res and infectivity (2, 14, 15). However, other studies have demonstrated the transmission of disease from infected animals that appear to lack significant levels of PrP-res (16–19). In such cases it has been suggested that a PK-sensitive form of PrP^{Sc} ($sPrP^{Sc}$) may represent the infectious agent (20–22). Hence it is possible that infectivity may be associated with a specific isoform of abnormal PrP. The identification of this specific conformer is imperative for the future of TSE diagnosis. If present, large amounts of PrP^{Sc} may be a clear indication of the presence of infectivity in a tissue sample. However, if TSE infectivity does not always associate with high levels of PrP^{Sc}, current diagnostic methods may fail to identify all animals with TSE disease and may not provide a realistic estimate of the level of infectivity in an infected tissue. For the purposes of this study, PrP^{Sc} is used to define all abnormal forms of PrP, whereas PrP-res specifically defines PK-resistant PrP, and $sPrP^{Sc}$ defines PK-sensitive forms of PrP^{Sc}.

We have previously identified two mouse models of TSE disease (18, 19) that indicate that the association between PrP-res and infectivity is not as straightforward as predicted by the prion hypothesis. Unlike wild-type controls, transgenic mice homozygous for a targeted mutation at amino acid 101 (proline to leucine) in endogenous murine PrP (101LL) develop clinical TSE disease following inoculation with hamster 263K scrapie or human Gerstmann Sträussler Scheinker (GSS) P102L disease (patient shown to contain vacuolar pathology and PrP-res at post-mortem) (18, 19). Pathological analysis of brain tissue from these mice (101LL/GSS and 101LL/263K) showed TSE-associated vacuolization, and the disease could be further transmitted to 101LL mice with short incubation times of 100–160 days (18, 19). Such incubation times were indicative of a high titer of infectivity in the 101LL/GSS and 101LL/263K tissues, yet analysis by immunoblot revealed that most animals contained extremely low levels of PrP-res, and several contained no detectable PrP-res at all (18, 19). However, the presence of high titers of infectivity cannot be proven by a short disease incubation time. To establish the true relationship between PrP^{Sc} and infectivity we have now performed detailed and quantitative analyses of the disease in these mice. The ID_{50} (dilution at which 50% of the animals become infected) and titer of infectivity in several 101LL/GSS- and 101LL/263K-infected brains have been established by bioassay. Corresponding levels of PrP-res in the same tissues have also been established semi-quantitatively by immunoblot. These analyses have shown no relationship between infectivity titer and PrP-res level. Moreover no other disease-associated forms of PrP were detectable in these tissues. Thus within our model system there is a clear dissociation between titer of infectivity and level of PrP^{Sc}.

EXPERIMENTAL PROCEDURES

Transgenic Mouse Lines and Tissues—Inbred gene-targeted transgenic mouse line 101LL and the corresponding inbred 129/Ola wild-type control line have been described previously (18). 101LL/GSS tissues were produced by inoculation of 101LL transgenic mice with 1% brain homogenate prepared from the occipital cortex of a GSS P102L brain showing numerous multicentric plaques and abundant PrP-res by immunoblot. The individual was methionine 129 homozygous with a confirmed proline to leucine mutation at codon 102.[5] 101LL/263K tissues were produced by inoculation of 101LL transgenic mice with 1% brain homogenate from a 263K-infected hamster. Control tissues were produced by ME7 inoculation of 129/Ola wild-type mice and 101LL transgenic mice.

Preparation of Inocula—Separate inocula were prepared from the brains of two 101LL/GSS- and three 101LL/263K-infected mice with terminal TSE disease, which had been shown by immunohistochemical (IHC) analysis to contain extremely low levels of PrP deposition. Inocula were also prepared from brains of one wild-type and one 101LL mouse with terminal ME7 scrapie as controls. A 10% homogenate of each sample was prepared in sterile saline prior to use as an inoculum. This inoculum was then used to produce a series of 10-fold dilutions from 10^{-2} to 10^{-9} in sterile saline. Each dilution (20 μl) was inoculated intracerebrally under anesthesia into groups of 101LL mice for 101LL/ME7, 101LL/GSS, and 101LL/263K tissues, or wild-type 129/Ola mice for Wt/ME7 tissue. All experimental protocols were submitted to the Local Ethical Review Committee for approval before mice were inoculated. All experiments were performed under license and in accordance with the UK Home Office Regulations (Animals (Scientific Procedures) Act of 1986).

Scoring of Clinical TSE Disease—The presence of clinical TSE disease was assessed as described previously (23). Animals were scored for clinical disease without reference to the genotype of the mouse. Genotypes were confirmed for each animal by PCR analysis of tail DNA at the end of the experiment. Incubation times were calculated as the interval between inoculation and cull due to terminal TSE disease. Mice were killed by cervical dislocation at the terminal stage of disease, at termination of the experiment (between 500–700 days), or for welfare reasons due to intercurrent illness. The proportion of mice showing positive vacuolar pathology was calculated for each group, and the ID_{50} (dilution at which 50% of the mice became infected) was determined using the Karber method (24). This value was used to calculate the number of infectious units per gram wet weight of tissue (IU/g).

Genotyping of Mouse Tail DNA—A 2- to 3-cm portion of tail was removed post-mortem from each mouse. DNA was prepared, and the PrP genotype of each mouse was confirmed as described previously (18).

Immunoblot Analysis and Quantification of PrP-res—For immunoblot analysis, residual inocula (10% saline homogenate) were mixed with an equal volume of 2× Nonidet P-40 buffer (2% Nonidet P-40, 1% sodium deoxycholate, 300 mM NaCl, 100 mM Tris/HCl, pH 7.5) and further homogenized in a microcentrifuge tube using 20–30 strokes with a pre-cooled centrifuge tube pestle (Anachem). The homogenate was centrifuged at 11,000 × *g* for 10 min at 10 °C to remove cellular debris, and the supernatant stored in 50-μl aliquots at −70 °C. For quantification of PrP-res levels in each tissue, homogenates were digested with 20 μg/ml PK at 37 °C for 1 h. Digested homogenates were diluted to 1%, and 2-fold serial dilutions were prepared using PK-treated normal brain homogenate as

[5] J. W. Ironside and M. W. Head, personal communication.

the diluent to keep overall protein concentrations constant. Diluted samples were mixed with sample loading buffer and sample reducing agent (Invitrogen) and loaded across two 12% Tris/glycine polyacrylamide gels (Invitrogen) at concentrations ranging from 1 mg/ml to 3.9 μg/ml (200 μg to 0.8 μg of wet weight tissue equivalent). 50 ng of recombinant PrP was loaded onto each gel as an internal control. After separation, proteins were transferred onto a polyvinylidene difluoride membrane by electroblotting, and PrP was detected with mAb 8H4 (West Dura ECL substrate, Pierce). Monoclonal antibody 7A12 and polyclonal antibody 1B3 were also used to confirm the low PrP-res levels in 101LL/GSS and 101LL/263K tissues. Images were captured on both x-ray film and by a Kodak Digital Image Station 440. Experiments were repeated in duplicate or triplicate depending on sample availability.

Digital images of each gel were analyzed using Kodak ID software, and PrP-res levels were expressed as pixel intensities. Samples were normalized across the two blots and quantified using the recombinant PrP controls as standards. Each value was multiplied by the dilution factor, and an average was taken for all samples run per tissue to determine the level of PrP-res per gram wet weight brain tissue in each model. This value, combined with the titer of TSE infectivity measured in each tissue (IU/g) was used to calculate the number of molecules of PrP-res per infectious unit for each tissue as in Equations 1–3.

$$\text{Number of PrP-res molecules per g of tissue} = n \quad \text{(Eq. 1)}$$

$$n = [\text{PrP-res per g/Avagadro's number } (6.02 \times 10^{23})]/\text{molecular weight PrP } (30{,}000) \quad \text{(Eq. 2)}$$

$$\text{Number of molecules PrP-res per infectious unit} = n/\text{titer (IU/g)} \quad \text{(Eq. 3)}$$

Measurement of Alternative Forms of PrP—The PK resistance of PrP in all samples was analyzed by digestion with a range of PK concentrations. Individual 9-μl aliquots of each 5% Nonidet P-40 brain homogenate were incubated at 37 °C for 1 h with PK concentrations ranging from 1 to 20 μg/ml. The reaction was terminated by addition of phenylmethylsulfonyl fluoride to 1 mM, and samples were analyzed by SDS-PAGE and immunoblotting as described above.

For "cold PK" digestion, samples (10% homogenate) were incubated with 250 μg/ml PK on ice for 1 h. Digestion was stopped by the addition of phenylmethylsulfonyl fluoride to 1 mM. Samples were de-glycosylated with peptide *N*-glycosidase F (New England Biolabs) following the manufacturer's instructions and analyzed by SDS-PAGE and immunoblotting.

CDI Analysis—Samples were analyzed for the presence of PrP^{Sc} using conformation-dependent immunoassay (CDI) as described by Safar *et al.* (20). Briefly, abnormal PrP was precipitated from brain homogenates of 101LL/GSS, 101LL/263K, and 101LL/ME7 infected mice and uninfected 101LL mice using sodium phosphotungstate, and pellets were resuspended in either distilled water or 4 M guanidine hydrochloride to produce native and denatured samples. 4 M guanidine hydrochloride samples were further heat-denatured at 80 °C for 6 min. Samples were added to 96-well plates coated with mAb FH11, and PrP levels were detected using europium-labeled mAb 7A12 and a Victor 2 ELISA plate reader (PerkinElmer Life Sciences). The ratio of denatured to native signal (d/n) was calculated for each tissue to determine the presence of PrP^{Sc}.

Immunoprecipitation of PrP^{Sc}—Laterally bisected brain halves from 101LL transgenic mice were homogenized at 10% (w/v) in Tris-buffered saline and diluted to reach a concentration of 5% (w/v) in Tris-buffered saline containing 1% Triton. Homogenates were sonicated for three pulses of 4 s and clarified by centrifugation at 400 × *g* for 10 min at 4 °C. Phenylmethylsulfonyl fluoride was added to all samples to a concentration of 2 mM. Each sample was analyzed by dot blot to estimate the total PrP content. Briefly, brain homogenates were serially diluted (1:1) in Tris-buffered saline containing 1% Triton then denatured in Tris-SDS sample buffer at 100 °C for 5 min. Equivalent amounts of each sample were then deposited on a nitrocellulose membrane and left until dry. The membrane was probed with mAb 6H4 (Prionics) and a horseradish peroxidase-labeled anti-mouse secondary antibody (Pierce). The resulting signals were compared semi-quantitatively. These data were used to ensure equal PrP input into each individual immunoprecipitation (IP) reaction. For each IP reaction, the motif grafted antibodies or control antibodies were incubated at 10 μg/ml final concentration for 2 h at room temperature in a reaction mixture with 1% Triton. Rabbit anti-human antibodies (Jackson) coupled to magnetic Dynabeads (Dynal) were used to capture the PrP-specific antibodies as described (25, 26). Immunoblot membranes were probed with mAb 6H4 and developed using the ECL femtomolar kit (Pierce).

RESULTS

101LL Mice Infected with 263K and GSS P102L Show Little PrP Deposition in Brain—Brain tissue from 101LL transgenic mice, which showed TSE clinical signs and TSE-associated vacuolar pathology following inoculation with hamster 263K scrapie or human GSS P102L (18, 19), was screened for PrP deposition by IHC using anti-PrP mAb 6H4. As previously demonstrated, 101LL/GSS- and 101LL/263K-infected mice had low levels of PrP deposition in the brain, despite having confirmed TSE disease. Three 101LL/263K- and two 101LL/GSS-infected tissues, which showed extremely low PrP deposition in the brain, were selected for further analysis by bioassay (Fig. 1 and Table 1). In each case, PrP deposition was restricted to the thalamus and, in most cases, was only visible as small grainy deposits under high power microscopy (Fig. 1, *F–H*). Low or undetectable levels of PrP-res in each brain homogenate were confirmed by immunoblot following PK treatment of residual inoculum (Fig. 2).

High Levels of Infectivity Can Be Measured by Bioassay of 101LL/GSS and 101LL/263K Brain Tissue—Although short incubation times in mice can be indicative of high levels of TSE infectivity in an inoculum, the actual level can only be determined by establishing the ID_{50} (dilution at which 50% of the animals become infected) for the inoculum. Infectivity titers were therefore established for the five selected tissues: 101LL/263K(a), 101LL/263K(b), 101LL/263K(c), 101LL/GSS(d), and 101LL/GSS(e) (Table 1). It was considered extremely important in these experiments that, as far as possible, a single brain be

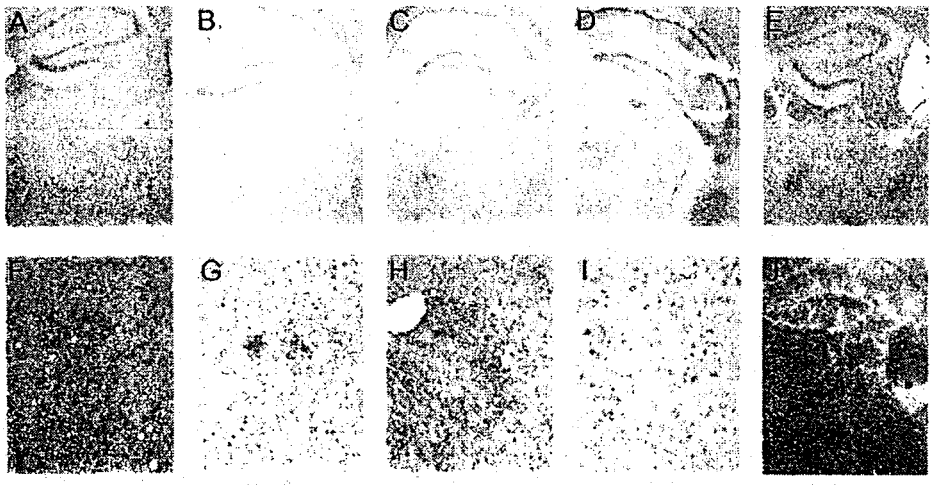

FIGURE 1. **Low levels of PrP deposition in 101LL/GSS- and 101LL/263K-infected brain.** Immunohistochemistry was performed on sections of brain from 101LL/263K- and 101LL/GSS-infected mice using mAb 6H4 to determine the levels of PrP deposition. ME7-infected control mouse brain was stained as control (*J*). Five brains shown in *A–E* (3× 101LL/263K and 2× 101LL/GSS) showing very low levels of deposition were selected for further analysis to quantify the levels of TSE infectivity and PrP^{Sc} in each tissue. Very low levels of PrP deposition were observed in brain tissue, which varied between each individual mouse brain. Deposition was mainly observed in the thalamus (*F–H*). Thalamus of an uninfected 101LL mouse is shown for background comparison (*I*). *A–E* and *J*, 4× magnification; *F–I*, 20× magnification. *A*, 101LL/263K(a); *B*, 101LL/263K(b); *C*, 101LL/263K(c); *D*, 101LL/GSS(d); *E*, 101LL/GSS(e); *F*, thalamus of 101LL/263K(a); *G*, thalamus of 101LL/263K(c); *H*, thalamus of 101LL/GSS(d); *I*, thalamus of 303-day-old uninfected 101LL mouse; and *J*, Wt/ME7 control.

TABLE 1

Tissues selected for analysis

Details of clinical disease and vacuolar pathology in the five tissues selected for analysis. All mice showed positive clinical and vacuolar signs of TSE disease and low levels of PrP deposition.

Tissue used for titration	Clinical TSE	Vacuolar pathology	PrP deposition[a]	Incubation period	
				Primary[b]	Secondary[c]
				days ± S.E.	
101LL/263K(a)	Positive	Positive	+	385	109 ± 2
101LL/263K(b)	Positive	Positive	+/−	464	129 ± 2
101LL/263K(c)	Positive	Positive	+/−	534	262 ± 4
101LL/GSS(d)	Positive	Positive	+	259	154 ± 3
101LL/GSS(e)	Positive	Positive	+/−	252	123 ± 1

[a] Scoring of PrP deposition: +++, high; ++, medium; +, low; +/−, very small grainy deposits.
[b] Incubation time of each individual mouse on primary transmission of either 263K or P102L GSS.
[c] Incubation time of 101LL mice inoculated with 1% brain homogenate from each specific 101LL/263K- or 101LL/GSS-infected tissue. Transmission of disease on subpass to 101LL mice was 100% in each case.

FIGURE 2. **Low or undetectable levels of PrP-res in 101LL/GSS- and 101LL/263K-infected brain.** Residual inoculum from the tissues selected for ID_{50} bioassay were analyzed by immunoblot following PK treatment to detect PrP-res. *Lanes 2, 4, 6, 8, 10,* and *12*, digested with PK at 20 μg/ml for 1 h at 37 °C; *lanes 1, 3, 5, 7, 9,* and *11*, no PK control; *lanes 1* and *2*, uninfected Wt 129/Ola mouse; *lanes 3* and *4*, 101LL/263K(a); *lanes 5* and *6*, 101LL/263K(b); *lanes 7* and *8*, 101LL/263K(c); *lanes 9* and *10*, 101LL/GSS(d); and *lanes 11* and *12*, 101LL/GSS(e). All samples were loaded at 10 mg/ml (w/v) wet weight tissue (200 μg per lane). Blots probed with mAb 8H4.

used for each series of procedures (ID_{50} determination, PK digestion, IHC, etc.). This allowed direct correlation to be made between the level of infectivity and PrP-res in each individual brain and avoided any variation that may occur between tissues, as is often observed on a primary transmission. Moreover this approach avoided the necessity of carrying out large numbers of titration experiments, which would have been both impractical and ethically unacceptable. Inocula were prepared from each individual tissue as 10% sterile saline homogenates and used to produce a series of 10-fold dilutions (10^{-2} to 10^{-9}) for inoculation. Wild-type control 129/Ola and transgenic 101LL mouse brains infected with the well characterized mouse scrapie strain ME7 (Wt/ME7 and 101LL/ME7, respectively) (18) were also assayed as controls. The seven samples were inoculated intracerebrally into groups of 129/Ola mice for Wt/ME7, and transgenic 101LL mice for all other samples. The percentage of mice that developed TSE pathology was calculated for each group in each dilution series, and the ID_{50} was determined using the Karber calculation (24). The numbers of infectious units per gram tissue (IU/g) for each individual mouse brain are shown in Table 2. Assuming a ±0.5 log error for each titer (24), all 101LL/GSS and 101LL/263K samples produced titers of infectivity ranging from ~10^7 to 10^9 IU/g. The highest titer ($10^{9.8}$) was identified in 101LL/GSS(d), however a titer of $10^{8.7}$ was also identified in 101LL/263K(a). Both of these brains showed low levels of PrP deposition by IHC, but titers were higher than that measured in control Wt/ME7 brain ($10^{8.5}$), which showed significantly more PrP deposition by IHC (Fig. 1). Titers in the other three tissues were similar ($10^{7.2}$ to $10^{7.5}$) and confirmed a high level of infectivity in the presence of extremely low or undetectable PrP deposition in the brain (Figs. 1 and 2). The results of the ID_{50} determination therefore prove the presence of high levels of infectivity in 101LL transgenic mice infected with P102L GSS or hamster 263K.

Little or No PrP-res Is Detected in Highly Infectious Tissue—IHC using anti-PrP monoclonal and polyclonal antibodies found little or no PrP deposition in brain tissue of 101LL/263K and 101LL/GSS infected mice (Fig. 1, and data not shown). However, IHC does not distinguish between different forms of PrP, therefore direct measurement of brain PrP-res levels was undertaken to determine the amount of PrP-res associated with titer of infectivity in each brain, listed in Table 1. Residual inoculum from each bioassay was mixed with detergent buffer and digested with PK (Fig. 2), and a 2-fold serial dilution from 1 mg/ml to 3.9 μg/ml (wet weight brain tissue) was analyzed by immunoblotting with mAb 8H4 (27). Recombinant PrP was loaded on each gel at 50 ng as an internal control. For the ME7-infected tissues, the limit of PrP-res detection was 15.6 μg/ml for Wt/ME7 homogenate and 31.3 μg/ml for 101LL/ME7 homogenate. Hence the same agent produced ~2-fold less PrP-

TABLE 2

Comparison of titer of infectivity and PrP-res level

PrP-res levels, quantified relative to recombinant PrP from digital immunoblot images, and infectivity titer, measured by ID_{50} bioassay. Detection limit of the immunoblot system was estimated to be equivalent to 25 μg of PrP-res/g wet weight brain.

Model	PrP-res	PrP-res	Titer[a]
	μg/g tissue[b]	% of ME7	IU/g tissue
Wt/ME7	1994	100	$10^{8.5}$
101LL/ME7	1040	52	$10^{7.8}$
101LL/263K(a)	498	25	$10^{8.7}$
101LL/263K(b)	<25	<1.3	$10^{7.3}$
101LL/263K(c)	<25	<1.3	$10^{7.5}$
101LL/GSS(d)	<25	<1.3	$10^{9.8}$
101LL/GSS(e)	<25	<1.3	$10^{7.2}$

[a] Titer of infectivity per gram of brain tissue as calculated from ID_{50} bioassay in mice using the Karber calculation.

[b] The actual amount of PrP-res quantified from the blots (0.5–2 mg/g) is higher than would be predicted for mouse tissue and may reflect the use of recombinant PrP for calibration, because this does not possess any post-translational modifications and may therefore display altered antibody affinity. However, this internal control acts to normalize each blot and, therefore, ensures that the relative proportions of PrP-res between each model are real, despite possible errors in the absolute quantification.

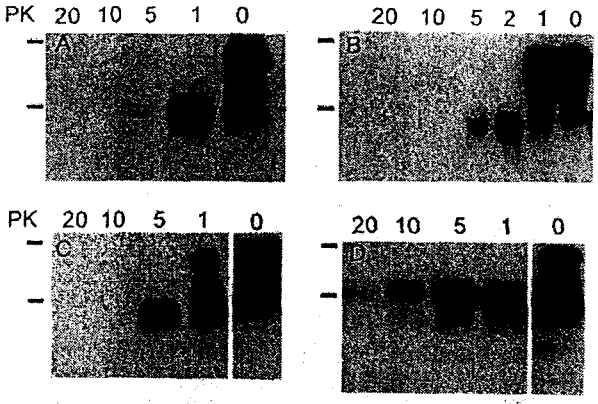

FIGURE 3. **PK resistance of PrP in 101LL/GSS and 101LL/263K brain tissue.** Brain homogenates in Nonidet P-40 lysis buffer were digested with varying concentrations of proteinase K at 37 °C for 1 h. Samples were subjected to SDS-PAGE and immunoblotting to determine the PK sensitivity of the PrP present in 101LL/GSS and 101LL/263K tissue. Representative images show: *A*, uninfected 101LL control mouse brain; *B*, uninfected Wt 129/Ola control mouse brain; *C*, 101LL/263K(b) mouse brain; and *D*, 101LL/263K(a) mouse brain. The PK concentration used for digestion is shown *above* each *lane* (micrograms/ml). Blots were probed with mAb 8H4. *Bars* indicate molecular mass markers of 36 and 30 kDa.

res in the 101LL transgenic mice compared with wild-type mice, although this was associated with a 0.7 log drop in titer (Table 2). In 101LL/263K(a) the limit of PrP-res detection was 62.5 μg/ml brain homogenate, which was approximately half the level in 101LL/ME7 and one quarter the level in Wt/ME7. For all other samples, no PrP-res was detectable in even the most concentrated (1 mg/ml) sample examined (Table 2, Fig. 2, and supplemental Fig. S1*A*). Digital imaging of immunoblots and quantitation of PrP-res relative to recombinant PrP control allowed the calculation of PrP^{Sc} concentration (mean PrP-res grams per gram wet weight of tissue) in each sample (Table 2). The level of sensitivity for the immunoblot, determined using recombinant PrP, was 5–10 ng, therefore the level of PrP-res in samples that showed no PK-resistant material must be below this threshold. Previous studies have shown that PrP-res from other well characterized rodent scrapie strains with titers ranging from $10^{5.5}$ to 10^{9} can be easily identified on immunoblot of 1% brain homogenate following PK treatment (supplemental Fig. S1*B*). These data would suggest that tissue containing titers of 10^{7} to 10^{9} IU/g should contain levels of PrP-res, which can be easily identified by immunoblot. However, for 101LL/GSS- and 101LL/263K-infected tissue this was clearly not the case. Although we cannot eliminate the possibility that PrP-res was indeed present below the threshold level of the immunoblot, a poor correlation between the level of infectivity and the amount of PrP-res in the brain is nevertheless clearly established. To confirm that the failure to detect PrP-res on these immunoblots was not simply a consequence of the loss of the monoclonal antibody epitope (8H4) duplicate blots were also probed with a second monoclonal antibody (7A12) and a polyclonal antibody (1B3), which detects multiple epitopes in PrP. These results confirmed the low PrP-res levels in 101LL/GSS and 101LL/263K tissues (data not shown). Although the combination of monoclonal and polyclonal antibodies used to examine these tissues makes it unlikely that a form of PrP-res exists that has not been detected in our immunoassays, this possibility has not been totally excluded and we continue to investigate these tissues with new antibodies.

Are Alternative Forms of PrP Associated with Infectivity?—Although PrP-res was present at low or undetectable levels in tissues from 101LL/GSS- and 101LL/263K-infected mice, it is possible that forms of PrP other than PrP-res may be infectious (28). Alternative forms of PrP such as transmembrane PrP (29, 30), cytoplasmic PrP (31, 32), and PrP with amino acid insertions or deletions (33–36) have been linked with disease. In addition, a PK-sensitive variant of PrP^{Sc}, $sPrP^{Sc}$, has been recently described (20–22) that may represent an intermediate in the refolding of PrP^{C} to PrP^{Sc} during the disease process and could therefore be associated with infectivity. To test whether $sPrP^{Sc}$ may account for the dissociation between PrP-res and infectivity in 101LL/263K and 101LL/GSS tissues we examined the protease resistance of PrP in such brains by digesting with a range of PK concentrations from 1 μg/ml to 20 μg/ml. Homogenates from Wt/ME7, 101LL/ME7, and uninfected 101LL and 129/Ola mice were also treated with varying PK concentrations as controls. Digestion was stopped by the addition of phenylmethylsulfonyl fluoride to 1 mM, and samples were analyzed by immunoblot (Fig. 3). In the positive controls (Wt/ME7 and 101LL/ME7) PrP-res was evident in all dilutions, with the PK-resistant core still visible after treatment with 20 μg/ml PK (data not shown). PrP in the uninfected controls was found to be sensitive to PK concentrations >5 μg/ml, and produced mildly PK-resistant fragments at PK concentrations of 2–5 μg/ml under the digestion conditions used here (Fig. 3). PrP in the 263K-infected 101LL brains showed variable PK resistance, in agreement with the level of PrP-res detectable in each homogenate. Thus, 101LL/263K(a) showed PrP-res at 20 μg/ml, but 101LL/263K(b) and -(c) showed a similar pattern of PK resistance to uninfected mice (Fig. 3). In addition, samples from both 101LL/GSS(d) and 101LL/GSS(e) showed a PK-sensitivity pattern identical to that of uninfected 101LL mice (data not shown).

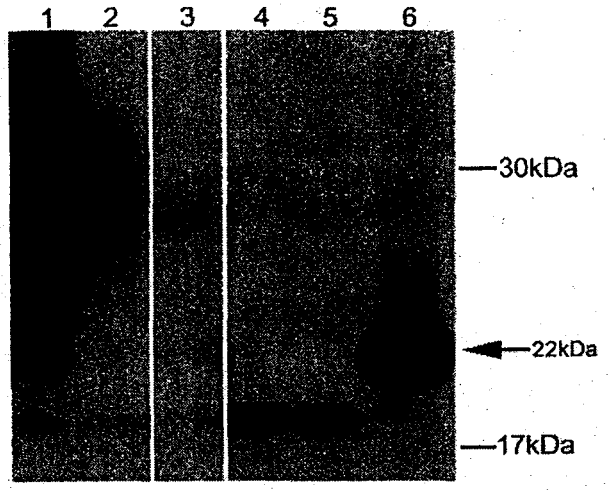

FIGURE 4. **Cold PK treatment of tissues from high titer/low PrP-res models.** 101LL/GSS and 101LL/263K tissues taken from the same transmission experiments as those shown in Table 1 were subjected to cold PK digestion on ice. Uninfected and Wt/ME7-infected brains were also digested as controls. *Lane 1*, undigested 101LL/GSS brain homogenate; *lane 2*, 101LL/263K(g); *lane 3*, 101LL/GSS(f); *lane 4*, 101LL uninfected control; *lane 5*, Wt129/Ola uninfected control; *lane 6*, Wt/ME7 infected control. *Lanes 2–6* were treated with 250 μg/ml PK on ice for 1 h and de-glycosylated with peptide *N*-glycosidase F. ME7 control was loaded at ~25% of the concentration of *lanes 2–5* to allow comparison. The blot was probed with mAb 7A12. The image has been cropped from a single blot to remove lanes with samples that are not relevant to this figure.

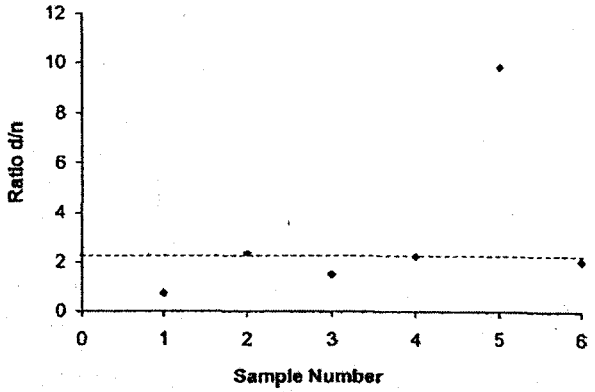

FIGURE 5. **CDI analysis of 101LL/GSS and 101LL/263K brain homogenate.** Samples of 101LL/GSS brain homogenate, 101LL/263K homogenate, and uninfected or ME7-infected controls were analyzed for the presence of PrP^{Sc} using a CDI. Samples were precipitated with sodium phosphotungstate, and pellets were resuspended in either distilled water or 4 M guanidinium-HCl to provide native and denatured samples. These were analyzed in a sandwich ELISA using mAb FH11 as capture and mAb 7A12 as detector. Ratio of denatured to native (*d/n*) signal plotted to show presence of PrP^{Sc}. *Sample 1*, 101LL/GSS(j); *sample 2*, 101LL/GSS(k); *sample 3*, 101LL/263K(m); *sample 4*, 101LL/263K(n); *sample 5*, 101LL/ME7; and *sample 6*, uninfected 101LL mouse. All samples were assayed in duplicate. *Dotted line* indicates cut-off value, which was calculated as the d/n ratio of the uninfected 101LL plus 10%.

The presence of $sPrP^{Sc}$ in brain tissue has also been demonstrated by performing cold PK digestion, *i.e.* PK digestion on ice (21, 22). $sPrP^{Sc}$ has been previously identified in samples that showed no PrP-res (using standard digestion conditions of 20 μg/ml for 1 h at 37 °C) by the presence of a 22-kDa band on immunoblot after digestion with PK on ice and subsequent de-glycosylation with peptide *N*-glycosidase F (21, 22). Although we aimed to perform all procedures on each individual mouse brain, the limited tissue size meant this was not possible for the cold PK analyses carried out here. However, cold PK digestion was performed on brain tissue taken from mice showing positive clinical and vacuolar signs of TSE, but low levels of PrP deposition in the same primary transmission experiments as those listed in Table 2 (details in supplemental Fig. S2 and Table S1). These tissues failed to demonstrate any marked increase in the 22-kDa PK-resistant PrP band after cold PK digestion (Fig. 4, *lanes 2* and *3*). When compared with the ME7 control (Fig. 4, *lane 6*, loaded at 25% concentration of *lanes 2–5*), the low levels of PrP apparent in *lanes 2* and *3* after digestion with PK on ice demonstrate that $sPrP^{Sc}$ cannot account for the high titer of infectivity in the 101LL/263K and 101LL/GSS models.

Although PrP^{Sc} is generally defined by its partial resistance to PK digestion, it can also be identified using immunoassays that exploit the differential binding of anti-PrP antibodies to PrP^{Sc} in the native and denatured state. Epitopes that are hidden in the native PrP^{Sc} conformation become exposed on denaturation in increasing concentrations of guanidine hydrochloride, leading to an increase in antibody binding. This observation is the basis of the CDI, where levels of PrP^{Sc} are calculated by measuring the ratio of the denatured to native signal (d/n ratio) in a sandwich ELISA (20, 37, 38). An increase in d/n ratio indicates the presence of PrP^{Sc}, which produces the increased signal obtained on denaturation of the sample. Because this assay does not use PK digestion to identify abnormal PrP, it can also be used to identify $sPrP^{Sc}$. To confirm the absence of large amounts of PrP-res or $sPrP^{Sc}$ in the models described here, CDI analysis was performed on tissues from 101LL/GSS- and 101LL/263K-infected mice. Tissue from animals detailed in Table 1 was not analyzed due to limited sample availability, but analysis was performed on tissues from 101LL/GSS- and 101LL/263K-infected mice with confirmed clinical and pathological TSE disease, but little or no PrP^{Sc} in the brain (supplemental Fig. S3 and Table S1). The d/n ratios obtained for all four infected animals ranged from 0.73 to 2.39, which were similar to or lower than the uninfected 101LL control (d/n ratio of 2.01). The 101LL/ME7 control gave a d/n ratio of 9.8 (Fig. 5). These data confirm the limited PK digestion studies, proving that no PrP^{Sc}-like conformers are present in 101LL/GSS- and 101LL/263K-infected tissues that could account for the observed titers of infectivity.

Immunoprecipitation Using PrP^{Sc}-specific Monoclonal Antibodies—Several mAbs have been generated that specifically bind PrP^{Sc} isoforms, but not PrP^{C}. These antibodies can therefore isolate PrP^{Sc} from non-PK-treated tissue homogenates by immunoprecipitation, ensuring that all abnormal PrP isoforms are identified. This technique has been used by others to demonstrate the presence of $sPrP^{Sc}$ in the brains of mice overexpressing 101L-PrP (22). Here, PrP^{Sc}-specific motif grafted mAbs 89–112 and 136–158 (25) were used to immunoprecipitate PrP from brain tissue homogenates of 101LL/GSS- and 101LL/263K-infected mice. Tissues analyzed were taken from mice showing positive clinical and vacuolar signs of TSE but low levels of PrP deposition in the same primary transmission experiments as those used to determine titer of infectivity in each model (details in supplemental Fig. S2 and Table S1). Positive control mAb D13 (which precipitates only the cellular form of PrP) and negative control mAb b12 were also

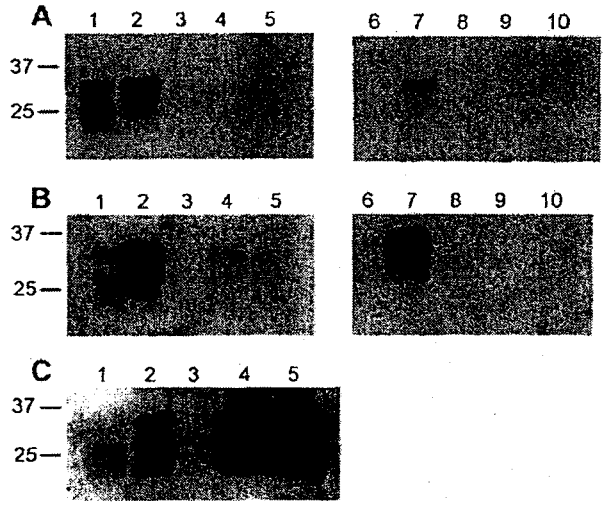

FIGURE 6. **Immunoprecipitation using PrP^{Sc}-specific monoclonal antibodies.** 101LL/GSS and 101LL/263K tissues taken from the same transmission experiments as those shown in Table 1 were subjected to IP using PrP^{Sc}-specific mAbs 89–112 and 136–158 to determine whether forms of PrP^{Sc}, which were sensitive to PK, were present in these tissues. mAb D13, which precipitates only cellular PrP, and mAb b12, which recognizes the HIV gp120 antigen, were used as IP controls. In *A*: *lanes 1–5*, 101LL/GSS(h); *lanes 6–10*, uninfected 101LL; in *B*: *lanes 1–5*, 101LL/263K(i); *lanes 6–10*, uninfected 101LL; in *C*: RML scrapie Wt control. *Lanes 1* and *6*, crude brain homogenate; *lanes 2* and *7*, IP with mAb D13 (positive control antibody); *lanes 3* and *8*, IP with mAb b12 (negative control antibody); *lanes 4* and *9*, IP with mAb 89–112; *lanes 5* and *10*, IP with mAb 136–158.

included in all experiments. For all 101LL/GSS and 101LL/263K tissues examined, extremely low levels of PrP^{Sc} were immunoprecipitated by both PrP^{Sc}-specific antibodies (Fig. 6). These levels were estimated by immunoblot to be 100- to 1000-fold less than those precipitated from control RML-infected mouse brain. Results from these immunoprecipitations therefore support our previous biochemical data, which show no evidence of PK-sensitive forms of PrP^{Sc} in brain tissue from 101LL/GSS- and 101LL/263K-infected mice.

DISCUSSION

PrP^{Sc} is thought to be the sole component of the prion, or TSE infectious agent. For this reason it has become the main target for TSE diagnostic assays, where identification of PrP^{Sc} in post-mortem brain tissue indicates a TSE-positive animal. However the relationship between PrP^{Sc} and TSE infectivity has not been definitively demonstrated, and concerns have been raised by earlier reports of disease transmission in the apparent absence of PrP-res (16, 18). In particular, 101LL gene-targeted transgenic mice inoculated with GSS P102L or 263K succumb to a disease, which is highly transmissible to both 101LL and wild-type mice but shows extremely low levels of PrP-res in the brain. Extended analyses of this model (described here) have now used quantitative assays to unequivocally demonstrate that titers of 10^7 to 10^9 IU/g can be present in brain tissue, which shows little or no abnormal PrP accumulation by standard immunoblot analysis, IHC, CDI, or immunoprecipitation. These titers are similar to or higher than those observed in our well characterized, high titer control strain ME7, but for 4 of 5 brains analyzed, PrP-res levels were below the limit of detection of our immunoblot assay (<1.3% of the amount of PrP-res in wild-type ME7 tissue). Previous studies have shown that PrP-res from other well characterized rodent scrapie strains with titers ranging from $10^{5.5}$ to 10^9 can be easily identified on immunoblot of 1% brain homogenate following PK treatment. Based on these previous data, it would be predicted that the tissues studied here should contain titers far below 10^5 IU/g tissue. However the transmission data clearly show that 101LL/GSS- and 101LL/263K-infected tissues contained high titers of infectivity, which exceed those measured in both 79V- and 22A-infected tissue (supplemental Fig. S1*B*). These data suggest that current diagnostic assay systems that rely on PrP^{Sc} detection might fail to identify some highly infectious tissues. To this end, tissues from 101LL/GSS- and 101LL/263K-infected mice are currently being assessed in several of these assay systems in our laboratory.

Several independent studies have previously shown that one TSE infectious unit is composed of ~10^5 PrP^{Sc} molecules (2, 14, 15). In contrast to these studies the data obtained from 101LL/GSS- and 101LL/263K-infected tissues indicate that the number of PrP^{Sc} molecules per unit of infectivity must display a wide range, with 101LL/GSS and 101LL/263K tissues showing between 10 to 1000 times fewer PrP-res molecules per unit infectivity than Wt/ME7. Alternatively, these data could indicate that only a very small proportion of PrP^{Sc} present in TSE-infected tissue is actually infectious. This lack of correlation between levels of PrP-res and infectivity does not support PrP-res as the infectious agent of TSE.

Because PrP-res does not appear to be a major component of infectivity in this study, it is possible that another form of PrP is responsible for disease in these mice. We have shown previously that 101LL mice can form PrP-res when inoculated with other rodent TSE strains (39); therefore, the lack of PrP^{Sc} in these models is not due to an inherent inability of 101L-PrP to convert to a protease-resistant isoform. In contrast to the gene-targeted transgenic 101LL mice described here, transgenic mice, which overexpress 101L-PrP at levels 8- to 16-fold higher than endogenous PrP, develop a spontaneous neurological disease that appears to be associated with a PK-sensitive form of PrP^{Sc} (21, 22). We have found no evidence of $sPrP^{Sc}$ in 101LL/GSS or 101LL/263K brain tissue by either limited PK digestion studies or CDI analysis. Additionally, motif-grafted mAbs 89–112 and 136–158, which specifically bind PrP^{Sc}, did not immunoprecipitate PK-sensitive forms of PrP^{Sc} from 101LL/GSS or 101LL/263K brain tissue, even though these mAbs have been shown to immunoprecipitate abnormal PK-sensitive PrP^{Sc} from 101L-overexpressing transgenic mice.[6] One possible reason for this discrepancy between models is that disease in 101LL/GSS and 101LL/263K mice is due to a TSE infection, which has been transmitted from a known infected source, and can be further passaged to both 101LL and wild-type 129/Ola mice (18, 19). In contrast the disease observed in transgenic mice overexpressing 101L PrP does not transmit to wild-type mice and only appears to accelerate the phenotype already present in mice expressing lower levels of the transgene (17, 22). This

[6] A. Bellon and R. A. Williamson, unpublished data.

suggests that $sPrP^{Sc}$ may instead be associated with overexpression or misfolding of 101L-PrP and not TSE. The species of abnormal PrP produced due to overexpression of 101L-PrP is therefore different from that produced by TSE infection. The nature of the infectious agent in the current study has yet to be established. We now aim to use this unique model to determine whether infectivity in these tissues is consistent with other abnormal conformations of PrP or with factors other than PrP.

The models of disease described herein demonstrate the potential for the existence of high levels of TSE infectivity with undetectable PrP-res in natural disease. Indeed, increased surveillance and sensitivity of testing methods has identified a new TSE of sheep, termed atypical scrapie. These animals were identified as TSE infected by one PrP^{Sc}-specific diagnostic ELISA, but could not be confirmed by other methods (40, 41). Such cases are now only identifiable using assays that require low concentrations of PK, or no PK, in the assay procedure. It is unknown whether this is truly a new TSE of sheep, or whether it has been present in sheep for some time (42) but was not detected due to the reduced PK resistance of PrP^{Sc}. However, the disease has been shown to be highly transmissible to transgenic mice expressing ovine PrP (43), indicating the presence of substantial levels of infectivity. The results of our study raise concern over the suitability of PrP^{Sc} as a sole diagnostic marker of TSE disease. It is vital that markers of TSE infectivity other than PrP^{Sc} are identified and validated in models such as those we have described and characterized here. We anticipate that such research will lead to the development of more robust diagnostic assays for TSE disease, which will have important implications for both animal and human health.

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