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Molecular Analysis of the Protease-Resistant Prion Protein in Scrapie and Bovine Spongiform Encephalopathy Transmitted to Ovine Transgenic and Wild-Type Mice

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The existence of different strains of infectious agents involved in scrapie, a transmissible spongiform encephalopathy (TSE) of sheep and goats, remains poorly explained. These strains can, however, be differentiated by characteristics of the disease in mice and also by the molecular features of the protease-resistant prion protein (PrP^{res}) that accumulates into the infected tissues. For further analysis, we first transmitted the disease from brain samples of TSE-infected sheep to ovine transgenic [Tg(OvPrP4)] and to wild-type (C57BL/6) mice. We show that, as in sheep, molecular differences of PrP^{res} detected by Western blotting can differentiate, in both ovine transgenic and wild-type mice, infection by the bovine spongiform encephalopathy (BSE) agent from most scrapie sources. Similarities of an experimental scrapie isolate (CH1641) with BSE were also likewise found following transmission in ovine transgenic mice. Secondly, we transmitted the disease to ovine transgenic mice by inoculation of brain samples of wild-type mice infected with different experimental scrapie strains (C506M3, 87V, 79A, and Chandler) or with BSE. Features of these strains in ovine transgenic mice were reminiscent of those previously described for wild-type mice, by both ratios and by molecular masses of the different PrP^{res} glycoforms. Moreover, these studies revealed the diversity of scrapie strains and their differences with BSE according to labeling by a monoclonal antibody (P4). These data, in an experimental model expressing the prion protein of the host of natural scrapie, further suggest a genuine diversity of TSE infectious agents and emphasize its linkage to the molecular features of the abnormal prion protein.

Transmissible spongiform encephalopathies (TSE) are fatal neurodegenerative diseases, affecting both human (Creutzfeldt-Jakob disease [CJD]) and animals, including mainly sheep and goats (scrapie), deer and elk (chronic wasting disease [CWD]), and cattle (BSE).

Whereas the nature of the infectious agent causing these diseases still remains controversial (17, 37, 45), a central event in their pathogenesis is the accumulation in infected tissues of an abnormal form of a host-encoded protein, the prion protein. The abnormal form of this protein (PrP Sc in scrapie) differs from its normal form by its biochemical properties, including insolubility in nondenaturing detergent and partial resistance to degradation by proteases. Whereas the normal cellular protein (PrP C) is fully sensitive to proteases (PrP^{sen}), the abnormal prion protein (PrP Sc) is only partly degraded (PrP^{res}), its amino-terminal end being removed. Also, whereas PrP C is predominantly α-helical, PrP Sc has a higher β-sheet content (40).

A key question regarding these diseases has been linked to the finding that the infectious agents involved in their transmission could show a biological diversity reminiscent of strains of other classical infectious agents, like viruses (13). The existence of different TSE strains has been essentially defined by the different features of the disease, including differences in the incubation periods and in the distribution of brain lesions, following TSE transmission in mice of different genotypes (24). Nevertheless, this has been most extensively demonstrated in natural scrapie; in contrast, a unique and stable BSE strain has been recognized in cattle BSE (11, 23). Scrapie strain diversity still remains a matter of controversy (51). Indeed, it remains to be determined how the infectious agent is carrying some strain-specific biological information, especially within the framework of the prion protein-only hypothesis.

However, different features of the PrP^{res} protein have been found in mice infected with different biological strains of scrapie, including different electrophoretic patterns demonstrated by Western blotting (4, 32, 34, 35, 50). This had also been described for strains isolated in hamster from transmissible mink encephalopathy (7–10). Criteria showing the molecular diversity of PrP^{res} include ratios of the di-, mono-, and unglycosylated forms, their respective molecular masses, and the long-term resistance of the protein to proteinase K digestion. While the involvement of informational molecular components other than PrP, putatively host independent, is still discussed (51), some studies have argued that such strains indeed differ when propagated into the same host species by the PrP Sc conformations (16, 44, 46).

In this context the relationship between the different infectious agent strains in the natural diseases remains poorly understood, although strain features can be used to follow the possible transmission of such infectious agents through different species (2, 11, 12). The demonstration that the BSE agent has almost certainly been transmitted from cattle to human

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TABLE 1. Sources of sheep with scrapie or BSE inoculated into ovine transgenic and wild-type mice

Sheep	Breed	Phenotype[a]	PrP^{res}-positive mice by Western blot[b]	
			Tg(OvPrP4) mice	C57BL/6 mice
Natural sources				
Scr 1	Charollais × Texel	VRQ/VRQ	4/5	6/11
Scr 2	Manech tête rousse	ARQ/VRQ	4/7	0/12
Scr 3	Blanc du Massif Central	ARQ/ARQ	6/7	0/12
Experimental sources				
BSE	Lacaune	ARQ/ARQ	10/13	11/11
SSBP/1	Mainly Cheviot (pool)	Mixed	7/16	16/24
CH1641	Cheviot (pool)	Mixed	6/12	0/25

[a] Amino-acids encoded at codons 136, 154, and 171 of the *pm-p* allele.
[b] Number of PrP^{res}-positive mice by Western blot detection/number of inoculated mice.

(14, 57) has triggered further molecular studies of the abnormal PrP in human and animal TSE, together with the characterization of the infectious agents in murine experimental models (5, 6, 12, 18, 27, 29–31, 52, 53). The recent development of transgenic mice expressing the prion protein of the natural hosts of TSE has offered new opportunities for the biological and molecular characterization of the infectious agents involved in these diseases (15, 20, 48, 54, 55).

In this study we investigated the molecular features of PrP^{res}, as analyzed by Western blotting, in wild-type and ovine-transgenic mice, following the transmission of some sheep scrapie and BSE isolates and of murine-adapted scrapie and BSE strains. We show that the molecular diversity characterizing PrP^{res}, in natural scrapie and experimental BSE in sheep but also in experimental murine models, is similarly found in transgenic mice expressing the prion protein of the ovine natural host of scrapie.

(Part of this research was presented at the International Conference on Transmissible Spongiform Encephalopathies, Edinburgh, Scotland, September 2002.)

MATERIALS AND METHODS

TSE sheep isolates and mouse-adapted strains. Experimental TSE sheep sources were either scrapie-infected sheep inoculated with sheep scrapie brain pool 1 (SSBP/1) and CH1641 scrapie brain pools (kindly provided by N. Hunter, Institute for Animal Health, Edinburgh, United Kingdom) or a BSE-infected sheep inoculated with a French BSE case in cattle (5). SSBP/1 has been maintained by serial passage mostly in Cheviot sheep, animals with a V136 Q171 allele showing higher susceptibility (25). CH1641 was initially derived from a Cheviot sheep in 1970 and has since been maintained in homozygous A136 Q171 Cheviot sheep only (22, 25). Three French natural scrapie cases (Scr 1 to 3) detected by the national clinical surveillance network of suspect clinical scrapie cases were also studied. Breeds and, when known, genotypes of the sheep at codons 136, 154, and 171 are recorded in Table 1.

Mouse-adapted scrapie strains were 87V from IM mice (kindly provided by M. Bruce, Institute for Animal Health, Edinburgh, United Kingdom) and strains C506M3 (kindly provided by D. Dormont, Commissariat à l'Energie Atomique, Fontenay-aux-Roses, France) (36) and 79A and Chandler (kindly provided by M. Groschup, Federal Research Centre for Virus Diseases of Animals, Germany) from C57BL/6 mice. Mouse-adapted BSE was a second passage in C57BL/6 mice from a French BSE case in cattle (4).

Mouse lines and experimental infections. The ovine transgenic mouse line Tg(OvPrP4) expressing the ovine prion protein (A136 R154 Q171 sequence) under the control of the neuron-specific enolase promoter, on a murine *prn-p* null background, has been previously described (20). These mice were used for transmissions of both sheep TSE isolates (19, 20) and mouse-adapted scrapie and BSE strains. Experimental and natural sheep scrapie isolates and BSE from the experimentally infected sheep were also inoculated in C57BL/6 mice (Charles Rivers, L'Arbreste, France). Mice were cared for and housed according to the guidelines of the French Ethical Committee (decree 87-848) and European Community Directive 86/609/EEC.

Four- to six-week-old female mice were inoculated intracerebrally with 10% (wt/vol) brain homogenates in glucose 5% in distilled water (20 μl per animal), and brains were sampled at the terminal stage of the disease or death of the animal for intercurrent disease or aging.

Extraction of PrP^{res}. PrP^{res} was obtained following concentration by ultracentrifugation, as previously described (4). Dissociation of half of the whole brain of mice was performed in 1 ml of 5% glucose in distilled water, using disposable blenders, and complete homogenization was obtained by forcing the brain suspension through a 0.4-mm-diameter needle. A 330-μl volume was completed to 1.2 ml in 5% glucose before incubation with proteinase K (10 μg/100 mg of brain tissue; Roche) for 1 h at 37°C. Thirty percent *N*-lauroyl sarcosyl (600 μl; Sigma) was added. After incubation at room temperature for 15 min, samples were then centrifuged at 200,000 × *g* for 2 h on a 10% sucrose cushion in a Beckman TL100 ultracentrifuge. Pellets were resuspended and heated for 5 min at 100°C in 50 μl of denaturing buffer (4% sodium dodecyl sulfate, 2% β-mercaptoethanol, 192 mM glycine, 25 mM Tris, 5% sucrose).

Western blot analysis. Samples were run in sodium dodecyl sulfate-15% polyacrylamide gel electrophoresis and electroblotted to nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 10% isopropanol) at a constant level of 400 mA for 1 h. The membranes were blocked during 1 h with 5% nonfat dried milk in phosphate-buffered saline–0.1% Tween 20 (PBST). After two washes in PBST, membranes were incubated (1 h at room temperature) with RB1 rabbit antiserum (1:2,500 in PBST), raised against synthetic bovine residues 106 to 121 (THGQWNKPSKPKTNMK) PrP peptide (3) or P4 monoclonal antibody (0.2 μg/ml in PBST), and raised against synthetic ovine residues 89 to 104 (GGGGWGQGGSHSQWNK) PrP peptide (r-biopharm, Darmstadt, Germany) (28). SAF 15 monoclonal antibody, raised against denatured scrapie-associated fibrils (SAFs) from a scrapie-infected hamster brain, was shown to recognize the entire octarepeat region in all mammalian PrP studied. It was used at 1/2,000 in PBST instead of P4 for the study of wild-type mice PrP^{res}. After three washes in PBST, the membranes were incubated (30 min at room temperature) with peroxidase-labeled conjugates against rabbit or mouse immunoglobulins (1/2,500 in PBST) (Clinisciences). After three washes in PBST, bound antibodies were detected either on films after exposure of the membranes on Biomax MR Kodak films (Sigma) by using ECL (Amersham) chemiluminescent substrate or, for quantitative studies, on pictures directly obtained with the Fluor-S Multimager (Bio-Rad) analysis system by using Supersignal (Pierce) chemiluminescent substrate. For quantitative studies of the glycoform ratios, chemiluminescent signals corresponding to the three glycoforms of the protein were quantified by using the Fluor S-Multimager software. Glycoform ratios were expressed as mean percentages (± standard deviations) of the total signal for the three glycoforms (high [H], low [L], and unglycosylated [U] forms), with at least 10 different runs obtained from a minimum of three different mice per experimental group and at least three separate gel runs per mouse. For comparisons of PrP^{res} migrations between experimental groups, repeated runs were performed in gels with samples from the different experimental groups that were compared. Comparisons involved from 1 to 5 PrP^{res}-positive mice in each experimental group. The molecular mass was precisely evaluated by comparison of the positions of each of the PrP^{res} bands with a biotinylated marker by using Quantity One (Bio-Rad) software in one mouse per experimental group from eight different runs of the same gel. The intensities of PrP^{res} signals were assessed by using the Fluor S-Multimager software, allowing comparison of reactivities with either RB1 or P4 antibodies, as previously described for sheep (38, 52). Immunological reactivities of PrP^{res} were scored according to the decrease of P4 labeling (−, no labeling; ±, light labeling; +, moderate labeling; ++, intense labeling) compared to RB1 labeling found on a same quantity of PrP^{res} loaded on a second gel. This differential labeling was assessed by quantification of total PrP^{res} signals obtained with both antibodies from repeated runs of the samples.

Statistical analysis. The difference between groups in terms of glycoform ratios and electrophoretic mobilities were studied by using analysis of variance. According to the data structure, because the factor ≪measure≫ was nested within the factor ≪individual mouse≫ and because this latter factor was nested within the factor ≪experimental group≫, the data were analyzed by using a two-level hierarchical model. Regarding the ratios of the three glycoforms, the

TABLE 2. Sources of scrapie strains inoculated from wild-type mice to ovine transgenic mice

Strain source	Mouse line at origin of inoculum	Western blot results[a]
BSE	C57BL/6	3/6
C506M3	C57BL/6	4/7
87V	IM	5/9
79A	C57BL/6	2/10
Chandler	C57BL/6	7/14

[a] Number of PrP^{res}-positive mice by Western blot detection/number of inoculated mice.

different experimental groups were compared through the factor ≪individual mouse≫ by using a mixed model adjusted on the ≪measure≫ as a random effect. Comparisons of electrophoretic mobilities were performed through the factor ≪individual mouse≫, taking into account that the factor ≪gel run≫ was crossed with the factor ≪experimental group≫, using a mixed model adjusted on the ≪gel run≫ and the ≪measure≫ factors as random effects. These data were analyzed by using S-Plus 2000 software.

RESULTS

We first transmitted the disease by inoculating ovine transgenic [Tg(OvPrP4)] and wild-type (C57BL/6) mice with three natural field scrapie isolates and with three experimental sheep TSE isolates, including scrapie sources (SSBP/1 and CH1641) and BSE. Results obtained for the detection of PrP^{res} by Western blot at the terminal stage of the disease or after natural death of mice (Table 1) show that each of these TSE isolates were transmitted in some Tg(OvPrP4) mice. In contrast, the CH1641 experimental scrapie isolate, as well as two of the three natural scrapie isolates, failed to transmit to C57BL/6 mice.

We then transmitted in Tg(OvPrP4) mice scrapie and BSE strains previously adapted and maintained by serial passages in wild-type mice. Results obtained for the detection of PrP^{res} by Western blotting are given in Table 2, showing that four scrapie strains (87V, C506M3, 79A, and Chandler) and BSE could be transmitted.

Comparison of PrP^{res} electrophoretic patterns from mice infected with sheep TSE isolates. We studied the electrophoretic patterns of PrP^{res} in Tg(OvPrP4) mice successfully infected with the sheep TSE isolates by analysis of both glycoform ratios and electrophoretic mobilities by using RB1 antiserum directed against a bovine peptidic sequence (residues 105 to 120) of PrP (sequence 98 to 113 in sheep). The proportions of diglycosylated PrP^{res} were higher than those of monoglycosylated PrP^{res} in all cases except that of SSBP/1-infected mice (Fig. 1A). The dominance of diglycosylated PrP^{res} was particularly important (66% ± 2.2%) in mice infected with experimental BSE in sheep. BSE was significantly different from both CH1641 [$P_{H(BSE\text{-}CH1641)} < 0.0001$; $P_{L(BSE\text{-}CH1641)} = 0.0001$; $P_{U(BSE\text{-}CH1641)} = 0.0048$] and SSBP/1 experimental

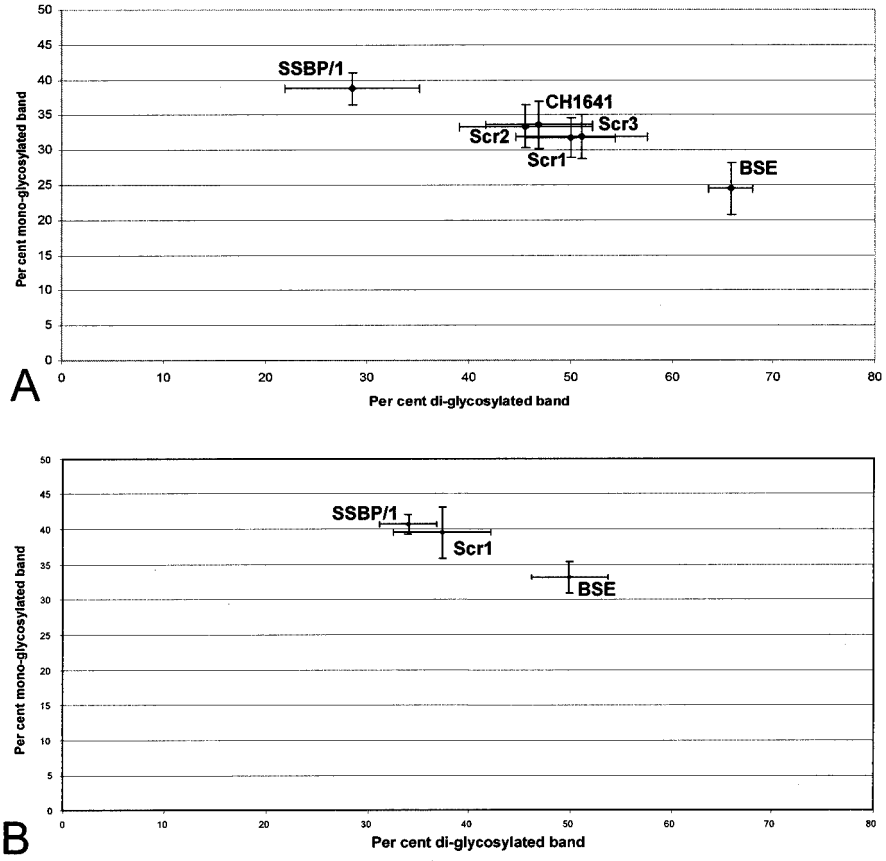

FIG. 1. Glycoform ratios of PrP^{res} (means ± standard deviations) detected by Western blotting with RB1 antiserum in Tg(OvPrP4) (A) or wild-type C57BL/6 (B) mice infected with natural or experimental TSE sheep isolates.

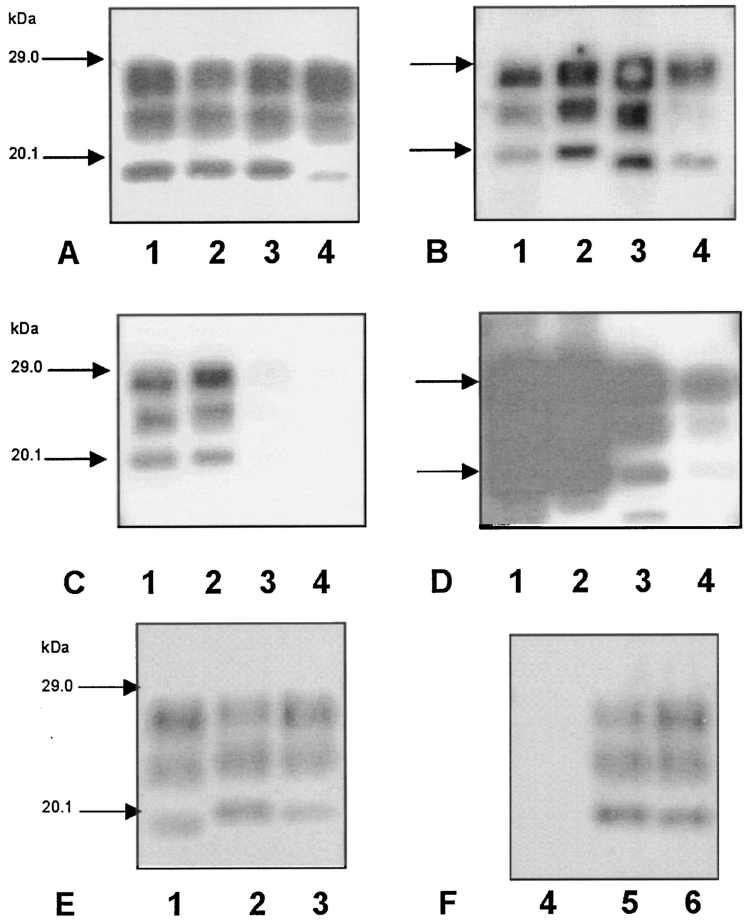

FIG. 2. Comparison of electrophoretic profiles and antibody labeling of PrP^{res} after Western blot detection by using RB1 antiserum (A, B, and E), monoclonal antibodies P4 (C and D), or SAF 15 (F). (A) Tg(OvPrP4) mice infected with natural scrapie cases Scr 1 to 3 (lanes 1 to 3) or experimental ovine BSE (lane 4). (B, C, and D) Sheep (lanes 1 and 3) or Tg(OvPrP4) mice (lanes 2 and 4) infected with natural scrapie (lanes 1 and 2) or experimental ovine BSE (lanes 3 and 4). Panels B and C were loaded with the same quantities of extracted PrP^{res} of each sample. Panels C and D are different exposures of the same membrane. (E and F) C57BL/6 mice infected with experimental ovine BSE (lanes 1 and 4), natural scrapie (Scr 1) (lanes 2 and 5), or SSBP/1 (lanes 3 and 6). Equal amounts of extracted PrP^{res} were loaded on both gels analyzed with RB1 (E) or SAF 15 (F) antibodies.

scrapie [$P_{H(BSE\text{-}SSBP/1)} < 0.0001$; $P_{L(BSE\text{-}SSBP/1)} < 0.0001$; $P_{U(BSE\text{-}SSBP/1)} < 0.0001$] sources, as well as from the three natural scrapie cases, such as Scr 1 [$P_{H(BSE\text{-}Scr\ 1)} = 0.0002$; $P_{L(BSE\text{-}Scr\ 1)} = 0.0019$; $P_{U(BSE\text{-}Scr\ 1)} = 0.0010$]. In contrast, glycoform ratios observed following the transmission of the natural scrapie cases were not significantly different in the 3 studied cases.

Electrophoretic profiles of PrP^{res} could also be studied in wild-type C57BL/6 mice infected with BSE in sheep and compared to those of a natural scrapie isolate (Scr 1) and to the experimental SSBP/1 scrapie isolate. As previously described for ovine transgenic mice, BSE also significantly differed by ratios of the three PrP^{res} glycoforms from both natural [$P_{H(BSE\text{-}Scr1)} = 0.0004$; $P_{L(BSE\text{-}Scr1)} = 0.0059$; $P_{U(BSE\text{-}Scr1)} = 0.0077$] and experimental scrapie [$P_{H(BSE\text{-}SSBP/1)} = 0.0001$; $P_{L(BSE\text{-}SSBP/1)} = 0.0035$; $P_{U(BSE\text{-}SSBP/1)} = 0.0027$] sources (Fig. 1B).

Analysis of the electrophoretic mobilities showed a clearly distinct pattern of PrP^{res} in BSE-infected Tg(OvPrP4) mice, with a consistently lower molecular mass of the unglycosylated band (0.5- to 1-kDa differences) compared to that observed for mice infected with the natural scrapie isolates (Scr 1 to 3) (Fig. 2A), as was also found when sheep with natural scrapie or experimental BSE were compared (Fig. 2B). Moreover, while close mobilities of the unglycosylated PrP^{res} were found in mice infected with SSBP/1 or natural scrapie, the migration of

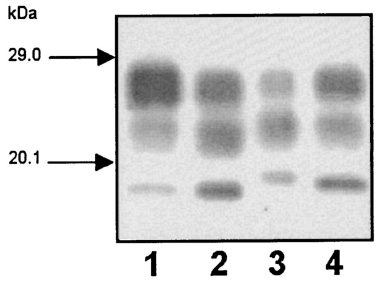

FIG. 3. Comparison of electrophoretic profiles of PrP^{res} detected by RB1 antiserum in ovine transgenic mice infected with experimental BSE in sheep (lane 1), CH1641 (lane 2), or SSBP/1 (lane 3) experimental scrapie isolates or in natural scrapie (Scr 1) (lane 4).

unglycosylated PrP^{res} in CH1641-infected Tg(OvPrP4) mice was close to that found in BSE-infected mice (Fig. 3), as previously described for experimental sheep TSE isolates inoculated in these mice (5).

Also, in C57BL/6 mice, the three PrP^{res} glycoforms showed a lower molecular mass in BSE-infected mice (0.4- to 1.1-kDa differences) than did mice infected with the natural-field scrapie isolate (Scr 1) or with the SSBP/1 experimental scrapie isolate (Fig. 2E).

Because two main different-sized fragments of PrP^{res} were observed in Tg(OvPrP4) ovine transgenic mice, as in sheep infected with either BSE-CH1641 or natural scrapie-SSBP/1, we further compared the PrP^{res} reactivities by using RB1 antiserum (residues 98 to 113 sheep PrP sequence) and P4 monoclonal antibody (directed against the sheep PrP 89 to 104 sequence) for further analysis of the protease cleavage site. PrP^{res} from Tg(OvPrP4) mice infected with ovine BSE or CH1641 scrapie showed a drastically lower reactivity with P4 antibody than did those infected with other scrapie sources (Fig. 2C and also see Fig. 5, lanes 1, respectively). Overexposure of films obtained by using P4 monoclonal antibody (Fig. 2D) nevertheless allowed the detection of PrP^{res} signals in the brains of Tg(OvPrP4) mice infected with sheep BSE as well as in the sheep BSE isolate that had been used for the inoculation, showing that P4 antibody can recognize a portion of PrP^{res} molecules in these cases. However, contrary to RB1 labeling, the migration of the P4-labeled unglycosylated band in BSE is no more different from that found in PrP^{res} from a natural scrapie source in both sheep and ovine transgenic mice.

In wild-type mice, SAF-15 antibody that reacts with mouse PrP was used instead of P4 antibody. Low labeling of PrP^{res} was also observed in BSE-infected C57BL/6 mice using SAF 15 monoclonal antibody compared to that of those infected with scrapie (Fig. 2F). In contrast with the different mobilities of PrP^{res} bands detected with RB1 antibody between BSE and scrapie (Fig. 2E), SAF 15-labeled PrP^{res} species migrated similarly in both BSE- and scrapie-infected mice, as shown by overexposure of films (data not shown).

Comparison of PrP^{res} electrophoretic patterns in ovine transgenic mice infected with mouse-adapted strains of scrapie and BSE and with CH1641. We studied the glycoform ratios of PrP^{res} in ovine transgenic mice infected with mouse-adapted scrapie and BSE strains, these strains being known to show distinct electrophoretic patterns in wild-type mice. This analysis, using RB1 antibody (Fig. 4A), showed clearly distinct ratios of the three different PrP^{res} glycoforms in Tg(OvPrP4) mice between some of these different strains, from heavily glycosylated forms (BSE) to highly aglycosyl forms (79A and Chandler). Statistical analysis of the data showed that BSE significantly differs from the four scrapie strains by ratios of the three different PrP^{res} glycoforms [$P_{H(BSE\text{-}C506M3)} = 0.0001$; $P_{L(BSE\text{-}C506M3)} = 0.0015$; $P_{U(BSE\text{-}C506M3)} = 0.0015$; $P_{H(BSE\text{-}79A)} < 0.0001$; $P_{L(BSE\text{-}79A)} < 0.0001$; $P_{U(BSE\text{-}79A)} < 0.0001$; $P_{H(BSE\text{-}87V)} = 0.0027$; $P_{L(BSE\text{-}87V)} = 0.0178$; $P_{U(BSE\text{-}87V)} = 0.0249$; $P_{H,\,L,\,U(BSE\text{-}Chandler)} < 0.0001$]. Among these scrapie strains two groups of two strains can be distinguished by the three glycoform ratios. These included the heavily glycosylated C506M3 and 87V strains on one side and the poorly glycosylated 79A and Chandler strains on the other side. The strains within each group were not significantly different. On the contrary, Chandler and 79A were significantly different from C506M3 [$P_{H(C506M3\text{-}79A)} = 0.0003$; $P_{L(C506M3\text{-}79A)} = 0.0064$; $P_{U(C506M3\text{-}79A)} = 0.0011$; $P_{H(C506M3\text{-}Chandler)} < 0.0001$; $P_{L(C506M3\text{-}Chandler)} = 0.0008$; $P_{U(C506M3\text{-}Chandler)} = 0.0007$] and from 87V [$P_{H(87V\text{-}79A)} < 0.0001$; $P_{L(87V\text{-}79A)} = 0.0004$; $P_{U(87V\text{-}79A)} < 0.0001$; $P_{H(87V\text{-}Chandler)} < 0.0001$; $P_{L(87V\text{-}Chandler)} < 0.0001$; $P_{U(87V\text{-}Chandler)} < 0.0001$]. Comparison of glycoform ratios with those observed following transmission of the CH1641 scrapie isolate also showed that C506M3 and 87V did not significantly differ from this scrapie source. In contrast, CH1641 was clearly distinct from the poorly glycosylated scrapie strains 79A [$P_{H(CH1641\text{-}79A)} = 0.0001$; $P_{L(CH1641\text{-}79A)} = 0.0029$; $P_{U(CH1641\text{-}79A)} = 0.0002$] and Chandler [$P_{H(CH1641\text{-}Chandler)} < 0.0001$; $P_{L(CH1641\text{-}Chandler)} = 0.0002$; $P_{U(CH1641\text{-}Chandler)} = 0.0001$] or from the highly glycosylated BSE strain [$P_{H(CH1641\text{-}BSE)} = 0.0005$; $P_{L(CH1641\text{-}BSE)} = 0.0034$; $P_{U(CH1641\text{-}BSE)} = 0.0074$].

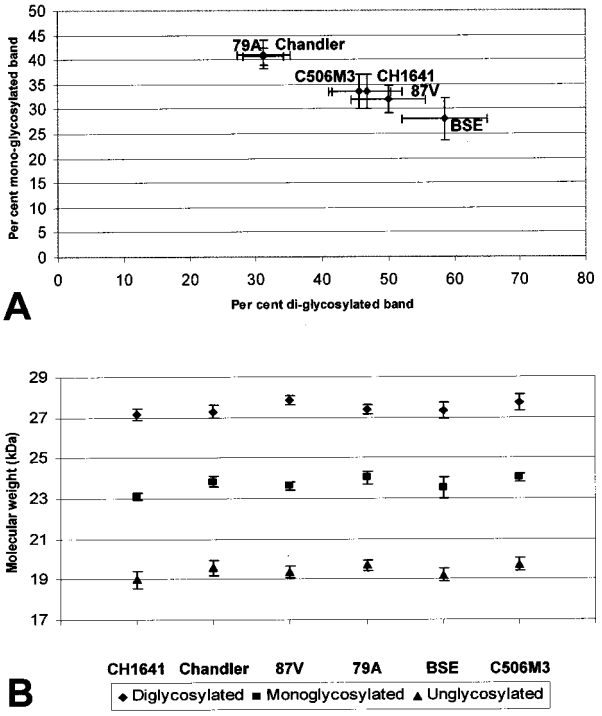

FIG. 4. Glycoform ratios (A) and molecular sizes (B) of PrP^{res} (means ± standard deviations) detected by Western blotting with RB1 antiserum in ovine transgenic mice infected with experimental scrapie and BSE strains from wild-type mice and with the CH1641 experimental sheep scrapie isolate.

We then studied the electrophoretic mobilities of PrP^{res} that can also distinguish BSE and some scrapie strains in wild-type mice. Similar results were observed when different mice from each of the experimental groups were compared (Fig. 5A and B). The mean molecular masses and standard deviations are shown in Fig. 4B. Statistical analysis of the data showed significantly lower molecular masses of both monoglycosylated and unglycosylated PrP^{res} in BSE-infected mice compared to that of animals infected with scrapie strains C506M3 [$P_{L(C506M3\text{-}BSE)} < 0.0001$; $P_{U(C506M3\text{-}BSE)} = 0.0005$], Chandler [$P_{L(Chandler\text{-}BSE)} = 0.0026$; $P_{U(Chandler\text{-}BSE)} = 0.0074$], and 79A [$P_{L(79A\text{-}BSE)} = 0.0001$; $P_{U(79A\text{-}BSE)} = 0.0096$] (Fig. 4B and 5A

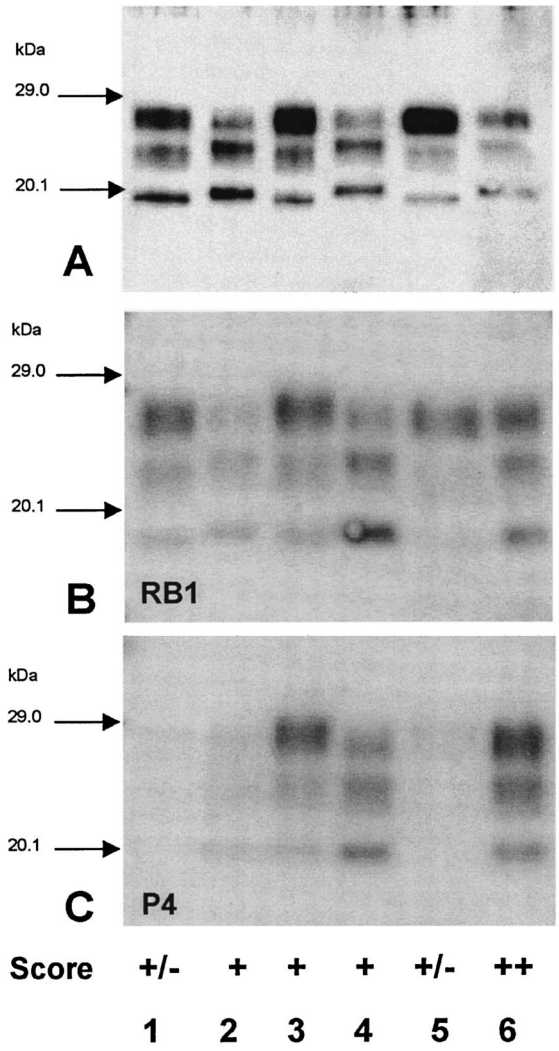

FIG. 5. Comparison of electrophoretic profiles and P4 labeling of PrP^{res} after Western blot detection by using RB1 antiserum (A and B) or P4 monoclonal antibody (C) in ovine transgenic mice infected with experimental scrapie isolate CH1641 (lane 1), mouse-adapted scrapie strains Chandler (lane 2), 87V (lane 3), 79A (lane 4), C506M3 (lane 6), and mouse-adapted BSE (lane 5). In panels A and B, two different mice from each of the experimental groups were loaded in lanes 2 to 6, while PrP^{res} from the same mouse was loaded in lanes 1 of both gels. In panels B and C equal amounts of extracted PrP^{res} were loaded on both gels analyzed with RB1 or P4 antibodies. Scoring of labeling by P4 monoclonal antibody from quantitative analysis of repeated runs of the samples, compared to that with RB1 antiserum, is indicated as described in Materials and Methods.

and B). On the other hand, the mobilities of monoglycosylated and unglycosylated PrP^{res} were not significantly different between BSE and 87V. The lowest molecular masses of both unglycosylated and monoglycosylated bands were observed in CH1641-infected mice, with results significantly different from those of the four scrapie strains [$P_{L,\ U(CH1641\text{-}C506M3)} < 0.0001$; $P_{L,\ U(CH1641\text{-}79A)} < 0.0001$; $P_{L,\ U(CH1641\text{-}Chandler)} < 0.0001$; $P_{L(CH1641\text{-}87V)} = 0.0001$; $P_{U(CH1641\text{-}87V)} = 0.0037$] and also from BSE [$P_{L(CH1641\text{-}BSE)} = 0.0021$; $P_{U(CH1641\text{-}BSE)} = 0.0056$]. This analysis also revealed a higher molecular mass of diglycosylated PrP^{res} in both 87V- and C506M3-infected mice compared to that of BSE [$P_{H(87V\text{-}BSE)} < 0.0001$; $P_{H(C506M3\text{-}BSE)} < 0.0002$] and scrapie source CH1641 [$P_{H(87V\text{-}CH1641)} < 0.0001$; $P_{H(C506M3\text{-}CH1641)} < 0.0001$].

Differential immunolabeling by RB1 and P4 antibodies, as illustrated in Fig. 5B and C, was assessed by a score reflecting the ratios of PrP^{res} signals obtained from repeated runs of the samples. This revealed that a single strain, C506M3, was consistently more strongly labeled by P4 antibody. Among cases showing the lower molecular masses, 87V was clearly labeled by P4 antibody while PrP^{res} signals were strongly decreased in CH1641- and in BSE-infected mice.

DISCUSSION

TSE strain diversity has been historically evidenced by the demonstration of distinct and specific behaviors in defined inbred mouse lines, following serial passages in wild-type mice of natural scrapie isolates (11). Strain characterization thus requires transmission in mice, but some scrapie isolates failed to transmit to wild-type mice of any genotype as the CH1641 experimental scrapie source (11, 22). Ovine transgenic mice facilitates disease transmission of sheep TSE (20, 55). In this study three sheep isolates that failed to transmit to C57BL/6 mice could indeed be transmitted to the ovine transgenic mouse line Tg(OvPrP4), including the CH1641 scrapie source. It is worth noting that, while BSE in sheep was readily transmitted in C57BL/6 mice from a homozygous A_{136} R_{154} Q_{171} sheep, only the homozygous V_{136} R_{154} Q_{171} isolate was transmitted in some of the wild-type mice among the three natural scrapie isolates studied here. An increased difficulty to transmit scrapie isolates from sheep homozygous for alanine at codon 136 had already been suggested in previous studies (12).

It is noteworthy that in some experimental groups, as previously described (20), a proportion of ovine transgenic mice did not show detectable levels of PrP^{res}. In mice inoculated with mouse-adapted strains, the reintroduction of a species barrier may prevent the transmission of the infectious agent and explain these observations. However, preliminary results also suggest that individual variations in the expression levels of the ovine PrP protein might play a role in susceptibility (data not shown). Individual variations in susceptibility, possibly associated with variations in PrP expression, may be linked to the mixed genetic background of these mice, with C57BL/6J, 129Sv, and OF1 contributions (20). Other studies have also described the selection of prions with different molecular and lesional features following transmission of cattle-BSE in human transgenic mice that might be associated with variations of the individual genetic backgrounds (1).

We then showed that the characteristic electrophoretic differences of PrP^{res} that can be observed by Western blot analysis between different sheep isolates are maintained following their transmission to ovine transgenic mice. In most cases, it has been difficult to identify different electrophoretic patterns in sheep with natural scrapie (6, 27, 30, 31, 52, 53), in contrast with the known diversity of PrP^{res} molecular features found among scrapie strains in wild-type mice (34, 35, 50). In sheep, PrP^{res} glycoform ratios did not clearly differ from that found in cattle-BSE and did not reveal distinct subgroups of natural scrapie (6, 27, 52, 53). In comparison with some sheep natural scrapie cases, significantly lower and higher levels of diglyco-

sylated PrP^{res} were reported in SSBP/1 and BSE experimentally infected sheep, respectively (52). However, in sheep the most striking molecular feature has been the lower molecular mass of the unglycosylated PrP^{res} in experimental infection with BSE or with scrapie CH1641 compared to that of most natural scrapie cases and with scrapie SSBP/1 (5, 30, 31, 39, 52). These data have not been described for large series of sheep infected by such TSE sources (52), but our results with ovine transgenic mice as well as with wild-type mice for sheep isolates that can be transmitted to these mice are consistent with these data and clearly show that these molecular differences are transmissible. Transmission of some differences in the PrP^{res} sizes has already been described in chimeric human-mouse transgenic mice infected by different human prion diseases (54), showing that different molecular features of inoculated PrP^{res} were replicated in mice with a unique genetic background. Nevertheless, differences in the PrP^{res} electrophoretic patterns in human prion diseases are often associated not only with distinct clinicopathological features but also with different prion protein gene PRNP genotypes and thus may not necessarily reflect the existence of different strains of infectious agents (41–43).

We were able to infect ovine transgenic mice with several strains of scrapie and with BSE previously maintained in wild-type mice, which were often shown to express a higher molecular diversity than that found in sheep (2, 4). Our data indeed show that the major differences observed in wild-type mice regarding electrophoretic features of these strains (4, 27, 35, 50) were maintained in ovine transgenic mice, including differences in both glycoform ratios and molecular sizes. In both lines of mice, BSE and 87V shared high levels of diglycosylated PrP^{res} and low molecular masses of the unglycosylated PrP^{res} (35, 50). In contrast, Chandler and 79A strains also maintained in ovine transgenic mice their characteristic low levels of diglycosylated PrP^{res}, as reported for wild-type mice (4, 35). A higher molecular diversity could thus be expected in natural scrapie, while so far uniform patterns were reported in most cases for sheep (6, 31, 39, 52, 53). Nevertheless, it has been shown that the molecular features of PrP^{res} can be strongly modified during infection of a single host by different strains (4), and the isolation of different strains from some sheep scrapie isolates has also been shown (12, 33). However, our data further support the idea that a genuine diversity of scrapie can be found independent of the *prn-p* genotype.

Importantly, the features of PrP^{res} were similar in ovine transgenic mice infected with the BSE agent inoculated either from an experimentally infected sheep or from a second passage in C57BL/6 mice. We did not find any evidence of modifications of the strain as described for some human transgenic mice infected with cattle-BSE in other studies (1), despite the previously mentioned individual variability of genetic backgrounds of the Tg(OvPrP4) mice. In human transgenic mice, such changes of the BSE phenotypic features were only observed following transmission of cattle-BSE, reproducing a bovine-human species barrier, but not following inoculation of human variant CJD. More generally, no major differences were found in our study when different mice were examined in the different experimental groups that were compared, including up to five mice per experimental group. Statistical analysis of glycoform ratios (performed on three different mice per experimental group when available) and molecular weights (performed on one mouse per experimental group) were thus computed assuming that the variability of indicators between different mice in an experimental group was not significant compared to the variability of measures for a given individual mouse.

It is particularly noteworthy that, for each of the different strains, glycoform ratios observed in ovine transgenic mice were consistent with those previously described for wild-type mice (4, 35, 50). A possible relation of strain-specific glycoform distribution with the neuroanatomical targeting of infection has been considered (21, 49), but conflicting results were discussed regarding some possible differences of PrP^{res} glycoform ratios between different neuroanatomical regions in a single TSE-infected host (35, 49). The distribution of abnormal PrP in our ovine transgenic mice is presently being studied, but it should be noticed that these mice express the sheep prion protein under the control of the neuron-specific enolase promoter, which may strongly influence the features of PrP C expression. Nevertheless, the different strains or isolates showed the same behavior, with regard to PrP^{res} glycosylation, that wild-type mice expressing mouse PrP C showed under the control of the *prn-p* promoter in its normal location into the mouse genome. Recent studies using in vitro conversion of 3F4 epitope-tagged PrP C extracted from a murine neuroblastoma cell line by different murine-adapted scrapie strains have shown that glycoform ratios were primarily dictated by PrP^{res} and did not require specific cell types (56). Our in vivo results now extend these observations.

Whereas the origin of variable but strain-specific glycoform ratios still remains an open question, the relationship between the molecular mass of PrP^{res} and the protease cleavage sites has clearly been demonstrated by amino acid sequencing or by epitope mapping (4, 27, 43, 52). Our data showed, in the same experimental host, both variable molecular masses of the three PrP glycoforms and variable reactivities with P4 monoclonal antibody directed against an epitope close to the protease cleavage site. Such variable reactivities may be linked to different truncations of PrP^{res} following proteinase K digestion. As previously described for sheep (52), low labeling of PrP^{res} in BSE- and CH1641-infected Tg(OvPrP4) mice was indeed found by using P4 monoclonal antibody, suggesting that the major cleavage site by proteinase K is within or is C terminal to the epitope recognized by P4 in these cases. This distinguished BSE from most other scrapie sources except the CH1641 experimental scrapie isolate. A portion of PrP^{res} molecules can, however, be labeled by P4 antibody in BSE- and CH1641-infected mice, consistent with previous data showing the ragged amino-terminal cleavage of PrP^{res} by proteinase K (4, 43). This P4-labeled subpopulation of PrP^{res} molecules migrate similarly in both scrapie and BSE or CH1641, with BSE or CH1641 only differing from other scrapie sources by the proportion of this subpopulation among PrP^{res} molecules. A similar phenomenon can be observed in wild-type mice by using SAF-15 monoclonal antibody.

However, our study also revealed variations among different scrapie strains, with the higher mean molecular mass of unglycosylated PrP^{res} in the C506M3 strain associated with the strongest labeling by P4 antibody (34) On the other hand, among transmissions in ovine transgenic mice showing a lower

molecular mass of unglycosylated PrP^{res}, decreased P4 labeling of PrP^{res} was found in CH1641 and BSE, but 87V remained strongly labeled by P4 antibody. Whether this might be associated with the higher molecular mass of diglycosylated PrP^{res} also found in this strain remains to be determined. Also, it was recently described that, by using immunohistochemical methods, different scrapie sources could differ in their labeling with different antibodies, including P4 monoclonal antibody (26). Immunological characterization of strains suggesting the existence of PrP conformational differences has already been reported for scrapie-infected hamsters and more recently for bovine transgenic mice infected with BSE or variant CJD by using a conformation-dependent immunological assay of abnormal PrP prior to proteinase K digestion (46, 47).

Together these data show the usefulness of PrP^{res} immunological characterization, which could allow the demonstration of a greater molecular diversity of scrapie strains than previously thought. Whereas further studies will describe the biological features of strains in this model, the experimental host expressed PrP from the natural host of the scrapie disease, thus importantly providing further insights in the poorly understood issue of the existence of different strains of TSE infectious agents in sheep disease.

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