mice inoculated with the same prion strain vary considerably, the disease patterns being host, as well as prion strain, dependent (Bruce, 1993). The neuropathology observed in SJL and RIIS mice inoculated with either BSE or vCJD showed only diffuse staining for PrP without florid or other PrP immunoreactive plaques (data not shown).

## **Discussion**

Prion propagation involves recruitment and conversion of host PrP<sup>C</sup> into PrP<sup>Sc</sup>, and the degree of primary structural similarity between inoculated PrP<sup>Sc</sup> and host PrP<sup>C</sup> is thought to be a key component of intermammalian transmission barriers (Prusiner *et al.*, 1990). It is clear, however, that prion strain type can also be crucial, as clearly demonstrated by the very distinctive transmission



**Fig. 4.** Scattergraph of proportions of protease-resistant PrP in higher molecular mass (diglycosylated) and low molecular mass (monoglycosylated) glycoforms seen in sporadic CJD, vCJD, BSE and in wild-type mice following challenge with vCJD and BSE. Data points are plotted as mean  $\pm$  SEM. (A–C) Human cases indicated as circles: sporadic CJD type 1 PrP<sup>Sc</sup>, light grey ( $n = 12$ ); sporadic CJD type 2 PrP<sup>Sc</sup>, mid-grey ( $n = 49$ ); sporadic CJD type 3 PrP<sup>Sc</sup>, dark grey ( $n = 22$ ); vCJD type-4 PrP<sup>Sc</sup>, yellow ( $n = 16$ ). Cattle BSE, black square ( $n = 3$ ). (A) Primary transmission of vCJD and BSE to wild-type mice: vCJD-inoculated FVB mice, green diamond ( $n = 19$ ); vCJD-inoculated SJL mice, green triangle ( $n = 4$ ); BSE-inoculated FVB mice, red diamond ( $n = 12$ ); BSE-inoculated SJL mice, red triangle ( $n = 7$ ); BSE-inoculated RIIS mice, red star ( $n = 4$ ); BSE-inoculated C57BL/6 mice, inverted red triangle ( $n = 3$ ). (B) Transmission of SJL-passaged BSE to further wild-type mice: SJL-passaged-BSE-inoculated FVB mice, blue diamond ( $n = 4$ ); BSE passaged twice in SJL mice, blue triangle ( $n = 3$ ); BSE passaged three times in SJL mice, open triangle ( $n = 3$ ). (C) Transmission of BSE passaged twice in C57BL/6 mice to further wild-type mice: C57BL/6-passaged BSE to FVB mice, orange diamond ( $n = 3$ ); C57BL/6-passaged BSE to SJL mice, orange triangle ( $n = 4$ ); C57BL/6-passaged BSE to RIIS mice, orange star ( $n = 3$ ); C57BL/6-passaged BSE to C57BL/6 mice, inverted orange triangle ( $n = 3$ ).

properties of sporadic CJD 129MM and vCJD 129MM prions (of identical PrP primary structure) in either 129VV Tg152 (Hill *et al.*, 1997; Collinge, 1999) or 129MM Tg35 mice. Prion strain type may also affect transmission barriers via an effect on PrP<sup>Sc</sup> tertiary structure and state of aggregation (Hill *et al.*, 1997; Collinge, 1999).

These 129MM Tg35 mice, in which human PrP<sup>Sc</sup> types can be propagated, have been used to study the BSE-to-human species barrier. The frequent presence of sub-clinical prion disease in vCJD- and BSE-inoculated

129MM Tg35 mice further argues for the need to reassess current definitions of 'species' or transmission barriers that limit prion transmission between different hosts (Hill *et al.*, 2000). Such barriers have hitherto been quantitated on the basis of either comparative end-point titrations in the two respective hosts, or by measuring the fall in incubation period between primary and subsequent passage as the prion strain adapts to the new host. Both methods rely on measurement of time to onset of a clinical syndrome. Modelling the BSE-to-human barrier in 129MM Tg35 mice would lead to the conclusion, on the basis of induced clinical disease, that a substantial barrier existed. However, it is clear that human PrP<sup>Sc</sup> propagation can be efficiently induced by inoculation with BSE or vCJD prions, suggesting a smaller barrier to infection (but not to clinical disease) than hitherto thought (Collinge *et al.*, 1995) in humans of the *PRNP* 129MM genotype. Humans infected with BSE prions, but who became asymptomatic carriers, may nevertheless pose a threat of iatrogenic transmission via medical and surgical procedures. Alternatively, it is possible that the lifespan of the laboratory mouse is insufficient to allow expression of clinical disease in most inoculated mice, whereas a higher proportion of infected humans might survive the incubation period to develop clinical signs of disease. Serial passage studies and titration of prions in these mice are in progress to study this further.

These studies further strengthen the evidence that vCJD is caused by a BSE-like prion strain. Also, remarkably, the key neuropathological hallmark of vCJD, the presence of abundant florid PrP plaques, can be recapitulated on BSE or vCJD transmission to these mice. However, the most surprising aspect of the studies was the finding that an alternate pattern of disease can be induced in 129MM Tg35 mice from primary transmission of BSE, with a molecular phenotype indistinguishable from that of a sub-type of sporadic CJD. This finding has important potential implications as it raises the possibility that some humans infected with BSE prions may develop a clinical disease indistinguishable from classical CJD associated with type 2 PrP<sup>Sc</sup>. This is, in our experience, the commonest molecular sub-type of sporadic CJD. In this regard, it is of interest that the reported incidence of sporadic CJD has risen in the UK since the 1970s (Cousens *et al.*, 1997). This has been attributed to improved case ascertainment, particularly as much of the rise is reported from elderly patients and similar rises in incidence were noted in other European countries without reported BSE (Will *et al.*, 1998). However, it is now clear that BSE is present in many European countries, albeit at a much lower incidence than was seen in the UK. While improved ascertainment is likely to be a major factor in this rise, that some of these additional cases may be related to BSE exposure cannot be ruled out. It is of interest in this regard that a 2-fold increase in the reported incidence of sporadic CJD in 2001 has recently been reported for Switzerland, a country that had the highest incidence of cattle BSE in continental Europe between 1990 and 2002 (Glatzel *et al.*, 2002). No epidemiological case-control studies with stratification of CJD cases by molecular sub-type have yet been reported. It will be important to review the incidence of sporadic CJD associated with PrP<sup>Sc</sup> type 2 and other molecular sub-types in both BSE-affected and unaffected countries in the

light of these findings. If human BSE prion infection can result in propagation of type 2 PrP<sup>Sc</sup>, it would be expected that such cases would be indistinguishable on clinical, pathological and molecular criteria from classical CJD. It may also be expected that such prions would behave biologically like those isolated from humans with sporadic CJD with type 2 PrP<sup>Sc</sup>. The transmission properties of prions associated with type 2 PrP<sup>Sc</sup> from BSE-inoculated 129MM Tg35 mice are being investigated by serial passage.

We consider these data inconsistent with contamination of some of the 129MM Tg35 mice with sporadic CJD prions. These transmission studies were performed according to rigorous biosafety protocols for preparation of inocula and both the inoculation and care of mice, which are all uniquely identified by sub-cutaneous transponders. However, crucially, the same BSE inocula have been used on 129VV Tg152 and 129MM Tg45 mice, which are highly sensitive to sporadic CJD but in which such transmissions producing type 2 PrP<sup>Sc</sup> were not observed. Furthermore, in an independent experiment, separate inbred lines of wild-type mice, which are highly resistant to sporadic CJD prions, also propagated two distinctive PrP<sup>Sc</sup> types on challenge with either BSE or vCJD. No evidence of spontaneous prion disease or PrP<sup>Sc</sup> has been seen in groups of uninoculated or mock-inoculated aged 129MM Tg35 mice.

While distinctive prion isolates have been derived from BSE passage in mice previously (designated 301C and 301V), these, in contrast to the data presented here, are propagated in mice expressing different prion proteins (Bruce *et al.*, 1994). It is unclear whether our findings indicate the existence of more than one prion strain in individual cattle with BSE, with selection and preferential replication of distinct strains by different hosts, or that 'mutation' of a unitary BSE strain occurs in some types of host. Western blot analysis of single BSE isolates has not shown evidence of the presence of a proportion of monoglycosylated dominant PrP<sup>Sc</sup> type in addition to the diglycosylated dominant pattern (data not shown). Extensive strain typing of large numbers of individual BSE-infected cattle either by biological or molecular methods has not been reported.

Presumably, the different genetic background of the different inbred mouse lines is crucial in determining which prion strain propagates on BSE inoculation. The transgenic mice described here have a mixed genetic background with contributions from FVB/N, C57BL/6 and 129Sv inbred lines; each mouse will therefore have a different genetic background. This may explain the differing response of individual 129MM Tg35 mice, and the difference between 129MM Tg35 and 129MM Tg45 mice, which are, like all transgenic lines, populations derived from single founders. Indeed, the consistent distinctive strain propagation in FVB and C57BL/6 versus SJL and RIIS lines may allow mapping of genes relevant to strain selection and propagation, and these studies are in progress.

That different prion strains can be consistently isolated in different inbred mouse lines challenged with BSE prions argues that other species exposed to BSE may develop prion diseases that are not recognizable as being caused by the BSE strain by either biological or molecular

strain typing methods. As with 129MM Tg35 mice, the prions replicating in such transmissions may be indistinguishable from naturally occurring prion strains. It remains of considerable concern whether BSE has transmitted to, and is being maintained in, European sheep flocks. Given the diversity of sheep breeds affected by scrapie, it has to be considered that some sheep might have become infected with BSE, but propagated a distinctive strain type indistinguishable from those of natural sheep scrapie.

## Materials and methods

### Generation of transgenic mice

The 759 bp human PrP ORF was amplified by PCR with *pfu* polymerase from genomic DNA encoding methionine at codon 129, using forward primer 5'-GTCGACCAGTCATTATGGCGAACCTT-3' and reverse primer 5'-CTCGAGAAGACCTTCCTCATCCACT-3'. Restriction sites *Sall* and *XhoI* (underlined) were introduced in the forward and reverse primers, respectively, for cloning. The sequence was confirmed and ligated into the cosmid vector CosSHaTet (Scott *et al.*, 1989). Microinjection of the purified DNA was carried out according to the standard protocol into single cell eggs of a strain of mice (FVB/N × Sv129 × C57BL/6) in which the murine PrP gene has been ablated (Bueler *et al.*, 1992). Genotyping was performed by PCR, and PrP expression levels estimated by western blot analysis.

### Transmission studies

Strict biosafety protocols were followed. Inocula were prepared, using disposable equipment for each inoculum, in a microbiological containment level 3 laboratory and inoculations performed within a class 1 microbiological safety cabinet. Five separate BSE inocula, each derived from single natural BSE-affected cow brainstems (I060, I062, I064, I066, I783), and a separate inoculum prepared from a pool of five natural BSE brainstems (I038) were studied. Aliquots of these (except I783) have been used in previously published studies (Collinge *et al.*, 1995; Hill *et al.*, 1997). BSE tissues were collected under strict aseptic conditions using sterile instrumentation, specifically for transmission studies, by the UK Central Veterinary Laboratory [now the Veterinary Laboratories Agency (VLA)]. The BSE pool homogenate was titrated into RIII wild-type mice at VLA with a resultant titre of 10<sup>3.3</sup> mouse intracerebral LD<sub>50</sub> units/g of tissue. Sporadic and vCJD inocula were prepared from brain tissue from neuropathologically confirmed cases. Consent for use of tissues for research was obtained. The genotype of each transgenic mouse was confirmed by PCR of tail DNA prior to inclusion and all mice were uniquely identified by sub-cutaneous transponders. RIIS/J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and SJL/OlaHsd mice were obtained from Harlan UK Ltd (Bicester, UK). Disposable cages were used, and all cage lids and water bottles were also uniquely identified by transponder and remained with each cage of mice throughout the incubation period. Care of the mice was according to institutional guidelines. Both transgenic and wild-type mice were anaesthetized with a mixture of halothane and O<sub>2</sub>, and intracerebrally inoculated into the right parietal lobe with 30 µl of a 1% brain homogenate prepared in PBS. Thereafter, all mice were examined daily for clinical signs of prion disease. Mice were killed if they were exhibiting any signs of distress or once a diagnosis of prion disease was established. Criteria for clinical diagnosis of scrapie in mice were as described previously (Carlson *et al.*, 1986).

### Neuropathology and immunohistochemistry

Mice were killed using CO<sub>2</sub> asphyxiation, brains fixed in 10% buffered formal-saline and then immersed in 98% formic acid for 1 h and paraffin wax embedded. Serial sections of 4 µm were pre-treated with autoclaving, formic acid and 4 M guanidine thiocyanate. Abnormal PrP accumulation was examined using an anti-PrP monoclonal IgG antibody raised against recombinant human PrP (ICSM 35; A.Khalili-Shirazi, unpublished data), followed by a biotinylated anti-mouse IgG secondary antibody and an avidin-biotin-horseradish peroxidase conjugate before development with 3',3'-diaminobenzidine tetrachloride as the chromogen. The extent of gliosis was determined by GFAP (Dako) staining. Slides were pre-treated by heating in the microwave (900 W) in citrate buffer pH 6.0 for 25 min, followed by overnight incubation (1:1000). Biotinylated swine anti-rabbit

immunoglobulins and avidin–biotin complex were applied as described above. Harris haematoxylin was used as the counterstain. Appropriate controls were used throughout.

### Western blotting

Preparation of brain homogenates (10% w/v in PBS), proteinase K digestion (50 or 100 µg of proteinase K for 1 h at 37°C) and subsequent western blotting were performed as described previously (Wadsworth *et al.*, 2001). For primary screening of both transgenic and wild-type mouse brain homogenates, blots were probed with a biotinylated anti-PrP monoclonal antibody which recognizes both human and mouse PrP (biotinylated-ICSM 35) in conjunction with an avidin–biotin–alkaline phosphatase conjugate (Dako) and development in chemiluminescent substrate (CDP-Star; Tropix Inc.). Primary screening of brain homogenates was performed blind to sample identity.

### Quantitation and analysis of PrP glycoforms

Western blotting was performed as above but using different primary and secondary detection reagents. For transgenic mice expressing human PrP, blots were incubated with anti-PrP monoclonal antibody 3F4 (Kasczak *et al.*, 1987), whereas for wild-type mice expressing mouse PrP, blots were incubated with anti-PrP monoclonal antibody 6H4 (Prionics, Switzerland), followed by incubation with goat anti-mouse IgG–alkaline phosphatase conjugate (Sigma) and development in chemi-fluorescent substrate (AttoPhos; Promega) and visualization on a Storm 840 PhosphorImager (Molecular Dynamics). Quantitation of PrP<sup>Sc</sup> glycoforms was performed using ImageQuant software (Molecular Dynamics).

### Acknowledgements

We thank G.Mallinson for biotinylation of antibodies, S.Brandner for neuropathological opinion, R.Bond and team for animal care, and R.Young for preparation of figures. We specially thank all patients and their families for generously consenting to use of human tissues in this research, and the UK neuropathologists who have kindly helped in providing these tissues. We thank R.Bradley, D.Matthews, S.A.C.Hawkins and colleagues at the UK VLA for providing BSE tissues. We thank C.Weissmann for critical review of the manuscript. This work was supported by the Wellcome Trust, Medical Research Council and the European Commission.

### References

Baker,H.E., Poulter,M., Crow,T.J., Frith,C.D., Lofthouse,R., Ridley, R.M. and Collinge,J. (1991) Aminoacid polymorphism in human prion protein and age at death in inherited prion disease. *Lancet*, **337**, 1286.

Bessen,R.A. and Marsh,R.F. (1992) Biochemical and physical properties of the prion protein from two strains of the transmissible mink encephalopathy agent. *J. Virol.*, **66**, 2096–2101.

Bessen,R.A. and Marsh,R.F. (1994) Distinct PrP properties suggest the molecular basis of strain variation in transmissible mink encephalopathy. *J. Virol.*, **68**, 7859–7868.

Bruce,M.E. (1993) Scrapie strain variation and mutation. *Br. Med. Bull.*, **49**, 822–838.

Bruce,M., Chree,A., McConnell,I., Foster,J., Pearson,G. and Fraser,H. (1994) Transmission of bovine spongiform encephalopathy and scrapie to mice: strain variation and the species barrier. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **343**, 405–411.

Bruce,M.E. *et al.* (1997) Transmissions to mice indicate that 'new variant' CJD is caused by the BSE agent. *Nature*, **389**, 498–501.

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

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

Collinge,J. (1999) Variant Creutzfeldt–Jakob disease. *Lancet*, **354**, 317–323.

Collinge,J., Palmer,M.S. and Dryden,A.J. (1991) Genetic predisposition to iatrogenic Creutzfeldt–Jakob disease. *Lancet*, **337**, 1441–1442.

Collinge,J. *et al.* (1995) Unaltered susceptibility to BSE in transgenic mice expressing human prion protein. *Nature*, **378**, 779–783.

Collinge,J., Beck,J., Campbell,T., Estibeiro,K. and Will,R.G. (1996a)

Prion protein gene analysis in new variant cases of Creutzfeldt–Jakob disease. *Lancet*, **348**, 56.

Collinge,J., Sidle,K.C.L., Meads,J., Ironside,J. and Hill,A.F. (1996b) Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. *Nature*, **383**, 685–690.

Cousens,S.N., Zeidler,M., Esmonde,T.F., De Silva,R., Wilesmith,J.W., Smith,P.G. and Will,R.G. (1997) Sporadic Creutzfeldt–Jakob disease in the United Kingdom: analysis of epidemiological surveillance data for 1970–96. *BMJ*, **315**, 389–395.

Crozet,C., Bencsik,A., Flamant,F., Lezmi,S., Samarut,J. and Baron,T. (2001) Florid plaques in ovine PrP transgenic mice infected with an experimental ovine BSE. *EMBO rep.*, **2**, 952–956.

Glatzel,M., Rogivue,C., Ghani,A., Streffer,J., Amsler,L. and Aguzzi,A. (2002) Incidence of Creutzfeldt–Jakob disease in Switzerland. *Lancet*, **360**, 139–141.

Hill,A.F., Desbruslais,M., Joiner,S., Sidle,K.C.L., Gowland,I., Doey,L., Lantos,P. and Collinge,J. (1997) The same prion strain causes vCJD and BSE. *Nature*, **389**, 448–450.

Hill,A.F., Joiner,S., Linehan,J., Desbruslais,M., Lantos,P.L. and Collinge,J. (2000) Species barrier independent prion replication in apparently resistant species. *Proc. Natl Acad. Sci. USA*, **97**, 10248–10253.

Kasczak,R.J., Rubenstein,R., Merz,P.A., Tonna DeMasi,M., Fersko,R., Carp,R.I., Wisniewski,H.M. and Diringer,H. (1987) Mouse polyclonal and monoclonal antibody to scrapie-associated fibril proteins. *J. Virol.*, **61**, 3688–3693.

Lasmézas,C.I., Deslys,J.-P., Demaimay,R., Adjou,K.T., Lamoury,F., Dormont,D., Robain,O., Ironside,J. and Hauw,J.-J. (1996) BSE transmission to macaques. *Nature*, **381**, 743–744.

Lloyd,S.E., Onwuazor,O.N., Beck,J.A., Mallinson,G., Farrall,M., Targonski,P., Collinge,J. and Fisher,E.M.C. (2001) Identification of multiple quantitative trait loci linked to prion disease incubation period in mice. *Proc. Natl Acad. Sci. USA*, **98**, 6279–6283.

McBride,P.A., Bruce,M.E. and Fraser,H. (1988) Immunostaining of scrapie cerebral amyloid plaques with antisera raised to scrapie-associated fibrils (SAF). *Neuropathol. Appl. Neurobiol.*, **14**, 325–336.

Palmer,M.S., Dryden,A.J., Hughes,J.T. and Collinge,J. (1991) Homo zygous prion protein genotype predisposes to sporadic Creutzfeldt–Jakob disease. *Nature*, **352**, 340–342.

Prusiner,S.B. *et al.* (1990) Transgenic studies implicate interactions between homologous PrP isoforms in scrapie prion replication. *Cell*, **63**, 673–686.

Race,R., Raines,A., Raymond,G.J., Caughey,B. and Chesebro,B. (2001) Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt–Jakob disease in humans. *J. Virol.*, **75**, 10106–10112.

Scott,M. *et al.* (1989) Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. *Cell*, **59**, 847–857.

Telling,G.C. *et al.* (1996) Evidence for the conformation of the pathologic isoform of the prion protein enciphering and propagating prion diversity. *Science*, **274**, 2079–2082.

Wadsworth,J.D.F., Hill,A.F., Joiner,S., Jackson,G.S., Clarke,A.R. and Collinge,J. (1999) Strain-specific prion-protein conformation determined by metal ions. *Nat. Cell Biol.*, **1**, 55–59.

Wadsworth,J.D.F., Joiner,S., Hill,A.F., Campbell,T.A., Desbruslais,M., Luthert,P.J. and Collinge,J. (2001) Tissue distribution of protease resistant prion protein in variant CJD using a highly sensitive immuno-blotting assay. *Lancet*, **358**, 171–180.

Will,R.G. *et al.* (1996) A new variant of Creutzfeldt–Jakob disease in the UK. *Lancet*, **347**, 921–925.

Will,R.G. *et al.* (1998) Descriptive epidemiology of Creutzfeldt–Jakob disease in six European countries, 1993–1995. *Ann. Neurol.*, **43**, 763–767.

Zeidler,M., Stewart,G., Cousens,S.N., Estebeiro,K. and Will,R.G. (1997) Codon 129 genotype and new variant CJD. *Lancet*, **350**, 668.

Received August 1, 2002; revised September 24, 2002;  
accepted October 17, 2002