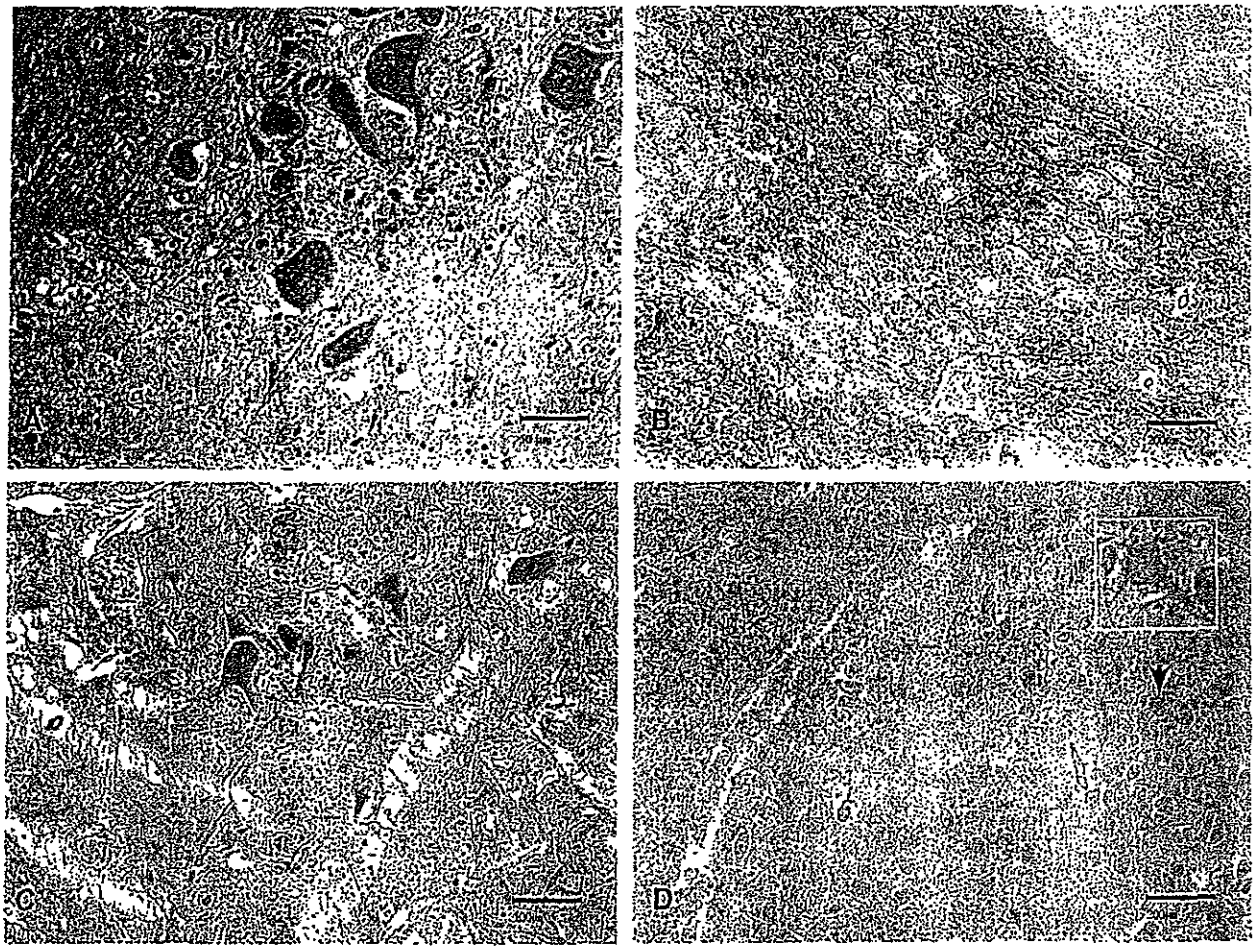

Figure 1. Histopathology and IHC on brain samples from US BSE cases. In both cases the medulla at the level of the obex was examined. US BSE case 1 is depicted in A, and B, US BSE case 2 is depicted in C, D, A; C = HE staining; B, D = IHC for PrP^{Sc} in red, counterstain with hematoxylin in blue; Arrow = Neuron surrounded by PrP^{Sc}; Inset = magnification of neuron depicted by arrow. Bar = 75 μm.

throughout the obex. This included most nuclei and gray mater neuropil. Both intracellular and intracellular staining was present. Case 2 also had widespread neuropil and intracellular immunostaining however the staining was markedly less intense than case 1 and more prominent in the lateral and ventral portions of the section as compared to case 1. The staining was most pronounced in the nucleus of the solitary tract and the nucleus of the spinal tract of the trigeminal nerve as well as the olivary nuclei.

Western blot analysis

Western blot analysis (employing the Prionics®-Check Western Kit®) of brainstem homogenates of case 1 revealed a strong positive reaction at 1 mg tissue equivalent (Fig. 2A–C). All 3 isoforms of PrP^{SC} were easily detected using the monoclonal antibody 6H4 and showed similar molecular masses when compared to the May 2003 Canadian and a Swiss BSE isolate (Fig. 2B). The glycoform profile of the 3 PrP^{Sc}-isoforms of case 1 was determined by analyzing 5 independent Western blots using the Typhoon™ imaging system employing software ImageQuant 5.2™. The diglycosylated isoform was the most prominent isoform (72.0% ± 4.7), followed by the monoglycosylated (23.3% ± 3.6) and the unglycosylated (4.0% ± 1.2) isoforms. The unglycosylated isoform of case 1 showed a lower molecular weight when compared with mule deer CWD, sheep-passaged mule deer CWD, elk CWD and sheep scrapie samples (Fig. 2A, B). Using a hybrid Western blot employing monoclonal antibodies P4 and 6H4,[44] all TSE-infected samples reacted well with antibody 6H4, however, US BSE case 1 and a cattle-passaged scrapie isolate did not react with antibody P4 (Fig. 2C, D). Sheep scrapie and mule deer CWD

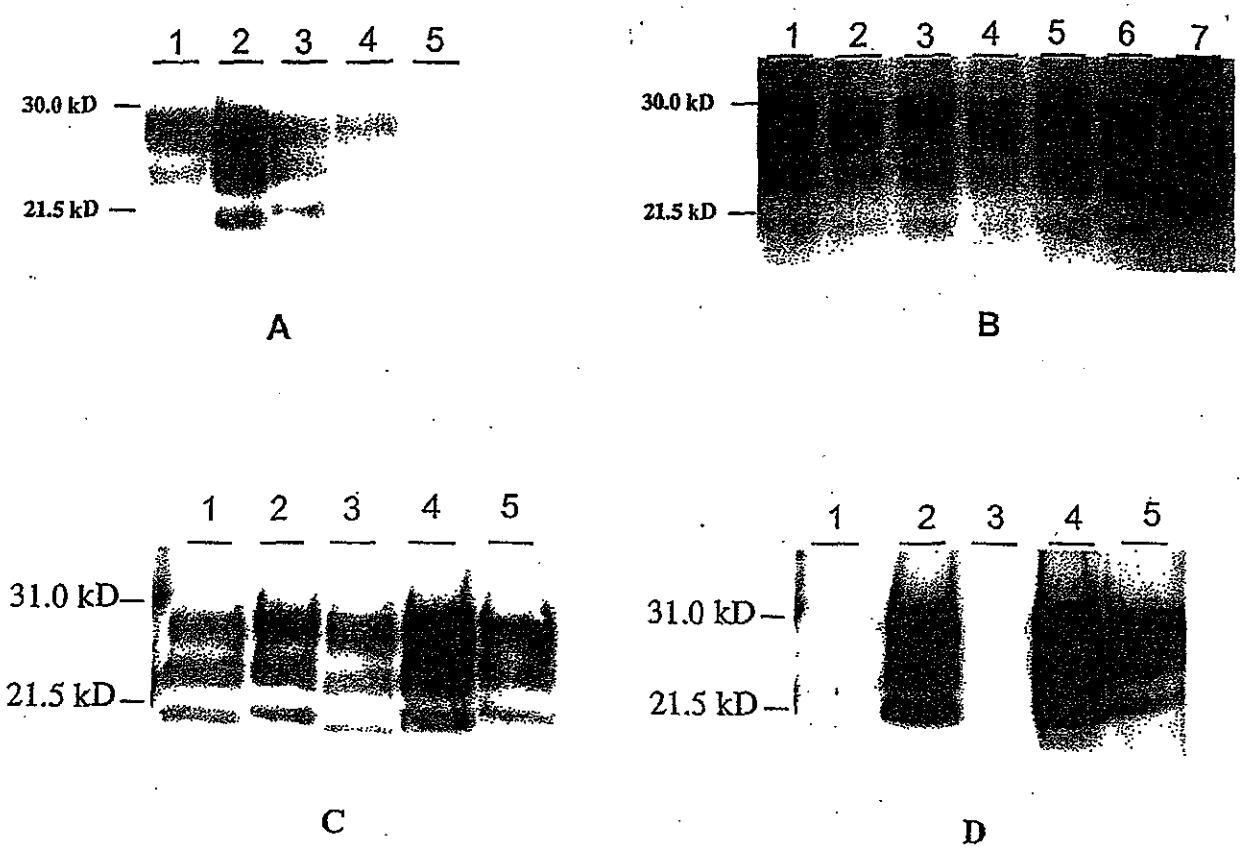

Figure 2. Western blot analysis of US BSE case 1. A, Antibody 6H4. All samples were loaded with 1-mg brain tissue equivalent. 1 = US BSE case 1; 2 = Sheep scrapie; 3 = CWD mule deer; 4 = sheep-passaged CWD mule deer; 5 = negative cattle CNS control. B, Antibody 6H4. All samples were loaded at 1-mg brain tissue equivalent. 1 = Canadian BSE isolate 2003; 2 = US BSE case 1; 3 = Canadian BSE isolate 2003; 4 = US BSE case 1; 5 = Swiss BSE isolate; 6 = US BSE case 1; 7 = elk CWD. C, and D, Hybrid Western blot analysis [44] using antibodies 6H4 (C) and P4 (D). One PAGE gel was loaded with 2 sets of identical samples (1-mg brain tissue equivalent) and cut in the middle before incubation with the respective antibodies. 1 = cattle-passaged scrapie; 2 =sheep scrapie; 3 = US BSE case 1; 4 = sheep scrapie; 5 = mule deer CWD. D. 1 = cattle-passaged scrapie; 2 = sheep scrapie; 3 = US BSE case 1; 4 = sheep scrapie; 5 = mule deer CWD.

reacted with both antibodies. As already discussed above (see Fig. 2A), the relative molecular mass of the unglycosylated isoform of the mule deer CWD isolate and the two sheep scrapie isolates was higher than the respective isoform of case 1 (Fig. 2C).

Brain material (brainstem and cerebellum) from case 2 was analyzed by Western blot with and without enrichment of PrP^{Sc}. It should be noted that it was not possible to determine the precise anatomical location where the samples were taken, since the material was frozen and thawed several times before Western blot analysis was performed. Nonenriched samples (Prionics®-Check Western Kit[f]) were analyzed at a concentration of 1–1.5 mg brain tissue equivalent (mg eq) and enriched samples (SAF Immunoblot) at a concentration of 10–150 mg eq. As shown in Fig. 3A, brainstem from case 2 was definitely positive after enrichment (≥20 mg eq), whereas the nonenriched sample (1 mg eq) was inconclusive. All 3 isoforms of PrP^{Sc} were definitely present at 20 mg eq with a strong reaction at 40 and 80 mg eq (Fig. 3A). In addition, brainstem and cerebellum samples were taken from different regions of the respective tissues and analyzed by enrichment Western blot. However, only 2 out of 4 cerebellum and 3 out of 6 brainstem samples were positive at 20 mg eq (data not shown). Glycoform profile analysis (analyzing 4 independent Western blots) revealed a significant proportion of the diglycosylated isoform (61.2% ± 2.9), and lesser amounts of the monoglycosylated (29.8% ± 2.3) and unglycosylated (9.0% ± 2.5) isoforms. Furthermore, cerebellum and brainstem samples from case 1 and case 2 were compared side by side employing monoclonal antibody 6H4. All of these samples, except for the sheep scrapie control sample, were enriched for PrP^{Sc}. The unglycosylated and monoglycosylated isoforms of PrP^{Sc} from case 2 migrated with an apparent molecular mass higher than the respective

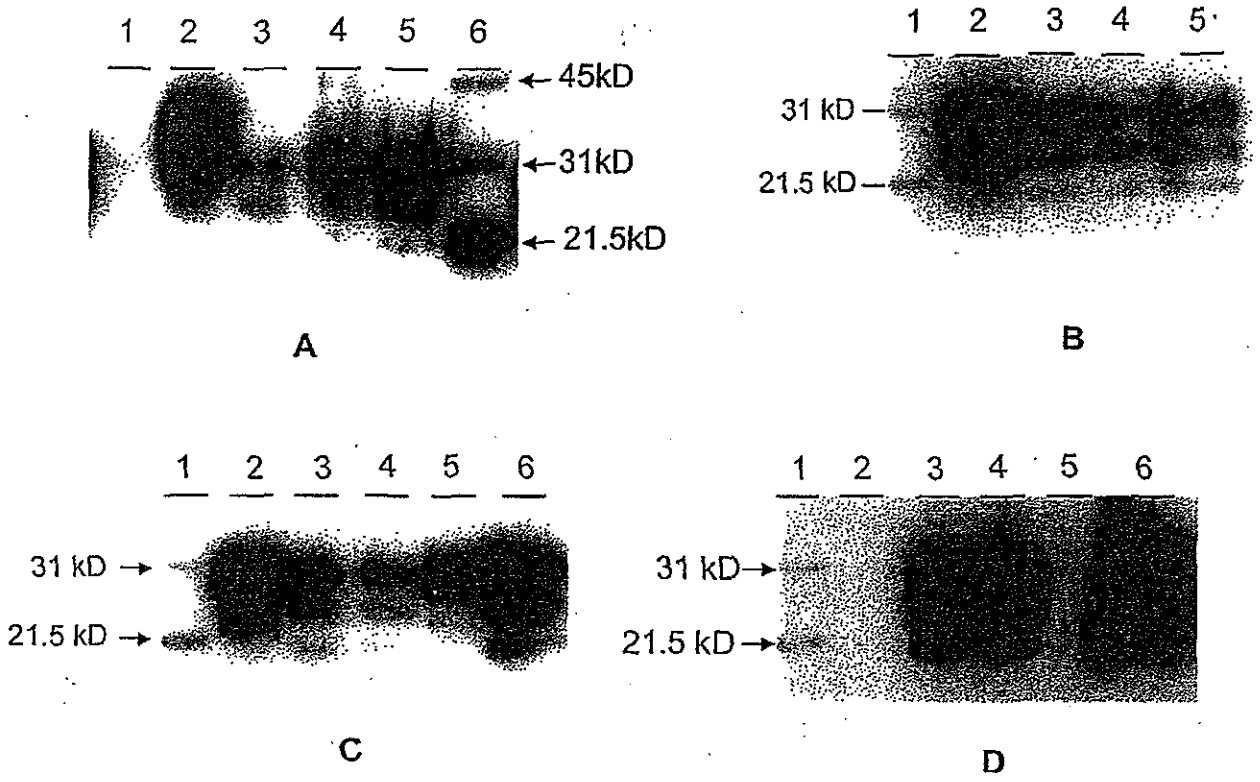

Figure 3. Western blot analysis of US BSE case 2. A, Brainstem material, antibody 6H4. 1 = US BSE case 2, 1 mg eq, PK-treated; 2 = US BSE case 2, 20 mg eq, non-PK-treated; 3 = US BSE case 2, 20 mg eq, PK-treated; 4 = US BSE case 2, 40 mg eq, PK-treated; 5 = US BSE case 2, 80 mg eq, PK-treated; 6 = Molecular weight marker™. B, Brainstem material, antibody 6H4. 1 = Molecular weight marker™; 2 = US BSE case 1, 20 mg eq; 3 = US BSE case 2, 65 mg eq; 4 = US BSE case 2, 25 mg eq; 5 = sheep scrapie, 1.5 mg eq. C, and D, Hybrid Western blot analysis [44] with cerebellum samples using antibodies 6H4 (C) and P4 (D). One PAGE gel was loaded with 2 sets of identical samples and cut in the middle before incubation with the respective antibodies. 1 = Molecular weight marker™; 2 = US BSE case 1, 3 mg eq; 3 = US BSE case 2, 50 mg eq; 4 = US BSE case 2, 50 mg eq; 5 = US BSE case 1, 3 mg eq; 6 = sheep scrapie, 1.5 mg eq.

isoforms of case 1 (Fig. 3B, 3C), indicating a relative difference in molecular mass between the two BSE cases. The reaction pattern was very similar, independent of the region of the brain (brainstem or cerebellum) used for analysis (Fig. 3B, 3C). In hybrid Western blots, enriched cerebellum samples from case 1 reacted strongly with antibody 6H4 (Fig. 3C) and either weakly or not at all with antibody P4 (Fig. 3D), a similar reaction pattern as described for non-enriched brainstem samples of case 1 (Fig. 2C, 2D). Cerebellum samples from case 2 reacted strongly with both antibodies, 6H4 and P4 (Fig. 3C, 3D). The intensity of the reaction of case 2 brain material with antibody P4 (Fig. 3D) was even stronger than with 6H4 (Fig. 3C) at similar milligram equivalent amounts. The sheep scrapie control sample showed a similar antibody reaction pattern as seen for case 2 (Fig. 3C, 3D).

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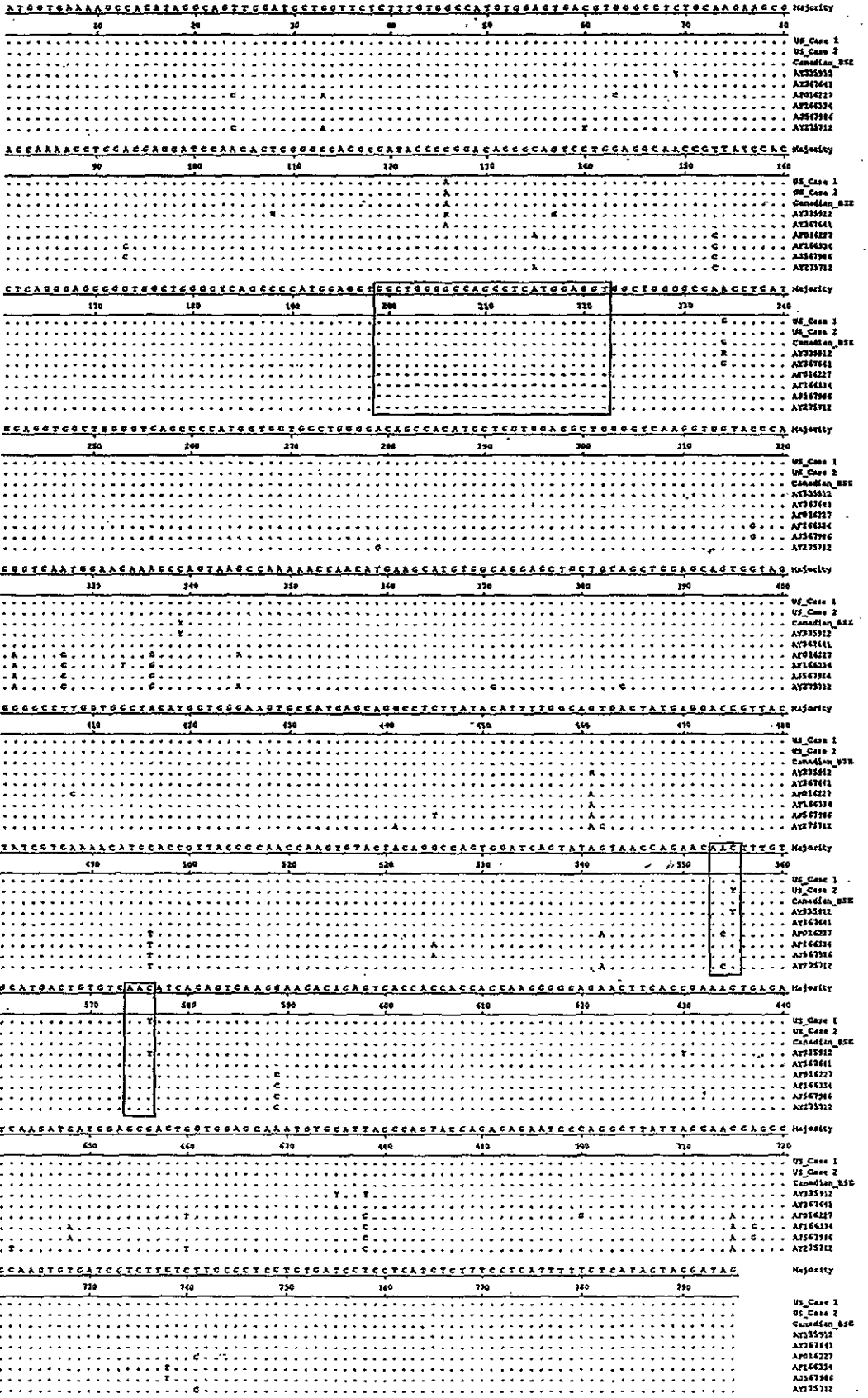
Figure 4. Alignment of bovine, ovine and cervid *Prnp* sequences including US BSE cases 1 and 2. A, Nucleotide sequences. Box enclosing nucleotides 199–222 (codons 67–74) represents an additional octapeptide-repeat region (total of 6 octapeptide repeats) but not in ovine and cervids (total of 5 octapeptide repeats). Box surrounding nucleotides 553–556 represents the synonymous polymorphism (AAC/AAT) located in the 3rd position of bovine codon 185 of US BSE Case 2. Box surrounding nucleotides 574–576 indicates the synonymous polymorphism at codon 192 (AAC/ AAT) of US BSE Case 1. Standard single letter codes are used for nucleotides. Y = C or T; R = A or G; K = G or T; W = A or T. B, Amino acid sequences. Standard IUPAC single letter codes are used for amino acids. Codon numbering refers to the most common 6-copy octapeptide repeat allele for *Bos Taurus*. The following bovine, cervid, and ovine GenBank entries were used for the alignments: US BSE Case 1, US BSE Case 2, Canadian May 2003 BSE case,[12] and GenBank accession numbers AY335912 (bovine: reports *Prnp* coding variation in a panel of 96 cattle chosen to represent most of the genetic diversity of the beef cattle breeds most commonly raised in North America), AY367641 (bovine), AF166334 (sheep), AJ567986 (sheep), AY275712 (white-tailed deer), and AF016227 (elk).



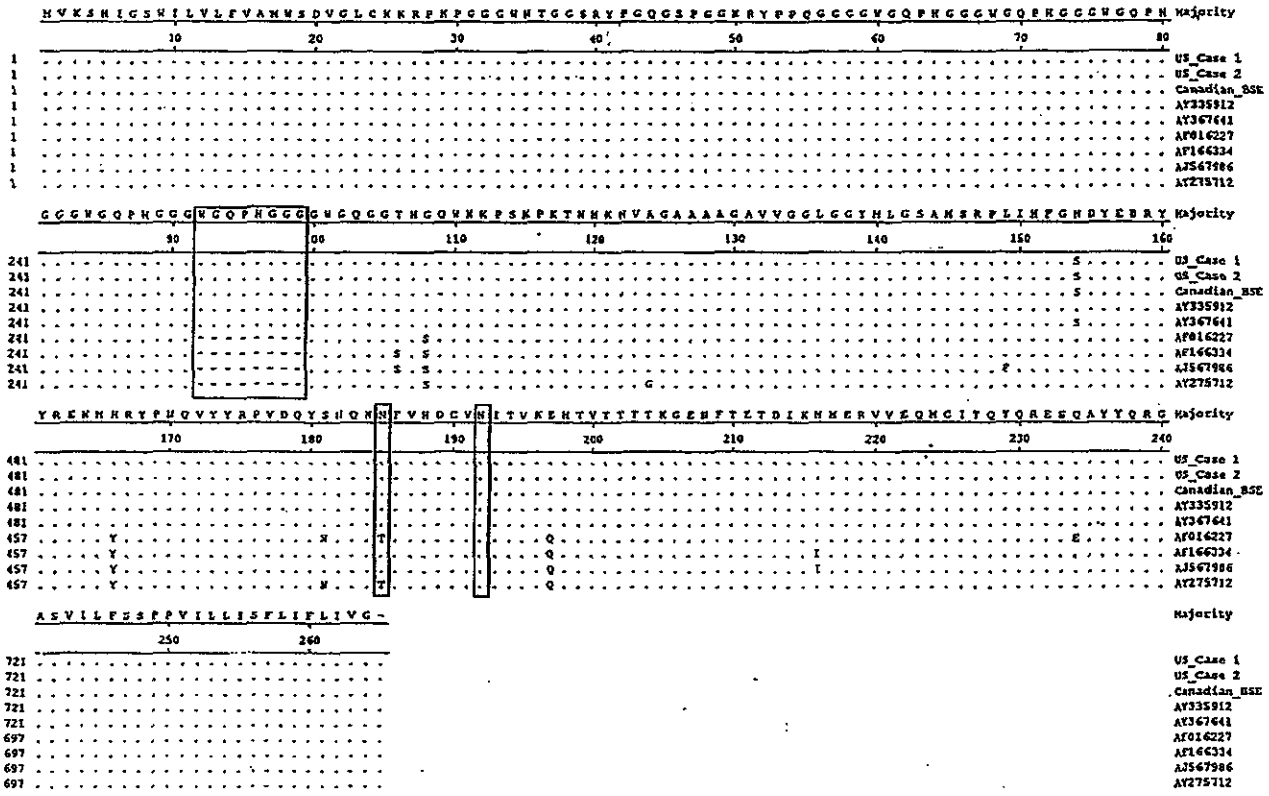


Figure 4. Continued.

Analysis of the Prnp

In order to confirm that the specimens from both cases were of cattle origin and to determine whether the might be associated with a spontaneous germline mutation, the full coding sequence from exon 3 of the *Prnp* was amplified and aligned with known PrP sequences from cattle, sheep, and cervids. DNA was isolated from brainstem material of both cases. As shown in Fig. 4A, the DNA sequence of both cases was identical to representative bovine *Prnp* sequences, but different from sheep and cervid sequences. The *Prnp* sequence of case 1 was found to be normal with a synonymous polymorphic position at codon 192 (T or C; no amino acid substitution). The animal had 6 copies of the octapeptide repeat region on both of their *Prnp* alleles. The *Prnp* gene of case 2 was also found to be normal with a synonymous polymorphism at codon 185 (T or C; no amino acid substitution), and both alleles contained the 6-copy octapeptide repeat region. The amino acid sequences of the 2 cases did not differ from each other and other bovine PrP protein sequences, however, there were differences when compared with the sheep and cervid sequences (Fig. 4B).

Discussion

The BSE epidemic in the United Kingdom was a food-borne disease in cattle, associated with feeding of meat and bone meal (MBM) that contained infected central nervous system tissue.[52] The cause of the original case or cases of bovine spongiform encephalopathy (BSE), however, remains an enigma. Sheep- or goat-derived scrapie-infected tissues included in the MBM rations fed to cattle or a previously undetected sporadic bovine TSE (maybe due to a germline mutation in the *Prnp* of affected cattle) have been considered as possible origins. Recently, another theory namely that BSE originated from a human TSE through animal feed containing imported mammalian raw materials contaminated with human remains from the Indian subcontinent has been brought forward.[12] Unlike CWD and scrapie, there is little evidence for either direct horizontal or vertical transmission of BSE among cattle.[2,53] Since the introduction of a ban on the use of ruminant proteins for ruminant feed in affected countries, the incidence rate of BSE in these countries has been steadily declining.[47]

This report presents the prion protein polypeptide profile and genotype from 2 cases of BSE diagnosed in the United States in 2003 and 2004. The obex area of the brainstem of case 1 was positive by a rapid BSE test, contained spongiform changes and had extensive deposition of the abnormal form of the prion protein, PrP^{Sc}, by IHC (Fig. 1A, 1B). Western blot analyses using brain material revealed a positive reaction using a 1-mg brain tissue equivalent (mg eq). The PrP^{Sc} polypeptide profile from BSE case 1 was characterized by 1) a lower molecular mass of the unglycosylated PrP^{Sc} polypeptide fragment compared to samples from sheep with scrapie and deer or elk with CWD, 2) good immunoreactivity with monoclonal antibody 6H4, and a lack of or weak staining with monoclonal antibody P4, and 3) a glycoform profile with a predominant proportion of the diglycosylated PrP^{BSE} isoform (see Fig. 2). Western blot comparison of the US BSE case 1 to the May 2003 Canadian and European BSE isolates revealed similar sized PrP^{Sc} polypeptide fragments (Fig. 2B). The 2003 Canadian BSE isolate from Alberta was reported to be a typical BSE isolate with similar molecular properties (including a lack of or weak staining with monoclonal antibody P4) to the PrP^{Sc} isolated from BSE cases in Switzerland (Fig. 2B) and the United Kingdom.[45] PrP^{Sc} isolated from elk with CWD had a higher molecular mass profile than did the corresponding PrP^{Sc} for the Canadian BSE case.[45] Similarly, PrP^{Sc} from mule deer and elk with CWD had a higher molecular mass profile than did the corresponding PrP^{Sc} for the US BSE case 1 (Fig. 2A, 2B).

United States BSE case 2 was born and raised in Texas, and represents the first native case of BSE in the United States. The brainstem of this animal reacted positive in the rapid BSE test used for BSE surveillance in the United States. The tissue of US BSE case 2 had been frozen before formalin fixation. Therefore, artifactual histopathological changes were present in the brainstem of this animal (Fig. 1C), however, unambiguous vacuolar changes diagnostic for BSE were not detected. PrP^{Sc} was detected in the brainstem by IHC (Fig. 1D). However, the staining intensity was less intense when compared with the signal found in the brainstem of case 1 (Fig. 1B). Moreover, the IHC staining pattern of case 2 was rather localized and not as diffuse as found with case 1. Interestingly, case 2 was only positive after formic acid treatment and extended antigen retrieval. Initially, case 2 was negative by IHC, even though the IHC positive control brainstem sections (from case 1) were strongly positive under the initial fixation and antigen retrieval conditions used. A positive Western blot reaction using antibody 6H4 was only seen after sample enrichment using the OIE SAF Immunoblot method and only 50% (5 out of 10) of brainstem or cerebellum samples were positive for the presence of PrP^{Sc} when tested at 20-mg brain tissue equivalent. This indicates that PrP^{Sc} was not uniformly distributed in the brainstem or cerebellum of case 2 and the PrP^{Sc} content per mg tissue equivalent was significantly lower (at least 20 times less) than found in brain material of case 1. Surprisingly, brain material from case 2 reacted strongly with antibody P4 (Fig. 3D). The intensity of the reaction of cerebellum and brainstem from case 2 with antibody P4 was even stronger than with 6H4 at similar mg tissue amounts (Fig. 3 C–D). A similar pattern was also seen with the sheep scrapie control sample (Fig. 3C–D). The unglycosylated and monoglycosylated isoforms of PrP^{Sc} from case 2 migrated higher than the respective isoforms of case 1 (Fig. 3B–C), indicating a difference in molecular mass between the 2 US BSE cases. The migration pattern of case 1 is typical for BSE (Fig. 2B), whereas the migration pattern of case 2 was reported as being unusual for BSE.[5] In addition, glycoform profile analysis of case 2 revealed a significant proportion of the diglycosylated isoform (approximately 61%), however, the percentage was lower than observed for the diglycosylated isoform of case 1 (approximately 72%). The monoglycosylated and unglycosylated isoforms of case 2 were more prominent (approximately 30% and 9%, respectively) than observed with the respective isoforms of case 1 (approximately 23% and 4%, respectively). The glycoform profile for case 1 is similar to that described for typical cases of BSE [5,28], whereas the profile found for case 2 is rather unusual.[5] The differences observed between case 1 and 2 were not caused by sampling of different regions of brain, since both, brainstem and cerebellum samples of case 1 and 2 showed the above described reaction patterns.

The *Prnp* gene of *Bos Taurus* contains multiple polymorphic sites: binary single nucleotide polymorphisms and an insertion-deletion polymorphism with 5, 6, or 7 repeats in the octapeptide-repeat region have been reported for exon 3.[13,25] The *Prnp* sequences of case 1 and 2 clearly fall within the range of cattle sequence diversity previously reported for cattle (Fig. 4A, 4B). Therefore, one etiological possibility for the disease condition of these animals, namely a germline mutation similar to one of the genetic forms of human prion disease can most likely be ruled out.[37] *Prnp* sequences from both US BSE cases contained 6 octapeptide-repeat regions on both alleles, a number found in cattle but not in either sheep or cervids. United States BSE case 1 had a polymorphic site at codon 192, a position previously reported to be variable in the bovine *Prnp* gene with