Reduced susceptibility to bovine spongiform encephalopathy prions in transgenic mice expressing a bovine PrP with five octapeptide repeats

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In this work, transgenic (Tg) mice were generated expressing a bovine prion protein containing five octarepeats (BoPrP 5OR -Tg). After intracerebral inoculation of bovine spongiform encephalopathy (BSE) inoculum, these mice suffered a BSE-like neuropathology but survived longer compared with homologous Tg mice expressing similar levels of a six octarepeat BoPrP protein (BoPrP 6OR -Tg). De novo-generated five octarepeat (5OR) PrP Sc showed no biochemical differences from 6OR-PrP Sc , and the proteinase K-resistant core (PrP res ) was biochemically indistinguishable from the 6OR counterpart. Lower susceptibility to BSE is suggested for BoPrP 5OR -Tg mice, as they were not as efficient at replicating BSE prions from the same natural source inoculum as BoPrP 6OR -Tg mice expressing similar PrP C levels. These results raise the possibility of selecting cattle breeds bearing the 5OR Prnp allele that are less susceptible to prion infection.

INTRODUCTION

In bovine spongiform encephalopathy (BSE), as in all known transmissible spongiform encephalopathies (TSEs), the central event appears to be the misfolding of the prion protein (PrP) and the subsequent accumulation of protease-resistant deposits of misfolded PrP (PrPSc) in the brain (Prusiner, 1991). Bovine PrP is the product of a single Prnp gene, which encodes a glycosylphosphatidylinositol-attached lipid raft-associated membrane glycoprotein of 250 aa (Oesch et al., 1985). A characteristic stretch of repeats of an octapeptide motif is present close to the N terminus of the protein. In cattle, some degree of polymorphism in the number of recurrent octapeptides has been described (Goldmann et al., 1991; Yoshimoto et al., 1992; Hunter et al., 1994; Neibergs et al., 1994; Schlapfer et al., 1999; Walawski & Czarnik, 2003). Previously, we and others have shown that the number of octapeptide repeats (ORs) can be inversely associated with incubation times after experimental intracerebral inoculation (Chiesa et al., 1998; Castilla et al., 2004, 2005). Thus, transgenic mice bearing a bovine 7OR- or 10OR-PrP showed reduced survival times compared with mice bearing the wild-type bovine 6OR protein. As the 5OR polymorphism is also present in cattle populations, we decided to assess the effect, if any, of this allelic variant on susceptibility to BSE infection. For this purpose, we generated transgenic mice lines expressing a bovine 5OR-PrP C , characterized the expression of 5OR-PrP C in these mice and performed infectivity studies to evaluate their susceptibility to disease.

METHODS

Plasmid constructs and generation of transgenic mice. The open reading (ORF) of the bovine PrP gene was amplified by PCR cycling on DNA purified from bovine muscle tissue, using primers including a XhoI (underlined) restriction enzyme site adjacent to the translation start codon (5'-CCGCTCGAGGCATCATGGTGAAAA-GCCATATAG-3') and to the stop site (5'-CGGCTCGAGCTATCC-TACTATGAG-3'). The 5' primer also included Kozak sequences. The PCR fragment was subcloned into a T-tailed vector (Promega) and the insert was sequenced, confirming the presence of five copies of the OR sequence and the absence of changes in the inferred amino acid sequence compared with previously reported bovine PrP sequences (GenBank accession no. AF455119). The 5OR-PrP ORF was excised from the T-tailed vector by XhoI digestion and inserted in the vector...
MoPrP.Xho (Borchelt et al., 1996), previously digested with XhoI. This vector contains the murine PrP promoter, exon 1, intron 1, exon 2 and 3′ untranslated sequences. Transgenic mice were generated by microinjection of mouse oocytes with linearized plasmid MoPrP.Xho as reported previously (Castilla et al., 2003, 2004, 2005). The presence of MoPrP.Xho bovine transgenes were identified by a PCR assay using specific primers for the mouse PrP exon 2 and bovine PrP ORF. The primers used were 5′-CCAGCTTCCACCCATGTGGC-3′ and 5′-CATTTCGCCCTCTTAGTTGACC-3′. The presence of a 291 bp PCR product was indicative of a MoPrP.Xho 50R transgene. The absence of the endogenous murine PrP ORF was confirmed by PCR using primers 5′-ATGGCCGACCTTGGCTACTGGC-3′ and 5′-GATTATGGTTACCCCTCCTTG-3′.

Source of inoculum: preparation of brain homogenates. BSE-infectious material for inoculation purposes was supplied by the Veterinary Laboratory Agency (VLA, Surrey, UK). A single BSE-infected brainstem (VLA reference Q 225: PG1199/00), designated BSE2, was used for comparative studies of infection in transgenic mice expressing either 50R- or 60R-PrPC. A pool of BSE material obtained from the brainstem of 49 BSE-infected calf (reference TSE/08/59), designated BSE0, was also used. The infectious titre of BSE0 was ~10^8 ID_{50} (g bovine brainstem)^{-1} (Castilla et al., 2003). No estimation of infectious titre was available from BSE2, but it contained 8- to 16-fold more PrP^Sc than the BSE0 inoculum as judged by immunoblot analysis (Castilla et al., 2004). A brain homogenate from a healthy cow diagnosed as negative for PrPres was used as a negative control. Brain homogenates (10% in PBS without CaCl_2 or MgCl_2) were prepared in sterile PBS without CaCl_2 or MgCl_2 using mechanical homogenization (OMNI International). To minimize the risk of bacterial infection, all inocula were cleared by centrifugation at 2000 g for 30 min. Samples were treated with 20 gM guanidine isothiocyanate for 2 h at 4°C, and treated with 98% formic acid for 15 min at room temperature. Samples for PrP^Sc labelling were rehydrated, pre-treated with 98% formic acid for 15 min at room temperature and 4 M guanidine isothiocyanate for 2 h at 4 °C, and treated with PK (4 µg ml^{-1} in Tris/HCl, pH 7.8) for 15 min at 37 °C. Tissue sections were rinsed in PBS (pH 7.4) and blocked with 10% normal goat serum (Sigma) for 30 min at room temperature. Samples were incubated overnight at 4 °C with mAb 2A11 (tissue culture supernatant diluted 1:400 in PBS). A secondary biotinylated goat anti-mouse IgG (Dako) diluted 1:200 in PBS and an avidin–peroxidase complex (Vector) were used. The reaction was visualized by the application of 3,3′-diaminobenzidine (1 mg ml^{-1}; Sigma) and H_2O_2, before counterstaining with Harris’s haematoxylin, dehydration and routine assembly. Samples for GFAP labelling were rehydrated without pre-treatment and blocked as described above. Sections were incubated overnight at 4 °C with primary anti-bovine GFAP polyclonal antibody (Dako) diluted 1:500 in PBS. For immunoblotting, mAb 2A11 (Brun et al., 2004) was used at a 1:1000 dilution and mAbs Sha31, 12B2 and 9A2 (Féraudet et al., 2004; Vuill et al., 2006) were used at a concentration of 1 µg ml^{-1}. Immuno complexes were detected using a horseradish peroxidase-conjugated anti-mouse IgG. Development of immunoblots was carried out using enhanced chemiluminescence. ELISA was used for the detection of PrP^Sc in brains using the commercial TeSeE assay (Bio-Rad). A modification of this assay in which the PK treatment and protein precipitation steps were omitted was used for measuring PrP^Sc content in transgenic brain samples.

Infection experiments. Groups of 8–12 mice (6–7 weeks old, weighing approximately 20 g) were housed following the guidelines of the Code for Methods and Welfare Considerations in Behavioural Research with Animals (Directive 86/609/EC). Inoculations were performed into the right parietal lobe using a 25-gauge disposable hypodermic needle. Twenty microlitres of 10% brain homogenate was delivered to each animal. When progression of the disease was evident, animals were sacrificed and their brains were removed for analysis. These samples were used to determine spongiform degeneration by histopathology and PrP^Sc accumulation in brain preparations by immunohistochemistry and Western blot.

Histopathology and immunohistochemistry. Formalin-fixed brains were cut into four pieces and immersed in 98% formic acid for 1 h before routine processing and embedding in paraffin wax. Serial sections of 4 µm nominal thickness were cut and stained with haematoxylin and eosin (H&E) for routine histopathological examination and for immunohistochemical techniques. Sections were taken from (a) the medulla oblongata at the level of the obex (myelencephalon); (b) the cerebellum; (c) the thalamus/hypothalamus (diencephalon); (d) the hippocampus; (e) the cerebral cortex (frontal and occipital areas); (f) the corpus striatum; and (g) the midbrain (mesencephalon). Sections were scored blindly for spongiform changes, gliosis (astrocytosis and microgliosis), eventual neuronal changes and PrP deposits at any level. The avidin–biotin–peroxidase complex technique was used for the immunohistochemical study of PrP^Sc and glial fibrillary acid protein (GFAP). After dewaxing and dehydration, endogenous peroxidase was quenched by incubation with 3% hydrogen peroxide in methanol for 30 min at room temperature. Samples for PrP^Sc labelling were rehydrated, pre-treated with 98% formic acid for 15 min at room temperature and 4 M guanidine isothiocyanate for 2 h at 4 °C, and treated with PK (4 µg ml^{-1} in Tris/HCl, pH 7.8) for 15 min at 37 °C. Tissue sections were rinsed in PBS (pH 7.4) and blocked with 10% normal goat serum (Sigma) for 30 min at room temperature. Samples were incubated overnight at 4 °C with mAb 2A11 (tissue culture supernatant diluted 1:400 in PBS). A secondary biotinylated goat anti-mouse IgG (Dako) diluted 1:200 in PBS and an avidin–peroxidase complex (Vector) were used. The reaction was visualized by the application of 3,3′-diaminobenzidine (1 mg ml^{-1}; Sigma) and H_2O_2, before counterstaining with Harris’s haematoxylin, dehydration and routine assembly. Samples for GFAP labelling were rehydrated without pre-treatment and blocked as described above. Sections were incubated overnight at 4 °C with primary anti-bovine GFAP polyclonal antibody (Dako) diluted 1:500 in PBS. A secondary goat anti-rabbit IgG (Vector) diluted 1:20 in PBS was used, and the rest of the technique was carried out as described above. Tomato (Lycopersicum esculentum) lectin was used for histochemical detection of glial cells. After dehydration and quenching of endogenous peroxidase, sections were incubated overnight with biotin-conjugated L. esculentum lectin (6 µg ml^{-1} in 10% normal goat serum; Sigma) at 4 °C. Sections were then incubated with avidin–peroxidase complex (Sigma) for 1 h at room temperature, washed three times in PBS and the rest of the technique carried out as described above. Specific primary antibodies were replaced by PBS, and non-immune mouse serum or non-immune rabbit serum in tissue sections was used as the negative control. All histological changes were graded semi-quantitatively on a scale of 0–3.

Statistical analysis. Data handling, analysis and graphical representation were performed using PRISM 2.01 (GraphPad Software). Statistical differences were determined using Student’s t-test for non-paired variants. Differences were considered significant at a value of P<0.05.
RESULTS

Generation of bovine Prp5OR-Tg mouse lines and Prp5 expression analysis

Four different lines (founders) carrying the bovine (bo) 5OR-PrPC (bo5ORPrPC) were obtained. All of these lines also bore wild-type murine (mo) 5OR-PrPC (mo5ORPrPC). The expression of PrPC in these lines carrying both endogenous mo5ORPrPC and bo5ORPrPC transgenes (PrP mo+/− bo+/−) was evaluated by subjecting brain homogenates to Western blotting analysis using mAb FH11, which does not recognize endogenous muPrP (not shown). Two PrP mo+/− bo+/− lines, BoPrP5OR-Tg004 and BoPrP5OR-Tg008, were selected based on their expression levels and were bred to homozygosity in a murine PrP null background (PrP mo−/−). This was achieved by crossing the selected lines with PrP-null mice to obtain transgene heterozygous lines (PrP mo−/− bo+/−) and then crossing heterozygous animals to obtained the transgene genotype (PrP mo−/− bo+/−). The absence of the murine PrP gene in these animals was confirmed by PCR using specific primers (data not shown).

Transgenic PrPC expression in homozygous mice was then checked by Western blotting using a specific anti-PrP monoclonal antibody (2A11). Equivalent total brain protein samples, confirmed by checking the actin content, were separated by SDS-PAGE (Fig. 1a). In all mouse lines tested, the SDS-PAGE banding pattern of PrP proteins was similar, although PrP expression levels varied among the different lines obtained. PrP levels from lines BoPrP5OR-Tg008 and BoPrP5OR-Tg004 were compared with those of mouse lines BoPrP6OR-Tg022 and BoPrP6OR-Tg078 expressing a bovine 6OR-PrP (Castilla et al., 2003). Whereas BoPrP5OR-Tg008 expressed higher levels of PrP, both BoPrP6OR-Tg022 and BoPrP5OR-Tg004 showed similar levels, although lower that those of BoPrP5OR-Tg008. The relative levels of BoPrP6OR-Tg022 were established previously to be half that of cattle brain PrP (Castilla et al., 2003). Therefore, for comparison purposes, we concluded that BoPrP5OR-Tg004 PrP expression levels were ~0.5-fold, whilst those of BoPrP5OR-Tg008 were ~1-fold. This conclusion was further supported using a brain PrP capture ELISA using serial dilutions of equivalent total protein concentrations (data not shown).

To confirm the theoretical difference in electrophoretic mobility, brain PrP from both BoPrP5OR-Tg and BoPrP6OR-Tg mice was subjected to deglycosylation experiments using PNGase F (Fig. 1b). As expected, following SDS-PAGE, brain PrP expressed in BoPrP5OR-Tg mice displayed a lower apparent relative molecular mass than BoPrP6OR-Tg mice. Moreover, the reduction of one OR did not alter the biochemical properties of PrP; it showed no increased solubility and showed a similar sensitivity to protease. These results indicated that PrP from BoPrP5OR-Tg mice is likely to be processed in a similar way to PrP from BoPrP6OR-Tg mice.

Fig. 1. Expression of bovine 5OR-PrP proteins in bovine Tg mouse lines. (a) Immunoblotting of brain extracts from BoPrP5OR-Tg mouse lines 004 and 008, and BoPrP6OR-Tg from mouse lines 022 and 078. Equivalent protein loads of brain homogenates were analysed by Western blotting using mAb 2A11 and anti-actin mAb. (b) Endoglycosidase treatment of equivalent protein brain extracts from BoPrP5OR-Tg and BoPrP6OR-Tg mice using PNGase F enzyme. Control: cow brain extract. Molecular mass markers (lane M) are shown in kDa. Arrows indicate the position of unglycosylated 5OR-PrPC and 6OR-PrPC.

Susceptibility of BoPrP5OR-Tg mice to BSE infection

To assess the effect of OR reduction on susceptibility to BSE prions, mice were inoculated with a 10% brain homogenate of 6OR-PrPSc-containing BSE2 inoculum into the right parietal lobe. As a control, mice were also inoculated with a homogenate prepared from a healthy cow brain that showed no detectable PrPSc. Whereas BoPrP5OR-Tg004 mice survived for 635 ± 31 days after inoculation (mean ± SEM), BoPrP6OR-Tg022 mice expressing similar levels of a bovine 6OR-PrP transgene survived for only 475 ± 24 days (Table 1). Thus, 6OR-PrPSc propagation in 5OR-PrPC-expressing mice was not as efficient as it was in 6OR-PrPSc mice. Kaplan–Meier representation of survival times reflected significant differences between both mouse groups (Fig. 2a). In contrast, no significant differences in survival times were found when comparing BoPrP5OR-Tg004 mice inoculated with BSE2 (635 ± 31 days) with those inoculated with the healthy brain control (622 ± 34 days) (Table 1 and Fig. 2b). These results were supported by the
fact that only 50% of 5OR mice showed detectable levels of PrPres in their brains (Table 1). In agreement with these results, BoPrP5OR-Tg004 mice inoculated with the inoculum containing less PrPSc (BSE1) survived for $691 \pm 24$ days with a similar attack rate (60%) (Fig. 2c and Table 1).

As expression levels modulate survival time after prion inoculation, the expected survival times for BoPrP5OR-Tg008 mice expressing 2-fold higher PrP C levels of BoPrP6OR-Tg022 should be lower. However, in spite of the different expression levels, the mean survival times

### Table 1. Susceptibility of BoPrP5OR-Tg mice to BSE prions

<table>
<thead>
<tr>
<th>Recipient*</th>
<th>Inoculum</th>
<th>Expression level (fold)†</th>
<th>Death (days ± SEM)</th>
<th>n/n₀‡</th>
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<tr>
<td>MoPrP₂⁻/⁻-Tg</td>
<td>BSE₂</td>
<td>0.0</td>
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<td>0/9</td>
</tr>
<tr>
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<td>$475 \pm 24$</td>
<td>9/9</td>
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<td>BoPrP5OR-Tg004</td>
<td>BSE₂</td>
<td>0.5</td>
<td>$635 \pm 31$</td>
<td>5/10</td>
</tr>
<tr>
<td>BoPrP5OR-Tg008</td>
<td>BSE₂</td>
<td>1.0</td>
<td>$700 \pm 35$</td>
<td>3/5</td>
</tr>
<tr>
<td>BoPrP5OR-Tg022</td>
<td>BSE₁</td>
<td>0.5</td>
<td>$500 \pm 24$</td>
<td>9/9</td>
</tr>
<tr>
<td>BoPrP5OR-Tg004</td>
<td>BSE₁</td>
<td>0.5</td>
<td>$691 \pm 24$</td>
<td>6/10</td>
</tr>
<tr>
<td>BoPrP5OR-Tg008</td>
<td>BSE₁</td>
<td>1.0</td>
<td>$555 \pm 31$</td>
<td>9/12</td>
</tr>
<tr>
<td>BoPrP5OR-Tg004</td>
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<td>$622 \pm 34$</td>
<td>0/11</td>
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<tr>
<td>BoPrP5OR-Tg008</td>
<td>Cattle brain</td>
<td>1.0</td>
<td>$634 \pm 14$</td>
<td>0/9</td>
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<tr>
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<td>0.5</td>
<td>$714 \pm 24$</td>
<td>0/7</td>
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<tr>
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<td>1.0</td>
<td>$739 \pm 17$</td>
<td>0/9</td>
</tr>
</tbody>
</table>

*All mice lines were established in a murine PrP⁺/⁺ background. †Relative to cattle PrP expression. ‡Number of mice with detectable PrPres/number of inoculated mice.

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Fig. 2. Kaplan–Meier representations of the survival of Tg mice after intracerebral BSE inoculation. (a, c) BoPrP5OR-Tg004 (solid line) and BoPrP5OR-Tg0022 (dotted line) mice groups inoculated with BSE₂ (a) or BSE₁ (c) inoculum; (b) BoPrP5OR-Tg004 mice inoculated with either BSE₂ (solid line) or healthy (dotted line) brain control; (d) BoPrP6OR-Tg022 (solid line) or BoPrP5OR-Tg008 (dotted line) mice inoculated with BSE₁ inoculum. P values for a 95% confidence interval are given.
of BoPrP<sup>5OR</sup>-Tg008 mice were higher than those of BoPrP<sup>6OR</sup>-Tg022 mice for both BSE inocula tested (Fig. 2d and Table 1). Moreover, and supporting our data, independent of the inoculum used, the attack rate in BoPrP<sup>5OR</sup>-Tg008 mice was lower (75%) than in BoPrP<sup>6OR</sup>-Tg022 mice (100%) (Table 1). Thus, we suggest that propagation of 6OR BSE prions in 5OR-PrP mice is not as efficient as in 6OR-PrP mice.

**Immunohistochemical analysis**

Western blotting and immunohistology showed similar numbers of BoPrP<sup>5OR</sup>-Tg animals with PrP<sup>Sc</sup>. BSE-infected BoPrP<sup>5OR</sup>-Tg mice showed bilateral and symmetrical vacuolization in all of the analysed areas. Lesion profiles were similar in both BoPrP<sup>5OR</sup>-Tg004 and BoPrP<sup>5OR</sup>-Tg008 mouse lines. The distribution of the vacuolation pattern in these mice mainly targeted the midbrain, thalamus and medulla oblongata at the level of the obex and showed no differences when compared with BoPrP<sup>6OR</sup>-Tg022 mice. Spongiosis was always found associated with gliosis, either astrocytosis or microgliosis; immunolabelling with anti-GFAP antibody [Fig. 3a(ii, vi and x)] and with *L. esculentum* [Fig. 3a(iii, vii and xi)] revealed that these cells were associated with PrP<sup>Sc</sup> deposits [Fig. 3a(iv, viii and xii)]. Fine to coarse granular deposits of PrP<sup>Sc</sup> were scattered in the perikarya of neurons along with larger ovoid deposits in the cytoplasm of glial cells, mainly in the brain stem (data not shown). Punctate neuropil labelling was observed in several brain sites [Fig. 3a(xii)]. However, the most prominent labelling was in the form of plaque-like deposits [Fig. 3a(iv and xii), arrowheads], which appeared scattered in all brain areas except the cerebral cortex.

To examine possible differences in PrPres profiling, PrPres deposits in seven different regions of each mouse brain from the two 5OR-PrPC mouse Tg lines were compared with profiling of a 6OR-PrPC-expressing mouse Tg line (Fig. 3b). We observed that PrPres deposition patterns for BSE-infected BoPrP<sup>5OR</sup>-Tg mice were strikingly similar to those of infected BoPrP<sup>6OR</sup>-Tg mice. No mice showed either ependymal or vascular patterns, and glial-associated PrPres staining was very light. In contrast, intracellular PrPres and neuropil deposits were abundant. Both 5OR-PrPC mouse Tg lines showed accumulation of large amounts of amyloid plaque-like deposits. Overall, no
differences in neuropathological findings could be found in the infected BoPrP5OR-Tg mice compared with infected BoPrP6OR-Tg mice.

**Characterization of new Bo5OR-PrPSc**

The newly formed Bo5OR-PrPSc was analysed biochemically for insolubility and PK sensitivity. No differences in insolubility or PK sensitivity were found (data not shown). To analyse in more detail the length of the protease-resistant fragment after PK treatment, different antibodies mapping near to the N-terminal site of the PK-resistant PrP core were used (Yull et al., 2006). As shown in Fig. 4(a), PrP(res) from both BoPrP5OR-Tg and BoPrP6OR-Tg mice was readily detected by mAb Sha31, which recognizes an epitope within the resistant core of PrP(res), and mAb 9A2, which recognizes an epitope at the N terminus of the PrP(res) fragment (Fig. 4b). However, little or no signal in either BoPrP5OR-Tg or BoPrP6OR-Tg mice was found when mAb 12B2 was used, indicating that PK cleavage resulted in loss of the 12B2 epitope seen in both sets of animals (mAb 12B2 recognizes the epitope in bovine PrP(C)) (data not shown). These results indicated that both 5OR-PrPSc and 6OR-PrPSc are similarly processed after PK treatment and, therefore, a similar structure is likely to be shared by PrPSc aggregates from both 5OR and 6OR mice. Moreover, analysis of the PrP(res) glycoform ratios did not reveal any significant differences (Fig. 4c), indicating that both proteins retained similar post-translational modifications.

**DISCUSSION**

It is known that, in some TSEs, the existence of genetic polymorphisms within the Prnp gene affects susceptibility to disease. An example is the known three-codon polymorphism in sheep that has formed the basis for the selection of scrapie-resistant breeds (Hunter, 1997). Such polymorphic point mutations have not been associated with bovine breeds, but the presence of polymorphic variations in the number of ORs within the Prnp gene has been reported. Both in observed clinical cases and by the use of transgenic mice, it has been shown that the number of ORs affects either spontaneous onset of the disease or strongly influences incubation times (Goldfarb et al., 1991; Poulter et al., 1992; Chiesa et al., 1998; Vital et al., 1998; Castilla et al., 2004, 2005). Thus, an inverse relation might exist in which the higher the number of ORs, the shorter the incubation/survival time. Mouse models have proved very useful in understanding inter- and/or intraspecific transmission barriers to prions. It is known that overexpression of PrP transgenes in mouse models can shorten the length of incubation times considerably and, consequently, shorten survival times. Our BoPrP5OR-Tg mouse lines expressed low levels of 5OR-PrPC contributing to lengthy survival times. However, when compared with other mouse Tg lines expressing similar, near to physiological, low levels of 6OR-PrPC, the survival times were significantly increased, in agreement with reported incubation times for gene-targeted bovine Tg mice (Bishop et al., 2006). Thus, considering expression levels as a major factor affecting the

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**Fig. 4.** Characterization of PrPSc from BSE-inoculated BoPrP5OR-Tg mice. (a) Comparison of electrophoretic profiles and antibody labeling of PrP(res) after Western blot detection using mAbs 12B2 (epitope 93WGQGG97), 9A2 (epitope 102WNK104) and Sha31 (epitope 148YEDRYYRE155). Equivalent PrP res quantities were loaded in each lane. The membrane was cut and immunoblotted with the indicated antibody. (b) Schematic diagram of the mature PrP polypeptide indicating the epitope locations of mAbs used in (a). Open boxes represent areas of α-helix structure and filled boxes represent predicted β-sheet structures. (c) Representation of Western blot profiles of the relative ratios of PrP(res) detected with mAb Sha31 in brain extracts from the indicated BSE-infected transgenic lines. Data shown are the means of six or more measurements obtained from density scans in at least two different Western blots. To interpret the plot, read the values for the diglycosyl, monoglycosyl and aglycosyl fractions along the bottom, right and left axes of the triangle respectively. For each point, the sum of the three values is 100.
length of incubation/survival times, it was possible to associate the extended survival rate with the presence of 5OR-PrPSc. One explanation for this could be that the transformation rate of 5OR-PrPSc to 5OR-PrPSc by means of an incoming 6OR-PrPSc is less efficient than that for 6OR-PrPSc; this is also supported by the limited number of inoculated BoPrP5OR-Tg mice that scored positive for PrPRes. The biochemical properties of 5OR-PrPSc were the same as 6OR-PrPSc in terms of insolubility, PK sensitivity, PrPRes glycoform ratio and PrPRes epitope mapping. Moreover, on the basis of immunohistological examination, it was not possible to determine differences in the pattern of brain lesions between BoPrP5OR-Tg and BoPrP6OR-Tg mice. In spite of the lack of biochemical and pathological differences between 5OR-PrPSc and 6OR-PrPSc, it remains to be determined whether the infectivity of both types of prion in homologous PrPC-expressing mice is similar. In other words, do the survival and attack rates for 5OR-PrPSc in BoPrP5OR-Tg mice differ from those for 6OR-PrPSc in BoPrP6OR-Tg mice? Experiments are in progress to assess the infectivity of both 5OR and 6OR bovine prions in homozygous 5OR/5OR and 6OR/6OR mice, as well as in heterozygous 5OR/6OR Tg mice.

In cattle, the presence of five, six and seven ORs in the Prnp gene has been reported (Goldmann et al., 1991; Brown et al., 1993; Neibergs et al., 1994; Ferguson et al., 1997; Schlapfer et al., 1999). Due to scarce epidemiological data, it remains to be established whether or not there is an association between these polymorphisms and the prevalence of BSE in cattle. However, some epidemiological considerations might support the evidence for a genetic basis in the reported BSE incidence. The dispersal of BSE cases has been shown to be different depending on the country affected (Hagenaars et al., 2000). In contrast, homogenous animals bearing 5OR Prnp alleles have never been found in BSE-affected cows (Goldmann et al., 1991; Yoshimoto et al., 1992; Neibergs et al., 1994), although the frequency of this genotype is usually very low, particularly in the Frisona-Holstein breeds (Brown et al., 1993). The allele encoding 6OR-PrP seems to be the most frequent in cattle. Based on these data, it is reasonable to assume that the presence in nature of 5OR prions may be less frequent than 6OR prions.

The significant delay in survival times and the reduced attack ratio observed in BoPrP5OR-Tg mice after inoculation of a BSE inoculum reflects a lower efficiency of prion replication, which is probably due to a reduced ability for transformation of 5OR-PrPSc into 5OR-PrPSc. This fact might substantiate the genetic basis for the low incidence of BSE in 5OR-PrPSc-expressing cattle.

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