GENETIC EVALUATION OF THE OVINE AND BOVINE PRION PROTEIN GENES (*PRNP*)

A Dissertation

by

CHRISTOPHER MARK SEABURY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Genetics

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ABSTRACT

Genetic Evaluation of the Ovine and Bovine Prion

Protein Genes (*PRNP*). (December 2004)

Christopher Mark Seabury, B.S.; M.S., Sul Ross State University

Chair of Advisory Committee: Dr. James N. Derr

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of inevitably fatal neurodegenerative diseases that occur in mammalian species. Ovine susceptibility to scrapie, the prototypical TSE, is predominantly modulated by nonsynonymous polymorphisms within exon 3 of the ovine prion protein gene (*PRNP*). Investigation of *PRNP* exon 3 for two hair-sheep breeds revealed a novel predicted amino acid substitution (P_{116}) associated with the ovine ARQ allele ($P_{116}A_{136}R_{154}Q_{171}$). Additionally, two novel ovine *PRNP* genotypes (PARQ/ARR; PARQ/ARQ) also were detected, and most of the hair sheep sampled possessed *PRNP* exon 3 genotypes associated with some degree of resistance to scrapie and/or experimental BSE (bovine spongiform encephalopathy).

Unlike sheep, expression of bovine spongiform encephalopathy (BSE) in cattle and other bovids has not been associated with nucleotide variation within bovine *PRNP* exon 3. However, BSE susceptibility has been tentatively associated with specific insertion-deletion (indel) polymorphisms within the putative bovine *PRNP* promoter, and to a lesser extent intron 1, for a few German cattle breeds. Evaluation of the patterns

of nucleotide variation associated with bovine *PRNP* exon 3 provided evidence that strong purifying selection has intensely constrained bovine exon 3 over the long-term evolutionary history of the subfamily Bovinae, as well as evidence for significant purifying selection in regions of bovine *PRNP* exon 3 that are considered to be of functional, structural, and pathogenic importance in other mammalian species. Evaluation of the frequencies of known indel polymorphisms within the putative bovine *PRNP* promoter for a panel of U. S. cattle sires revealed no significant differences in the distribution of promoter alleles and/or genotypes between U. S. cattle sires and BSE-affected German cattle.

Notably, a nonsynonymous *PRNP* exon 3 polymorphism (T50C) identified in American bison (*Bison bison*) was tentatively associated with *Brucella* spp. seropositivity. Specifically, a significant overabundance ($P = 0.021$) of Yellowstone National Park bison possessing the CC genotype were *Brucella* spp. seropositive. Furthermore, the T-allele and TT genotype were observed at significantly higher frequencies in three bison populations that were either founded from *Brucella* spp. seronegative stock or previously subjected to test-and-slaughter management to eradicate brucellosis.

DEDICATION

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TABLE OF CONTENTS

Page

ABSTRACT…….. iii

DEDICATION…….. v

ACKNOWLEDGEMENTS…….. vi

TABLE OF CONTENTS…….. vii

LIST OF FIGURES……... ix

LIST OF TABLES……. x

INTRODUCTION……. 1

Prion diseases: A historical perspective…….. 1
The transmissible agent of prion diseases……... 3
Prion disease and the *PRNP* gene…… 4
The physiological function of PrP^C……. 5
Study objectives……...…....……..... 7

IDENTIFICATION OF A NOVEL OVINE *PRNP* POLYMORPHISM AND SCRAPIE RESISTANT GENOTYPES FOR ST. CROIX WHITE AND A RELATED COMPOSITE BREED…… 9

Introduction…….. 9
Materials and methods……. 11
Results and discussion……. 14

NOVEL PRION PROTEIN GENE (*PRNP*) VARIANTS AND EVIDENCE FOR STRONG PURIFYING SELECTION IN FUNCTIONALLY IMPORTANT REGIONS OF BOVINE EXON 3……. 18

Introduction…….. 18

Page

Materials and methods……..... 20
Results…….…… 24
Discussion……... 33

COMPARATIVE *PRNP* GENOTYPING OF U. S. CATTLE SIRES FOR

POTENTIAL ASSOCIATION WITH BSE…….……..... 39

Introduction…….…….... 39
Materials and methods…...……....…… 41
Results…….…… 43
Discussion……...…… 44

BISON *PRNP* GENOTYPING AND POTENTIAL ASSOCIATION WITH

BRUCELLA SPP. SEROPREVALENCE…….……. 51

Introduction…….. 51
Materials and methods……...…….. 53
Results……...……... 57
Discussion…… 61

CONCLUSIONS AND FUTURE INVESTIGATIONS…… 64

REFERENCES……..……. 68

VITA..……. 87

LIST OF FIGURES

FIGURE Page

1 Extreme conservation associated with specific regions of the mammalian prion protein……..……...……... 17

2 Sliding window analyses of Tajima's (*D*) and Fu and Li's tests (D^*, F^*) for cattle *PRNP* exon 3 (repeats R3, R4, RN2 deleted by gap handling)…..…….. 29

3 Phylogeny used for conducting tests of selection on bovine *PRNP* exon 3 sequences……..... 32

4 Graphical depiction of the multiplexed PCR products corresponding to indel polymorphisms in the bovine *PRNP* putative promoter, intron 1, and 3' UTR..… 45

LIST OF TABLES

TABLE		Page
1	Observed ovine *PRNP* allelic and genotypic frequencies…….....	14
2	Bovinae *PRNP* exon 3 polymorphic nucleotide sites……	26
3	Observed octapeptide repeat genotypes…….	27
4	*PRNP* exon 3 allelic diversity within domestic cattle breeds excluding gaps…...	28
5	McDonald-Kreitman tests for *PRNP* exon 3 of domestic cattle and non-domestic taxa of Bovinae……	31
6	Observed frequencies for bovine *PRNP* indel polymorphisms in the putative promoter, intron 1, and 3' UTR……..	46
7	Observed *PRNP* indel allele frequencies for cattle breeds where 3 or more sires were sampled …….....	47
8	Distribution of bison samples by local and sex……..	54
9	Distribution of Yellowstone bison samples by sex, serological status (*Brucella* spp. seronegative/seropositive), and age……	58
10	Observed bison *PRNP* exon 3 allelic and genotypic frequencies…….....	59

INTRODUCTION

Prion diseases: A historical perspective

Prion diseases, also termed transmissible spongiform encephalopathies (TSEs), are a group of neurodegenerative diseases that affect humans as well as both domestic and wild animals (Collinge 2001). Historically, prion diseases have been commonly referred to as slow viruses, transmissible dementias, and/or subacute spongiform encephalopathies (Collinge 2001). Scrapie, a naturally occurring TSE affecting sheep and goats worldwide is considered the prototypical TSE, and has been recognized in Europe for more than 200 years (Collinge 2001; McGowan 1922). Several additional animal TSEs have since been recognized including chronic wasting disease (CWD) in deer and elk (Williams and Young 1980; Williams et al. 1982), bovine spongiform encephalopathy (BSE) (Wells et al. 1987), feline spongiform encephalopathy (Wyatt et al. 1991), and transmissible mink encephalopathy (Marsh 1992).

In humans, prion diseases have customarily been classified as Creutzfeld-Jakob disease (CJD), Gerstmann-Sträusler-Scheinker disease (GSS), and kuru, with more recent classification into BSE-derived variant CJD (vCJD) (Collinge 2001). Classical CJD has often been thought of as a "sporadic" disorder consisting of several subtypes, while vCJD has been linked to consumption of beef derived from BSE-infected cattle

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This dissertation follows the style and format of the journal Mammalian Genome.

(Bruce et al. 1997; Scott et al. 1999; Collinge 2001; Asante et al. 2002). However, a recent study demonstrated biochemical and pathological similarities between one distinct subtype of sporadic CJD and a newly described form of bovine spongiform encephalopathy (Casalone et al. 2004). GSS, a human hereditary spongiform encephalopathy, is known to be an autosomal dominant disorder that includes a wide variety of clinical presentations (Ghetti et al 1995; Collinge 2001). Notably, as many as 20 pathogenic mutations resulting in hereditary prion disease have been described within exon two of the human prion protein gene (*PRNP*), with six mutations that embody the traditional understanding and description of GSS (Hsiao et al. 1989; Ghetti et al. 1995; Collinge 2001).

Kuru was initially recognized in humans during a 1950s epidemic of progressive ataxia among the Fore linguistic group of Papua New Guinea (Collinge 2001). Subsequent kuru field studies during the 1950s suggested that the disease was transmitted via cannibalistic mortuary feasts (Collinge 2001). The transmissibility of kuru was first demonstrated experimentally in 1966 via intracerebral inoculation of chimpanzees with infected brain homogenates (Gajdusek et al. 1966). Demonstrations of the transmissibility of both CJD and GSS subsequently followed (Gibbs et al. 1968; Masters et al. 1981). Collectively, these three experiments provided enormous insight into the infectious potential of prion diseases.

The transmissible agent of prion diseases

The nature of the infectious agent which modulates prion diseases has been a topic of heated debate among the scientific community for many years (Collinge 2001). Initial suggestions that the infectious agent was a virus of some kind have largely been stamped out, although a small proportion of studies involving prion diseases are still conveyed within general virology journals. Moreover, failure of the scientific community to isolate a virus from infected material as well as the inability of ultraviolet radiation or nuclease treatment to inactivate the agent (Collinge 2001) has lead researchers to ponder alternate hypotheses including the nobel prize winning prion-only hypothesis (Prusiner 1982, 1991, 1998). Through enriching homogenated brain material for infectivity, Bolton et al (1982) was able to isolate a protease-resistant glycoprotein which was subsequently termed the prion protein (PrP; Collinge 2001). The word "prion" (proteinaceous infectious particle) was coined by Stanley Prusiner (1982) and used to distinguish the infectious agent identified from viruses (Collinge 2001).

To date, all evidence indicates that the infectious agent(s) of prion diseases are abnormal protease-resistant isoforms (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{C}) (Prusiner 1982; Oesch et al. 1985; Liao et al. 1986; Sparkes et al. 1986; Prusiner 1991; Collinge 2001; Legname et al. 2004). The *PRNP* gene has been mapped to HSA 20 (*Homo sapien* autosome; Sparkes et al. 1986) in humans and consists of two exons, the second of which encodes the 253-amino acid protein (Puckett et al. 1991; Lee et al. 1998; Makrinou et al. 2002). Mapping studies in animals have placed the *PRNP* gene, consisting of three exons whereby the third exon encodes the prion protein, on autosome

13 for cattle (Ryan and Womack 1993), sheep, and goats (Castiglioni et al. 1998; Iannuzzi et. al. 1998). Prion disease propagation entails the conversion of PrP^C, predominantly consisting of alpha-helical structure, into a disease-associated isoform characterized by increased β-sheet formation and protease-resistant properties (Bolton et al. 1982; Prusiner 1982, 1991; Pan et al. 1993; Prusiner 1998; Collinge 2001; Legname et al. 2004). In addition, recent studies of synthetic mammalian prion proteins indicate that PrP^C is both necessary and sufficient for infectivity (Legname et al. 2004).

Prion disease and the PRNP gene

To date, the only gene identified in mammals that has conclusively been linked to the expression of prion diseases such as CJD, GSS, scrapie, CWD, and BSE, is the *PRNP* gene (Goldmann et al. 1990; Belt et al. 1995; Hunter et al. 1997; O'Rourke et al. 1999; Collinge 2001; Billinis et al. 2002; Johnson et al. 2003; O'Rourke et al. 2004; Sander et al. 2004). Four nonsynonymous mutations (P102L, P105L, A117V, F198S) encoded by exon two of the human *PRNP* gene are known to modulate the expression of GSS in humans and have been identified in multiple individuals from several unrelated families (Ghetti et al. 1995; Collinge 2001). In addition, heterozygosity at human *PRNP* codon 129 (M129V) has been associated with protection against aquired (vCJD, kuru), sporadic (CJD), and some hereditary (GSS) prion diseases (Collinge et al. 1991; Palmer et al. 1991; Ghetti et al. 1995; Collinge 2001; Mead et al. 2003). To date, only one individual heterozygous at codon 129 (M129V) is known to have aquired vCJD (Peden et al. 2004). However, this individual aquired vCJD via blood transfusion, perhaps bypassing and/or

overwhelming the protective mechanism(s) associated with human *PRNP* codon 129 heterozygosity.

The involvement of the *PRNP* gene in the expression of prion diseases in animals has also been widely explored, especially in wool-bearing sheep. Previous studies have demonstrated that amino acid variation encoded by codons 136 and 171 of ovine *PRNP* exon 3 largely modulate the expression of scrapie and experimental BSE in sheep (Belt et al. 1995; Hunter 1997; Jeffrey et al. 2001; Baylis et al. 2002a; Baylis et al. 2002b). Likewise, *PRNP* exon 3 polymorphisms have also been associated with the expression of scrapie in goats, (Goldman et al. 1996; Goldman et al. 1998; Billinis et al. 2002) and CWD in deer and elk (O'Rourke et al. 1999; Johnson et al. 2003; O'Rourke et al. 2004). To date, no association between variation encoded by bovine *PRNP* exon 3 and BSE has been established in domestic cattle (Hunter et al. 1994; Neibergs et al. 1994; Hernandez-Sanchez et al. 2002). However, two indel polymorphisms within the putative bovine *PRNP* promoter and intron 1 have been significantly associated with BSE susceptibility in a few German cattle breeds (Sander et al. 2004).

The physiological function of PrP^C

Perhaps the most intriguing yet difficult aspect of prion biology has been to define the precise physiological function(s) of PrP^C. Moreover, no consensus among the scientific community has been reached regarding the normal function(s) of PrP^C (Aguzzi and Hardt 2003; Collinge 2001). Interestingly, PrP^C has been suggested to perform just about every biochemical function imaginable, including nothing at all (Büeler et al.

1992; Lledo et al. 1996; Aguzzi and Hardt 2003). For example, PrP^C knockout mice (*Prnp*$^{0/0}$) display no overt developmental defects or reduced life expectancy and are resistant to experimental scrapie (Büeler et al. 1992; Büeler et al. 1993), yet PrP^C has been reported to modulate synaptic homeostasis (Collinge et al. 1994), promote neurite outgrowth and neuronal survival (Chen et al. 2003), and act as a cell-surface receptor for signal transduction (Mouillet-Richard et al. 2000). Importantly, the hypothesis that PrP^C may function as cell surface receptor is supported by the rapid cycling PrP^C between the cell surface and the early endosome, which is also a process indicative of many other cell-surface receptors (Shyng et al. 1994; van Rheede et al. 2003).

Perhaps one of the most provocative findings with respect to the physiological function of PrP^C stems from its proposed involvement in the establishment of *Brucella abortus* infection into mouse macrophages (Watarai et al. 2003; Watarai et al. 2004; Aguzzi and Hardt). Interestingly, several different lines of evidence all point to a relationship between PrP^C and *B. abortus* heat shock protein (Hsp) 60. First, immunofluorescence microscopy demonstrates distinct PrP^C tail formation upon internalization of *B. abortus* into mouse macrophages (Watarai et al. 2003; Watarai 2004). Second, when mouse macrophage lysates were added to pull-down assays utilizing Hsp 60 and/or PrP^C conjugated beads, the affinity of PrP^C and Hsp 60 to one another was noted (Watarai et al. 2003; Watarai 2004). Analysis of the proteins recovered via pull-down assays by immunoblotting with anti-PrP^C or Hsp 60 antibodies demonstrates that the 29 kilodalton (k-Da) PrP^C is associated with Hsp 60, but not the beads alone (Watarai et al. 2003; Watarai 2004). Notably, this association was further

characterized via immunoblotting assays using an antibody specific to *B*. *abortus* Hsp 60 (Watarai et al. 2003; Watarai 2004). Finally, when control mice (BALB/c and C57BL/6) and PrP^C deficient mice (Sakuguchi et al. 1996) were challenged with *B*. *abortus*, many bacteria (based on colony forming units) were recovered from the spleens of the control mice 10 days post-challenge, but few bacteria were recovered from the spleens of PrP^C deficient mice (Watarai et al. 2003; Watarai 2004). These results led Watarai and colleagues (2004) to suggest that replicative phagosome formation and proliferation of *B*. *abortus* in mice requires an uptake pathway associated with PrP^C.

Study objectives

Previous studies of ovine *PRNP* exon 3 and scrapie have predominantly focused on wool-bearing sheep breeds, with little or no regard to ovine breeds collectively referred to as hair sheep. Therefore, the first objective of this study was to examine *PRNP* exon 3 genotypes and allelic variants for hair sheep to facilitate comparative analyses as well as ascertain whether or not novel nucleotide and/or amino acid variants exist within these breeds.

Unlike sheep, exon three of the bovine *PRNP* gene has not been intensely investigated among diverse bovine breeds and/or species. As such, the second major objective of this study was to provide a comprehensive investigation of genetic variation within bovine *PRNP* exon 3 for both domesticated and non-domesticated species of the subfamily Bovinae. In addition, the evolutionary processes that may have influenced *PRNP* exon 3 variation within and between bovine species, as well as the potential for

structural and/or functional constraints exerted upon the bovine prion protein, were investigated using two large bovine DNA panels.

The third objective of the present study centers around the significant association noted between bovine *PRNP* indel polymorphisms in the putative promoter as well as intron 1 and BSE susceptibility in a few German cattle breeds (Sander et al. 2004). In response to this finding, a comparative investigation of the frequencies of BSE-associated *PRNP* indels for a diverse panel of commercial U. S. cattle sires was initiated in conjunction with the development of a PCR protocol incorporating fluorescently labeled primer combinations to produce a single multiplexed assay (*PRNP*MPLX) for high-throughput interrogation of bovine *PRNP* indels in the putative promoter, intron 1, and the 3' untranslated region. The potential applications for the *PRNP*MPLX assay include the facilitation of polymorphism analyses as well as marker-assisted selection.

The fourth and final objective of the present study was to evaluate the potential role of PrP^C in the natural resistance of American bison (*Bison bison*) to brucellosis (*B. abortus*) infection using serological data, *PRNP* exon 3 polymorphism data, and *PRNP*MPLX data generated for Yellowstone National Park (NP) bison as well as bison sampled from herds that were either founded from *Brucella* spp. seronegative stock and/or subjected to test-and-slaughter management to eradicate brucellosis.

IDENTIFICATION OF A NOVEL OVINE *PRNP* POLYMORPHISM AND SCRAPIE-RESISTANT GENOTYPES FOR ST. CROIX WHITE AND A RELATED COMPOSITE BREED*

Introduction

Scrapie is an inevitably fatal transmissible spongiform encephalopathy (TSE) affecting sheep and goats. Polymorphisms within exon three of the ovine host-encoded prion protein gene (*PRNP*) at codons 136 (Alanine or Valine; A,V), 154 (Histidine or Arginine; H,R), and 171 (Glutamine, Arginine, or Histidine; Q, R, or H) are associated with variation in the phenotypic expression of scrapie including incubation period, clinical signs, and pathology (Bossers et al. 1996; Bossers et al. 2000; reviewed by Hunter 1997). Of the twelve possible alleles derivable from these polymorphisms, only five are commonly seen: $A_{136}R_{154}R_{171}$ (hereafter ARR), ARQ, VRQ, AHQ, and ARH (Belt et al. 1995). It should also be noted that seven additional ovine *PRNP* polymorphisms, exhibiting little or no association with the phenotypic expression of scrapie, have been described at codons 112, 127, 137, 138, 141, 151, and 211 (as referenced in Bossers et al. 2000). High susceptibility to scrapie is associated with the ovine VRQ allele, while the ARR allele is associated with resistance (Westaway et al. 1994; Belt et al. 1995; Hunter et al. 1996; Sabuncu et al. 2003). The AHQ allele may be associated with resistance in some ovine breeds, but not others, while the ARH allele is

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likely to be neutral (Dawson et al. 1998; Baylis et al. 2002a). In the U.S. sheep population scrapie has only been confirmed in sheep homozygous for the *PRNP* allele encoding glutamine at codon 171 (Q/Q), regardless of breed (Westaway et al. 1994; O'Rourke et al. 1996, O'Rourke et al. 1997; O'Rourke et al. 2002). Moreover, the ovine *PRNP* genotype ARR/ARR is known to confer resistance to scrapie and experimental BSE (for review of genotypes see Belt et al. 1995; Hunter 1997; Baylis et al. 2002a, Baylis et al. 2002b). The ARR/AHQ and ARR/ARQ genotypes are associated with a high degree of resistance to natural scrapie worldwide as well as incubation periods of >5 years following intracerebral challenge (IC) with BSE (Foster et al. 2001; Jeffrey et al. 2001; Baylis et al. 2002a, Baylis et al. 2002b). The ARQ/ARQ genotype is generally associated with increased risk of scrapie worldwide, although some breeds (e.g. Cheviot Sheep, UK) are relatively resistant (Baylis et al. 2002b). Sheep possessing the ARQ/VRQ genotype are at high risk of scrapie and experimental BSE (Baylis et al. 2002b). The ARR/VRQ genotype, somewhat variable by breed, is generally associated with rare to slightly elevated risk of scrapie as well as incubation periods of >5 years following IC with BSE (Belt et. al. 1995; Foster et al. 2001; Jeffrey et al. 2001; Baylis et al. 2002a, Baylis et al. 2002b).

The St. Croix White (SCW) and White Dorper (WD) breeds are members of a larger group of sheep commonly referred to as hair sheep or hair breeds. Collectively, hair breeds make up a relatively small portion of the overall world sheep population and

as a result have often escaped studies pertaining to scrapie or the *PRNP* locus in general, yet they are the predominant breed type found throughout the Caribbean and other tropical regions (Shelton 1991; Godfrey and Collins 1999). Additionally, hair breeds are commonly utilized in tropical regions worldwide for meat production and are valued for their resistance to Trichostrongyle (Mazzola 1990; Godfrey and Collins 1999). In this study we investigated *PRNP* exon 3 genotypes and allelic variants for SCW as well as a related composite breed (CMP) developed for commercial meat production.

Material and methods

Study animals. A total sampling of 33 sheep from Dorpcroix Sheep Farm (Hermleigh, TX USA) consisted of the following: six unrelated adult SCW (ewes) previously utilized as breeding stock for CMP, one full-blooded adult WD (ram) utilized as breeding stock for CMP, and 26 CMP (20 adult ewes, three ewe-lambs, and three adult rams). Composite animals (26 of $n \geq 500$ total farm) were developed for commercial meat production in 1998 and represent a synthetic breed resulting from an initial cross (SCW ewes × WD rams) followed by selection and crossing of animals exhibiting economically important traits such as overall hardiness and robust body stature. The WD and SCW sampled do not represent the sole founding stock for CMP. Study animals had no previous history or symptoms of scrapie at the time of publication.

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DNA isolation and PRNP amplification. Genomic DNA was isolated from whole blood samples either by spotting whole blood on Whatman Bioscience FTA® Classic Cards, and following the preparation protocol provided by the manufacturer (Whatman Inc., Clifton, NJ), or through utilization of the SUPER QUICK-GENE DNA Isolation kit (Analytical Genetic Testing Center, Denver, CO). The entire coding region for exon three of the ovine *PRNP* gene was amplified via PCR with the flanking synthetic oligonucleotides SAF1 and SAF2 (Prusiner et al. 1993). Thermal cycling parameters, as optimized in our laboratory, were as follows: 2 min at 96°C; 4 cycles × 30 s at 96°C, 30 s at 58°C (–1°C/cycle), 90 s at 65ºC; 31 cycles × 30 s at 96ºC, 30 s at 54ºC, 90 s at 65ºC; 15 min at 65ºC. Each 25 μl reaction included a 1.2 mm FTA punch or 100 ng genomic DNA, 400 μM dNTPs, 2.0 mM $MgCl_2$, 0.28 μM each primer, 1× reaction buffer, 1× MasterAmp™ PCR Enhancer (Epicentre, Madison, WI) and 1.0 unit *Taq* polymerase (Promega). PCR products were examined through agarose gel electrophoresis and purified using a Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA).

Sequencing. Purified PCR products were directly sequenced using a Big Dye™ Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), the aforementioned PCR primers, and the following thermal parameters: 2 min at 96°C; 35 cycles × 30 s at 96ºC, 20 s at 54ºC, 4 min at 60ºC; 5 min at 60ºC. Each 10 μl sequencing

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reaction included: 60 ng purified PCR product, 2 μl Big Dye™, 0.8 μM primer and 0.5× MasterAmp™ PCR Enhancer. Reactions were purified with G-50 sephadex columns (Biomax, Odenton, MD). Sequence fragments were separated and analyzed using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA), and are available through GenBank accession no. (AY350241–AY350275).

Validation techniques. Most samples were directly sequenced more than once. Representative alleles from each genotypic class with more than one single nucleotide polymorphism (SNP) were validated through cloning using a TOPO TA Cloning kit according to the manufacturer's recommendations (Invitrogen, Carlsbad CA). Plasmid DNA was isolated and purified using a Qiagen Plasmid Mini Kit as directed by the manufacturer (Qiagen Inc., Valencia CA). Insert sequencing for 12 clones was carried out via the sequencing method previously described with the following exceptions: 400 ng/reaction plasmid DNA, 50ºC anneal temperature, and (6.2 pmol/reaction) M13 forward and reverse primers.

Computer software and analysis. Ovine *PRNP* exon three genotypes and allelic variants were visualized using ABI PRISM SeqScape SNP Discovery and Validation Software version 1.01 (Applied Biosystems, Foster City, CA). Allele frequencies and tests of genic differentiation were calculated in GENEPOP (Raymond and Rousset 1995).

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Results and discussion

The frequencies of the five most common ovine *PRNP* exon 3 alleles (ARR, ARQ, VRQ, AHQ, and ARH), as verified via cloning, as well as a new allele ($P_{116}A_{136}R_{154}Q_{171}$; hereafter PARQ) for SCW and CMP are presented in Table 1. The novel (P_{116}) polymorphism associated with the PARQ allele is the result of a SNP

Table 1. Observed ovine *PRNP* allelic and genotypic frequencies

Breed [a]		*PRNP* [b] allele	Total	Frequency	Genotype (obs.)	Total	Frequency %
WD		ARQ	2	1.0000	ARQ/ARQ	1	100
WD	Sum		2	1.0000		1	100
SCW		ARR	9	0.7500	ARR/ARR	3	50.0
		ARQ	0	0.0000	P_{116}ARQ/ARR	1	16.7
		VRQ	1	0.0833	ARR/AHQ	1	16.7
		AHQ	1	0.0833	ARR/VRQ	1	16.7
		ARH	0	0.0000			
		P_{116}ARQ[c]	1	0.0833			
SCW	Sum		12	1.0000		6	100
CMP		ARR	22	0.4231	ARR/ARR	4	15.4
		ARQ	23	0.4423	ARR/ARQ	12	46.2
		VRQ	2	0.0385	P_{116}ARQ/ARQ	4	15.4
		AHQ	1	0.0192	ARQ/ARQ	3	11.5
		ARH	0	0.0000	ARR/AHQ	1	3.8
		P_{116}ARQ[c]	4	0.0769	ARR/VRQ	1	3.8
					ARQ/VRQ	1	3.8
CMP	Sum		52	1.0000		26	100

[a] WD, White Dorper; SCW, St. Croix White; CMP, composite breed
[b] Ovine *PRNP* exon 3
[c] Alanine is the wild-type amino acid at ovine position 116

(G→C) at ovine nucleotide position 346. The origin of the PARQ allele is likely SCW since the allele is present in SCW and CMP, but not in the WD sampled (Table 1).

We did not detect the ARH allele in our SCW, WD, and CMP samples (Table 1). Absence of the ARH allele has previously been reported in Scottish Blackface, Welsh Mountain, Swaledale, and Beulah breeds in the UK (Arnold et al. 2002). Of the 15 possible genotypes derivable from the five most common ovine *PRNP* exon 3 alleles we determined our samples for WD, SCW, and CMP to possess only six, collectively (Table 1). However, two new *PRNP* genotypes (PARQ/ARR and PARQ/ARQ) were detected for the SCW and CMP samples, thereby increasing the total number of distinct *PRNP* genotypes detected in this study to eight (Table 1). The distribution of *PRNP* exon 3 genotypes within the SCW, CMP, and WD sampled are depicted in Table 1. Absence of the ARQ allele combined with the relatively high frequency of the ARR allele in the SCW sampled results in significant (P = 0.0123; S.E. = 0.00113) genic differentiation between SCW and CMP.

The relationship between scrapie susceptibility or resistance, the novel PARQ allele, and/or the associated genotypes (PARQ/ARR; PARQ/ARQ), is presently unknown. However, the proline polymorphism noted at ovine amino acid position 116 occurs between the N-terminal cleavage site (between Lys_{112} and His_{113}; human numbering) and the hydrophobic region of the prion protein, a region exhibiting extreme conservation across mammalian groups (Fig. 1). The functional ability of the normal

cellular prion protein (PrP^C) as a potential cell-surface receptor is most likely modulated by the proteolytic cleavage and removal of the N-terminal region of the protein (Harris et al. 1993; van Rheede et al. 2003). Furthermore, the amino acid residues immediately flanking the ovine (P_{116}) polymorphism are considered to play a major role in the interface between (PrP^C) and the pathogenic isoform (PrP^{Sc}) (Cohen and Prusiner 1998). Currently, three pathogenic human mutations causing GSS (Gerstmann-Sträusler-Scheinker syndrome; P102L; P105L; and A117V) and one human mutation strongly associated with the phenotypic expression of vCJD (variant Creutzfeld-Jakob disease, M129V) have been described within the regions of the prion protein immediately flanking the ovine (P_{116}) polymorphism (Fig. 1; for review see Collinge 2001 and van Rheede et al. 2003).

In conclusion, we have demonstrated that genotypes associated with moderate to high levels of resistance to scrapie and experimental BSE exist for the majority of the CMP (65.4%) and SCW (66.7%) sampled, while the WD ram was determined to possess a susceptible genotype (ARQ/ARQ) (Table 1). Additionally, the identification of a predicted novel ovine PrP polymorphism provides an opportunity for future challenge experiments to investigate the potential effect(s) of the PARQ allele as well as the PARQ/ARR and PARQ/ARQ genotypes.

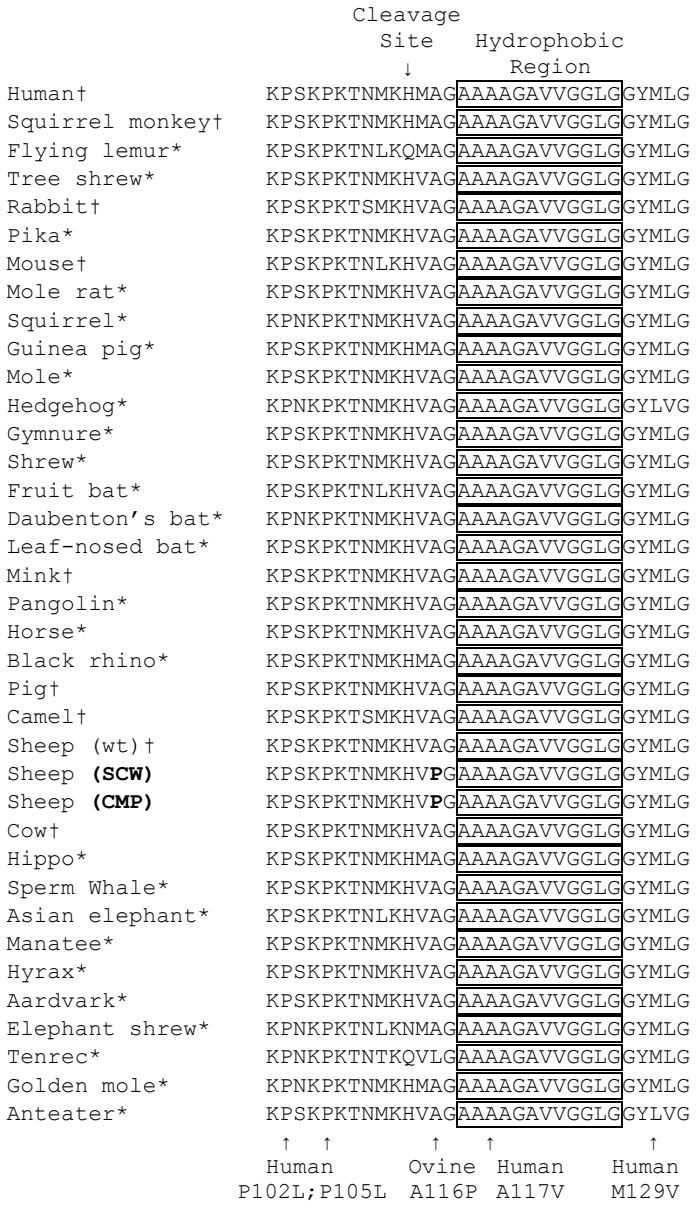

Fig. 1. Extreme conservation associated with specific regions of the mammalian prion protein. Proximally relevant human mutations also depicted (GSS mutations: P102L, P105L, and A117V; M129V associated with vCJD). Asterisk (*) indicates sequences from van Rheede et al. (2003). Sequences obtained from GenBank, as referenced and utilized in van Rheede et al. (2003), are indicated by a cross (†).

NOVEL PRION PROTEIN GENE (*PRNP*) VARIANTS AND EVIDENCE FOR STRONG PURIFYING SELECTION IN FUNCTIONALLY IMPORTANT REGIONS OF BOVINE EXON 3*

Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are inevitably fatal, neurodegenerative diseases that occur in humans as well as domestic and wild animals (Prusiner 1982; Prusiner 1991; Collinge 2001). Traditionally, human spongiform encephalopathies have been classified into Creutzfeld-Jakob disease (CJD), Gerstmann-Sträusler-Scheinker disease (GSS), and kuru, with more recent classification into variant CJD (vCJD) (Collinge 2001). Animal TSEs include transmissible mink encephalopathy, scrapie of sheep and goats, chronic wasting disease of deer and elk, feline spongiform encephalopathy, and bovine spongiform encephalopathy (BSE) (Collinge 2001). Central to the development of these diseases is the accumulation of an infectious protease-resistant isoform (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{C}) in tissues of the central nervous system (Griffith 1967; Prusiner 1982; Bossers et al. 2000).

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The prototypical TSE, scrapie, has been observed in European sheep for over 200 years, while BSE in domestic cattle (*Bos taurus*, *Bos indicus*; hereafter referred to as cattle) dates to 1986, presumably resulting from scrapie and/or BSE infected cattle feed (McGowan 1922; Wells et al. 1987; Wilesmith 1991; Hunter 1997). Thus, BSE seems to be a more recent phenomenon associated with modern agricultural practices. This idea is supported by several lines of evidence. First, at least 14 amino acid polymorphism encoded by exon 3 of the ovine prion protein gene (*PRNP*) have been described (Goldmann et al. 1990; Goldmann et al. 1991a; Laplanche et al. 1993; Belt et al. 1995; Belt et al. 1996; Bossers et al. 1996; Hunter et al. 1996; Tranulis et al. 1999; Gombojav et al. 2003; Heaton et al. 2003; Seabury and Derr 2003), and those associated with codons 136 and 171 have been shown to influence expression of scrapie (Belt et al. 1995; Hunter 1997). However, no amino acid polymorphisms associated with BSE have been identified in cattle, although an insertion-deletion polymorphism (indel) in the putative bovine *PRNP* promoter was shown to exhibit an association with BSE in a few German cattle breeds (Sander et al. 2004). Second, recorded patterns of variation within cattle *PRNP* exon 3 are markedly different from those observed in sheep, with variability in cattle primarily restricted to 11 synonymous nucleotide sites and two nonsynonymous sites where low-frequency variation has been observed (Goldmann et al. 1991b; Wopfner et al. 1999; Humeny et al. 2002; Heaton et al. 2003; Hills et al. 2003; Takasuga et al. 2003; Casalone et al. 2004). Notably, a substantially higher number of nonsynonymous polymorphic sites have been recorded for sheep (Goldmann et al. 1990; Goldmann et al. 1991a; Laplanche et al. 1993; Belt et al. 1995; Belt et al. 1996; Bossers

et al. 1996; Hunter et al. 1996; Tranulis et al. 1999; Gombojav et al. 2003; Heaton et al. 2003; Seabury and Derr 2003). Additionally, insertion-deletion polymorphism has not been observed within the octapeptide repeat region of ovine *PRNP* exon 3, whereas studies of cattle and other bovine species have yielded three indel isoforms possessing five to seven octapeptide repeats (Hunter et al. 1994; Neibergs et al. 1994; Goldmann et al. 1991b; Schlapfer et al. 1999; Wopfner et al. 1999; Premzl et al. 2000; Heaton et al. 2003; Hills et al. 2003; Takasuga et al. 2003).

Despite the importance of cattle to both agricultural practices worldwide and the global economy, surprisingly little is known about *PRNP* allelic diversity for cattle, collectively, and/or how this gene evolves in this lineage. In addition, while several non-domesticated species of Bovinae contracted TSE-like diseases contemporaneous with the BSE epidemic (Collinge 2001), even less is known regarding how the *PRNP* gene evolves in these species. Herein we provide a detailed investigation of genetic variation within *PRNP* exon 3 for 36 breeds of cattle and 10 additional species of Bovinae. In particular, we examine patterns of variation across *PRNP* exon 3 and evaluate the dynamics of this variation in terms of evolutionary processes that may have brought about changes at the amino acid level observed between species, especially as they relate to structural and/or functional constraints on the prion protein.

Materials and methods

DNA samples. To evaluate *PRNP* exon 3 we utilized a DNA panel of 119 artificial insemination (AI) sires from 36 cattle breeds. The source of DNA was spermatozoa.

Breed names and sample sizes (in parentheses) are as follows: Angus (4); Beefalo (1); Beefmaster (4); Belgian Blue (4); Blonde D'Aquitaine (5); Braford (4); Brahman (2); Brahmousin (2); Brangus (2); Braunvieh (5); Brown Swiss (4); Charolais (5); Chianina-Chiangus (5); Corriente (1); Gelbvieh (4); Hereford (3); Holstein (4); Limousin (3); Maine Anjou (4); Murray Grey (2); Nelore (8); Normande (1); Piedmontese (2); Red Angus (4); Red Brangus (1); Red Poll (1); Romagnola (2); Salers (3); Santa Gertrudis (2); Scottish Highland (1); Senepol (2); Shorthorn (5); Simbrah (3); Simmental (8); Tarentaise (1); Texas Longhorn (4); Three-way-cross (2); White Park (1). Six Nelore were not AI sires. Care was taken to select unrelated sires.

For comparison, we utilized a second DNA panel of 286 members of the subfamily Bovinae. Species included and sample sizes (in parentheses) are as follows: *Tragelaphus strepsiceros*, greater kudu (1); *Tragelaphus imberbis*, lesser kudu (1); *Boselaphus tragocamelus*, nilgai (1); *Bubalus bubalis*, Asian water buffalo (2); *Bubalus depressicornis*, lowland anoa (1); *Syncerus c. caffer*, African buffalo (1); *Syncerus c. nanus*, forest buffalo (1); *Bos javanicus*, banteng (2); *Bos gaurus*, gaur (2); *Bison bison*, American bison (274). American bison were sampled from four U. S. federal and one private herd. For banteng, gaur, and bison, mtDNA analysis was employed to exclude individuals showing evidence of interspecific hybridization with cattle (Ward et al. 1999). Likewise, Asian water buffalo that displayed karyotypic evidence of hybridization (river x swamp; Chowdhary et al. 1989) were excluded from statistical analyses. One blackbuck, *Antilope cervicapra*, was sampled and utilized as a reference species outside of the subfamily Bovinae.

PRNP amplification, sequencing, and SNP detection. Flanking primers SAF1 and SAF2 (Prusiner et al. 1993) were used to PCR amplify and sequence *PRNP* exon 3. For American bison, 96 were sequenced bi-directionally and 178 were sequenced using SAF1 only. Amplification, amplicon sequencing, and SNP detection followed previously published methods (Seabury and Derr 2003). Representative alleles from each genotypic class with more than one SNP were validated by a second PCR amplification, cloning, and bi-directional sequencing of multiple clones (Seabury and Derr 2003). This method also was utilized to assign indels to their respective alleles.

Population and evolutionary analyses. *PRNP* exon 3 sequences for all Bovinae taxa (all alleles) were aligned using ClustalX version 1.81 (Thompson et al. 1997). The alignment was corrected using the published cattle octapeptide repeat units as a guide (Goldmann et al. 1991b; Schlapfer et al. 1999). Using an intraspecific file of all cattle alleles, DnaSP ver. 3.53 (Rozas and Rozas 1999) was used to compute estimates of the number of segregating sites (S; Watterson 1975) and nucleotide diversity (π; Nei 1987). Estimates of Watterson's genetic diversity parameter (θ) were based on the number of segregating sites (Watterson 1975). The degree of non-random association between nucleotide variants (parsimony informative sites only; excluding gaps) for all cattle alleles was estimated using linkage disequilibrium parameters implemented in DnaSP (Rozas and Rozas 1999), and the significance of the associations was evaluated using Bonferroni-corrected two-tailed Fisher's exact tests (Sokal and Rohlf 1981). Estimates for the recombination parameter (R) per gene and between adjacent sites (Hudson 1987),

as well as the minimum number of recombination events (R_M; Hudson and Kaplan 1985) for all cattle alleles (excluding gaps), were computed in DnaSP (Rozas and Rozas 1999).

We used several approaches to evaluate the degree of selective constraint exerted upon bovine *PRNP* exon 3. Tajima's *D* (Tajima 1989) and Fu and Li's F^* and D^* (Fu and Li 1993) were calculated using all cattle alleles as implemented in DnaSP (Rozas and Rozas 1999). To further assess the potential for selective constraint within cattle *PRNP* exon 3, these statistics were computed using a sliding windows approach (window size = 100 base-pairs (bp); step size = 25 bp). The McDonald and Kreitman (MK) test (McDonald and Kreitman 1991) was performed to assess intraspecific patterns of selective constraint in cattle and bison using various wild bovine species as outgroup taxa. Estimates of the numbers of synonymous substitutions per synonymous site (d_S) and nonsynonymous substitutions per nonsynonymous site (d_N) were calculated using MEGA version 2.1 (Kumar et al. 2001) using the modified Nei-Gojobori method (Zhang et al. 1998) with Jukes-Cantor correction and complete deletion of gaps. Standard errors for d_S and d_N were estimated from 1,000 bootstrap pseudoreplicates. A phylogenetic approach (Zhang et al. 1997; Rooney and Zhang 1999) was used to examine the difference between d_S and d_N on both terminal and interior branches of the bovine phylogeny. In short, interior branch sequences were reconstructed using a distance-based Bayesian method (Zhang and Nei 1997), allowing the observed numbers of synonymous (*s*) and nonsynonymous (*n*) substitutions to be plotted on each branch in the phylogeny and compared to the potential numbers of synonymous (*S*) and

nonsynonymous (*N*) substitutions. The statistical significance of the difference between *s/(S-s)* and *n/(N-n)* was assessed using Fisher's exact test.

Results

Assessment of PRNP allelic differences. *PRNP* exon 3 analysis of 812 chromosomes and more than 643,000 bp across all taxa investigated yielded 45 polymorphic sites, including polymorphisms within octapeptide repeats where indel variation also was detected. The distribution of polymorphic sites, nucleotide variants associated with each site, and predicted amino acid replacements are presented in Table 2. *PRNP* exon 3 alleles possessing four to seven octapeptide repeats were observed for cattle (Table 3). The unprecedented 4 octapeptide repeat allele was detected in a single Brown Swiss sire where repeats R3 and R4 (Goldmann et al. 1991b) were predicted to be deleted by multiple sequence alignment. The seven octapeptide repeat allele (Schlapfer et al. 1999) was only observed for Brown Swiss cattle, while alleles possessing 5 and 6 repeats were noted for other domestic breeds and species of Bovinae (Table 3).

Evaluation of the degree of non-random association between polymorphic sites for cattle *PRNP* exon 3 based on Fisher's exact test and the Bonferroni procedure revealed 12 significant ($P < 0.001$; Bonferroni significant for $\alpha' = 0.05$) comparisons among all parsimony informative sites. These non-random associations correspond to polymorphic sites in three cattle breeds: Nelore (n = 5) 69/555, 69/630, 555/630; Brahman (n = 1) and Brahmousin (n = 1) 75/108, 75/126, 75/461, 108/126, 108/461, 108/678, 126/461, 126/678, 461/678 (see Table 2). Significant associations result from

several unique low frequency *PRNP* exon 3 alleles detected in seven individuals of three cattle breeds, including three unique alleles possessing the G461A mutation (predicted amino acid replacement S154N).

PRNP exon 3 sequence analysis for other species of Bovinae yielded 19 amino acid replacements not found in cattle. The S154N amino acid replacement predicted for three of 238 (Freq = 0.0126) cattle *PRNP* exon 3 alleles was observed in all alleles for the lesser kudu, nilgai, Asian water buffalo, lowland anoa, African buffalo, and forest buffalo. Fixed amino acid replacements observed for non-domestic species when compared to cattle are as follows: lowland anoa, S4R, A16V, P54S, G108S, V123M, F257L; Asian water buffalo, S4R, A16V, P54S, G108S, V123M, F257L; greater kudu, G22A, P54S, H166Y, E197Q, Q234E; lesser kudu, P54S, H166Y, E197Q, Q234E; African buffalo, A16V, P54S, I214V; forest buffalo, A16V, P54S, N185S, I214V; and nilgai, G59S, G75S, S146N, H188R, R231T, I244V, I252V.

Analyses of intraspecific polymorphism and interspecific tests of selection. Overall, genetic variability in cattle was low. Excluding regions with gaps due to indels (Goldmann et al. 1991b, Schlapfer et al. 1999), 22 unique *PRNP* exon 3 alleles were determined for our panel of cattle. Most of these alleles possessed one to three synonymous changes, and only one polymorphic site noted for cattle (G461A; Table 2) was associated with a predicted amino acid replacement (S154N). Thirteen of the 26 segregating sites observed, excluding gaps, were singletons. Furthermore, estimates of

Table 2. **Bovinae *PRNP* exon 3 polymorphic nucleotide sites**

Bovinae species	Polymorphic nucleotide sites and SNPs (IUPAC/IUB Codes)[a, b]									Total
Bos taurus; B. indicus	57(Y)	69(Y)	75(R)	108(W)	126(R)	183(Y)	189(Y)	195(W)	207(M)	30
	210(R)	231(Y)	234(R)	237(Y)	255(W)	261(M)	267(Y)	270(Y)	294(Y)	
	315(Y)	327(R)	339(Y)	378(Y)	405(B)	**461**(R)	534(Y)	555(Y)	576(Y)	
	630(Y)	675(Y)	678(Y)							
Bos javanicus	**110**(K)	255(Y)	267(W)	**554**(R)						4
Bison bison	**50**(Y)									1
Syncerus c. caffer	231(Y)	234(R)	237(Y)	255(W)	261(M)	267(W)	270(W)	**554**(R)	585(M)	11
	693(R)	**700**(S)								
Syncerus c. nanus	**679**(W)	**751**(R)								2
Bubalus depressicornis	**38**(Y)	351(R)	**548**(R)							3
Bubalus bubalis	126(R)	**127**(R)	234(R)	285(R)	**322**(R)	678(Y)				6
Boselap. tragocamelus	423(R)									1
								Total unique polymorphic sites:		45

[a] **Bolded** nucleotide sites are predicted to result in amino acid replacements; all others represent synonymous variation. Numbering was derived from the 6-octapeptide cattle allele (25; Genbank X55882). Nucleotide sites and variation underlined are shared among taxa.
[b] SNPs and predicted amino acid replacements are as follows: **461A**(S154N); **110T**(G37V); **554G**(N185S); **50C**(M17T); **700G**(Q234E); **679T**(T227S); **751A**(V251M); **38C**(L13P); **548G**(Q183R); **127A**(G43R); **322A**(G108S). Novel cattle polymorphisms include: 183(Y), 189(Y), 195(W), 207(M), 231(Y), 237(Y), 255(W), 261(M), 267(Y), 270(Y), 294(Y), 315(Y), 327(R), 378(Y), 405(B), 534(Y).

Table 3. Observed octapeptide repeat genotypes

Species	Observed genotype	(n)	Observed frequency	Indel repeat unit [a]
B. taurus, B. indicus	4:6	1	0.0084	R3, R4 del
	5:5	1	0.0084	R3 del
	6:5	5	0.0420	R3 del
	6:6	109	0.9160	
	6:7	1	0.0084	RN2 ins
	7:7	2	0.0168	RN2 ins
B. javanicus	6:5	2	1.0000	R4 del

[a] The repeat unit deleted (del) or inserted (ins) as predicted via multiple sequence alignment and previous studies (25, 30). Samples for all other species investigated were fixed for the 6:6 genotype. n = sample size.

θ and R for cattle exon 3 alleles (Table 4) are low in comparison to what has been observed for other nuclear genes (Nei 1987). Thus, it is not surprising that Tajima's test yielded a significantly negative D value (–2.14, $P < 0.01$; Table 4), as did Fu and Li's tests employing both the D^* and F^* statistics (– 4.12, $P < 0.02$; – 3.99, $P < 0.02$, respectively).

Application of a sliding windows approach to Tajima's and Fu and Li's tests revealed several significantly negative 100 bp windows (Fig. 2) within cattle *PRNP* exon 3 (Tajima's, $P < 0.05$; Fu and Li's, $P < 0.05$) that correspond to known structural features of the mammalian prion protein (van Rheede et al. 2003). Significantly negative windows by all tests correspond to the amino acid residues preceding the proline hydroxylation site, the octapeptide repeat region (repeat units R1-R6; RN2;

Table 4. *PRNP* exon 3 allelic diversity within domestic cattle breeds excluding gaps

Total Alleles	Unique Alleles	Segregating Sites	Total Mutations	θ (± SD) [a]	π (± SD) [a]	Tajima's D [b]	$R(4Nr/\text{bp})$ $R(4Nr/\text{gene})$
238	22	26	27	0.00578 (0.00163)	0.00125 (0.00014)	-2.14	0.0000 0.0010

[a] SD = standard deviation.
[b] Significant at the $P < 0.01$ level.

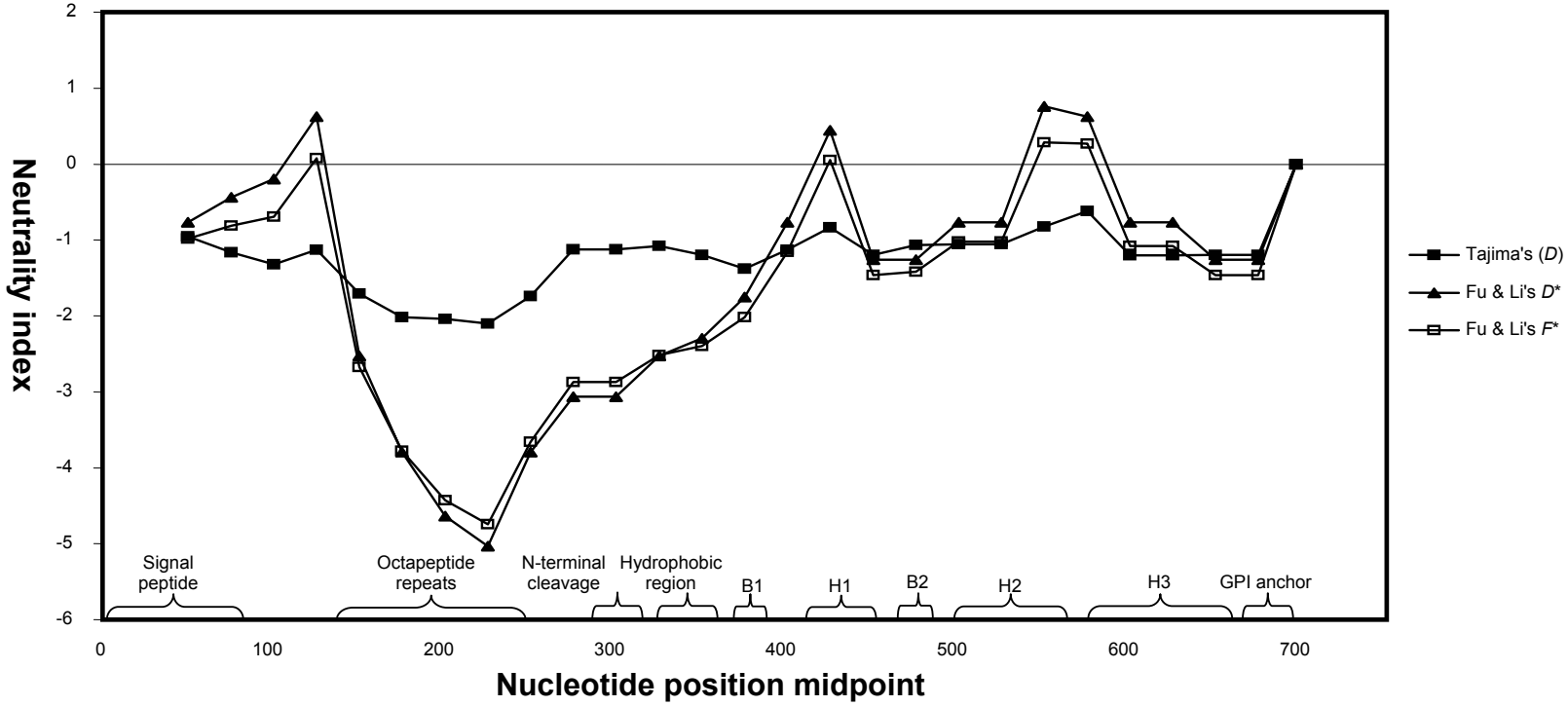

Fig. 2. Sliding window analyses of Tajima's (*D*) and Fu and Li's tests (D^*, F^*) for cattle *PRNP* exon 3 (repeats R3, R4, RN2 deleted by gap handling). Significantly negative 100 bp windows ($P < 0.05$; all tests) extend from the amino acid residues preceding the proline hydroxylation site, through the octapeptide repeat region, and into the region C-terminal to R6. Fu and Li's tests also revealed significantly negative windows corresponding to the hydrophobic region and B1 (beta region 1).

Goldmann et al. 1991b; Schlapfer et al. 1999), and the region C-terminal to R6 (R3, R4, RN2 deleted by gap handling). In addition, Fu and Li's tests yielded significantly negative values for a window corresponding to the region of cattle *PRNP* exon 3 extending from R5 to the amino acid residues preceding the N-terminal cleavage site (Fu and Li's tests, $P < 0.02$; Tajima's *D*, $P < 0.10$ for cleavage site). Fu and Li's tests also revealed several significantly negative windows ($P < 0.05$) corresponding to the hydrophobic region (trans-membrane domain component) and B1 (beta region 1) of cattle *PRNP* exon 3.

Examination of the pattern of synonymous and nonsynonymous substitutions were indicative of strong purifying selection, which is unsurprising given the low levels of polymorphism observed and the results obtained from Tajima's and Fu and Li's tests. The average values of d_S ($\bar{d}_S$) and d_N ($\bar{d}_N$), respectively, were 0.0045 ± 0.0015 and 0.000046 ± 0.000045 for all cattle *PRNP* exon 3 alleles, yielding a $\bar{d}_N / \bar{d}_S$ ratio of 0.01. All MK tests between cattle and other species of Bovinae were significant, except in cases when cattle were compared to closely related species (i.e., bison, gaur, and banteng; Table 5). These species diverged from cattle very recently, probably within the last 1-2 million years (Janecek et al. 1996; Ritz et al. 2000), which explains the lack of fixed differences between them. MK tests performed between *Bison bison* and all other taxa were not significant.

The observed numbers of synonymous and nonsynonymous changes were plotted for each branch in the bovine phylogeny (Fig. 3). Clearly these numbers are small, indicating that an intense level of selection constrains the number of variable sites within

Table 5. McDonald-Kreitman tests for *PRNP* exon 3 of domestic cattle and non-domestic taxa of Bovinae

Comparison species [a]	Fisher's [b]	G-test [c]	Substitutions	Fixed	Polymorphic
Syncerus c. nanus	0.0271	0.0266	Synonymous	4	26
			Nonsynonymous	4	3
Syncerus c. caffer	0.0359	0.0392	Synonymous	3	30
			Nonsynonymous	3	3
Bubalus bubalis	0.0006	0.0004	Synonymous	4	26
			Nonsynonymous	6	1
Bubalus depressicornis	0.0033	0.0028	Synonymous	4	27
			Nonsynonymous	6	3
Boselaphus tragocamelus	0.0009	0.0006	Synonymous	5	27
			Nonsynonymous	6	1
Tragelaphus strepsiceros	0.0127	0.0098	Synonymous	9	26
			Nonsynonymous	5	1
Tragelaphus imberbis	0.0136	0.0114	Synonymous	6	26
			Nonsynonymous	4	1
Bison bison	—	—	Synonymous	0	26
			Nonsynonymous	0	2
Bos javanicus	1.0000	—	Synonymous	1	27
			Nonsynonymous	0	3
Bos gaurus	—	—	Synonymous	0	26
			Nonsynonymous	0	1

[a] Between species comparisons were made with domestic cattle with complete deletion of gaps.
[b] P-value for Fisher's exact test.
[c] P-value for G-test with Williams correction. Dash indicates that statistical tests could not be performed. No complex codons were encountered.

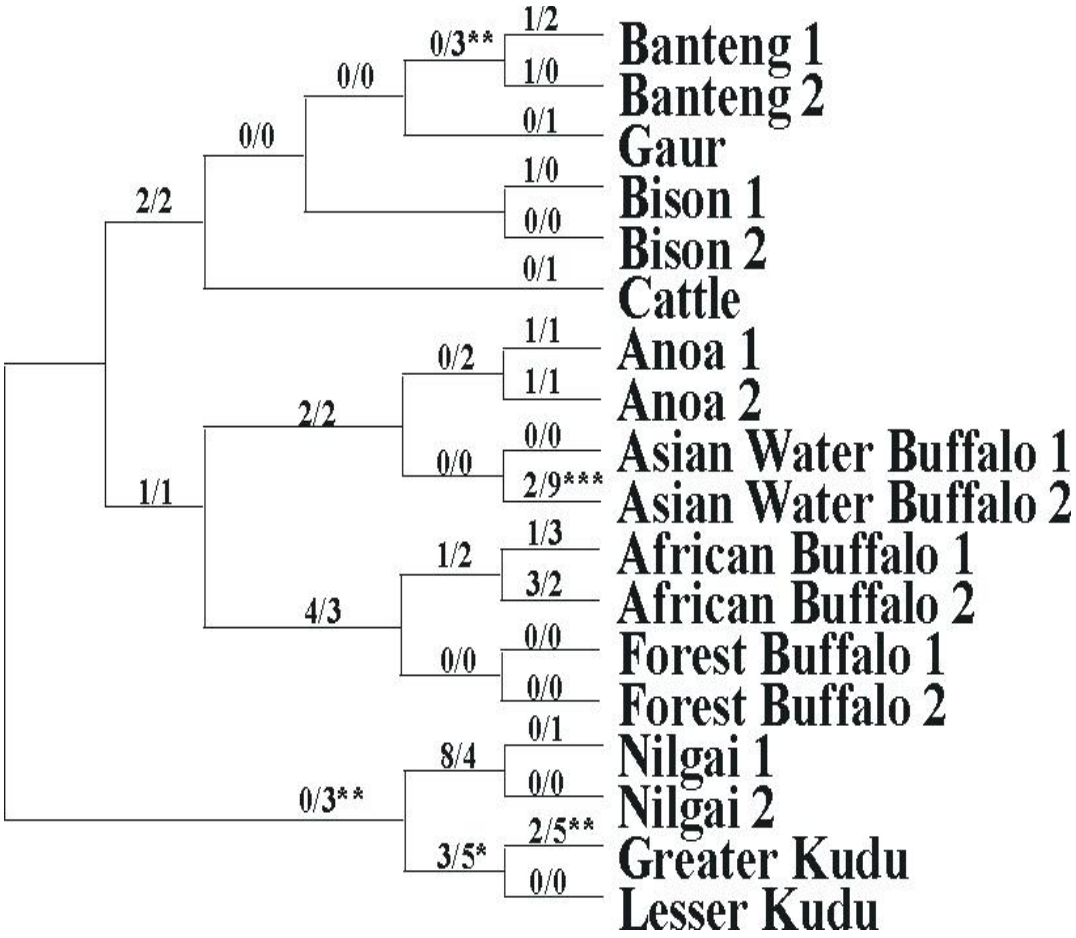

Fig. 3. Phylogeny used for conducting tests of selection on bovine *PRNP* exon 3 sequences. The phylogeny is based on published molecular and morphological studies (51-52). The numbers along branches represent observed *n* and *s*, respectively. The average values of *S* and *N* for all extant taxa were 226 and 563. The posterior probabilities for all inferred ancestral sequences were ≥ 99%. Note that the use of alternative alleles and/or tree topologies did not change the interpretation of the results. *0.02<P≤0.05; **0.005<*P*≤0.02; ***$P \leq 0.004$.

PRNP exon 3. Thus, when the differences between levels of synonymous and nonsynonymous changes are compared, Fisher's exact test must be used (Zhang et al. 1997). When we conducted this test, we found evidence for strong purifying selection ($P << 0.001$) when levels of synonymous and nonsynonymous nucleotide substitution were compared over the entire phylogenetic tree. On the other hand, with few exceptions

levels of synonymous and nonsynonymous substitution were not different from each other along individual branches, particularly at shallow levels (i.e., terminal branches). There are two possible explanations for this. First, all variable sites observed are selectively neutral. This is consistent with the fact that no amino acid polymorphisms have been identified to date that either confer resistance to BSE (advantageous mutations) or augment expression of BSE (deleterious mutations). The second explanation for the observed pattern is that there is a lack of power to detect selection along individual branches due to only a very small number (e.g., 1-3) of variable sites. This is the most likely explanation.

Discussion

While rare, detection of the predicted S154N polymorphism encoded by cattle *PRNP* exon 3 is not unprecedented, as this polymorphism was previously detected at low frequencies (Heaton et al. 2003; Takasuga et al. 2003). Given the low frequency of the S154N polymorphism and the overall pattern of nucleotide variation observed for cattle *PRNP* exon 3, additional functional, transgenic, and challenge experiments seem appropriate. Moreover, amino acid 154 (143, human numbering) has previously been implicated in the susceptibility of humans to cattle derived prions (Krakauer et al. 1996). Significant non-random associations between polymorphic nucleotides of the three unique cattle *PRNP* alleles possessing the G461A mutation (S154N; Table 2) is unusual, but not surprising given the low estimates obtained for recombination (Table 4). Also of interest is the commonality of the amino acid asparagine at position 154 (N154) for other

species of Bovinae, as well as among other mammalian taxa (van Rheede et al. 2003). Notably, bovine amino acid position 154 (6 octapeptide repeat allele) corresponds to ovine position 146, which has not been described as polymorphic (N146) in sheep nor been implicated in the expression of scrapie. Therefore, additional studies are needed to evaluate what effects, if any, the bovine S154N polymorphism has on BSE expression.

The distribution of observed *PRNP* exon 3 octapeptide repeat genotypes for cattle was somewhat unexpected (Table 3), as we anticipated more individuals of the 6:5 genotype based on earlier studies (Hunter et al. 1994; Neibergs et al. 1994; Schlapfer et al. 1999; Premzl et al. 2000; Heaton et al. 2003). The underlying reason for this may be due to the fact that our cattle samples are almost exclusively from AI sires. However, the observed genotypic frequency of the 6:6 genotype for young bulls (0.894) in a study of Polish Black-and-White cattle (Walawski and Czarnik 2003) was approaching that observed in this study (0.916; Table 3). Furthermore, a study of full families of Polish Black-and-White cattle demonstrated abnormal segregation of octapeptide repeat alleles, as evidenced by nearly twice the expected number of 6:6 genotypes (Walawski et al. 2003).

Frequency-distribution tests provided significant statistical support for an excess of rare alleles and/or singletons in our overall sample of cattle *PRNP* exon 3 alleles. Significantly negative values for Tajima's *D* and Fu and Li's tests (D^*; F^*) are often interpreted as purifying or directional selection, but may also indicate violations of the mutation-drift equilibrium assumptions (Tajima 1989) and/or the random sample requirement (Fu and Li 1993) of these tests. For cattle *PRNP* exon 3, all of the

singletons and most of the rare alleles (19/22; 86%) resulted from synonymous variation, which is unlikely to be subject to directional selection (Glatt et al. 2001). Such an excess of rare synonymous variants, given a theoretically large random mating population, might indicate recent population expansion followed by insufficient time to establish a balance between the occurrence of new mutations and their loss via genetic drift (Glatt et al. 2001). Nevertheless, the pattern of nucleotide variation observed for cattle *PRNP* exon 3 overall and within significantly negative 100 bp windows corresponding to regions preceding the N-terminal cleavage site, the cleavage site itself, the hydrophobic region, and B1 suggests that selection may be acting to preserve the amino acid sequence of cattle PrP^C within regions of functional, structural, or potential pathogenic importance.

Interestingly, amino acid substitutions associated with human hereditary and sporadic spongiform encephalopathies form two clusters: (1) region C-terminal to the octapeptide repeat region and N-terminal to the first alpha-helix; (2) the second and third alpha-helices (Collinge 2001, Krakauer et al. 1998; Fig. 2). Several significantly negative windows obtained for cattle *PRNP* exon 3 correspond to regions where three human mutations (P102L; P105L; A117V) associated with GSS, a human hereditary spongiform encephalopathy, have been identified (Collinge 2001). Synonymous variation (C339T) was observed at the third position of the cattle equivalent to human codon 102 in our study and in previous studies (Humeny et al. 2002; Heaton et al. 2003). Significantly negative windows also were obtained for the region of cattle *PRNP* exon 3 corresponding to human codon 129 (Collinge 2001). Also intriguing is the inclusion of

B1 within significantly negative windows identified by sliding window analysis, given that the conversion of PrP^{C} to PrP^{Sc} is considered to bring about changes in secondary structure hallmarked by increased beta sheet formation (Collinge 2001).

While the total number of fixed amino acid changes is relatively small in any given comparison, it is interesting to note the number of shared fixations among the taxa in our study. For example, our samples for lowland anoa and Asian water buffalo possessed the same fixed changes (S4R, A16V, P54S, G108S, V123M, F257L) when compared to cattle. Likewise, both subspecies of *Syncerus* (African and forest buffalo) shared the A16V, P54S, and I214V fixed changes, and three fixed replacements also were observed for both species of *Tragelaphus* when compared to cattle. Previous phylogenetic studies suggested convergence between great apes and cattle at specific amino acid residues (Krakauer et al. 1996; Krakauer et al. 1998). Although convergence remains possible, shared amino acid replacements between bovine species may represent shared primitive characters, given the short time frame since these species diverged from one another (Ritz et al. 2000; Janecek et al. 1996).

Several fixed amino acid replacements noted in between-species comparisons with cattle warrant further investigation in light of previous studies on ovine scrapie and PrP^{C} biogenesis (Belt et al. 1995; Hunter 1997; Hegde et al. 1998; Hegde et al. 1999; Kim et al. 2001; Kim et al. 2002). The fixed replacement H188R in nilgai, equivalent to codon 180 in sheep, is proximal to the ovine Q171R polymorphism associated with scrapie resistance (Belt et al. 1995; Hunter 1997). Furthermore, a study of amino acid replacements within the signal peptide of PrP^{C}, proximally relevant to fixed

replacements S4R and A16V, has demonstrated that signal peptide mutations influence the ratio of three topological forms in which PrP^{C} is synthesized at the endoplasmic reticulum (ER; Kim et al. 2001). The predominant form (^{sec}PrP) is fully translocated into the ER lumen, whereas the other two forms (^{Ntm}PrP and ^{Ctm}PrP) are single-spanning membrane proteins named to reflect the terminus inserted into the lumen (Hegde et al. 1998; Hegde et al. 1999; Kim et al. 2001). Signal sequence mutations may increase or decrease the ratio of ^{Ctm}PrP relative to the other topological forms (Kim et al. 2001; Kim et al. 2002). In addition, mutations that increase the generation of ^{Ctm}PrP have been associated with neurodegenerative disease (Hegde et al. 1998; Hegde et al. 1999; Kim et al. 2001). Therefore, it is important to evaluate the amino acid replacements M17T and L13P, observed within the signal sequences of bison and lowland anoa, respectively (Table 2).

Several other nonsynonymous *PRNP* exon 3 polymorphisms observed for taxa of Bovinae warrant investigation based on ovine *PRNP* and scrapie (Belt et al. 1995; Hunter 1997). For example, the amino acid replacement Q183R observed for lowland anoa corresponds to ovine position 175, which is near the ovine Q171R polymorphism. Likewise, the N185S replacement, polymorphic for banteng (Takasuga et al. 2003) but fixed for our sample of forest buffalo, is equivalent to ovine position 177, which is also proximal to ovine Q171R. Predicted amino acid replacements presented herein that are proximal to ovine amino acid 171 represent suitable candidates for future challenge experiments related to BSE resistance and/or susceptibility.

Conclusions. We have further documented and evaluated the emerging pattern of nucleotide variation for cattle *PRNP* exon 3, revealing evidence for highly intense purifying selection within regions previously suggested and/or demonstrated to be of functional, structural, or pathogenic importance in humans and other mammalian species (Belt et al. 1995; Hunter 1997; Collinge 2001; van Rheede et al. 2003). In addition, several novel polymorphic sites and corresponding amino acid replacements for taxa not included in previous studies were identified (Table 2). These polymorphisms as well as the fixed amino acid replacements identified in between-species comparisons with cattle provide an opportunity to evaluate a new battery of amino acid residues with respect to prion disease in domestic and wild bovids.

Perhaps the most interesting outcome of this study concerns the nature of selection on *PRNP* exon 3. Knock-out mice suffer either very subtle or no deleterious effects upon losing the *PRNP* gene (Estibeiro 1996; Collinge 2001), suggesting that *PRNP* may be an evolutionary "appendix" not necessarily needed by the body. Yet, if this were true, why would purifying selection be so intense on this protein? Such strong levels of purifying selection are normally only seen among proteins such as histones that are essential to eukaryotic life (Piontkivska et al. 2002). Thus, what is the driving force behind such intense purifying selection on *PRNP*? The answer to this question is currently unknown.

COMPARATIVE *PRNP* GENOTYPING OF U. S. CATTLE SIRES FOR POTENTIAL ASSOCIATION WITH BSE*

Introduction

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are inevitably fatal neurodegenerative diseases that occur in a variety of mammalian species, including humans as well as domestic and wild animals, and are often characterized by dementia and/or ataxia (Collinge 2001; Prusiner 1998). The pathogenic agents of prion diseases are infectious, protease-resistant proteins which arise through modification of the host-encoded normal cellular prion protein (PrP^C) (Prusiner 1982; Collinge 2001; Legname et al. 2004). Moreover, prion diseases may occur as genetic, infectious, or sporadic disorders (Prusiner 1998; Collinge 2001). Additionally, while no definitive consensus regarding the precise function of PrP^C has been reached to date (Aguzzi and Hardt 2003; Collinge 2001), it has been suggested to promote synaptic homeostasis (Collinge et al. 1994), aid in neurite outgrowth and neuronal survival (Chen et al. 2003), and function as a cell-surface receptor for signal transduction (Mouillet-Richard et al. 2000).

Bovine spongiform encephalopathy (BSE), resulting from ingestion of scrapie and/or BSE infected meat and bone meal, has also been implicated in the development of variant Creutzfeld-Jakob disease (vCJD) in humans via consumption of beef from BSE-affected cattle (Bruce et al. 1997; Scott et al. 1999; Collinge 2001; Asante et al. 2002).

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To date, nonsynonymous single nucleotide polymorphisms (SNPs) within the human and ovine prion protein genes (*PRNP*) have been notably associated with resistance and/or susceptibility to prion diseases (for review see Belt et al. 1995; Collinge 2001; Baylis et al. 2002a; Baylis et al. 2002b), and marker-assisted selection programs aimed at enriching the frequency of resistant ovine *PRNP* alleles in Dutch and British sheep has ensued (Schreuder et al. 1997; Arnold et al. 2002). Notably, a novel association was recently documented between BSE susceptibility and specific bovine *PRNP* insertion/deletion (indel) polymorphisms within the putative promoter as well as intron 1 of a few German cattle breeds, supporting the hypothesis that mutations potentially influencing the level of bovine *PRNP* expression might also influence incubation time and susceptibility to BSE (Bossers et al. 1996; Sander et al. 2004).

In this study we investigated the frequencies of BSE-associated *PRNP* indels for a diverse panel of commercial U. S. artificial insemination (AI) sires consisting of 39 distinct breeds and compared them to those recently described for healthy and BSE-affected German cattle breeds (Sander et al. 2004). Additionally, using published primer sequences (Sander et al. 2004) we developed and utilized a PCR protocol incorporating fluorescently labeled primer combinations to produce a multiplexed assay for high-throughput interrogation of bovine *PRNP* indels in the putative promoter, intron 1, and the 3' untranslated region (UTR) (Hills et al. 2001; Hills et al. 2003; Sander et al. 2004).

Materials and methods

To evaluate the frequencies of bovine *PRNP* indels within the putative promoter, intron 1, and the 3' UTR, we utilized a DNA panel consisting of 132 AI sires from 39 domestic cattle breeds. The source of DNA was spermatozoa purchased through commercial dealers. Names of breeds and sample sizes (n) are as follows: Angus (4); Beefalo (1); Beefmaster (5); Belgian Blue (4); Blonde D'Aquitaine (5); Braford (4); Brahman (4); Brahmousin (2); Brangus (5); Braunvieh (5); Brown Swiss (4); Charolais (5); Chianina-Chiangus (5); Corriente (1); Gelbvieh (4); Hereford (3); Holstein (4); Jersey (1); Limousin (3); Maine Anjou (4); Murray Grey (2); Nelore (8); Normande (1); Piedmontese (2); Pinzgauer (1); Red Angus (4); Red Brangus (2); Red Poll (1); Romagnola (2); Salers (3); Santa Gertrudis (4); Scottish Highland (1); Senepol (2); Shorthorn (5); Simbrah (3); Simmental (8); Tarentaise (1); Texas Longhorn (4); Three-way-cross (4); White Park (1). Note, six of the Nelore were not AI sires. Care was taken to select unrelated sires.

Multiplexed PCR assay and validation techniques. Forward and reverse primer pairs (Sander et al. 2004) targeting known indel polymorphisms in the bovine *PRNP* putative promoter (23 bp insertion; Sander et al. 2004), intron 1 (12 bp insertion; Hills et al. 2001), and the 3' UTR (14 bp insertion; Hills et al. 2003) were utilized together in single multiplexed 5 µl volume reactions. Forward primers, respectively, were synthesized with 5' fluorescent labels as follows: *PRNP*47784F 5' NED (Applied Biosystems, Foster City, CA); *PRNP*49686F 5' 6-FAM (Sigma- Genosys, The Woodlands, TX);

*PRNP*67976F 5' HEX (Sigma Genosys). All multiplexed PCR reactions were carried out on GeneAmp 9700 PCR Systems (Applied Biosystems) and consisted of the following: 50-100 ng DNA, 0.375 units *Taq* polymerase (Promega, Madison, WI), 0.64 μM *PRNP*47784F-NED, 0.64 μM *PRNP*477883R, 0.64 μM *PRNP*49686F-6-FAM, 0.64 μM *PRNP*49777R, 0.52 μM *PRNP*67976F-HEX, 0.52 μM *PRNP*68070R, 3 mM $MgCl_2$ (Promega), 500 μM dNTPs (Promega), 1X MasterAmp™ PCR Enhancer (Epicentre, Madison, WI), and 1.5X Reaction Buffer (Promega). Thermal cycling parameters, as optimized in our laboratory, were as follows: 2 min at 96° C; 4 cycles × 30 s at 96° C, 30 s at 58° C (–1 ° C/cycle), 90 s at 65° C; 31 cycles × 30 s at 96° C, 30 s at 54° C, 90 s at 65° C; 15 min at 65° C. Multiplexed PCR products were separated and analyzed on an ABI 3100 and/or 310 Genetic Analyzer (Applied Biosytems) and sized relative to an internal size standard (MAPMARKER LOW, Bioventures). For comparison of consistency and validation of the multiplex developed, 10 sires were also genotyped via agarose gel electrophoresis following the methods of Sander et al. (2004).

***Statistical analysis*.** The distributions of *PRNP* alleles between all German cattle (pooled healthy + BSE-affected; Sander et al. 2004) and U. S. cattle sires, healthy German cattle and U. S. cattle sires, and BSE-affected German cattle and U. S. cattle sires were tested for significant differences using Fisher's exact test within the program STAT-SAK (G. E. Dallal; freeware). In addition, tests of genic (Raymond and Rousset 1995) and genotypic (Goudet et al. 1996) differentiation between the aforementioned groups were carried out within the program GENEPOP 3.1d (Raymond and Rousset

1995) using the default parameters. In all cases, $P < 0.05$ was considered statistically significant.

Results

The multiplexed PCR assay developed for the rapid genotyping of BSE-associated bovine *PRNP* indel polymorphisms in the putative promoter and intron 1, as well as known indel polmorphisms within the 3' UTR, is illustrated in Figure 4. No significant differences in the distributions of bovine *PRNP* alleles and/or genotypes corresponding to the promoter, intron 1, and 3' UTR were noted between German cattle as a whole (pooled healthy + BSE-affected) and our panel of U. S. cattle sires. However, significant differences in the allelic and genotypic distributions of bovine *PRNP* indels in the promoter and intron 1 were noted when U. S. cattle sires were compared to healthy as well as BSE-affected German cattle (Table 6; Sander et al. 2004). Specifically, significant differences were detected between the distributions of *PRNP* promoter alleles for healthy German cattle and a panel of U.S. cattle sires (Table 6). Moreover, the frequency of the 23 bp promoter allele observed for our panel of U. S. cattle sires strongly resembled that previously reported for BSE-affected German cattle (Table 6; Sander et al. 2004). No significant difference was detected in the distribution of *PRNP* promoter genotypes between healthy German cattle and our panel of U. S. cattle sires ($P = 0.0510$; Table 6). In contrast, the frequency of the 12 bp intron 1 allele observed for our panel of U. S. cattle sires was identical to that previously reported for healthy German cattle (Table 6; Sander et al. 2004). Significant differences were

detected between the distributions of intron 1 alleles and genotypes for BSE-affected German cattle and our panel of U. S. cattle sires (Table 6). No significant differences were detected in the distributions of alleles or genotypes corresponding to the 14 bp indel within the *PRNP* 3' UTR, and the frequency of the 14 bp allele (+) and corresponding + + genotype was comparable to that reported for healthy German cattle (Table 6; Sander et al. 2004). Observed *PRNP* indel allele and genotype frequencies, subdivided by domestic breed for those breeds where three or more sires were sampled, are depicted in Table 7.

Discussion

Unlike previous bovine *PRNP* studies involving U. S. cattle (Ryan and Womack 1993; Neibergs et al. 1994; Heaton et al., 2003), the study presented and described here provides the first comprehensive survey of bovine *PRNP* indel polymorphisms corresponding to the promoter, intron 1, and 3' UTR, facilitated through the utilization of a large and diverse panel of commercial U. S. AI sires from 39 domestic breeds.

While *PRNP* polymorphisms associated with resistance to prion diseases have previously been described for humans, sheep, and goats (Belt et al. 1995; Collinge 2001; Billinis et al. 2002), a similar association has only recently been extended to domestic cattle (Sander et al. 2004). Additionally, it should be emphasized that the strength of the association uncovered in German cattle still remains to be evaluated through experimental challenge. Nevertheless, the results of Sander et al. (2004) clearly

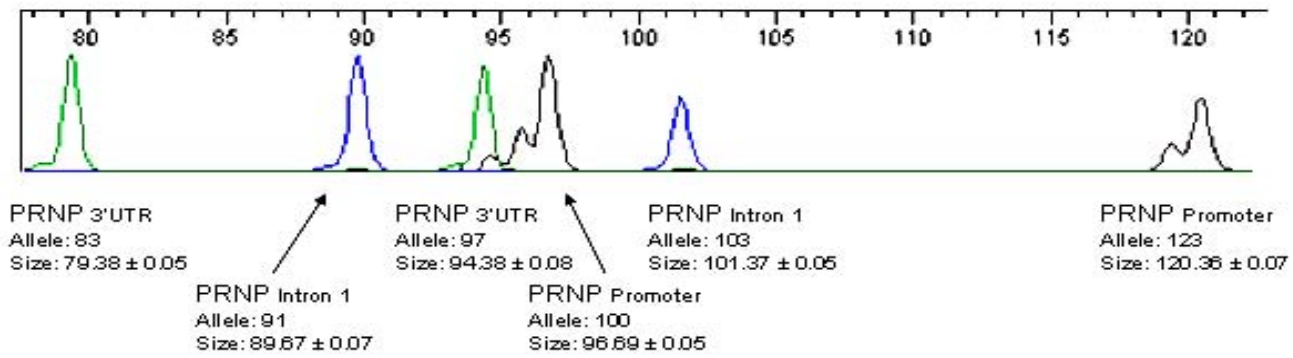

Fig. 4. Graphical depiction of the multiplexed PCR products corresponding to indel polymorphisms in the bovine *PRNP* putative promoter, intron 1, and 3' UTR. Average allele sizes (bp) relative to an internal size standard (Mapmarker LOW, Bioventures) and the standard deviation are given beneath each of the PCR products generated.

Table 6. Observed frequencies for bovine *PRNP* indel polymorphisms in the putative promoter, intron 1, and 3' UTR. Differences among healthy and BSE-affected German cattle (Sander et al. 2004) and a panel of commercial U. S. sires were evaluated. (P < 0.05 depicted in bold).

			Allele		Genotype					
PRNP		(n)	+	−	++	+−	−−	Healthy[a]	Affected[b]	U. S. sires
Promoter 23 bp indel	Healthy[a]	48	0.43	0.57	0.21	0.44	0.35	−	**0.0296**[c]; **0.0316**[d]	**0.0321**[c]; **0.0340**[d]
	Affected[b]	43	0.27	0.73	0.05	0.44	0.51	**0.0330**[e]	−	0.5871[c]; 0.5895[d]
	U. S. Sires	132	0.30	0.70	0.14	0.32	0.54	0.0510[e]	0.6141[e]	−
Intron 1 12 bp indel	Healthy[a]	48	0.49	0.51	0.21	0.56	0.23	−	**0.0344**[c]; **0.0355**[d]	1.0000[c]; 1.0000[d]
	Affected[b]	43	0.33	0.67	0.09	0.47	0.44	**0.0294**[e]	−	**0.0086**[c]; **0.0092**[d]
	U. S. Sires	132	0.49	0.51	0.32	0.35	0.33	1.0000[e]	**0.0198**[e]	−
3' UTR [f] 14 bp indel	Healthy[a]	48	0.95	0.05	0.90	0.10	0.00	−	0.5530[c]; 0.5565 [d]	1.0000[c]; 1.0000[d]
	Affected[b]	43	0.92	0.08	0.86	0.12	0.02	0.5773[e]	−	0.4632[c]; 0.4661[d]
	U. S. Sires	132	0.94	0.06	0.89	0.11	0.00[g]	0.8137[e]	0.6366[e]	−

[a] Healthy German cattle and corresponding data from Sander et al. (2004).
[b] BSE-affected German cattle and corresponding data from Sander et al. (2004).
[c] Probability obtained from Fisher's exact test (Genic) in STAT-SAK (G. E. Dallal).
[d] Probability obtained from Genic differentiation analysis (Raymond and Rousset 1995).
[e] Probability obtained from Genotypic differentiation analysis using the G-based exact test of Goudet et al. (1996).
[f] 14 bp indel consisting of 1 (−) or 2 (+) repeats.
[g] One sire out of 132 (actual observed frequency = 0.0075).

Table 7. Observed *PRNP* indel allele frequencies for cattle breeds where 3 or more sires were sampled.

Domestic Cattle Breed [a]		Promoter 23 bp indel		Intron 1 12 bp indel		3' UTR 14 bp indel	
	(n)	+	−	+	−	+	−
Angus [b]	8	0.188	0.812	0.188	0.812	1.000	0.000
Beefmaster	5	0.000	1.000	0.200	0.800	1.000	0.000
Belgian Blue	4	0.250	0.750	0.250	0.750	1.000	0.000
Blonde d'[c]	5	0.500	0.500	0.600	0.400	0.900	0.100
Braford	4	0.125	0.875	0.250	0.750	0.500	0.500
Brahman	4	0.250	0.750	1.000	0.000	0.875	0.125
Brangus [d]	7	0.071	0.929	0.571	0.429	1.000	0.000
Braunvieh	5	0.400	0.600	0.700	0.300	0.900	0.100
Brown Swiss	4	1.000	0.000	1.000	0.000	1.000	0.000
Charolais	5	0.600	0.400	0.700	0.300	1.000	0.000
Chianina [e]	5	0.500	0.500	0.500	0.500	0.900	0.100
Gelbvieh	4	0.250	0.750	0.375	0.625	1.000	0.000
Hereford	3	0.500	0.500	0.500	0.500	1.000	0.000
Holstein	4	0.375	0.625	0.375	0.625	1.000	0.000
Limousin	3	0.500	0.500	0.833	0.167	1.000	0.000
Maine Anjou	4	0.250	0.750	0.375	0.625	1.000	0.000
Nelore	8	0.000	1.000	1.000	0.000	1.000	0.000
Salers	3	0.333	0.667	0.333	0.667	0.833	0.167

Table 7. Continued.

Domestic Cattle Breed [a]		Promoter 23 bp indel		Intron 1 12 bp indel		3' UTR 14 bp indel	
	(*n*)	+	−	+	−	+	−
Santa Gertrudis	4	0.000	1.000	0.375	0.625	1.000	0.000
Shorthorn	5	0.100	0.900	0.100	0.900	1.000	0.000
Simbrah	3	0.333	0.667	0.833	0.167	0.833	0.167
Simmental	8	0.000	1.000	0.000	1.000	0.875	0.125
TX Longhorn	4	0.625	0.375	0.750	0.250	1.000	0.000

[a] Allele frequencies depicted herein are not intended to represent entire breeds
[b] Black and Red Angus combined
[c] Blonde d' Aquitaine
[d] Black and Red Brangus combined
[e] Chianina/Chiangus (Chianina X Angus)

demonstrate that domestic cattle possess *PRNP* alleles and genotypes significantly associated with susceptibility to BSE. Therefore, an assay capable of the rapid identification of cattle possessing *PRNP* genotypes significantly associated with the phenotypic expression of BSE is potentially invaluable to both the domestic cattle industry as well as human health. Herein we have provided such an assay based on data generated for healthy and BSE-affected German cattle (Figure 4; Sander et al. 2004). Additionally, we have also provided an initial survey of the allelic and genotypic frequencies corresponding to *PRNP* indels previously associated with BSE for specific domestic cattle breeds (Table 7). However, while care was taken to select unrelated sires from each respective breed, caution is necessary when interpreting the relationship between the observed frequencies depicted in Table 7 and potential susceptibility and/or

resistance to BSE. Future studies utilizing larger sample sizes are necessary to comprehensively evaluate breed-specific frequencies of *PRNP* indel polymorphisms within the promoter, intron 1, and 3' UTR for commercial U. S. cattle breeds.

The origin of the significant differences detected between *PRNP* allele and/or genotype distributions for our panel of U. S. cattle sires and German cattle is currently unclear (Table 6). Given that the newly described 23 bp bovine *PRNP* promoter indel exhibited the most significant association with BSE in German cattle, Sander et al. (2004) subsequently attributed the statistical significance noted between the 12 bp intron 1 indel and BSE to tight linkage between the promoter and intron 1. However, the potential effects and/or role(s) of each polymorphism with respect to bovine *PRNP* expression and BSE susceptibility still remain to be completely elucidated. Furthermore, it should be noted that the 12 bp intron 1 indel was previously determined to lie within a region important for retention of full *PRNP* promoter activity in cultured bovine CKT-1 cells, and the 12 bp deletion was determined to remove a putative binding site for the transcription factor SP1 (Inoue et al. 1997; Hills et al. 2001). Interestingly, our panel of U. S. cattle sires possesses a significantly higher frequency of the 12 bp (+) intron 1 allele and corresponding + + genotype than BSE-affected German cattle (Table 6), but differs significantly from healthy German cattle with respect to the frequency of the 23 bp (+) promoter allele which previously exhibited the most significant association with BSE status. If our analysis of these data is interpreted with strict adherence to the results of Sander et al. (2004), it appears that our panel of U. S. cattle sires, collectively, are largely susceptible to BSE based on polymorphism data generated for the bovine *PRNP*

promoter. However, additional studies focusing on the regulatory effects of *PRNP* indels in the promoter as well as intron 1 are necessary to fully evaluate the implications of the significantly different distributions of BSE-associated *PRNP* alleles and/or genotypes between commercial U. S. cattle sires and German cattle.

BISON *PRNP* GENOTYPING AND POTENTIAL ASSOCIATION WITH *BRUCELLA* SPP. SEROPREVALENCE

Introduction

Brucella abortus is a gram-negative facultative intracellular pathogen associated with brucellosis infection in mammals, including undulant fever in humans and abortion and/or infertility in many domestic and wild animals. Although classical breeding studies in domestic cattle (*Bos taurus*) have indicated that natural resistance to *B. abortus* is controlled by two or more genes (Templeton et al. 1988), few genes have been investigated and only one, *NRAMP1*, has been implicated in natural resistance to brucellosis in domestic cattle (Feng et al. 1996). *NRAMP1* is an integral protein hypothesized to be involved in phagosome acidification and/or phagosome-lysosome fusion processes within macrophages and has been associated with infectious and autoimmune diseases (Blackwell et al. 2001). To date, an association between *NRAMP1* and natural resistance to brucellosis has not been demonstrated in American bison (*Bison bison*), a member of the closely related sister genus to *Bos*.

Recently, *B. abortus* heat shock protein 60 was demonstrated to interact with host-encoded cellular prion protein (PrP^C), aiding in the establishment of infection into mouse macrophages and implicating PrP^C as a potential receptor for *B. abortus* (Watarai et al. 2003; Watarai 2004). While no consensus regarding the physiological function of PrP^C has been reached to date (Collinge 2001; Aguzzi and Hardt 2003), it may function as a cell-surface receptor for signal transduction (Mouillet-Richard et al. 2000).

Moreover, the rapid cycling of PrP^C between the cell surface and the early endosome via clathrin-coated vesicles is a physiological process shared by many other cell-surface receptors (Shyng et al. 1994; van Rheede et al. 2003). Notably, amino acid polymorphisms within the *PRNP* gene are known to influence the phenotypic expression of scrapie in sheep and goats (Belt et al. 1995; Billinis et al. 2002), Creutzfeld Jakob disease, Gerstman Sträussler Scheinker disease, and Kuru in humans (Collinge 2001), and chronic wasting disease in deer and elk (O'Rourke et al. 1999; Johnson et al. 2003; O'Rourke et al. 2004). In addition, an association was recently demonstrated between susceptibility to bovine spongiform encephalopathy (BSE) and specific insertion/ deletion (indel) polymorphisms within the putative *PRNP* promoter and intron 1 for several German cattle breeds, supporting the hypothesis that mutations potentially influencing the level of bovine *PRNP* expression may also influence incubation time and susceptibility to BSE (Bossers et al. 1996; Sander et al. 2004).

While brucellosis has been effectively eliminated from most bison populations in North America, two substantial reservoirs of brucellosis-infected bison remain in Yellowstone National Park (NP) (ID, MT, WY, U. S. A.) and Wood Buffalo NP (AB, Canada; Meyer and Meagher 1995; Joly and Messier 2004), also representing the only continuously wild bison populations in existence (Coder 1975). Following the initial identification of brucellosis in Yellowstone NP bison (Mohler 1917), multiple studies have documented *Brucella* spp. antibodies (Rush 1932; Tunnicliff and Marsh 1935) as well as *B. abortus* biovars 1 and 2 (Roffe et al. 1999; Rhyan et al. 2001) in Yellowstone NP bison. Moreover, exposure to *Brucella* spp. has remained relatively high (> 60%)

among Yellowstone NP bison for the past 70 years (Tunnicliff and Marsh 1935; Rhyan et al. 2001).

Herein, we evaluate the frequencies of bison *PRNP* exon 3 allelic variants and genotypes for 6 U.S. bison populations (4 federal, 1 private, and 1 state) and investigate whether any particular exon 3 allele and/or genotype is significantly associated with *Brucella* spp. antibodies in Yellowstone NP bison. The distribution of *PRNP* exon 3 alleles and genotypes for 3 populations that were either founded from *Brucella* spp. seronegative stock or subjected to test-and-slaughter management to eradicate brucellosis are compared to those observed for Yellowstone NP bison. Additionally, we investigate the frequencies of known bovine *PRNP* indel polymorphisms within the putative bison *PRNP* promoter, intron 1, and 3' untranslated region (UTR) (Hills et al. 2001; Hills et al. 2003; Sander et al. 2004).

Materials and methods

Bison sampling and serology. Collectively, 198 bison from 4 U. S. national parks, 1 private, and 1 state herd were utilized to investigate the frequencies of *PRNP* allelic variants and genotypes (Table 8). Yellowstone NP samples were obtained non-selectively with regard to pregnancy and/or *Brucella* spp. serology from bison subjected to management actions as they moved to the park boundaries near West Yellowstone NP, Montana (MT). From these samples, the following selection criteria were used: approximately equal sex ratio, proportionate sampling from ages 1-10^{+} as determined by

Table 8. Distribution of bison samples by local and sex

Bison Herd	Abbreviation	Location	Total	Male	Female
Castle Rock[a]	CR	NM	20	5	15
Grand Teton National Park[b]	GT	WY	15	4	11
Henry Mountains[c]	HM	UT	20	7	13
Theodore Roosevelt National Park[d]	TR	ND	10	3	7
Wind Cave National Park[e]	WC	SD	16	3	13
Yellowstone National Park[f]	YNP	ID, MT, WY	117	58	59
Total			198	80	118

[a] Private herd primarily established from seronegative YNP stock (D. Hunter, pers. comm.).
[b] Established from YNP bison in 1948 and supplemented with TR bison in 1964 (NPS 1996); population chronically infected with brucellosis (Meyer and Meagher 1995).
[c] Founded exclusively from YNP bison in 1940s; brucellosis eradicated through test-and-slaughter management in 1960s (Dowling 1990).
[d] South unit; no current or historical evidence of brucellosis (M. Oehler, pers. comm.).
[e] Founded in part from YNP (Coder 1975); brucellosis eradicated through test-and-slaughter management during the 1950s-1970s (B. Muenchau, pers. comm.).
[f] Yellowstone National Park, Montana; population chronically infected with brucellosis (Meyer and Meagher 1995).

established aging methods (Fuller 1959; Moffitt 1998), sampling from various collection dates and West Yellowstone NP locals, and approximately equal distributions of *Brucella* spp. serological phenotypes (seropositive/seronegative). Samples from the remaining 5 herds were chosen randomly from previously established repositories.

Brucella spp. antibodies were detected in serum samples from Yellowstone NP bison postmortem from 1997 (n = 27) and 1999 (n = 39) using the complement fixation, Rivanol precipitation, buffered acidified plate antigen**,** standard plate agglutination**,** standard tube agglutination, and card tests (Nielsen 2002). Because the effectiveness of individual serological tests vary in bison (Williams et al. 1997), only samples with consensus serological results were utilized. Fluorescence polarization assay (Gall et al. 2000) was used to evaluate the presence of *Brucella* spp. antibodies in samples collected in 2002 (n = 44) and 2003 (n = 7).

PRNP exon 3 amplification and sequencing. Genomic DNA was isolated by spotting whole blood on Whatman Bioscience FTA® Classic Cards following the recommended protocol (Whatman Inc., Clifton, NJ), by using the SUPER QUICK-GENE DNA Isolation kit (AGTC Inc., Denver, CO) on white blood cell isolates, or via pulverization/proteinase K treatment of tissues (Halbert et al. 2004). Flanking primers SAF1 and SAF2 (Prusiner et al. 1993) were used to PCR amplify and sequence bison *PRNP* exon 3 (Seabury and Derr 2003). Bidirectional sequencing of 96 bison samples from 4 U. S. federal and 1 private bison herd was previously employed to evaluate the number of variable sites within bison *PRNP* exon 3 (Seabury et al. 2004a), with 71 of

those samples included in the present study. Herein, 127 additional samples were sequenced using SAF1 only. *PRNP* exon 3 genotypes and allelic variants were determined using SeqScape version 1.01 (Applied Biosystems, Foster City, CA), and representative exon 3 alleles were validated via cloning and subsequent sequencing following Seabury and Derr 2003.

PRNP promoter, intron 1, and 3' UTR genotyping. Fluorescent dye-labeled primer combinations (Seabury et al. 2004b) targeting known indel polymorphisms in the bovine *PRNP* promoter (+ 23 bp; Sander et al. 2004), intron 1 (+ 12 bp; Hills et al. 2001), and 3'UTR (+ 14 bp; Hills et al. 2003) were utilized to generate genotypic data for bison from Yellowstone NP (n = 64; 24 seropositive, 40 seronegative), Wind Cave National Park NP (n = 20), Castle Rock (n = 20), and Henry Mountains (n = 20). PCR reactions and thermal cycling parameters followed Seabury et al. (2004b) with the following modifications: 5 µl reaction with primers *PRNP* 47784F, 47803R, 67976F, and 68070R with a 52° C annealing temperature, and a second 5 µl reaction using primers *PRNP* 49686F and 49777R with the cycling profile of Sander et al. (2004). Resulting fragments were co-loaded into a single injection for separation on an ABI 3100 (Applied Biosystems, Foster City, CA) using an internal size standard (Mapmarker LOW, Bioventures, Inc, Murfreesboro, TN).

Statistical analysis. Bison *PRNP* allele frequencies, genic and genotypic differentiation using both the probability test (Fisher's exact test) and the G-based exact test of Goudet et al. (1996), as well as Hardy-Weinberg equilibrium, were calculated within GENEPOP 3.1d (Raymond and Rousset 1995). *PRNP* exon 3 data for Yellowstone NP bison were subdivided into 2 groups based on serology (Table 9) and tested for genic and genotypic differentiation. Pairwise population differentiation was examined among all bison herds sampled, including Yellowstone NP both with and without subdivision. Because disease cases often differ from controls within individual genotypic classes, we also used a variation of the Z_{max} test to evaluate associations between specific genotypic classes and serological status in Yellowstone NP bison (Lange 2002). $P < 0.05$ was considered statistically significant.

Results

All bison *PRNP* exon 3 alleles examined possessed 6 octapeptide repeats and were identical in sequence with the exception of 1 nonsynonymous single nucleotide polymorphism (SNP) at nucleotide position 50 (T50C). The T50C SNP is predicted to result in the amino acid replacement M17T. None of the tested populations deviated from Hardy-Weinberg expectations for this SNP. Observed *PRNP* exon 3 allelic and genotypic frequencies for each bison population examined as well as pairwise tests of genic and genotypic differentiation are depicted in Table 10. Significant differences in the distributions of *PRNP* exon 3 alleles were detected between Yellowstone NP bison and 3 other bison populations, each either founded from *Brucella* spp. seronegative stock

Table 9. Distribution of Yellowstone bison samples by sex, serological status (*Brucella* spp. seronegative/seropositive), and age.

	Age class [a]										
	Total	1	2	3	4	5	6	7	8	9	10^{+}
Yellowstone											
Female seronegative	23	1	1	8	3	3	1	4	1		1
Female seropositive	36	1	1	7	5	7		3	2	1	9
Male seronegative	21	4	1	7	3	1		1		2	2
Male seropositive	37	1	1	16	9	1	1	5		1	2
Sum	117	7	4	38	20	12	2	13	3	4	14

[a] Bison scored in field as 'adult' considered here as 3 year-olds.

Table 10. Observed bison *PRNP* exon 3 allelic and genotypic frequencies. Yellowstone NP bison (YNP) are subdivided by *Brucella* spp. serology (seropositive, YNP+ and seronegative, YNP-). Probabilities for pairwise tests of genic and genotypic differentiation are depicted above and below the diagonal, respectively, in the last 7 columns (P < 0.05 illustrated in bold).

	Allele [a]			Genotype [a]			Genic and Genotypic Differentiation							
Herd	**(n)**	**T**	**C**	**TT**	**TC**	**CC**	**YNP+**	**YNP-**	**YNP**	**GT**	**TR**	**CR**	**WC**	**HM**
YNP+	73	0.514	0.486	0.301	0.425	0.274	–	0.05359	NA[b]	0.42297	0.47863	**0.00026**	**0.00583**	**0.01257**
YNP-	44	0.648	0.352	0.409	0.477	0.114	0.06994	–	NA[b]	0.66664	0.79848	0.05440	0.18667	0.30602
YNP	117	0.564	0.436	0.342	0.444	0.214	NA[b]	NA[b]	–	0.84522	0.81513	**0.00208**	**0.02145**	**0.03399**
GT	15	0.600	0.400	0.267	0.667	0.067	0.43923	0.65242	0.85227	–	1.00000	0.05578	0.16801	0.20469
TR	10	0.600	0.400	0.300	0.600	0.100	0.51240	0.78990	0.82476	1.00000	–	0.11272	0.21174	0.25001
CR	20	0.825	0.175	0.700	0.250	0.050	**0.00183**	0.06113	**0.00233**	**0.04905**	0.12022	–	0.76515	0.57814
WC	16	0.781	0.219	0.625	0.313	0.063	**0.01063**	0.18886	**0.03151**	0.14260	0.21646	0.78500	–	0.78844
HM	20	0.750	0.250	0.600	0.300	0.100	**0.01307**	0.31785	**0.04908**	0.19035	0.38893	0.61824	0.80418	–

[a] Frequencies rounded to 3 decimal places.
[b] Not an appropriate pairwise test.

or subjected to test-and-slaughter management (Table 8). Specifically, the Castle Rock, Wind Cave NP, and Henry Mountains bison herds had a significantly higher frequency of the T-allele and TT-genotype than bison obtained from Yellowstone NP, (Table 10). No significant genic or genotypic differentiation was detected in pairwise comparisons between Yellowstone NP, Grand Teton NP, and Theodore Roosevelt NP bison (Table 10).

A significant overabundance (20/25; 80%; Z_{max} $P = 0.021$) of *Brucella* spp. seropositive bison were detected within the CC genotypic class in Yellowstone NP. However, no significant overall genic or genotypic (P = 0.05359; 0.06994) differentiation was detected between seropositive and seronegative Yellowstone NP bison (Table 10). No significant sex- and/or age-specific associations were detected for seropositive and/or seronegative Yellowstone NP bison with respect to *PRNP* (data not shown), although small sample sizes may have precluded detection (Table 9). *PRNP* exon 3 allelic and genotypic frequencies were significantly different between seropositive Yellowstone NP bison and our samples for the Castle Rock, Wind Cave NP, and Henry Mountains bison populations (Table 10).

No indel polymorphisms were detected within the targeted regions of the putative bison *PRNP* promoter, intron 1, or 3' UTR. Based on our fluorescent genotyping assay, all bison were fixed for the following alleles (compared directly with sizes in domestic cattle; Seabury et al. 2004b): *PRNP* promoter, 100-bp allele; intron 1, 103-bp allele; 3'UTR, 97-bp allele.

Discussion

The pattern of nucleotide variation observed for bison *PRNP* exon 3, consisting of 1 nonsynonymous SNP of moderate frequency within the signal sequence, is in marked contrast to domestic cattle (Heaton et al. 2003; Seabury et al. 2004a). Furthermore, although no bison *PRNP* indel variation was detected, exon 3 alleles with 4 to 7 octapeptide repeats (Goldmann et al. 1991; Schläpfer et al. 1998; Seabury et al. 2004a) and indel variation within the promoter, intron 1, and 3'UTR (Hills et al. 2001; Hills et al. 2003; Sander et al. 2004; Seabury et al. 2004b) have been documented for domestic cattle. Other regions of the bison *PRNP* gene remain to be examined to better establish the overall pattern of nucleotide variation. The bison *PRNP* exon 3 T50C nonsynonymous SNP and predicted amino acid substitution M17T have not been detected in domestic cattle (Heaton et al. 2003; Seabury et al. 2004a) or any other bovid species (Wopfner et al. 1999; Takasuga et al. 2003; Seabury et al. 2004a). However, the amino acid threonine is widely distributed at the corresponding signal peptide residue for most other mammalian species surveyed (van Rheede et al. 2003).

Previous studies have illustrated the sensitivity of PrP^{C} topology to mutations in the signal sequence and/or transmembrane domain (Hegde et al. 1998, 1999; Kim et al. 2001; Kim and Hegde 2002), with signal sequence mutations primarily altering the ratio of three topological forms (Hegde et al. 1998, 1999) in which PrP^{C} is synthesized at the endoplasmic reticulum (Kim et al. 2001). Furthermore, an association has been suggested between one topological form (^{Ctm}PrP), an uncleaved signal peptide, and neurodegenerative disease (Hegde et al. 1998, 1999; Stewart et al. 2001; Kim and Hegde

2002). In addition, Lundberg and colleagues (2002) demonstrated that the N-terminal region of mouse PrP^C (residues 1-28), when uncleaved, is a cell-penetrating peptide capable of transporting large hydrophilic cargoes through a cell membrane (Lundberg et al. 2002). Therefore, the predicted amino acid polymorphism M17T may have functional implications related to PrP^C biogenesis and/or entry of *Brucella* spp. into bison host cells.

The collective body of evidence presented herein is consistent with a tentative association between the T/C bison *PRNP* exon 3 alleles and seronegative/seropositive *Brucella* spp. serological status, respectively (Table 10). The genotypic differentiation noted between seropositive Yellowstone NP bison and the Castle Rock, Wind Cave NP, and Henry Mountains bison populations results from high TT-genotype frequencies observed in these populations (Table 10), consistent with the detection of a significant overabundance of seropositive Yellowstone NP bison within the CC-genotypic class. Since the Grand Teton NP, Wind Cave NP, Castle Rock, and Henry Mountains populations were all founded either in part or entirely from Yellowstone NP bison (Table 8), it is unlikely that unique genetic backgrounds lead to the observed differences in *PRNP* exon 3 allele and genotype frequencies among populations. However, marked differences do exist in the incidence of brucellosis and previous disease management strategies among these populations (Table 8). It is possible that test-and-slaughter management in both the Wind Cave NP and Henry Mountains bison populations, and the seronegative founder event establishing the Castle Rock population, effectively altered

the genetic composition of these herds with respect to genes involved in natural resistance to brucellosis.

To date, a cellular receptor for *Brucella* spp. organisms has not been conclusively identified (Gorvel and Moreno 2002). The association noted herein between bison *PRNP* exon 3 and *Brucella* spp. serological status indirectly supports the findings of Watarai et al. (2003), further implicating PrP^C as a potential cellular receptor and/or portal protein for *B. abortus*. However, much remains to be understood about the potential relationship between PrP^C and *B. abortus* infection. The opportunistic sampling of Yellowstone NP bison introduced several uncontrollable factors such as variation in the timing and rate of exposure and use of different serological tests among collection years, perhaps somewhat obscuring the true level of association between *PRNP* exon 3 variation and immune response to *Brucella* spp. organisms. Moreover, while both the susceptibility and immune response to brucellosis infection in bison and domestic cattle have traditionally been assumed equivalent, there are clear differences in the etiology of *B. abortus* infection (Rhyan et al. 2001) and efficacy of vaccination with strains 19 (Davis et al. 1991) and RB51 (Olsen et al. 2003). While other loci are likely involved in the establishment of brucellosis infection in bison and other bovids, future experimental challenge, RNA interference, and association studies aimed at evaluating variation within the *PRNP* gene for domestic and wild bovids are necessary to further evaluate the magnitude of the relationship between PrP^C and *B. abortus* infection.

CONCLUSIONS AND FUTURE INVESTIGATIONS

In our study of hair sheep, including samples for both the St. Croix White and the Royal White breed (CMP), we have determined that *PRNP* genotypes associated with a high level of resistance to natural scrapie, experimental scrapie, and experimental BSE do in fact exist at moderate to high frequencies among the hair sheep sampled in this study. Additionally, our study of *PRNP* in hair sheep has lead to the discovery and description of a novel ovine *PRNP* allele ($P_{116}A_{136}R_{154}Q_{171}$) as well as two novel genotypes (PARQ/ARR; PARQ/ARQ). These data provide the immediate opportunity to evaluate two new ovine *PRNP* genotypes with respect to TSEs, both in sheep as well as in transgenic mice expressing the relevant ovine *PRNP* allele(s).

From our comprehensive investigation of bovine *PRNP* we have uncovered evidence for intense purifying selection exerted over the long-term evolutionary history of the subfamily Bovinae, as well as significant purifying selection within regions previously demonstrated and/or suggested to be of structural, functional, or pathogenic importance in humans and other mammalian species. In addition, several novel amino acid substitutions were predicted which may ultimately lead to a more thorough understanding of the relationship between bovine PrP^C and BSE given appropriate experimental challenge as well as transgenic studies. However, the intense level of purifying selection detected within bovine *PRNP* is indisputably the most provocative result of the aforementioned study, especially given that loss of the *PRNP* gene in mice does not seem to bring about overt developmental defects (Estibeiro 1996; Collinge

2001). While this result may be interpreted in several different ways, it seems logical that bovine PrP^C, encoded by bovine *PRNP*, must perform some biological function in order to warrant the intense level of purifying selection detected. This idea is supported by human and transgenic mouse data which demonstrate that perturbations of the *PRNP* coding sequence often leads to the development of neurodegenerative disease and inevitable death (for review see Ghetti et al. 1995; Hegde et al. 1998; Collinge 2001; Aguzzi and Hardt 2003). Therefore, it is important to continue the quest to uncover the molecular and/or physiological role(s) of PrP^C.

The third study presented herein demonstrated no significant differences in the distribution of putative *PRNP* promoter alleles and/or genotypes between our panel of commercial U. S. AI sires, collectively, and 43 BSE-affected German cattle (Sander et al. 2004). This result was particularly interesting given that the 23 bp indel polymorphism within the putative bovine *PRNP* promoter displayed the most significant association with BSE susceptibility in German cattle (Sander et al. 2004). However, the results of Sander et al. (2004) have not yet been fully clarified with respect bovine *PRNP* expression, nor have they been independently verified. Therefore, quantitative PCR experiments are needed to ascertain whether or not the 23 bp indel within the putative bovine *PRNP* promoter augments the expression of bovine *PRNP*. The necessity of quantitative PCR experiments can further be evidenced by the results of previous studies which indicate that the incubation period of TSEs in transgenic mice expressing either ovine and/or bovine *PRNP* is inversely proportional to the level of PrP^C produced in the host brain (Prusiner et al. 1990; Vilotte et al. 2001; Castilla et al. 2004).

In our final study, we demonstrated a statistically significant association between a specific bison *PRNP* genotype (CC) and *Brucella* spp. seropositivity for Yellowstone NP bison. This tentative association was further reinforced by significantly higher frequencies of the T-allele and TT-genotype among samples obtained from three bison populations that were either founded from *Brucella* spp. seronegative stock or subjected to test-and-slaughter management to eradicate brucellosis. In addition, *PRNP* genotyping using the *PRNP*MPLX fluorescent genotyping assay revealed that bison were fixed for the following genotypes: putative promoter (100 bp/100 bp); intron 1 (103 bp/ 103 bp); 3' UTR (97 bp/ 97 bp). This result is very interesting for the following reasons: The 100 bp promoter allele and corresponding genotype (100/100) were both significantly associated with susceptibility to BSE in German cattle (Sander et al. 2004); An inverse relationship is known to exist between BSE incubation period and the level of bovine *PRNP* expression (Castilla et al. 2004); Both PrP^{C} as well as *PRNP* nucleotide variation have recently been either directly and/or indirectly associated with brucellosis in mice (Watarai et al. 2003; Watarai 2004) and bison. Therefore, the next logical step would be to test the hypothesis that the 100 bp putative *PRNP* promoter allele and corresponding genotype (100/100) are associated with an increased level of *PRNP* expression in both domestic cattle as well as bison using quantitative PCR technologies. In addition, the *PRNP*MPLX fluorescent genotyping assay should be utilized to ascertain whether any *PRNP* allele and/or genotype (putative promoter, intron 1, 3' UTR) is significantly associated with brucellosis infection in domestic cattle.

In conclusion, the studies presented herein provide comprehensive insight into bovine *PRNP*, with descriptions of novel variants, significant selective forces, indel distributions, and novel associations uncovered during the course of three discrete studies. Likewise, a novel variant is also described for ovine *PRNP* exon 3. Information derived from these studies will aid in the fundamental understanding of bovine *PRNP* evolution and bovine prion biology.

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