

Molecular Characterization of the Rocky Mountain Elk (*Cervus elaphus nelsoni*) *PRNP* Putative Promoter

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Abstract

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE) affecting deer (*Odocoileus* spp.), moose (*Alces alces*), and Rocky Mountain elk (*Cervus elaphus nelsoni*). Leucine homozygosity at elk *PRNP* codon 132 has been associated with reduced CWD susceptibility. However, naturally acquired CWD has been detected in elk possessing the 132 Leu/Leu genotype. Recent human and bovine studies indicate that *PRNP* regulatory polymorphisms may also influence TSE occurrence. Therefore, we generated sequences for the elk *PRNP* putative promoter (2.2 kb), exon 1 (predicted; 54 bp), intron 1 (predicted; 193 bp), and exon 3 (771 bp). Promoter prediction analysis using CpGProD yielded a single elk *PRNP* promoter that was homologous to regions of known promoter activity in cow and sheep. Molecular interrogation of the elk *PRNP* putative promoter revealed 32 diallelic single-nucleotide polymorphisms (SNPs). No variation was detected within the predicted exon 1 or intron 1 sequences. Evaluation of elk *PRNP* exon 3 revealed 3 SNPs (63Y, 312R, 394W → Met/Leu). Bayesian haplotype reconstruction resulted in 3 elk *PRNP* haplotypes, with complete linkage disequilibrium observed between all *PRNP* putative promoter SNPs and codon 132. The results of this study provide the initial genomic foundation for future comparative and haplotype-based elk *PRNP* studies.

Transmissible spongiform encephalopathies (TSEs), or prion diseases, are a group of inevitably fatal neurodegenerative diseases of humans and animals often generally characterized by progressive dementia and/or ataxia (Prusiner 1998; Collinge 2001). The pathogenic agents of prion diseases are infectious, protease-resistant proteins (PrP^{Sc}) generated via aberrant refolding of the normal host-encoded cellular prion protein (PrP^C) in a susceptible host (Prusiner 1982; Prusiner 2004). Notably, prion diseases may occur as genetic, infectious, or sporadic disorders (for review, see Prusiner 2004). Presently, no overwhelming consensus exists regarding the precise function of PrP^C. However, previous studies suggest that PrP^C may play a role in immunoregulation, signal transduction, copper binding, synaptic homeostasis, apoptosis, and antiapoptosis (for review, see Aguzzi and Polymenidou 2004; Aguzzi and Heikenwalder 2006).

Chronic wasting disease (CWD) is a TSE occurring in both captive and free-ranging mule deer (*Odocoileus hemionus*),

white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*) primarily within the western United States of America (Williams 2005). Recent hunter-harvest surveillance efforts in the state of Colorado have also detected CWD in wild moose (*Alces alces*; <http://wildlife.state.co.us/news/press.asp?pressid=3645>). Clinical signs of CWD in elk include polydipsia, polyuria, increased salivation, weight loss, teeth grinding, wide body stance, and behavioral changes (Williams and Young 1982; Williams 2005). Although the precise origin and natural history of CWD is largely unknown, CWD differs from scrapie and bovine spongiform encephalopathy (BSE) in that it readily occurs among nondomestic free-ranging species detached from modern agricultural practices (Williams 2005). However, it should also be noted that many similarities exist between CWD and scrapie, including the apparent efficiency of horizontal transmission (for review, see Williams 2005; Mathiason et al. 2006).

To date, several nonsynonymous single-nucleotide polymorphisms (SNPs) within the prion protein gene (*PRNP*) have been associated with enhanced putative resistance, prolonged incubation periods, and/or enhanced susceptibility to TSEs in humans, sheep, goats, deer, and elk (for review, see Belt et al. 1995; O'Rourke et al. 1999; Collinge 2001; Billinis et al. 2002; O'Rourke et al. 2004; Jewell et al. 2005; Williams 2005; Hamir et al. 2006; Johnson et al. 2006; Vaccari et al. 2006). However, unlike several other TSE-susceptible mammals, only one nonsynonymous SNP and corresponding amino acid substitution (Met→Leu; elk *PRNP* codon 132) has been detected in Rocky Mountain elk (*C. elaphus nelsoni*; hereafter elk; O'Rourke et al. 1999). Both case-control (O'Rourke et al. 1999) and experimental oral infection studies (Hamir et al. 2006) provide evidence of enhanced CWD susceptibility among elk possessing the Met/Met *PRNP* codon 132 genotype and enhanced putative resistance (or prolonged incubation periods) among elk possessing the Leu/Leu codon 132 genotype. In addition to associations between nonsynonymous *PRNP* polymorphisms and TSE occurrence, an inverse relationship between TSE incubation period and the level of host *PRNP* gene expression has also been established (Vilotte et al. 2001; Castilla et al. 2004; Safar et al. 2005; Sander et al. 2005; Scott et al. 2005). Therefore, enhanced susceptibility and/or resistance to TSEs in mammals is likely influenced by at least 4 genetic factors: 1) genetic variation within the *PRNP* coding region and the associated genotypes, 2) genetic variation within the *PRNP* regulatory regions and the associated genotypes, 3) the combined qualitative and quantitative effects of genetic variation within the *PRNP* coding and regulatory regions, and 4) genetic variation within genes other than *PRNP*. Although many of these genetic factors have been explored with respect to TSEs in sheep, cattle, mice, and humans, no such basic information currently exists regarding the sequence composition and/or the frequency of genetic polymorphism within the putative regulatory regions of the elk *PRNP* gene. A recent study demonstrated that bovine *PRNP* expression in vitro is modulated by genetic variation within the bovine *PRNP* promoter (Sander et al. 2005). To date, insertion-deletion (indel) polymorphisms within the bovine *PRNP* promoter and intron 1 have been associated with BSE incidence in several domestic cattle populations (Sander et al. 2004, 2005; Juling et al. 2006). In addition, previous studies indicate that human *PRNP* polymorphisms in both the intronic and upstream regulatory regions may be associated with sporadic Creutzfeldt-Jakob disease (CJD; McCormack et al. 2002; Bratosiewicz-Wasik et al. 2007). Therefore, mutations potentially influencing the level of elk *PRNP* expression may also influence CWD incubation period and thus overall resistance and/or susceptibility.

In this study, we utilized a comparative genomics approach employing both the cow (*Bos taurus*) *PRNP* sequence (Genbank AJ298878; Hills et al. 2001) as well as bovine oligonucleotide polymerase chain reaction (PCR) primers (Sander et al. 2004) to facilitate the development and characterization of the elk *PRNP* putative promoter

sequence. Herein, we also provide the first detailed polymorphism study, corresponding haplotype analysis, and subsequent comparative analysis with respect to the elk *PRNP* putative promoter. The results of this study provide a robust framework for future comparative and evolutionary analyses while also aiding in the characterization of genomic regions potentially influencing the regulation of elk *PRNP*.

Materials and Methods

Study Animals and DNA Isolation

To characterize and evaluate the frequency of genetic polymorphism within the elk *PRNP* gene, we developed and utilized a DNA panel consisting of $n = 29$ elk. Eighteen of the study elk (10 cows and 8 bulls) were obtained from the Cervid Research and Recovery Institute (CRRRI, Durango, CO). Information regarding the *PRNP* codon 132 genotypes for all CRRRI elk was available (see O'Rourke et al. [1999], for a description of the 3 elk codon 132 genotypes). Therefore, efforts were made to select unrelated elk representative of the 3 *PRNP* codon 132 genotypic classes. DNA was isolated and purified from whole blood using the MasterPure Genomic DNA Purification Kit (Epicentre, Madison, WI) according to the manufacturer's recommended protocol. Additionally, 11 DNA samples from an unrelated wild elk population (11 cows; Yellowstone National Park, Northwest Wyoming and South-Central Montana) were randomly selected from a local repository and included in our DNA panel. None of the elk utilized in this study had any known history or clinical symptoms of CWD.

Elk *PRNP* Amplification

Oligonucleotide primers flanking the bovine *PRNP* putative promoter (*PRNP* 47784F and *PRNP* 49673R; Sander et al. 2004) were utilized in 50- μ l PCRs to generate a single 2447-bp amplicon for the homologous region of the elk *PRNP* gene. All 50- μ l PCRs were carried out using GeneAmp 9700 PCR Systems (Applied Biosystems, Foster City, CA) and consisted of the following: 125 ng genomic DNA, 0.2 mM each deoxynucleoside triphosphate, 0.8 μ M each primer (*PRNP* 47784F and *PRNP* 49673; Sander et al. 2004), 1 \times Master Amp PCR Enhancer (Epicentre), 1 \times GeneAmp PCR Gold Buffer with MgCl₂ (1.5 mM MgCl₂; Applied Biosystems), and 3.0 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). Thermal cycling parameters, as optimized in our laboratory, were as follows: 5 min at 95 °C; 8 cycles \times 30 s at 95 °C, 30 s at 60 °C (–1 °C/cycle), 2.20 min at 72 °C; 45 cycles \times 30 s at 95 °C, 30 s at 52 °C, 2.20 min at 72 °C; 10 min at 65 °C. Additionally, flanking primers SAF1 and SAF2 (Prusiner et al. 1993) were utilized in 25- μ l PCRs to amplify *PRNP* exon 3 for all study elk as previously described (Seabury and Derr 2003). All elk *PRNP* amplicons were examined via agarose gel electrophoresis and subsequently purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, Valencia, CA) according to the manufacturer's recommended protocol.

Table 1. Oligonucleotides for Rocky Mountain elk (*Cervus elaphus nelsoni*) *PRNP* putative promoter sequencing

Oligo name	DNA sequence (5' → 3')	Sequence origin	Orientation
<i>PRNP</i> 47784F ^a	GTGCCAGCCATGTAAGTG	Cow (<i>Bos taurus</i>) ^a	Forward
CSEA-ELK2R-1	AGCATCTCTTTGAGTCTTGAGC	Elk (<i>C. elaphus nelsoni</i>) ^b	Reverse
CSEA-ELK2R-2	AGAGCCTTACCTTCTTTCCCTCA	Elk (<i>C. elaphus nelsoni</i>) ^b	Reverse
CSEA-ELK2F-1	GCTCAAGACTCAAAGAGATGCT	Elk (<i>C. elaphus nelsoni</i>) ^b	Forward
CSEA-ELK2F-2	TGAGGAAAGAAGGTAAGGCTCT	Elk (<i>C. elaphus nelsoni</i>) ^b	Forward
CSEA-ELK3F	AGTGTAACCTTGGGCTTATTGA	Elk (<i>C. elaphus nelsoni</i>) ^b	Forward
CSEA-ELK3R	TCAATAAGCCCAAGGTTACACT	Elk (<i>C. elaphus nelsoni</i>) ^b	Reverse
CSEA-ELK4F	ATACCCATTGACCCAGCATA	Elk (<i>C. elaphus nelsoni</i>) ^b	Forward
CSEA-ELK4R	TATGCTGGGTCAATGGGTAT	Elk (<i>C. elaphus nelsoni</i>) ^b	Reverse
CSEA-ELK5F	GACTTAGATTCTGGGTCTGC	Elk (<i>C. elaphus nelsoni</i>) ^b	Forward
<i>PRNP</i> 49673R ^{a,c}	GCTGGCAAAAACCAACGAG	Cow (<i>B. taurus</i>) ^a	Reverse

^a Primers derived from Sander et al. (2004).

^b Elk-specific (*C. elaphus nelsoni*) primers designed and utilized in this study.

^c *PRNP* 49673R was only utilized during a preliminary sequencing effort to ascertain feasibility.

Elk *PRNP* Sequencing

All purified elk *PRNP* amplicons were directly sequenced using Big Dye Terminator Cycle Sequencing technology in conjunction with GeneAmp 9700 PCR Systems (Applied Biosystems) in 10- μ l reaction volumes. Elk *PRNP* putative promoter amplicons were initially sequenced using the primers *PRNP* 47784F and *PRNP* 49673R (Sander et al. 2004). Thereafter, internal sequencing primers were designed using the online utility Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and utilized in a stepwise fashion to generate the remaining elk *PRNP* promoter sequences. Elk *PRNP* putative promoter primers used for amplicon sequencing are depicted in Table 1. Internal sequencing primers were designed to facilitate generous overlaps, thereby enabling both correct assemblies and verification of observed SNPs. For the elk *PRNP* putative promoter amplicons, each 10- μ l sequencing reaction consisted of the following: 150 ng purified amplicon, 2 μ l Big Dye, 2 μ l HalfBD (Genetix, Boston, MA), 1 μ M primer, and 0.5 \times Master Amp PCR Enhancer. Thermal cycling parameters followed those previously described (Seabury and Derr 2003) with the exception that a 50 °C annealing temperature was utilized in combination with 50 total cycles. Likewise, purified elk *PRNP* exon 3 amplicons were also directly sequenced using the aforementioned thermal cycling parameters with reaction concentrations as previously described (Seabury and Derr 2003).

Sequence Analysis and Validation Techniques

Most elk *PRNP* regions were sequenced more than once to ensure correct assemblies and confirm observed SNPs. Likewise, this strategy also allowed for confirmation of *PRNP* codon 132 genotypes in CRRI elk. All elk *PRNP* sequences were assembled and analyzed within the program Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI) using the more rigorous default assembly parameters. Sequence quality was examined by manual inspection of electropherograms and confirmed via Sequencher 4.7 quality

analysis score. Heterozygous nucleotides were flagged manually during initial electropherogram inspection and subsequently confirmed by Sequencher 4.7 analysis of multiple overlapping sequences. All heterozygous nucleotides were annotated with the appropriate International Union of Pure and Applied Chemistry–International Union of Biochemistry and Molecular Biology code for heterozygosity within Sequencher 4.7, and the final consensus sequences were exported for further analysis.

Haplotype Analysis

PRNP genotypes (unphased) for all elk were assembled into a single data set. Each variable site was enumerated based on its position in the putative elk *PRNP* promoter sequence developed herein and/or the elk *PRNP* exon 3 reference sequence (Genbank AF016227; coding sequence). Because deviations from Hardy–Weinberg equilibrium (HWE) may enhance the error rate associated with haplotype inference algorithms (Niu et al. 2002; Stephens and Donnelly 2003), HWE for the entire elk *PRNP* data set was assessed via the exact test of Guo and Thompson (1992) online (GENEPOP; <http://wbiomed.curtin.edu.au/genepop/>) and/or the chi-square test (<http://www.genes.org.uk/software/hardy-weinberg.shtml>). Estimates for the elk *PRNP* haplotype frequencies and the most likely pairs of haplotypes for each individual were reconstructed using a Bayesian statistical method implemented within the software package PHASE 2.1 (Stephens et al. 2001; Stephens and Donnelly 2003). Only elk *PRNP* SNPs with genotype distributions similar to HWE expectations and minor allele frequencies greater than 0.10 were included in haplotype reconstruction analyses. Estimates of recombination across the elk *PRNP* putative promoter and exon 3 were computed using PHASE 2.1 as previously described (Li and Stephens 2003). The physical distance (base pairs) between variable sites within the elk *PRNP* putative promoter and exon 3 was estimated using the mule deer *PRNP* sequence (GenBank AY330343). Moderate changes in distance approximations did not change the outcome of PHASE 2.1 analyses.

Promoter Prediction and Comparative Analysis

Two approaches were utilized to predict the elk *PRNP* putative promoter regions via the primary sequence data generated. The first analysis was conducted using the online utility PROSCAN version 1.7, an application for identifying mammalian promoters based on homology scoring with putative eukaryotic Pol II promoter sequences (Prestridge 1995; <http://thr.cit.nih.gov/molbio/proscan/>). Additionally, because approximately 60% of all promoters in humans and mice colocalize to CpG islands (Bird 2002; Antequera 2003), a second promoter analysis was performed using the mammalian-specific program CpGProD online (Ponger and Mouchiroud 2002; http://pbil.univ-lyon1.fr/software/cpgprod_query.html). The results of both analyses were compared for consistency. Likewise, both PROSCAN 1.7 and CpGProD were utilized to predict *PRNP* promoter regions for mule deer (*O. hemionus*; GenBank AY330343; Brayton et al. 2004), cow (*B. taurus*; GenBank AJ298878; Hills et al. 2001), and sheep (*Ovis aries*; GenBank DQ077504; Green et al. 2006) for comparison with elk. Because *PRNP* regions possessing promoter activity in cattle and sheep have previously been identified (Inoue et al. 1997; O'Neill et al. 2003; Sander et al. 2005), promoter prediction analysis for these species was useful for tentatively assessing the relative accuracy of PROSCAN 1.7 and CpGProD. The online BLAST utilities bl2seq and blastn (<http://www.ncbi.nlm.nih.gov/blast/>) were routinely employed for comparative analysis of the elk *PRNP* putative promoter sequences with the *PRNP* sequence for cow (GenBank AJ298878; DQ457195), sheep (GenBank DQ077504), and mule deer (GenBank AY330343). Elk and mule deer (AY330343) *PRNP* sequences were also evaluated for the presence of 4 conserved motifs previously identified within the *PRNP* promoter sequences for human, Syrian golden hamster, sheep, mouse, rat, and cow (Westaway et al. 1994; Saeki et al. 1996; Inoue et al. 1997).

Results

Collectively, we generated and analyzed more than 93 000 bp of *PRNP* nucleotide sequence data for 29 elk derived from 2 populations. Elk *PRNP* amplification success was 100% using sheep and cattle *PRNP* primers previously described (Prusiner et al. 1993; Sander et al. 2004). BLASTN alignment (bl2seq) of the elk *PRNP* sequence (2447 bp) generated herein with the complete mule deer *PRNP* sequence (AY330343; Brayton et al. 2004) resulted in significant similarity beginning 2.2 kb upstream of mule deer *PRNP* exon 1, extending through exon 1 (54 bp), and terminating 193 bp into intron 1. The maximum sequence identity (blastn) between the 2447-bp elk *PRNP* sequence and the corresponding mule deer (AY330343), cow (DQ457195), and sheep (DQ077504) *PRNP* sequences were 94%, 91%, and 92%, respectively. Molecular interrogation of the 2.2-kb putative elk *PRNP* promoter sequences resulted in the identification of 32 previously unreported diallelic SNPs (Table 2), indicating an average

density of one SNP for every 69 bp sequenced. The predicted elk *PRNP* exon 1 (54 bp) and partial intron 1 (193 bp) sequences were monomorphic among the study samples. Notably, the predicted elk *PRNP* exon 1 sequences were identical to those previously reported for mule deer (AY330343; Brayton et al. 2004). Pairwise sequence identities between the cow (AJ298878) and sheep (DQ077504) *PRNP* exon 1 sequences and the predicted elk exon 1 sequence were 92% and 94%, respectively. Analysis of electropherograms and sequence alignments for all elk *PRNP* sequences yielded no evidence of indel polymorphism among the study samples. Molecular

Table 2. Rocky Mountain elk (*Cervus elaphus nelsoni*) *PRNP* polymorphisms

Elk <i>PRNP</i> position ^a	Elk <i>PRNP</i> region ^b	SNPs; alleles observed ^c	Elk <i>PRNP</i> major/minor allele frequencies
91	Promoter	R; (A,G)	0.55/0.45
93	Promoter	Y; (T,C)	0.55/0.45
226	Promoter	W; (T,A)	0.55/0.45
275	Promoter	R; (A,G)	0.55/0.45
283	Promoter	Y; (C,T)	0.55/0.45
359	Promoter	Y; (C,T)	0.55/0.45
622	Promoter	Y; (C,T)	0.55/0.45
639	Promoter	Y; (T,C)	0.55/0.45
645	Promoter	R; (G,A)	0.55/0.45
705	Promoter	Y; (T,C)	0.55/0.45
766	Promoter	Y; (C,T)	0.55/0.45
772	Promoter	Y; (C,T)	0.55/0.45
917	Promoter	Y; (C,T)	0.55/0.45
935	Promoter	Y; (C,T)	0.55/0.45
942	Promoter	R; (A,G)	0.55/0.45
959	Promoter	Y; (T,C)	0.55/0.45
997	Promoter	R; (A,G)	0.55/0.45
1133	Promoter	S; (C,G)	0.55/0.45
1155	Promoter	S; (C,G)	0.55/0.45
1243	Promoter	R; (G,A)	0.55/0.45
1274	Promoter	R; (A,G)	0.55/0.45
1329	Promoter	S; (G,C)	0.55/0.45
1331	Promoter	Y; (T,C)	0.55/0.45
1333	Promoter	R; (A,G)	0.55/0.45
1501	Promoter	Y; (T,C)	0.55/0.45
1513	Promoter	M; (A,C)	0.55/0.45
1615	Promoter	Y; (C,T)	0.55/0.45
1616	Promoter	R; (G,A)	0.55/0.45
1723	Promoter	Y; (C,T)	0.55/0.45
1772	Promoter	R; (G,A)	0.55/0.45
1946	Promoter	S; (G,C)	0.55/0.45
2059	Promoter	K; (G,T)	0.55/0.45
63	Exon 3 (codon 21)	Y; (C,T)	0.53/0.47
312 ^d	Exon 3 (codon 104)	R; (G,A)	0.98/0.02
394 ^d	Exon 3 (codon 132)	W; (A,T)	0.55/0.45

^a Elk *PRNP* promoter sequence from this study; exon 3 position from AF016227.

^b Based on alignment with cow, mule deer, and/or elk (AJ298878, AY330343, AF016227).

^c SNPs depicted using International Union of Pure and Applied Chemistry codes; alleles depicted as major, minor.

^d SNPs previously described (codons 104, 132 Met/Val; O'Rourke et al. 1998, 1999).

Table 3. Reconstructed Rocky Mountain elk (*Cervus elaphus nelsoni*) *PRNP* haplotypes and estimates of the sample haplotype frequencies

Haplotype name ^a	Haplotype sequence ^b	Estimated (frequency)	SE
Elk <i>PRNP</i> -1	ATTACCCTGTCCCCATACCGAGTATACGCGGG TA	0.017241	0.000000
Elk <i>PRNP</i> -2	ATTACCCTGTCCCCATACCGAGTATACGCGGG CA	0.534483	0.000000
Elk <i>PRNP</i> -3	GCAGTTTCACTTTTGCGGGAGCCGCCTATACT TT	0.448276	0.000000

SE, standard error.

^a The PHASE 2.1 best overall reconstruction and best pairs analysis supports the existence of all 3 haplotypes among the study animals.

^b Haplotype sequences are defined by diallelic SNPs within the elk *PRNP* putative promoter and exon 3. SNPs within elk *PRNP* exon 3 appear in bold text. Enumeration of the SNPs depicted for each haplotype are as follows—promoter: 91, 93, 226, 275, 283, 359, 622, 639, 645, 705, 766, 772, 917, 935, 942, 959, 997, 1133, 1155, 1243, 1274, 1329, 1331, 1333, 1501, 1513, 1615, 1616, 1723, 1772, 1946, 2059; exon 3: 63, 394.

interrogation of elk *PRNP* exon 3 resulted in the identification of 1 nonsynonymous (394W; codon 132 Met/Leu) and 2 synonymous SNPs (63Y; 312R; Table 2). The synonymous SNP at position 312 was observed in a single elk derived from Yellowstone National Park. Elk *PRNP* exon 3 SNPs at positions 312 and 394 have previously been reported (O'Rourke et al. 1998, 1999; Hamir et al. 2006).

Of the 35 diallelic elk *PRNP* SNP loci observed, only one (*PRNP* exon 3 position 312; Table 2) displayed a minor allele frequency less than 0.10 (observed allele frequencies: 312A = 0.02; 312G = 0.98) and was therefore excluded from haplotype reconstruction analysis. The major and minor allele frequencies observed for all elk *PRNP* SNPs are depicted in Table 2. Of the 35 SNPs observed within the elk *PRNP* gene, 34 had genotype distributions similar to HWE. The exact test for HWE (Guo and Thompson 1992) could not be performed for SNP variation associated with *PRNP* exon 3 position 312 using GENEPOP online. Three elk *PRNP* haplotypes were predicted via the PHASE 2.1 best reconstruction and best pairs analysis. The 3 predicted elk *PRNP* haplotypes and estimates of the sample haplotype frequencies are depicted in Table 3. Phase probabilities for all elk *PRNP* variable sites were 1.0. Likewise, probabilities for the best pairs of *PRNP* haplotypes for all study elk also were 1.0.

No evidence of recombination was detected using the general model for varying recombination rate (Li and Stephens 2003) for the elk *PRNP* region spanning the putative promoter (2.2 kb), exon 1 (54 bp; predicted), intron 1 (193 bp; predicted), and exon 3 (771 bp) for our study samples. Moreover, complete linkage disequilibrium (LD) was observed for all diallelic SNPs within the elk *PRNP* promoter, resulting in the segregation of 2 high-frequency promoter haplotypes (see Table 3). Additionally, complete LD was also noted between all elk *PRNP* promoter SNPs and nonsynonymous exon 3 variation (394W codon 132 Met/Leu; O'Rourke et al. 1998, 1999) for all study elk ($n = 29$). Therefore, the *PRNP* exon 3 codon 132 genotypes (Met/Met, Met/Leu, Leu/Leu) for all study elk could be retrospectively ascertained with 100% accuracy by genotyping any one of the 32 diallelic promoter SNPs (see Table 2).

The 4 conserved *PRNP* promoter motifs previously described for mouse, sheep, human, Syrian golden hamster,

rat, and cow (Westaway et al. 1994; Saeki et al. 1996; Baybutt and Manson 1997; Inoue et al. 1997) were also conserved within the elk and mule deer (AY330343) *PRNP* sequences (Figure 1). No genetic variation was observed within the 4 elk *PRNP* promoter motifs. *PRNP* promoter prediction analyses for elk, mule deer (AY330343), sheep (DQ077504), and cow (AJ298878) are depicted in Table 4. Notably, CpGProD predicted a single plus-strand *PRNP* promoter for all species evaluated, whereas PROSCAN 1.7 routinely predicted multiple *PRNP* promoter regions on both the plus and minus strands (see Table 4). The results of *PRNP* promoter prediction analyses using both CpGProD and PROSCAN 1.7 were not congruent for all species evaluated (see Table 4). The elk *PRNP* promoter region predicted by CpGProD included 2 potential binding sites for the transcription factor AP-2 (Mitchell et al. 1987) and 4 consensus Sp1 transcription factor-binding sites in the following orientation—AP-2: CCCCCGGGC 2104–2111, putative promoter; Sp1: TCCCCGCCCC 2152–2161, putative promoter; Sp1: CCGCCC 2175–2180, putative promoter; Sp1: CCGCCC 2191–2196, putative promoter; Sp1: CCGCCC 2293–2298, predicted intron 1; and AP-2: CCCCCGGGC 2323–2330, predicted intron 1. The corresponding elk *PRNP* promoter region predicted by PROSCAN 1.7 did not include the second potential binding site for AP-2 (2323–2330; Table 4). Moreover, PROSCAN 1.7 also predicted a second *PRNP* promoter (minus strand) for elk possessing the codon 132 MM and 132 LM genotypes (Table 4). Similar to the elk, 2 potential binding sites for AP-2 (Mitchell et al. 1987) and 3 consensus Sp1 binding sites, arranged in the same general orientation as the elk, were also noted within the mule deer *PRNP* promoter regions predicted by CpGProD and PROSCAN 1.7 (Table 4). *PRNP* promoter regions predicted by CpGProD for cow and sheep included regions of known promoter activity (see Table 4). Cow and sheep *PRNP* promoters predicted by PROSCAN 1.7 were not homologous with sequence regions known to possess transcriptional activity (see Table 4). The elk and mule deer *PRNP* promoter regions homologous with regions of known promoter activity in both cow and sheep are defined in Table 4. Likewise, the elk *PRNP* promoter region corresponding to the CpGProD-predicted promoter for mule deer is also presented in Table 4. Nucleotide sequence

	Motif 1	Motif 2	Motif 3	Motif 4
	5'			3'
Mouse	CTTTCATTTTCTC...CCATTAT T GTAACG...TAAAGATGATTTTTA...TCAGGGAG			
Rat	CTTTCATTTTCTC...CCATTAT T GTAACG...TAAAGATGAC C TTTTA...TCAG C AGAG			
SHa	CTTTCATTTTCTC...CCATTAT T GTAACG...TAAAGATGATTTTTA...TCAGGGAG			
Sheep	CTTTCATTTTCTC...CCATTAT T GTAACG...TAAAGATGATTTTTA...TCAGGGAG			
Cow	CTTTCATTTTCTC...CCATTAT C GTAACG...TAAAGATGATTTTTA...TCAGGGAG			
Human	CTTTCATTTTCTC...CCATTAT T GTAACG...TAAAGATGATTTTTA...TCAGGGAG			
Elk	CTTT T ATTTTCTC... C TATTAT C GTAACG...TAAAGATGATTTTTA...TC G GGGAG			
Mule Deer	CTTTCATTTTCTC... C TATTAT C GTAACG...TAAAGATGATTTTTA...TCAGGGAG			

Figure 1. Comparative sequence alignment of 4 conserved *PRNP* promoter motifs (Westaway et al. 1994) among 8 mammalian taxa. Imperfect conservation is illustrated in bold, underlined text. *PRNP* promoter sequences were derived from both Westaway et al. (1994) and the following GenBank accessions: U52821 (mouse), D50093 (rat), M14054 (SHa, Syrian golden hamster), DQ077504 (sheep), AJ298878 (cow), AF315723 (human), and AY330343 (mule deer). The representative Rocky Mountain elk (*Cervus elaphus nelsoni*) *PRNP* promoter sequence depicted here was derived from this study. The 4 *PRNP* promoter motifs were monomorphic for all study elk.

alignment of the predicted elk *PRNP* promoter (CpGProD; Table 4) with the *PRNP* region of known promoter activity in sheep (O'Neill et al. 2003) revealed 8 total diallelic SNPs (sheep DQ077504: 5354M, 5525M, 5622S, 5700Y, 5739K; elk: 1772R, 1946S, 2059K). Similarly, comparative alignments made between the *PRNP* regions of known promoter activity in cow (Inoue et al. 1997) and the predicted elk *PRNP* promoter (CpGProD; Table 4) revealed 7 total diallelic SNPs (cow DQ457195: 3966R, 4065K, 4186Y, 4262R; elk: 1772R, 1946S, 2059K). No *PRNP* SNPs were in common among sheep, cow, and elk. No corresponding mule deer and/or white-tailed deer (*O. virginianus*) *PRNP* polymorphism data were available for similar comparisons.

Discussion

Herein, we have provided the first molecular characterization and polymorphism analysis for the elk *PRNP* putative promoter, thereby also representing the most comprehensive assemblage of elk *PRNP* data to date. Collectively, 32 novel SNPs were identified within the genomic region encompassing the elk *PRNP* putative promoter. Additionally, one novel synonymous SNP (63Y) was also identified within exon 3 of the elk *PRNP* gene. Comparative sequence analyses between the 2447-bp elk *PRNP* sequences and the *PRNP* sequences of mule deer, cow, and sheep yielded evidence of both good conservation (>90% sequence identity) as well as regions of elk-specific *PRNP* sequence (data not shown). Conservation of the 4 distinct *PRNP* promoter motifs (Westaway et al. 1994) across 8 divergent mammalian taxa (Figure 1) implies some level of biological significance despite the corresponding lack of transcriptional activity noted during reporter gene deletion studies in mouse, cow, and rat (Saeki et al. 1996; Baybutt and Manson 1997; Inoue et al. 1997). Given the known limitations of reporter gene deletion studies, previous authors have suggested that the conserved promoter region may regulate *PRNP* expression in different tissues and/or during different stages of development (McCormack et al. 2002).

Identification of LD within the elk *PRNP* putative promoter was not completely unexpected given recent evidence of high LD within the promoter of both cow and sheep (Clawson et al. 2006; Green et al. 2006). However, detection of complete LD between all elk *PRNP* putative promoter SNPs ($n = 32$; Table 2) for elk obtained from 2 distinct populations was not anticipated. Moreover, the identification of complete LD between all elk *PRNP* putative promoter SNPs ($n = 32$) and nonsynonymous exon 3 variation (394W; codon 132 Met/Leu; Table 2) is unprecedented among *PRNP* studies of artiodactyls. Interestingly, elk is the only TSE-susceptible artiodactyl known to possess an amino acid substitution equivalent to the human *PRNP* codon 129 polymorphism (Owen et al. 1990; O'Rourke et al. 1999; note, human codon 129 = elk codon 132). Similar to the results presented here, previous studies of human *PRNP* noted complete LD between 2 upstream regulatory SNPs and human codon 129 (129 Met/Val; McCormack et al. 2002; Bratosiewicz-Wasik et al. 2007). Specifically, the -101G SNP, previously determined to be in complete disequilibrium with methionine at human codon 129, has been associated with sporadic CJD in humans (McCormack et al. 2002; Bratosiewicz-Wasik et al. 2007). Comparative alignment of the human *PRNP* promoter sequence (AF315723; McCormack et al. 2002) with the elk *PRNP* sequence revealed a proximal relationship between the elk 2059K SNP (Table 2) and the human -101G SNP (AF315723; McCormack et al. 2002). The functional significance of the 32 elk *PRNP* putative promoter SNPs, and/or the 2 corresponding high-frequency *PRNP* haplotypes (Table 3), remains to be determined.

PRNP promoter prediction results generated using the program CpGProD consistently appeared more plausible and potentially more accurate than PROSCAN 1.7 based on *a priori* knowledge of *PRNP* regions possessing promoter activity in cow and sheep (Inoue et al. 1997; O'Neill et al. 2003; Table 4). Moreover, a CpG island-related *PRNP* promoter was predicted for all mammalian species analyzed. Notably, the *PRNP* promoters predicted by CpGProD for elk and mule deer (AY330343) were homologous with

Table 4. *PRNP* promoter prediction and comparative analyses

Species <i>PRNP</i> compared ^a	CpGProD-predicted promoter (strand) ^b	PROSCAN 1.7-predicted promoter (strand) ^c	<i>PRNP</i> region of known promoter activity ^d	Homologous elk <i>PRNP</i> promoter sequence ^e	Homologous mule deer <i>PRNP</i> promoter sequence ^f
Cow (A1298878)	48984–50686 (plus)	50780–51030 (plus) ^g	49339–49400; 49551–50313	2145–2172; 2323–2447	24954–24981; 25545–25894
Sheep (DQ077504)	5193–6852 (plus)	7008–7258 (plus) ^h	5226–5747	1761–2279	24569–25093
Mule deer (AY330343)	24605–26231 (plus)	24907–25157 (plus) ⁱ	NA	1797–2447	NA
Rocky Mountain elk (This report)	1730–2447 (plus)	1948–2322 (plus) ^j	NA	NA	24538–25256

NA, not applicable.

^a GenBank accessions in parentheses. The elk (*Cervus elaphus nelsoni*) *PRNP* sequence was derived from this study.

^b Promoter prediction via CpGProD (Ponger and Mouchiroud 2002).

^c Promoter prediction via PROSCAN 1.7 (Prestridge 1995).

^d Regions of known *PRNP* promoter activity for cow and sheep as previously described (Inoue et al. 1997; O'Neill et al. 2003).

^e Elk *PRNP* promoter regions homologous with regions of known promoter activity in cow and sheep and with the CpGProD-predicted promoter for mule deer (defined via *bl2seq* online; <http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>).

^f Mule deer *PRNP* promoter regions homologous with regions of known promoter activity in cow and sheep and with the CpGProD-predicted promoter for elk (defined via *bl2seq* online; <http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi>).

^g Additional promoters predicted: 5048–5298 (plus), 32029–32279 (plus), 37064–37314 (plus), 37553–37303 (minus), 26626–26373 (minus), 22254–22004 (minus), 5303–5053 (minus).

^h Additional promoters predicted: 14111–14361 (plus), 29900–29650 (minus), 5977–5727 (minus), 4973–4723 (minus).

ⁱ Additional promoters predicted: 43486–43236 (minus), 40736–40486 (minus), 37498–37248 (minus), 1406–1156 (minus).

^j Additional promoter predicted for codon 132 MM and 132 LM elk: 1516–1266 (minus).

sequence regions known to possess promoter activity in both cow and sheep (Inoue et al. 1997; O'Neill et al. 2003; Table 4). Therefore, additional studies are necessary to ascertain whether the *PRNP* promoter regions predicted for elk and mule deer possess significant transcriptional activity. Additionally, given the relative density of SNPs previously reported within regions of known promoter activity for cow and sheep, future studies are generally needed to assess the significance of the relationship between *PRNP* regulatory SNPs and relevant TSEs among ruminant species.

Herein, we report 33 novel elk *PRNP* polymorphisms and 2447 bp of previously unreported elk *PRNP* genomic sequence. The nucleotide sequence data and corresponding haplotype analysis reported in this study provide the initial foundation for further characterizing elk *PRNP* haplotype structure while also providing a natural segue toward future haplotype-based case-control studies with respect to CWD in elk. Additionally, the prediction of *PRNP* regions potentially possessing promoter activity for both elk and mule deer provides an immediate opportunity for reporter gene assays. Future studies targeting the entire elk *PRNP* gene for additional populations are currently needed to comprehensively evaluate the overall patterns of polymorphism while also further assessing the distribution and magnitude of LD.

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