JOURNAL OF VIROLOGY, May 2007, p. 4533–4539 Vol. 81, No. 9
0022-538X/07/$08.00+0 doi:10.1128/JVI.02762-06
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Resistance to Chronic Wasting Disease in Transgenic Mice Expressing a Naturally Occurring Allelic Variant of Deer Prion Protein▽

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Received 14 December 2006/Accepted 14 February 2007

Prion protein (PrP) is a required factor for susceptibility to transmissible spongiform encephalopathy or prion diseases. In transgenic mice, expression of prion protein (PrP) from another species often confers susceptibility to prion disease from that donor species. For example, expression of deer or elk PrP in transgenic mice has induced susceptibility to chronic wasting disease (CWD), the prion disease of cervids. In the current experiments, transgenic mice expressing two naturally occurring allelic variants of deer PrP with either glycine (G) or serine (S) at residue 96 were found to differ in susceptibility to CWD infection. G96 mice were highly susceptible to infection, and disease appeared starting as early as 160 days postinfection. In contrast, S96 mice showed no evidence of disease or generation of disease-associated protease-resistant PrP (PrPres) over a 600-day period. At the time of clinical disease, G96 mice showed typical vacuolar pathology and deposition of PrPres in many brain regions, and in some individuals, extensive neuronal loss and apoptosis were noted in the hippocampus and cerebellum. Extraneural accumulation of PrPres was also noted in spleen and intestinal tissue of clinically ill G96 mice. These results demonstrate the importance of deer PrP polymorphisms in susceptibility to CWD infection. Furthermore, this deer PrP transgenic model is the first to demonstrate extraneural accumulation of PrPres in spleen and intestinal tissue and thus may prove useful in studies of CWD pathogenesis and transmission by oral or other natural routes of infection.

Prion diseases, also known as transmissible spongiform encephalopathies (TSE diseases), are progressive brain diseases characterized by spongiform neurodegeneration of the central nervous system. These diseases include scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in deer and elk of North America, and Creutzfeldt-Jakob disease (CJD) in humans. In all prion diseases, the pathogenic process is associated with the presence of an abnormally folded partially protease-resistant protein, PrPres, derived from the normal isoform of protease-sensitive host-encoded prion protein, PrPsen. PrPres is likely to be an important trigger of the neurodegenerative process. Many in the field believe that ingestion or inoculation of PrPres itself can transmit the disease, but this remains controversial (1, 9, 31). Although TSE agents show species specificity, in certain situations, the infectious agents can cross into new species and can eventually adapt to new species by continued passage (32).

Sheep scrapie has been recognized for at least 200 years, and horizontal spread is known to occur from mothers to offspring as well as via contaminated pastures. Other forms of prion disease were not recognized until the 1900s. The origin of all these diseases is unclear. There has been much speculation that the diseases in humans, cattle, and cervids might have originated from an adapted form of sheep scrapie; however, this hypothesis has not been proven (38). CWD was first described in captive mule deer in Colorado in 1964 and has since been detected in both captive and wild deer and elk herds in a widening area of the United States and Canada (38). CWD spreads horizontally, and infection appears to be enhanced by crowding, which occurs in farmed herds (27). In some areas, CWD spread in wild deer has also been extensive. In wild elk, CWD infection can also occur, but the extent of horizontal spread is uncertain at present. There is also concern over the possibility that CWD might be able to spread to humans or domestic animals exposed to cervids or cervid tissues. Therefore, it is of importance to understand the host genetic factors that might influence CWD spread among cervids and other species.

Normal mice are resistant to CWD from deer or elk, but recently, several publications have described successful CWD infection in transgenic (Tg) mice expressing PrPsen of deer or elk (7, 21, 22, 35). Therefore, cervid PrP appears to be an important species-specific factor in susceptibility to deer or elk prion disease. Furthermore, in deer populations, different PrP molecules with glycine or serine at position 96 (30) and serine or phenylalanine at position 225 (18) have been described. Some of these PrP sequence variations might modify resistance or susceptibility to CWD in deer because variations at several positions in sheep PrP are known to influence resistance to

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▽ Published ahead of print on 21 February 2007.

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scrapie (3, 5, 14). However, all four of the previous studies of cervid PrP Tg mice used deer or elk PrP alleles with glycine at position 96. In the current study, in order to test whether the allelic variation at this position in deer PrP might be important for susceptibility, we generated deer PrP Tg mice with either glycine (G) or serine (S) at position 96. Following intracerebral infection, mice expressing G96 deer PrP were susceptible to four deer or elk CWD pools. In contrast, mice expressing S96 deer PrP were resistant to these same pools. These results suggest that allelic variation in deer PrP may be an important factor in resistance to CWD infection in vivo.

MATERIALS AND METHODS

Generation of deer PrP transgenic mice. Transgenic mice expressing deer PrP were generated using standard procedures. Plasmid DNA encoding the half genomic mouse PrP (pHGPrP) (12) was modified to encode deer PrP with either the G96 or S96 allele. A 5-kb portion of unsequenced DNA was removed from the promoter region of pHGPrP by digestion with BamHI followed by religation. Unique SalI and XhoI sites were removed by digestion with SalI and XhoI followed by religation. Most of the mouse PrP open reading frame (ORF) was removed by digestion with AgeI and SfoI, and this region was replaced by an oligonucleotide polylinker containing AgeI, NsiI, BstEII, MluI, XhoI, and SfoI restriction enzyme sites as described previously (10). The remaining mouse PrP ORF downstream from the signal peptide was removed by digestion with PshAI (in the mouse PrP signal peptide sequence) and XhoI (in the polylinker). The 5′ portion of the deer PrP ORF was inserted in this position between PshAI and XhoI by ligation of two upper strand and two lower strand oligonucleotides as follows: 5′ATGTCGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAG GATGGAACACCG3′ (upper1), 5′GTGGGAGCCGATACCCGGGACGCGT GCATGCCTCGAG3′ (upper2), 5′TCGAGGCATGCACGCGTCCCGGGTA TCGGCTCCCACCGGTGTTCCAT 3′ (lower1), and 5′CCTCCTCCAGGTTT TGGTCGCTTCTTGCAGAGGCCGACAT3′ (lower2).

After reannealing, these oligonucleotides encoded deer PrP from the PshAI site in the mouse PrP signal peptide to the SmaI site in deer PrP and also contained MluI, SphI,and XhoI sites at the 3′ end. To replace the unsequenced mouse PrP DNA removed in the first step above, the plasmid was digested with NotI and BspEI, and a 6.2-kb NotI-to-BspEI fragment from pHGPrP was inserted. To introduce the remainder of the 3′ deer PrP ORF, a 0.67-kb SmaI-to-SalI fragment encoding the 3′ two-thirds of deer PrP G96 or S96 excised from the respective pTZnot clones (33) was inserted from SmaI to XhoI. The GenBank accession number for the G96 clone was AF156185, and the accession number for the S96 clone was AF156184. To remove the bacterial plasmid DNA, the final plasmids, p65-7 (G96 allele) and p97-7 (S96 allele), were digested with NotI and SbfI, and the large eukaryotic DNA fragment was purified and used to generate transgenic mice using PrP null mice (25). The transgene was maintained with the mouse PrP null genotype by selective crossing and PCR analysis as described previously (10). The PrP produced from this transgene has the mouse PrP signal peptide followed by the deer PrP ORF, but after cleavage of the signal peptide, the mature PrP is identical to the PrP from deer.

CWD pools. Four CWD pools used in these studies were from the following groups. The Deer 1 group consisted of captive white-tailed deer, deer 94W05803(WY), 95W10089(WY), 97W6570(CO), 97W6571(CO), 97W07231(CO), 98W00770(WY), and 98W06348(CO) (Elizabeth Williams, Department of Veterinary Sciences, University of Wyoming, Laramie, WY). The Deer 2 group consisted of free-ranging mule deer, deer 02W643, 01W10027, 00W8741, 00W13445, 01W11102, and 00W11673 (E. Williams). The Deer 3 group consisted of captive Colorado mule deer, deer 03-407541, 03-465081, 03-464506, and 03-460516 (Michael Miller, Colorado Division of Wildlife, Wildlife Research Center, Ft. Collins, CO). The Elk group consisted of free-ranging elk, elk 98W12019 and 01W12716 (E. Williams). In elk and mule deer, the G96 PrP is the only type detected (6, 33). In contrast, white-tailed deer express S96 PrP and/or G96 PrP (19, 30). Thus, the brains of the white-tailed deer in the Deer 1 pool used in this study might contain either one or both alleles. Unfortunately, it was not possible to genotype the animals used in this pool.

Immunoblotting. Brain samples were prepared for analysis of PrPsen as previously described (10). Samples were frozen at −20°C until electrophoresed on a 16% acrylamide gel (Invitrogen) in 25 mM Tris, 250 mM glycine, and 0.1% sodium dodecyl sulfate (SDS). Each lane contained 2.7 mg brain equivalents. Immunoblots were probed first with anti-PrP monoclonal antibody L42 (R-Biopharm) (36) at 0.2 μg/ml. This antibody recognizes PrP sequences from residues 145 to 163 in sheep (23) and cross-reacts strongly with deer and elk PrP. Blots were next probed with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin G at 0.25 μg/ml (Amersham). After the development of enhanced chemiluminescence according to the manufacturer's recommendations (Amersham), blots were exposed to film for visualization of protein bands.

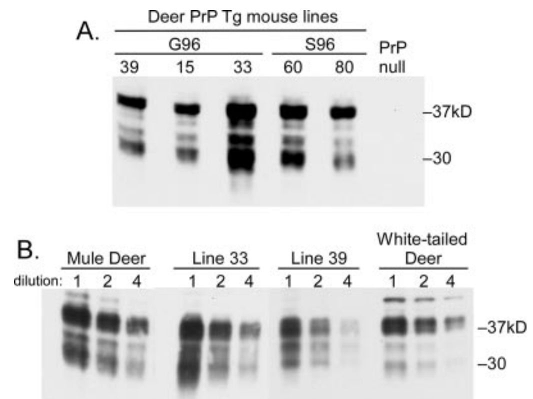

FIG. 1. (A) Immunoblot detection of brain expression of PrPsen in transgenic mouse lines expressing deer PrP. Each lane was loaded with 2.7 mg equivalents of brain homogenate. (B) Immunoblot comparison of PrPsen levels in brains of mule deer, white-tailed deer, and line 33 and line 39 Tg heterozygous mice expressing deer PrP. In panel B, dilutions were 1:1, 1:2, and 1:4. Lanes with 1:1 dilutions had 0.8 mg equivalent of brain. Two or three mice from each line were tested in two or more replicate experiments with three different monoclonal antibodies (L42, D18, and F99/97.6.1). The results were consistent in all these tests, so only the L42 data were presented.

To demonstrate PrPres, tissues were made into a 20% (wt/vol) homogenate in 0.01 M Tris HCl (pH 7.4) and 0.005 M MgCl and then sonicated in a cup-horn sonicator for 1 min. Twenty microliters of the 20% homogenate was adjusted to 100 mM Tris HCl (pH 8.3), 1% Triton X-100, and 1% sodium deoxycholate in 31 μl. Samples were treated with 50 μg/ml concentration of proteinase K (PK) for 1/2 h at 37°C. The reaction was stopped by adding 2 μl of 0.1 M phenylmethylsulfonyl fluoride and placed on ice for 5 min. An equal volume of 2× sample buffer was added, then tubes were boiled 5 min and run on SDS-polyacrylamide gels, and blots were probed with anti-PrP monoclonal L42. For detection of extraneural PrPres, the ultracentrifugation method was used to concentrate the PrPres (10), and 20 mg equivalents of original tissue were added to each lane. For *N*-glycosidase F (PNGase F) treatment (New England Biolabs) of PrPsen and PrPres samples, reaction conditions were as recommended by the manufacturer except denaturing of 4 mg brain equivalents was done in SDS-polyacrylamide gel electrophoresis sample buffer. Each sample was digested using 2,500 U of PNGase F and incubated overnight at 37°C. Samples were frozen at −20°C until analyzed by immunoblotting.

Histopathology. Tissues were removed, fixed in neutral buffered formalin, and embedded in paraffin (20). PrPres was detected by immunostaining with monoclonal anti-PrP antibody F99/97.6.1 (28) plus biotinylated rabbit anti-mouse $F(ab)_2$ (Jackson Labs) followed by horseradish peroxidase-conjugated streptavidin using an autoclaving protocol (20). Hematoxylin and eosin staining was done using routine methods. Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) staining (green) with 4′,6′-diamidino-2-phenylindole (DAPI) nuclear staining (blue) was done using the Roche in situ cell death detection kit. Formalin-fixed deparaffinized sections were permeabilized with Neuropore (Trevigen) for 20 min and rinsed three times with phosphate-buffered saline prior to exposure to kit reagents for 1 h at 25°C. After the sections were rinsed, they were mounted in Prolong Gold Anti-Fade reagent with DAPI (Invitrogen) and examined in a fluorescence microscope.

Animal studies. Each 4- to 6-week-old mouse received 50 μl of a 1% brain homogenate of one of the four CWD pools described above. This inoculum was administered by injection through the skull into the parietal region of the brain using a 27-gauge needle. Mice were observed daily for signs of clinical disease. The earliest signs were usually mild gait abnormalities. This progressed to severe wobbling gait, tremors, ataxia, and weakness with a leg clasping reflex. Subsequently, there was a state of marked inactivity with maintenance of a hunched posture in spite of stimuli. This latter status was defined as severe clinical disease

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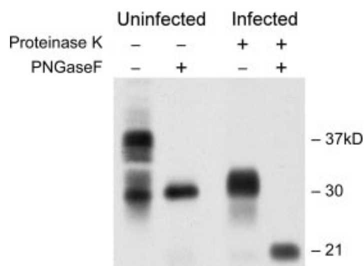

FIG. 2. Comparison of PrPsen and PrPres in Tg mice expressing deer PrP (line 33). The brain from an uninfected Tg mouse expressing deer PrP was used for analysis of PrPsen. Brain homogenate from a Tg mouse infected 301 days earlier with Deer 1 CWD pool was treated with proteinase K (+) to detect PrPres. Both samples were analyzed with (+) and without (−) digestion with PNGase F to remove carbohydrates.

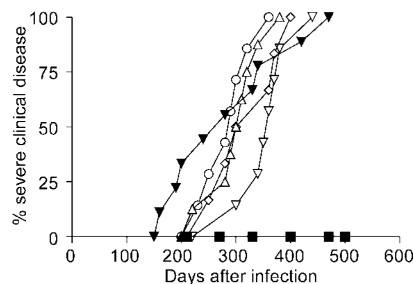

FIG. 3. Survival curves for transgenic mice expressing deer PrP inoculated intracerebrally with four different CWD brain pools. Line 33 G96 Tg mice were inoculated with Deer 1 CWD pool (n = 12) (○), Deer 2 CWD pool (n = 20) (◇), Deer 3 (n = 13) CWD pool (△), and elk CWD pool (n = 14) (▽). For line 15 G96 Tg mice (n = 19) (▼) and lines 60 and 80 of S96 Tg mice (n = 26) (■), pooled data from mice inoculated with all four CWD pools are shown. The graph shows time from inoculation to severe clinical neurological disease. Brain tissue was tested by immunoblotting for PrPres to confirm the diagnosis of CWD. Age-matched line 33 Tg mice, which were inoculated with healthy brain homogenate or sham inoculated, showed no signs of clinical disease, and brain PrPres was not detected by immunoblotting. The time of earliest detectable clinical disease, usually mild gait abnormality, was 30 to 50 days earlier than the time to severe clinical disease which was reported in this figure. Curves were compared by the Kruskal-Wallis test, and no significant differences among the G96 (line 33 and line 15) mice were found.

and was recorded as the end point in the data presented. Typically, mice in this condition were sacrificed, as they would progress to a moribund status within 2 or 3 days. In these assays, the duration of clinical signs from onset to end stage was from 30 to 50 days, which was longer than usually observed with standard mouse scrapie strains, such as 22L and RML. Prion disease was confirmed by analysis of brain tissue for PrPres by immunoblotting.

RESULTS AND DISCUSSION

Analysis of PrPsen expression. Transgenic founder mice were identified by DNA hybridization and PCR. Deer PrP expression was analyzed by reverse transcription-PCR using mRNA from adult brain tissue of Tg+/− mice. By this assay, high expression was found in G96 Tg lines 15, 33, and 39 as well as in S96 Tg lines 60 and 80 (data not shown). By Western blot analysis, brain deer PrPsen protein expression was highest in line 33, was about 30% lower in line 60, and was about 50% lower in lines 15, 39, and 80 (Fig. 1A). Brain PrPsen level in line 33 was slightly higher than the levels seen in brains of uninfected mule deer and white-tailed deer, and the levels in line 39 were similar to the levels in both types of deer (Fig. 1B).

CWD infection of deer PrP transgenic mice. Mice from all five Tg lines were inoculated with four different sources of CWD infectivity. Brain tissue was analyzed for the presence of PrPres by Western blotting to confirm the clinical diagnosis of CWD. PrPres showed partial protease resistance associated with the approximate 6- to 9-kDa reduction in molecular size typically seen in CWD and other prion diseases (Fig. 2). This size difference was more accurately visualized when samples were also digested with PNGase F to remove carbohydrates (Fig. 2, compare lanes 2 and 4).

In these experiments, all three Tg lines expressing the G96 deer PrP molecules appeared susceptible to clinical CWD induced by all four CWD pools tested. Line 33 mice developed severe disease from 200 to 400 days postinfection (dpi), and the differences among the mice with CWD induced by the four CWD pools were minimal (Fig. 3). Line 15 mice which expressed about 50% less deer PrP in brain developed severe disease from 160 to 450 dpi. Line 39 mice also developed severe clinical CWD with detectable brain PrPres at times similar to those of lines 33 and 15, but many of these mice succumbed to dermatitis prior to the onset of CWD signs (data not shown). In contrast to the G96 deer PrP mice, lines 60 and 80, which expressed S96 deer PrP, showed no signs of clinical CWD after inoculation of any of the four pools up to 600 dpi (Fig. 3). Furthermore, random sacrifice of 33 infected mice between 276 and 600 dpi showed no mice with PrPres in brain detectable by immunoblotting. In addition, five mice sacrificed at 600 dpi showed no PrPres in the spleen or gut by immunoblotting. The lack of disease and PrPres in S96 deer PrP Tg mice did not appear to be due to low PrP expression levels because the levels in both line 60 and 80 were similar to or higher than the levels in line 39 (G96) and line 15 (G96) mice (Fig. 1). Therefore, G96 deer PrPsen appeared to facilitate susceptibility of transgenic mice to CWD infection by these four pools, whereas S96 deer PrPsen did not.

Brain PrPres glycoform patterns. In some cases, strain variation in prion diseases has been correlated with different PrPres banding patterns on immunoblot analysis (8, 11). To look for CWD strains in our model, immunoblots of brain PrPres from line 33 mice infected with the four different CWD pools were compared. These blots did not show any differences

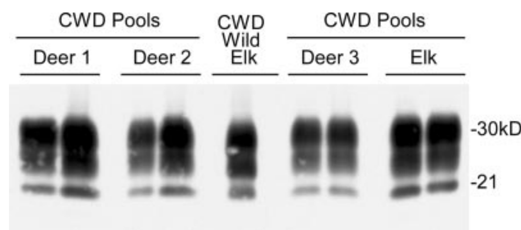

FIG. 4. Comparison of PrPres from line 33 Tg mice expressing deer PrP infected with four different CWD pools. Pairs of mice infected with each pool were sacrificed at the time of clinical disease from 301 to 371 dpi. None of the Tg mice appeared to have different PrPres banding patterns. Elk brain from a wild elk with clinical CWD was used for comparison, and its pattern differed only slightly from that of the CWD-infected Tg mice.

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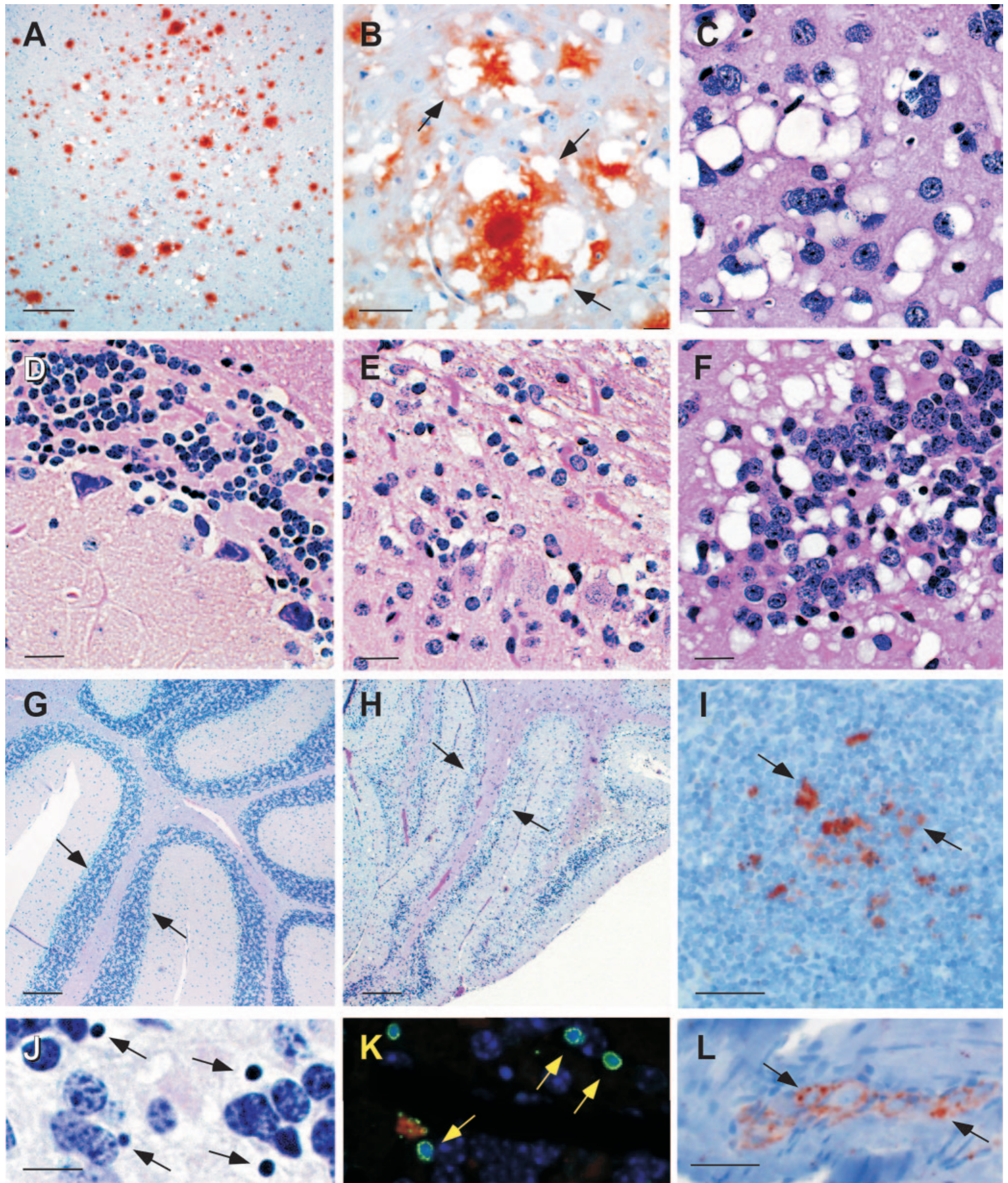

FIG. 5. PrPres detection and pathology in brain tissue of line 33 Tg mice expressing deer PrP at various times after CWD infection. (A) Dense PrPres accumulations in the brain stem detected by monoclonal antibody F99/97.6.1 at 371dpi; (B) PrPres "florid" plaque surrounded by vacuoles (arrows) in anterior cerebral cortex at 371 dpi, detected as described above for panel A. (C to H) hematoxylin-and-eosin-stained sections; (C) large vacuoles in anterior cerebral cortex at 337 dpi; (D) normal cerebellar granule cell layer in uninfected mouse; (E) abnormal cerebellar granule cell layer at 413 dpi; (F) vacuoles and neuronal damage in dentate gyrus of hippocampus at 337 dpi; (G) normal cerebellar granule layer neurons (arrows) in uninfected mouse; (H) loss of cerebellar granule layer neurons (arrows) at 371 dpi. (I) Staining of PrPres (arrows) with monoclonal antibody F99/97.6.1 in a spleen follicle of deer PrP Tg mouse 288 days after infection with Deer 1 CWD pool. (J) Apoptotic bodies and small

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TABLE 1. Regional distribution of PrPres, vacuolar pathology, and neuronal loss in the brains of transgenic mice expressing deer PrP with G96 inoculated with four CWD pools

Brain region	Regional distribution[a] of:		
	PrPres	Vacuoles	Neuronal loss
Olfactory bulb	+++	++++	++
Forebrain	++++	++++	+
Cerebral cortex	+++	+++	+
Corpus callosum	++	+	±
Hippocampus	+++	++++	++++
Thalamus	+++	++	+
Habenula	++	++++	+
Hypothalamus	+++	+	±
Superior colliculus	++	+++	+
Cerebellum	++	++	++++
Brain stem	+++	+++	±

[a] Using three or four mice inoculated with each CWD pool, PrPres, vacuoles, and neuronal loss in various regions were graded on a relative scale from ± (little) to ++++ (abundant or striking). Examples are shown in Fig. 5: ++++ vacuoles in forebrain and hippocampus in Fig. 5C and F; ++ vacuoles in cerebellum in Fig. 5E; ++++ neuronal loss in cerebellum in Fig. 5E and H; ++++ PrPres in forebrain in Fig. 5B. No apparent differences in regional pathology were noted among the four CWD pools.

in PrPres banding patterns among mice infected with the different pools (Fig. 4). Thus, by this test, there was no evidence for the presence of different CWD strains in the elk and deer pools tested. Control mice inoculated with normal brain homogenate and sham-inoculated mice had no detectable PrPres.

Brain tissues from Tg mice inoculated with CWD pools or with normal brain homogenate or sham-inoculated mice were examined histologically for sites of PrPres deposition and pathology. Control mice showed no evidence of PrPres. CWD-inoculated mice had plaque-like PrPres accumulation and vacuolar pathology in numerous areas of the central nervous system (Fig. 5A and B and Table 1). G96 deer PrP Tg mice inoculated with each of the four CWD pools showed similar regional distribution of pathology and PrPres deposition. Although the PrPres deposits were often in dense disc-like accumulations resembling amyloid plaques, staining with Thioflavin S or Congo red was negative, suggesting that this material was not a classical amyloid in nature. This result differed from that reported by others using different lines of cervid PrP Tg mice (7, 35).

Brain histopathology and PrPres distribution. In different brain regions, there appeared to be evidence for two types of histological damage: vacuolation of the gray matter and neuronal apoptosis. These two processes were not always coincident. The cerebellar granular layer had massive neuronal loss (Fig. 5E and H) compared to this layer in age-matched control mice (Fig. 5G). Apoptotic bodies were often seen together with positive TUNEL staining (Fig. 5J and K), but in the cerebellum, this process was associated with only moderate vacuolation and PrPres deposition (Table 1). In one transgenic elk PrP model, infection with elk CWD was also reported to induce marked apoptosis and neuronal loss in the cerebellar granule cell layer (21). In contrast to our results in the cerebellum, the hippocampus had very prominent vacuolation (Fig. 5F), as well as evidence for apoptosis and neuronal loss (not shown). The deeper forebrain regions also had striking vacuolation (Fig. 5C) which was often associated with dense round 20- to 30-micron PrPres deposits (florid plaques) (Fig. 5B), but neuronal loss was not obvious, and only minimal TUNEL staining was observed (not shown). Both of these types of pathology were seen in mice infected with all four CWD pools studied. However, neuronal loss and apoptosis were less prominent in animals that had a more rapid clinical onset and were sacrificed at earlier time points, suggesting that apoptosis was not the primary process leading to clinical disease in this model.

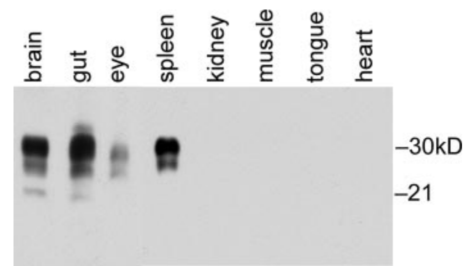

FIG. 6. Immunoblot analysis of PrPres in various tissues of CWD-infected line 33 Tg mice expressing deer PrP. PrPres was concentrated by the ultracentrifuge method as described in Materials and Methods, and 20 mg equivalents of tissue were added to each lane. Results are representative of data from five mice infected with each of the four CWD pools tested.

Detection of PrPres outside brain. In many prion diseases, including CWD, PrPres can be detected in extraneural sites, such as tonsil, spleen, and lymph nodes. Therefore, we examined several tissues for evidence of PrPres in the G96 deer PrP Tg mice intracerebrally infected with CWD. By immunoblotting using monoclonal antibody L42, significant PrPres signals were found in brain, eye, spleen and intestinal tissue, but kidney, tongue, heart, and skeletal muscle were all negative (Fig. 6). Liver, testes, and lung were also negative (not shown). By immunohistochemistry, PrPres in spleen was consistent with possible location in follicular dendritic cells and macrophages associated with follicles (Fig. 5I). PrPres in intestine appeared to be located primarily in neuronal ganglia between the muscular layers (Fig. 5L), but PrPres was also noted in mesenteric lymph nodes and Peyer's patches in some mice (not shown).

Using sensitive biological infectivity assays, the CWD infectious agent was recently found in saliva and blood (26) and skeletal muscle (2) of CWD-infected deer. However, by immunoblotting, which is less sensitive, we failed to detect PrPres in

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pyknotic nuclei (arrows) in cerebellar granule cell layer at 289 dpi. (K) TUNEL-positive nuclei (yellow arrows) in cerebellar granular cell layer at 289 dpi. TUNEL-positive cells were not seen in uninfected mice. (L) Punctate PrPres staining in neuronal ganglion cells in lower gut of deer PrP Tg mouse 288 days after infection with Deer 1 CWD pool. Bars, 200 microns (A, G, and H), 50 microns (B, J, K, and L), 20 microns (C to F), and 10 microns (I).

skeletal muscle of CWD-infected Tg mice (Fig. 6). We also failed to detect PrPres in heart tissue of these mice, similar to results with CWD-infected mule deer heart but differing from results with elk heart and white-tailed deer heart (17). Therefore, there are likely to be species differences among cervids and Tg mice which account for these discrepancies.

None of the four previous studies of cervid PrP transgenic mice reported on studies of extraneural PrPres. One paper stated that no PrPsen was found in non-brain tissues and ascribed this finding to the use of a modified promoter (7). The finding of PrPres in spleen and intestinal tissue in our line 33 Tg mice indicated that there was sufficient extraneural PrPsen in these tissues to allow PrPres generation. Thus, these mice should prove very useful in studies of peripheral CWD pathogenesis by oral or other possible natural routes of infection.

The most striking discovery from the present studies was the finding that two deer PrP alleles differed in the ability to confer sensitivity to CWD. Previous transgenic mouse studies have analyzed only G96 deer or elk PrP alleles, which were found to confer susceptibility to deer and elk CWD in all studies so far. In contrast, this is the first study testing the S96 deer PrP allele in transgenic mice, and it was surprising to find that this allele failed to confer susceptibility to any of the four CWD pools tested. Of course, it is possible that these mice will develop disease or PrPres at a later time point past the present 600-day observation period.

Our results are similar but not identical to results of naturally occurring CWD in white-tailed deer where S96 homozygous deer appear to be partially resistant to CWD. Nevertheless, at least three such deer have been documented to develop CWD as diagnosed by immunohistochemical PrPres detection in the brain stem (obex) (19, 30).

It is presently unclear exactly how the allelic variation at PrP residue 96 might influence prion disease susceptibility. At a molecular level, deer and elk PrP residue 96 is located in the flexible disordered tail of the N-terminal domain near the globular domain (15). In transgenic mice, expression of PrP constructs with various deletions indicated that the region around residue 96 was important for scrapie susceptibility (13, 34, 37). In addition, molecular PrP conversion studies using N-terminus-deleted PrP showed that the region between residues 94 and 106 influenced the type and amount of PrP conversion (24). However, in other cell-free conversion studies, G96 and S96 deer PrP were found to convert equally well when incubated with CWD from several cervid sources (33). Therefore, in CWD-infected deer, the influence of differing amino acid residues at position 96 probably does not act by alteration of the PrP conversion process.

An alternative explanation of the resistance of S96 PrP Tg mice to CWD infection is that deer expressing G96 or S96 PrP might be preferentially infected by different CWD strains. This selectivity for different strains or isolates has been found in sheep scrapie. Sheep carrying the so-called PrP VRQ allele are more susceptible to VRQ-derived isolates, like SSBP/1, but are more resistant to non-VRQ-derived isolates, like CH1641. In contrast, PrP ARQ carriers or breeds usually resist SSBP/1 but are highly susceptible to CH1641 (14, 16). This selectivity has been further supported by the underlying molecular conversion process in which VRQ and ARQ prion proteins are more rapidly converted by their homologous VRQ or ARQ PrPres molecules (4).

Although no distinct CWD strains have been identified so far, a recent study of CWD infection in cervid PrP Tg mice described a mule deer CWD pool which differed from other pools by its inability to induce vacuolation in the anterior cerebral cortex and olfactory bulb (22). Such regional pathology differences might be due to the existence of a variant CWD strain.

In our experiments, the CWD pools, Deer 2, Deer 3, and Elk, are likely to have come from G96 animals because both mule deer and elk appear to be homozygous for G96 PrP (6, 29). In uninfected white-tailed deer, the S96/S96 homozygous genotype exists naturally at a low frequency (5 to 9%), but its incidence is even lower (0.3 to 3%) in CWD-infected animals (19, 30). Therefore, homozygous S96 PrP deer may not be represented in our Deer 1 pool of CWD-infected white-tailed deer. In future experiments, CWD agent derived from homozygous S96 PrP white-tailed deer will have to be tested in our S96 deer PrP Tg mice to search for a unique CWD strain.

ACKNOWLEDGMENTS

We thank Teng Chao, Anita Mora, Gary Hettrick, Scott Hughes, and Lisa Kercher for technical and graphic advice and assistance, and we thank Jan Langeveld, Byron Caughey, Suzette Priola, and John Portis for helpful comments concerning the manuscript. We also thank Lionel Feigenbaum for assistance in generating transgenic mice, Edward Schreckengust for assistance with animal care, and the Colorado and Wyoming Departments of Fish and Game for help with cervid sample collection.

REFERENCES

1. **Aguzzi, A.** 2006. Prion diseases of humans and farm animals: epidemiology, genetics, and pathogenesis. J. Neurochem. **97:**1726–1739.
2. **Angers, R. C., S. R. Browning, T. S. Seward, C. J. Sigurdson, M. W. Miller, E. A. Hoover, and G. C. Telling.** 2006. Prions in skeletal muscles of deer with chronic wasting disease. Science **311:**1117.
3. **Baylis, M., and W. Goldmann.** 2004. The genetics of scrapie in sheep and goats. Curr. Mol. Med. **4:**385–396.
4. **Bossers, A., P. B. G. M. Belt, G. J. Raymond, B. Caughey, R. de Vries, and M. A. Smits.** 1997. Scrapie susceptibility-linked polymorphisms modulate the *in vitro* conversion of sheep prion protein to protease-resistant forms. Proc. Natl. Acad. Sci. USA **94:**4931–4936.
5. **Bossers, A., B. E. Schreuder, I. H. Muileman, P. B. Belt, and M. A. Smits.** 1996. PrP genotype contributes to determining survival times of sheep with natural scrapie. J. Gen. Virol. **77:**2669–2673.
6. **Brayton, K. A., K. I. O'Rourke, A. K. Lyda, M. W. Miller, and D. P. Knowles.** 2004. A processed pseudogene contributes to apparent mule deer prion gene heterogeneity. Gene **326:**167–173.
7. **Browning, S. R., G. L. Mason, T. Seward, M. Green, G. A. Eliason, C. Mathiason, M. W. Miller, E. S. Williams, E. Hoover, and G. C. Telling.** 2004. Transmission of prions from mule deer and elk with chronic wasting disease to transgenic mice expressing cervid PrP. J. Virol. **78:**13345–13350.
8. **Cali, I., R. Castellani, J. Yuan, A. Al-Shekhlee, M. L. Cohen, X. Xiao, F. J. Moleres, P. Parchi, W. Q. Zou, and P. Gambetti.** 2006. Classification of sporadic Creutzfeldt-Jakob disease revisited. Brain **129:**2266–2277.
9. **Chesebro, B.** 2003. Introduction to the transmissible spongiform encephalopathies or prion diseases. Br. Med. Bull. **66:**1–20.
10. **Chesebro, B., M. Trifilo, R. Race, K. Meade-White, C. Teng, R. LaCasse, L. Raymond, C. Favara, G. Baron, S. Priola, B. Caughey, E. Masliah, and M. Oldstone.** 2005. Anchorless prion protein results in infectious amyloid disease without clinical scrapie. Science **308:**1435–1439.
11. **Collinge, J., K. C. L. Sidle, J. Meads, J. Ironside, and A. F. Hill.** 1996. Molecular analysis of prion strain variation and the aetiology of "new variant" CJD. Nature **383:**685–690.
12. **Fischer, M., T. Rulicke, A. Raeber, A. Sailer, M. Moser, B. Oesch, S. Brandner, A. Aguzzi, and C. Weissmann.** 1996. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. EMBO J. **15:**1255–1264.
13. **Flechsig, E., D. Shmerling, I. Hegyi, A. J. Raeber, M. Fischer, A. Cozzio, C. von Mering, A. Aguzzi, and C. Weissmann.** 2000. Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. Neuron **27:**399–408.

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14. **Goldmann, W., N. Hunter, G. Smith, J. Foster, and J. Hope.** 1994. PrP genotype and agent effects in scrapie: change in allelic interaction with different isolates of agent in sheep, a natural host of scrapie. J. Gen. Virol. **75:**989–995.
15. **Gossert, A. D., S. Bonjour, D. A. Lysek, F. Fiorito, and K. Wuthrich.** 2005. Prion protein NMR structures of elk and of mouse/elk hybrids. Proc. Natl. Acad. Sci. USA **102:**646–650.
16. **Hunter, N., J. D. Foster, W. Goldmann, M. J. Stear, J. Hope, and C. Bostock.** 1996. Natural scrapie in a closed flock of Cheviot sheep occurs only in specific PrP genotypes. Arch. Virol. **141:**809–824.
17. **Jewell, J. E., J. Brown, T. Kreeger, and E. S. Williams.** 2006. Prion protein in cardiac muscle of elk (Cervus elaphus nelsoni) and white-tailed deer (Odocoileus virginianus) infected with chronic wasting disease. J. Gen. Virol. **87:**3443–3450.
18. **Jewell, J. E., M. M. Conner, L. L. Wolfe, M. W. Miller, and E. S. Williams.** 2005. Low frequency of PrP genotype 225SF among free-ranging mule deer (Odocoileus hemionus) with chronic wasting disease. J. Gen. Virol. **86:**2127–2134.
19. **Johnson, C., J. Johnson, J. P. Vanderloo, D. Keane, J. M. Aiken, and D. McKenzie.** 2006. Prion protein polymorphisms in white-tailed deer influence susceptibility to chronic wasting disease. J. Gen. Virol. **87:**2109–2114.
20. **Kercher, L., C. Favara, C. C. Chan, R. Race, and B. Chesebro.** 2004. Differences in scrapie-induced pathology of the retina and brain in transgenic mice that express hamster prion protein in neurons, astrocytes, or multiple cell types. Am. J. Pathol. **165:**2055–2067.
21. **Kong, Q., S. Huang, W. Zou, D. Vanegas, M. Wang, D. Wu, J. Yuan, M. Zheng, H. Bai, H. Deng, K. Chen, A. L. Jenny, K. O'Rourke, E. D. Belay, L. B. Schonberger, R. B. Petersen, M. S. Sy, S. G. Chen, and P. Gambetti.** 2005. Chronic wasting disease of elk: transmissibility to humans examined by transgenic mouse models. J. Neurosci. **25:**7944–7949.
22. **LaFauci, G., R. I. Carp, H. C. Meeker, X. Ye, J. I. Kim, M. Natelli, M. Cedeno, R. B. Petersen, R. Kascsak, and R. Rubenstein.** 2006. Passage of chronic wasting disease prion into transgenic mice expressing Rocky Mountain elk (Cervus elaphus nelsoni) PrPC. J. Gen. Virol. **87:**3773–3780.
23. **Langeveld, J. P., J. G. Jacobs, J. H. Erkens, A. Bossers, F. G. van Zijderveld, and L. J. van Keulen.** 2006. Rapid and discriminatory diagnosis of scrapie and BSE in retro-pharyngeal lymph nodes of sheep. BMC Vet. Res. **2:**19.
24. **Lawson, V. A., S. A. Priola, K. Meade-White, M. Lawson, and B. Chesebro.** 2004. Flexible N-terminal region of prion protein influences conformation of protease-resistant prion protein isoforms associated with cross-species scrapie infection in vivo and in vitro. J. Biol. Chem. **279:**13689–13695.
25. **Manson, J. C., A. R. Clarke, M. L. Hooper, L. Aitchison, I. McConnell, and J. Hope.** 1994. 129/Ola mice carrying a null mutation in PrP that abolishes mRNA production are developmentally normal. Mol. Neurobiol. **8:**121–127.
26. **Mathiason, C. K., J. G. Powers, S. J. Dahmes, D. A. Osborn, K. V. Miller, R. J. Warren, G. L. Mason, S. A. Hays, J. Hayes-Klug, D. M. Seelig, M. A. Wild, L. L. Wolfe, T. R. Spraker, M. W. Miller, C. J. Sigurdson, G. C. Telling, and E. A. Hoover.** 2006. Infectious prions in the saliva and blood of deer with chronic wasting disease. Science **314:**133–136.
27. **Miller, M. W., and E. S. Williams.** 2003. Prion disease: horizontal prion transmission in mule deer. Nature **425:**35–36.
28. **O'Rourke, K. I., T. V. Baszler, T. E. Besser, J. M. Miller, R. C. Cutlip, G. A. Wells, S. J. Ryder, S. M. Parish, A. N. Hamir, N. E. Cockett, A. Jenny, and D. P. Knowles.** 2000. Preclinical diagnosis of scrapie by immunohistochemistry of third eyelid lymphoid tissue. J. Clin. Microbiol. **38:**3254–3259.
29. **O'Rourke, K. I., T. E. Besser, M. W. Miller, T. F. Cline, T. R. Spraker, A. L. Jenny, M. A. Wild, G. L. Zebarth, and E. S. Williams.** 1999. PrP genotypes of captive and free-ranging Rocky Mountain elk (Cervus elaphus nelsoni) with chronic wasting disease. J. Gen. Virol. **80:**2765–2769.
30. **O'Rourke, K. I., T. R. Spraker, L. K. Hamburg, T. E. Besser, K. A. Brayton, and D. P. Knowles.** 2004. Polymorphisms in the prion precursor functional gene but not the pseudogene are associated with susceptibility to chronic wasting disease in white-tailed deer. J. Gen. Virol. **85:**1339–1346.
31. **Prusiner, S. B.** 1982. Novel proteinaceous infectious particles cause scrapie. Science **216:**136–144.
32. **Race, R., A. Raines, G. L. Raymond, B. Caughey, and B. Chesebro.** 2001. Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J. Virol. **75:**10106–10112.
33. **Raymond, G. J., A. Bossers, L. D. Raymond, K. I. O'Rourke, L. E. McHolland, P. K. Bryant III, M. W. Miller, E. S. Williams, M. Smits, and B. Caughey.** 2000. Evidence of a molecular barrier limiting susceptibility of humans, cattle and sheep to chronic wasting disease. EMBO J. **19:**4425–4430.
34. **Shmerling, D., I. Hegyi, M. Fischer, T. Blattler, S. Brandner, J. Gotz, T. Rulicke, E. Flechsig, A. Cozzio, C. von Mering, C. Hangartner, A. Aguzzi, and C. Weissmann.** 1998. Expression of amino-terminally truncated PrP in the mouse leading to ataxia and specific cerebellar lesions. Cell **93:**203–214.
35. **Tamgüney, G., K. Giles, E. Bouzamondo-Bernstein, P. J. Bosque, M. W. Miller, J. Safar, S. J. DeArmond, and S. B. Prusiner.** 2006. Transmission of elk and deer prions to transgenic mice. J. Virol. **80:**9104–9114.
36. **Vorberg, I., A. Buschmann, S. Harmeyer, A. Saalmuller, E. Pfaff, and M. H. Groschup.** 1999. A novel epitope for the specific detection of exogenous prion proteins in transgenic mice and transfected murine cell lines. Virology **255:**26–31.
37. **Weissmann, C.** 1999. Molecular genetics of transmissible spongiform encephalopathies. J. Biol. Chem. **274:**3–6.
38. **Williams, E. S.** 2005. Chronic wasting disease. Vet. Pathol. **42:**530–549.

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