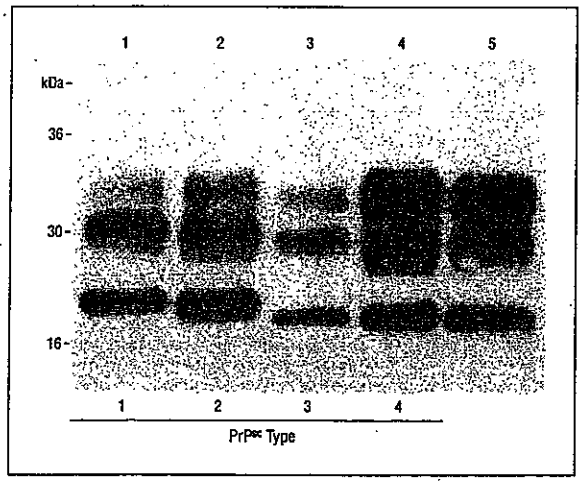

Figure 3. Immunoblotting of 10% brain homogenate after limited proteinase K digestion using anti–prion protein (PrP) monoclonal antibody 3F4. Lanes 1, 2, and 3 show 3 types of PrP^{Sc} (the scrapie isoform of PrP) seen in sporadic and iatrogenic cases of Creutzfeldt-Jakob disease; lane 4 shows PrP^{Sc} type 4, which is uniquely seen in brain tissue from patients with variant Creutzfeldt-Jakob disease.[21] Lane 5 shows PrP^{Sc} from the cerebellum of our patient demonstrating the same predominance of the high-molecular-mass diglycosylated PrP glycoform and a molecular mass of all PrP fragments similar to those of PrP^{Sc} type 4.

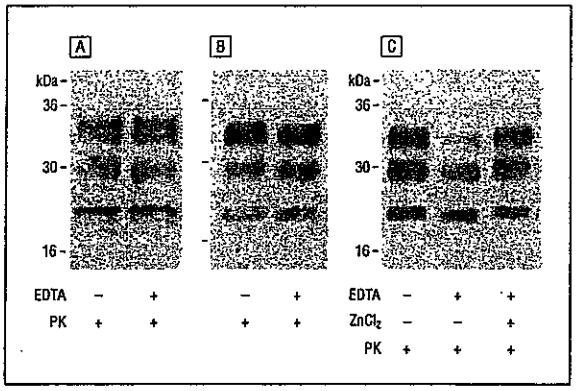

Figure 4. Immunoblotting of 10% brain homogenate after limited proteinase K (PK) digestion using anti–prion protein (PrP) monoclonal antibody 3F4. A, Cerebellum from a patient with variant Creutzfeldt-Jakob disease demonstrating a doublet of low-molecular-mass nonglycosylated bands of PrP^{Sc} (the scrapie isoform of PrP) with an identical pattern of PrP fragments observed after proteolysis in the presence of 25mM EDTA. B, Cerebellum from our patient demonstrating a doublet of low-molecular-mass nonglycosylated PrP^{Sc} bands. All bands migrate with lower apparent molecular mass following proteolysis in the presence of 25mM EDTA. C, Aliquots of cerebellum homogenate from our patient digested directly with proteinase K or after treatment with 25mM EDTA and sequential washing of insoluble pellets with *N*-ethyl morpholine buffer either lacking (–) or containing (+) 20μM zinc chloride ($ZnCl_2$).[19]

tained from separately analyzed tissue samples from opposite poles of the cerebellum. The glycoform ratio and fragment sizes resembled PrP^{Sc} type 4 seen in vCJD (**Figure 3**). The nonglycosylated band was seen as a doublet, as is seen for PrP^{Sc} in the cerebellum in vCJD (**Figure 4**). The effect of adding the metal ion chelator EDTA to the cerebellum homogenate before proteinase K cleavage was to reduce the apparent molecular weight of PrP^{Sc} fragments. This reflects the involvement of metal ions (most likely copper and zinc) in the conformation of PrP and determination of accessible protease cleavage sites.[19] This deduction was verified by showing that application of zinc ions to EDTA-treated samples before proteolysis resulted in preservation of the original PrP^{Sc} fragment size (Figure 4C). Although similar dependence on metal ions is observed for some PrP^{Sc} conformers associated with sporadic CJD,[19,21] this is not observed with PrP^{Sc} type 4 propagated in vCJD[19,21] (Figure 4). Therefore, these findings reflect a novel PrP^{Sc} type when compared with the diversity we and others have so far documented.[21,22]

COMMENT

Does the PrP^{Sc} typing suggest a BSE-related cause, or can our findings be accommodated by the spectrum seen in sporadic CJD cases worldwide? The molecular strain typing of the patient's brain material demonstrated a novel PrP^{Sc} type when compared with our archived cases.[21] There is as yet no internationally agreed-on classification of PrP^{Sc} type. Parchi and colleagues[23] identified 2 PrP^{Sc} types in sporadic CJD. However, Hill et al[21] described 3 PrP^{Sc} types associated with sporadic and iatrogenic CJD (types 1-3) and PrP^{Sc} type 4 associated with vCJD. The PrP^{Sc} type 5 has, to our knowledge, been observed only in mice expressing human PrP 129V inoculated with vCJD.[3,12] Hill et al[21] recently described a novel PrP^{Sc} type 6 in sporadic CJD.

The PrP^{Sc} type from our case has features similar to PrP^{Sc} type 4 (vCJD) in the predominance of the diglycosylated band; however, it is distinct from PrP^{Sc} type 4 in the dependence of the protease cleavage pattern of PrP^{Sc} on metal ions, suggesting a distinct PrP^{Sc} conformation. Unfortunately, only cerebellum was available for Western blotting in this case, although in vCJD cases from which whole brain was available we have not found evidence of any regional variation in PrP^{Sc} type. Others have reported coexistence of Gambetti PrP^{Sc} type 1 in the brain from patients with vCJD as a minority component.[24] It would also have been interesting to look for peripheral lymphoreticular PrP deposition because this is prominent in vCJD, but that tissue was not available for analysis. Transmission of BSE isolates to transgenic mice expressing human PrP 129 valine results in clinical prion disease with undetectable PrP^{Sc}; however, transmission of vCJD isolates to the same mice produces PrP^{Sc} type 5 that shares the same predominance of diglycosylated PrP^{Sc} to that of PrP^{Sc} type 4, and these data suggest that the molecular signature of BSE may be preserved after BSE transmission to *PRNP* codon 129 VV humans.[3,12] Transmission studies of the current case in transgenic mice are now being undertaken to investigate transmission characteristics.

We have described a novel PrP^{Sc} type that would be designated type 7 by our classification. A firm connection between novel PrP^{Sc} types and BSE cannot be made on the basis of a single case, and it will be important to see whether other similar cases occur in the United Kingdom and other BSE-exposed countries but not elsewhere and to perform detailed transmission studies of prions from this patient into transgenic and conventional mice to compare with BSE-derived isolates from

cattle and other species. Two other cases of prion disease with valine homozygosity and atypical features have been reported in the United Kingdom and the Netherlands. One of these cases was atypical because of very young onset and a protracted psychiatric history[25]; the other was notable because certain clinical and molecular features of the case overlapped with those of vCJD, including Western blot analysis of autopsied brain showing a predominance of a diglycosylated PrP^{Sc} isoform.[26]

We recommend keeping an open mind about the etiology of such cases during the ensuing years. These cases emphasize the importance both of continued surveillance of prion disease and the further development and refinement of molecular classification of prion diseases of humans and animals. It will also be important to assess lymphoreticular involvement in subsequent cases either at diagnostic tonsil biopsy or at autopsy.

Accepted for Publication: February 22, 2006.
Correspondence: John Collinge, FRS, MRC Prion Unit and Department of Neurodegenerative Disease, Institute of Neurology, University College London, National Hospital for Neurology and Neurosurgery, Queen Square, London WC1N 3BG, England (j.collinge@prion.ucl.ac.uk).
Author Contributions: *Study concept and design:* Mead and Collinge. *Acquisition of data:* Mead, Joiner, Desbruslais, O'Donoghue, Lantos, Wadsworth, and Collinge. *Analysis and interpretation of data:* Mead, Joiner, Desbruslais, Beck, Wadsworth, and Collinge. *Drafting of the manuscript:* Mead, Desbruslais, Beck, Lantos, Wadsworth, and Collinge. *Critical revision of the manuscript for important intellectual content:* Mead, Joiner, O'Donoghue, Wadsworth, and Collinge. *Statistical analysis:* Mead. *Obtained funding:* Collinge. *Administrative, technical, and material support:* Desbruslais, Beck, Lantos, and Collinge. *Study supervision:* Wadsworth and Collinge.
Financial Disclosure: None reported.
Funding/Support: This work was funded by the MRC and undertaken at the University College London Hospitals and University College London, who received a proportion of funding from the Department of Health's National Institute for Health Research Biomedical Research Centres funding scheme.
Additional Contributions: Ray Young assisted with figure design. The MRC London Neurodegenerative Diseases Brain Bank (Institute of Psychiatry) provided pathological material. We also acknowledge the many clinicians involved in the care of this patient.

REFERENCES

1. Will RG, Ironside JW, Zeidler M, et al. A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet.* 1996;347(9006):921-925.
2. Collinge J, Sidle KCL, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of "new variant" CJD. *Nature.* 1996;383(6602):685-690.
3. Hill AF, Desbruslais M, Joiner S, Sidle KCL, Gowland I, Collinge J. The same prion strain causes vCJD and BSE. *Nature.* 1997;389(6650):448-450.
4. Bruce ME, Will RG, Ironside JW, et al. Transmissions to mice indicate that "new variant" CJD is caused by the BSE agent. *Nature.* 1997;389(6650):498-501.
5. Hill AF, Zeidler M, Ironside J, Collinge J. Diagnosis of new variant Creutzfeldt-Jakob disease by tonsil biopsy. *Lancet.* 1997;349(9045):99-100.
6. Hill AF, Butterworth RJ, Joiner S, et al. Investigation of variant Creutzfeldt-Jakob disease and other human prion diseases with tonsil biopsy samples. *Lancet.* 1999;353(9148):183-189.
7. Wadsworth JDF, Joiner S, Hill AF, et al. Tissue distribution of protease resistant prion protein in variant CJD using a highly sensitive immuno-blotting assay. *Lancet.* 2001;358(9277):171-180.
8. Collinge J, Whitfield J, McKintosh E, et al. Kuru in the 21st century—an acquired human prion disease with very long incubation periods. *Lancet.* 2006;367(9528):2068-2074.
9. Collinge J, Beck J, Campbell T, Estibeiro K, Will RG. Prion protein gene analysis in new variant cases of Creutzfeldt-Jakob disease. *Lancet.* 1996;348(9019):56.
10. Collinge J. Variant Creutzfeldt-Jakob disease. *Lancet.* 1999;354(9175):317-323.
11. Mead S, Stumpf MP, Whitfield J, et al. Balancing selection at the prion protein gene consistent with prehistoric kurulike epidemics. *Science.* 2003;300(5619):640-643.
12. Wadsworth JD, Asante EA, Desbruslais M, et al. Human prion protein with valine 129 prevents expression of variant CJD phenotype. *Science.* 2004;306(5702):1793-1796.
13. Bishop MT, Hart P, Aitchison L, et al. Predicting susceptibility and incubation time of human-to-human transmission of vCJD. *Lancet Neurol.* 2006;5(5):393-398.
14. Asante EA, Linehan JM, Gowland I, et al. Dissociation of pathological and molecular phenotype of variant Creutzfeldt-Jakob disease in transgenic human prion protein 129 heterozygous mice. *Proc Natl Acad Sci U S A.* 2006;103(28):10759-10764.
15. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet.* 2004;364(9433):527-529.
16. Ironside JW, Bishop MT, Connolly K, et al. Variant Creutzfeldt-Jakob disease: prion protein genotype analysis of positive appendix tissue samples from a retrospective prevalence study [published correction appears in *BMJ.* 2006;333(7565):416]. *BMJ.* 2006;332(7551):1186-1188.
17. Kascsak RJ, Rubenstein R, Merz PA, et al. Mouse polyclonal and monoclonal antibody to scrapie-associated-fibril proteins. *J Virol.* 1987;61(12):3688-3693.
18. Hill AF, Joiner S, Beck JA, et al. Distinct glycoform ratios of protease resistant prion protein associated with *PRNP* point mutations. *Brain.* 2006;129(pt 3):676-685.
19. Wadsworth JDF, Hill AF, Joiner S, Jackson GS, Clarke AR, Collinge J. Strain-specific prion-protein conformation determined by metal ions. *Nat Cell Biol.* 1999;1(1):55-59.
20. Poulter M, Baker HF, Frith CD, et al. Inherited prion disease with 144 base pair gene insertion, I: genealogical and molecular studies. *Brain.* 1992;115:675-685.
21. Hill AF, Joiner S, Wadsworth JD, et al. Molecular classification of sporadic Creutzfeldt-Jakob disease. *Brain.* 2003;126(pt 6):1333-1346.
22. Parchi P, Giese A, Capellari S, et al. Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. *Ann Neurol.* 1999;46(2):224-233.
23. Parchi P, Castellani R, Capellari S, et al. Molecular basis of phenotypic variability in sporadic Creutzfeldt-Jakob disease. *Ann Neurol.* 1996;39(6):767-778.
24. Yull HM, Ritchie DL, Langeveld JP, et al. Detection of type 1 prion protein in variant Creutzfeldt-Jakob disease. *Am J Pathol.* 2006;168(1):151-157.
25. Hillier CE, Llewelyn JG, Neal JW, Ironside JW. Creutzfeldt-Jakob disease in a young person with valine homozygosity at codon 129: sporadic or variant? *J Neurol Neurosurg Psychiatry.* 2001;70(1):134-135.
26. Head MW, Tissingh G, Uitdehaag BMJ, et al. Sporadic Creutzfeldt-Jakob disease in a young Dutch valine homozygote: atypical molecular phenotype. *Ann Neurol.* 2001;50(2):258-261.

別紙様式第 2-1

医薬品
医薬部外品　研究報告　調査報告書
化粧品

別紙 3-1

識別番号・報告回数		回	報告日 年　月　日	第一報入手日 2007 年 11 月 14 日	新医薬品等の区分 該当なし	総合機構処理欄
一般的名称			研究報告の公表状況	Species barrier for chronic wasting disease by in vitro conversion of prion protein. Li, L. et al, Biochem. Biophys. Res. Com. 364(4), 796-800 (2007)	公表国 カナダ	
販売名（企業名）						

研究報告の概要	使用上の注意記載状況・その他参考事項等
本稿の著者らは，慢性消耗性疾患（北米シカに影響を及ぼす伝染性海綿状脳症）は，in vitro アッセイにおいてある特定の条件下で種の壁をすり抜けて感染することを明らかにした。本アッセイは，異種動物からの正常な脳ホモジネート（正常 PrP^{C}）を基質として，エルク（ヨーロッパヘラジカ）の異常プリオンタンパク質（PrP^{Sc}）とともにインキュベートするものである。標準の条件（pH 7.4）下では，エルク（ヨーロッパヘラジカ）PrP^{Sc} は同種系列〔トナカイ，ムース（アメリカヘラジカ），カリブー及びエルク（ヨーロッパヘラジカ）〕の PrP^{C} をタンパク質分解酵素耐性アイソフォームへと変換させたが，異種 PrP^{C}（ヒト，マウス，ヒツジ，ウシ，ハムスター）については，PrP^{C} のタンパク質配列が全ての種で 90%以上保持されているにもかかわらずタンパク質分解酵素耐性アイソフォームへ変換されたものは僅かであった。しかしながら，低 pH（3.5）による部分変性の条件下では，PrP^{Sc} によるタンパク質分解酵素耐性アイソフォームへの変換は全ての種で劇的に増大した。これより，基質の部分変性によって構造上の変化が起こり，遠隔種間の種の壁を越えることが示唆される。	BYL-2008-0303

報告企業の意見	今後の対応
異常プリオン PrP^{Sc} によるアイソフォーム変換への感度および耐性は，基質である PrP^{C} の立体構造が重要であるとしているが，生物学的な関連性については疑問が残る。	現時点で新たな安全対策上の措置を講じる必要はないと考える。

32

Available online at www.sciencedirect.com
ScienceDirect
ELSEVIER
Biochemical and Biophysical Research Communications 364 (2007) 796–800
BBRC
www.elsevier.com/locate/ybbrc

Species barriers for chronic wasting disease by *in vitro* conversion of prion protein

Li Li [a], Michael B. Coulthart [b], Aru Balachandran [c], Avi Chakrabartty [d], Neil R. Cashman [a,*]

[a] *Brain Research Centre, Division of Neurology, Department of Medicine, University of British Columbia and Vancouver Coastal Health, UBC Hospital, 2211 Wesbrook Mall, Vancouver, BC, Canada V6T 2B5*
[b] *Prion Diseases Program, National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, Man., Canada R3E 3R2*
[c] *National Reference Laboratory for Scrapie and CWD, Animal Diseases Research Institute, Canadian Food Inspection Agency, 3851 Fallowfield Road, Nepean, Ont., Canada K2H 8P9*
[d] *University Health Network, Department of Medical Biophysics, University of Toronto, Toronto, Ont., Canada M5G 1L7*

Received 6 October 2007
Available online 25 October 2007

Abstract

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy that can affect North American cervids (deer, elk, and moose). Using a novel *in vitro* conversion system based on incubation of prions with normal brain homogenates, we now report that PrP^{CWD} of elk can readily induce the conversion of normal cervid PrP (PrP^{C}) molecules to a protease-resistant form, but is less efficient in converting the PrP^{C} of other species, such as human, bovine, hamster, and mouse. However, when substrate brain homogenates are partially denatured by acidic conditions (pH 3.5), PrP^{CWD}-induced conversion can be greatly enhanced in all species. Our results demonstrate that PrP^{C} from cervids (including moose) can be efficiently converted to a protease-resistant form by incubation with elk CWD prions, presumably due to sequence and structural similarities between these species. Moreover, partial denaturation of substrate PrP^{C} can apparently overcome the structural barriers between more distant species.

Keywords: CWD; PrP^{C}; PrP^{Sc}; *In vitro* conversion; Species barrier

Chronic wasting disease (CWD) is a cervid form of transmissible spongiform encephalopathy (TSE) or prion disease. CWD's rapid spread from' Colorado to other states [1,2], to Canadian provinces (Alberta, Saskatchewan) [1] and to Korea [2,3] has raised concerns about its species tropism [4–6]. CWD has been transmitted to cattle via intracerebral inoculation [7], and to other animals, including ferrets, mink, and goats [8,9]. Reports documenting CWD prions in the muscle [10,11], blood, and saliva [12] of infected cervids, have heightened interest in the disease by public health agencies [13].

CWD and other TSEs are believed to be due to the template-directed accumulation of disease-associated prion protein, generically designated PrP^{Sc}. PrP^{C} in brain homogenates can be converted to a protease-resistant form by incubation with PrP^{Sc} "seeds" which are thought to recapitulate the template-directed misfolding of prion protein in disease [14,15], including protein misfolding cyclic amplification (PMCA) [15]. We have previously reported that partially denatured human brain PrP^{C} (which may mimic a PrP conversion intermediate [16]) is a superior substrate for templated *in vitro* conversion compared with untreated PrP^{C} in an incubation-shaking assay that does not utilize PMCA sonication [17].

Materials and methods

Reagents and antibodies. Proteinase K (PK) was purchased from Invitrogen. Mouse monoclonal antibody 6H4 was from Prionics Co. (Zürich, Switzerland). Horseradish peroxidase-conjugated sheep anti-

* Corresponding author. Fax: +1 604 822 7299.
E-mail address: Neil.Cashman@vch.ca (N.R. Cashman).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.10.087

mouse antibody was purchased from Amersham Biosciences. All other chemicals were purchased from Sigma unless specified otherwise.

Brain tissues and homogenate preparation. All brain samples were obtained from the disease control and surveillance programs of the Canadian Food Inspection Agency (CFIA) and were harvested within 24 h of death. The normal brain tissue was determined to be free of neurological disorders on the basis of neuropathological examination. The presence of PrP^{Sc} in brain tissue from an elk with clinical chronic wasting disease (CWD) was confirmed by immunohistochemistry and PK resistance on immunoblotting analysis. All tissues were frozen immediately after collection and stored at −80 °C. Ten percent (w/v) brain homogenates were prepared in lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 10 mM Tris–HCl, pH 7.5) as previously described [17].

Preparation of acid/GdnHCl-treated PrP^{C}. The preparation was followed as previously described [17], in brief, 100 µl of 10% brain homogenate was mixed with an equal volume of 3.0 M guanidine hydrochloride GdnHCl (final concentration of 1.5 M) in PBS at pH 7.4 or pH 3.5 adjusted with 1 M HCl, and incubated for 5 h at room temperature with shaking. After that, samples were precipitated with methanol and resuspended in 100 µl of PBS (pH 7.4) with 0.05% SDS, 0.5% Triton X-100.

In vitro conversion of acid/GdnHCl-treated PrP^{C}. In vitro conversion was performed in a 50 µl volume of the appropriate test substrate material (49 µl of normal brain homogenate + 1 µl CWD brain homogenate in a 1:50 dilution as the prion template). The sample was then incubated in a thermomixer at 37 °C for 12 h with shaking. After PK digestion and boiling in the loading buffer, the samples were subjected to SDS–PAGE and immunoblotting.

Proteinase K resistance and immunoblotting. To determine the PK-resistance of the PrP, 20 µl of the sample was incubated with PK at 100 µg/ml for 1 h at 37 °C, and the digestion reaction was terminated by addition of PMSF to 2 mM of final concentration. Proteins were separated by NuPAGE 4–12% pre-cast Bis–Tris gel (Invitrogen) and electrotransferred onto PVDF membranes. 6H4 was used as primary antibody (1:5000) and horseradish peroxidase-conjugated sheep anti-mouse IgG as secondary antibody. The proteins were visualized by enhanced chemiluminescence + Plus (ECL + Plus, Amersham Biosciences), the blots were scanned and were analyzed by Quantity One (Bio-Rad) software. At least eight experiments were performed on each species.

Results and discussion

Sequence alignment of prion protein

CWD appears to be freely transmitted among susceptible species of cervids by direct or environmentally medi-

A

```
    1                                                   50
Rangifer    MVKSHIGSWI LVLFVAMWSD VGLCKKRPKP GGGWNTGGSR YPGQGSPGGN
Elk         MVKSHIGSWI LVLFVAMWSD VGLCKKRPKP GGGWNTGGSR YPGQGSPGGN
Moose       MVKSHIGSWI LVLFVAMWSD VGLCKKRPKP GGGWNTGGSR YPGQGSPGGN

   51                                                  100
Rangifer    RYPPQGGGGW GQPHGGGWGQ PHGGGWGQPH GGGWGQPHGG GGWGQGGTHS
Elk         RYPPQGGGGW GQPHGGGWGQ PHGGGWGQPH GGGWGQPHGG GGWGQGGAHS
Moose       RYPPQGGGGW GQPHGGGWGQ PHGGGWGQPH GGGWGQPHGG GGWGQGGIHS

  101                                                  150
Rangifer    QWNKPSKPKT NMKHVAGAAA AGAVVGGLGG YMLGSAMSRP LIHFGNDYED
Elk         QWNKPSKPKT NMKHVAGAAA AGAVVGGLGG YMLGSAMSRP LIHFGNDYED
Moose       QWNKPSKPKT NMKHVAGAAA AGAVVGGLGG YMLGSAMSRP LIHFGNDYED

  151                                                  200
Rangifer    RYYRENMYRY PNQVYYRPVD QYNNQNTFVH DCVNITVKQH TVTTTTKGEN
Elk         RYYRENMYRY PNQVYYRPVD QYNNQNTFVH DCVNITVKQH TVTTTTKGEN
Moose       RYYRENMYRY PNQVYYRPVD QYNNQNTFVH DCVNITVKQH TVTTTTKGEN

  201                                                  250
Rangifer    FTETDIKMME RVVEQMCITQ YQRESQAYYQ RGASVILFSS PPVILLISFL
Elk         FTETDIKMME RVVEQMCITQ YQRESQAYYQ RGASVILFSS PPVILLISFL
Moose       FTETDIKMME RVVEQMCITQ YQRESQAYYQ RGASVILFSS PPVILLISFL

  251  256
Rangifer    IFLIVG
Elk         IFLIVG
Moose       IFLIVG
```

Fig. 1. Prion protein amino acid sequence alignment. (A) Prion protein sequence alignment of caribou/reindeer (rangifer), elk and moose. Protein sequences of PrP^{C} in cervid group are highly conserved, except for one amino acid polymorphism boxed in grey. (B) Prion protein sequence alignment of elk and other species (hamster, human, mouse, bovine, and sheep). PrP is >90% conserved.