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Doctoral Course in Biotechnology Applied
to Veterinary and Animal Sciences

TSE GENETICS IN GOATS.
LOOKING OUTSIDE THE *PRNP* OPEN READING FRAME: MOLECULAR CHARACTERIZATION OF THE *PRNP* REGULATORY REGIONS AND ASSESSMENT OF THE ROLE OF THE *SPRN* GENE

Doctoral Thesis

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Index

Introduction

1.1 Prion diseases

1.1.1 Human prion diseases

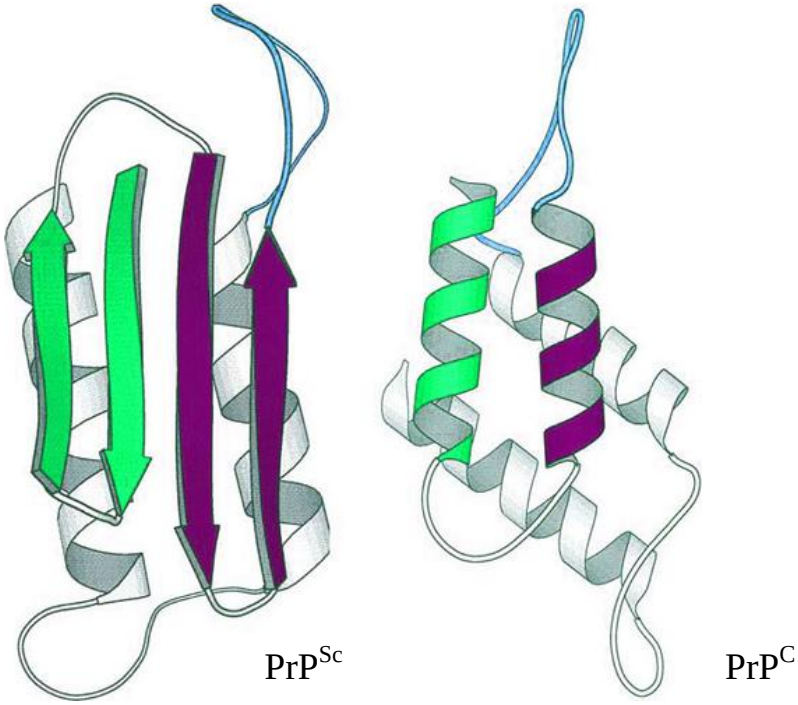
Prion diseases occur as sporadic, genetic, and transmissible disease in humans (Table 1). Although infectious forms of prion disease are most well known to the general public, sporadic and heritable forms of the disease occur much more frequently in humans, with sporadic (s) Creutzfeldt-Jakob Disease (CJD) accounting for approximately 85% of cases. sCJD has no known cause although spontaneous misfolding of the cellular isoform of the prion protein (PrP^{C}) into an aberrantly folded, β-sheet-rich isoform (PrP^{Sc}) is a leading hypothesis (Prusiner 1989; Hsiao et al. 1991) (Fig. 1). The brains of sCJD patients harbour infectious prions that are transmissible to experimental animals (Gibbs et al. 1968; Brown et al. 1994). In humans, virtually all forms of prion disease feature

neuropathological changes including vacuolation (resulting in the spongiform appearance of brain tissue), astrocytic gliosis, and PrP deposition. The morphology of vacuoles and PrP deposits varies depending on the prion strain and host, as do the regions of the brain affected.

Table 1. Prion diseases of humans and animals.

Disease	Host	Mechanism of pathogenesis
Kuru	humans (Fore people)	infection through ritualistic cannibalism
Iatrogenic CJD	humans	infection from prion-contaminated HGH, medical equipment, etc.
Variant CJD	humans	infection from bovine prions
Familial CJD	humans	germline mutations in the *PRNP* gene
GSS	humans	germline mutations in the *PRNP* gene
FFI	humans	germline mutations in the *PRNP* gene
Sporadic CJD	humans	somatic mutation or spontaneous conversion of PrP^{C} to PrP^{Sc}
sFI	humans	somatic mutation or spontaneous conversion of PrP^{C} to PrP^{Sc}
Scrapie	sheep	infection
BSE	cattle	infection or sporadic
TME	mink	infection with prions from sheep or cattle
CWD	deer, elk	infection
FSE	cats	infection with prion-contaminated bovine tissues or MBM
Exotic ungulate encephalopathy	greater kudu, nyala, oryx	infection with prion-contaminated MBM

Fig. 1. Prion structures



To date, over 40 different mutations of the PrP gene (*PRNP*) have been shown to segregate with the heritable human prion diseases. The resulting diseases have been classified as Gerstmann–Sträussler–Scheinker syndrome (GSS), familial (f) CJD, or fatal familial insomnia (FFI) according to the clinical symptoms, although all result from *PRNP* mutations. At the time when the discoveries were reported that fCJD and GSS could be transmitted to apes and monkeys, many still thought that scrapie, CJD, and related disorders were caused by slow viruses (Roos et al. 1973; Masters et al. 1981). Only the discovery that a proline-to-leucine mutation at codon 102 of the human *PRNP* gene was genetically linked to some GSS pedigrees permitted the unprecedented conclusion that prion disease can have both genetic and infectious etiologies (Hsiao et al. 1989; Prusiner 1989). This mutation has been found in unrelated families from several countries, and other mutations causing GSS have since been identified (Dlouhy et al. 1992; Petersen et al. 1992; Poulter et al. 1992; Rosenmann et al. 1998). Likewise, several different

mutations have also been discovered to cause fCJD. A repeat expansion in the amino-terminal region of PrP, which in the healthy population contains five repetitive sequences of eight residues each (octarepeats), has been genetically linked to fCJD. Insertions of two to nine additional octarepeats have been found in individuals within fCJD pedigrees (Owen et al. 1989; Goldfarb et al. 1991a). Molecular genetic investigations have revealed that Libyan and Tunisian Jews with fCJD have a *PRNP* gene point mutation at codon 200, resulting in a glutamic acid-tolysine substitution (Goldfarb et al. 1990a; Hsiao et al. 1991b), a mutation that has since been identified in fCJD pedigrees in many locations (Goldfarb et al. 1990a; Goldfarb et al. 1990b; Bertoni et al. 1992).

The D178N mutation can cause either fCJD or FFI, depending on the polymorphism present at codon 129, where both methionine and valine are commonly found. D178N coupled with V129 produces fCJD, in which patients present with dementia and widespread deposition of PrP^{Sc} (Goldfarb et al. 1991c). If the disease mutation is coupled with M129, however, FFI results and patients present with a progressive sleep disorder that is ultimately fatal. *Post mortem* analysis of FFI brains revealed deposition of PrP^{Sc} confined largely to specific regions of the thalamus (Lugaresi et al. 1986; Gambetti et al. 1995).

Infectious forms of prion diseases include kuru, iatrogenic (i) CJD, and variant (v) CJD. Kuru in the highlands of New Guineawas transmitted by ritualistic cannibalism, as people in the region ate the brains of their dead relatives in an attempt to immortalize them (Glasse 1967; Alpers 1968; Gajdusek 1977). Iatrogenic transmissions include prion-tainted human growth hormone and gonadotropin, duramater grafts, and transplants of corneas obtained from people who died of CJD (Koch et al. 1985; PHS 1997). In addition, CJD cases have been recorded after neurosurgical procedures in which ineffectively sterilized depth electrodes or instruments were used. More than 200 teenagers

and young adults have died of vCJD, mostly in Britain (Spencer et al. 2002; Will 2003) while two confirmed cases have been reported in Italy so far. Both epidemiologic and experimental studies have built a convincing case that vCJD resulted from prions being transmitted from cattle with bovine spongiform encephalopathy (BSE, or "mad cow" disease) to humans through consumption of contaminated beef products (Chazot et al. 1996; Will et al. 1996; Cousens et al. 1997). Until recently, all of the vCJD-affected individuals were identified to express methionine homozygously at codon 129. A single case of vCJD in a patient heterozygous at codon 129 has been reported, raising the possibility of a second wave of "mad cow"–related deaths (Kaski et al. 2009).

1.1.2 Animal prion diseases

Prion diseases occur naturally in many mammals, including scrapie of sheep and goats, BSE, transmissible mink encephalopathy (TME), chronic wasting disease (CWD) of mule deer and elk, feline spongiform encephalopathy, and exotic ungulate encephalopathy (Table 1). Unlike in humans, prion diseases in animals

mainly occur as infectious disorders. As in humans, prion disease in animals is characterized by neuropathologic changes, including vacuolation, astrocytic gliosis, and PrP deposition.

Scrapie of sheep has been documented in Europe for hundreds of years. Despite efforts attempting to link scrapie to CJD, no evidence exists to establish a relationship (Chatelain et al. 1981). Polymorphisms in sheep PrP modulate susceptibility to scrapie, rendering some breeds more resistant to infection than others (Goldmann et al. 1991). As scrapie prions can persist in soil for years (Palsson 1979; Brown and Gajdusek 1991), selective breeding programs may be the most effective means to eradicate scrapie. In part because scrapie is not infectious for humans, hamster- and mouse-adapted scrapie

strains, such as Sc237 and RML, are important laboratory tools for studying prions.

During the BSE epidemic in Britain, it was estimated that nearly one million cattle were infected with prions (Anderson et al. 1996; Nathanson et al. 1997). The mean incubation time for BSE is approximately 5 years. Most cattle were slaughtered between 2 and 3 years of age, and therefore, in a presymptomatic phase of infection (Stekel et al. 1996). BSE is a massive common-source epidemic caused by meat and bone meal (MBM) fed primarily to dairy cows (Wilesmith et al. 1991; Nathanson et al. 1997). MBM was prepared from the offal of sheep, cattle, pigs, and chickens as a high-protein nutritional supplement. In the late 1970s, the hydrocarbon-solvent extraction method used in the rendering of offal began to be abandoned, resulting in MBM with a much higher fat content (Wilesmith et al. 1991; Muller et al. 2007). It is now thought that this change allowed scrapie prions from sheep or low levels of bovine prions generated sporadically to survive the rendering process, resulting in the widespread infection of cattle. Changes in the methods used for feeding cattle have since eliminated the epidemic, although sporadic BSE cases arise occasionally.

Mule deer, white-tailed deer, and elk have been reported to develop CWD. As the only prion disease identified in free-ranging animals, CWD appears to be far more communicable than other forms of prion disease. CWD was first described in 1967 and was reported to be a spongiform encephalopathy in 1978 on the basis of histopathology of the brain. Originally detected in the American West, CWD has spread across much of North America and has been reported also in South Korea. In captive populations, up to 90% of mule deer have been reported to be positive for prions (Williams and Young 1980). The incidence of CWD in cervids living in the wild has been estimated to be as high as 15% (Miller et al. 2000). The development of transgenic (Tg) mice expressing cervid PrP, and thus susceptible to CWD, has enhanced detection of CWD and

the estimation of prion titers (Browning et al. 2004; Tamugey et al. 2006). Shedding of prions in the feces, even in presymptomatic deer, has been identified as a likely source of infection for these grazing animals (Williams and Miller 2002; Tamguney et al. 2009b). CWD has been transmitted to cattle after intracerebral inoculation, although the infection rate was low (4 of 13 animals [Hamir et al. 2001]). This finding raised concerns that CWD prions might be transmitted to cattle grazing in contaminated pastures.

1.2 The prion protein gene (*PRNP*) in ruminants

The *PRNP* gene in sheep, goats and cattle has been mapped to chromosome 13. The genomic sequence available around the *PRNP* gene locus is 32 kb in sheep (accession No. U67922), 27 kb in goat (accession No. EU870890), 78 kb in cattle (accession No. AJ298878) and 65 kb in mule deer (accession No. AY330343). Only the genomes of species in the genus *Odocoileus* (e.g. mule deer, white-tailed deer) contain a pseudogene (PrPψ, accession No. AY371694) with all the features of a classical retro-element (Brayton et al. 2004). The functional length of the *PRNP* gene is approximately 21 kb and it is composed of three exons. Exons I and II are small (100 bp) and form the non-coding 5'UTR of the transcript. Exon III (~ 4 kb) contains the full open reading frame and the 3'UTR of the transcript. Introns I and II are approximately 2.5 kb and 14 kb in length, respectively.

All four genomes produce a messenger RNA of about 4.6 kb, but sheep and goat also generate in peripheral tissues by alternative polyadenylation a second mRNA of ~ 2.1 kb (Goldmann et al., 1999; Horiuchi et al., 1998). This short mRNA is produced at significant level and may be differently regulated compared to the long mRNA. No 2.1 kb mRNA has been detected in cattle. Whether the deer transcript shows alternative polyadenylation is not known. The specific functions of exons I and II in the 5'UTR are also unknown. Differential splicing of exon II has not yet been observed, however cattle have a particular alternative splicing at exon I which creates either a 5'UTR with an additional 115 bases (exon Ib) (Horiuchi et al., 1997) or a 5'UTR without exon I (Haigh et al., 2007). Again, their role is unidentified but one could speculate that the different mRNA are recognised in different regulatory pathways and in that way protein expression could be regulated at various cellular levels.

The gene promoter region regulates production of messenger RNA and consequently controls to a large extent protein expression. The amount of PrP

protein, PrP tissue distribution and temporal availability are therefore all genetically controlled. The *PRNP* gene promoter is active in most tissues and PrP mRNA can be readily detected throughout development in most ruminants (Goldmann et al., 1999). The ruminant *PRNP* promoter contains several well characterised transcription factor motifs within its core (e.g. SP-1, AP-2). Upstream of the core sequence are four motifs highly conserved in sequence and position between species (Westaway et al., 1994) and present in all known *PRNP* promoters; they are probably involved in the regulation of transcription.

1.3 The genetics of scrapie in goats

1.3.1 Role of the PRNP coding region

Natural scrapie in goats was reported for the first time in France (Chelle, 1942), afterwards other cases were described worldwide: a state of the art review on goat scrapie in the European Union, including epidemiology, has been published by Vaccari et al. (2009). Goats are susceptible not only to classical scrapie, but also atypical/Nor98 scrapie cases have been detected, characterized, as in sheep, by distinctive features regarding epidemiology (i.e. occurrence as single cases in the affected herds), molecular pattern, and distribution of histopathological lesions. Moreover the only two confirmed cases of natural BSE in small ruminants were reported in goats, in France (Eloit et al., 2005) and in a retrospective study in UK (Jeffrey et al., 2006).

In Italy the first case of scrapie in goat was diagnosed in 1997 (Capucchio et al., 1998) and, since then, 65 outbreaks have been detected in total (51 of classical scrapie and 14 of Nor98 scrapie), in which, according to the foreseen measures for the eradication of the disease, 13.000 goats have been culled.

Although scrapie is an infectious disease, the susceptibility of sheep is strongly influenced by polymorphisms of the prion protein gene (*PRNP*). *PRNP* haplotypes valine/arginine/glutamine (VRQ) and alanine/arginine/glutamine (ARQ) at codons 136, 154, 171, respectively, are associated with high susceptibility to classical scrapie, whereas the ARR haplotype has been linked to resistance (Belt et al., 1995; Bossers et al., 1996; Hunter et al., 1996; Hunter et al., 1997) (Fig. 2). Accordingly, the EU has implemented breeding programs to increase scrapie resistance in sheep populations. In compliance with regulation (EC) 999/2001, as amended, several Member States are now increasing the frequency of the ARR haplotype. A similar approach is not applied in goats yet but it would be desirable in this species too, given that

scrapie poses a problem for the economy and for animal welfare, and that goats, often bred in mixed flocks with sheep, can have a role in maintaining the circulation of scrapie strain and the consequent sheep exposure. Genetic analysis of the goat *PRNP* gene revealed 42 polymorphisms in the open reading frame (Vaccari et al., 2009), including silent mutations and a *PRNP* variant containing three instead of the usual five octapeptide repeats (Goldmann et al., 1998) (Table 2). Various European studies have suggested that several polymorphisms can modulate scrapie susceptibility in goats as well. The presence of methionine (M) at codon 142 was associated with increased incubation periods after experimental challenge of BSE and scrapie strains (Goldmann et al., 1996) and in natural scrapie outbreaks (Barillet et al, 2009; González et al., 2010). A reduced susceptibility to natural scrapie has also been reported for goats carrying arginine (R) at codon 143, histidine (H) at codon 154 (Vaccari et al., 2006; Billinis et al., 2002) or glutamine (Q) at codon 211 (Barillet et al., 2009). However, H154 has clearly been suggested to be a risk factor for Nor98 goat scrapie (Colussi et al., 2008). The most promising results have been obtained for codon 146, carrying serine (S) or aspartic acid (D), which was linked to high resistance in Cyprus (Papasavva-Stylianou et al., 2007; 2011), and for codon 222, carrying lysine (K), which in Italy was first reported as conferring resistance, being associated only with healthy animals (Acutis et al., 2006; Vaccari et al., 2006). Association of K222 with a protective effect was afterwards found also in France and Greece (Barillet et al., 2009; Bouzalas et al., 2010). All these results are encouraging to consider supporting breeding programs for resistance in goats against classical scrapie in all EU Member States, as stated by the EFSA Panel on Biological Hazards in the "Opinion on genetic TSE resistance in goats in all EU Member States" (2009). Accordingly, the European Commission recently approved the Regulation (EC) 189/2011, amending the Regulation (EC) 999/2001, that foresees the possibility of delaying the killing and complete destruction of

dairy goats in scrapie outbreaks for a maximum period of 18 months or until the 31 December 2012 if the index case has been confirmed before 1 July 2011. In the preliminary remarks is explicitly stated that genetic resistance to scrapie in goats could exist and that possibly in 2013 exemption from the killing of animals that may be considered resistant in the near future may be appropriate.

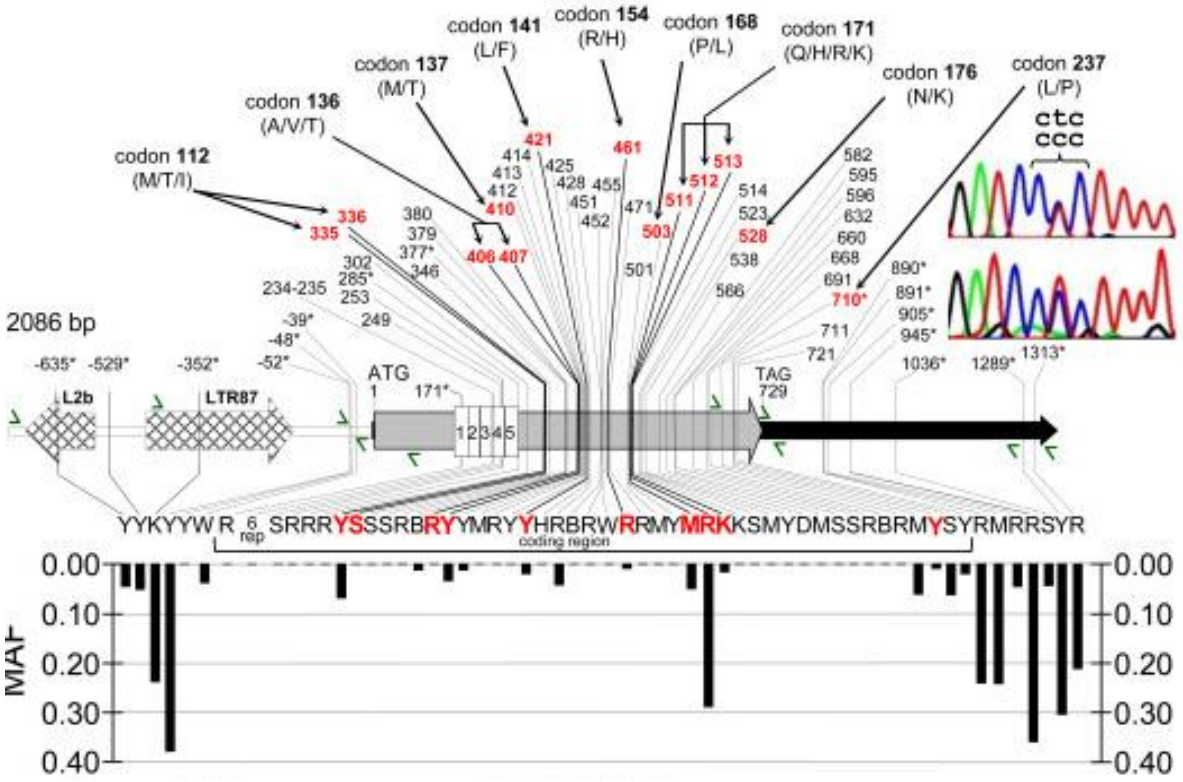


Fig. 2. Physical maps of the ovine *PRNP* coding sequence and polymorphisms

Features include: thick shaded arrow, coding sequence; black arrow, 3' untranslated region of exon 3; hatched arrows, ovine repetitive elements; white numbered vertical rectangles, octapeptide repeats; vertical lines, positions of SNPs; green single headed arrows, PCR amplification and/or sequencing primers (GenBank AY326330). SNP position numbers are distance to the first base of the PRNP start codon. Letters below SNPs are IUB ambiguity codes (R = a/g, Y = c/t, M = a/c, K = g/t, S = c/g, W = a/t, B = c/g/t, H = a/c/t, D = a/g/t). Red numbers and letters indicate sites affected by nonsynonymous substitutions at codons 112, 136, 154, 171, and 237. *PRNP* octapeptide repeats at positions 160 to 285 have either five or six repeats (5rep or 6rep). MAF histograms correspond to genotypes from approximately 950 sheep available at http://cgemm.louisville.edu/USDA/index.html website.

Table 2. Known caprine *PRNP* gene polymorphisms[a].

Polymorphism	EU countries reported from	Non-EU countries reported from
W18R	ES	
V21A	GR	
L23P	GR	
G37V	IT	
P42P	UK, IT, FR, GR	US, CN, JP, PK
G49S	GR	
Q101R	UK	
Q101Q	NL	
W102G	UK	CN, JP
K107K	GR	
T110P	IT	
V125V		CN
G127S	IT, UK, ES, FR	US, CN, JP
L133Q	IT	
M137I	IT	
S138S	UK, IT, FR, CY, GR	US, CN, JP, PK
I142M	UK, FR, ES	US, JP
I142T	IT	
I142I	NL	
H143R	UK, IT, GR, NL	US, CN, JP
N146D	CY	
N146S	CY, UK	US, CN, JP
R151H	CY	
R154H	GR, IT, ES, CY, FR	US, CN
P168Q	IT, GR, CY	
V179V	CY	
D181D	CY	
T194P	IT	
F201F	ES	
T202T	IT	
K207K	GR	
R211Q	UK, FR, ES	US, CN, JP
R211G		CN
I218L		CN
T219I	ES	CN
T219T	IT	
Q220H	FR, CY, GR	
Q222K	IT, FR, UK, ES	US, CN
Q222Q	NL	
G232W	ES	
G232G	IT	
S240P	IT, FR, UK, GR, ES, CY, NL	US, CN, JP, PK

[a]Coding polymorphisms in regular print, silent mutations in italics.

1.3.2 Role of the PRNP regulatory regions

In addition to associations between non-synonymous *PRNP* polymorphisms and scrapie occurrence, an inverse relationship between TSE incubation period and the level of host *PRNP* gene expression has also been established in mice and cattle (Vilotte et al. 2001; Safar et al. 2005; Sander et al. 2005; Scott et al. 2005). Two SNPs within the human *PRNP* gene promoter are considered a risk factor associated with spontaneous Creutzfeldt-Jacob disease (CJD) (McCormack et al. 2002). In sheep, a significant imbalance in the incidence of one SNP (5354-C/A) within the promoter region was found in the closed NPU (IAH Edinburgh) Cheviot flock (Hunter et al. 1996; O'Neill et al., 2005); the variant carrying adenine created consensus sequences for STAT transcription factor. Electrophoretic mobility shift assays (EMSA) using ovine or murine cells and tissues demonstrated differential binding of transcription factors to polymorphic variants of the ovine *PRNP* promoter, suggesting that the prion protein expression pattern and the distribution of TSE infectivity may be associated with *PRNP* promoter genotype (Burgess et al. 2009). Studies of the regulatory regions of bovine *PRNP* gene have revealed 46 polymorphisms within a 5.4 kb fragment ranging from promoter I to exon II, based on the analysis of about 70 animals (Sander et al. 2004). A 23 bp and a 12 bp 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). Both sequences contain transcription factor binding motifs, and a functional promoter analysis of the different bovine *PRNP* promoter alleles by reporter gene assays demonstrated that bovine *PRNP* expression in vitro is modulated by genetic variation within these regulatory regions (Sander et al., 2005). Although the complete goat *PRNP* gene was recently sequenced (Van Poucke et al., 2009) no basic information currently exists regarding the regulation of

PRNP transcription and frequency of genetic polymorphism within the putative regulatory regions of the goat *PRNP* gene.

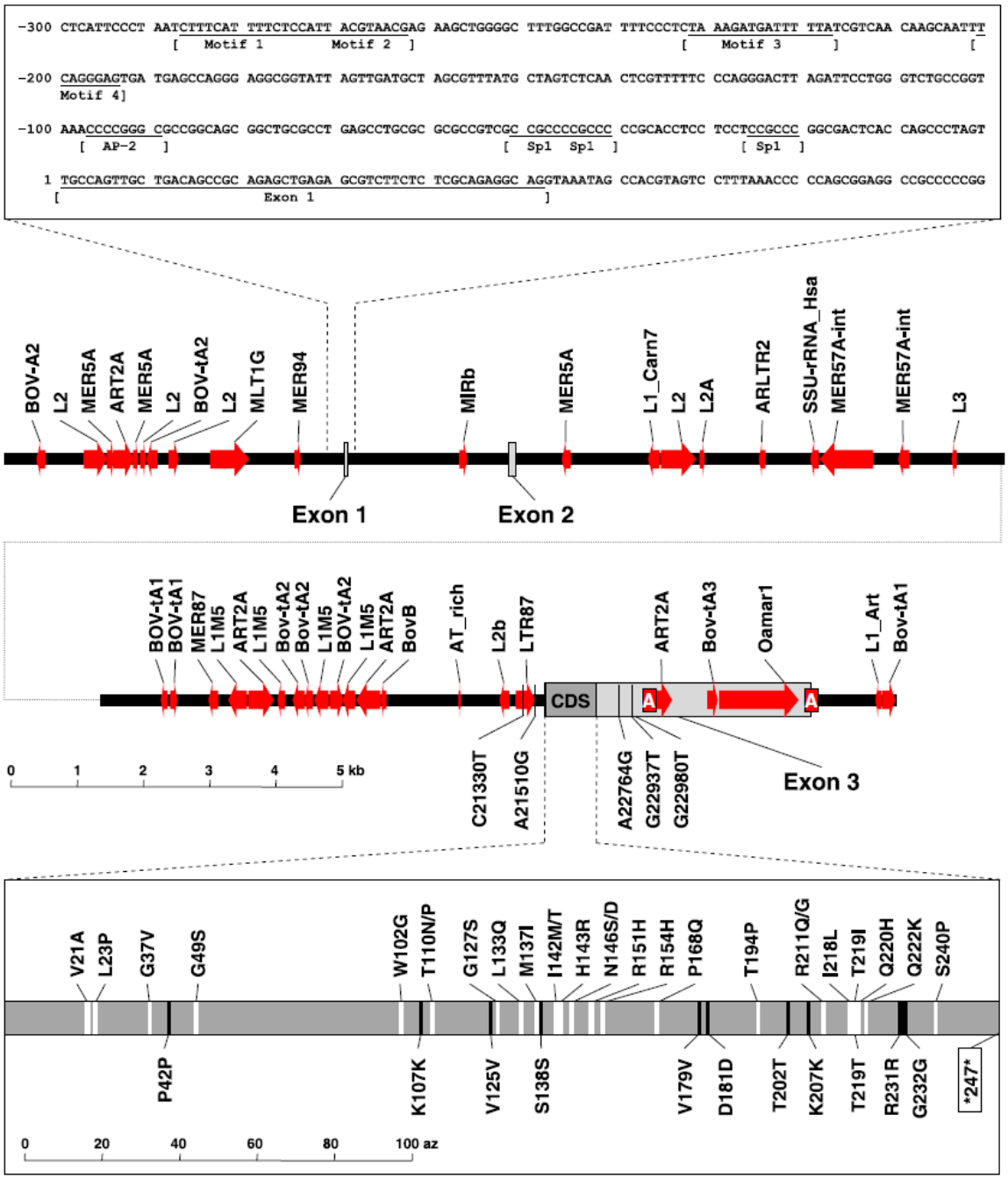


Fig. 3. Schematic overview of the goat *PRNP* gene organization.
The thick black line in the middle represents the goat *PRNP* sequence with gray boxes as putative exons, CDS as coding sequence, arrows as repeat sequences, 'A' as putative polyadenylation signal, and black vertical lines as mutations found by goat *PRNP* database sequence comparison. The upper window represents the putative promoter sequence with conserved motives and putative transcription factor binding sites. The lower window represents all known missense (white lines) and silent (black lines) mutations in the CDS.

1.4 The Shadow of the Prion Protein gene (*SPRN*)

It is clear that *PRNP* is the major genetic determinant of prion disease incubation time; however, it is also apparent that other genes also make a significant contribution to the natural variation observed between different mouse lines. The greatest amount of incubation time data exists for wild type mice (Carlson et al., 1986; Westaway et al., 1987; Lloyd & Collinge, 2005; Carlson et al., 1988; Race et al., 1990). Within this group, where the *PRNP* coding sequence is identical, the shortest incubation time described for intracerebral inoculation of Chandler/RML prions is 105 ± 4 in SJL/J mice and the longest is 221 ± 5 in PWK/Pas mice. This suggests that the combined effect of other genes can extend the incubation time by over 100 days, effectively doubling the incubation time. Similarly, not all sheep with the same *PRNP* genotype are equally susceptible or develop pathology in the same way (Goldmann, 2008) suggesting the involvement of other host genes, amongst which the prion family proteins are primary candidates. Beside *PRNP*, the mammalian prion protein gene family currently contains other two known genes: *PRND* (encoding doppel protein, Dpl) and *SPRN* (encoding shadoo protein, Sho) (Premzl et al., 2003; 2004; Premzl & Gamulin, 2007; Watts & Westaway, 2007). PrP and Sho are highly expressed in the neurons of the central nervous system (Hu et al., 2007; Watts et al., 2007), whereas Dpl appears to be specific for the reproductive system (Genoud et al., 2003). The more recently discovered Sho protein bears similarity to two features within PrP: the hydrophobic domain and a series of tandem repeats with positively charged residues. Both of these regions in PrP^{C} are considered to be unstructured, or at least less structured than the carboxy-terminal domain (Fig. 4). Interest in Sho was catalyzed by the observation that it is expressed predominantly in the CNS and is perpetuated by a number of PrP-like properties (Table 3), beyond mere sequence homology, and by an intriguing

downregulation phenomenon in prion infected cells. Dpl, discovered several years earlier, bears a resemblance to the carboxyl-terminal globular domain of PrP^{C}. Dpl is expressed in the male reproductive tract and investigations into its biology have centred not only upon reproduction (predictably) but also on the unexpected neurotoxic effects of ectopic Dpl expression in the CNS.

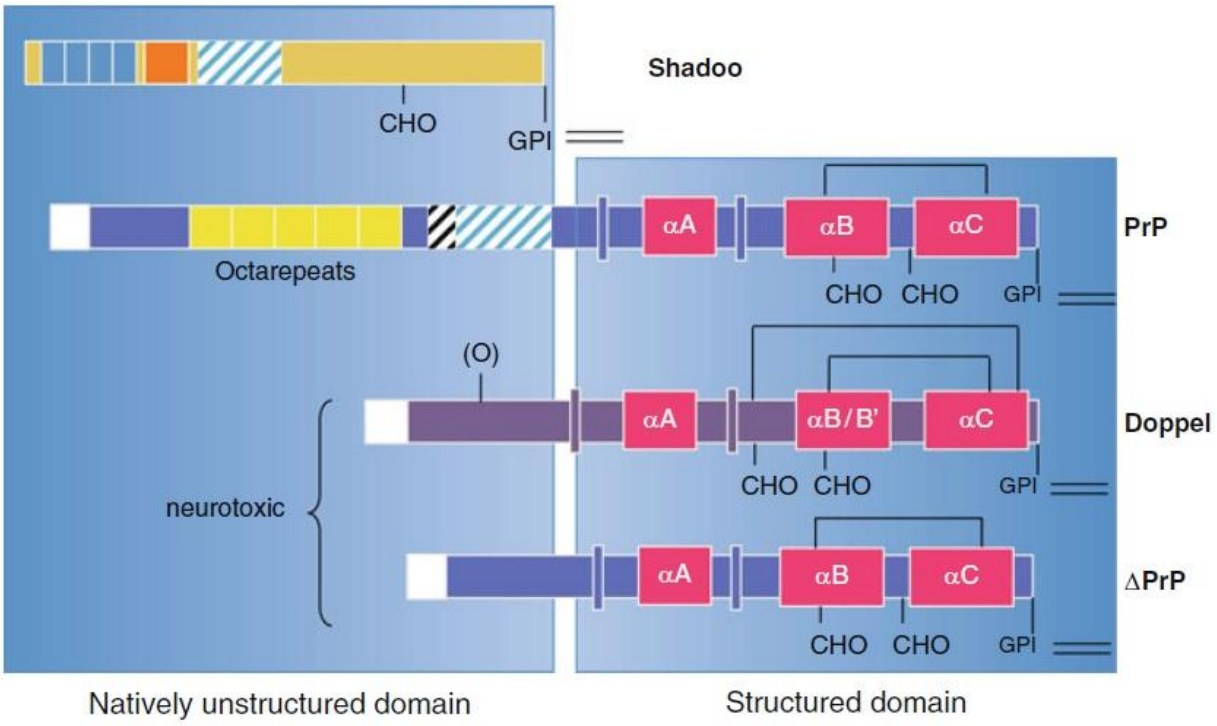


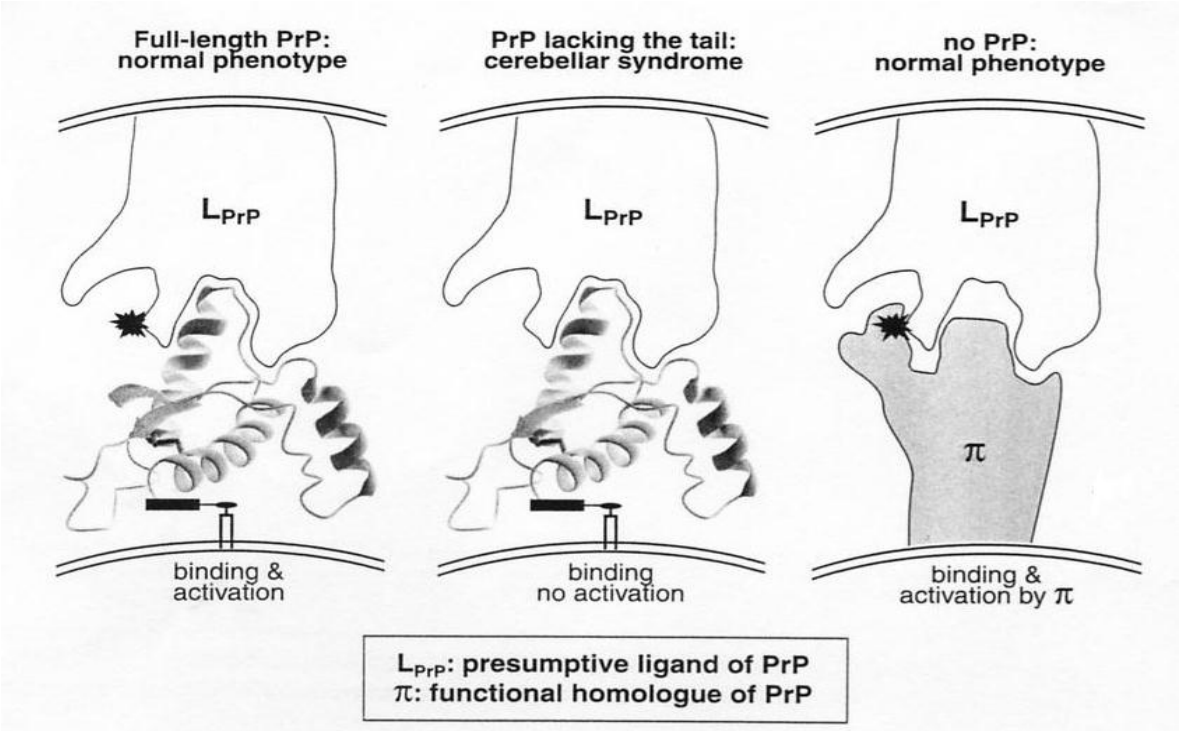
Fig. 4. Domain structure of the PrP protein family.
Sho, PrP, Dpl, and internally deleted forms of PrP, noted here, generically, as DPrP are shown. The proteins are organized around natively unstructured and a-helical structured domains. The exact boundaries between the domains are approximate. The central hydrophobic tract is indicated by blue diagonal shading. For the Sho protein the blue shaded rectangles indicate degenerate arginine-glycine-glycine (RGG) repeats. The orange shaded rectangle encompasses a different type of arginine-rich tract of the general form RVRVRPAPR. White boxes in PrP and Dpl indicate an N-terminal tract of charged residues. The diagonally shaded box with black stripes indicates a second type of charged patch. CHO indicates N-linked carbohydrates. Disulfide linkages are shown by black lines. Alpha helices are shown as crimson boxes (aA, aB, and aC) while narrower boxes denote b-strands. GPI denotes the glycosylphosphatidyl inositol anchor.

Table 3. Properties of PrP, Sho, and Dpl.

Property	PrP^C	Sho	Dpl
Tissue with high levels of expression	Brain and many others including, lung, cardiac muscle, circulating lymphocytes	Brain	Male reproductive tract
Natively unstructured region?	N-terminal half of the molecule	Whole protein?	Small N-terminal tract?
α-Helical domain	C-terminal	Not described	C-terminal
Allelic variants linked to human prion disease?	Yes (many)	Yes (two)	Controversial (unlikely)
Refolds to β-sheet form in vitro?	Yes	Yes	No
Refolds to β-sheet form in vivo?	Yes	No	No
Protein level drops during prion disease pathogenesis?	No overt change	Yes	No
Infectious form known?	Yes-PrP^{Sc}	Unknown	No

Recently, Watts et al. (2007) hypothesized how the *SPRN* gene and Sho protein may be involved in prion disease, proposing that Sho and PrP bind to the same cellular receptor in their neuroprotective role and that perturbation of Sho expression during prion disease enhances neuronal death (Fig. 5).

Fig. 5. Molecular model of interaction as hypothesized by Charles Weissmann (2001).



Association of *SPRN* with human prion diseases has been demonstrated by Beck et al. (2008) who found that a frameshift mutation in *SPRN*, resulting in reduced expression of Sho, is associated with vCJD and that another coding mutation (T7M) may be a risk factor for sporadic CJD. A close correlation between *PRNP* and *SPRN* expression in sheep brain tissue has been suggested by Lampo et al. (2009) and Gossner et al. (2009). These findings have emphasized the need to understand the genetics of both genes. In sheep, the PrP-homologous hydrophobic region of Sho is polymorphic (Fig. 6) and an indel polymorphism detected in this region, resulting in the insertion/deletion

of two alanine residues, has been associated with susceptibility to classical scrapie (Lampo et al., 2010).

Mouse Sho is post-translationally processed from an ORF of 147 codons, it appears in cells as a 18 kDa GPI-anchored membrane protein with one N-linked carbohydrate (Watts et al., 2007). Lampo et al. (2007) published an ovine *SPRN* ORF encoding a protein of similar overall structure to mouse Sho. The most homologous region to PrP is the 20 aa hydrophobic sequence AAAGAAAGAAAGAAAGLAAG in sheep Sho protein. In PrP, a 16 aa hydrophobic sequence VAGAAAAGAVVGGLGG (sheep codons 115–130) is the most conserved part of the protein. Variants of this sequence are very rare, only 11 are known in almost 400 PrP variants from over 130 species. There are no insertions/deletions in this hydrophobic PrP sequence. It has been suggested that the hydrophobic core of PrP is the binding site for other proteins, such as stress-inducible protein 1 (STI1) (Zanata et al., 2002), that it modulates the formation of transmembrane PrP isoforms (Hegde et al., 1998) and that it is important for basolateral sorting of PrP in a cell (Uelhoff et al., 2005). Furthermore, the AGAAAAGA palindrome may play a part in the disease-specific PrP^{Sc}–PrP^{C} interaction (Norstrom & Mastrianni, 2005): peptides containing this sequence are neurotoxic (Forloni et al., 1993) and amyloidogenic (Gasset et al., 1992), and the variant AGAAVAGA is linked to Gerstmann–Sträussler–Scheinker syndrome, a genetic form of human prion disease (Mallucci et al., 1999). The homology in this region between PrP and Sho implies that there is similar cellular sorting and ligands and that they use similar mechanisms to promote their neuroprotective properties (Chiarini et al., 2002; Watts et al., 2007). Given the overall degree of conservation of the Sho hydrophobic domain it is therefore of considerable interest that deletion and insertion variants have been found in this region, in both sheep and bovine species and, more rarely, in humans (Daude et al., 2009; Stewart et al., 2009) (Fig. 6). These genetic variations may arise because mammalian coding

sequences for the Sho hydrophobic domain are arranged as tandem repeats, a situation slightly different from the PrP hydrophobic domain, which is palindromic.

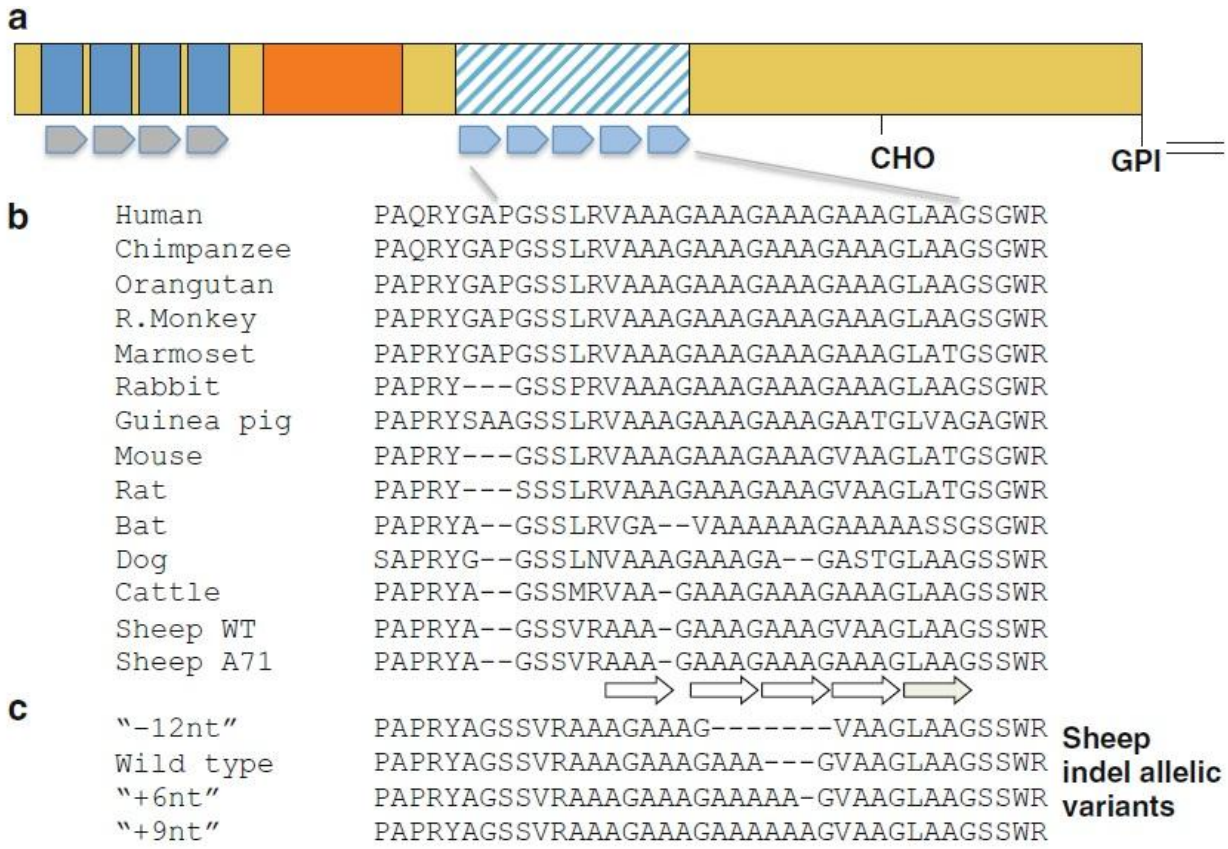


Fig. 6. Tandem repeats and the structure of the Sho protein.
(a) Domain structure of the Sho protein. Blue shaded rectangles indicate degenerate arginine-glycine-glycine (RGG) repeats with tandem head-to-tail organization indicated by gray arrows. An orange shaded rectangle encompasses a different type of arginine-rich tract of the general form RVRVRPAPR. A central hydrophobic tract is indicated by diagonal shading and is itself made up of tandem repeats composed of four amino acids (blue arrows). (b) Expanded view and sequence alignment of the hydrophobic tract from different species to demonstrate tandem repeat organization. (c) Insertion/deletion (indel) allelic variants of the hydrophobic tract seen in sheep. Similar, albeit rare, indel variants are also described in humans (not shown).

Regarding *SPRN* in goats data are still limited and, at the time of writing, there is only one paper in literature by Stewart et al. who sequenced caprine *SPRN* from 135 goats of different breeds and origins. Caprine Sho is 3 aa longer than ovine Sho due to an insertion of Leu-Arg-Pro at the very end of the ORF (Fig. 7). The four identified DNA polymorphisms 144G→A, 177G→C, 300C→T and 433C→A (numbered relative to the ORF) were all silent mutations, no amino acid changes were detected. The same Authors did not detect amino acid polymorphisms in samples from wapiti; domestic cattle and deer samples were also very stable, with three allelic variants amongst them. The same applies to laboratory mouse strains which, according to the analysis of the strains used for scrapie experiments, all express the same Sho.

Fig. 7. Alignment of Sho amino acid sequences.
Dots, Identical amino acids; dashes, amino acid deletions; grey shading, amino acid polymorphisms in sheep.

1.5 Bioinformatics tools

Bioinformatics is a relatively recent science and can be defined as the application of computer science and information technology to the field of biology and medicine. Several bioinformatic tools are currently available for the researcher and many of them are freely available on the web. Aim of this section of the thesis is to provide an overview of the bioinformatic software that has been used to analyse the sequence data generated from the experiments.

1.5.1 CpGProD

CpGProD is a program dedicated to the prediction of promoters associated with CpG islands (CGIs) in mammalian genomic sequences. CpGProD is available either via a web server (http://pbil.univ-lyon1.fr/software/cpgprod_query.html), useful for a small dataset, or as a standalone application for a larger dataset. The entry sequence (or file) must be submitted in FASTA format. In vertebrate genomes, the CpG dinucleotides are present at about 25% of their expected frequency. This deficiency is due to the methylation of cytosine at CpG dinucleotides and the very high mutation rate of the methylated cytosines.

CGIs are stretches of DNA escaping methylation and exhibiting a high G+C content and CpG frequency relative to the bulk DNA. The CGIs are several hundreds base to several kilobase long and are dispersed throughout the genome. 50%-60% of the human genes exhibit a CGI over their Transcription Start Site (TSS) but all the CGIs are not associated with a TSS.

Some studies (Ioshikhes and Zhang, 2000; Ponger et al., 2001) have shown that the CGIs located over the TSS (start CGIs) are characterized by a particular structure compared to other CGIs (no-start CGIs): the start CGIs are longer and display a greater CpGo/e ratio and G+C level than no-start CGIs.

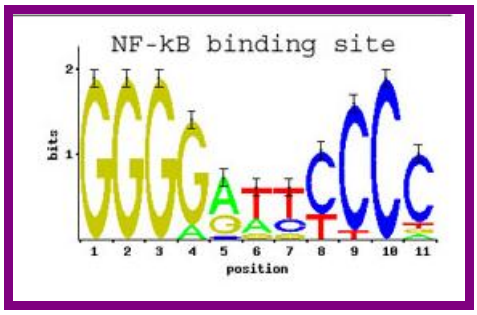
CpGProD computes a score corresponding to the probability to be over the TSS (start-p value) from the length, the G+C content and the CpGo/e ratio of each CGI.

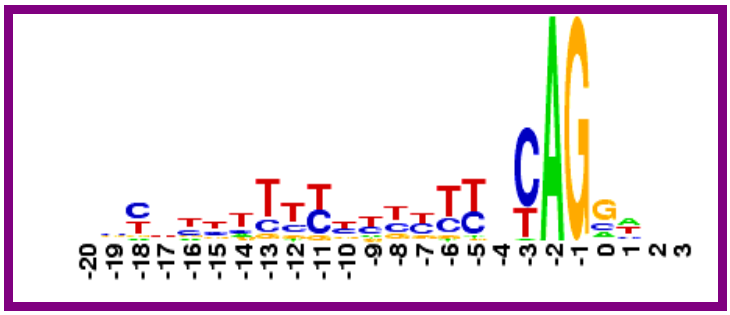
Moreover, two compositional biases between the plus and the minus strand of the start CGIs were observed in the start CGIs (Ponger, unpublished data). The CGIs located over the plus strand exhibit an excess of T compared to A and an excess of G compared to C. On the contrary, the CGIs located over the minus strand exhibit a depletion of T compared to A and a depletion of G compared to C. These biases are estimated by using two parameters, the AT-skew and the GC-skew. CpGProD calculates these parameters to predict the strand of each potential promoter and the probability to be over this strand.

1.5.2 TFSEARCH

The TFSEARCH program was written by Yutaka Akiyama (Kyoto University, currently at RWCP) in 1995. The TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) searches highly correlated sequence fragments against TFMATRIX transcription factor binding site profile database in the TRANSFAC databases developed at GBF-Braunschweig, Germany. TRANSFAC contains data on transcription factors, their experimentelly-proven binding sites, and regulated genes. Its broad compilation of binding sites allows the derivation of positional weight matrices (Fig. 8). Search results are summarized in a tabular form and presented directly on the client's web browser.

Fig. 8. Examples of positional weight matrices as used by TFSEARCH.





1.5.3 PHASE

The program PHASE (http://stephenslab.uchicago.edu/software.html) implements a Bayesian statistical method for reconstructing haplotypes from population genotype data. The software can deal with SNP, microsatellite, and other multi-allelic loci (eg tri-allelic SNPs, and HLA alleles), in any combination, and missing data are allowed. The current version (v2.1) implements extensions to the original method that was described in Stephens et al. (2001). Experiments with the software on both real and simulated data indicate that PHASE can provide an improvement over other existing methods for reconstructing haplotypes - for example, in simulation experiments in Stephens et al. (2001) the mean error rate using PHASE was about half that obtained by the EM algorithm. PHASE algorithm starts by dividing the data into segments of consecutive loci. It then computes a list of plausible

haplotypes within each segment, and then iteratively combines segments to obtain a list of plausible haplotypes, and a best guess for each pair of haplotypes, across the whole region. Moreover, PHASE can perform a permutation test for significant differences in haplotype frequencies in case and control groups. More precisely, PHASE tests the null hypothesis that the case and control haplotypes are a random sample from a single set of haplotype frequencies, versus the alternative that cases are more similar to other cases than to controls.

1.5.4 PATROCLES

Patrocles (www.patrocles.org) is a database of polymorphic miRNA-target interactions. Patrocles compiles SNPs in the 3′UTR of human and mouse genes that either create or destroy miRNA target sites. miRNA target sites are defined as octamer motifs that are expected to show perfect Watson-Crick complementarity with the 5′ end of mature miRNAs. The web server provides a tool (Patrocles finder) that allows the user to determine whether his favorite mutation/polymorphism may perturb miRNA-mediated gene regulation of custom target sequences. Specifically, Patrocles finder allows convenient examination of the miRNA target site content of a sequence of interest and examination of the effect of genetic variants in that sequence on target-site content. Patrocles finder analyzes both isolated sequences as well as alignments of orthologous sequences in FASTA format. When selecting the latter option, Patrocles finder provides direct information about the conservation or not of the identified miRNA target sites.

Aim of the thesis

The research project that I presented at the beginning of my doctorate was planned to focus on the study of genetic factors involved in TSE resistance/susceptibility in goats. In 2008, when the doctoral course started, knowledge on the role of *PRNP* genetics in goats was preliminary, especially if compared with data available for sheep. In the meantime, some studies on goat *PRNP* genetics were published, all looking at the PrP coding sequence. Results from these studies clearly indicated the existence of polymorphisms in the *PRNP* open reading frame conferring resistance or, at least, lower susceptibility to TSEs in goats. However, the situation in goats seems to be different from sheep. Actually, the polymorphisms at codons 136, 154 and 171 of the ovine *PRNP* gene are generally diffused in the sheep population and this allowed the implementation by the European Union of breeding programmes towards scrapie resistance in each Member State according to regulation (EC) 999/2001. Currently, in goats, the allele carrying lysine (K) at the PrP codon 222 is the best candidate polymorphism proposed to enhance resistance to scrapie and proved to have a protective role both by case-control studies and, very recently, by experimental inoculation. Nevertheless, the frequency of the K222 mutation is very low or even absent in a number of goat breeds limiting so far the feasibility of a genetic approach for controlling scrapie also in this species. Therefore, the knowledge of other genetic factors involved in the modulation of the response to the scrapie agent in goats could represent an effective control tool as they could be employed in association with the genetic selection based on *PRNP* genetics. For instance, in breeds where the frequency of the desired *PRNP* polymorphisms is very low or null, the existence of other genetic targets could allow to initiate disease control and, at the same time, to increase/introduce 222K carriers balancing selection towards scrapie resistance and preservation of genetic diversity.

On these basis, the main aim of my research project was to look for TSE genetic factors outside the goat *PRNP* open reading frame by the molecular

characterization of the *PRNP* gene regulatory regions and the assessment of the role of the Shadow of the Prion Protein gene (*SPRN*), a recently discovered member of the prion family. In the first part of the research project, the first detailed polymorphism study and corresponding haplotype analysis was performed to characterize the goat *PRNP* regulatory regions within 3 kb of the transcription start site. A bioinformatic analysis has been applied to the primary sequence data generated to identify putative transcription motifs and the impact of polymorphisms possibly involving putative transcription motifs has been evaluated. Finally, a comparative analysis with respect to other mammalian species has been performed. In the second part of the study, the analysis of the *SPRN* genes of goats from several scrapie outbreaks was carried out in order to detect polymorphisms and to look for association between *SPRN* alleles and the occurrence of scrapie by a case-control study approach. The *SPRN* gene is a third discovered member of the prion protein gene family along with *PRNP* and *PRND* (encoding Doppel protein, Dpl). *SPRN* encodes Shadoo protein (Sho) which shares several structural and biochemical features with PrP. As an association of *SPRN* with human prion diseases has been already demonstrated, this gene represent a promising candidate to play a role in prion diseases also in other species. However, at the time of writing, data on *SPRN* variability in goats are still very limited and the existence of a potential link between *SPRN* mutations and scrapie completely unknown.

Materials and Methods

3.1 Animals and DNA isolation

To characterize the goat *PRNP* promoter a DNA panel consisting of n = 32 goats belonging to the following European breeds: Camosciata, Garganica, Maltese, Siriana, Ionica, Moncaina, Pirenaica, Retinta and the Hellenic breed (*Capra prisca*) was used (Table 4). Information regarding the *PRNP* open reading frame (*ORF*) genotypes for all goats were available. Animals included in the study were unrelated. Genomic DNA was isolated from whole blood using the GenElute Mammalian Genomic DNA kit (Sigma-Aldrich) according to the manufacturer's protocol. None of the goats utilized in this study had any known history or clinical symptoms of TSE.

To study the relationship between the *SPRN* gene and scrapie, a panel of 424 goats was enrolled in the study, including 21 scrapie-positive cases (age range, 3-10 years) and a control group represented by 403 scrapie negative goats taken from ten herds (animals per herd range, 3-103) that had scrapie outbreaks between 2002 and 2010. For scrapie diagnosis, the obex region was examined by histopathology, immunohistochemistry and/or Western blotting. The animals were primarily of the Maltese, Red Mediterranean, Capra dei Nebrodi and crossed breeds. Their age ranged from 1 to 9 years. Routine rapid tests were performed on the obex region of control animals. Table 5 summarizes the geographic distribution of the herds and goat breeds.

Table 4. Animals used for the study of the *PRNP* regulatory regions.

Breed	Samples (n)	Country
Camosciata	8	Italy - IZSPLV
Garganica	3	Italy - IZSPLV
Maltese	3	Italy - IZSPLV
Siriana	3	Italy - IZSPLV
Ionica	6	Italy - ISS
Capra prisca	3	Greece - CERTH-INA
Moncaina	2	Spain - UNIZAR
Pirenaica	2	Spain - UNIZAR
Retinta	2	Spain - UNIZAR

Table 5. Herds enrolled in the *SPRN* case-control study.

Herd	Region	Breed
1	Sicilia	Capra dei Nebrodi
2	Basilicata	Incrocio
3	Sicilia	Capra dei Nebrodi
4	Marche	Incrocio
5	Lazio	-
6	Lombardia	Saanen
7	Sicilia	Incrocio

3.2 Goat *PRNP* and *SPRN* PCR amplification

The regulatory regions of the *PRNP* gene were analysed by generation of two overlapping amplicons covering 2 kb upstream and 1 kb downstream of the gene transcription start site (Fig. 9). This 3 kb gene sequence included exon I and the putative *PRNP* promoter. Oligonucleotide primers were obtained from Invitrogen (Carlsbad, USA) and the first pair [1F (+) 5'-GAGAAGTGGCAATGTAGCTTCC-3'; 1R (-) 5'-GGGGGTTTAAAGGACTACGTG-3'] was designed starting from a multiple alignment of ovine and bovine promoter sequences. The second primer pair was based on the bovine sequence as previously published [PRNP49443_F (+) 5'-AGCCGCAGAGCTGAGAG-3'; PRNP50640_R (-) 5'-ACCCGGCCATGAGATAAG-3'; Sander et al. 2004]. The two amplified fragments align to the target *PRNP* DNA at corresponding nucleotides 3807-5747 (1F-1R), 5680-6869 (PRNP49443_F-PRNP50640_R) of the ovine *PRNP* sequence (GenBank acc. no. DQ077504). PCR reactions were carried out using GeneAmp 9700 PCR Systems (Applied Biosystems, Foster City, USA) with the following conditions: 100 ng of genomic DNA, 50 pmol of each primer, 100 μM of dNTPs (Fermentas), 1,5 unit of Taq polymerase (HotStarTaq, Qiagen) in a final volume of 50 μl of buffer consisting of Tris·Cl, KCl, $(NH_4)_2SO_4$ additioned of Q-solution (Qiagen) and 1.5 mM $MgCl_2$. Thermocycling parameters consisted of an initial denaturation step (95 °C, 15 min) followed by 40 cycles of denaturation (94 °C, 1 min), annealing (58 °C, 1 min) and extension (72 °C, 1 min). Additionally, primers p8(+) and p9(-) annealing on the *PRNP* exon 3 were used to amplify the open reading frame (*ORF*) for all studied goats as previously described (Bossers et al., 1996).

A region of the caprine *SPRN* gene, including the complete ORF and fragments of the 5'UTR and 3'UTR (Fig. 10), was amplified by setting up 25 μl PCR reactions containing: 0,4 mM dNTPs (Fermentas), 10x reaction buffer

(Roche), 1,5 mM $MgCl_2$, 5x GC-rich solution (Roche), 2U FastStartTaq DNA Polymerase (Roche) and 0,4 µM of each primer SPRN-117d (5'-TGTGCCTGGGCCCTGA-3') and SPRN+166u (5'-CCGGCGGGAGGTGTCACA-3'); 100-200 ng of genomic DNA were used as template. The following thermal profile was employed: activation of Taq polymerase (95°C, 10 min.), followed firstly by 7 cycles using a touch-down protocol (95°C, 1 min. - 68°C, 30 sec. - 72°C for 1 min.), and then by 35 cycles at 95°C for 1 min., 61°C for 30 sec., 72°C for 1 min., with a final extension at 72°C for 10 mins..

All goat *PRNP* and *SPRN* amplicons were examined by 1.5% agarose gel electrophoresis and products with the appropriate length were directly purified by the silica column method (NucleoSpin Extract II, Macherey-Nagel) according to the manufacturer's protocol.

Fig. 9. Organization of the caprine *PRNP* gene and PCR amplification strategy.

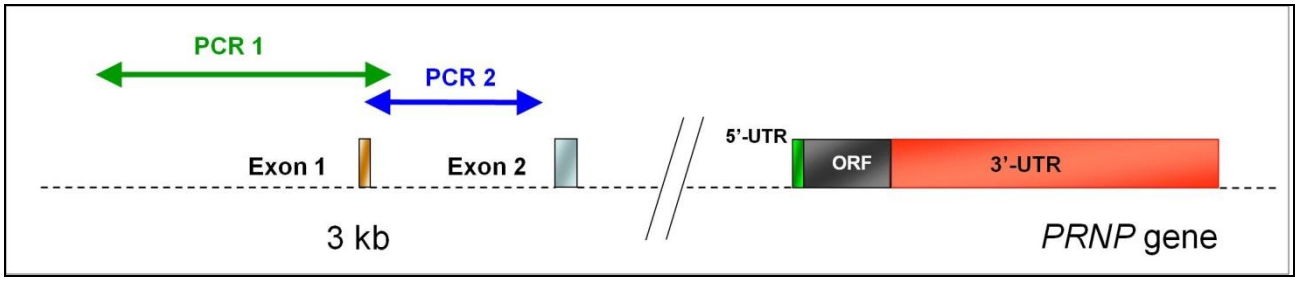
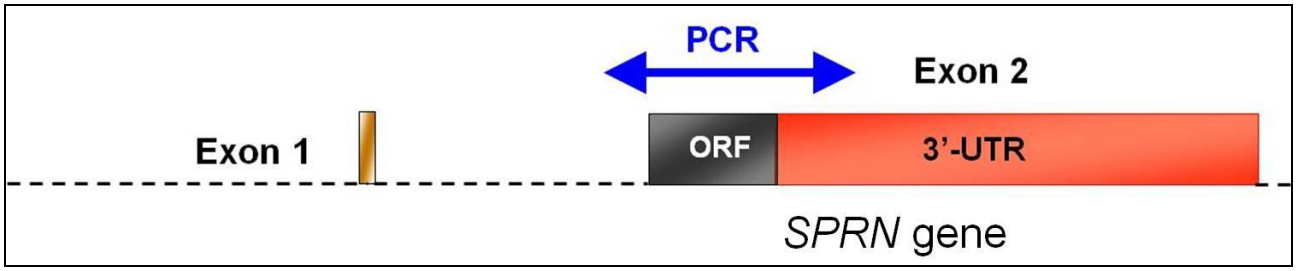


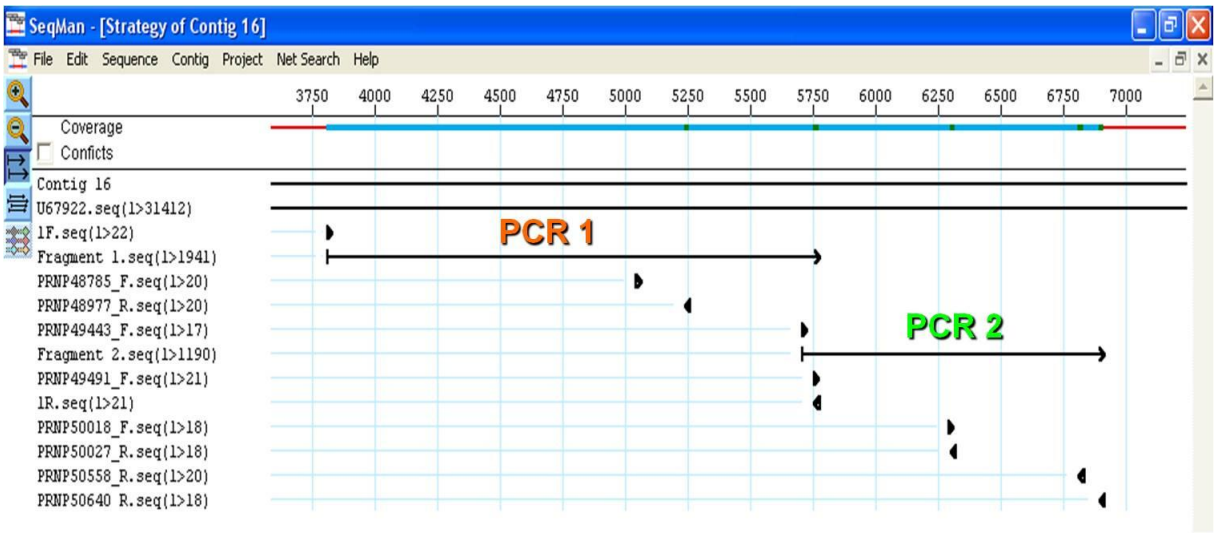
Fig. 10. Organization of the caprine *SPRN* gene and PCR amplification strategy.



3.3 Cloning and sequencing of the goat *PRNP* and *SPRN* genes

The goat *PRNP* and *SPRN* sequences were determined by direct DNA sequencing of the PCR products by Big Dye terminator cycle-sequencing using the amplification primer pairs (*SPRN*) and eight internal sequencing primers (*PRNP*). Sequencing strategy was designed to ensure electropherogram overlap (Fig. 11), thereby enabling both correct assemblies and verification of observed SNPs. For *PRNP* sequencing, primers annealing on the first fragment were 4219F (+) 5'-AGAATAGGACCAAAGGC-3', 5034F (+) 5'-CCATCCTTCACATGAGA-3', 4593R (-) 5'-GGCTGTTTTCTATCCAG-3' and 5320R (-) 5'-CAAAAATTCTACTACCCTG-3'. Primers annealing on the second fragment were PRNP49491_F, PRNP50027_R, PRNP50018_F and PRNP50558_R (Sander *et al.*, 2004).

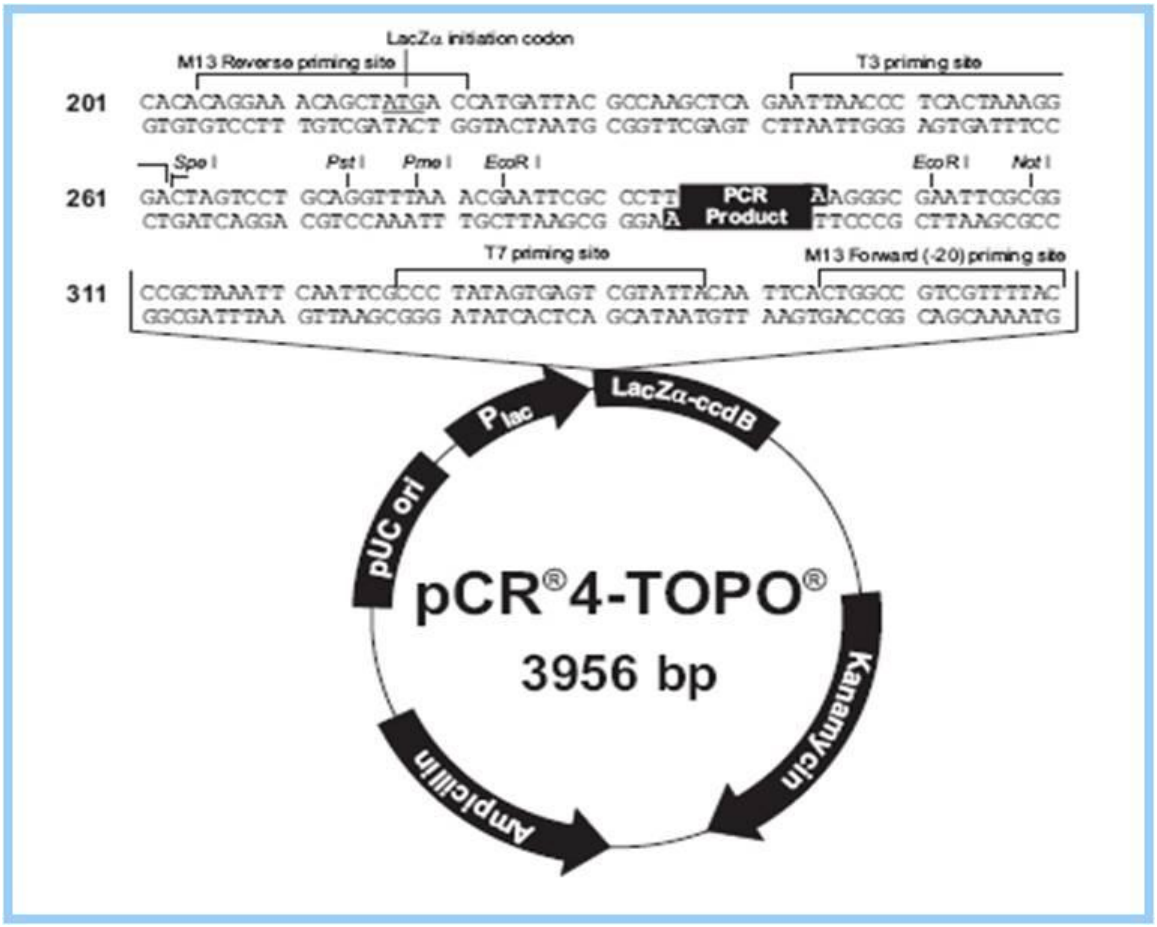
Fig. 11. Sequencing strategy of the caprine *PRNP* regulatory regions.



Cycle-sequencing reactions were prepared in a final volume of 10 µl as follows: 150 ng of purified amplicon, 2 µl Big Dye v. 3.1 (Applied Biosystems), 1 µl Sequencing Buffer, 3.2 pmol primer. Thermal cycling parameters followed those recommended by the manufacturer. Purified goat *PRNP* and *SPRN* amplicons were directly sequenced using the aforementioned

internal primers on an ABI Prism® 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For *PRNP* analysis, PCR products from selected heterozygous samples were cloned into TOPO TA cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions (Fig. 12).

Fig. 12. Map showing the features of TOPO TA vector and the sequence surrounding the cloning site.



At least 12 recombinant cloned plasmids were analysed by multiple sense sequencing adding to the employed sequencing primer panel the oligos provided with the kit [M13 forward (+) 5'-CGCCAGGGTTTTCCCAGTCACGA-3' and M13 reverse (-) 5'-

AGCGGATAACAATTTCACACAGGA-3’] annealing on the vector sequence and flanking the insert.

3.4 Sequence analysis

Goat *PRNP* and *SPRN* sequences were analysed using the program Sequencing Analysis 5.2 (Applied Biosystems). Electropherograms were evaluated by manual inspection and looking at the Sequencing Analysis quality score. Uncertain bases and presumed heterozygous nucleotides were flagged automatically by the program and subsequently assigned by analysis of multiple overlapping sequences. All goat *PRNP* and *SPRN* sequences were assembled using the program SeqMan II v. 5.03 (DNASTAR inc., Madison, USA) in order to obtain a consensus sequence for each sample. All heterozygous nucleotides were annotated according to the International Union of Pure and Applied Chemistry (IUPAC) and the final consensus sequences were assembled into a single data set. Each variable site was enumerated based on the corresponding position in the ovine promoter *PRNP* and caprine *SPRN* reference sequences (Genbank DQ077504 and EU559165, respectively).

3.5 *PRNP* haplotype analysis

A dataset including all the individual goat *PRNP* genotypes was set up. Deviations from Hardy-Weinberg equilibrium (HWE) have been reported to increase the error rate associated with haplotype inference algorithms (Niu et al., 2002; Stephens and Donnelly, 2003). In order to minimize the error rate HWE was assessed for each polymorphism by the chi-square test (http://www.genes.org.uk/software/hardy-weinberg.shtml). Estimates for the goat *PRNP* haplotype frequencies and the most likely haplotypes were reconstructed using a Bayesan statistical method implemented within the software package PHASE 2.1 (Stephens et al., 2001; Stephens and Donnelly,

2003). Only goat polymorphisms with genotype distributions similar to HWE expectations and minor allele frequencies greater than 0.10 were included in the haplotype reconstruction analyses.

3.6 *PRNP* promoter prediction and comparative analysis

A final *PRNP* consensus sequence, representative of the goat panel analysed in this study, was obtained by the multiple alignment of the consensi derived from each sample using BIOEDIT 7.0.5.3 program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The primary sequence data generated were screened against a library of repetitive elements using Repeat Masker web-based computer service (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) and submitted to the mammalian-specific program CpGProD (Ponger and Mouchiroud 2002; http://pbil.univ-lyon1.fr/software/cpgprod_query.html) to perform promoter prediction analysis. CpGProD has been previously reported to give accurate results based on the homology of *PRNP* regulatory regions predicted for elk and mule deer with sequence regions known to possess promoter activity in both cow and sheep (Seabury et al., 2007). Additional bioinformatic analyses were performed using TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) to study the promoter and look for possible transcriptional regulatory elements. The goat *PRNP* consensus sequence was edited manually using EditSeq 5.03 (DNASTAR inc., Madison, USA) in order to insert singularly each polymorphic variant before submission to TFSEARCH analysis. This approach was aimed to look at the influence of the different variants on the creation/deletion of potential transcription factor binding motifs. Goat *PRNP* sequences were also evaluated for the presence of four conserved motifs previously identified within the

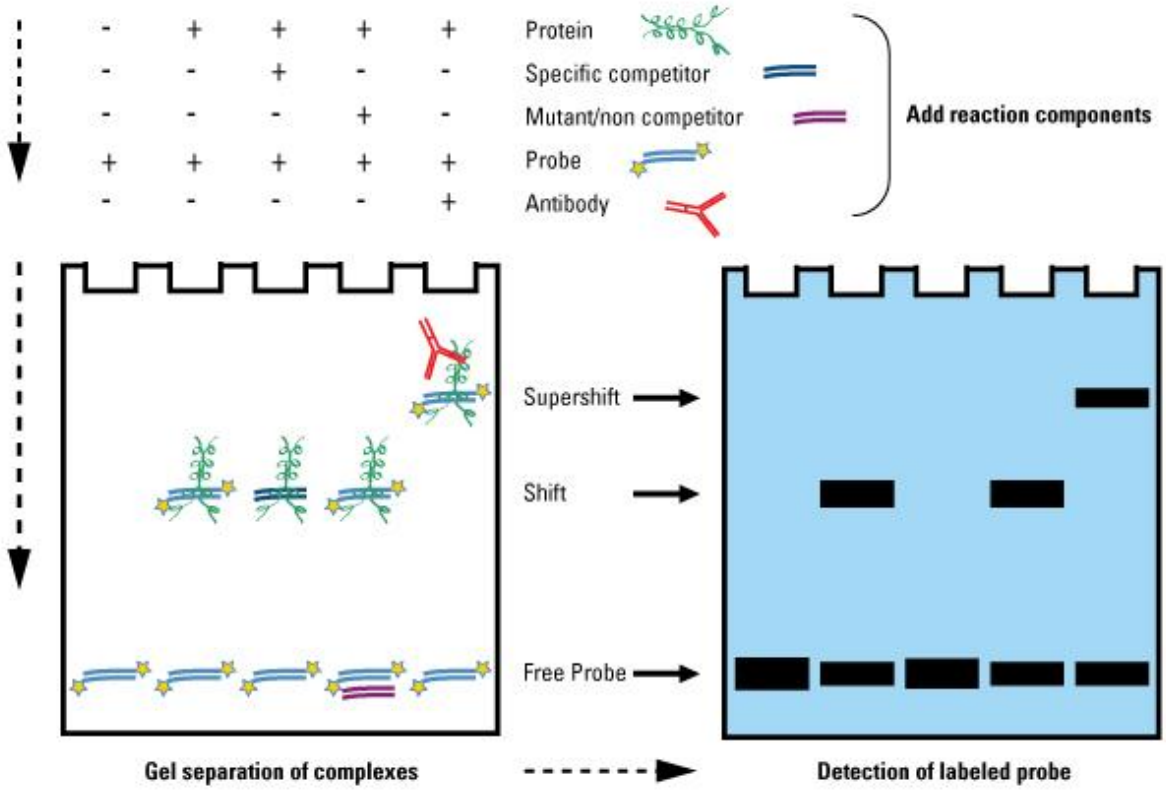
PRNP promoter sequences for human, Syrian golden hamster, sheep, mouse, rat, and cow (Westaway et al. 1994; Saeki et al. 1996; Inoue et al. 1997).

3.7 Electrophoretic mobility shift assays (EMSA)

The EMSA technique is based on the observation that protein:DNA complexes migrate more slowly than free linear DNA fragments when subjected to non-denaturing polyacrylamide or agarose gel electrophoresis (Fig. 13) . Because the rate of DNA migration is shifted or retarded when bound to protein, the assay is also referred to as a gel shift or gel retardation assay. The ability to resolve protein:DNA complexes depends largely upon the stability of the complex during each step of the procedure. During electrophoresis, the protein:DNA complexes are quickly resolved from free DNA, providing a "snapshot" of the equilibrium between bound and free DNA in the original sample. The gel matrix provides a "caging" effect that helps to stabilize the interaction complexes: even if the components of the interaction complex dissociate, their localized concentrations remain high, promoting prompt reassociation. Additionally the relatively low ionic strength of the electrophoresis buffer helps to stabilized transient interactions, permitting even labile complexes to be resolved and analyzed by this method.

The gel shift assay consists of three key steps: (1) binding reactions, (2) electrophoresis, (3) probe detection. The order of component-addition for the binding reaction is often critical. Completed binding reactions are best electrophoresed immediately to preserve potentially labile complexes for detection. This idealized example shows complete elimination of the protein:probe complex with addition of specific competitor or protein specific antibody. However, only a reduction in intensity is observed rather than the complete elimination of bands (Fig. 13).

Fig. 13. Overview of the Gel Shift Assay Method.



Typically, linear DNA fragments containing the binding sequence(s) of interest are used in EMSAs. If the target DNA is short (20-50 bp) and well defined, complementary oligonucleotides bearing the specific sequence can be economically synthesized and annealed to form a duplex. For most applications and sequences, standard desalting of the oligonucleotides yields sufficient purity for use in EMSA. However, for sequences that form strong secondary structure or have long repeats, gel- or HPLC-purification may be required to ensure that the majority of the product is of the correct length and sequence for the experiment.

In our experiments, complimentary pairs of oligonucleotides (Eurofins MWG, Germany) biotinylated at the 3' end and containing the conserved motif sequences were annealed forming short double-stranded DNA fragments (probes). The following probes (only top strand sequence given) were used:

Goat1, 5′-CTCCACCCAGCCCAAGACT-Bio-3′; Goat2, 5′-CTCCACCCAGCTCAAGACT-Bio-3′; Goat1FL, 5′-GGACTGCGGGCTTCCGGCGGGCGCGGGGAACGT-Bio-3′.

DNA probes with known binding sites were used as positive controls. Transcription factors were contained in commercially available nuclear extracts generated from fibroblast cell line 3T3, from macrophage cell line RAW and from total mouse brain (all Active Motif, LaHulpe, Belgium). EMSAs were performed using the gel shift assay kit (Thermo Scientific). Reactions consisted of 2 µl of 5× binding buffer (250 mm NaCl, 50 mm Tris-HCl, pH 7.5, 2.5 mm EDTA, 2.5 mm dithiothreitol, 5 mm $MgCl^2$, 20% glycerol, 0.5 µg poly(dI·dC)·poly(dI·dC)), 1–2 µl (10–20 µg) NE, and dH_2O to a total volume of 20 µl incubated at 25 °C for 10 min. To the reaction mixture 1 µl of biotnylated double-stranded DNA probe (0.035 pmol, ~10,000–50,000 cpm/10–100 fmol) was added and further incubated at 25 °C for 20 min. For competition experiments a 200-fold molar excess of unlabeled competitor and/or non-competitor (NC-1) double-stranded DNA probes were added. The reactions were electrophoresed in a 6% non-denaturing polyacrylamide gel in 0.5× Tris-buffered EDTA at 100 mA at room temperature for 1 h. DNA was then blotted onto nylon membrane for 40 min at 380mA. After UV cross-linking, biotin/strepavidine interaction was monitored by chemiluminescence detected by exposure to x-ray film.

3.8 Statistical analysis (*SPRN* case-control study)

To assess the role of *SPRN* polymorphisms in the susceptibility of goats to scrapie a case-control study was carried out. The "case" was considered as an animal diagnosed definitively scrapie positive after the compulsory confirmatory diagnostic tests. The "control" was an animal coming from the same herds as the cases and therefore exposed to the scrapie agent, but resulted negative for scrapie by routine rapid testing. To estimate the relationship between the polymorphisms in the host *SPRN* gene and the occurrence of scrapie we carried out a preliminary univariate analysis computing Pearson χ^2 test and then a logistic regression model was fitted including the variables resulted positively associated with the outcome (i.e. the scrapie status). We considered information about *SPRN* gene polymorphisms, goat breed, herd and age. In the regression model the number of the positive cases was included as dependent variable to estimate the role of the polymorphisms in the host *SPRN* gene. The animal breed was included into the model as covariate in order to assess its potential effect. After analysing the distribution of the cases and the controls for the different breeds, we aggregated all the pure breeds in an unique category and we compared its effect with the cross-bred animals. To avoid biased results due to the prevailing effect of *PRNP* polymorphisms in the control group, 49 controls that resulted to be carriers of the protective 222K allele were excluded from the analysis. Finally, 375 animals were entered in the model. As the *PRNP* genotyping was available only for codon 222 in the control animals, we took into account the possible occurrence of histidine (H) at codon 154 (another protective allele in goats) by a simulation model considering the frequencies of the 154H allele reported for different Italian breeds (Acutis et al., 2008). Specifically, we considered 154H allele frequencies of 11.3% for the Camosciata delle Alpi, 0% for the Saanen, 11.2% for the Garganica, 6% for the Maltese, 7.4% for the Ionica, 5.3% for the

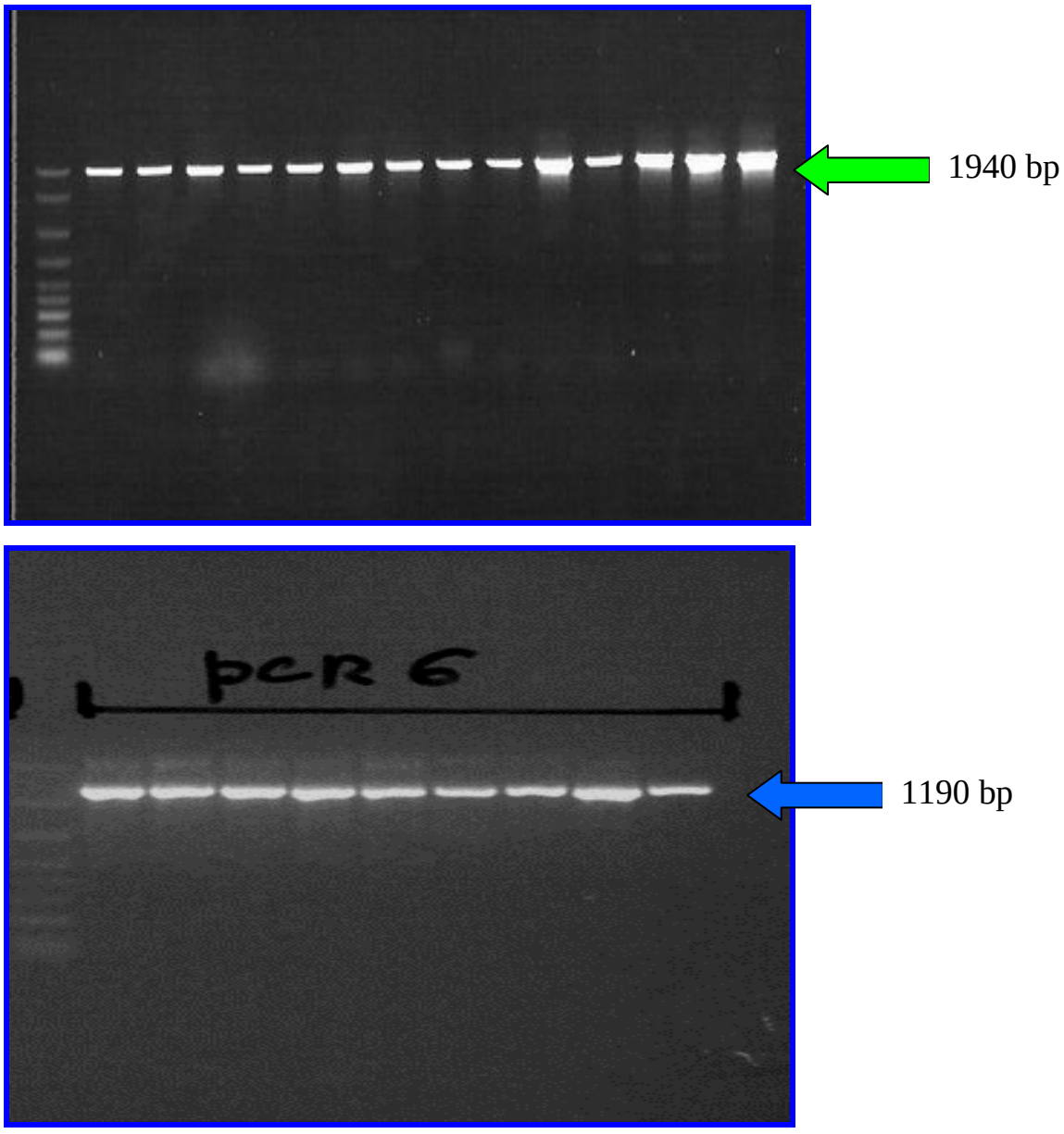
Siriana, 8% for the Capra dei Nebrodi and 5% for the cross-bred. For other goat breeds we set 154H frequencies equal to 50%. A Monte Carlo simulation was performed to simulate the occurrence of the 154H allele in the control group executing 5000 replications and a logistic regression for each simulation. In the regression model the number of the positive cases was included as dependent variable while the animal breed (all pure breeds vs cross-bred) and the simulated 154H allele frequencies were included as covariates. The mean of the estimated odds ratios and their significance for the included variables was analysed for the total simulations. All descriptive statistics and data manipulation were performed using Stata Statistical Software (StataCorp LP).

Results and Discussion

4.1 Molecular characterization of the putative regulatory regions of the prion protein gene (*PRNP*) in goats

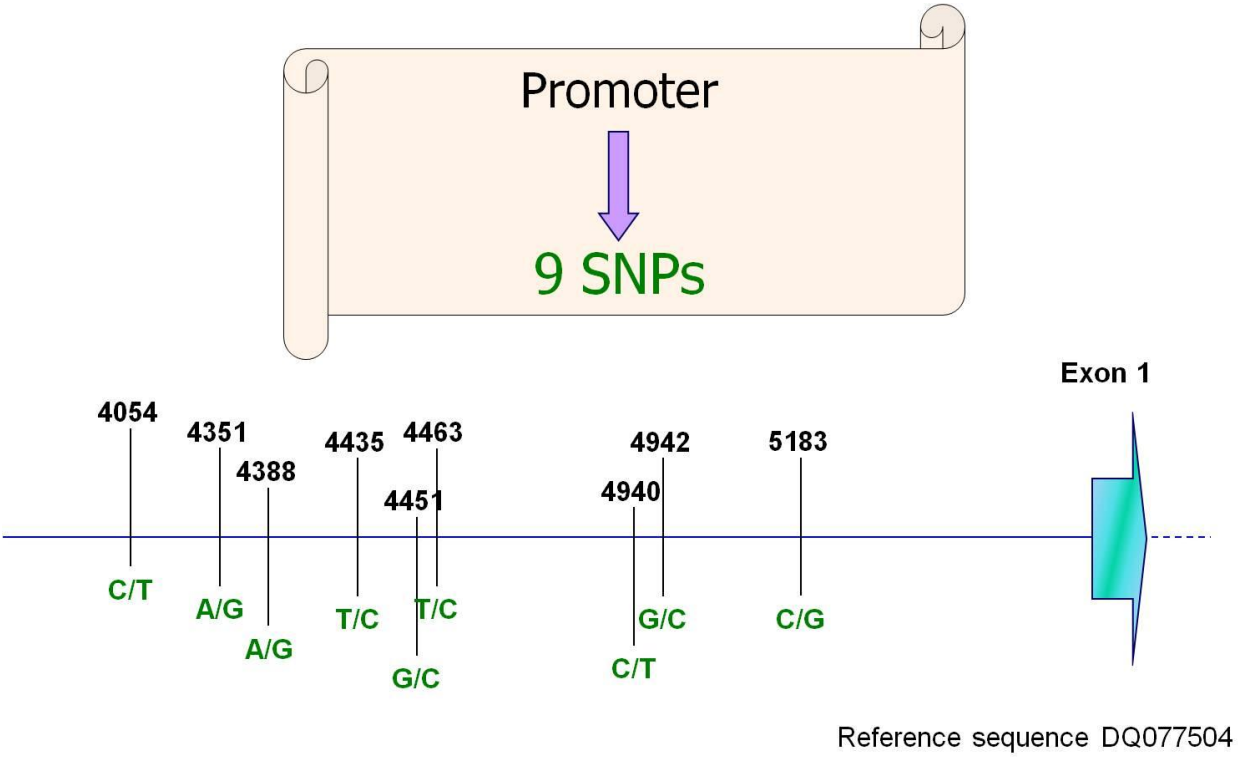
PCR products of the expected molecular size (approximately 1940 bp and 1190 bp, respectively) were obtained using primers and optimized protocols described in the Methods section (Fig. 14).

Fig. 14. PCR products corresponding to the putative goat *PRNP* regulatory regions.



Sequence analysis of 3 kb *PRNP* non coding region of goats from different European breeds resulted in the identification of 26 previously unreported diallelic SNPs (Fig. 14 and Fig. 15).

Fig. 14. Graphical representation of the polymorphisms identified in the goat *PRNP* promoter (numbering referred to ovine ref. seq. DQ077504).



Only one SNP was detected within the predicted exon 1 of a goat belonging to Ionica breed. Moreover, analysis of electropherograms and sequence alignments yielded evidence of three novel indels among the studied samples (Fig. 18). Indel polymorphisms were not found in homozygosis in the analysed animals therefore they were firstly observed as frame shifts in the sequence electropherograms (Fig. 15) and then confirmed by cloning of heterozygous samples (Fig. 16) and alignment of single chromosomal sequences (Fig. 17).

Fig. 15. Electropherogram presenting a frame shift (arrow) due to an indel polymorphism in heterozygosis.

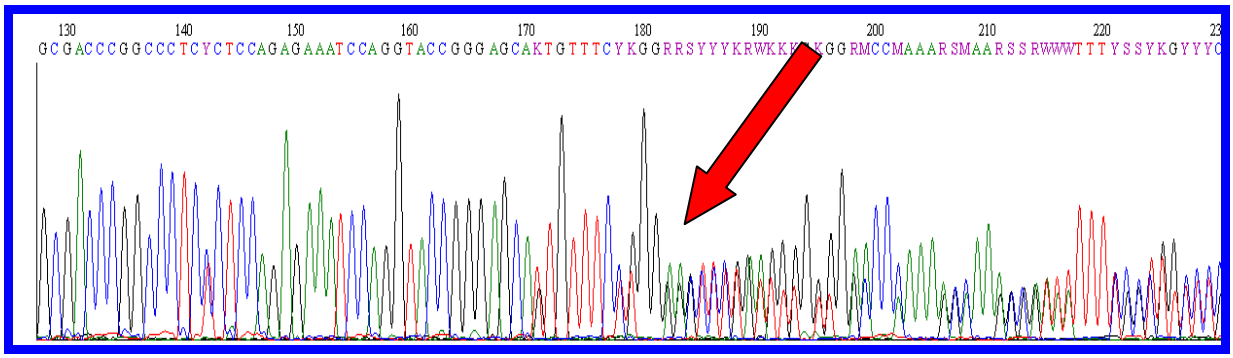


Fig. 16. PCR performed on recombinant *E.coli* colonies for *PRNP* indel characterization.

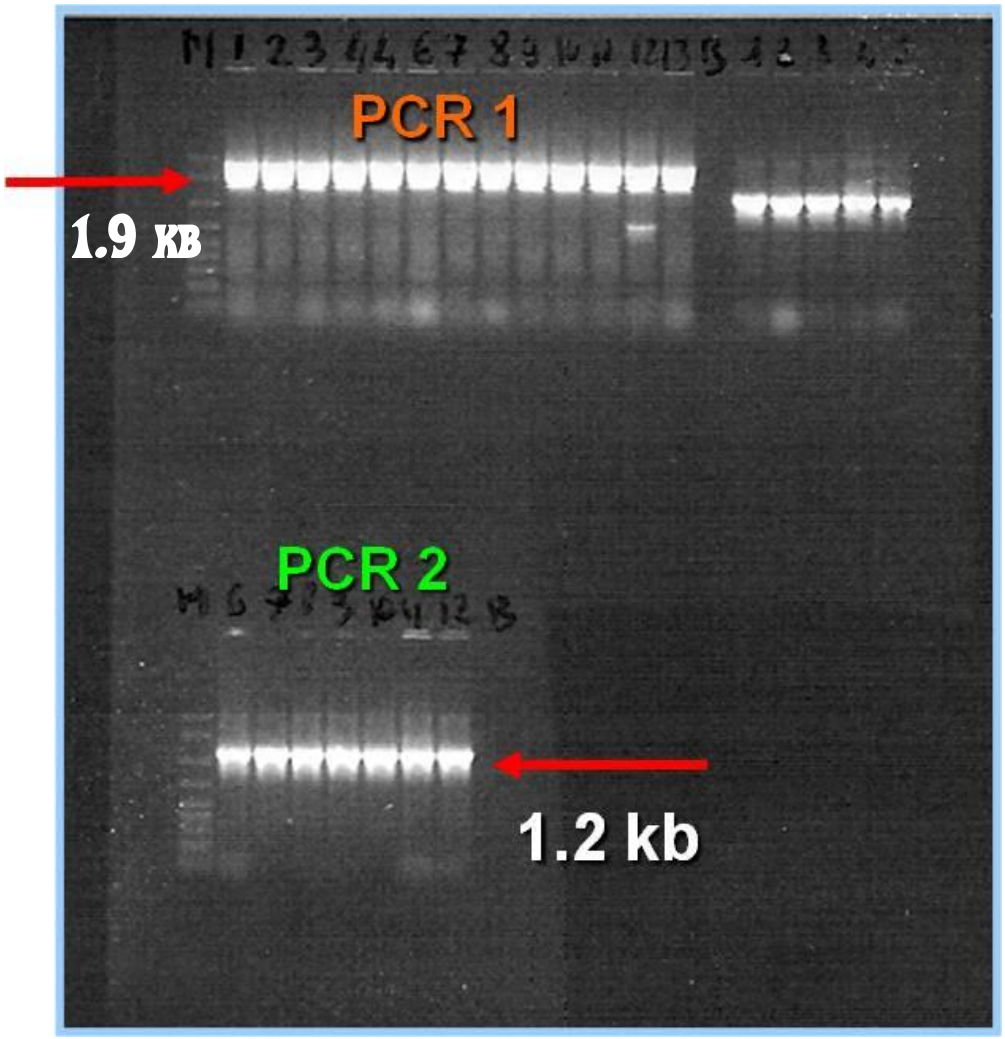
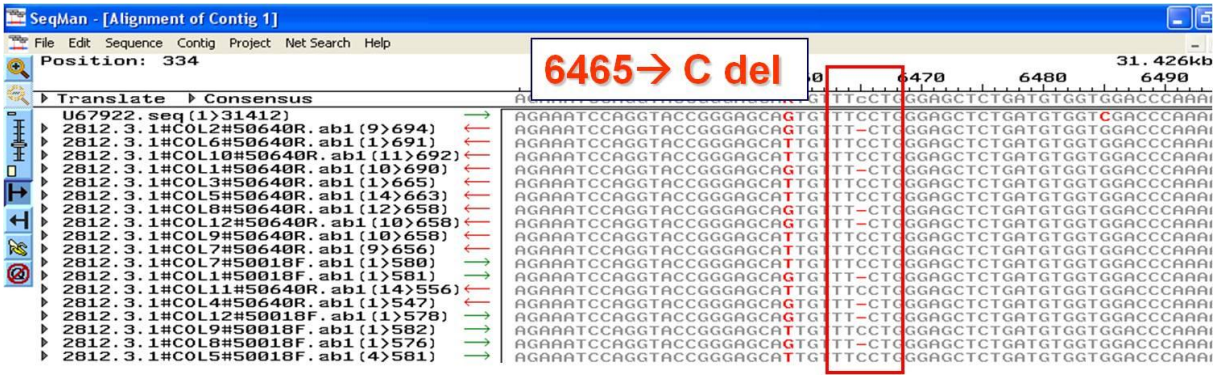
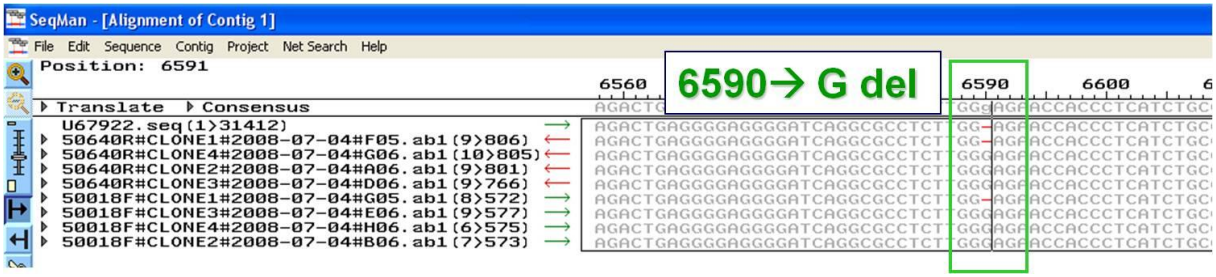


Fig. 17. Alignment of sequences derived from recombinant *E coli* clones. Each panel (A, B, C) corresponds to an indel polymorphism identified in the goat *PRNP* intron I. Numbering is referred to ovine ref. seq. DQ077504.

A.



B.



C.

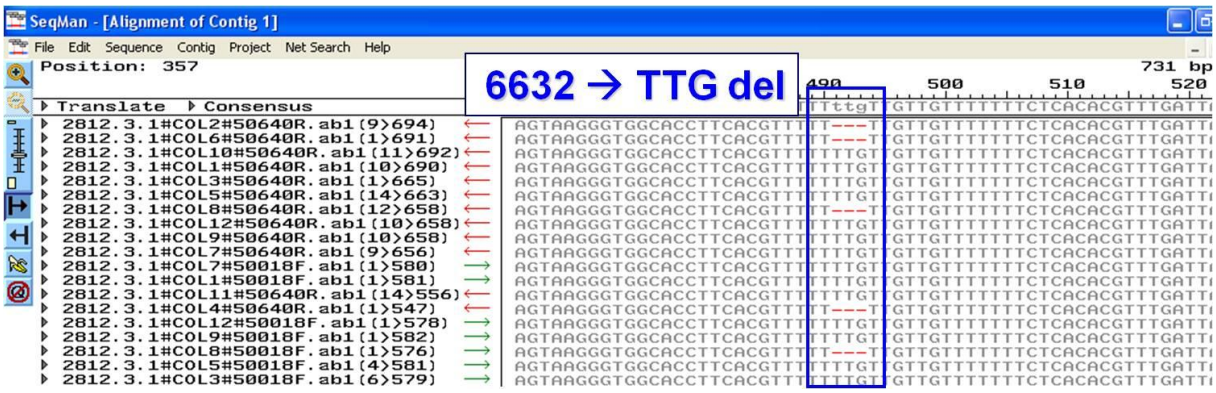
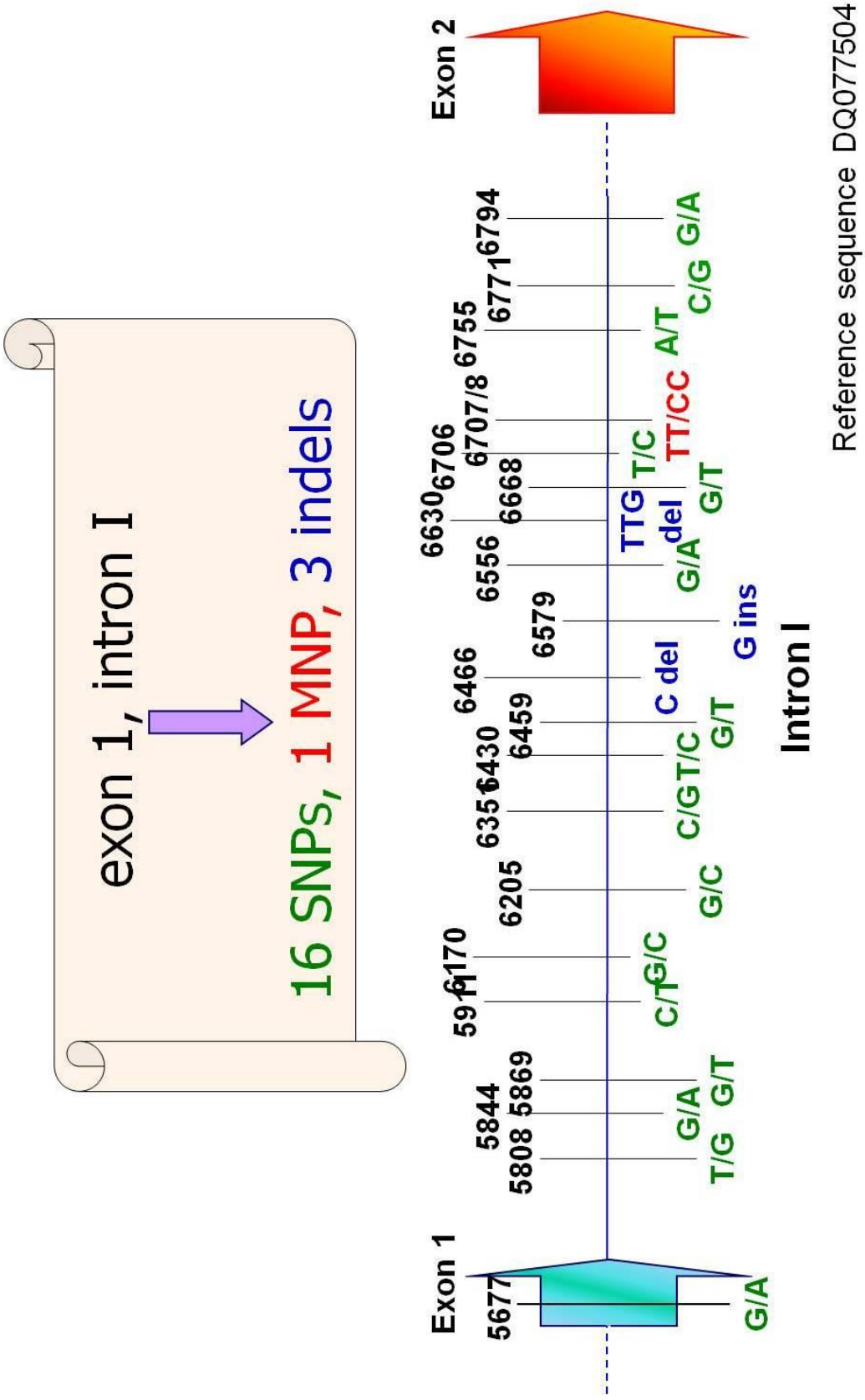


Fig. 18. Graphical representation of the polymorphisms identified in the goat *PRNP* exon 1 and Intron I (numbering referred to ovine ref. seq. DQ077504).



A list of the identified polymorphisms with the indication of their position in the goat *PRNP* and relative allele frequencies is shown in Table 6.

Table 6.

Goat *PRNP* position (*U67922*)	Goat *PRNP* region (Ref Seq DQ077504)	SNPs; alleles observed	Goat *PRNP* major/minor allele *f*
4054	Promoter	Y; (C/T)	0.91/0.09
4351	Promoter	R; (A/G)	0.27/0.73
4388	Promoter	R; (A/G)	0.35/0.65
4435	Promoter	Y; (T/C)	0.86/0.14
4451	Promoter	S; (G/C)	0.49/0.51
4463	Promoter	Y; (T/C)	0.56/0.44
4940	Promoter	Y; (C/T)	0.48/0.52
4942	Promoter	S; (G/C)	0.48/0.52
5183	Promoter	S; (C/G)	0.92/0.08
5677	Exon 1	R; (G/A)	0.98/0.02
5808	Intron I	K; (T/G)	0.74/0.36
5844	Intron I	R; (G/A)	0.74/0.36
5869	Intron I	K; (G/T)	0.94/0.06
5911	Intron I	Y; (C/T)	0.98/0.02
6171	Intron I	S; (G/C)	0.72/0.28
6206	Intron I	S; (G/C)	0.49/0.51
6352	Intron I	S; (C/G)	0.67/0.33
6430	Intron I	Y; (T/C)	0.97/0.03
6459	Intron I	K; (G/T)	0.56/0.44
6465	Intron I	C del	0.92/0.08
6556	Intron I	R; (G/A)	0.51/0.49
6590	Intron I	G del	0.92/0.08
6632	Intron I	TTG del	0.82/0.18
6668	Intron I	K; (G/T)	0.95/0.05
6706	Intron I	Y; (T/C)	0.82/0.18
6707/8	Intron I	TT/CC	0.69/0.31
6755	Intron I	W; (A/T)	0.55/0.45
6771	Intron I	S; (C/G)	0.53/0.47
6794	Intron I	R; (G/A)	0.98/0.02

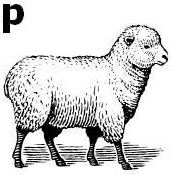
No genetic variation was observed within the four conserved *PRNP* promoter motifs previously described for mouse, sheep, human, Syrian golden hamster, rat, and cow (Westaway et al., 1994; Saeki et al.1996; Baybutt and Manson 1997; Inoue et al. 1997) (Fig. 19).

Fig. 19. Alignment of four promoter motifs conserved across mammalian *PRNP* sequences.

	Motif 1	Motif 2	Motif 3	Motif 4
	5’			3’
Mouse	CTTTCATTTTCTC	CCATTATGTAACG	TAAAGATGATTTTTA	TCAGGGAG
Rat	CTTTCATTTTCTC	CCATTATGTAACG	TAAAGATGACTTTTA	TCACAGAG
SHa	CTTTCATTTTCTC	CCATTATGTAACG	TAAAGATGATTTTTA	TCAGGGAG
Sheep	CTTTCATTTTCTC	CCATTACGTAACG	TAAAGATGATTTTTA	TCAGGGAG
Cow	CTTTCATTTTCTC	CCATTACGTAACG	TAAAGATGATTTTTA	TCAGGGAG
Human	CTTTCATTTTCTC	CCATTATGTAACG	TAAAGATGATTTTTA	TCAGGGAG
Elk	CTTTTATTTTCTC	CTATTACGTAACG	TAAAGATGATTTTTA	TCGGGGAG
Mule deer	CTTTCATTTTCTC	CTATTACGTAACG	TAAAGATGATTTTTA	TCAGGGAG
Goat	CTTTCATTTTCTC	CCATTACGTAACG	TAAAGATGATTTTTA	TCAGGGAG

PRNP promoter prediction analyses for cow (AJ298878), sheep (DQ077504), and goat are depicted in Table 7. CpGProD software predicted a single plus-strand goat *PRNP* promoter with a predicted probability to be located over this strand corresponding of 0.8857 (strand-p). The putative regulatory region was identified by the software between nucleotides 1405 and 3005 of the query (3005 nt masked consensus sequence submitted) corresponding to bases 5265-6859 and 49028(3743)-50630(5361) of the homologous sheep and cattle *PRNP* sequences, respectively.

Table 7. *PRNP* promoter prediction and comparative analyses.

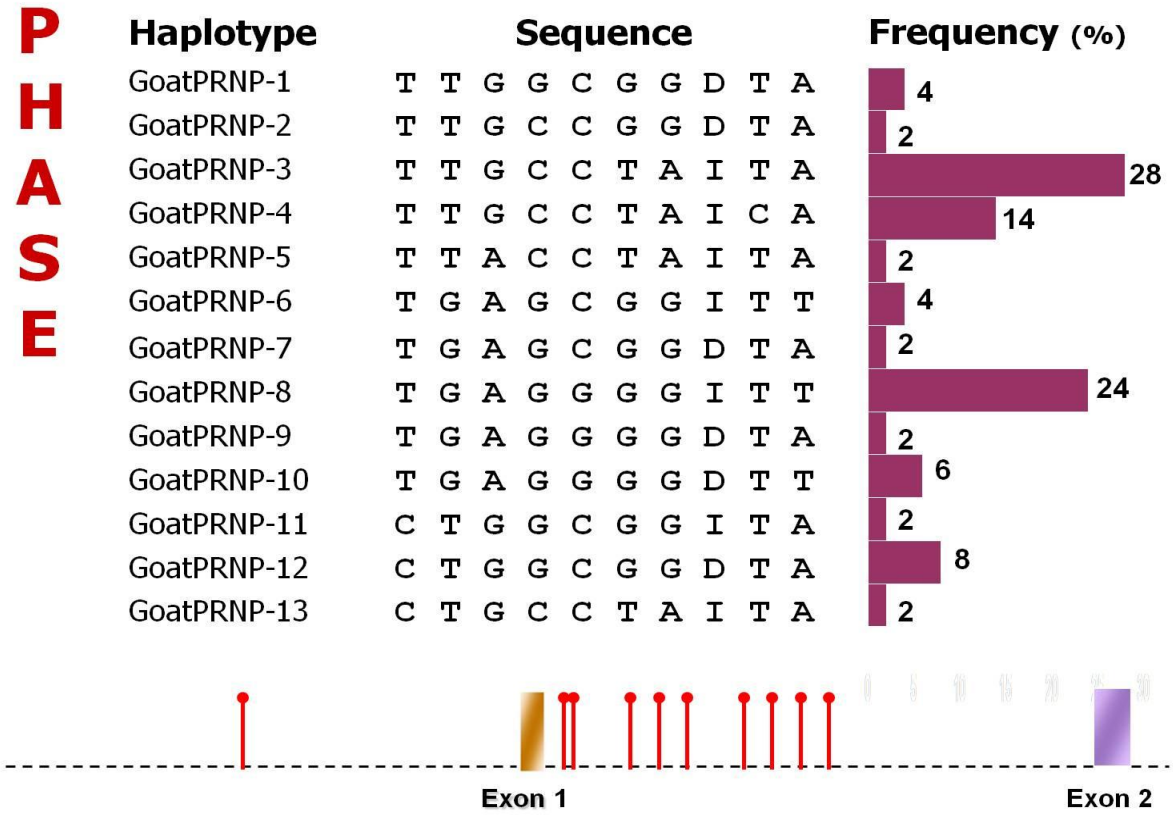
Species (*PRNP*)	CpGProD	*PRNP* region of known promoter activity	Homologous goat PRNP promoter sequence
Cow 	48984-50686 (plus)	49339-49400; 49551-50313	1693-1750 1899-2646
Sheep 	5193-6852 (plus)	5226-5747	1365-1887
Goat 	1411-3012 (plus)	-	-

Nucleotide sequence alignment of the predicted goat *PRNP* promoter (CpGProD) with the *PRNP* region of known promoter activity in sheep (O'Neill et al., 2003) revealed the presence of one SNP in the homologous region of goat (5677-G/A, Table 6). In such region SNPs 5354-C/A and 5622-C/G are linked to transcription factor binding motif profile in sheep. Variants 5454-A and 5622-G create additional STAT and Sp1 transcription factor binding sites (O'Neill et al., 2003). All analysed caprine sequences carry 5354-A and 5622-G. Likewise human and cattle also goats do not seem to be polymorphic at these sites based on our results. Similarly, comparative alignments made between the *PRNP* regions of known promoter activity in cow (Inoue et al., 1997) and the predicted goat *PRNP* promoter revealed nine SNPs and one indel (5808-T/G, 5844-G/A, 5869-G/T, 5911-C/T, 6171-G/C,

6206-G/C, 6352-C/G, 6431-T/C, 6460-G/T, 6466 C-del) in the homologous caprine sequence. No *PRNP* SNPs were in common among sheep, cow and goat. However, looking at the complete alignment assembly it appears that sheep and goat share a polymorphic site flanking the 3' end of the region known to have promoter activity in sheep (5808-C/G in sheep, 5808-T/G in goat).

Ten out of the 29 observed polymorphisms displayed a minor allele frequency less than 0.10 (Table 6) and were therefore excluded from haplotype analysis. The remaining polymorphisms were tested for genotype distribution similar to HWE and nine resulted to deviate from HWE expectations. Based on 10 polymorphic sites (9 SNPs and one indel), thirteen goat *PRNP* haplotypes were predicted using PHASE 2.1 best reconstruction and best pairs analysis. Figure 20 shows the predicted haplotypes (n = 13) and estimates of the sample haplotype frequencies. PHASE 2.1 analysis retrieved also the most likely haplotype pair for each goat included in the study (data not shown).

Fig. 20. PHASE haplotype reconstruction and frequencies.



Analysis performed using TFSEARCH aimed to evaluate the presence of transcription factor binding sites within the region of interest. In the present study sequencing of the goat *PRNP* gene was extended 2 kb upstream and 1 kb downstream the transcription start site. This allowed us to perform *in silico* identification of putative transcription binding sites outside the core promoter region. TFSEARCH analysis was done through two approaches as described below.

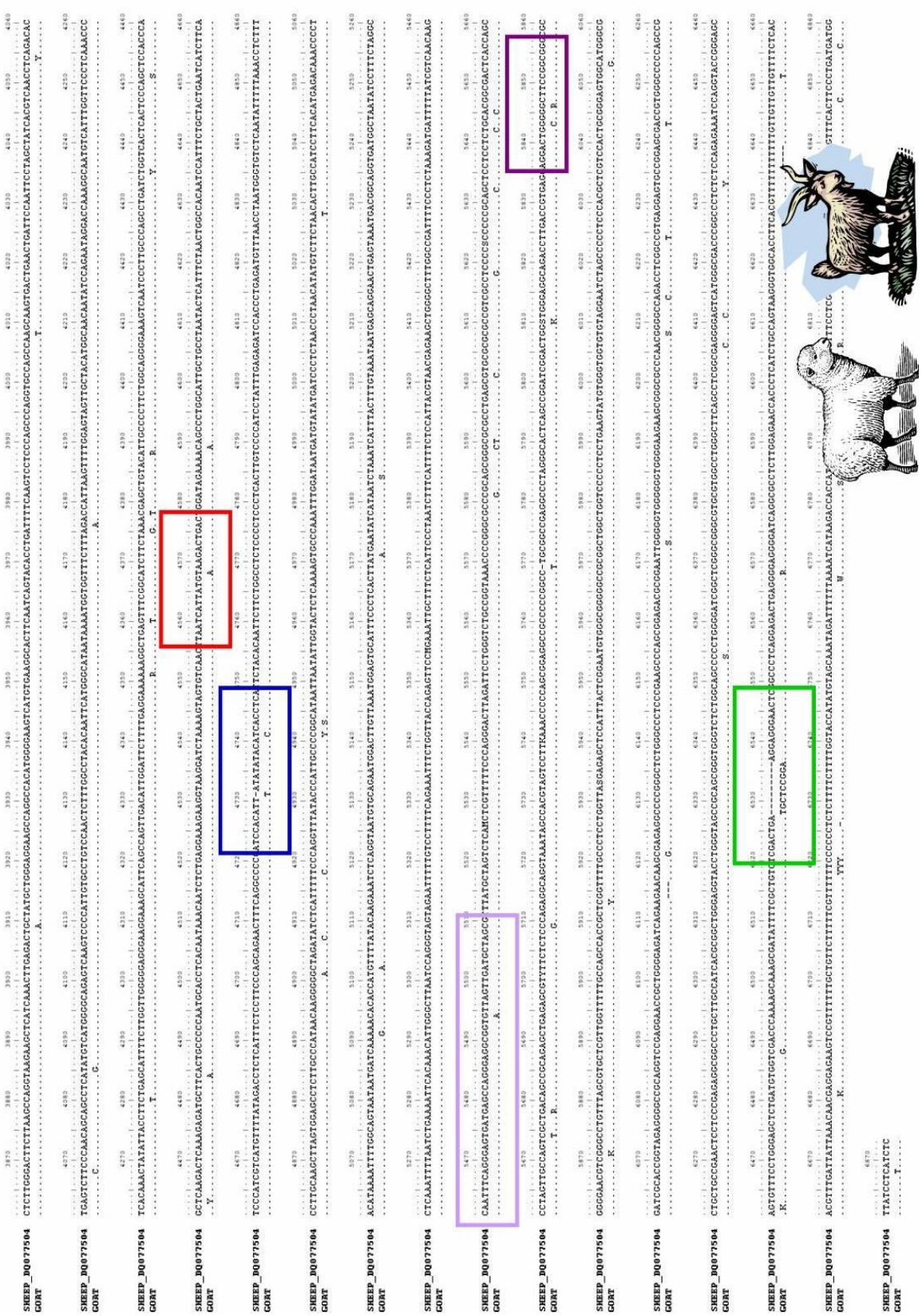
4.1.1 Alignment of the goat PRNP sequence with the ovine homologous sequence and evaluation of the presence of goat additional or defective transcription factor binding sites based on basic nucleotide differences among the considered consensus sequences.

Alignment of ovine and goat homologous sequences showed several mismatches and some of them potentially differentiate the binding site profile of the two species (Fig. 21). At position 4068 (numbering according to ovine DQ077504) the ovine sequence carries a T, responsible for potential Ikaros (Ik-2, Ik-3) binding sites on minus strand, while the caprine sequence carries a C, creating a consensus motif for AP-1 binding site lying on the same strand. The presence of adenine at position 4484 of the goat sequence (4484-C in sheep) generate a putative binding site for the cardiac transcription factor Nkx-2.5 (Evans, 1999). The consensus sequences Nkx-2.5 in the upstream promoter region might also be relevant for the regulation of cell-specific promoter activity since PrP^{C} mRNA and protein are also expressed in the heart (Manson et al, 1992; Bendheim et al., 1992). At position 4568 the sheep reference sequence carries a T, while the obtained goat consensus carries a A. This change leads to the loss of a potential E4BP4 binding site in goat (Fig. 21, —). E4BP4 protein was recently recognized as transcription factor binding to conserved motif 2 by electrophoretic mobility shift assays. Functional promoter analysis of four different promoter variants revealed that motif 2 (E4BP4) was linked to a slight enhancing activity (Burgess et al., 2009). Conversely, the insertion of a T at position 4730 of the goat sequence enhance the binding site profile in this species creating consensus motifs for Oct-1 (plus strand) (Fig. 21, —) and TATA (minus strand) transcription factors. Oct-1 is a ubiquitous transcription factor but also controls tissue-specific genes including several in brain or lymphatic cells (reviewed by Sytina and Pankratova, 2003). Another caprine potential Oct-1 binding site is given by the combination of

nucleotide changes at positions 4906 and 4917 (thymines in sheep are replaced by cytosines at both positions in goat). Interestingly, the lack of the G at position 5494 in the goat sequence (A substitution) affects the consensus sequence for a putative Sp1 binding site (Fig. 21, —). Even though this Sp1 motif is not located so close to the transcription start site, it could compensate the minor number of Sp1 binding factors present in the core promoter of sheep *PRNP* with respect to other related ruminant species. In fact, the only Sp1 site proximal to the sheep *PRNP* exon 1 start site is subjected to a change (5622-C) that creates an AP2 consensus sequence (Dynan et al., 1986). In contrast to the sheep *PRNP* sequence, three Sp1 sites have been identified in the homologous region of the bovine and caprine *PRNP* sequences (Inoue et al., 1996; Van Poucke et al., 2009). A potential Lyf-1 motif on the minus strand of sheep *PRNP* exon 1 is abolished in goat by C→G change at position 5708. The same happens for the c-Rel binding site in Intron I of sheep which is affected by G→C change at position 5841 of goat *PRNP* (Fig. 21, —). C-Rel has been characterized as a potential candidate transcription factor in the *PRNP* promoter of human neuronal cells (Funke-Kaiser et al., 2001). NF-κB/Rel proteins might be relevant during amyloid-related neurodegenerative processes (Grilli and Memo, 1999). Position 6051 seems to be important in the *PRNP* transcription binding site profile because the presence of guanine in goat (replacing cytosine at the homologous sheep position) potentially creates three overlapping binding sites in this species: GATA-1, GATA-2 and p300. Transcription factors of the GATA family have been implicated in hematopoiesis and cardiac development (Charron and Nemer, 1999) but are also expressed in embryonic and adult central nervous system (Pandolfi et al., 1995; Nardelli et al., 1998). Homozygous knock-out mice lacking GATA-2 exhibit, for example, neurogenesis defects in the hindbrain (Nardelli et al., 1998). p300 is a transcriptional coactivator that interact with a large number of transcription factors and regulate transcription through multiple mechanisms.

Conditional transgenic mice expressing an inhibitory truncated form of p300 have impaired long-term recognition memory and contextual fear memory (Oliveira et al., 2007). At positions 6404 and 6409 two close nucleotide mismatches have the effect of deleting a putative MZF-1 binding site in goat. MZF-1 regulates cellular proliferation, but it is also a negative regulator of expression of the mouse extracellular superoxide dismutase (EC-SOD or SOD3) (Zelko and Folz, 2003). At position 6486 the goat consensus sequence lacks a potential CP2 binding site (cytosine with guanine substitution). CP2 is a ubiquitously expressed transcription factor present in regulatory regions of multiple erythroid genes. In these regions, the CP2 binding site is usually adjacent to a site for the erythroid factor GATA-1. Interestingly, the sheep *PRNP* binding motif profile displays the same organization with a GATA-1 motif flanking the predicted CP2 binding site. The disruption of a potential interaction between these two binding factors in goat could have a significance considering that PrP^{C} is expressed on hematopoietic cells, including erythroid precursors and that the expression of the erythroid differentiation-related factor (EDRF) is reduced in lymphatic tissues of rodents and cattle as well as in whole blood of sheep that suffer from TSEs (Miele et al., 2001; Brown et al., 2007).

Fig. 21. Alignment of sheep and goat *PRNP* regions under study.



*Squares indicate putative TF binding sites created by sequence differences. References in the text to each site are pointed out by coloured bars (▬).

A nine base pair TGCTCCGGA insertion starting from position 6525 is present in all the analysed goat and creates the consensus sequence for a potential Elk-1 binding site (Fig. 21, —). Elk-1 is an ETS-like gene that codes for a DNA binding protein that is involved in transcriptional activation, proliferation and differentiation. Elk-1 is an activator of Mitogen-Activated Protein (MAP) kinases and induces apoptosis (Rao et al., 1989; Hipskind et al., 1991; Rao and Reddy, 1994; Shao et al., 1998). At position 6834 the goat sequence shows a T→C substitution with respect to sheep. This change turns out in the loss of a potential Interferon-Stimulated Response Element (ISRE) in goat. Regulatory regions of interferon-inducible genes often contain ISRE which is predominantly stimulated by type 1 interferons. Microglial transcriptional changes are detectable beginning at 30 days after innoculation in CJD brain. In particular, 10 interferon-sensitive genes were upregulated at very early stages of infection (Baker et al., 2004). In mouse models, one mechanism has been suggested by which increased interferon responsive gene expression may enhance disease progression (Stobart et al., 2007).

4.1.2 Submission to TFSEARCH of the identified variants of the goat PRNP gene to evaluate the possible effect of polymorphisms on the transcription binding site profile.

A putative AML-1A binding site (minus strand) was identified by TFSEARCH linked to the 4054-C variant. The AML1 gene encodes several transcription factors, including AML1A, which bind to DNA via a TGT/cGGT consensus sequence that is found in several promoters (Meyers et al., 1993). Polymorphism at position 4463 gives rise to an additional Sp1 transcription factor binding site when the variant carrying cytosine is present, adding a fourth enhancer motif to the three Sp1 binding sites present immediately upstream exon 1 in goat (Van Poucke et al., 2009). The Sp1 transcription factor has been shown experimentally to play a role in transcriptional regulation of *PRNP* (Saeki et al., 1996; Baybutt & Manson 1997; Inoue et al., 1997; Mahal et al., 2001).

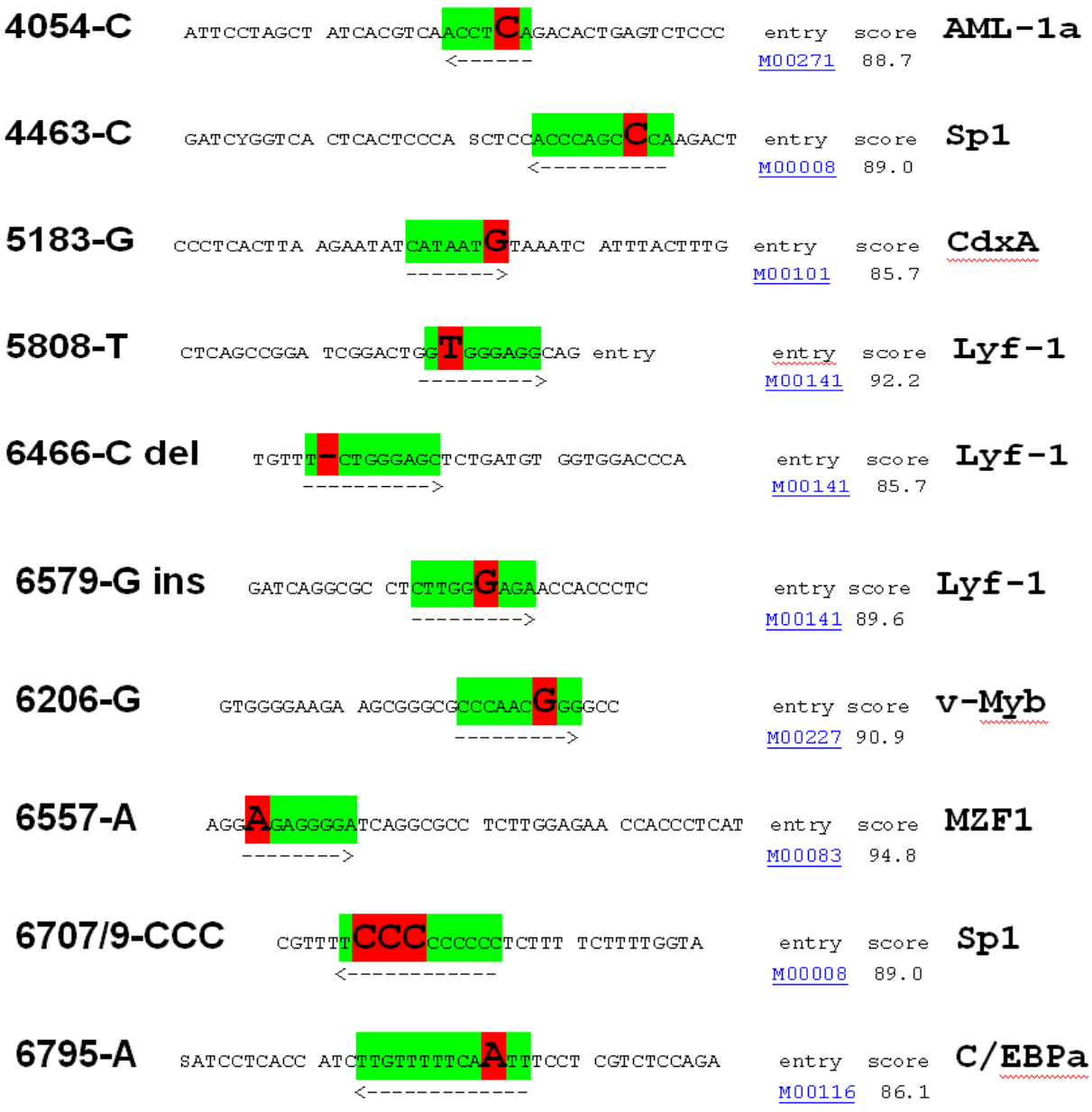
Variant 5183-G leads to a putative CdxA transcription factor binding site on the plus strand and two additional CdxA motifs on the minus strand. Another CdxA motif is created by 6756-T variant in Intron I (minus strand). Interestingly, the SNP at position 5808 (5808-T) and the two single nucleotide indel polymorphisms (6466-C deletion and 6579-G insertion) identified in Intron I, could have a similar influence on the regulation of *PRNP* expression. In fact, they all give origin to putative LyF-1 transcription factor binding sites. The transcription factor LyF-1 can be considered a candidate for binding to the *PRNP* promoter region, because the consensus binding site TCAGGGAG is identical to the conserved motif 4 (TCAGGGAG) (Lee et al., 1998). At the polymorphic position 6206 the presence of glycine creates a putative binding motif for Myb transcription factor. The Myb proteins bind to DNA and regulate gene expression. These proteins are defined by the Myb domain, a sequence of approximately 50 amino acids with a structure similar to the helix-turn-helix motif of prokaryotic transcriptional repressors and eukaryotic

homeodomains (Ganter et al., 1999). Interestingly, the variant carrying adenosine at position 6557 affects a myeloid zinc finger-1 (MZF-1) potential regulatory element. This motif was one of the seven motifs (among 51 short conserved sites) found fully conserved within Intron I of human, mouse, bovine, ovine, and tamar wallaby from Premzl et al. (Premzl et al., 2004). When SNP at position 6707 and adjacent MNP at position 6708 were combined to give a CCC triplet an additional Sp1 binding site was recognized by TFSEARCH, lying in Intron I on the minus strand. The occurrence of the less frequent variant of polymorphism at position 6795 (6795-A) originates a CCAAT/Enhancer-binding Protein (C/EBP) alfa binding site on the minus strand. C/EBPs comprise a family of transcription factors that are critical for normal cellular differentiation and function in a variety of tissues (Lekstrom-Himes and Xanthopoulos, 1998). Results of the TFSEARCH analyses and identified putative transcription factors with the frequency of the binding and non-binding alleles are summarized in Table 8. A graphical representation is also shown in Fig. 22. The putative Sp1 binding site created by the C/T polymorphism and located in the promoter region at 1203 bp from the transcription start site (highlighted in Table 8) was selected for the subsequent EMSA experiments.

Table 8. Polymorphisms within the goat *PRNP* regulatory regions that affect transcription factor-binding sites.

T.F.	Binding site pos.	Strand	Binding Allele (*f*)	Non-binding Allele (*f*)
ALM-1a	-1612	–	acctCa (0.92)	acctTa (0.08)
Sp1	-1203	–	acccagcCca (0.44)	acccagcTca (0.56)
CdxA	-483	+	cataatG (0.08)	cataatC (0.92)
Lyf-1	+142	+	gTgggagg (0.63)	gGgggagg (0.37)
v-Myb	+540	+	cccaacGgg (0.55)	cccaacCgg (0.45)
Lyf-1	+800	+	t–ctgggagc (0.08)	tCctgggagc (0.92)
MZF1	+891	+	Agagggga (0.49)	Ggagggga (0.51)
Lyf-1	+913	+	cttggGaga (0.08)	cttgg-aga (0.92)
Sp1	+1041	–	tCCCcccccc (0.04)	tCTTcccccc (0.96) tTTTcccccc tTCCcccccc
C/EBPa	+1129	–	ttgtttttcaAtt (0.02)	ttgtttttcaGtt (0.98)

Fig. 22. Graphical representation of transcription factor-binding sites affected by polymorphisms within the goat *PRNP* regulatory regions.



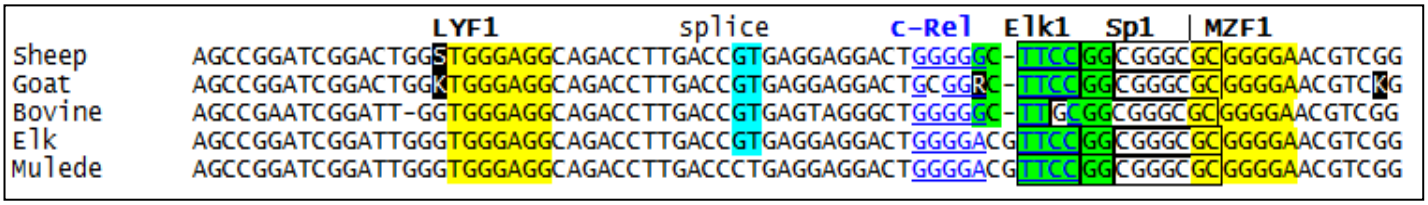
4.2 EMSA analysis of putative TF binding motifs in the *PRNP* promoter and Intron I sequence

As described above sequence analysis of the non coding regions of the caprine Prnp gene and interspecies comparison of this gene region allowed the identification of putative TF binding motifs. Some of these putative motifs were polymorphic in the caprine gene and others are species specific when compared with other ruminants. A molecular understanding of the *PRNP* gene regulation would benefit the evaluation of polymorphisms and the potential relevance for disease phenotypes. It was decided to perform "electrophoretic mobility shift assays (EMSA)" to establish molecular interaction of TFs with *PRNP* promoter and intron 1 regions based on the caprine gene sequence.

Two putative motifs were selected:

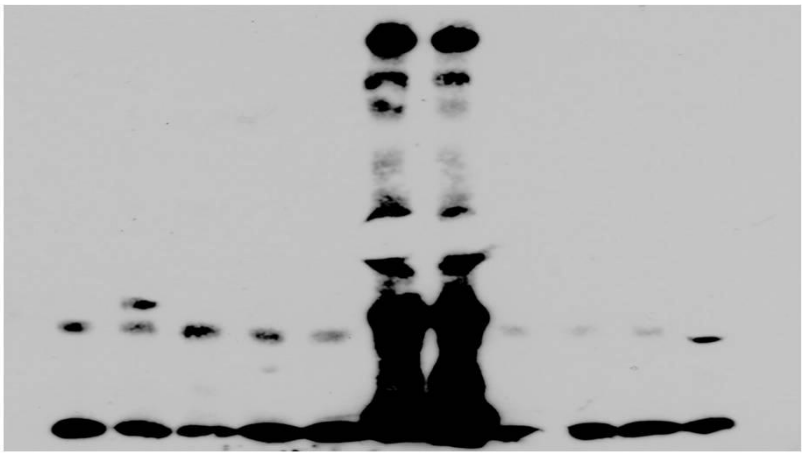
1) A Sp1 putative binding site identified in the goat promoter (-1203 from the transcription start site, Table 6) and linked to the C allele of the 4463T/C polymorphism (Table 8, Fig. 22). The haplotype frequency for this polymorphism has 44% C to 56% T. We decided to include this site for EMSA experiments as it appeared to be highly variable in the studied goats and it is known that Sp1 is a common regulator protein, which has been indicated in cattle to be associated with different *PRNP* expression and possibly BSE susceptibility.
2) A region of Intron I close to exon 1 showing conservation by comparative alignment of ruminant species (Fig. 23) and a striking overlap of not less than four putative TF binding sites, including c-Rel, ELK1, Sp1 and MZF1. This region also contains a polymorphisms decribed previously by us for the caprine gene and a species specific difference between goats and sheep on the one side and cattle on the other.

Fig. 23. Alignment of intron 1 from *PRNP* gene sequences from different ruminant species.



The EMSA targeting the -1203 Sp1 binding site demonstrated that, under standard conditions, no shift was observable using 3T3 cell line extract for both alleles. As control the biotinylated probe carrying the Sp1 consensus motif showed a characteristic band shifted to the top of the blot (gel) (Fig. 24). This control shift was also confirmed as Sp1-related by comparison with previous EMSA data. We conclude that neither of the caprine *PRNP* haplotypes in this position is able to bind Sp1 with significant efficiency and therefore Sp1 is not likely to control *PRNP* gene expression through the -1203 position.

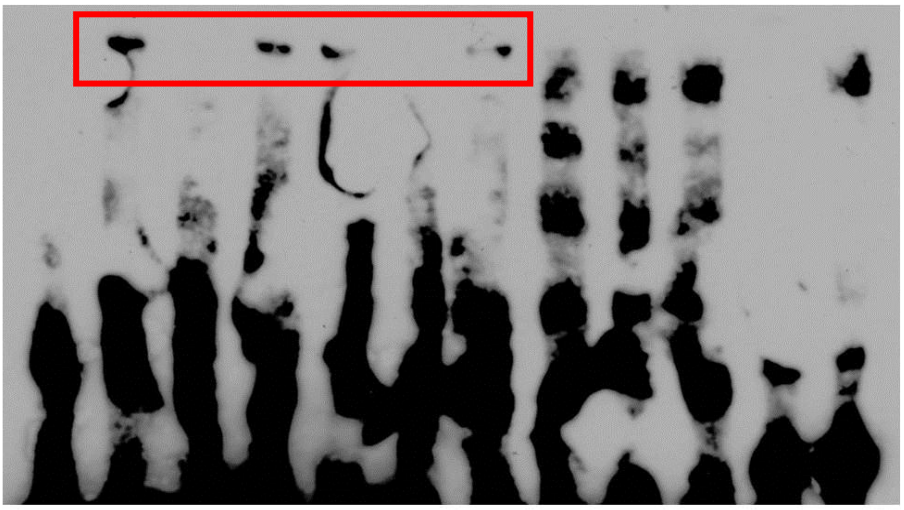
Fig. 24. Gel shift assay targeting the putative -1203 Sp1 binding site.



Even though this experiment seem to exclude binding of the predicted Sp1 factor at this site, it must be taken into account that EMSA is very sensible to experimental conditions and adjustments to the protocol could be introduced to assess whether this is the real result or binding can occur under optimized conditions.

In contrast to the above EMSA result, when using the intron 1 ELK1 containing sequence we were able to demonstrate the formation of a complex with the two celll line extracts (RAW & 3T3). This complex was competed by the ELK1 unlabeled probe (Fig. 25). However, it is also likely that other TFs bind to this position as predicted from the alignment. RAW cell extracts showed also additional smaller complexes with Goat1FL probe which were similar to Sp1 complexes in size but might not consist of Sp1. Subsequently, the Goat1FL probe and biotinylated Sp1c control were also loaded with recombinant Sp1 TF showing that Goat1FL does not bind Sp1. In conclusion, the analysed conserved sequence from the *PRNP* promoter represented by the Goat1FL oligo binds ELK1 TF and probably two other factors, probably cREL and MZF, which need to be tested in future experiments.

Fig. 25. Gel shift assay targeting the Intron I sequence.



Goat1FL	+	+	+	+	+	+	+	+	+	+		
Sp1c											+	+
NE RAW		+	+	+	+	+	+				+	
NE moBr								+	+	+		+
sh1+sh2 comp			+									
Sp1c comp				+					+			
C-rel comp					+							
Elk1 comp						+				+		
Mzf1 comp							+					

4.3 Role of the *SPRN* gene in goat susceptibility/resistance to classical scrapie

4.3.1 Genetic variability of the caprine SPRN gene

A total of 11 mutations were found in the caprine *SPRN* gene; ten were SNPs and one was an indel polymorphism (Table 9). A polymorphism was considered as such when it was found in three or more animals.

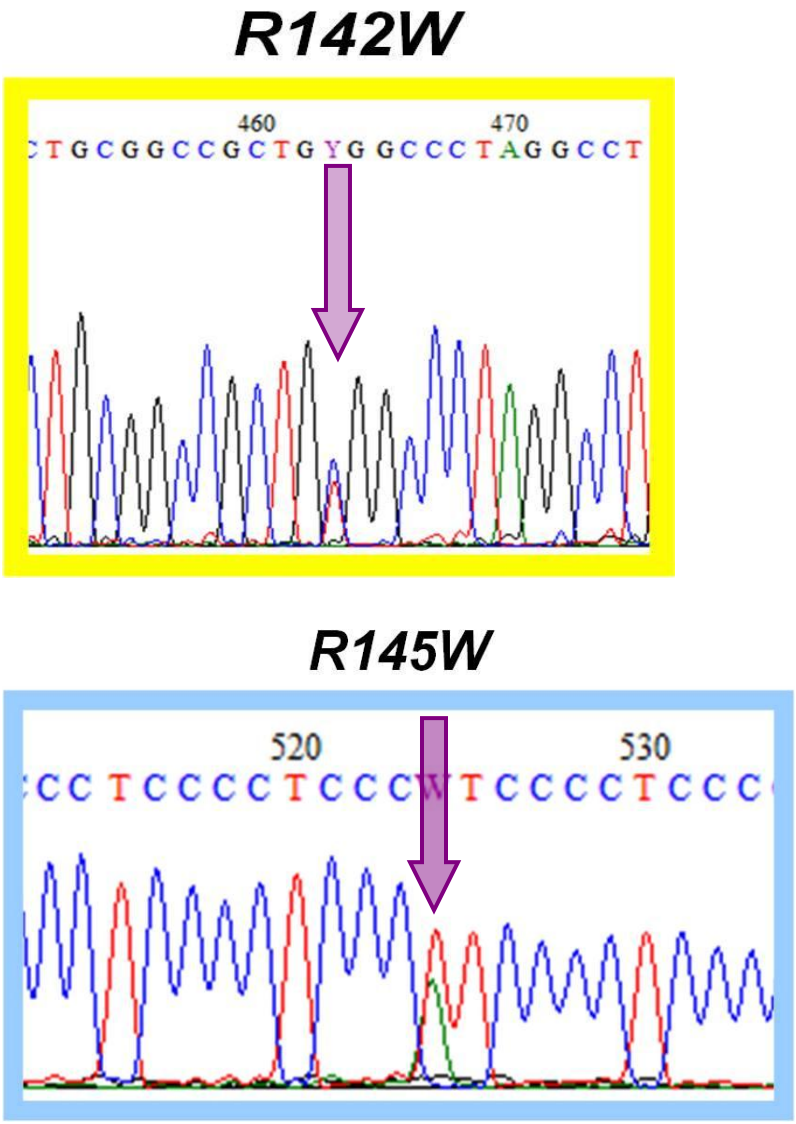
Table 9.

		Allele frequency (%)		
Codon	**Alleles**	***Scrapie***	***Controls***	***P-value***
48	*ccG* (Pro)	76.2	66.7	*0.203*
	ccA (Pro)	23.8	33.3	
59	*gcG* (Ala)	76.2	66.7	*0.206*
	gcC (Ala)	23.8	33.3	
61	*gcG* (Ala)	100	99.7	-
	gcC (Ala)	0	0.3	
100	*ccT* (Pro)	76.2	68.3	*0.288*
	ccC (Pro)	23.8	31.7	
142	*Cgg* (Arg)	100	99.8	-
	Tgg (Trp)	0	0.2	
145	*Cgg* (Arg)	88.1	93.3	
	Agg (Arg)	11.9	6.4	*0.164*
	Tgg (Trp)	0	0.3	

Five synonymous SNPs in the *SPRN* ORF (codon (c.) 48G>A, c.59G>C, c.61G>C, c.100T>C, c.145C>A) and four polymorphisms in the 3'UTR (positions 573G>A, 602_606insCTCCC, 624G>T, 645G>T) had been reported previously (Stewart et al. 2009), while two novel non-synonymous mutations

were identified. They were located in the ORF and were detected at very low incidence: at codon 142, a CGG→TGG substitution caused an amino acid change of Arg to Trp, and at codon 145, an AGG→TGG substitution led to the amino acid change Arg to Trp (Fig. 26). No change was found in the hydrophobic alanine region of the gene.

Fig. 26. Electropherograms showing two novel coding mutations detected in the caprine *SPRN* ORF.



4.3.2 SPRN case-control study

Table 10 shows the cases and the controls breeds and their proportion (data were not available for 12 animals). The cross-bred animals represented 27.4% of the studied sample.

Table 10. Case and control breeds and their proportion.

Breed	Cases (%)	Controls (%)	Total (%)
Alpina	0 (0)	16 (4.0)	16 (3.8)
Bionda Adamello	0 (0)	1 (0.30)	1 (0.24)
Camosciata	1 (4.8)	48 (11.9)	49 (11.6)
Capra dei Nebrodi	1 (4.8)	95 (23.6)	96 (22.6)
Frisa	0 (0)	6 (1.5)	6 (1.4)
Garganica	1 (4.8)	0 (0)	1 (0.24)
Maltese	1 (4.8)	79 (19.6)	80 (18.9)
Saanen	0 (0)	24 (6.0)	24 (5.7)
Siriana	0 (0)	23 (5.7)	23 (5.4)
Cross-bred	16 (76.2)	100 (24.8)	116 (27.4)
Missing	1 (4.8)	11 (2.8)	12 (2.8)
Total	**21 (100)**	**403 (100)**	**424 (100)**

The results of the bivariate analysis are shown in the Table 11. In the 3'UTR, a polymorphism causing the insertion of five nucleotides (602_606insCTCCC) was found to be associated with susceptibility to scrapie in goats ($p = 0.028$). The genotype *SPRN* and the breed showed a significant association with the disease.

Table 11. Distribution of considered factors among cases and controls.

	Category	Cases (%)	Controls (%)	P-value (*)
Genotype *SPRN*				0.028
	1. No mutation	11 (52.4)	299 (74.2)	
	2. Mutation	10 (47.6)	104 (25.8)	
Genotype *PRNP*				0.089
	1. No mutation	0 (0)	49 (12.2)	
	2. Mutation	21 (100)	354 (87.8)	
Breed				0.00
	1. Pure breeds	4 (20)	292 (74.5)	
	2. Cross-breed	16 (80)	100 (25.5)	

(*) Pearson χ^2

Table 12 shows the results of the logistic regression model. Even including the breed as covariate, the polymorphisms in the host *SPRN* gene resulted associated with the disease.

Table 12. Results of the logistic regression model analysis.

Covariate	Category	OR	95% CI
Genotype *SPRN*	1. No mutation	Referent	
	2. Mutation	2.5	1.03-6.11
Breed	1. Pure breeds	Referent	
	2. Cross-breed	12.1	3.88-37.59

OR=odds ratios, CI: confidence interval. Pseudo R^2: 0.18; LR χ^2, p-value: 0.00

In the Table 13 a descriptive analysis of the results of the models, including the Monte Carlo simulation (5000 replications) of occurrence of histidine at codon 154 of the *PRNP* gene, is presented. In 96% of the models the polymorphisms in the host *SPRN* gene was still associated with the disease while the presence of the 154H allele was unlikely to affect *SPRN* association with scrapie.

Table 13.

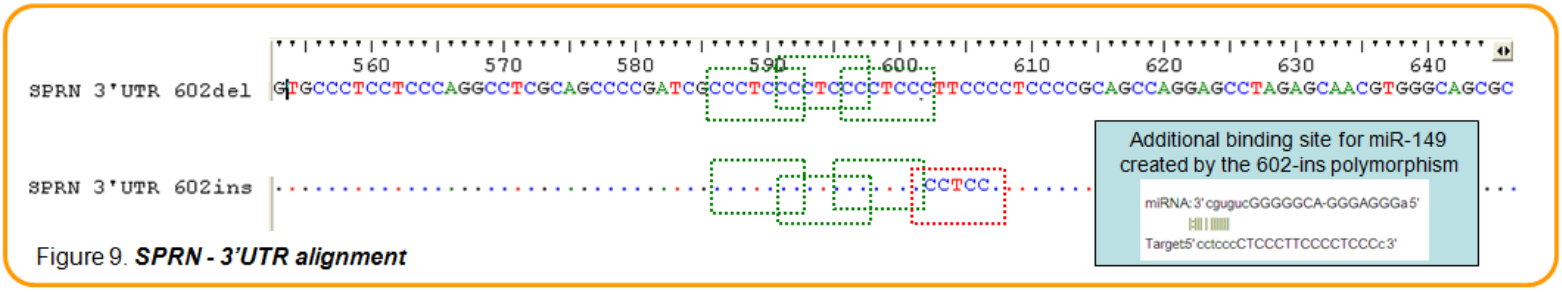
Variable	Mean of the estimated OR	Min. observed	Max observed	Significant OR / total estimated OR *100
Genotype *SPRN* (mutation vs. no mutation)	2.9	2.0	3.6	96%
Breed (cross-breed vs. pure breed)	12.7	10.0	49.9	100%
154H allele (presence vs. absence)	0.84	0.03	3.5	6.7%

OR=odds ratios

4.3.3 microRNA target prediction

In silico analysis for microRNA targets using the program PATROCLES (http://www.patrocles.org) predicted multiple putative binding sites for miR-149* in this specific region of the *SPRN* gene (Fig. 27). The allele carrying the 602_606CTCCC insertion created an additional target site for miR-149*, a recently identified cognate miRNA of miR-149 (Lin et al., 2010).

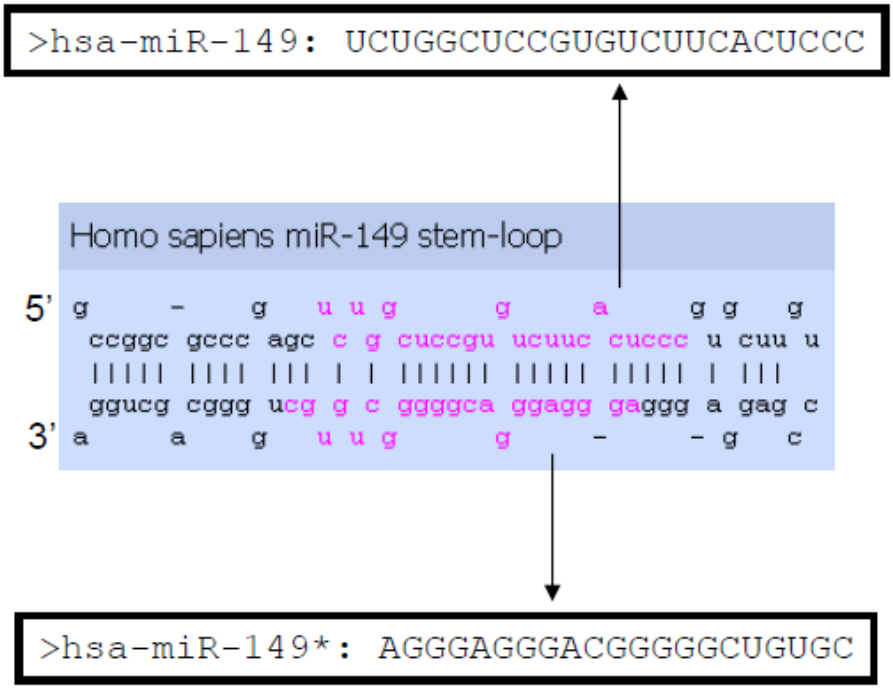
Fig. 27. Graphical representation of prediction analysis of microRNA target sites in the *SPRN* 3'UTR.



MicroRNAs (miRNAs) are ~22 nt noncoding RNAs that negatively regulate target gene expression and play important roles in multiple biological and metabolic processes of eukaryotic organisms and in disease (e.g. cancer). Most miRNA genes are first transcribed into long transcripts of primary miRNAs (pri-miRNAs) by RNA polymerase II. The pri-miRNAs are then processed by Drosha to generate the ~80 nt precursor miRNAs (pre-miRNAs) with the stem-loop structure. After being exported from nucleus into cytoplasm, premiRNAs are further processed by Dicer to produce the mature miRNAs. Sometimes, there are two mature miRNAs originated from the opposite arms of the same pre-miRNA, which may be differentially expressed. In that case, the mature sequence of the predominant product is assigned as the specific ‘‘miRNA’’ and the minor product as ‘‘miRNA*’’ (Fig. 28). So far, the mutual relationship and interactions of miRNA and its cognate miRNA* have not been elucidated yet.

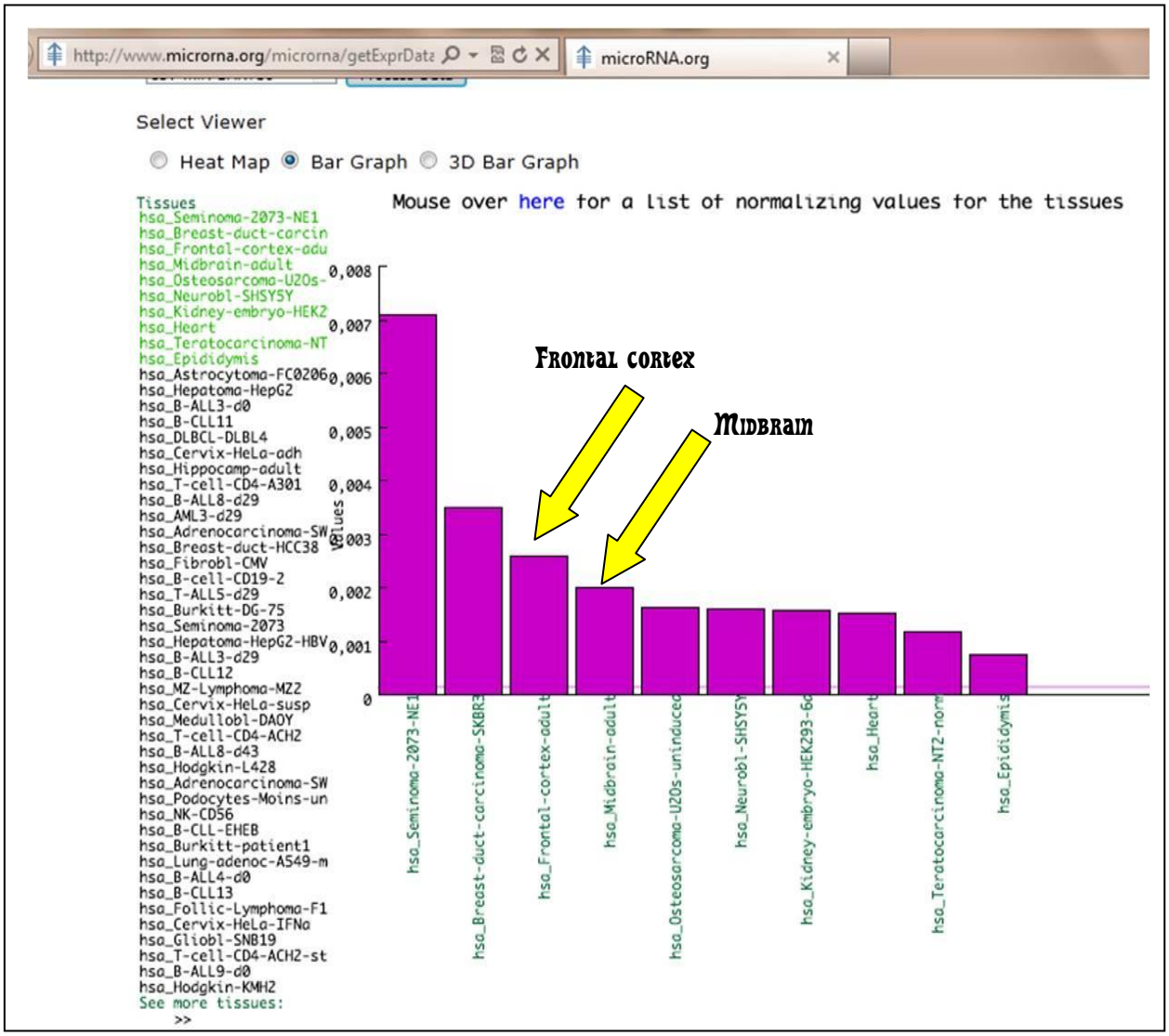
Fig. 28. Human precursor miR-149 stem-loop is processed into two miRNAs, miR-149 and miR-149* (adapted from the miRBase; http://microrna.sanger.ac.uk/sequences/index.shtml).

Lin *et al.*



To our knowledge information on the physiological tissue expression profile of miR-149* are currently not available, however, looking at the expression profile of the predominant miR-149, as reported by the microRNA.org database (www.microrna.org) it can be noticed that, among normal tissues, it is highly expressed in nervous tissues (Fig. 29). Even though this finding is obviously far to be a proof of an analogous expression profile for miR-149*, it is intriguing when associated to the observation that the expression of miR-149* has been demonstrated to inhibit the growth of a neuroblastoma cell line, Be2C cells and HeLa cells by inducing apoptosis (Lin et al., 2010).

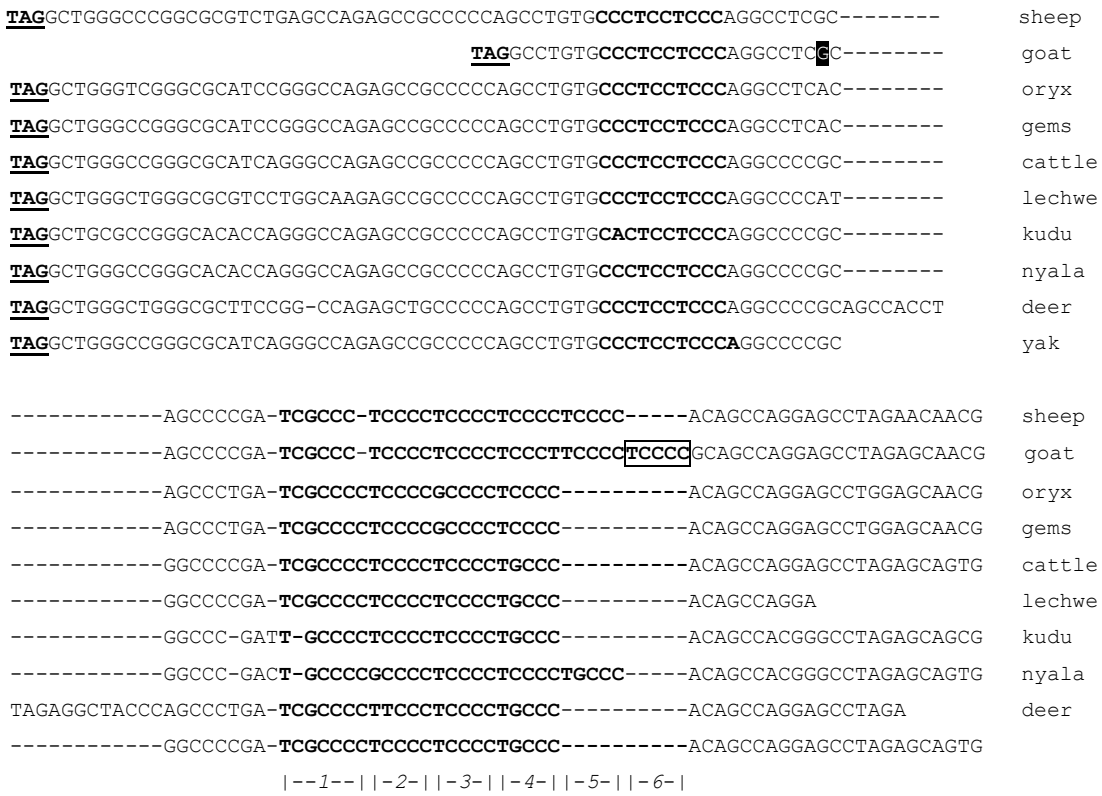
Fig. 29. miR-149 expression levels at various tissues.



Moreover, a comparison of the surrounding region of the 3'UTR revealed large insertions / deletions between species and also as polymorphism in caprine *SPRN* (Fig. 30). Caprine *SPRN* has a deletion of 33 bp compared to all other sequences whereas a 21 bp insertion characterized the cervine *SPRN* 3'UTR. Within the approximately 120 bp 3'UTR which was sequenced here we found a (T/G)CCC motif repeated between nine and eleven times depending on species and allele. The sequence GCCCTCCTCCC is highly conserved in these ruminants whereas further downstream the number of direct repeats of

this motif vary between four and six between species or in the case of the caprine 3'UTR between two alleles. These motifs are conserved in human *SPRN*, but not in mouse *SPRN* (not shown). It remains to be tested whether they have a role in post-transcriptional or translational modulation of gene expression.

Fig. 30. Variation in the 3' UTR of the *SPRN* gene across ruminants.



Conclusions

The results of this study provide further insight in the field of goat TSE genetics as it represents the first polymorphism analysis of the goat *PRNP* putative regulatory regions and the first association study investigating the role of the goat *SPRN* gene in scrapie occurrence.

Twenty-nine novel polymorphisms were identified within a 3 Kb region encompassing the *PRNP* transcription start site in goats belonging to nine goat breeds coming from Italy, Greece and Spain. None of the identified changes were identical to bovine and ovine variants demonstrated to be involved in gene expression. The goat *PRNP* promoter prediction, generated using the program CpGProD online, was homologous to the regions of known promoter activity of both cattle and sheep. This result was not unexpected given that BLAST analysis yielded that the analysed goat *PRNP* region has the best nucleotide homology with sheep and cow (max identity of 96% and 92%, respectively). Haplotype analysis, performed on the study goats using bioinformatics, predicted 13 haplotypes based on ten polymorphic sites. The estimated haplotype frequencies indicate that three major haplotypes (numbered 3, 4 and 8 in Fig. 20) represent over 65% of the genetic variability in the study goats. Most of the polymorphisms with an influence on the presence/absence of transcription factor binding sites were found in Intron 1. Only three identified SNPs are located upstream the transcription start site (4054-C, 4463-C, 5183-G) and they are all outside of the core promoter region and of the predicted CpGProD promoter. However, the finding of control elements inside Intron 1 is not unusual. Many genes have conserved regulatory regions located within the intron sequences, which may contribute to the control of gene expression. Control elements are preferentially found located in the 5’ introns with less control elements found in the introns located further downstream. The enhancer effects of these control regions are so essential that some genes will not be expressed in their absence. In addition, these control elements may be involved in the tissue specific expression of the gene product.

Actually, our preliminary functional studies have also demonstrated that a conserved region in the *PRNP* intron I is able to bind different TFs, among which the binding of ELK1 has been assessed by EMSA experiments. Therefore it appears that ELK1 could be a regulator of *PRNP* transcription, which has been previously suggested based on cell experiments. Moreover, bioinformatics analysis identified ten polymorphisms that could have an effect on regulation of *PRNP* expression level in goats. They could be selected as primary targets to be investigated in functional assays as well as in case-control studies.

In the course of the research project, novel data about the genetic variability of the caprine *SPRN* gene have also been generated showing that, similarly to cattle, the Shadoo protein appears to be highly stable in goats. Conservation of the Shadoo protein in goats could be regarded as unexpected if one consider the parallelism proposed by other Authors (Daude et al., 2009) between genetic variability of *PRNP* and *SPRN* genes. This was based on the observation that low degrees of nucleotide diversity were found in human and mouse *SPRN* and *PRNP* genes, while a number of coding alterations occur both in *PRNP* and *SPRN* in sheep. Therefore a common selection pressure acting on these two genes of the prion family was hypothesized. On these basis, as the caprine *PRNP* gene carries many missense polymorphisms, a similar scenario might be expected for the *SPRN* gene; nevertheless, the lack of coding polymorphisms in the studied goat sample, indicate that, al least in Italy, this seems not to be the case. The most remarkable finding from the *SPRN* study is the identification of an indel polymorphism in the 3'UTR (602_606insCTCCC) associated with the susceptibility to classical scrapie in goats from Italian outbreaks. This indel occurs in a region of the *SPRN* 3'UTR highly conserved in ruminants and characterized by repetitive motifs. Bioinformatics analyses suggest the hypothesis that the detected polymorphism may contribute to modulation of susceptibility to the disease via gene

expression regulation by a miRNA-mediated post-transcriptional mechanism. Even though TSE association studies are notoriously difficult and require confirmation by experimental exposure to different TSE strains of animal carrying the alleles of interest, the identification of a genetic target outside the *PRNP* gene could improve future genetics-based scrapie control approaches (e.g., by helping to overcome limitations due to the low frequency of the desired *PRNP* polymorphisms in some goat breeds). However, further studies are needed to assess whether genetic differences in the 3'UTR of the *SPRN* gene could modulate other aspects of disease such as pathology or subclinical infection.

In conclusion, the results presented in this Doctoral thesis provide novel knowledge on genetic factors outside the *PRNP* open reading frame investigating their influence in the modulation of scrapie susceptibility in goats. These data could provide a significant contribution to the control of classical scrapie in goats as well as further research opportunities on other aspects related to the function and pathogenicity of the prion protein.

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