FEBS 30096 FEBS Letters 579 (2005) 6237–6246

# Transgenic mice expressing bovine PrP with a four extra repeat octapeptide insert mutation show a spontaneous, non-transmissible, neurodegenerative disease and an expedited course of BSE infection

J. Castilla[a], A. Gutiérrez-Adán[b], A. Brun[a], B. Pintado[b], F.J. Salguero[a], B. Parra[a], F. Díaz San Segundo[a], M.A. Ramírez[b], A. Rábano[c], M.J. Cano[a], J.M. Torres[a,*]

[a] *Centro de Investigación en Sanidad Animal, INIA, 28130 Valdeolmos, Madrid, Spain*
[b] *Departamento de Reproducción Animal y Conservación de Recursos Zoogenéticos, INIA, 28040 Madrid, Spain*
[c] *Laboratorio de Neuropatología, Fundación Hospital Alcorcón, 28922 Alcorcón, Madrid, Spain*

Received 26 July 2005; revised 1 September 2005; accepted 22 September 2005

Available online 19 October 2005

Edited by Berend Wieringa

**Abstract** **Transgenic (Tg) mice carrying four extra octapeptide repeats (OR) in the bovine PrP gene (10OR instead of 6) have been generated. In these mice, neuropathological changes were observed depending upon the level of transgene expression. These changes primarily involved a slowly advancing neurological disorder, characterized clinically by ataxia, and neuropathologically, by vacuolization in different brain areas, gliosis, and loss of cerebellar granule cells. Accumulation of insoluble bovine 10OR-PrP (bo10OR-PrP) was observed depending on the level of expression but no infectivity was found associated with this insoluble form. We also compared the behavior of bo6OR-PrP and bo10OR-PrP Tg mouse lines in response to BSE infection. BSE-inoculated bo10ORTg mice showed an altered course of BSE infection, reflected by reduced incubation times when compared to bo6ORTg mice expressing similar levels of the wild type 6OR-PrP. In BSE-inoculated mice, it was possible to detect $PrP^{res}$ in 100% of the animals. While insoluble bo10OR-PrP from non-inoculated bo10ORTg mice was non-infectious, brain homogenates from BSE-inoculated bo10ORTg mice were highly infectious in all the Tg mouse lines tested. This Tg mouse model constitutes a new way of understanding the pathobiology of bovine transmissible spongiform encephalopathy. Its potential applications include the assessment of new therapies against prion diseases.**


*Keywords:* Genetic encephalopathy; Transgenic; Prion; Octapeptide repeats; Scrapie; PrP; Neuropathology

## 1. Introduction

Transmissible spongiform encephalopathies (TSEs), also termed prion diseases, comprise a group of neurodegenerative disorders in which spongiosis, astrocytosis, microglial activation and neuronal loss are common pathological events of the central nervous system (CNS). Prion diseases of infectious aetiology have been described in animals, i.e., BSE and Scrapie [1–3] as well as in humans, i.e., kuru and vCJD [4,5]. Spontaneous and familial TSE aetiology have been only described in humans including Creutzfeldt–Jakob disease (CJD) [6], Gerstsmann–Sträussler–Scheinker syndrome [7,8] and fatal familial insomnia [9–13]. It is generally accepted that misfolding of the cellular prion protein ($PrP^{C}$) leads to the accumulation in the brain of an insoluble, toxic PrP isoform ($PrP^{Sc}$). PrP is the product of the *Prnp* gene constitutively expressed in the brain as a cell-surface, GPI anchored, glycoprotein containing a region of octapeptide repeats (OR) close to the N-terminus. Point mutations and extra octapeptide insertions in the PrP protein have been found in familial TSEs. Several lines of transgenic (Tg) mice have been developed to model the pathophysiology of these mutation-associated TSE disorders [14,15]. In these mice spontaneous neurological illness develops, resembling common features of prion disorders. In addition, the severity of the spontaneous prion disease is influenced by the number of extra OR inserted [16].

A pathogenic insertion has never been described in cattle. Tg mice expressing bovine PrP with one extra OR insertion (7OR-PrP) show reduced incubation and survival times after BSE prion inoculation compared to boTg mice with the wild type (wt) 6OR-PrP [17], yet no neuropathological disorders have been related to natural cases of cows with 7OR-PrP or observed in Tg mice [18,19].

In this study, we show the generation of a Tg mouse carrying a transgene encoding bovine PrP with four extra OR insertion (10OR-PrP) as a model of "genetic" bovine encephalopathy. A slowly progressive neurological disorder characterized by ataxia and neuropathological changes was observed when the level of 10OR-PrP expression was high. A new insoluble and weakly proteinase-K resistant bovine 10OR-PrP form was found but no infectivity could be associated to it. Tg mice expressing low levels of 10OR-PrP, did not show any neurological sign but showed reduced incubation and survival times after BSE prion inoculation compared to Tg mice expressing the wt bovine 6OR-PrP. These results confirm that human PrP-associated insertional mutations confer identical neuropathological phenotypes within the context of a bovine PrP. In addition, these findings suggest a role for the OR gene region in toxicitypathology and in efficiency of prion conversion and propagation.

*Corresponding author. Fax: +34 91 620 22 47.
*E-mail address:* jmtorres@inia.es (J.M. Torres).

0014-5793/$30.00 
doi:10.1016/j.febslet.2005.09.099

## 2. Materials and methods

### 2.1. Plasmid constructs

The open reading (ORF) of the bovine PrP gene was isolated by PCR amplification from bovine DNA using primers that create a *Xho*I restriction enzyme site adjacent to the translation start codon (5′-CCGCTCGAGGCCATCATGGTGAAAAGCCATATAG-3′) and to the stop site (5′-CGGCTCGAGCTATCCTACTATGAG-3′). The 5′ primer also included Kozak sequences [20]. The PCR fragment was subcloned into a T-tailed vector and the insert was sequenced, confirming six copies of the OR sequence and no changes in the inferred amino acid sequence with respect to previously sequenced bovine PrP genes (GenBank Accession No. AF455119). The four extra octarepeats were introduced into the six octarepeat bovine PrP gene as follows: 5′-CATGGAGGTGGCTGGGGCCAGCCC-3′ and 5′-CATGGGGCTGGCCCCAGCCACCTC-3′ primers were used to obtain a tandem of 4 artificial octarepeats containing ends compatible with the *Nco*I restriction enzyme site. The PrP ORF was partially cut with *Nco*I to avoid eliminating the constitutive OR, and the tandem of 4 artificial octarepeats containing extremes compatible with *Nco*I was inserted (supplemental figure 1). The six (6OR) and ten (10OR) octarepeat PrP ORFs were excised from the T-tailed vector with the restriction enzyme *Xho*I and inserted into MoPrP · Xho [21], also digested with *Xho*I. This vector contains the murine PrP promoter and exon 1, intron 1, exon 2 and 3′ untranslated sequences.

### 2.2. Generation of Tg mice

MoPrP · Xho bovine transgene (6OR and 10OR) containing fragments were excised from the plasmid vector with the restriction endonuclease *Not*I to give rise to a 12.1 kb DNA fragment that was subsequently purified using sodium chloride gradients as previously described [39]. Finally, the DNA for microinjection was resuspended in TE (10 mM Tris, pH 7.4, 0.1 mM EDTA) at a final concentration of 2–6 μg/ml and used to microinject 350 or 257 pronuclear stage ova for the 6OR or 10OR constructs, respectively. One-cell embryos were obtained from superovulated B6CBAf1 females mated to 129/Ola males carrying a null mutation in endogenous PrP [22] and were microinjected. Homozygous Tg lines were established in two steps. Founders were backcrossed to homozygous null animals muPrP$^{-/-}$ (*Prnp*$^{-/-}$) to obtain homozygosity for the null mutation. Interbreeding within the same Tg line was performed to obtain homozygosity for the bovine PrP constructs. Hybrids B6CBA × 129/Ola mice and *Prnp*$^{-/-}$ mice were used as controls in all the infectivity studies.

### 2.3. Screening of founders

DNA was prepared from tail biopsies as previously described [23]. The MoPrP · Xho bovine transgenes were identified by a PCR assay using specific primers for the mouse PrP exon 2 and the bovine PrP open reading frame. The primers used were 5′-CCAGCCTCCACCACCATGTGGC-3′ and 5′-CATTCTGCCTTCCTAGTGGTACC-3′. The presence of PCR amplified products of 315 and 411 nucleotides was indicative of MoPrP · Xho 6OR and 10OR bovine transgenes, respectively. The absence of murine PrP ORFs in Tg mice was confirmed by PCR using the primers 5′-ATGGCGAACCTTGGCTACTGGC-3′ and 5′-GATTATGGGTACCCCCTCCTTGG-3′.

### 2.4. Source of BSE inoculum – Preparation of brain homogenates

A pool of BSE material (TSE/08/59) obtained from the brainstem of 49 BSE infected cattle and supplied by the Veterinary Laboratory Agency (VLA) (New Haw, Addlestone, Surrey, UK) was used for most of the experimental inoculations and was denoted $BSE_1$. A different BSE inoculum (RQ 225:PG1199/00), $BSE_2$, obtained from one BSE infected brainstem supplied by the VLA was used for comparative infection studies along with $BSE_1$. The $BSE_2$ inoculum contains 8- to 16-fold more $PrP^{res}$ than $BSE_1$, as detected by immunoblot analysis [18]. Pools of brain homogenates from a non-inoculated bo10ORTg mouse line sacrificed 150 days after birth containing high amounts of insoluble 10OR-PrP were used in infectivity experiments and denoted bo10ORTg006. Finally, pools from a first passage of $BSE_1$ inoculum in the mouse line bo10ORTg012 were used for some experiments and referred to as bo10ORTg$BSE_1$. The $BSE_2$ inoculum also contains a greater amount of $PrP^{res}$ than the bo10ORTg$BSE_1$ inoculum, as detected by immunoblot analysis (supplemental figure 2). In all the experiments, phosphate buffered saline (PBS) was used as a negative inoculation control and brain homogenates (10% wt/vol) were prepared in sterile PBS lacking $Ca^{2+}$ or $Mg^{2+}$ by mechanical homogenization (OMNI International, Warrenton, VA, USA). To minimize the risk of bacterial infection, all inocula were preheated for 10 min at 70 °C before being used to inoculate mice.

### 2.5. Infection experiments

Groups of 4–13 mice (six to seven weeks old, weighing approximately 20 g) were inoculated in the right parietal lobe using a 25 gauge disposable hypodermic syringe. 20 μl of 10% brain homogenate were delivered to each animal. To evaluate the clinical signs appearing after inoculation, mice were observed daily and their neurological status was assessed twice-weekly. The presence of two or three signs of neurological dysfunction (using 10 different diagnostic criteria) [24,25] was necessary to score a mouse positive for prion disease. When progression of the disease was evident, animals were culled for ethical reasons and their brains harvested for analysis. These specimens were used to determine $PrP^{res}$ deposits in brain preparations by either Western blot or immunohistochemistry (IHC) a for histopathology and IHC studies.

### 2.6. Histopathology and IHC

Brains were fixed by immersion in 10% buffered formalin for 1 week and then cut into four and placed in 98% formic acid for 1 h before routine processing and paraffin wax embedding. 5 μm-thick sections were cut and stained with hematoxylin and eosin (HE) or Nissl's stain for routine histopathological examination and for immunohistochemical techniques. Sections were examined from: (a) the medulla oblongata at the level of the obex and the pontine area; (b) the cerebellum; (c) the diencephalon including thalamus; (d) the hippocampus and (e) the cerebral cortex. We assessed spongiform changes, astrocytosis, eventual neuronal changes and PrP deposits at any level. All histological changes were graded semi quantitatively on a 0–3 scale basis.

The avidin–biotin–peroxidase complex technique was used for the immunohistochemical study of $PrP^{res}$ 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 $PrP^{res}$ labeling were rehydrated, pretreated with 98% formic acid for 15 min at room temperature, 4 M guanidine isothiocyanate for 2 h at 4 °C, and proteinase K (Roche, Germany) treated (4 μg/ml in Tris–HCl, pH 7.8) for 15 min at 37 °C. Tissue sections were rinsed in phosphate buffered saline (PBS, pH 7.4, 0.01 M) and blocked with 10% normal goat serum (Sigma Chemical Co.) for 30 min at room temperature. Samples were incubated overnight at 4 °C with primary 6H4 monoclonal antibody (mAb) (Prionics, Switzerland) or 2A11 mAb [26] diluted 1:400 in PBS. A secondary biotinylated goat anti-mouse Ig G (Dako, Glostrup, Denmark) antibody diluted 1:20 in PBS and an avidin–peroxidase complex (Vector, Burlingame, UK) were used. Sections were developed using a 3,3′-diaminobenzidine tetrahydrochloride substrate (Sigma). The slides were then counterstained with Mayer's hematoxylin for 1 min, dehydrated and routinely assembled.

Samples for GFAP labeling were rehydrated without pretreatment and blocked as described above. Sections were incubated overnight at 4 °C with the primary anti-bovine GFAP polyclonal antibody (Dako) diluted 1:500 in PBS. Goat anti-rabbit Ig G (Vector) diluted 1:200 in PBS was used as the secondary antibody and the rest of the technique was continued as described above. As negative controls, specific primary antibodies were replaced with PBS, and non-immune mouse serum or non-immune rabbit serum were used in tissue sections.

### 2.7. Insolubility and PK resistance studies of $PrP^{C}$ and $PrP^{res}$ from non-inoculated or BSE-inoculated bo6ORTg and bo10ORTg mouse lines

For the insolubility studies, brain homogenates from non-inoculated bo6ORTg and bo10ORTg mouse lines were homogenized in 10% Sarkosyl in PBS, pH 7.4 and the complete™ cocktail of protease inhibitors (Roche). 100 μl of the samples were pre-cleared by centrifugation at 2000 × *g*. Soluble (S) and insoluble (P) fractions were obtained by ultracentrifugation at 100 000 × *g* for 1 h. Insoluble fractions were washed exhaustively in 5% Sarkosyl in PBS, pH 7.4 and subjected to a further ultracentrifugation step. For PK resistance studies, non-inoculated and BSE-inoculated brain homogenates from bo6ORTg110 [27] or bo10ORTg012 mouse lines and from cattle were digested with 0, 7.5, 15, 30, 60, 125, 250, 500 and 1000 μg/ml of PK for 60 min at

37 °C. An equal volume of 2× SDS sample loading buffer was then added to all the samples and each was boiled for 5 min before loading onto a SDS/12% polyacrylamide gel. The Mab 2A11 was used at a 1/1000 dilution for immunoblotting. Immunocomplexes were detected using a horseradish peroxidase conjugated anti mouse IgG (Sigma Chemical Co.) antibody. Immunoblots were developed according to enhanced chemiluminescence protocols.

### 2.8. Western blots

The analysis of $PrP^C$ expression in Tg mice was performed as follows. Brains from mice were homogenized in extraction buffer (0.5% NP40, 1% sodium deoxycholate, 10 mM EDTA in PBS, pH 7.4) and the complete™ cocktail of protease inhibitors (Roche) or in 10% Sarkosyl in PBS and the complete™ cocktail of protease inhibitors (Roche). Samples were pre-cleared by centrifugation at $2000 \times g$ for 5 min and either treated with 20 μg/ml of proteinase K (Roche) for 60 min at 37 °C or left untreated. An equal volume of 2× SDS reducing sample loading buffer was added to all samples and each one was boiled for 5 min before loading on a SDS/12% polyacrylamide gel. MAbs 6H4 and 2A11 were used at 1/5000 and 1/1500 dilutions, respectively, for immunoblotting. Immunocomplexes were detected with horseradish peroxidase conjugated anti mouse IgG. Immunoblots were developed by enhanced chemiluminescence.

### 2.9. Statistical analysis

The statistics SPSS 10.0 package was used to generate all the Kaplan–Meier curves and statistical data. Data were analyzed using the Student's $t$ test for non-paired variants. Incubation times were analyzed after testing for normality (Kolmogorov–Smirnow test) and for equal variance (Levene Median test) by one way ANOVA followed by multiple pair wise comparison using Fisher's LSD test. The level of significance was set at $P < 0.05$.

## 3. Results

### 3.1. Bo10ORPrP$^C$ expression in Tg mice

We obtained four different lines (founders) carrying bovine (bo) 10OR-$PrP^C$ (bo10OR$PrP^C$). All these lines also bore wt murine (mo) $PrP^C$ with 5 octarepeats (mo5OR$PrP^C$). The expression of $PrP^C$ in these lines carrying both murine 5OR-$PrP^C$ and bovine 10OR-$PrP^C$ transgenes (PrP $mo^{+/-}$ $bo^{+/-}$) was evaluated by subjecting brain homogenates to Western blot analysis using the 2A11 mAb (Fig. 1A), which recognizes both bo$PrP^C$ and mo$PrP^C$ (9). It was possible to distinguish between wt murine (5OR) and bovine (10OR) PrP according to their relative electrophoretic mobility. As shown in Fig. 1A, the increased number of OR corresponds to an increased relative molecular mass.

Bo10ORTg010 mice died in their fourth week of life and failed to breed. The remaining Tg mouse lines (PrP $mo^{+/-}$ $bo^{+/-}$), bo10ORTg006, bo10ORTg012 and bo10ORTg029 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 heterozygous transgene lines (PrP $mo^{-/-}$ $bo^{+/-}$). Then, by crossing heterozygous animals, we 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). Next, we established transgene expression levels in each mouse line by serial dilution of brain homogenates and compared these to $PrP^C$ levels in bovine brain homogenates. $PrP^C$ expression

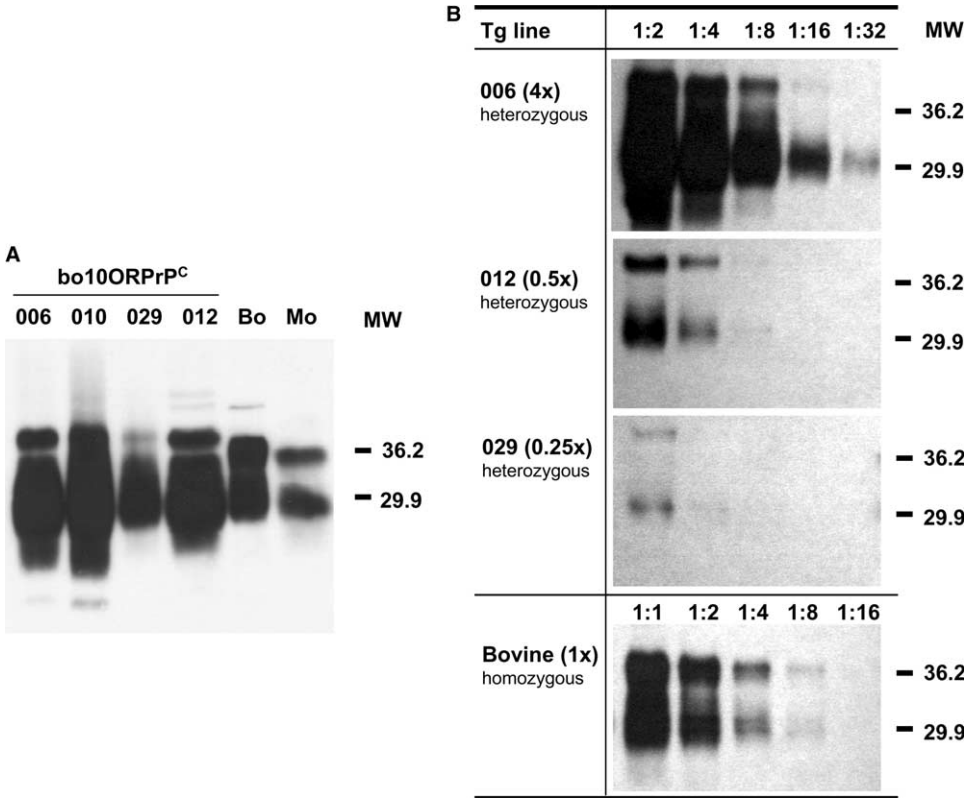


Fig. 1. Expression of bovine 10OR-PrP proteins in heterozygous ($mo^{+/-}$ $bo^{+/-}$) boTg mouse lines. Immunoblotting of brain extracts from bo10ORTg mouse lines 006, 010, 012 and 029 using the monoclonal antibody 2A11. Equivalent amounts of total protein were loaded onto each lane (A). Serial dilutions of heterozygous ($mo^{+/-}$ $bo^{+/-}$) bo10ORTg (006, 012 and 029 mouse lines) and bovine brain homogenates were analyzed by Western blotting using monoclonal antibody 2A11 (B). Bo, cow brain extract; Mo, B6CBAx129/Ola brain extract. Relative molecular mass in kilodaltons.

levels for the three one-month-old heterozygous Tg lines bo10ORTg006, bo10ORTg012 and bo10ORTg029 were found to be 4×, 0.5× and 0.25×, respectively (Fig. 1B). Further, all the homozygous transgene lines tested showed double the PrP expression levels detected in their heterozygous counterparts (data not shown).

The behavior and phenotype of the homozygous bo10ORTg-012 and bo10ORTg029 mouse lines showing 10OR-PrP expression levels of 1×, 0.5×, respectively, were identical to those of normal mice. Mean survival times of bo10ORTg012 and bo10ORTg029 were 685 ± 42 (mean days ± S.E.M.) and >600, respectively. However, the bo10ORTg010 and bo10ORTg006 lines, with PrP expression levels of around 4–8× showed extensive neurological damage. Bo10ORTg010 mice died within the first month of life presenting severe ataxia and bo10ORTg006 mice showed motor impairment with ataxia mainly affecting the hind limbs and also had breeding difficulties. This line was unable to yield homozygous individuals for the bovine 10OR-PrP transgene. The lifespan of this Tg mouse line was only 180 ± 10 (mean days ± S.E.M.) and the onset of clinical signs occurred on day 118 ± 6.

### 3.2. Neuropathological alterations in bo10ORTg mouse lines

The bo10ORTg010 and bo10ORTg006 lines with PrP expression levels of around 4–8× showing extensive neurological damage were used to characterize the genetic/spontaneous prion-related disease observed. Although for these two lines the clinical signs were visually apparent and used for the diagnosis of the disease, the most important features were the histological changes in the cerebellum, characterized by atrophy of cerebellar folia with an intense loss of granular cells, but with preservation of the Purkinje cells (Figs. 2A(1) and A(2)). This specific severe degeneration of cerebellar granular cells was not associated with conspicuous vacuolation (Fig. 2C(1)). GFAP immunostaining detected a band of astrocytosis outlining the granular cell layer (Fig. 2C(2)). Other morphological changes such as spongiosis associated with the dentate gyrus were observed in the hippocampus but there was no evidence of neuronal loss at this level. In this area, the dentate gyrus, spongiosis was also related to several intraneuronal vacuoles (Fig. 3).

The bo10ORTg006 mouse line showed a highly consistent neuropathological phenotype, with intense loss of granular cell layer neurons and relative preservation of Purkinje cells, but no PrP deposits could be detected through IHC. Moreover, Gallyas and tau immunostaining (data not shown) indicated no neurofibrillary pathology.

In order to establish the kinetics of neuropathological alterations, as well as the earliest time point at which they can be detected, we used bo10ORTg006, bo10ORTg012 and bo10ORTg029 mouse lines expressing 4-, 1- and 0.5-fold $PrP^C$ levels, respectively. Animals were grouped according to pre-established culling times (60, 90, 120, 150, 180, 240, 300 and 400 days after birth). Mice were sacrificed at the indicated time points independently of the onset of neurological signs. As shown in Table 1, neuropathological alterations were assessed at different time points. Neurological diagnosis was achieved using lesion profiles including alterations in the cerebellum, brainstem, thalamus, striatum and hippocampus. Animals carrying the PrP-10OR insert mutation from the bo10ORTg006 mouse line mainly showed the lesion profile

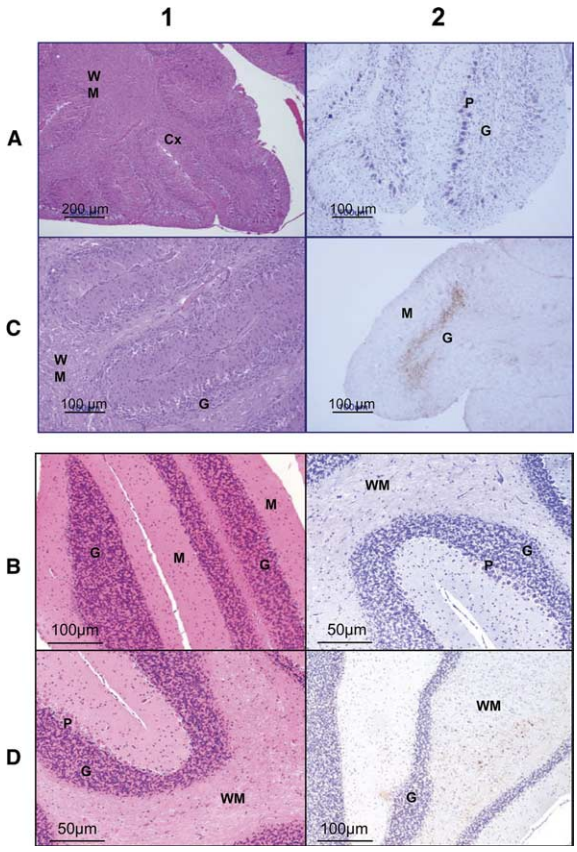


Fig. 2. Histological changes in the cerebellum of bo10ORTg compared to bo6ORTg mice. (A1) Low-power view of atrophy of the cerebellar folia, showing white matter (WM) and cortex (Cx) (H/E, 4×). (A2) Nissl staining reveals the intense loss of granular cells (G), with preservation of Purkinje cells (P) (Nissl, 10×). (C1) Specific severe degeneration of cerebellar granular cells is not associated with conspicuous vacuolation (H/E, 10×). (C2) Immunostaining for GFAP shows a band of astrocytosis defining the granular cell layer (GFAP, 10×). (B1) Low-power view demonstrating the normal size of the cerebellar layers (H/E, 10×). (B2) Nissl staining shows a normal granular layer and Purkinje cells (Nissl, 20×). (D1) H&E staining reveals a normal granular layer and Purkinje cells and lack of vacuolation (H/E, 20×). (D2) Immunostaining for GFAP shows several astrocytes mostly in the cerebellar white matter (GFAP, 10×). Bo10ORTg and bo6ORTg mice were sacrificed a 180 after birth.

scored as 3 (Table 1), and in most cases, pathological changes were confined to the cerebellum and the hippocampus. Although clinical signs and neurological alterations determined by IHC were well correlated, at early times such as 60–90 days after birth, individual animals from the bo10ORTg006 mouse line showed the first histopathological changes consistent with slight cerebellar and hippocampal damage. At 120 days after birth, when clinical signs were evident, the cerebellar cortex showed a moderate to intense loss of granule cells, with signs of karyorrhexis in several cases, and preservation of Purkinje cells. GFAP staining demonstrated a band of astrocytosis outlining the atrophic granular layer. However, there were no spongiform changes observed at this level. The hippocampal formation showed mild to severe focal spongiosis involving the dentate gyrus, not associated with conspicuous neuronal loss or astrocytosis. No PrP deposits in the brain tissue of any of the three groups of animals were observed using immunohistochemical stains.

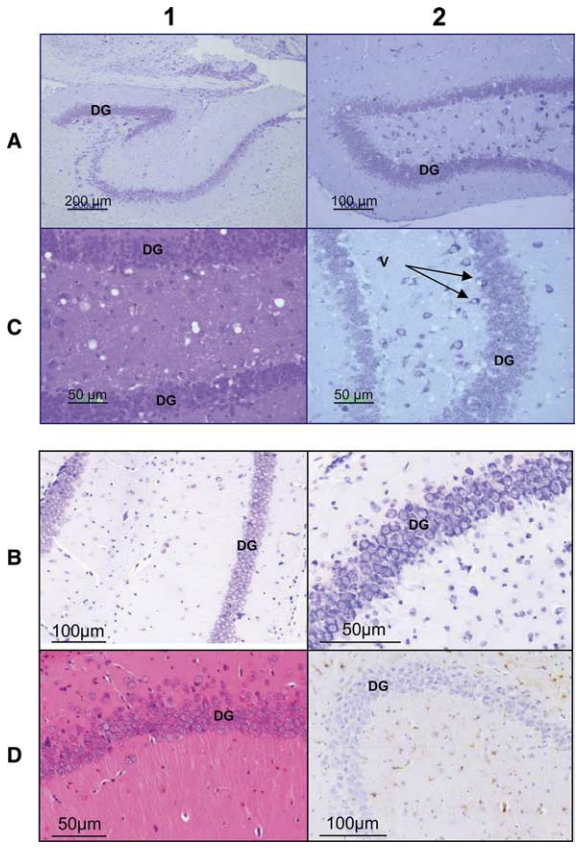


Fig. 3. Morphological changes observed in the hippocampus of bo10ORTg compared to bo6ORTg mice. (A1) Low-power image shows focal spongiosis associated with the dentate gyrus (DG) (Nissl, 4×). (A2) Intermediate-power image shows spongiosis with no evidence of neuronal loss at this level (Nissl, 10×). (C1) High-power view of a similar area shows spongiosis along with several hypertrophic astrocytes (H/E, 20×). (C2) A similar area of the dentate gyrus shows spongiosis and several intraneuronal vacuoles (V) (Nissl, 20×). (B1) Normal structure of the dentate gyrus (Nissl, 10×). (B2) Nissl staining shows no evidence of neuronal loss or vacuolation (Nissl, 20×). (D1) H&E staining shows a normal dentate gyrus, absence of vacuolation and no hypertrophic astrocytes (H/E, 20×). (D2) Immunostaining for GFAP shows normal astrocytes in the dentate gyrus and other layers of the hippocampus (GFAP, 10×). Bo10ORTg and bo6ORTg mice were sacrificed a 180 after birth.

Despite carrying the 10OR-PrP transgene, the bo10ORTg-012 and bo10ORTg029 mouse lines, however, failed to show any histopathological signs at least up to 400 days after birth (Table 1). These observations indicate that the rate at which the illness progresses is strongly related to the expression level of bo10OR-PrP$^{C}$.

### 3.3. Insolubility and proteinase-K sensitivity studies on 10OR-PrP$^{C}$

Since 10OR-PrP$^{C}$ contents in the brain of Tg mice produced neurological alterations even at levels where wt 6OR-PrP$^{C}$ does not cause these signs [27], we then tried to establish whether the insertion of four extra octarepeats would modify the biochemical properties of the bo10OR-PrP protein, in relation to the wt bo6OR-PrP protein. To this end, brain homogenates from bo6ORTg and bo10ORTg (line 006) mouse lines were solubilized in extraction buffer and ultracentrifuged at 100 000 × *g* for 1 h as described in Section 2. Subsequent Western blotting of the soluble and insoluble proteins indicated differential biochemical behavior of the bo6OR-PrP and bo10OR-PrP proteins (Fig. 4A). While the addition of one extra octarepeat did not alter the biochemical properties of PrP [17], the presence of four extra octarepeats rendered a more insoluble protein. This insolubility was detected early on (30 days after birth) in the lifespan of the mouse, indicating that quantification of the 10OR-PrP could reflect a cumulative effect (data not shown).

To evaluate the protease sensitivity of the mutant 10OR-PrP$^{C}$ compared to wt 6OR-PrP$^{C}$, brain homogenates were treated with different concentrations of proteinase-K (PK) (see Section 2). While all bovine 6OR-PrP$^{C}$ was completely digested at PK concentrations higher than 7.5 µg/ml, bovine 10OR-PrP$^{C}$ was slightly more resistant to lower concentrations of PK (Fig. 4B). However, the degree of resistance was much lower than the one shown by the pathogenic isoform 10OR-PrP$^{Sc}$ obtained after BSE inoculation (Fig. 4B).

### 3.4. Brain homogenates from spontaneously ill bo10ORTg mice do not contain detectable infectivity

To test the infectivity in the brain of bo10OR-PrP Tg mice, brain homogenates were prepared from bo10ORTg006 mice sacrificed after spontaneous development of clinical signs. These homogenates were intracerebrally inoculated in five different Tg mouse lines: three lines expressing different levels of wt 6OR-PrP$^{C}$ as well as bo10ORTg012 and bo10ORTg029 mouse lines. The PrP expressed by the bo10ORTg lines 012 and 029 should be a particularly efficient substrate for assessing infectivity, because it has the same amino acid sequence as the PrP present in the inocula. The inoculum used showed a high amount of insoluble bo10OR-PrP$^{C}$ (supplemental figure 2). After inoculation, mice were weekly observed for the appearance of neurological signs and at the end of their lifespan survival times were determined. The brains were removed and checked for the presence of PrP$^{res}$ by Western blotting and IHC. None of the inoculated animals showed any change in survival times (Table 2) compared to PBS-inoculated control mice (Table 3). PrP$^{res}$ was not detected neither by Western blotting nor by IHC indicating the absence of PK resistant PrP in the inoculated ($n = 28$) animals (data not shown). These data demonstrate that bo10ORTg mice do not spontaneously generate detectable levels of infectious prions in their brains indicating that the spontaneous disease observed in these mice is not transmissible at least in a first passage. Re-inoculation studies using brain homogenates from these PrP$^{res}$ negative animals are currently underway.

### 3.5. Intracerebral inoculation of bo10ORTg mice with BSE prions cause BSE

We next investigate the susceptibility of the bo10ORTg mice to infectious BSE prions. Two different inocula ($BSE_1$ and $BSE_2$) were intracerebrally inoculated (see Section 2 and supplemental figure 2) in the bo10ORTg012 and bo10ORTg029 mouse lines. The bo10ORTg006 mouse line could not be used in infectivity studies due to its shorter lifespan (180 ± 10 days), compared to more than 600 days for the rest of the bo10ORTg mouse lines used (Table 3).

Recently, we reported that Tg mice expressing bovine PrP bearing an additional octapeptide insertion with respect to the wt (7OR instead of 6) showed an altered course of BSE infection, reflected as reduced incubation times when

Table 1
Histopathological studies in non-inoculated bolOORTg mice

| Tg line | Days after birth | Neurological diagnosis $\pm(n/n_0)$[b] | Lesion profile[c] | Cerebellum | Brainstem | Thalamus | Striatum | Hippocampus | Clinical signs |
|---|---|---|---|---|---|---|---|---|---|
| bo10ORTg006$^{+/-}$ (4×)[a] | 60 | +(4/4) | 3 | 1[d] | 0[d] | 0[d] | 0[d] | 1[d] | – |
| | 90 | +(5/5) | 3 | 2 | 0 | 0 | 0 | 1 | – |
| | 120 | +(6/6) | 3 | 2–3 | 0 | 0 | 0 | 1–2 | +[e] |
| | 150 | +(9/9) | 3 | 2–3 | 0–1 | 0 | 0 | 2 | +[e] |
| | 180 | +(16/16) | 3 | 2–3 | 1–2 | 0–2 | 0–2 | 2 | +[e] |
| bo10ORTg012$^{+/+}$ (1×)[a] | 90 | −(0/6) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 120 | −(0/4) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 150 | −(0/6) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 180 | −(0/6) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 240 | −(0/8) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 300 | +(3/10) | 1 | 1 | 0–1 | 0 | 0 | 0 | – |
| bo10ORTg029$^{+/+}$ (0.5×)[a] | 120 | −(0/4) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 180 | −(0/6) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 240 | −(0/8) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 300 | −(0/6) | 0 | 0 | 0 | 0 | 0 | 0 | – |
| | 400 | −(0/6) | 0 | 0 | 0 | 0 | 0 | 0 | – |

$^{+/+}$ or $^{+/-}$ Homozygotic or heterozygotic for the 10OR bovine prnp gene. All Tg animals are murine *prnp*$^{-/-}$.
[a]Relative to cattle PrP expression.
[b]Number of animals considered as positive for histopathological changes with respect to the total of animals.
[c]The lesion profiles observed were categorized descriptively as follows: 0, no pathological changes; 1, spongiform change and astrocytosis in cerebellum (both cortex and white matter), brainstem, striatum and thalamus; 2, changes limited to cerebellum and brainstem; 3, marked changes in cerebellum, with intense loss of granule cells, and lesser changes in hippocampus.
[d]The intensity of pathological changes was graded semi quantitatively as: 0, absent; 1, mild; 2, moderate; 3, severe.
[e]Neurological signs characterized by motor impairment.

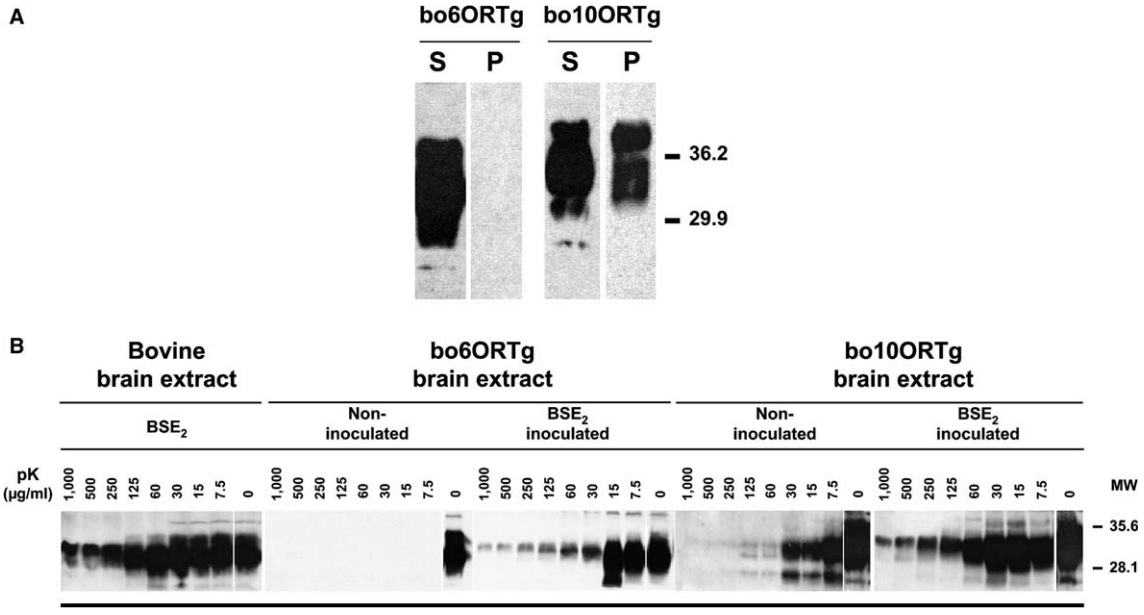


Fig. 4. Insolubility and PK resistance studies of $PrP^{C}$ and $PrP^{res}$ from non-inoculated or BSE-inoculated bo10ORTg and bo6ORTg mouse lines. (A) Western blot analysis of the soluble (S) and insoluble (P) fractions obtained from non-inoculated bo6ORTg and bo10ORTg brain extracts (10% Sarkosyl in PBS, pH 7.4 previously pre-cleared by centrifugation at 2000 × *g*) after ultracentrifugation at 100 000 *g* for 1 h. Insoluble fractions were washed exhaustively with 5% Sarkosyl in PBS, pH 7.4 and subjected to a further ultracentrifugation step. (B) Western blotting of non-inoculated or $BSE_2$-inoculated brain homogenates from bo6ORTg110 or bo10ORTg012 mouse lines and cattle were digested with 0, 7.5, 15, 30, 60, 125, 250, 500 and 1000 μg/ml of PK for 60 min at 37 °C. An equal volume of 2× SDS sample loading buffer was added to all samples and each was boiled for 5 min before loading onto a SDS/12% polyacrylamide gel. Mab 2A11 was used at a 1/1000 dilution for immunoblotting. Relative molecular mass in kilodaltons.

compared with mice expressing similar levels of the wt six-octapeptide protein [17]. To explore if the number of OR influences the propagation of BSE prions, we also inoculated the same inocula in mice previously described [27] expressing similar or higher levels of the wt six octapeptide protein. When disease progression was evident the animals were sacrificed

Table 2
Infectivity studies of bovine 10OR Tg mice brain homogenates

| Recipient | Inoculum | Transgene expression[a] | Death (days ± S.E.M.) | $n/n_0$[b] |
|---|---|---|---|---|
| bo6ORTg110$^{+/+}$[c] | Non-inoculated bo10ORTg006 | 8× | >600 | 0/6 |
| bo6ORTg135$^{+/+}$[c] | Non-inoculated bo10ORTg006 | 6× | >600 | 0/5 |
| bo6ORTg113$^{+/+}$[c] | Non-inoculated bo10ORTg006 | 3× | 617 ± 78 | 0/3 |
| bo10ORTg012$^{+/+}$ | Non-inoculated bo10ORTg006 | 1× | 633 ± 33 | 0/7 |
| bo10ORTg029$^{+/+}$ | Non-inoculated bo10ORTg006 | 0.5× | 632 ± 23 | 0/7 |

$^{+/+}$ or $^{+/-}$ Homozygous or heterozygous for the 6OR or 10OR bovine *prnp* gene. All Tg animals are murine *prnp*$^{-/-}$.
[a]Relative to cattle PrP expression.
[b]Number of animals with PrP$^{res}$ with respect to the number of inoculated animals.
[c]These bovine PrP Tg mouse lines have been described previously [17,18,27].

Table 3
Susceptibility of bovine 6OR and 10OR Tg mice to BSE prions

| Recipient | Inoculum | Transgene expression[a] | Death (days ± S.E.M.) | $n/n_0$[b] |
|---|---|---|---|---|
| Non-Tg (B6xCBAx129Ola) | $BSE_1$ | 1× (endogenous mouse PrP) | 656 ± 30 | 5/12 |
| Non-Tg (*prnp*$^{-/-}$) | $BSE_1$ | 0× | 688 ± 35 | 0/6 |
| bo6ORTg110$^{+/+}$[d] | $BSE_1$ | 8x | 326 ± 18 | 13/13 |
| bo6ORTg110$^{+/-}$[d] | $BSE_1$ | 4× | 359 ± 15 | 11/11 |
| bo6ORTg113$^{+/+}$[d] | $BSE_1$ | 3× | 410 ± 7 | 10/10 |
| bo6ORTg078$^{+/+}$[d] | $BSE_1$ | 2× | 436 ± 14 | 10/10 |
| bo6ORTg022$^{+/+}$[d] | $BSE_1$ | 0.5× | 513 ± 29 | 9/9 |
| bo10ORTg012$^{+/+}$ | $BSE_1$ | 1× | 276 ± 14 | 10/10 |
| bo10ORTg029$^{+/+}$ | $BSE_1$ | 0.5× | 320 ± 20 | 7/7 |
| bo6ORTg110$^{+/+}$[d] | $BSE_2$ | 8× | 308 ± 5 | 5/5 |
| bo6ORTg113$^{+/+}$[d] | $BSE_2$ | 3× | 377 ± 12 | 7/7 |
| bo10ORTg012$^{+/+}$ | $BSE_2$ | 1× | 261 ± 8 | 10/10 |
| bo10ORTg029$^{+/+}$ | $BSE_2$ | 0.5× | 260 ± 22 | 13/13 |
| bo6ORTg110$^{+/+}$[d] | bol0ORTg$BSE_1$ | 8× | 342 ± 15 | 11/11 |
| bo10ORTg012$^{+/+}$ | bol0ORTg$BSE_1$ | 1× | 327 ± 15 | 6/6 |
| bo10ORTg029$^{+/+}$ | bol0ORTg$BSE_1$ | 0.5× | 378 ± 33 | 5/5 |
| bo6ORTg110$^{+/+}$[d] | PBS-inoculated | 8× | >600 | 0/4 |
| bo6ORTg113$^{+/+}$[d] | PBS-inoculated | 3× | 581 ± 39 | 0/4 |
| bo6ORTg078$^{+/+}$[d] | PBS-inoculated | 2× | >500 | 0/6 |
| bo6ORTg022$^{+/+}$[d] | PBS-inoculated | 0.5× | 707 ± 18 | 0/6 |
| bo10ORTg006$^{+/-}$ | PBS-inoculated | 4× | 180 ± 10[c] | 0/13 |
| bo10ORTg012$^{+/+}$ | PBS-inoculated | 1× | 685 ± 42 | 0/9 |
| bo10ORTg029$^{+/+}$ | PBS-inoculated | 0.5× | >600 | 0/13 |

$^{+/+}$ or $^{+/-}$ Homozygous or heterozygous for the bovine 6OR or 10OR *prnp* gene. All Tg animals are murine *prnp*$^{-/-}$.
[a]Relative to cattle PrP expression.
[b]Number of animals with PrP$^{res}$ with respect to the number of inoculated animals.
[c]Animals were sacrificed for ethical reason.
[d]These bovine PrP Tg mouse lines have been described previously [17,18,27].

and their brains used for Western blotting (Fig. 5A) or IHC studies (Fig. 5B).

In these experiments, the bo10ORTg mice consistently showed reduced survival times compared to the bo6ORTg expressing similar or higher levels of the wt PrP$^{C}$ (Table 1). This reduction in survival times was observed in all the lines expressing different amounts of bo10ORPrP$^{C}$ and for both the inocula used. For instance, the bo6ORTg022 mouse line (0.5×) inoculated with $BSE_1$ showed a survival time of 513 ± 29 days, while this time was 320 ± 20 days for the bo10ORTg029 mouse line, which expresses identical levels of the 10OR bovine prion protein (Table 3). Similarly, the bo6ORTg113 (3×) and bo10ORTg012 (1×) mouse lines inoculated with $BSE_2$ showed incubation times of 377 ± 12 and 261 ± 8 days, respectively, although the bo6ORTg113 mouse line expresses 3 times more bo6OR-PrP than the bo10ORTg012 mouse line (Table 3).

### 3.6. Characterizing the new bo10OR-PrP$^{Sc}$

After intracerebral $BSE_1$ inoculation in bo10ORTg012 and bo10ORTg029 mouse lines, a new 10OR-PrP$^{Sc}$ was obtained. We named this new prion bo10ORTg$BSE_1$ and then tried to determine if the four extra octarepeat insert mutation modifies the biochemical properties and the infectious character of the new bo10OR-PrP$^{Sc}$ protein compared to the wt bo6OR-PrP$^{Sc}$ by assessing insolubility and PK sensitivity as described in Section 2. In these studies, the mutant bo10OR-PrP from brains of spontaneously ill (non-inoculated) bo10ORTg mice was also assayed. The two PrP$^{Sc}$ (bo6OR-PrP$^{Sc}$ and bo10OR-PrP$^{Sc}$) and the bo10OR-PrP$^{C}$ showed similar degree of insolubility (data not shown). However, while PrP$^{Sc}$ from both $BSE_1$ inoculated bo6ORTg and bo10ORTg mouse lines showed identical PK resistance, bo10OR-PrP$^{C}$ from non-inoculated bo10ORTg mouse lines showed reduced PK resistance (Fig. 4B).

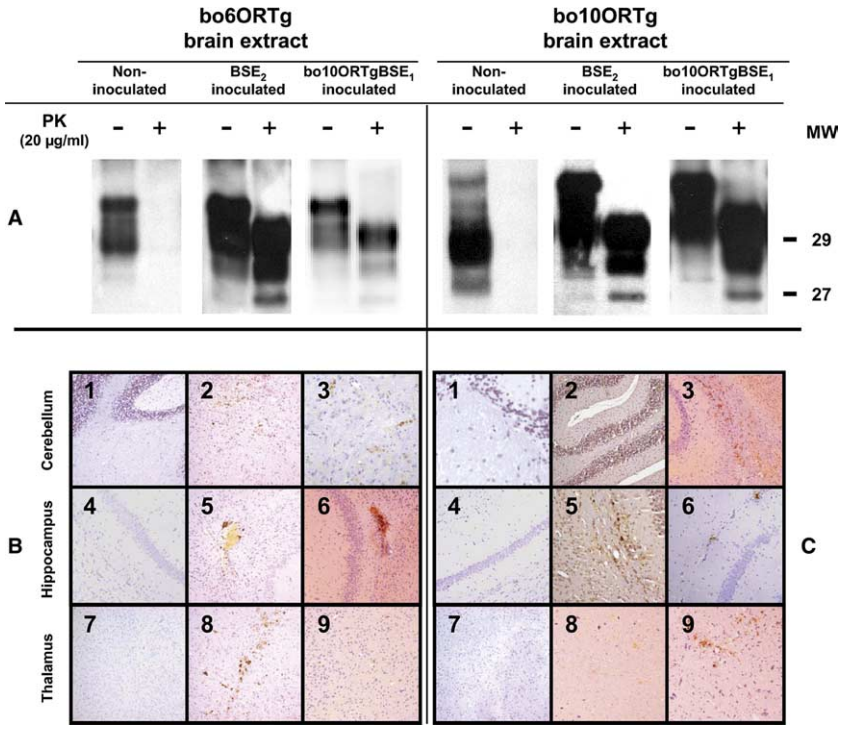


Fig. 5. Detection of the $PrP^{res}$ protein in bo6ORTg and bo10ORTg mice after BSE inoculation. (A) Western blot of bo6ORTg brain extracts from mice intracerebrally inoculated with different inocula. (B,C) $PrP^{res}$ immunodetection using mAb 2A11 in the deep layers of the cerebellum (1, 2 and 3), hippocampus (4, 5 and 6) and thalamus (7, 8 and 9) of bo6ORTg (B) or bo10ORTg (C) mice inoculated with $BSE_2$ and $bo10ORTgBSE_1$ inocula (~250 days postinoculation). Monoclonal antibody 2A11 was used at a 1:1500 dilution. Relative molecular mass in kilodaltons.

In a further set of experiments, we analyzed the new generated $PrP^{res}$ by Western blotted detergent extracts of infected Tg mice brain homogenates after standard proteinase-K treatment. The band patterns for the different BSE inocula were identical for the two different passages, regardless of the transgene (data not shown).

In addition, the $bo10ORTgBSE_1$ (bo10OR-$PrP^{Sc}$) inoculum containing similar amounts of PK resistant protein to the $BSE_2$ inoculum (supplemental figure 2) was intracerebrally inoculated in bo10ORTg mouse lines bo10ORTg012 and bo10ORTg029 and in mice expressing the wt six octapeptide protein (bo6ORTg110), to compare the course of BSE infection. In all cases, the new bo10OR-$PrP^{Sc}$ was able to infect 100% of the Tg mice independently of the type of bovine PrP expressed (Table 3), and developed identical signs of CNS dysfunction after BSE inoculation. Moreover, western blotting (Fig. 5A) and IHC studies (Fig. 5B) revealed the identical behavior of the new bo10OR prion used as inoculum. However, the onset of signs was slightly delayed when these studies were compared to the standard $BSE_2$ inoculum in the three different lines. Bo10ORTg mice consistently showed reduced survival times (Table 3) compared to the bo6ORTg mouse line. For instance, the bo6ORTg110 mouse line inoculated with $BSE_2$ showed a survival time of 308 ± 5 days, while this time was 342 ± 15 days when the animals were inoculated with $bo10ORTgBSE_1$, indicative of a slower propagation. Similarly, the bo10ORTg012 and bo10ORTg029 mouse lines inoculated with $BSE_2$ showed incubation times of 261 ± 8 and 260 ± 22 days, respectively, while these figures were 327 ± 15 and 378 ± 33 days when $bo10ORTgPrP^{Sc}$ was used (Table 3).

In line with the biochemical data, histopathological and immunohistological studies revealed $PrP^{res}$ deposition in brain sections of mice injected with each of the three types of BSE inoculum. The histological pattern observed for the BSE inoculum or first passage BSE in bo6ORTg and bo10ORTg mice (Fig. 5B) was indistinguishable from that of classic BSE in cattle; mainly vacuolization of the neuropil mostly in the brain stem, hippocampus and cerebellar white matter (Fig. 5B and C). However, we observed several patterns indicating immunopositivity and correlating Western blot patterns for $PrP^{res}$. The most common were fine granular and punctate neuropil labeling, and stellate labeling foci, which apparently associated with glial cells. However, we also observed granular staining in neuronal cytoplasm and around neurons, occasionally as plaque-like deposits. These labeling patterns were mostly observed in the neuropil of the rostral brain stem, in cerebellar nuclei and both in the deep layers of cerebral cortex and in the hippocampus. This pattern was identical for the bo6ORTg and bo10ORTg lines, irrespective of the inoculum used (Fig. 5B and C). Severe vacuolization and $PrP^{res}$ deposition were accompanied by astrocytic gliosis, as observed by the immunohistochemical detection of GFAP in different structures of the encephalic area. Vacuoles were often enveloped by astrocytic prolongations, and astrocytosis was also observed as an enlarged astrocyte cytoplasm. No histopathological changes in $PrP^{res}$ deposition (Fig. $5B_{1,4\text{ and }7}$ and $C_{1,4\text{ and }7}$) or in astrocyte reactions (data not shown) were observed in the non-inoculated mice used as negative controls.

## 4. Discussion

Although an infectious origin is the most likely cause of BSE, other explanations such as a spontaneous/genetic origin,

cannot be ruled out. Spontaneous and inherited prion diseases have been characterized, mainly in humans, associated with the presence of an increased number of OR within the PrP ORF [16,28,29]. Tg mice models over-expressing PrP containing extra octarepeats confirmed the pathogenicity of this mutation [15,30].

Although bovine PrP with a four extra octarepeat insertional mutation has not yet been naturally found in bovines it is interesting to assess whether or not this mutation could potentially manifest as a neurodegenerative syndrome in a bovine PrP context. For this purpose we analyzed the phenotype of a Tg mouse model expressing a ten octarepeat bovine PrP gene.

Herein we show that the four extra repeat mutation in the bovine PrP gene triggers a spontaneous neurodegenerative disease. Tg mice showed motor impairment with ataxia affecting mainly the hind limbs but, although clinical signs were visually manifest, the most important features were the histological changes in the cerebellum, characterized by atrophy of cerebellar folia with an intense loss of granular cells but preservation of Purkinje cells. The results also showed that the rate at which the illness progresses is strongly related to the expression level of bo10OR-PrP$^{C}$. These data correlate well with clinical observations in patients with a four extra repeat mutation in the PrP gene who are initially affected by progressive cerebellar and brain stem signs [31]. This neuropathology, with astrogliosis and massive damage to cerebellar granule cells, is common in other Tg mice models expressing PrP containing extra octarepeats [15,30] or other point mutations [32]. Most of these Tg mice models have confirmed that the presence of these mutations triggers spontaneous disease.

We suggest that the addition of octarepeats generates different protein conformations with a varying capacity for pathogenic conversion and specific biochemical properties. Hence, whereas 7OR-PrP$^{C}$ shows similar protease sensitivity and solubility in non-denaturing detergents to homologous 6OR-PrP$^{C}$ [17], the addition of four extra octarepeats rendered a bo10OR-PrP$^{C}$ showing both a reduced protease sensitivity (Fig. 4B) and a reduced solubility (Fig. 4A) in the same conditions. An increased number of OR has also been related to enhanced aggregation and PK resistance of the mutant PrP protein in hamster [19].

The four extra OR insertion mutation in the PrP could give rise to a different PrP structure with respect to wt PrP. This conformation, denoted PrP$^{toxic}$ by other authors [33], show special biochemical properties, are difficult to degrade and are prone to build up, triggering a spontaneous disease when expression levels exceed the clearance threshold. The enhanced aggregation and/or protease-resistance properties of the mutant PrP could in some way affect the appearance of the disease associated with these mutations. It is clear that a greater understanding of PrP structure and the effects of adding octarepeats to its coding region is required. In effect, it has been proposed that the special properties (toxicity, insolubility and high aggregation capacity) of PrPs with more than 7OR could be directly related to their increased number of OR. The mechanisms through which extra copies of the OR may influence the aggregation properties of PrP are unclear. However, it has been suggested that the repeat region of the protein may itself act as a site promoting PrP self-aggregation or aggregation between PrP and other cell factors [14,34].

On the other hand, Tg mice expressing bovine PrP with ten octarepeat do not spontaneously generate detectable levels of infectious prions in their brains (Table 1) indicating that the spontaneous disease observed in these mice is not transmissible. While several mutant PrP$^{res}$ like molecules have been generated in Tg mice and some of these can acquire several biochemical properties of PrP$^{res}$, so far none have been shown to be infectious [14,35] and the transmissibility of these new pathogenic isoforms is questionable. These data reinforce the idea that PrP can induce neurodegeneration without being converted to conventional PrP$^{Sc}$.

Insertional mutation-derived spontaneous human TSEs show different phenotypes and behavior related to the number of additional OR within the PrP region. Thus, the number of OR has been shown to affect the severity of symptoms and the time of onset of clinical signs in humans [29]. Although it is known that the region comprising the OR is not essential for mediating the pathogenesis of prions, it does modulate the incubation time leading to disease [36–38].

In the present study, we also evaluated the effects of the four extra octarepeats insertion mutation in the bovine PrP on the efficiency of BSE prion propagation. Results showed that BSE transmission was highly efficient in bo10OR Tg mice, in agreement with the lack of an effective species barrier. In addition, bo10ORTg mice show an altered course of BSE infection, resulting in highly reduced survival times when compared to bo6ORTg mice expressing similar or higher levels of the wt bovine PrP (Fig. 5 and Table 3). Consistent with the lack of a species barrier to BSE infection all inoculated mice showed the typical signs of CNS spongiform degeneration (data not shown) and PrP$^{res}$ deposits in brain (Fig. 5). The histological and Western blot patterns observed in both bo6ORTg and bo10ORTg mice for the different BSE inocula used were indistinguishable from those of classic BSE in cattle. These findings correlate well with previous observations in Tg mice expressing bo7OR-PrP [17], in which significant differences in the times of onset of clinical signs and survival were also reduced after BSE inoculation.

Despite the BSE agent being considered a slow prion when propagated in bovine Tg mice requiring models that overexpress the PrP protein, here we report a bovine PrP Tg mouse line (bo10ORTg012) with half the normal expression level (0.5×) and reduced incubation times to disease onset of around 250 days (Table 3). These data suggest that the 10OR-PrP shows a new conformation that is more efficiently converted into a pathogenic conformation than its 6OR-PrP homologue (wt). Although, we can not rule out that the new generated 10OR-PrP$^{Sc}$ could be more toxic than its homologue 6OR-PrP$^{Sc}$ at equivalent molarity. The existence of seeding mechanisms could explain the fact that low levels of PrP expression yield reduced incubation times after prion inoculation. 10OR-PrP, a molecule with an evident tendency to aggregate, could be a good initiator of the conversion–propagation event triggered by an exogenous prion.

Although the infectivity of the newly formed 10OR-PrP$^{Sc}$ was patent in both bo6ORTg and bo10ORTg inoculated mice, the survival times of both Tg mice after 10OR-PrP$^{Sc}$ inoculation were slightly longer than the survival times after 6OR-PrP$^{Sc}$ inoculation. This once again suggests that the new pathogenic isoform with 10OR might have a different conformation with respect to the wt isoform bearing 6OR. These infectivity differences could be modulated by the initial

PrP conformations, as shown by their different biochemical properties. Our histopathological studies, on the other hand, revealed the identical behavior of both bovine prions (6OR-$PrP^{Sc}$ and 10OR-$PrP^{Sc}$).

In summary, the addition of OR could give rise to a different PrP structure with respect to wt PrP, capable of acquiring properties according to the number of OR added. The presence of four extra OR seems to generate a new 10OR-PrP structure that can be transformed more efficiently than its 6OR-PrP homologue and has a tendency for aggregation. This ability could induce a seeding process, or the new conformation could intrinsically be more susceptible to be transformed into another pathogenic isoform. However, this new conformation was in no case able to transmit the disease to other mice, even to homologous 10OR-PrP Tg mice. Thus, if BSE had a genetic origin, it is unlikely that the causal mutation would be related to the number of OR.

*Acknowledgments:* This work was supported by two national grants from Spain (SC00-055 and OT02-008). The authors thank J.C. Manson for supplying the *Prnp*$^{-/-}$ mice, D.R. Borchelt for providing MoPrP · Xho plasmid and the Veterinary Laboratory Agency (Weybridge, UK) for BSE brain material.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2005.09.099.

## References

[1] Adams, D.H. (1986) The nature of the scrapie agent. Med. Hypoth. 20, 37–50.
[2] Prusiner, S.B. (1998) Prions. Proc. Natl. Acad. Sci. USA 95, 13363–13383.
[3] Alpers, M. and Rail, L. (1971) Kuru and Creutzfeldt–Jakob disease: clinical and aetiological aspects. Proc. Aust. Assoc. Neurol. 8, 7–15.
[4] Will, R.G. et al. (1996) A new variant of Creutzfeldt–Jakob disease in the UK. Lancet 347, 921–925.
[5] Hill, A.F. et al. (1997) The same prion strain causes vCJD and BSE. Nature 389, 448–450, 526.
[6] Beck, E. et al. (1969) Creutzfeldt–Jakob disease. The neuropathology of a transmission experiment. Brain 92, 699–716.
[7] Bendheim, P.E. (1984) The human spongiform encephalopathies. Neurol. Clin. 2, 281–298.
[8] Collinge, J. et al. (1989) Diagnosis of Gerstmann–Straussler syndrome in familial dementia with prion protein gene analysis. Lancet 2, 15–17.
[9] Goldfarb, L.G. et al. (1992) Fatal familial insomnia and familial Creutzfeldt–Jakob disease: disease phenotype determined by a DNA polymorphism. Science 258, 806–808.
[10] Manetto, V. et al. (1992) Fatal familial insomnia: clinical and pathologic study of five new cases. Neurology 42, 312–319.
[11] Medori, R. et al. (1992) Fatal familial insomnia, a prion disease with a mutation at codon 178 of the prion protein gene. N. Engl. J. Med. 326, 444–449.
[12] Goldfarb, L.G. et al. (1994) Molecular genetic studies of Creutzfeldt–Jakob disease. Mol. Neurobiol. 8, 89–97.
[13] Dormont, D. (1994) Human transmissible subacute spongiform encephalopathy. Bull. Acad. Natl. Med. 178, 887–903.
[14] Chiesa, R. et al. (1998) Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. Neuron 21, 1339–1351.
[15] Chiesa, R. et al. (2000) Accumulation of protease-resistant prion protein (PrP) and apoptosis of cerebellar granule cells in transgenic mice expressing a PrP insertional mutation. Proc. Natl. Acad. Sci. USA 97, 5574–5579.
[16] Campbell, T.A. et al. (1996) A prion disease with a novel 96-base pair insertional mutation in the prion protein gene. Neurology 46, 761–766.
[17] Castilla, J. et al. (2004) Different behavior toward bovine spongiform encephalopathy infection of bovine prion protein transgenic mice with one extra repeat octapeptide insert mutation. J. Neurosci. 24, 2156–2164.
[18] Castilla, J. et al. (2004) Subclinical bovine spongiform encephalopathy infection in transgenic mice expressing porcine prion protein. J. Neurosci. 24, 5063–5069.
[19] Priola, S.A. and Chesebro, B. (1998) Abnormal properties of prion protein with insertional mutations in different cell types. J. Biol. Chem. 273, 11980–11985.
[20] Kozak, M. (1989) Context effects and inefficient initiation at non-AUG codons in eucaryotic cell-free translation systems. Mol. Cell. Biol. 9, 5073–5080.
[21] Borchelt, D.R. et al. (1996) A vector for expressing foreign genes in the brains and hearts of transgenic mice. Genet. Anal. 13, 159–163.
[22] Manson, J.C. et al. (1994) 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. Mol. Neurobiol. 8, 121–127.
[23] Hogan, B. and Williams, J. (1981) Integration of foreign genes into the mammalian germ line: genetic engineering enters a new era. Nature 294, 9–10.
[24] Scott, M. et al. (1989) Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. Cell 59, 847–857.
[25] Scott, M. et al. (1993) Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. Cell 73, 979–988.
[26] Brun, A. et al. (2004) Proteinase K enhanced immunoreactivity of the prion protein-specific monoclonal antibody 2A11. Neurosci. Res. 48, 75–83.
[27] Castilla, J. et al. (2003) Early detection of PrPres in BSE-infected bovine PrP transgenic mice. Arch. Virol. 148, 677–691.
[28] Krasemann, S. et al. (1995) Prion disease associated with a novel nine octapeptide repeat insertion in the PRNP gene. Brain Res. Mol. Brain Res. 34, 173–176.
[29] Cochran, E.J. et al. (1996) Familial Creutzfeldt–Jakob disease with a five-repeat octapeptide insert mutation. Neurology 47, 727–733.
[30] Chiesa, R. et al. (2001) Primary myopathy and accumulation of PrPSc-like molecules in peripheral tissues of transgenic mice expressing a prion protein insertional mutation. Neurobiol. Dis. 8, 279–288.
[31] Yanagihara, C. et al. (2002) Rapidly progressive dementia syndrome associated with a novel four extra repeat mutation in the prion protein gene. J. Neurol. Neurosurg. Psychiatry 72, 788–791.
[32] Hsiao, K. et al. (1991) Spontaneous neurodegeneration in transgenic mice with prion protein codon 101 proline–leucine substitution. Ann. N.Y. Acad. Sci. 640, 166–170.
[33] Chiesa, R. and Harris, D.A. (2001) Prion diseases: what is the neurotoxic molecule?. Neurobiol. Dis. 8, 743–763.
[34] Jarrett, J.T. and Lansbury Jr., P.T. (1993) Seeding one-dimensional crystallization'' of amyloid: a pathogenic mechanism in Alzheimer's disease and scrapie?. Cell 73, 1055–1058.
[35] Lehmann, S. and Harris, D.A. (1996) Two mutant prion proteins expressed in cultured cells acquire biochemical properties reminiscent of the scrapie isoform. Proc. Natl. Acad. Sci. USA 93, 5610–5614.
[36] Goldmann, W. et al. (1998) The shortest known prion protein gene allele occurs in goats, has only three octapeptide repeats and is non-pathogenic. J. Gen. Virol. 79 (Pt 12), 3173–3176.
[37] Flechsig, E. et al. (2000) Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. Neuron 27, 399–408.
[38] Beck, J.A. et al. (2001) Two-octapeptide repeat deletion of prion protein associated with rapidly progressive dementia. Neurology 57, 354–356.
[39] Fink, P.S. (1991) Using sodium chloride step gradients to fractionate DNA fragments. Biotechniques 10, 446, 448, 450.