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Genomic organization, comparative analysis, and genetic polymorphisms of the bovine and ovine prion Doppel genes (*PRND*)

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Abstract. The doppel protein (Dpl) is a prion-like protein encoded by the gene *PRND*, which has been found downstream of the prion gene, *PRNP*, in human and mouse. This paper describes the isolation and structural organization of the bovine and ovine *PRND* genes, which are composed of two exons compared with the three of human and mouse. Intergenic distances between *PRNP* and *PRND* were covered by means of long-range PCR and found to be 16.8 and 20 kb, in cattle and sheep respectively. The 5′ and 3′ untranslated regions (UTR) were analyzed to identify transcription regulatory sequences and compared with those from the *PRND* and *PRNP* sequences published for other species. Three polymorphisms (R50H, N110H, and R132Q) were revealed in the cattle coding region; two synonymous substitutions (I12I, A26A) were found in sheep. None of the polymorphisms was significantly associated with either Bovine Spongiform Encephalopathy (BSE) in cattle or scrapie in sheep.

Introduction

Prion diseases comprise a group of fatal neurodegenerative pathologies such as Creutzfeldt Jakobs disease (CJD) in humans, BSE in cattle, and scrapie in sheep and goat. As these diseases develop, an abnormal conformer of the host-encoded prion protein (PrP) accumulates in the brain, accompanied by neurodegeneration (Prusiner et al. 1998).

It has proved difficult to identify factors other than PrP that could be directly involved in the pathogenesis of prion diseases. However, *PRNP* has a paralog, *PRND*, localized about 12 and 21 kbp downstream of the murine and human prion genes, respectively (Moore et al. 1999). *PRND*, referred to as Doppel, encodes a protein (Dpl) that is similar to the C-terminal globular domain of PrP. The murine *PRND* encodes a polypeptide of 179 amino acids with 19% identity and 50% amino acid similarity to the C-terminal globular domain of PrP. The human gene (PRND, OMIM 604263) maps to chromosomal location 20p12-pter, like *PRNP*. Structural predictions for Dpl suggest that it is anchored at the cell surface, like PrP, by a glycosylphosphatidylinositol anchor (Silverman et al. 2000). *PRNP* and *PRND* are transcribed separately, but chimeric transcripts are also generated by intergenic splicing (Moore et al. 1999). Recently, the presence of the *PRND* gene in cattle and sheep has been shown by cloning and sequencing of the cDNA (Tranulis et al. 2001).

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Here we describe the organization of *PRND* in cattle and sheep, position them with respect to *PRNP*, and report on the analysis of genetic polymorphisms in the ruminants' coding regions in terms of association with TSE.

Materials and methods

Amplification of introns and long-range PCR. Intron sequences were detected by using primers BP1/U (CCAGGAGTATGTGCAGAAGGT) and M2/L (AAACTGCCAATAGTTGGCCTCATAGT), corresponding to nt 15–35 and 355–330, and 1–21 and 341–316, of cattle and sheep cDNA sequences respectively (AJ278011 and AJ278010). The amplified fragments were cloned by using the TOPO TA Cloning Kit (Invitrogen) and were sequenced on both strands.

Long-PCR fragments were produced from two YACs containing the bovine and sheep prion loci with primers 104/U (TTCCCAGATGGTGCCATGCT at position 30315–30334 of the sheep *PRNP* region, acc. U67922) and B1/L (ACCTTCTGCACATACTCCTGG), corresponding to nt 35–15 and 21–1 of bovine and sheep cDNA sequences, respectively, by using the Takara LA Long-PCR Kit (Takara). PCR products were cloned into TOPO PCR Cloning XL Kit (Invitrogen).

FISH mapping. Cattle and sheep chromosome spreads were prepared and FISH localization of *PRND* was performed as described in Castiglioni et al. (1998) with YAC clones containing the entire prion locus as probes.

Polymorphism analysis. Fresian cattle from the UK (45 BSE-affected and 48 controls), Italian Sarda sheep (32 scrapie-affected and 24 controls), and Norwegian Rygja (16 scrapie-affected and 34 controls) were studied.

The Dpl coding region of cattle and Sarda sheep was amplified from 50 ng genomic DNA with primers BP2/U (ATGAGGAAACATCTGGGTGGATG) and BP2/L (TTATTTCACAATAAACCAAATGAAAAC), corresponding to nt 101–123 and 623–597, 87–109 and 623–597, respectively, of cattle and sheep cDNA sequences. In the case of the Rygja sheep, primers were GT1F (CGACACAATGAGGAAACATCTG) and GT2R (TTGCCTGCAAGCTTATTTCACA), corresponding to nt 80–101 and 635–614 of the cDNA sequence.

DNA sequencing and analyses. Reactions were performed by dye-terminator cycle sequencing and were analyzed on an ABI310 (Applied Biosystems) on both strands of either cloned PCR fragments or PCR products. Genome-wide repeats and low complexity regions were identified with the program RepeatMasker (http://ftp.genome.washington.edu/RM/RepeatMasker.html). Database searches were performed with BLAST programs (Altschul et al. 1990; http://www.ncbi.nlm.nih.gov/blast/), and analyses of non-coding regions were performed with BLASTN Advantage options (settings: -W7 -G1 -e 3.00 -q-1). Potential coding regions were searched with GRAIL (Uberbacher and Mural 1991) and GENESCAN (http://genome.dkfz-heidelberg.de/cgi-bin/GENSCAN/genscan.cgi). Pro-

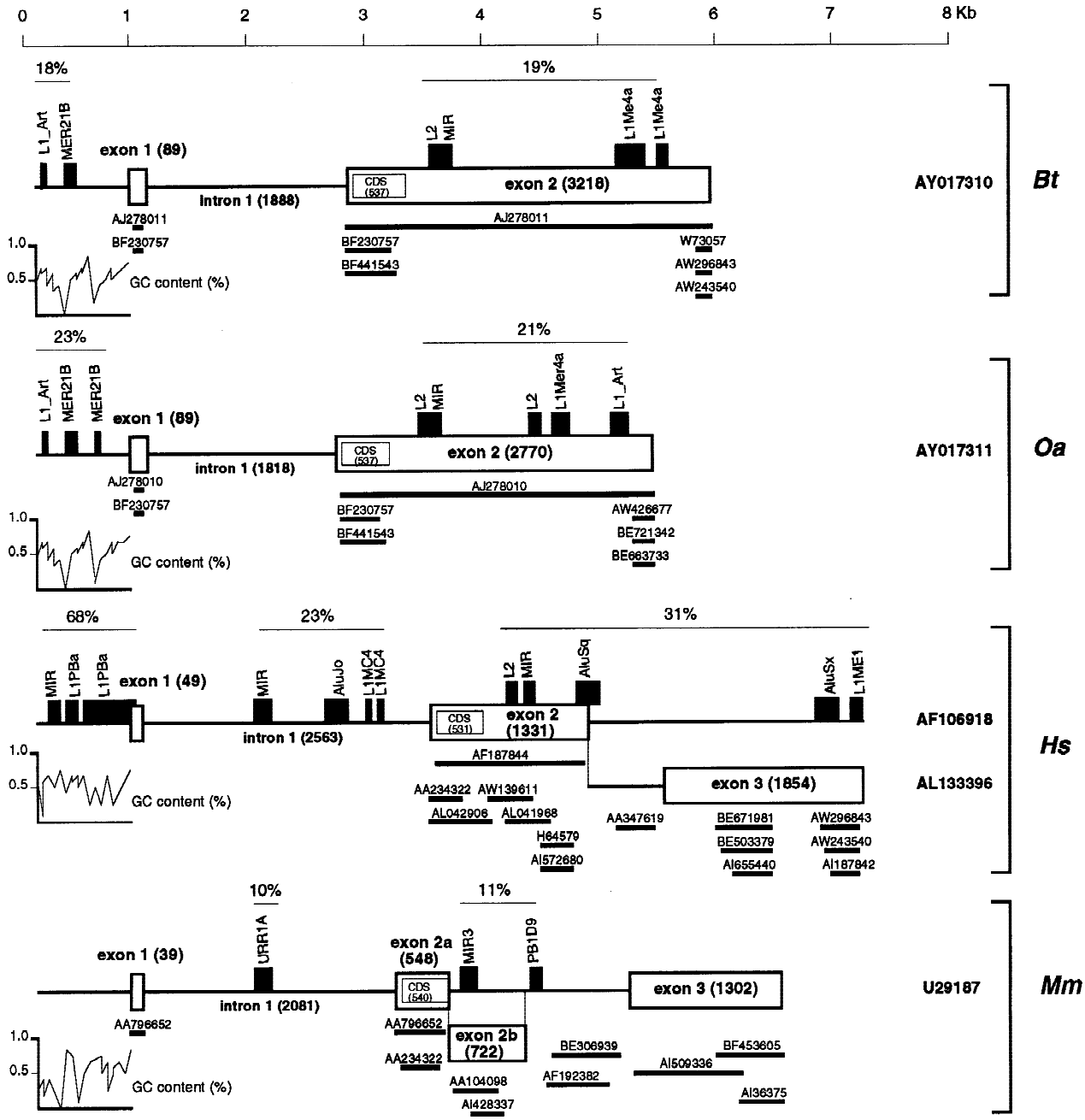

Fig. 1. Genomic organization of the cattle and sheep *PRND* genes. The cattle (Bt) and sheep (Oa) genes are compared with the human (Hs) and murine (Mm) genes as inferred from the sequences deposited in GenBank (accession numbers to the left of the species symbol). The exons are shown as open boxes; the length of exons and introns is shown in bp; CDS is the coding region of *PRND*. The GC content of the promoter region is shown in the graphical inserts for each gene. Repetitive elements are shown as black boxes with the names above the sequence and the frequency distribution in introns and exons indicated with a percentage. cDNA and EST sequences (black bars) with GenBank accession numbers are aligned below the genes.

moter analyses were performed with Web Signal Scan (Prestridge 1991) and Web Promoter Scan (Prestridge 1995) accessed at http://bimas.dcrt.nih.gov/molbio/signal/. CpG composition, multiple alignments, poly-adenylation signal searches and primer design were performed with the Vector NTI suite version V.6 (Informax, North Bethesda, MD, USA). Dpl structural features were predicted by using PSIpred V.2.0 server (http://insulin.brunel.ac.uk/psiform.html) and PSORT server (http://psort.nibb.ac.jp/form2.html).

The sequences reported in this paper have been deposited in GenBank with the accession numbers AYO17310 (cattle) and AYO17311 (sheep).

Results

On the basis of the sequences of bovine and ovine testicular cDNA (Tranulis et al. 2001), a preliminary identification of the coding regions of *PRND* was performed, by means of comparison with the human and mouse mRNAs (Moore et al. 1999). Putative uninterrupted exons were identified in cattle and sheep that were similar in length and composition to the homologous human and murine exons (see below). Specific primers were designed and used to amplify genomic DNA in the two ruminant species, to determine the intron/exon structure of *PRND*. The only discontinuity found between the cDNA and genomic DNA separated exons 1 and 2. The amplified fragments were sequenced on both strands, revealing introns of 1888 and 1818 nucleotides, for cattle and sheep respectively. No other introns were found in the cDNA sequence. Thus, a likely bovine and ovine *PRND* structure constituted by two exons was deduced and compared with the other known Doppel

genes (Fig. 1). The ruminant genes exhibit a similar structure, with a non-coding 5′ exon (confirmed by the cDNAs and by the AJ278011 and BF230757 EST sequences) longer than that found in human and mouse. The entire coding sequence is found within a larger second exon that also contains the 3′UTR region. In contrast, Moore et al. (1999) reported a three-exon structure for the mouse gene (GenBank acc. U29187), with exon 2a and 2b variants and with ESTs (BE306939 and AF192382) that might suggest the existence of read-through transcripts. The human gene also has a potential three-exon structure, as deduced from comparison of the nucleotide sequence of *PRND* mRNA and gene. Thus, aligning the available human *PRND* sequences (GenBank acc. AL133396 and AF106918) with ESTs (such as AW139611, H64579, AW296843, AW243540, and Al187842) allows the delineation of potential boundaries of the second and third exons (Fig. 1). In both cattle and sheep, the *PRND* maps to the same chromosomal region as *PRNP* (Castiglioni et al. 1998), namely, BTA13q17 and OA13q17/18 (data not shown).

Oligonucleotides were designed from the 3′ UTR of ovine *PRNP*, which is conserved in cattle, and from a common region of *PRND* exon 1 of cattle and sheep (104/U and B1/L; see Materials and methods). PCR products were cloned and digested with restriction endonucleases in order to size them. Thus, a distance of 16.8 kb in cattle and 20 kb in sheep between *PRNP* and *PRND* was estimated.

Promoter regions were investigated by sequencing the intergenic long-range PCR products. Comparison of a region 1 kb upstream of exon 1 with the equivalent in human and mouse revealed a low level of similarity, whereas for cattle and sheep a similarity of 65% was found. Using BLASTN with parameter settings that allow matching of non-coding sequences allowed the identification of two conserved boxes between the ruminants and the human regions, showing 74% and 79% of nucleotide similarity (Fig. 2). Computer analysis found different putative promoter sequences, such as a TATA-box in the mouse, 45 bp upstream of the transcription start site, and initiator sites (lnr) in the ruminants. However, BLAST searches of the same regions against the eukaryotic promoter database (EPD) did not find significant similarity with known promoters. Several transcription factor binding sites, common in the four species, were found by computer analysis, such as GATA-1, Sp1, and glucorticoid hormone receptor binding sites (Fig. 2). In general, the Doppel promoter regions did not show similarity to the corresponding prion regions within species. Furthermore, none of the prion-specific, conserved promoter motifs (Saeki et al. 1996) was found in the Doppel regions studied. The average GC content of the sequence was about 50%, with a significant increase only in the proximity of exon 1 (Fig. 1). These regions should, however, not be considered as "CpG islands" because they are different from the corresponding prion promoter regions (Lee et al. 1999) and do not have a high frequency of sites for rare cutting restriction endonucleases.

The first exon showed 96% nucleotide similarity between cattle and sheep, much less (25%) when the ruminant sequences were compared with either human or mouse, owing to a 52-nucleotide segment at the beginning of the ruminant exons (data not shown). Gene-finding programs (GRAIL and GENESCAN) did not correctly identify the exon 1 sequence, whereas larger putative exons of 212 and 114 nucleotides—comprising the actual exon 1—were found for cattle and sheep, respectively. In contrast, no exon 1 was identified for human and mouse. Database searches of the extended ruminants' putative exons failed to reveal matches in dbEST.

Intron 1 has a low density of interspersed repeats in human and mouse, which are absent in the ruminants (Fig. 1). The ovine sequence has few deletions compared with the bovine and shares with it a remarkably high nucleotide similarity (84%). In the four species examined, the overall nucleotide similarity drops to 22%, but it stays up to a significant 65% in the first kilobase segment of

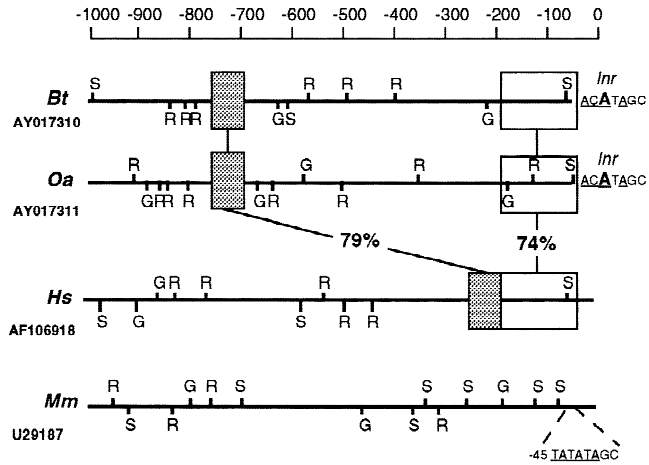

Fig. 2. Analysis of the promoter region of the *PRND* gene in cattle and sheep. 1 kb DNA sequence 5′ of the cattle and sheep *PRND* exon 1 was analyzed and compared with the available sequence in human (AF106918 and AL133396) and mouse (U29187). The coordinates are calculated, taking the first nucleotide of exon 1 (A in boldface) as +1. The position of significant DNA elements is shown with small vertical bars above (sense strand) or below (non-sense strand) the sequences, with a letter code: G, GATA1; R, Glucorticoid Responsive Element; S, Sp1. Putative initiator sequences (lnr) and TATA-box are also shown, with nucleotides of the consensus sequence underlined. Two blocks of 79% and 74% nucleotide similarity between the ruminant and human regions are indicated. The nucleotide coordinates in this case refer to entry AF106918 and are as follows: grey block, nt 18057–18132; white block, nt 18119–18310.

the introns (data not shown). Two microsatellite motifs were found only in the human sequence. Differences in intron sizes between ruminants, human, and mouse arise from insertions/deletions of repetitive elements. GRAIL and GENESCAN revealed putative internal exons within intron 1 in the ruminants, but those did not match the cDNA or any entry in dbEST. BLAST searches against the EPD found significant similarity with promoters only in the case of the human intron sequence, namely, with the promoters of snRNP E (83% of nucleotide similarity), CD3-g (83%), and renin-b (82%). Exon-intron junctions match the consensus GT and AG nucleotides at the intron donor and acceptor sites, respectively (Table 1).

Exon 2 is the most conserved part of the *PRND* examined. The ruminant sequences 5′ of exon 2 are very similar (bovine, ATCCCGACACA*ATG;* ovine, ATTCCGACACA*ATG*), while they diverge from that in human (GTTCTGACGCG*ATG*) and mouse (ATTCACC*ATG*). There is a 57–78% similarity to Kozak's consensus sequence (GCCGCCa/gCC*ATG*), and all the sequences have a purine at position −3 of the initiator ATG. The nucleotide and amino acid identity between cattle and sheep is 98% and 95%, respectively. At the amino acid level, the ruminants' Dpl exhibited a 76% and 75% identity with human and mouse. When comparing PrP and Dpl proteins in cattle and sheep, an identity score of 24% was found. The remainder of exon 2 sequence in the ruminants is the 3′ UTR. In human, two sequences (AF106918 and AL133396) describe the organization of *PRND*, annotated with a two-exon structure. However, human ESTs can be used to delineate potential boundaries of a third exon, although this still leaves an undefined exon/intron composition as outlined in Fig. 1. On the basis of expression data, mouse exon 2 has alternative 3′UTR sequences designated exon 2a and exon 2b (Moore et al. 1999).

The ruminant UTRs have been determined in testicular tissue cDNA (Tranulis et al. 2001), and the human and mouse 3′ UTRs have been previously described (Moore et al. 1999). As a whole, the UTR regions are quite different between mouse and ruminants, whereas the bovine and sheep sequences are 75% identical, with an additional bovine segment of about 400 nucleotides that explains

Table 1. Exon-intron sequence of the exon 1 splice donor and the exon 2 splice acceptor sites in the Doppel genes.

Exon 1 (length)	Species	Splice donor	Intron 1 (length)	Splice acceptor
89	Bt	AGACCCTAAG**gt**atgtgggg	1888	cagccct**aag**ATCCCGACACAATG
89	Oa	AGACCCTAAG**gt**atgtgggg	1818	tcgacctat**ag**ATTCCGACACAATG
49	Hs	GAGAGCCAAG**gt**acgtggg	2563	tctctggc**ag**GTTCTGACGCGATG
39	Mm	GAGAACCGAG**gt**atgtggcg	2081	tcccttgc**ag**ATTCACCATG

Exon and intron sequences are in uppercase and in lowercase letters, respectively. The splice consensus nucleotides **gt** and **ag** are shown in boldface, while initiator methionine is underlined. Exons and introns lengths are expressed in nucleotides.

the difference in length between the two species. Ruminant and human sequences share a 69% nucleotide similarity and also exhibit similar frequencies of interspersed elements (Fig. 1). In contrast to the 3′UTRs of the cattle and sheep *PRNP*, no long relic mariner sequences (Lee et al. 1999) were found in the 3′UTRs of *PRND*. The 3′UTRs of the four species were also analyzed for the presence of poly-A signals and elements that might affect the stability of the mRNAs. This revealed several putative poly-A signals as well as destabilizing elements, particularly in the mRNAs of the ruminants (data not shown).

The coding region of cattle and sheep *PRND* was analyzed for polymorphisms. Fresian BSE-affected and healthy animals from the UK and two populations of sheep, scrapie-affected and controls, were analyzed, Italian Sarda and Norwegian Rygja. Three polymorphisms were detected leading to amino acid substitutions (R50H, N110H, R132Q) in the bovine *PRND*, whereas in only one of the ovine breeds (Sarda) two silent mutations were found at codons 12 and 26. The *PRND*-genotypes, summarized in Table 2, are distributed equally in affected and unaffected cattle. However, for a low-frequency genotype (R50H, N110N, R132R, f = 0.09), a *p*-value of 0.051 was obtained by Fischer's exact test. The distribution and frequencies of *PRND* and *PRNP*-genotypes for the Sarda and Rygja breed are reported in Table 3. The latter showed no polymorphism in the Dpl-ORF, while the silent mutations at codons 12 and 26 in the Sarda breed appeared unrelated to PrP-polymorphisms and incidence of scrapie.

Table 2. *PRND* genotypes and *PRNP* repeat copies in UK cattle.

	PRND genotypes		*PRNP* octapeptide repeats 6:6		5:6	
	N	f	N	f	N	f
BSE:						
R50R N110N R132R wt	35	0.78	32	0.71	3	0.07
R50H N110N R132R	4[a]	0.09	3	0.07	1	0.02
R50R N110H R132R	1	0.02	1	0.02	0	0
R50R N110N R132Q	5	0.11	5	0.11	0	0
Total	45	1.00	41	0.91	4	0.09
Controls:						
R50R N110N R132R wt	42	0.87	31	0.65	11	0.23
R50H N110N R132R	0	0	0	0	0	0
R50R N110H R132R	0	0	0	0	0	0
R50R N110N R132Q	6	0.13	5	0.10	1	0.02
Total	48	1.00	36	0.75	12	0.25

[a] Fisher's exact test: $p = 0.051$. N and f indicate the numbers and the observed frequencies.

Discussion

Several questions arose from the discovery of the mouse *PRND*, a paralog of *PRNP* (Moore et al. 1999), such as whether the gene is found in other species and whether it is involved in TSE. EST mapping and sequencing of HSA20 revealed that *PRND* is placed approximately 21 kb from *PRNP*. More recently, sheep and cattle cDNAs were isolated and sequenced (AJ278010 and AJ278011; Tranulis et al. 2001).

In this paper we describe the genomic structure of *PRND* in cattle and sheep and its relation to *PRNP*. Despite a remarkable difference in the number of exons (two in cattle and sheep, three in mouse and most likely in human), *PRND* is of similar size in the four species, including the intron 1 sequence. Cattle and sheep *PRND* are also very similar in nucleotide composition, the main difference being an additional 400 bp in the 3'UTR of the bovine gene. The high similarity in the ruminants reinforces the observation of a high level of sequence conservation throughout the whole prion locus in the two species (Comincini et al. 2000).

Before predictions on a function for Dpl can be made, an important issue was to establish whether variations in *PRND* might play a role in TSE, as is the case for PRNP in human. The analysis of polymorphisms in human PRND suggested that variations do not seem to play a major role in the pathogenesis of human TSEs (Peoc'h et al. 2000; Mead et al. 2000). It is well established that the ovine PrP coding region is highly polymorphic and that some polymorphisms strongly affect scrapie susceptibility (Hunter 1998). In the bovine PrP coding region, however, genetic variation is limited to a modest fluctuation in the number of octarepeats, but bearing no association with the incidence of BSE (Hunter et al. 1994). As shown herein, the situation with *PRND* appears different because polymorphisms were detected only in cattle. The R50H variation in cattle is found in the same position in wild-type sheep. Similarly, the codon 132 polymorphism restores the mouse wt Arginine 133. One polymorphism that may affect the biochemical properties of Dpl is N110H, because it abolishes a possible asparagine-linked glycosylation site in the protein. In general, our data do not show any clear-cut association between bovine *PRND*-polymorphisms and BSE. However, the R50H and N110H polymorphisms merit further studies.

Breed differences in scrapie susceptibility may indicate strain variation in the scrapie agent or the result of genetic influences, other than *PRNP* (Hunter 1998). In many breeds the major determinant for susceptibility is the V136A polymorphism, often referred to as the "Cheviot-type" of genetic modulation. Conversely, in breeds defined as "Suffolk-type", a Q171R polymorphism is the major regulator of scrapie. In a search for *PRND* polymorphisms related to the disease, we studied one "Cheviot-type" of breed, the Norwegian Rygja (Tranulis et al. 1999), and one "Suffolk-type", the Italian Sarda. In contrast to the highly polymorphic *PRNP*, only two silent *PRND* mutations were detected in the Sarda breed, but none in the Rygja. It is still possible that polymorphisms in non-coding regions of *PRND* could play a role in scrapie susceptibility.

Expression analysis of *PRND* in different tissues (Tranulis et al. 2001) revealed mRNA profiles similar to the mouse (Moore et al. 1999), that is, high levels in testis and lower in mammary gland, spleen, and heart. Since these patterns could be the result of common regulatory pathways, promoter and UTR regions were compared between species to identify putative regulatory signals. The promoter regions in human, mouse, and ruminants showed marked differences in nucleotide composition, although similarities were seen between cattle and sheep and also between ruminants and human. Several putative regulatory protein binding sites were identified, in particular for the glucorticoid responsive elements

Table 3. *PRND* and *PRNP* genotypes in Italian and Norwegian sheep.

Italian Sheep (SARDA)

			PRNP genotypes								
			codon 136			codon 154			codon 171		
PRND genotypes			VV	VA	AA	RR	RH	HH	QQ	RQ	RR
	N	f	f	f	f	f	f	f	f	f	f
Scrapie:											
I12I A26A wt	21	0.66	0	0	0.66	0.59	0.06	0	0.66	0	0
I12I A26A	1	0.03	0	0	0.03	0.03	0	0	0.03	0	0
I12I A26A	9	0.28	0	0	0.28	0.25	0.03	0	0.28	0	0
I12I A26A*	1	0.03	0	0	0.03	0.03	0	0	0.03	0	0
Total	32	1.00	0	0	1.00	0.91	0.09	0	1.00	0	0
Controls:											
I12I A26A wt	22	0.92	0	0	0.96	0.88	0.04	0	0.21	0.46	0.25
I12I A26A	0	0	0	0	0	0	0	0	0	0	0
I12I A26A	2	0.08	0	0	0.04	0.08	0	0	0	0.08	0
I12I A26A*	0	0	0	0	0	0	0	0	0	0	0
Total	24	1.00	0	0	1.00	0.96	0.04	0	0.21	0.54	0.25

Norwegian sheep (RYGJA)

			PRNP genotypes								
			codon 136			codon 154			codon 171		
PRND genotypes			VV	VA	AA	RR	RH	HH	QQ	RQ	RR
	N	f	f	f	f	f	f	f	f	f	f
Scrapie:											
I12I A26A wt	16	1.00	0.47	0.17	0.36	0.88	0	0.12	0.88	0.12	0
Controls:											
I12I A26A wt	34	1.00	0	0.27	0.73	0.79	0.21	0	0.35	0.53	0.12

The underlined or asterisked amino acids letters indicate synonymous heterozygous or homozygous DNA polymorphisms, respectively. N and f indicate the numbers and the observed frequencies.

(GRE) and a fairly large number of putative poly-adenylation signals and stretches of mRNA destabilizing elements within the 3′UTRs. It has been proposed that *PRNP* expression in sheep may be modulated in tissues via alternative polyadenylation of prion mRNA (Goldmann et al. 1999; Moore et al. 1999). Further investigation is required to assess the relevance of changes in the pattern of *PRND* expression to prion diseases.

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