Journal of General Virology (2000), **81**, 1615–1620. Printed in Great Britain

Characterization of the murine BSE infectious agent

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Bovine spongiform encephalopathy (BSE) is a prion-associated disease where the infectious agent is thought to be a host-encoded protein with a protease-resistant conformation (PrP^{Sc}). Here, data are presented on the solubilization of purified murine BSE material, using guanidine–HCl as a denaturing agent. This treatment led to loss of infectivity, which was partially recovered on renaturation after dialysis to remove the chaotropic agent. The renatured product was then fractionated on an isopycnic sucrose-density gradient and the fractions were analysed for the presence of PrP^{Sc}, nucleic acids and infectivity. It was found that the major part of PrP^{Sc} ($> 90\%$) and the endogenous nucleic acids did not contribute towards the formation of infectious particles on renaturation. Infectivity was distributed in the top three, low-density fractions. Among these, the presence of considerable infectivity in the fraction of lowest density, with barely detectable PrP^{Sc}, is of particular interest.

Introduction

Scrapie, Creutzfeldt–Jakob disease (CJD) and bovine spongiform encephalopathy (BSE) are slow, progressive neurological disorders belonging to the group of transmissible spongiform encephalopathies (TSEs) that are caused by infectious entities termed prions. These diseases lead to the lethal decline of cognitive and motor functions. As early as 1966, Alper and colleagues had suggested, from the anomalous resistance of scrapie agent to ionizing irradiation, that its size was too small for a viral genome and that it behaved more like a protein of the order of 100 kDa (Alper *et al.*, 1966). In 1982, Prusiner (1982) proposed that the scrapie agent was not a virus but a proteinaceous infectious agent, which he called a 'prion', thus starting a worldwide controversy. In the years that followed, detection of nucleic acids in highly purified prion preparations prompted scientists to undertake direct and quantitative nucleic acid analysis on the size and number of nucleic acid molecules present. One study demonstrated that nucleic acids of virus origin with significant molecular mass co-purified with infectivity (Akowitz *et al.*, 1994), whereas another study claimed that only nucleic acids smaller than 80 nucleotides (Kellings *et al.*, 1992) could be part of the infectious agent and, therefore, the infectious agent could not be a virus. In the meantime, the prion hypothesis, claiming that a protein was the predominant component of the agent, was supported by the formulation of the 'protein-only' hypothesis, which stated that the prion protein alone represents the infectious agent. According to this model, either the cellular prion (PrP^{C}) molecules spontaneously underwent transition under certain conditions or these were transformed into the pathogenic isoform by a catalytic reaction triggered by the latter, leading to the propagation of the pathogen itself.

It has been shown that denaturation of PrP^{Sc} aggregates *in vitro* leads to the final loss of infectivity (Manuelidis *et al.*, 1995; Riesner *et al.*, 1996). The same does not necessarily apply in the case of a report by Safar *et al.* (1993), where conformational transitions did not affect infectivity. However, a model for the transformation of PrP^{C} into infectious PrP^{Sc} aggregates would be the ultimate proof of prion propagation. Several approaches have been reported in the literature, but so far none has achieved such a transformation conclusively with

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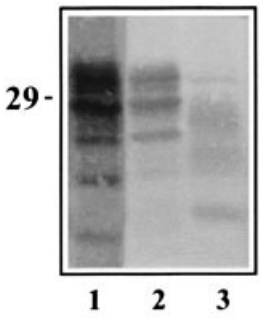

Fig. 1. Western blot analysis of the murine BSE fraction p215-MN. Lanes: 1, gold staining for total protein; 2, PrP-specific staining with the polyclonal rabbit antibody 78295; 3, PrP-specific staining following treatment with proteinase K. Each lane contains 100 mg brain tissue equivalent.

measurable infectivity (Gasset *et al.*, 1993; Kocisko *et al.*, 1994; Kaneko *et al.*, 1995; Riesner *et al.*, 1996). Moreover, qualitative and conformational studies of the infectious material are complicated by the limited solubility of prions. So far, attempts to solubilize scrapie infectivity have not been successful (Prusiner *et al.*, 1980; Millson & Manning, 1979). Interestingly, the very property of insolubility of the TSE infectious agents is now used for the enrichment of infectivity, together with the introduction of proteinase K digestion in the purification protocols, thus giving rise to the PrP27–30 fraction (Prusiner *et al.*, 1982). Even though this fraction is infectious, its infectivity when separated on SDS–PAGE (Brown *et al.*, 1990) has not been confirmed (Prusiner *et al.*, 1993).

Attempts to renature scrapie infectivity from guanidine (Gdn)–thiocyanide- or urea-treated samples were unsuccessful (Prusiner *et al.*, 1993). There is considerable loss of infectivity (99·5 %) in the soluble fraction of hamster CJD infectious agent upon denaturation with 2·5 M Gdn–HCl (Manuelidis *et al.*, 1995). It has also been reported that, on renaturation, PrP^{Sc} from the 263K strain of hamster scrapie that had been treated with 3 M Gdn–HCl regained resistance to proteinase K (Kocisko *et al.*, 1994; Kaneko *et al.*, 1995); however, as shown by subsequent investigators, this may not be the only factor governing infectivity (Hill *et al.*, 1999).

In the light of these findings, we have looked at the possibility of reconstructing the mouse-passaged BSE infectious agent after denaturation with Gdn–HCl and investigated the properties of the newly acquired product, including infectivity.

Methods

■ **Purification of the infectious material.** Brain tissue from BSE-infected mice was obtained frozen from the Central Veterinary Laboratory (Weybridge, UK). The procedures used for partial purification of the prion infectious agent have been described previously in detail (Sklaviadis *et al.*, 1989). In brief, frozen material was homogenized (10% w/v in 25 mM Tris–HCl, pH 7·6, 10% Sarkosyl and 5 mM PMSF) and spun at 25 000 ***g*** for 20 min at 20 °C. The supernatant was centrifuged again at 215 000 ***g*** for 2 h at 20 °C. The pellet, p215, was washed for 2 h at room temperature in 25 mM Tris–HCl, pH 7·6, containing 0·05 % Sarkosyl and then treated with 50 U micrococcal nuclease (MN) per g tissue equivalent in 20 mM Tris–HCl, pH 8·0, 4 mM $CaCl_2$ for 24 h at 37 °C under mild agitation in order to digest all accessible endogenous nucleotides. Another aliquot of MN was added, followed by further incubation for 1 h, and the reaction was terminated by the addition of a final concentration of 5 mM EGTA. After centrifugation at 13 000 ***g*** for 30 min, the pellet was washed twice with 25 mM Tris–HCl, pH 7·6. The washed pellet was designated as p215-MN. For digestion with proteinase K, 0·5 g brain equivalent of p215-MN was resuspended in 25 mM Tris–HCl, pH 7·6, and treated with 5 μg/ml proteinase K for 1 h at 37 °C with mild agitation. The digestion was terminated by adding a cocktail of 1 mM PMSF, 1 μM pepstatin, 1 μg/ml aprotinin and 1 μg/ml leupeptin (final concentrations) and spun at 13 000 ***g*** for 30 min. The pellet, designated as p215-PK, was washed and used for denaturation experiments. Unless stated, all experiments were done with p215-MN.

■ **Denaturation, renaturation and isopycnic fractionation.** For denaturation, 1 g wet tissue equivalent of p215-MN was resuspended in 340 μl of a solution containing 2·5 M Gdn–HCl in 25 mM Tris–HCl, pH 8·9, 0·05 % Sarkosyl and 0·5 mM PMSF and bath-sonicated five times (30 min each) with alternate vortexing (45 min each). The samples were then centrifuged at 13 000 ***g*** for 30 min. The pellet, designated as pG, was kept for further use, while the supernatant, sG, was dialysed exhaustively at room temperature against 25 mM Tris–HCl, pH 7·6, 1 mM PMSF and 0·05 % Sarkosyl in a Gibco BRL Microdialysis system with a membrane cut-off of 12–14 kDa. A linear, 30–86 % (w/v) sucrose gradient (3 ml) was poured over a cushion of 500 μl 86 % (w/v) sucrose and the dialysed material (dsG) in 800 μl dialysis buffer, equivalent to 2 g tissue, was loaded on the sucrose gradient for isopycnic fractionation. Denaturation, dialysis and isopycnic fractionation took place at room temperature in the presence of 1 mM PMSF. The samples were spun at 220 000 ***g*** for 41 h at 25 °C. At the end of the run, eight fractions of 500 μl each were collected carefully from the gradient and kept frozen. Blank sucrose gradients were also run in order to estimate the density of sucrose in each fraction by refractive index measurements (Index Instruments).

All fractions, including those from the sucrose gradient, were divided into three aliquots for the estimation of PrP^{Sc}, nucleic acid analysis and bioassays.

■ **Estimation of PrP^{Sc}.** Proteins were separated on 13 % SDS–PAGE and blotted onto a PVDF membrane and stained with AuroDye Forte (Amersham Life Science) for the estimation of total protein. For PrP^{Sc}-specific staining, the membranes were blocked (1 h, room temperature) in 3 % BSA, 1 % Ficoll and 1 % polyvinyl pyrrolidone and incubated with a polyclonal anti-PrP primary antibody (78295; Kascsak *et al.*, 1997), a generous gift of R. Kascsak, diluted 1:5000 in the blocking solution, for 1 h at room temperature, washed three times for 10 min in 100 ml PBS, 0·1 % Tween and incubated further with a goat anti-rabbit secondary antibody conjugated with alkaline phosphatase (1:3000). NBT and BCIP were used as substrates to visualize the proteins. Quantification of PrP^{Sc} on PVDF membranes (34–19 kDa bands) was performed by densitometry (ScanPack version 3.0, Biometra Biomedizinische Analytik). Proteins from the sucrose-density fractions were concentrated by precipitating with 10 vols ethanol before immunoblotting.

■ **Analysis of nucleic acids.** Nucleic acids were isolated from the second portion of each fraction. The procedure that was employed has been reported previously in detail (Sklaviadis *et al.*, 1990; Akowitz *et al.*, 1994). In brief, nucleic acids were extracted with phenol–chloroform,

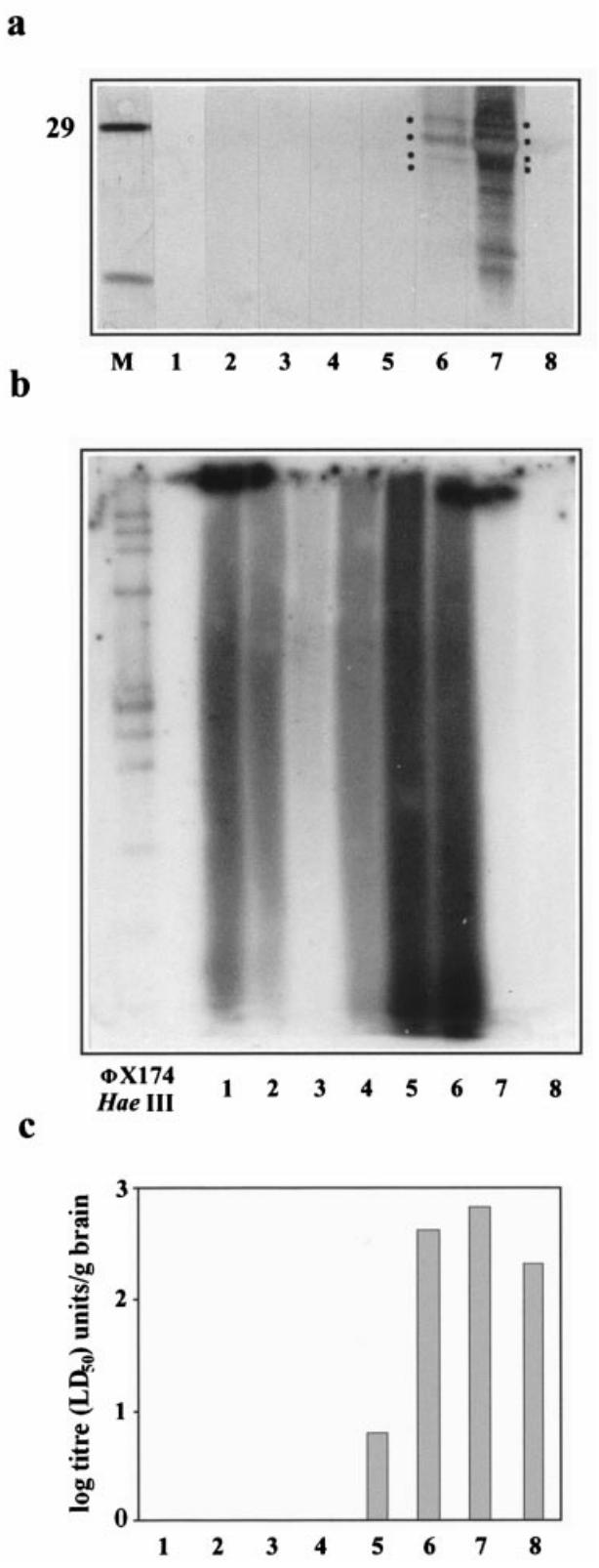

Fig. 2. Characterization of the fractions generated on a 30–86% linear sucrose-density gradient. Lanes 1–8 show fractions 1–8 from the gradient (densities in g/cm^3: 1·30, 1·29, 1·25, 1·21, 1·17, 1·11, 1·06 and 1·03). (*a*) Immunoblot of sucrose gradient fractions detected with the polyclonal rabbit antibody 78295. Each lane represents 2 g brain tissue equivalent. M, Molecular mass marker. Dots indicate PrP-specific bands that were used for densitometry analyses and comparison among the fractions. (*b*) Autoradiograph of ^{32}P-end-labelled nucleic acids that were isolated from the sucrose gradient fractions. ϕX174 *Hae*III restriction fragments were used as markers. (*c*) Infectivity results presented as $\log_{10}$ LD_{50} titres per g brain tissue equivalent. Twenty mice of IM strain were included in each group. The value for each group was obtained from the mean incubation period (±SD; Table 1) for that group and was based on a standard dose response curve for the 301V strain of mouse BSE in VM mice (same genotype as in IM mice used in this study), as determined by Spearman–Karber analysis.

precipitated with ethanol and end-labelled with ^{32}P by standard methods. After further precipitation with ethanol and separation on 1% agarose gels, the nucleic acids were analysed by densitometry (ScanPack version 3.0) on autoradiographs.

■ **Bioassays.** The third portion from each fraction was suspended in normal saline and used for bioassays in 3-week-old IM (*Sinc*^{p7}) mice, in groups of 20 per fraction. Each mouse received the inoculum through two routes, 20 µl intra-cranially and 100 µl intra-peritoneally (Bruce *et al.*, 1994). Mice in the control group were inoculated intra-cranially with normal saline. Mice were scored from day 90 post-inoculation for early clinical and terminal signs of disease over a period of up to 400 days. Infectious units were calculated from mean incubation time for each group, based on a titration curve obtained from mice of the same genotype infected with the mouse BSE strain 301V (D. Taylor, Institute of Animal Health, Neuropathogenesis Unit, Edinburgh, UK, personal communication).

Results

Extraction of PrP^{Sc}-enriched material and dispersion of prion aggregates

High enrichment for PrP^{Sc} was achieved in the p215 fraction. This was retained after treatment with MN in p215-MN, the major preparation used in this study (Fig. 1, lanes 1 and 2).

Subsequent to its treatment with 2·5 M Gdn–HCl, a major part of p215-MN was collected as soluble supernatant (Manuelidis *et al.*, 1995), sG, while a very small portion remained in the form of a pellet, pG. Exhaustive dialysis of sG to remove Gdn–HCl generated dsG, which was then analysed on an isopycnic sucrose gradient for further characterization.

Composition of the sucrose gradient fractions

The sucrose-density gradient provided eight fractions, with densities ranging from 1·30 to 1·03 g/cm^3. PrP^{Sc} was found to be concentrated mainly in fractions 6, 7 and 8, with densities of 1·11, 1·09 and 1·03 g/cm^3, respectively. Most of it moved into fractions 6 and 7 and only traces of the protein were seen in fraction 8 (Fig. 2*a*, lanes 6–8).

In contrast to the PrP^{Sc} distribution, nucleic acids were found clustered in high-density fractions 1 and 2 and fractions 4, 5 and 6 (Fig. 2*b*). Small amounts were noticed in fraction 3 and fractions 7 and 8 were devoid of detectable nucleic acids. Nucleic acids were found associated with PrP^{Sc} only in fraction 6 (Fig. 2*a*, *b*; lanes 6). Nucleic acids in the high-density

Table 1. Infectivity assay of fractions used in this study

The various inocula are indicated as: p215, precipitated infectious material; p215-MN, MN-treated material; sG, supernatant after Gdn–HCl treatment; pG, pellet after Gdn–HCl treatment; dsG, dialysed sG; fr1–fr8, sucrose fractions 1–8; PK, proteinase K-treated p215-MN.

Inoculum	Dead animals*	Incubation time (days) (mean ± SD)	Infectious units (per g brain tissue)	Log titre (log_{10} LD_{50} units per g brain tissue)
p215	20/20	134 ± 3	638000	5·8
p215-MN	20/20	141 ± 5	270000	5·4
sG	0/20	–	–	–
pG	6/20	240 ± 47	86	1·9
dsG	19/20	186 ± 14	3200	3·5
fr1	0/20	–	–	–
fr2	0/20	–	–	–
fr3	0/20	–	–	–
fr4	0/20	–	–	–
fr5	6/20	329 ± 48	6	0·8
fr6	13/20	264 ± 49	411	2·6
fr7	19/20	208 ± 20	680	2·8
fr8	16/20	264 ± 72	218	2·3
PK	20/20	150 ± 21	34000	4·5

* Number of dead animals with clinical and pathological symptoms per group of 20 IM mice.

fractions must be 'naked', while those in fractions 4 and 5 are likely to be associated with other macromolecules in the form of protein–nucleic acid complexes.

Bioassays

Data on infectivity in the various fractions (Table 1) showed that p215 itself was highly infectious (5·8 log_{10} LD_{50} units), with no considerable loss of infectivity in the p215-MN fraction (5·4 log_{10} LD_{50} units). Following exposure of p215-MN to Gdn–HCl, the pG fraction exhibited low but measurable infectivity (1·9 log_{10} LD_{50} units), while the corresponding soluble sG fraction was not infectious. Partial infectivity (3·5 log_{10} LD_{50} units, two log units less than that of the pre-denatured material) was recovered in dsG, however, the dialysed supernatant.

Only fractions 6 (2·6 log_{10} LD_{50} units), 7 (2·8 log_{10} LD_{50} units) and 8 (2·3 log_{10} LD_{50} units) from the sucrose-density gradient were infectious (Tables 1 and 2, Fig. 2*c*). Infectivity that was detected in fraction 5 (Fig. 2*c*) had to be from residual material from the fraction(s) above because, in addition to the negligible infectivity (0·8 log_{10} LD_{50} units) that this fraction showed, only six of 20 mice were infected, with an incubation period of 329 ± 48 days, way beyond those presented by other infectious fractions. For these reasons, it was decided to not consider this fraction for further analysis. Comparison of the infectious fractions in terms of percentages (Table 2) showed that fraction 6, where nearly all of the nucleic acids were found, contained 0·6% of the total PrP^{Sc} and 31% of the total infectivity. Fraction 7, with no detectable nucleic acids, contained 99·2% of PrP^{Sc} and 52% of total infectivity, while fraction 8, with no detectable nucleic acids and only 0·2% of PrP^{Sc}, showed 17% of infectivity. Obviously, the majority of the nucleic acids did not contribute towards infectivity, because both fractions 7 and 8 were infectious without detectable nucleic acids. The important observation is that fractions 6 and 8 were infective but had only meagre amounts of PrP^{Sc} (0·6 and 0·2%, respectively), thus making it clear that infectivity was not associated directly with abundance of PrP^{Sc}. The absence of infectivity in fractions 1–4 precludes any cross-contamination among the fractions due to handling (Table 1). These fractions also served as internal negative controls.

Table 2. Analysis of the three low-density fractions from the sucrose gradient

Parameter	fr6	fr7	fr8
PrP^{Sc} (%)*	0·6	99·2	0·2
Nucleic acids (%)	100	ND	ND
Infectivity (%)	31	52	17

* Percentages of PrP^{Sc} were calculated by densitometry from bands marked with dots in Fig. 2(*a*), as explained in Methods.
ND, Not detectable by the detection protocol applied (less than picogram quantities).

As shown above, the analysis of dsG showed that, even though they were not linked to infectivity in the fractions,

endogenous nucleic acids were definitely associated with renatured PrP^{Sc}. This association was, however, found by using gel-retardation electrophoresis to be not specific (data not shown).

Discussion

The infectious nature of the pathogenic agent that causes prion diseases is still far from certain. In this study, an attempt has been made for the first time to define the properties of infectivity, in the mouse BSE agent. In order to achieve this, the infectious agent that was enriched in the form of the p215-MN fraction was denatured chaotropically under defined conditions in the presence of 2·5 M Gdn–HCl (Manuelidis *et al*., 1995; Kocisko *et al*., 1996) and renatured by gradual removal of the chaotropic agent. The renatured material was then checked for its properties in terms of composition and infectivity.

It was observed that, on treatment with 2·5 M Gdn–HCl, there was complete loss of infectivity in the supernatant, and only residual infectivity was detected in the pellet. These results are similar to those of Manuelidis *et al*. (1995), where a loss of 99·5% of the starting infectivity, under similar conditions, was reported with hamster CJD preparations. In other studies, where the 263K strain of hamster scrapie has been denatured with 2·5 M Gdn–HCl, partial disaggregation of PrP^{Sc} has been noticed (Kocisko *et al*., 1996) and, in a more recent work (Caughey *et al*., 1997), a $>$ 1000-fold reduction in infectivity has been observed without complete loss in the supernatant. None of the above workers, however, attempted to recover infectivity from the denatured preparations. Interestingly, in our hands, we have regained a portion of the infectivity after the removal of Gdn–HCl by dialysis (Fig. 2*c*; dsG). The differences between the above findings and those of our own study might be attributable to the different strains used in the studies. On the other hand, similarities that the results of this work present to those of Manuelidis *et al*. (1995), where hamster CJD was used, may be explained by a common and an increased sensitivity of the two agents to chaotropic agents. The implications of such a similarity on the passing of the BSE agent to humans to cause variant CJD remains to be seen.

On further analysis with an isopycnic sucrose-density gradient, it was discovered that, in the renatured fraction, only a portion of the total PrP^{Sc} was responsible for infectivity (Table 2). It has been reported earlier that overexpression of PrP^{Sc} was not a criterion for the onset of prion diseases in $PrP^{+/-}$ mice (Bueler *et al*., 1994). It has also been shown that the BSE infectious agent can be transmitted to mice in the absence of detectable prion protein (Somerville & Dunn, 1996; Lasmezas *et al*., 1997). Therefore, in accordance with the findings of other workers and using experiments *in vitro*, the present work confirms that, during infection, pathogenicity of the infectious agent (murine BSE in this study) is dependent on the correct conformation *in vivo* and not on the bulk of PrP^{Sc} in the inoculum. A role of other macromolecules, not detected under the present conditions, as part of the infectious agent cannot be excluded. However, as shown by gold staining (Fig. 1, lane 1), the relative purity of the starting preparation in terms of contamination with other proteins precludes such a possibility. In order to use a more defined starting material, we repeated our experiments with p215-MN after digestion with proteinase K (Fig. 1, lane 3), but the preparation was not easily denatured with 2·5 M Gdn–HCl. Denaturation with 5 M Gdn–HCl to overcome this difficulty led to unrecoverable loss of infectivity (data not shown). It was also observed that only a part of the total infectivity in the starting material was recovered at the end of the denaturation–renaturation steps (Table 1). This could be due to only partial recovery of the correct conformation that may contribute structurally towards infectivity.

Although the nature of the prion itself is proteinaceous (Prusiner, 1982), its ability to associate with nucleic acids (Akowitz *et al*., 1994) and also with RNA aptamers (Weiss *et al*., 1997) has been demonstrated earlier. Our own experiments have demonstrated the capability of PrP^{Sc}-enriched preparations to bind nucleic acids *in vitro* (unpublished data). In this study, dsG was found to be associated with nucleic acids that were structurally protected, since dsG was derived from the p215-MN (micrococcal nuclease-treated) preparation. Even though their presence has been demonstrated for the first time in mouse BSE preparations, these endogenous nucleic acids did not seem to contribute towards infectivity and were bound non-specifically to the infectious complexes.

It is likely that novel states of the murine BSE agent were generated under the denaturing–renaturing conditions used in this study. Such unfolded/refolded stages of the agent may be extremely useful as templates for studying *in vitro* protein–protein interactions of PrP^{Sc} with its own cellular isoform or even other macromolecules from the same or different species, since these steps are known to precede/accompany transmission of prion diseases.

This work was supported by the AIR 3-CT93-0859 programme of the European Community Specific Programme for Research. The authors wish to thank Dr R. Kascsak, Institute for Basic Research in Developmental Disabilities, Staten Island, NY, USA, for providing the rabbit polyclonal antibody 78295, Dr Cindy Humphrey-Panagiotidis for her comments on the manuscript, Mr Vasilis Karageorgis for help with the scanning and Mr I. Dexter and Mr R. Green for their technical assistance.

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Received 11 January 2000; Accepted 22 February 2000