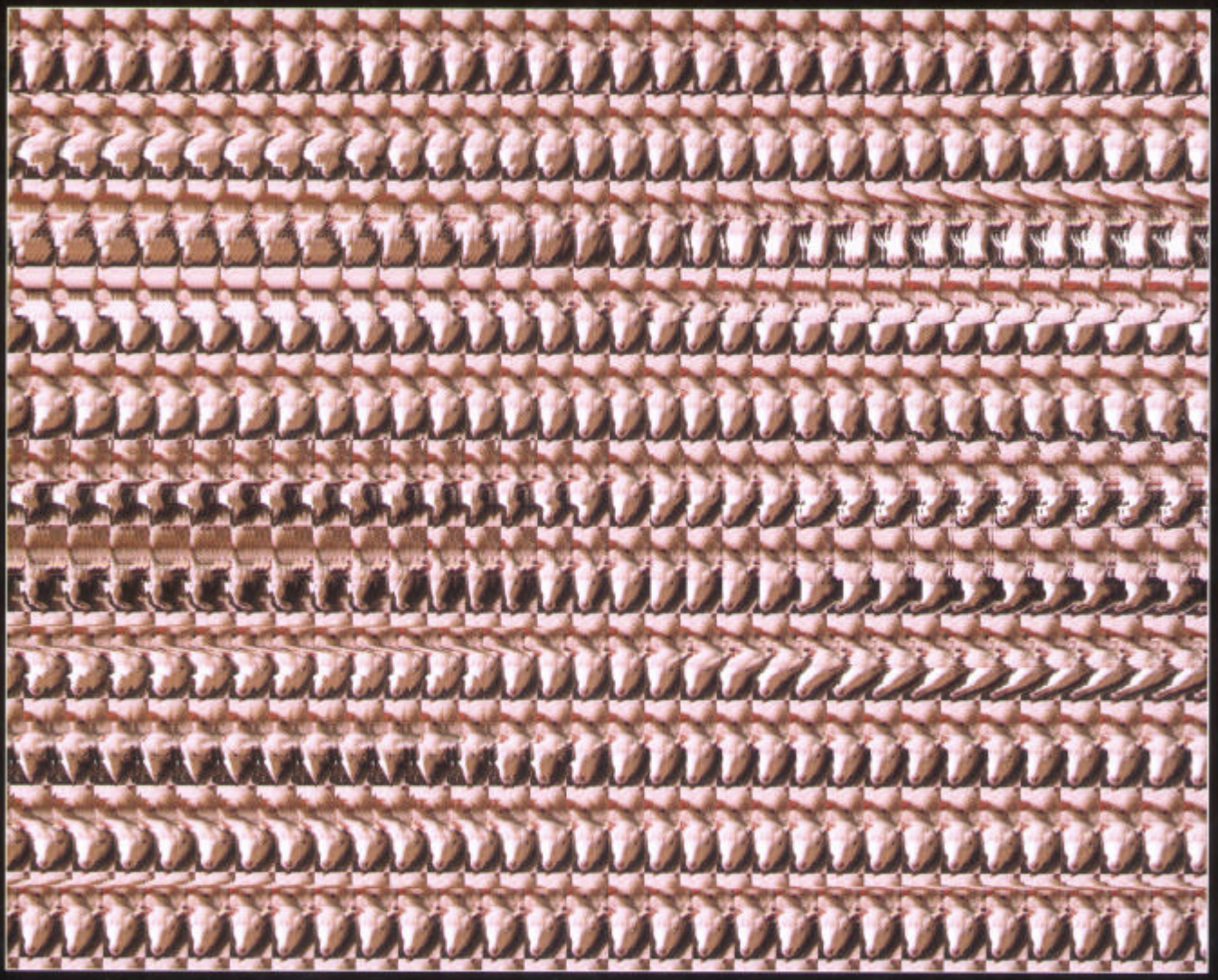
Prion diseases: Susceptibility and Transmissibility

In vivo and *in vitro* studies with sheep scrapie

Alex Bossers



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1999

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In vivo and *in vitro* studies with sheep scrapie

Prionziekten: Gevoeligheid en Overdraagbaarheid

In vivo en *in vitro* studies met schapen scrapie

(met samenvatting in het Nederlands)

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The only limits,
are the limits of
our imaginations.

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CHAPTER 1

General Introduction

1.1 Introduction.

Prion diseases or transmissible spongiform encephalopathies (TSEs) are fatal neuro-degenerative diseases of man and animals which can be transmitted by natural and/or experimental infections. Scrapie is the archetype of prion diseases in animals and was first recognised in sheep more then 200 years ago (Parry, 1983). In the early 1950's the first spongiform encephalopathy in man, called kuru, was recognised in the Fore tribe of New Guinea by Carleton Gajdusek and colleagues. This disease had great similarities with scrapie in sheep (Gajdusek and Zigas, 1957). Subsequent work demonstrated that kuru, as well as Creutzfeldt-Jacob disease (CJD), also a fatal spongiform encephalopathy in humans, were transmissible to (non-human) primates. This clearly demonstrated the infectious nature of these diseases (Gajdusek *et al.* 1966). The properties of the infectious agent for TSEs appeared to be highly unusual. Based on the results of early purification and characterisation studies, Griffith *et al.* (1967) proposed a proteinaceous nature for the TSE agent. Supported by further characterisation and isolation studies, the prion hypothesis was formulated (prion = proteinaceous infectious particle) (Prusiner *et al.* 1982). This hypothesis states that the infectious agent in human and animal TSEs is composed of a single kind of protein designated PrP^{Sc} devoid of any nucleic acid and that the major if not the only component of the infectious agent is PrP^{Sc}. This PrP^{Sc} molecule is a misfolded pathological isoform that originates from folding transitions of a normal host encoded glycoprotein designated PrP^{C}.

Currently, a great deal of impressive evidence supports the prion hypothesis. It is evident that the prion protein (PrP) plays a critical role in the disease process and it has become increasingly difficult to explain all the available data by a viral theory of pathogenesis. However, the ultimate test to prove that this provocative protein-only model is correct, i.e. production of infectivity in a test tube by experimental manipulation of synthetic or recombinant PrP, has not been performed yet. The absence of this evidence, however, does not necessarily invalidate the prion hypothesis, it only demonstrates the difficulties of reproducing *in vitro* a remarkable kind of molecular transformation.

Public awareness of prion diseases has increased in the last decade by emerging prion diseases like bovine spongiform encephalopathy ('mad-cow disease') in the UK (over 175,000 confirmed cases). This epidemic had profound economical, medical, and political ramifications. The consequences for human health became even more profound after the appearance of a new-variant form of Creutzfeldt-Jakob disease (nvCJD or vCJD) in humans in the UK late 1995 (Will *et al.* 1996). These nvCJD cases occurred mainly in younger adults and were suspected to be caused by ingestion of BSE-contaminated cattle products. Recently, it has been demonstrated that a similar TSE strain is involved in both BSE and

nvCJD (Bruce *et al.*1997; Collinge *et al.*1996). Sceptics fear that the number of nvCJD cases (39 cases in the UK and one French case at the beginning of 1999) may develop into an epidemic of biblical proportions. Whether this fear is ungrounded is not known yet.

1.2 Transmissible Spongiform Encephalopathies.

TSEs or prion diseases such as CJD, Gerstmann-Sträussler-Scheinker syndrome (GSS), kuru, bovine spongiform encephalopathy (BSE), and scrapie are transmissible neurodegenerative diseases of humans and animals. Table 1 shows different types of prion diseases, their natural hosts, year of first description, and year of experimental transmission.

Prion diseases are always fatal and are characterised by disturbances in behaviour and movement, extreme long incubation periods, absence of immunological responses, astroglyosis, degeneration (sponge formation) in tissues of the central nervous system (CNS), and the accumulation of an abnormal isoform of the host-encoded cellular prion protein in tissues of the CNS and to a minor extent in lymphoid tissues.

Table 1. *Main TSEs of man and animals*

Natural host	Disease	Year of first description/transmission[§]
Humans	Kuru	1957[1] / 1966[2]
	Creutzfeldt-Jakob disease (CJD)	1920[3] / 1969[4]
	New-variant CJD (nvCJD)	1996[5] / 1996[6]
	Gerstmann-Sträussler-Scheinker syndrome (GSS)	1928[7] / 1981[8]
	Fatal Familial Insomnia (FFI)	1986[9] / 1995[10]
Sheep	Scrapie	~1750[11] / 1936[12]
Goat		1942[13] / 1976[14]
Moufflon		1992[15] / n.d.
Mule/Whitetail deer	Chronic Wasting Disease (CWD)	1980[16] / 1982[17]
Elk		1982[18] / n.d.
Mink (farmed)	Transmissible Mink Encephalopathy (TME)	1965[19] / 1967[20]
Cattle (domestic)	Bovine Spongiform Encephalopathy (BSE)	1987[21] / 1988[22]
Greater kudu		1990[23] / 1992[24]
Cat (domestic)	Feline Spongiform Encephalopathy (FSE)	1990[25] / 1993[26]

n.d.: experimental transmission not (yet) demonstrated.

[§] References are indicated in superscript: [1](Gajdusek and Zigas, 1957), [2](Gajdusek *et al.*, 1966), [3](Creutzfeldt, 1920), [4](Gibbs *et al.*, 1968), [5](Will *et al.*, 1996), [6](Bruce *et al.*, 1996), [7](Gerstmann, 1928), [8](Masters *et al.*, 1981), [9](Lugaresi *et al.*, 1986), [10](Tateishi *et al.*, 1995), [11](Parry, 1983), [12](Cuillè and Chelle, 1936), [13](Chelle, 1942), [14](Dickinson, 1976), [15](Wood *et al.*, 1992), [16](Williams and Young, 1980), [17](Williams *et al.*, 1982), [18](Williams and Young, 1982), [19](Hartsough and Burger, 1965), [20](Zlotnik and Barlow, 1967), [21](Wells *et al.*, 1987), [22](Fraser *et al.*, 1988), [23](Kirkwood *et al.*, 1990), [24](Jeffrey *et al.*, 1992), [25](Wyatt *et al.*, 1990), [26](Bruce, 1993).

During disease development normal protease-sensitive PrP (PrP^C) converts into abnormal protease-resistant PrP^{Sc} (from scrapie) isoform by a post-translational process involving profound changes in the secondary and tertiary structure of PrP. This conformational change in protein structure appears to be a fundamental event in the propagation of the causative agent and the pathogenesis of TSEs.

Prion diseases can manifest as contagious, sporadic, and/or inherited disorders. Infectious prion diseases are usually transmitted horizontally although vertical transmission cannot be excluded. Owing to the so-called species barrier the transmission between members of the same species is in general more efficient than the transmission between members of different species. In the inherited forms of prion diseases several mutations have been found in the PrP gene that are associated with the disease. For the sporadic forms it has been hypothesised that unrecognised transmission, somatic mutations in the PrP gene, or spontaneous conversion of PrP^C into PrP^{Sc} may trigger disease development. Like the infectious forms, the sporadic and inherited forms are (experimentally) transmissible.

The factor that causes infectious prion diseases has some of the attributes of a virus, such as transmissibility and strain variation, but it resists procedures that normally destroy nucleic acids and conventional viruses. Many attempts to characterise the infectious agent have led to the suggestion that it differs from a virus and that it is devoid of nucleic acid. The term "prion" was introduced to emphasise the proteinaceous nature of the infectious unit. Prion particles are thought to be composed largely, if not entirely, of these PrP^{Sc} molecules. The current prion hypothesis states that the infectious agent 'replicates' by protein-only and that the actual TSE-agent is an abnormal isoform of the host encoded prion protein, i.e. PrP^{Sc}.

1.3 Prion Protein.

The term "prion" is a synonym for the agent of TSEs or prion diseases. The term "prion protein" is used to indicate the host-encoded PrP molecule. The normal PrP (PrP^C) itself is a harmless glycoprotein of approximately 35 kDa that sticks into the membrane of cells in the brain and other tissues by a glycolipid anchor (Stahl *et al.* 1993). PrP gene organisation and structure are well conserved among mammalian species. The PrP open reading frame (PrP-ORF) of sheep, encoding a polypeptide of 256 amino acids, is completely localised within exon III of the PrP-gene without being interrupted by intron sequences (Goldmann *et al.* 1993). The primary translation product of the PrP-ORF contains an N-terminal signal sequence for translocation of mature PrP into the endoplasmatic reticulum and a C-terminal signal sequence for the addition of a glycolipid anchor (Figure 1). The C-terminal GPI signal sequence is also thought to be involved in the

translocation of PrP^C to caveolae-like domains on the surface of the cell (Kaneko *et al.*1997). After deletion of this C-terminal signal sequence, the PrP^C molecules no longer stay on the cell surface but are secreted into the medium. Post/co-translational modifications of PrP include cleavage of the signal peptides, addition of the GPI-anchor, disulphide bridge formation and asparagine linked glycosylation at two glycosylation sites (Figure 1 and 3).

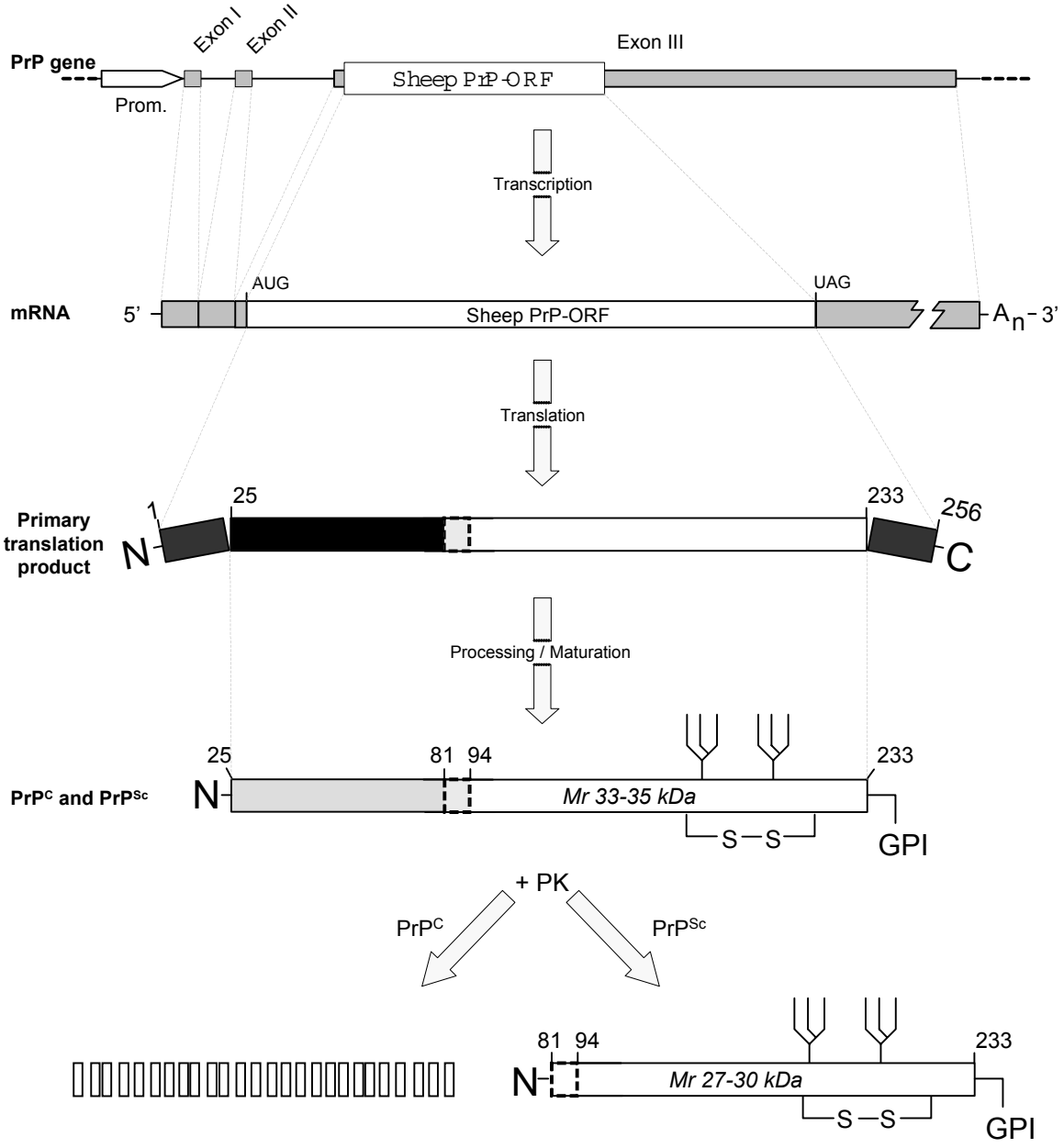


Figure 1. Organisation of the sheep PrP gene and protein. After RNA transcription of the PrP gene and splicing of intron sequences, the mRNA is translated by ribosomes. The primary translation product is further processed (cleavage of signal sequences, glycosylation, disulphide-bridge formation, and addition of a GPI-anchor). Mature PrP^C has a molecular weight of about 33-35 kDa and is fully hydrolysed by proteinase-K. PrP^{Sc} only differs from PrP^C in conformation and not in molecular weight or other posttranslational modifications. Proteinase-K cleaves about 6 kDa from the N-terminus of PrP^{Sc} dependent on conformation approximately between codons 81 and 94.

The normal cellular form of PrP has a half-life of approximately 6 hours after which it is internalised into the cell by endocytic vesicles from where the protein is recycled onto the plasma membrane or is degraded by fusion with non-acidic compartments bound to cholesterol-rich membranes (Bennet *et al.* 1992).

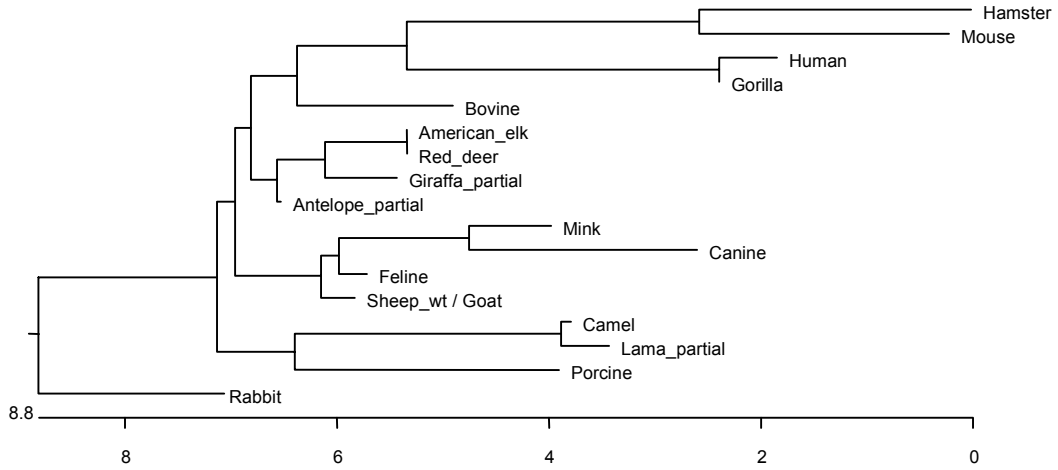
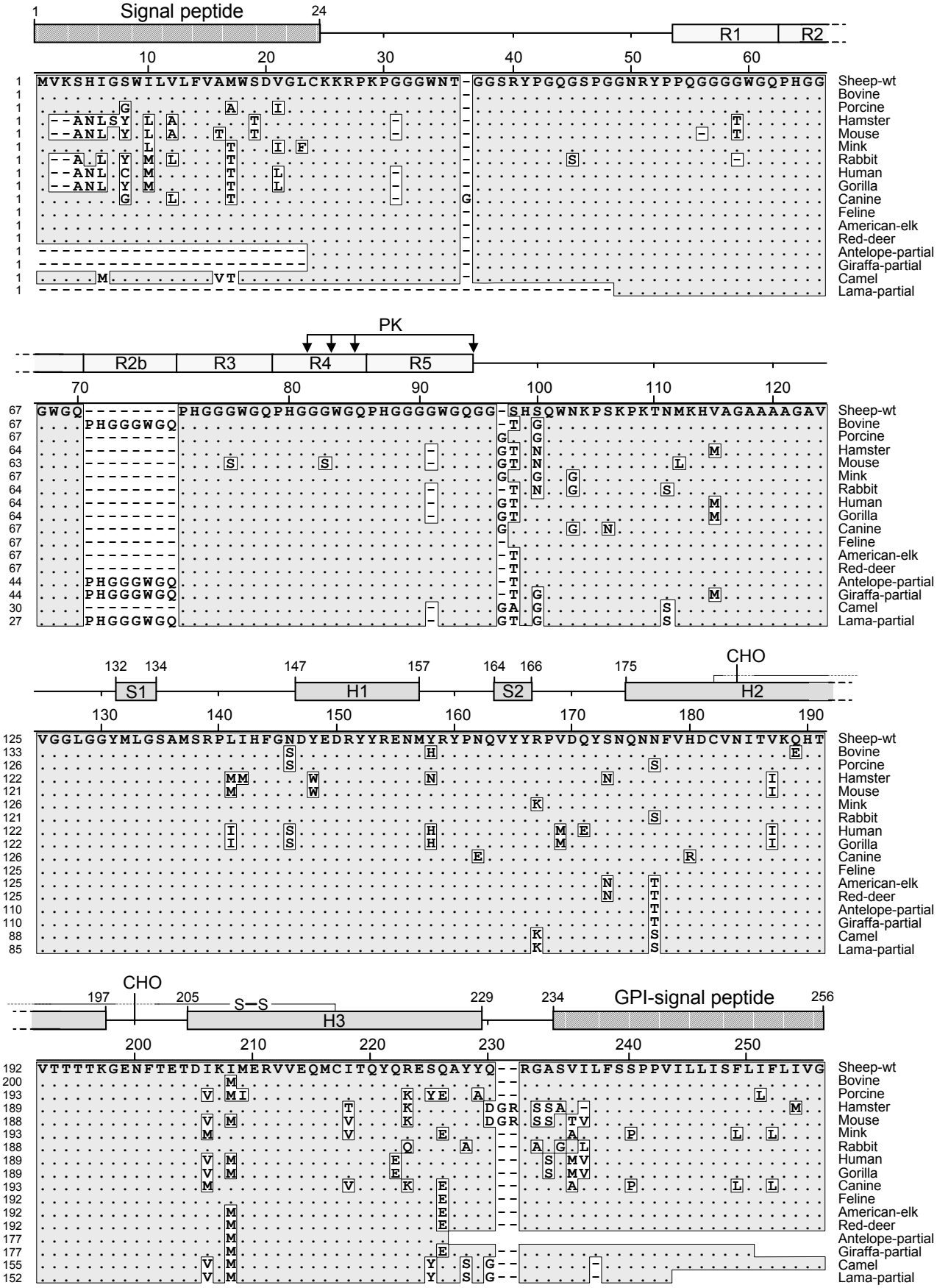


Figure 2. Phylogenetic tree after alignment (Clustal algorithm) of PrP protein sequences truncated for the N-terminal signal sequence and the C-terminal GPI-signal sequence (amino acid 1-24 and 234-256 respectively; sheep PrP-ORF numbering). The units at the bottom of the tree indicate the number of substitution events. Database accession numbers: Sheep_wt (wildtype), AJ00739; Goat, AAD19987; Bovine, X55882; Porcine, L07623; Hamster, K02234; Mouse, M18070; Mink, S46825; Rabbit, AF015603; Human, M13899; Gorilla, U15166; Canine (dog), AF042843; Feline (cat), AF03087; American_Elk, U21210; Red_Deer, Y09761; Pronghorn-Antelope, AF090852; Giraffa, AF113942; Camel, Y09760 and Lama, AF113943.

Although the PrP gene, structure, and organisation is highly conserved between species, suggesting that its function is important (Figure 2), knock-out mice devoid of prion protein develop normally and show only minor abnormalities (Büeler *et al.* 1992, 1993; Collinge *et al.* 1994; Sakaguchi *et al.* 1996; Sailer *et al.* 1994; Tobler *et al.* 1996). Different strains of PrP knock-out mice show different phenotypes, i.e. modification of circadian sleep rhythm, mild electrophysiological abnormalities, and loss of cerebellar Purkinje cells. The exact function of normal PrP is still unknown although it is thought to be involved in copper metabolism as a recycling receptor for uptake of copper from the extracellular

Figure 3 (next page). Multiple amino acid alignment of PrP of the most important species (accession numbers as in figure 2). Residues different from sheep PrP are boxed. Numbering is according to sheep PrP. Above the alignment a schematic representation is shown of the various regions or domains within prion proteins (PrP^C). Structural element boundaries as determined by homology modelling (Swissmodel, Peitsch, 1996) of sheep PrP on basis of hamster and mouse structural data. Putative glycosylation sites (CHO) as well as octarepeat regions (R), α-helices (H), β-sheets (S), and the disulphide-bond (S-S) are indicated separately (α-helices and β-sheets only account for PrP^C). The proteinase-K cleavage site in PrP^{Sc} (conformational structure dependent) is indicated at region 82-94.


Signal peptide
R1
R2
PK
R2b
R3
R4
R5
S1
H1
S2
H2
CHO
S–S
H3
GPI-signal peptide
Sheep-wt
Bovine
Porcine
Hamster
Mouse
Mink
Rabbit
Human
Gorilla
Canine
Feline
American-elk
Red-deer
Antelope-partial
Giraffa-partial
Camel
Lama-partial

milieu and PrP might be involved in the long-term survival of Purkinje neurons (Hornshaw *et al.* 1995; Brown *et al.* 1997; Brown *et al.* 1998; Stockel *et al.* 1998; McKenzie *et al.* 1998; Pauly and Harris, 1998; Sakaguchi *et al.* 1996).

The PrP amino acid sequence is well conserved between the different species with interspecies similarities of generally 90% or higher. Figure 2 and 3 show the results of the alignment of PrP amino acid sequences of 17 species of the more than 60 (sub-)species PrP protein sequences deposited in public databases. The expected clustering of hamster with mouse, human with gorilla, and American-elk with red-deer and antelope is obvious. The most distinct species seems to be rabbit. Rabbits appear to be resistant to natural as well as experimental infections with many, if not all, different TSEs (Barlow and Rennie, 1976). Other more distinct species like pig and camel are also thought to have a low susceptibility to TSE (Dawson *et al.* 1990; Kaluz *et al.* 1997).

Most species carry PrP with 5 octarepeats near the N-terminus (Figure 3) while in some species, like bovine (Goldmann *et al.* 1991) and pronghorn antelope, the six octarepeat variant is most common (R2b in Figure 3). This octapeptide region is probably involved in binding of bivalent metal ions like Cu^{2+} or Zn^{2+}. An extra octarepeat in cattle seems not to be associated with differences in TSE susceptibility (Neiberg *et al.* 1994). In contrast, extra octapeptide insertions (five up to nine extra) have been detected in humans where they all are associated with familial forms of Creutzfeldt-Jakob disease (Goldfarb *et al.*1991, 1993; Laplanche *et al.*1995; Campbell *et al.*1996). Studies in tissue culture cells revealed that insertion of at least 4 to 6 extra octapeptides in hamster PrP^{C} were necessary to find aberrant localisation, spontaneous aggregation and increased protease-resistance of PrP (Priola and Chesebro, 1998). In goats, a short PrP allelic variant with only 3 of these octarepeats has been reported as causing no spontaneous pathological effects (Goldmann *et al.* 1998).

PrP^{C} is soluble in mild detergents and fully degradable by proteinase K. In contrast, PrP^{Sc} forms insoluble aggregates and is partially resistant to proteinase K, which removes only 60-70 amino acid residues from the N-terminus of PrP^{Sc} (Figure 1 and 3) and occasionally a few amino acids from the C-terminus (Parchi *et al.*1998). No covalent chemical differences have been detected between PrP^{C} and PrP^{Sc} that can explain their differences in aggregation properties and proteinase K sensitivities (Caughey *et al.* 1995; Stahl *et al.* 1993). However, PrP^{C} has a high α-helix content and is almost devoid of β-sheet structure, whereas PrP^{Sc} has a lower α-helix content but a high ß-sheet content (Pan *et al.* 1993). Table 2, summarises the main differences between PrP^{C} and PrP^{Sc}.

Table 2. *Differences between PrP^C and PrP^{Sc}.*

PrP^C	**PrP^{Sc}**
Normal cellular form	Pathological form
Protease sensitive M_r (-PK) 33-35 kDa M_r (+PK) fully degraded	Protease resistant (partly) M_r (-PK) 33-35 kDa M_r (+PK) 27-30 kDa
Halflife ~6 hours	Halflife »years
Soluble	Insoluble (precipitates/aggregates)
Monomer	Polymer (Scrapie Associated Fibrils)
Mainly alpha-helical structure	Predominant beta-sheeted structure
Not infectious	Infectious

Recently 3D structures for recombinant mouse (codons 121 to 231) and hamster (codons 90 to 231) PrP expressed in *Escherichia coli* have been generated on the basis of NMR data (Figure 4 left panel) (Riek *et al.* 1996; Donne *et al.* 1997;James *et al.* 1997). From these NMR structures of PrP^C and based on many experimental parameters, models for PrP^{Sc} have been constructed (Figure 4 right panel) (Huang *et al.* 1996).

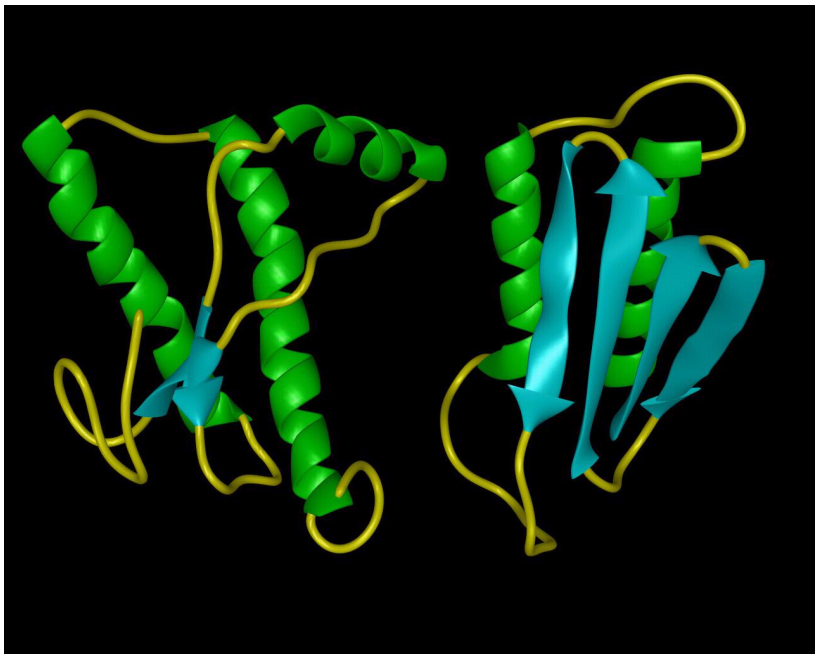


Figure 4. Three dimensional models of PrP. The left model represents the structure as determined by NMR for recombinant hamster PrP^C (James *et al.* 1997). The right model represents the theoretical model of monomeric hamster PrP^{Sc} (Cohen *et al.*, Cellular & Molecular Pharmacology, University of California, San Fransisco USA).

PrP^C has three α-helices and two smaller β-sheets (predicted locations of these α-helices and β-sheets for sheep PrP are depicted in the alignment figure 3). These two small β-sheets are thought to form the interface between PrP^C and PrP^{Sc}. PrP^{Sc} is formed from PrP^C by profound changes in the secondary and tertiary structure of PrP. It is supposed that from these small β-sheets in PrP^C the first α-helix (S1, S2, and H1 in figure 3) is converted into an anti-parallel organised β-sheeted structure by a zipper like mechanism. These changes allow PrP^{Sc} to aggregate into compact fibril-like structures. No modifications other than changes in the three dimensional structure result in the formation of PrP^{Sc} from PrP^C. Studies involving the full-length mouse (23-231) or hamster (29-231) recombinant PrP's showed that the N-termini are highly flexible without any identifiable defined structure (Riek *et al.* 1997; Donne *et al.* 1997).

1.4 Prion protein conversion.

It is now generally accepted that PrP^{Sc} is derived from PrP^C by a post-translational process involving conformational changes only. According to the (protein-only) prion hypothesis, infectious prion particles consist of PrP^{Sc} molecules which are able to convert the harmless host PrP^C into additional copies of PrP^{Sc}, thus replicating itself in the absence of nucleic acid (Fig. 5 panel 1). The interaction between PrP^C and PrP^{Sc} and the subsequent conversion are most efficient when the two isoforms have the same primary amino acid sequence, giving a molecular basis to the species barrier (Prusiner *et al.* 1996). In inherited prion diseases, the mutated host PrP^C is thought to convert spontaneously into the pathogenic PrP^{Sc}, thereby generating a "seed" for further conversion of PrP^C into PrP^{Sc} (Fig. 5 panel 2). Some PrP-ORF mutations may make PrP^C more prone to convert into PrP^{Sc} after infection with TSE agent, while other mutations may make PrP^C less prone to convert into PrP^{Sc} (Fig. 5 panel 3). Such mutations cause intra-species variability in the susceptibility for prion diseases and differences in the incubation periods.

Two different mechanisms have been proposed for the conversion of PrP^C into PrP^{Sc}. In the first mechanism, PrP^{Sc} can exist as a monomer while in the second it only exists as a polymer. Much experimental evidence indicates the validity of the nucleated polymerisation mechanism (Caughey *et al.* 1995, 1997; Come *et al.* 1993; Gajdusek 1988; Kocisko *et al.* 1996; Lansbury and Caughey 1995). Others consider the aggregation and polymerisation as being an artefact of PrP^{Sc} isolation and favour the dimerisation mechanism (Kaneko *et al.* 1995).

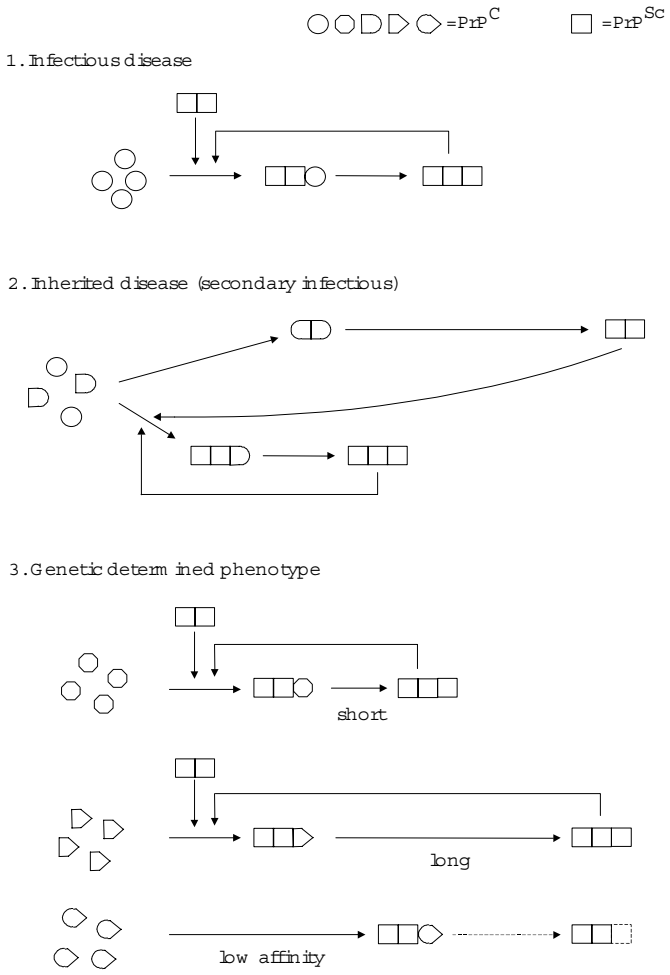


Figure 5. Schematic representation of the central pathogenic events in infectious (panel 1) and inherited (panel 2) forms according to the prion hypothesis. These inherited forms can also be transmitted causing a secondary infection. Panel 3 demonstrates schematically how polymorphisms in PrP can modulate the susceptibility of an individual which results in different incubation times.

Results of experiments with transgenic mice suggest that other so-far unknown host-encoded factors may function in the formation of new PrP^{Sc} molecules (Telling *et al.* 1995). Specific chaperones like GroEL and to a lesser extend Hsp104 have been identified *in vitro* as factors that are able to promote the conversion process from PrP^{C} into PrP^{Sc} (DebBurman *et al.* 1997). However, the conversion of PrP^{C} into protease-resistant forms in a cell-free system does not seem to require other factors (Figure 6)(Caughey *et al.* 1995; Kocisko *et al.* 1994).

Basically, cell-free conversion reactions require only the mixing of purified (and radio-labelled) PrP^{C} with PrP^{Sc} isolated from TSE affected brains (Figure 6). The radiolabelled protease-sensitive PrP^{C} is converted by the protease-resistant PrP^{Sc} into new radiolabelled protease-resistant PrP without the biosynthesis of new macromolecules (Kocisko *et al.*

1994). This cell-free conversion is generally most efficient when input PrP^{C} and PrP^{Sc} have the same primary amino acid sequence, an observation that could account for the existence of the species barriers (Kocisko *et al.* 1995). Furthermore, it has been observed that in the cell-free system one and the same PrP^{C} molecule can be converted into at least two different conformational structures (Bessen *et al.* 1995).

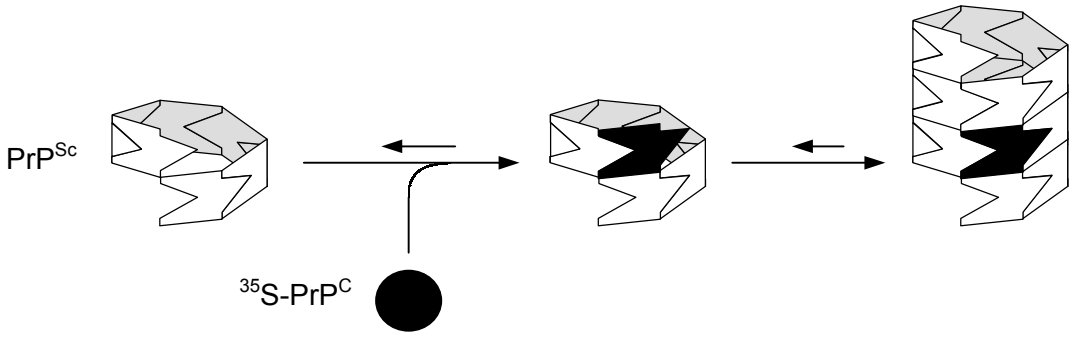


Figure 6. Cell-free (*in vitro*) formation of protease-resistant prion protein (Kocisko *et al.* 1994). Immuno-purified and radio-labelled hamster PrP^{C} (proteinase-K sensitive) is mixed and incubated with partially denatured hamster PrP^{Sc} (proteinase-K resistant). After proteinase-K digestion of the reaction products radiolabelled proteinase-K resistant PrP is formed.

This cell-free conversion system reproduced many biological parameters like species specificity and the non-genetic propagation of TSE strains (Kocisko *et al.* 1995; Bessen *et al.* 1995). The existence of different conformational structures for PrP^{Sc} and the copying of these structures to recruited PrP^{C} molecules is an essential prerequisite for the prion hypothesis to explain strain-specific properties such as differences in incubation period or different lesion patterns in the brain. No evidence has been obtained yet that detectable *de novo* prion infectivity has been generated in this cell-free system however (Kaneko *et al.* 1995; Kocisko *et al.* 1994; Hill *et al.* 1999). Final acceptation of the prion hypothesis awaits experiments in which such *de novo* infectivity is generated from PrP^{C} molecules under conditions guaranteed to be free of any nucleic acid, exogenous TSE infectivity, and which is generated under defined chaperone conditions.

1.5 Linkage between PrP and barriers to transmission.

Transmission of TSEs between hosts of the same species (homologous transmissions) are per definition more efficient than transmissions between hosts of different species (heterologous transmissions). The amino acid sequence homology between the PrP^{Sc} constituent of the agent and PrP^{C} of the host is an important parameter in determining the level of this species-barrier. An absolute species barrier was found in mice lacking the normal prion protein. Since these knock-out mice are resistant to prion disease,

this indicates that PrP expression is required for the development of the disease (Büeler *et al.* 1993; Sailer *et al.* 1994). Experiments with transgenic mice have further indicated that the level of PrP^{C} expression is directly proportional to the rate of PrP^{Sc} formation and the length of the incubation period (Manson *et al.* 1995).

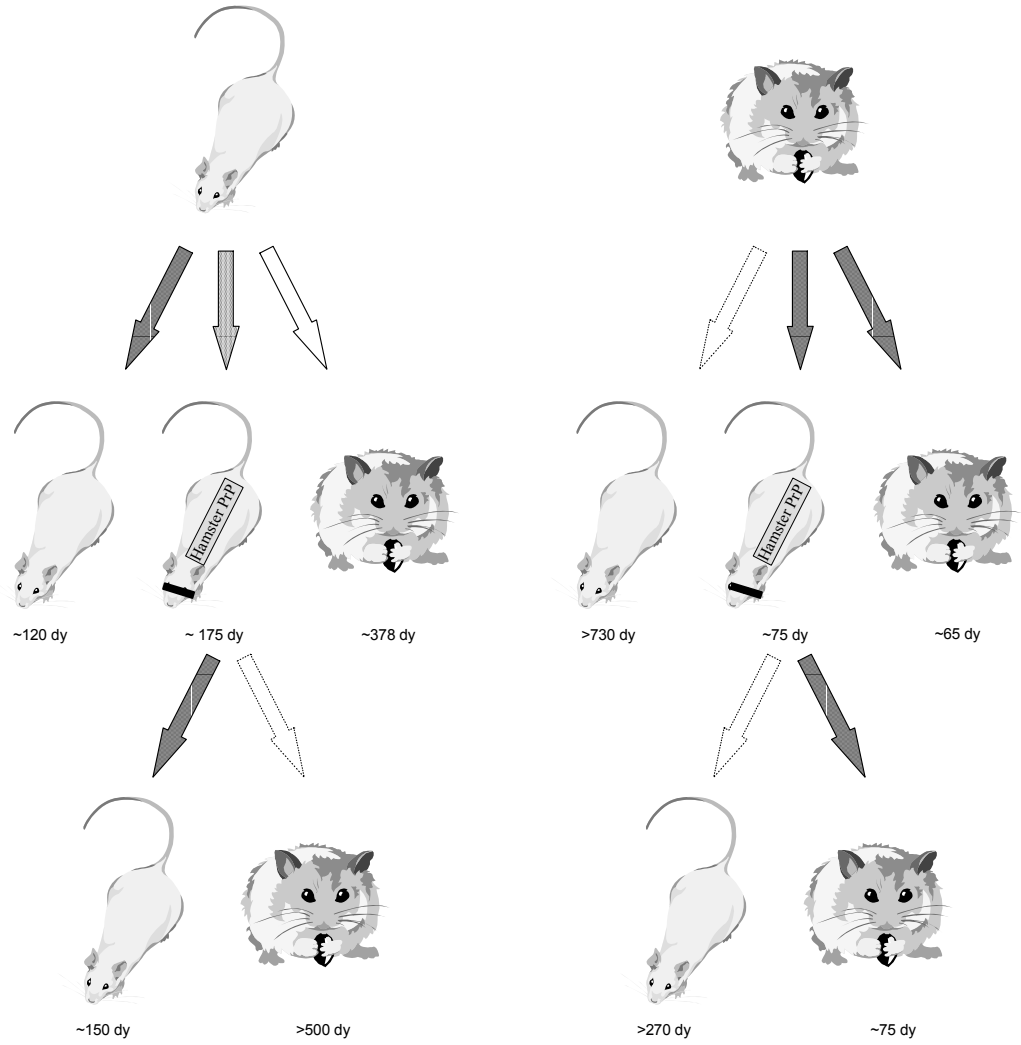


Figure 7. Experimental transmission of Chandler mouse or hamster 263K scrapie to mice, hamsters, or transgenic mice overexpressing a hamster PrP transgene. Efficiencies of transmissions are indicated by the arrows (the darker the arrow, the more efficient the transmission). Average incubation periods between time of inoculation and onset of clinical disease are indicated in days.

The barrier to interspecies transmissions can result either in a complete resistance to transmission (as found in PrP knock-out mice) or to an increased incubation period between the time of infection and onset of clinical disease. The level of this 'species barrier' varies depending on the species being infected, the species in which the agent was formed (replicated), the specific strain of TSE agent involved, and some other unidentified factors. Until now there are no reports on TSE isolates that have shorter incubation periods

in heterologous hosts than in their homologous host. For instance, Chandler mouse scrapie can be transmitted to mice with incubation periods of about 120 days, while Syrian hamsters have an incubation period for this isolate of about 378 days (Figure 7) (Kimberlin and Walker 1978; Kimberlin *et al.* 1987). Conversely, the hamster 263K strain of scrapie has only a 65 days incubation period in Syrian hamsters but has until now not successfully been transmitted to mice (no clinical disease after 2 years) (Kimberlin *et al.* 1989). If however, hamster PrP is overexpressed in transgenic mice, these mice do become susceptible to hamster TSE but propagate hamster instead of mouse prion agent (Figure 7) (Scott *et al.* 1989). The same situation is applicable to transmission of human TSE to mice. Normal mice are relatively resistant to human TSE, while mice carrying human PrP transgenes are highly susceptible to human TSE especially when endogenous mouse PrP gene is not functional (Telling *et al.* 1995).

Not only experiments with transgenic mice but also experimental infections and genetic studies in different species have indicated that the PrP genotype strongly influences the host susceptibility and donor transmissibility of TSE agents from and to different species (Carlson *et al.* 1986; Goldmann *et al.* 1994; Race *et al.* 1988). For instance, sheep with the 'wildtype' PrP genotype (prion protein EMBL accession no. AJ000739) are more susceptible to BSE than sheep that have other allelic forms of PrP like a valine at codon 136 or an arginine at codon 171 (EMBL accession no. AJ000738 and AJ000736 respectively) (Goldmann *et al.* 1994). Other studies have indicated that a high homology between PrP^{C} and PrP^{Sc} molecules between amino acid positions 90-225, is preferred for efficient PrP^{Sc} accumulation (Goldmann *et al.* 1994; Priola *et al.* 1994, 1995; Scott *et al.* 1992; Telling *et al.* 1994).

Furthermore, the N-terminal protease sensitive part of PrP^{Sc} and the region including α-helix 1 and β-sheet 2 of the mouse prion protein (H1 (codons 25 to 94) and S2 (codons 136 to 173) respectively using sheep PrP numbering in Figure 3) could be deleted without loss of PrP^{Sc} formation in transgenic mice or scrapie infected neuroblastoma cells (ScN2a cells) (Rogers *et al.* 1993; Muramoto *et al.* 1996). PrP^{C} lacking the GPI-anchor by deleting the C-terminal signal sequence, could also convert into PrP^{Sc} in ScN2a cells (Rogers *et al.* 1993), although with reduced efficiency. Deletion of the regions containing either β-sheet-1 (S1 Figure 3, codons 117 to 136) or one of the cysteins involved in disulphide-bridge formation (codon regions 173 to 197 or 197 to 214 respectively), prevented PrP^{Sc} formation in ScN2a cells (Muramoto *et al.* 1996). However, interference with one or both of the asparagine-linked glycosylation sites (codons 184 and 200 respectively, Figure 3) by site-directed mutagenesis or by inhibiting glycosylation with tunicamycin-D in ScN2a cells or cell-free systems, showed that aglycosyl PrP^{C} can readily

be converted into protease-resistant forms of PrP (Taraboulos *et al.* 1990; Kocisko *et al.* 1994; this thesis). Mutations that disrupt the N-linked glycosylation sites causes the mutant protein (especially at codon 184) to accumulate inside the cell (Lehmann and Harris, 1997). It is still unknown whether these mutant forms of PrP^{C} can be converted into PrP^{Sc}. Until now only one natural mutation disrupting an N-linked glycosylation site (consensus N-X-T/S) has been described at codon 183 (Thr to Ala) of human-PrP (sheep codon 184). This mutation seems to be linked to a familial form of CJD with some atypical characteristics (Nitrini *et al.* 1997).

Not only deletions, insertions or secondary structure modifications in PrP are involved in determining species barriers but also changes of a single amino acid residue can have dramatic effect on TSE susceptibility and transmissibility. The most striking example of this is the hamster-mouse species barrier. These species have very similar amino acid sequences but still encounter a high species-barrier (compare Figure 2 with Figure 7). Using chimeric hamster-mouse PrP molecules in ScN2a cells, this hamster-mouse species-barrier effect seemed to be determined by a single amino acid mismatch between hamster and mouse PrP at codon 138 of mouse PrP (Priola and Chesebro, 1995). This stresses the importance of the type and location of the polymorphisms in the primary amino acid sequence in determining species barriers.

Single amino acid changes may also determine the phenotypic expressions of TSE's (see Young *et al.* 1999 for a complete review). The most striking example is probably the behaviour of the polymorphism combination at amino acid positions 178 and 129 of human PrP. If codon 178 encodes an asparagine and codon 129 a methionine the patient develops FFI, while patients having asparagine at codon 178 and valine at codon 129 develop CJD (Goldfarb *et al.* 1992). The codon 129 polymorphism itself is rather common in humans and not associated with TSE (Collinge *et al.* 1991; Palmer *et al.* 1991). However, it does alter the phenotypic expression of the TSE associated with the codon 178 polymorphism.

In the PrP gene of sheep several polymorphisms have been detected (Goldmann *et al.* 1990; Goldmann *et al.* 1991; LaPlanche *et al.* 1993). A polymorphism at codon 136 was found to be associated with susceptibility in both experimental (Goldmann *et al.* 1991; Goldmann *et al.* 1994; Maciulis *et al.* 1992) and natural scrapie (Laplanche *et al.* 1993; Hunter *et al.* 1993). A polymorphism at codon 171 is also associated with susceptibility in Cheviot sheep for experimental (Goldmann *et al.* 1994) and in Suffolk sheep for natural scrapie (Westaway *et al.* 1994). Polymorphisms at codons 112 and 154, are rare and have not been significantly associated with scrapie susceptibility yet. The first chapters of this thesis will further describe the quest to find and associate polymorphisms in the sheep-PrP gene with scrapie susceptibility, transmissibility, and survival times. In the following

chapters the effect of different polymorphisms in either PrP^{C} or PrP^{Sc} of sheep on the 'susceptibility and transmissibility' of sheep scrapie will be discussed.

1.6 TSE strain variation.

TSE strain variation is an important phenomenon that determines differences in pathology and differences in inter- and intra-species transmission efficiencies of TSEs, independent of the host PrP amino acid sequences. Strains of TSE agent can be distinguished from each other on the basis of species tropism, clinical disease, incubation period, neuropathological manifestations, PrP^{Sc} distribution in brain tissue, and sometimes on basis of small biochemical differences. Over 20 phenotypically different strains have been isolated in mice and hamsters by serially passaging natural TSE sources like sheep scrapie, BSE or TME from a wide range of species in an animal with a given *Sinc* (for *S*crapie *inc*ubation gene) or PrP genotype (Kimberlin *et al.* 1978; Carlson *et al.* 1986, 1987; Hunter *et al.* 1987; Bruce 1993; Race *et al.* 1990; Bessen and Marsh, 1992). Many different TSE strains can be stably propagated even within inbred hosts. In the context of the protein-only hypothesis this requires that the propagation of the properties of the strain must be mediated by stable variations in PrP^{Sc} independent of the host-encoded PrP amino acid sequence. In conventional micro-organisms such properties would have their genotype or 'blue-print' in agent-specific nucleic acid.

According to the protein-only hypothesis this blue-print for the phenotype-specific properties of TSE strains is encoded in the three dimensional structure of PrP^{Sc} itself rather than in an agent-specific nucleic acid. There is evidence that PrP^{Sc} molecules can induce a 3D folding pattern onto newly recruited PrP^{C} molecules that is identical to the 3D folding structure of the inducing PrP^{Sc} itself (Kascsak *et al.* 1986; Bessen and Marsh, 1994; Parchi *et al.* 1996; Caughey *et al.* 1998). Molecularely well characterised strains are the two hamster adapted strains of TME (from mink), hyper (HY) and drowsy (DY) respectively (Bessen and Marsh, 1992). These two strains differ in their phenotypic expression in hamsters but also have distinct PrP properties (Bessen and Marsh, 1994). HY-TME causes a disease characterised by hyperexcitability, incoordination, ataxia, and short incubation periods of about 65 days. In contrast, DY-TME is characterised by a progressive lethargy and longer incubation periods of about 165 days. PrP^{Sc} isolated from HY-TME infected isogenic hamster brains is biochemically distinct from DY-PrP^{Sc} (Bessen and Marsh, 1992; Caughey *et al.* 1998). These two PrP^{Sc} isoforms could be readily distinguished by SDS-PAGE after limited proteinase-K digestion (molecular weight differences of about 1-2 kDa). Proteinase-K hydrolyses different fragments from the N-terminal part of PrP^{Sc}, indicating distinct conformational structures. Experiments using these two strains showed

that the strain specific properties could be propagated in inbred hosts *in vivo* (Bessen and Marsh, 1992, 1994) but could also be non-genetically propagated *in vitro* (Figure 8) (Bessen *et al.* 1995).

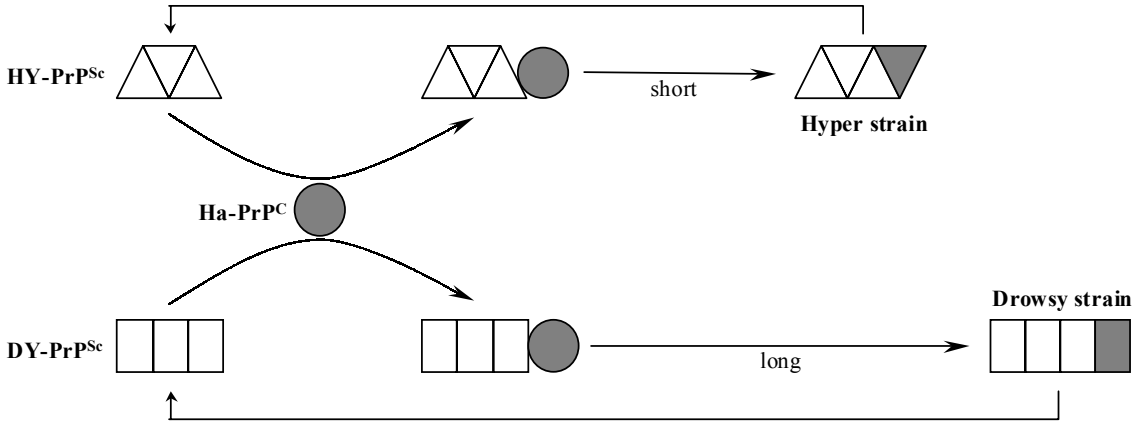


Figure 8: Non-genetic propagation of the hamster adapted hyper (HY) and drowsy (DY) strains of TME. HY-PrP^{Sc}, DY-PrP^{Sc}, and hamster PrP^{C} have all the same primary amino acid sequence but differ in three dimensional conformation. The conformation of HY-PrP^{Sc} or DY-PrP^{Sc} is 'inherited' by the one and the same hamster-PrP^{C} molecule.

In vitro hamster PrP^{C} could be converted in two distinct forms of protease-resistant PrP corresponding to the input type of PrP^{Sc}. This indicated that self-propagation of PrP^{Sc} with distinct three-dimensional structures could be the molecular basis of TSE strains and that the 3D structure itself is the 'blueprint' for strain specific properties.

Another strain distinguishing tool, next to strain typing in mice, are the differences in molecular heterogeneity of PrP^{Sc} due to differential glycosylation and partial endogenous or exogenous proteolysis (Kascsak *et al.* 1985; Hope *et al.* 1988; Somerville *et al.* 1990; Parchi *et al.* 1996; Collinge *et al.* 1996). Strains that have such different endogenous or exogenous PrP proteolysis patterns, are for instance HY and DY of hamster passaged TME (see previous paragraphs) or fatal familial insomnia (FFI) (Bessen and Marsh, 1994; Gambetti *et al.*1995; Telling *et al.*1996). More striking however, are the featured differences in glycosylation of PrP^{Sc}, the so called ‘glycoprofile’. These glycoprofiles originate from differential glycosylation at both glycosylation sites, at only one of the two glycosylation sites, or at neither site. The resulting three-banding pattern and their relative intensities after SDS-PAGE and Western blotting determines the 'glycoprofile' which segregate with different TSE strains. It is proposed that this glycoprofile is the 'blueprint' for the propagation of strain specific phenotypes. The different TSE strains might be targeted to those cells that produce PrP^{C} with the strain specific glycoprofile. Alternatively differential glycosylation may only allow the PrP^{C} molecule to rearrange its three

dimensional structure in limited set of ways. Although the exact mechanism is still unresolved, the glycoprofiles are useful for the identification of different TSE strains. Using this method, several scrapie strains could be differentiated from each other (Somerville *et al.* 1997).

In natural cases of sheep scrapie it is unknown which and how many strains circulate in the various flocks at different times and in different countries. It is thought that in sheep scrapie a mixture of strains is present in the same flock at the same time, and maybe even in the same animal. Whether specific strains of sheep scrapie prefer particular sheep PrP genotypes or sheep breeds is unknown. Strain typing studies of scrapie sheep from the UK revealed that at least three different strains, 82A, ME7 and 87V were present (Bruce, 1993). Although not completely characterised, strain typing of Icelandic scrapie revealed also three distinct scrapie strains which clearly differed from the UK strains (Fraser, 1983).

In contrast to the high degree of variation found between sheep scrapie strains, the agent circulating in UK cattle affected with BSE seems to be very uniform (Fraser *et al.* 1988, 1992; review Bruce *et al.* 1994). This indicates that each cow is probably infected with the same strain of agent. BSE experimentally transmitted to either sheep, goats, or pigs and subsequently to mice, show similar strain specific properties compared to BSE from cattle (review Bruce *et al.* 1994). Analysis of BSE transmissions to various species revealed a remarkable stable glycoprofile clearly different from other strains and this glycoprofile matched the glycoprofile of nvCJD (Collinge *et al.* 1996). The unique glycoprofile of BSE and nvCJD did not match any of the already known glycoprofiles of for instance, sheep scrapie, iatrogenic- or sporadic-CJD. In addition, transmissions of nvCJD to mice revealed a strain type matching with BSE transmissions to mice (Bruce *et al.* 1997). These matching glycoprofiles and strain types indicate that BSE caused nvCJD or that BSE and nvCJD share a common source of TSE.

1.7 Aims and outline of this thesis.

From the public health perspective it is important to know whether particular TSE strains can be naturally transmitted to humans by the use or consumption of animal products. From epidemiological studies of scrapie in sheep and TSEs in humans there are no indications that transmission of scrapie from sheep to humans can occur. This suggests the existence of a very high or even absolute species-barrier for transmissions of scrapie from sheep to humans. The appearance of nvCJD in the UK, that is caused by a strain of TSE indistinguishable from the BSE strain (Bruce *et al.* 1997), and suspected to be caused by infections from BSE infected cattle products, indicates that BSE from infected cattle might have crossed the species-barrier from cattle to humans. Also from the animal health perspective it is important to know the characteristics of transmissions within species but also whether TSE agents from particular animal species can be transmitted to other animal species. For example with regard to the legislation on the use of meat-and-bone-meal in the animal food chain. For these reasons, but also from a scientific point of view, it is important to understand the molecular mechanisms underlying the differences in transmission efficiencies within and between species, and the effects that polymorphisms have on the conversion of prion proteins in abnormal isoforms. These insights will allow us to quantify and predict the levels of inter- and intraspecies-barriers of TSE transmissions and to assess the risks for different species to develop some form of TSE more accurately. This knowledge is necessary to develop methods and strategies to prevent and control TSE diseases.

This thesis describes research towards the identification of polymorphisms in the sheep PrP gene and their association with scrapie susceptibility, transmissibility, and differences in survival times. It describes whether the absence of TSEs means the absence of susceptible PrP genotypes. It also describes efforts to characterise the underlying molecular mechanisms by which polymorphisms determine susceptibility/transmissibility and influence the conversion of normal prion protein into isoforms associated with TSEs. Furthermore it depicts how we used this knowledge to explore and predict the susceptibility of sheep for scrapie having particular PrP genotypes and to assess the potential transmissibilities of scrapie and BSE. The research described in this thesis contributes to understanding the basic mechanisms involved in determining the susceptibility of different hosts for TSEs and mechanisms involved in the inter- and intra-species transmissibilities of TSE agents.

Chapter 2, *PrP allelic variants associated with natural scrapie*, describes how different combinations of polymorphisms (allelic variants) of the sheep PrP gene were identified, characterised, and associated with natural scrapie. Therefore the different PrP allelic variants were determined in scrapie affected sheep, healthy sheep from scrapie-free flocks and in healthy sheep from scrapie-affected flocks. All sheep were predominantly of the Texel breed. To analyse these PrP genes, we applied 'denaturing gradient gel electrophoresis' (DGGE) allowing the separation of DNA molecules differing by as little as a single base change and in addition allowing us to determine hitherto unidentified polymorphisms. Six different PrP allelic variants were found of which at least two could be significantly associated either with increased susceptibility or with resistance.

Chapter 3, *PrP genotype contributes to determining survival times of sheep with natural scrapie*, describes whether specific PrP allelic variants could be associated with different survival times in scrapie affected sheep. Using DGGE, the PrP genotypes of sheep were determined within in a single scrapie affected flock which were all born in the same year ('within-flock' study). Several PrP genotypes could be associated with differences in survival times and all scrapie affected sheep from this flock carried at least one copy of a particular PrP allelic variant (PrP^{VRQ} respectively). Two hitherto unidentified PrP allelic variants were also identified.

Chapter 4, *PrP genotype frequencies of the most dominant sheep breed in a country free from scrapie*. This chapter describes the determination of PrP genotypes (by DGGE) of sheep residing in an environment absolutely free of scrapie (New Zealand). We found that PrP allelic variants associated with highest scrapie susceptibility and short survival times were present, which indicates the requirement of infectious prion agent in the environment for TSE development but also demonstrates the effectiveness of the measures taken by New Zealand to stay free from scrapie.

Chapter 5, *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms*. This chapter describes studies to explore whether sheep PrP^{C} can be converted *in vitro* to protease-resistant forms resembling PrP^{Sc}. In addition it was investigated whether the various sheep PrP allelic variants have different cell-free conversion characteristics and whether these characteristics reflect the observed differences in sheep scrapie susceptibility and the observed differences in survival times of scrapie-affected sheep.

Chapter 6, *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine natural occurring variants of PrP.* In this study the effects of natural occurring sheep PrP polymorphisms on the cell-free conversion efficiencies of PrP^{C} into protease-resistant isoforms were further explored. The reactions, induced by different sheep PrP^{Sc} variants,

revealed a profile of relative susceptibility of sheep having one of these different PrP^C variants. These results clearly indicated that not only the PrP genotype of the acceptor is important but also the PrP genotype of the donor that produced the agent. In more general terms; scrapie susceptibility is not only dependent on the genetic background of the host but also dependent on the PrP genotypes of sheep in the direct environment. The results strengthen our views on polymorphism barriers and has further implications for scrapie-control/eradication programs by breeding strategies.

Chapter 7, *Molecular assessment of the potential transmissibilities of BSE and scrapie to humans*. This chapter describes efforts to assess on a molecular level the potential danger that BSE may have crossed the species barrier to humans by the human food chain. Therefore, the relative convertibility of human PrP^C by BSE and scrapie was compared, the latter being no detectable risk to humans. The *in vitro* conversion results of many different species' PrP^C with PrP^{Sc} from different species revealed a clear correlation of conversion efficiencies with *in vivo* susceptibility and transmissibility of TSEs. Subsequently it was demonstrated that the convertibility of human PrP^C by either BSE or sheep scrapie was finite but similarly low.

Chapter 8, *Summary and General discussion*. In this chapter the results and conclusions of the present work are briefly summarised and discussed with special emphasis on the current view and understanding of polymorphism-barriers in the inter- and intra-species transmission of TSE's. Additionally, topics for future research are highlighted to gain more insight in the molecular interactions between prion proteins, to identify potential prion protein binding sites, and/or to develop potential therapeutics will be discussed.

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CHAPTER 2

PrP Allelic Variants Associated With Natural Scrapie

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Summary

To determine whether there exist an association between specific combinations of polymorphisms in the PrP gene of sheep and natural scrapie, we applied denaturing gradient gel electrophoresis (DGGE) to analyze the PrP genes of 69 scrapie-affected and 176 healthy sheep predominantly of the Texel breed. We found six PrP allelic variants representing different combinations of polymorphisms at codons 136, 154, 171, and 211. Based on the triplet sequences present at codons 136, 154, and 171 these alleles were designated PrP^{VRQ}, PrP^{ARR}, PrP^{ARQ}, PrP^{ARH}, PrP^{AHQ}, and $PrP^{ARQQ211}$. Among the 245 sheep 15 different PrP genotypes or allele-pairs were present. The distribution of the allelic variants revealed that the PrP^{VRQ} allele is associated with a high incidence of natural scrapie and the PrP^{ARR} allele with a low incidence. The PrP^{ARQ} and PrP^{ARH} alleles were not associated with disease incidence. Nevertheless a number of scrapie-affected sheep were homozygous for the PrP^{ARQ} allele. Whether in these sheep other factors than the PrP genotype play a more decisive role in inducing the disease than in sheep with a PrP^{VRQ} allele remains to be established. The frequencies of the PrP^{AHQ} and the $PrP^{ARQQ211}$ alleles were to low to draw any conclusions.

Introduction

Scrapie is a fatal neurodegenerative disease of the central nervous system that occurs naturally in sheep. It is the archetype of a group of disorders known as transmissible spongiform encephalopathies or prion diseases. Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI) of man and bovine spongiform encephalopathy (BSE) are also members of this group. The etiology of scrapie has been debated for many years but the precise etiology remains unknown. Some considered natural scrapie as a genetic disorder, caused by an autosomal recessive gene, that just happens to be experimentally transmissible (1). Others considered scrapie as a virus-like disease, the outcome of which is influenced by host genetic components (2, 3, 4). The current view is that scrapie is an infectious disease where host genetic factors play an important role. By experimental infection of sheep with scrapie Dickinson and Outram (5) demonstrated that disease susceptibility and disease incubation period is controlled by a single sheep gene.

The PrP gene of the host is the gene that is most closely associated with features of prion diseases such as susceptibility, incubation period, incidence, and pathology (6, 7). In man at least 10 missense, one nonsense, and 7 insert mutations in the PrP gene segregate with prion diseases (7). A common polymorphism at codon 129 is associated with susceptibility to iatrogenic and sporadic CJD (8, 9). This polymorphism also determines the phenotype of the disease in familial CJD and FFI patients who have a PrP mutation at codon 178 (10). This suggests that specific combinations of polymorphisms within the PrP gene are associated with particular features of prion diseases.

In the PrP gene of sheep several polymorphisms have been detected (11, 12, 13). A polymorphism at codon 136 is associated with scrapie susceptibility in both experimental (12, 14, 15) and natural scrapie (13, 16). A polymorphism at codon 171 is also associated with susceptibility of Cheviot sheep for experimental scrapie (14) and Suffolk sheep for natural scrapie (17). Other polymorphisms, including polymorphisms at codons 112 and 154, are rare and have not been associated with scrapie.

In this study we identified and characterized different combinations of polymorphisms (allelic variants) of the sheep PrP gene and we investigated their association with natural scrapie. To this end we determined the PrP allelic variants of 69 scrapie-affected and 176 healthy sheep by denaturing gradient gel electrophoresis (DGGE). This technique allows the separation of DNA molecules differing by as little as a single base change.

Materials and methods

Sheep.

We used 210 sheep of the Texel breed, 34 were scrapie-affected, 91 were healthy and derived from scrapie-free flocks, and 85 were healthy and derived from scrapie-affected flocks. In addition we used 35 scrapie-affected sheep of seven other breeds. The scrapie-affected sheep from 18 different flocks were collected throughout the Netherlands from August 1991 to October 1992, with ages between 2 and 5 years. All scrapie cases were confirmed by histology and immunocytochemistry as described elsewhere (18). The age matched healthy control sheep derived from scrapie-free flocks (free from scrapie for the last five years) were collected from 15 different flocks in the Netherlands and were collected in the same period. The age matched healthy control sheep derived from scrapie-affected flocks were collected from 13 different flocks in the Netherlands. The 35 scrapie-affected sheep of seven other breeds were collected in the Nertherlands in the same period as the Texel sheep and were of the same age (detailed data will be published elsewhere). About 4 to 8% of the Dutch flocks suffer from scrapie, with an incidence of 1 in 100 sheep/ year (19). From all sheep we collected blood samples in EDTA vacutainers (Venoject, Terumo Europe N.V.) and they were frozen at -20°C until further use.

DNA amplification.

High-molecular-weight DNA was isolated from blood as described by Sambrook et al. (20). The PrP open reading frame, which resides on a single exon (11), was amplified by PCR. Amplification reactions were done in 50 µl containing 100 ng genomic DNA, 25 mM Tris-HCl pH 8.7, 2 mM $MgCl_2$, 0.005% gelatine, 200 µM dNTP's, 1 µM of primers, and 1 unit of Taq DNA polymerase (Perkin-Elmer-Cetus). The sequences of the oligonucleotide primers and their positions in the PrP gene have been described (21).The amplification reactions were performed in a Perkin-Elmer-Cetus DNA Thermal Cycler for 35 cycles of 1 min. 92°C, 1.5 min. 58°C, 1.5 min. 72°C with a hot start (80°C).

DGGE analysis.

Sheep genomic DNA was amplified as described above and 15 µl was digested with the restriction enzyme HinfI in a total volume of 20 µl. Subsequently the amplified DNA was denatured for 10 min. at 100°C and renatured at room temperature. The DNA was separated on DGGE gels containing 6% polyacrylamide (37.5:1, acrylamide:bisacrylamide) with a linear gradient from top to bottom of 45% to 65% denaturant (100% denaturant = 7 M urea/40% formamide (vol/vol), gellength = 17 cm) in 40 mM Tris-acetate, 1 mM EDTA,

pH 7.4. Electrophoresis was done at 50 V for 24 hours at 60°C in 40 mM Tris-acetate, 1 mM EDTA, pH 7.4 in an electrophoresis tank as described by Myers et al. (21). Gels were stained with ethidium bromide and examined by UV transillumination.

Cloning and sequencing.

For cloning and sequencing purposes, the coding region of the PrP allelic variants was amplified by PCR using Vent DNA polymerase (New England Biolabs) as described previously (21). Amplified fragments were inserted into the EcoRI and XbaI site of pGEM7 (Promega). Clones, representing different allelic variants of the PrP gene, were distinguished from each other by DGGE analysis as described above. Sequencing was done using the chain termination reaction (23). At least two independent clones of each allelic variant were sequenced to exclude PCR artefacts.

Statistical analysis.

Results were analyzed using the χ^2 test of association by comparing genotype frequencies in the scrapie-affected sheep and the healthy control sheep.

Results

Identification of PrP allelic variants and novel polymorphisms by DGGE analysis.

To identify and characterize allelic variants of the PrP gene of sheep and to identify hitherto unknown polymorphisms, we applied DGGE on PCR amplified DNA from codon position 1 to 223 of the PrP-gene (11). A single DGGE run allowed us to detect polymorphisms in a region that covers almost 90% of the coding region for PrP 27-30 (21). On the basis of the DGGE patterns we identified five different PrP allelic variants in 210 scrapie-affected and healthy sheep of the Texel breed. We cloned and sequenced these variants to identify the base changes responsible for their characteristic mobilities in the DGGE gel (Figure 1, lanes 2-6). Based on the sequence differences found at codon positions 136 (alanine (A) or valine (V)), 154 (arginine (R) or A), and 171 (glutamine (Q), histidine (H), or R), the first variant was designated PrP^{ARR} (Figure 1, lane 2). The second variant was designated PrP^{ARQ} and the third PrP^{VRQ} (Figure 1, lanes 3 and 4).

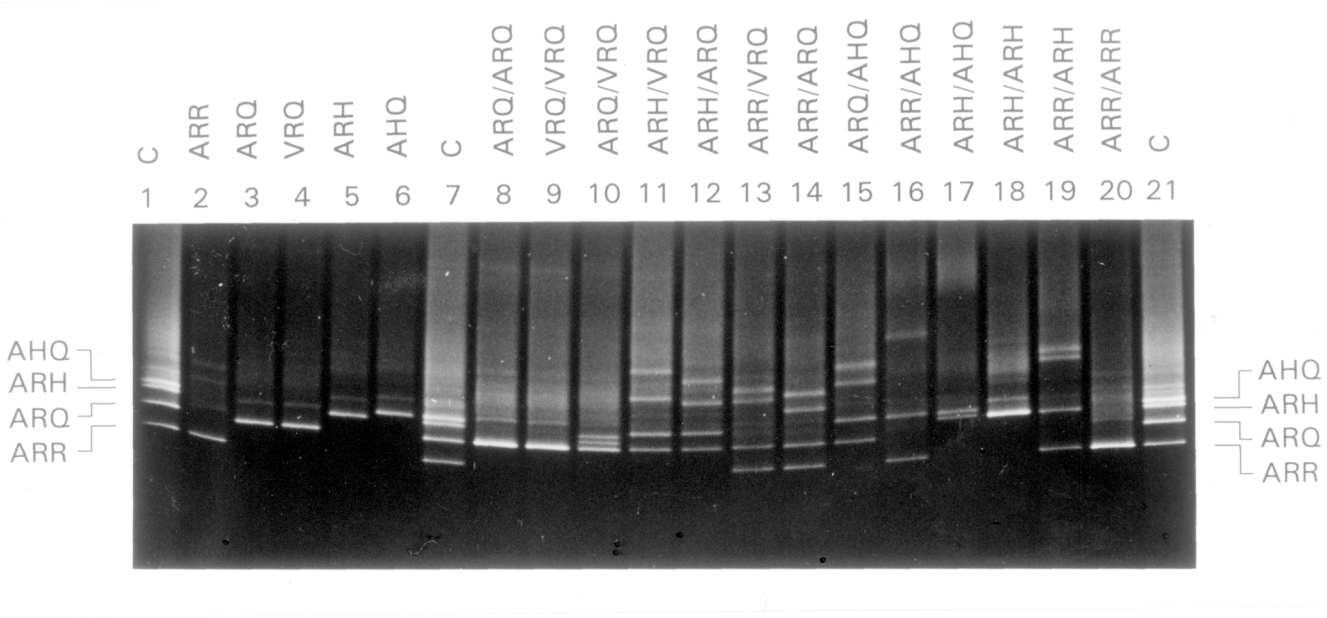

Figure 1. DGGE analysis of PCR amplified DNA. Lanes 2-6: DGGE patterns obtained from five cloned and sequenced allelic variants. Lanes 8-20: DGGE patterns representing 13 different genotypes present in sheep of the Texel breed. Lanes 1, 7, and 21 are control lanes that contain 4 combined allelic variants which were not denatured and renatured before loading. Allele variants are indicated on the right and on the left. Genotypes are indicated above the lanes. Figure is reproduced with permission of the Journal of General Virology.

Although the homoduplex bands of the PrP^{ARQ} and PrP^{VRQ} variants could not be distinguished from each other, they could be distinguished when heteroduplex molecules were formed with these variants (compare for example lanes 11 and 12). The fourth variant was designated PrP^{ARH} (Figure 1, lane 5). From the sequence analysis it appeared that also two silent mutations occurred in the PrP^{ARH} variant, one at codon 231 (AGG->CGG) and one at codon 237 (CTC->CTG). These mutations are however located outside the fragment analyzed by DGGE and therefore can not contribute to the shift in electrophoretic mobility. The fifth allelic variant was designated PrP^{AHQ} (Figure 1, lane 6) .

We also analyzed the PrP genes of 35 scrapie-affected sheep of seven other breeds. In this group we found four DGGE patterns identical to those found in the sheep of the Texel breed, and one DGGE pattern that differed (Figure 2). The homoduplex bands of this latter variant resembled the PrP^{ARH}/PrP^{ARQ} or the PrP^{ARH}/PrP^{VRQ} genotypes (Figure 2, lane 6). The heteroduplex bands revealed however a hitherto unknown DGGE mobility pattern (compare Figure 1, lanes 11 and 12, and Figure 2, lanes 6 and 7). By cloning and sequencing we found that this sheep contained one PrP^{VRQ} allele and a PrP^{ARQ} allele with an additional G->A transition at codon 211 resulting in an R to Q substitution. The latter allelic variant was therefore designated $PrP^{ARQQ211}$.

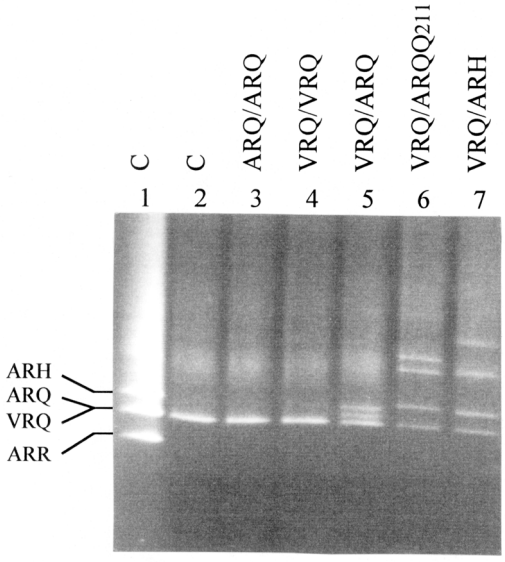

Figure 2. DGGE analysis of PCR amplified DNA. Lanes 3-7: DGGE patterns representing the 5 different genotypes present in the 35 scrapie-affected sheep from breeds other than the Texel breed. Lane 1 contains 3 combined allelic variants which were not denatured and renatured before loading. Lane 2 contains the PrP^{VRQ} variant. The haplotypes are indicated on the left. Genotypes are indicated above the lanes.

PrP allelic variants and their association with natural scrapie.

To investigate the association of the PrP allelic variants with natural scrapie, we identified the PrP alleles of 210 sheep of the Texel breed. Of these sheep 34 were scrapie-affected, 91 were healthy and derived from scrapie-free flocks, and 85 were healthy and derived from scrapie-affected flocks. In addition we determined the PrP genotypes of 35 scrapie-affected sheep of seven other breeds. In the Texel breed we found 14 different DGGE patterns (12 patterns are shown in Figure 1, lanes 8-20), all of which could be reproduced by mixing two of the five cloned and sequenced alleles (data not shown). Since heteroduplex bands with the PrP^{ARQ} and PrP^{VRQ} variants could be distinguished from each other and homoduplex bands not (see above), all samples showing homoduplex bands were mixed with another variant and analyzed again by DGGE. After this analysis (data not shown) we ended up with 15 different sets of PrP alleles (genotypes) in the sheep of the Texel breed.

Table 1. *Frequencies of PrP genotypes in scrapie-affected and healthy control sheep derived from scrapie-free flocks (h-sff) or scrapie-affected flocks (h-saf) of the Texel breed.*

	scrapie		h- sff		h-saf	
Genotype	n	%	n	%	n	%
PrP^{VRQ}/PrP^{VRQ}	3	9	0	0	0	0
PrP^{VRQ}/PrP^{ARQ}	12	35	4	4	4	5
PrP^{VRQ}/PrP^{ARH}	15	44	2	2	4	5
PrP^{VRQ}/PrP^{AHQ}	0	0	0	0	2	2
PrP^{VRQ}/PrP^{ARR}	1	3	4	4	11	13
PrP^{ARR}/PrP^{ARR}	0	0	15	17	8	9
PrP^{ARR}/PrP^{ARQ}	0	0	22	24	23	27
PrP^{ARR}/PrP^{ARH}	0	0	6	7	7	8
PrP^{ARR}/PrP^{AHQ}	0	0	2	2	3	4
PrP^{ARQ}/PrP^{ARQ}	3	9	10	11	3	4
PrP^{ARQ}/PrP^{ARH}	0	0	14	15	12	14
PrP^{ARQ}/PrP^{AHQ}	0	0	4	4	1	1
PrP^{ARH}/PrP^{ARH}	0	0	6	7	4	5
PrP^{ARH}/PrP^{AHQ}	0	0	2	2	2	2
PrP^{AHQ}/PrP^{AHQ}	0	0	0	0	1	1

* Parts of this table have been reproduced with permission of the *J. Gen. Virol.*

The frequencies of these genotypes in the three groups of sheep are summarized in Table 1. The PrP^{VRQ} variant was present in 91% of the scrapie-affected sheep, in 10% of the healthy sheep derived from scrapie-free flocks, and in 25% of the healthy sheep derived from scrapie-affected flocks. The PrP^{VRQ} homozygotes were present exclusively in the group of scrapie-affected sheep. We concluded that, at least in the Texel breed, the PrP^{VRQ} allele is associated with a high incidence of natural scrapie (Table 2, χ^2= 73.18, P< 0.0001 for scrapie vs healthy sheep derived from scrapie-free flocks and χ^2= 46.16, P< 0.0001 for scrapie vs healthy sheep derived from scrapie-affected flocks). There is also a slight but significant difference in the presence of the PrP^{VRQ} allele between the healthy sheep from scrapie-affected flocks and scrapie-free flocks (Table 2, χ^2= 5.70, P< 0.0170 for healthy sheep derived from scrapie-affected flocks vs healthy sheep derived from scrapie-free flocks). In the Texel breed only 9% of the scrapie-affected sheep did not possess a PrP^{VRQ} allele, instead these animals were homozygous for PrP^{ARQ}. However this PrP^{ARQ} allele did not show any significant association with disease incidence (Table 2) since it is almost equally distributed among the three groups.

	Scrapie<->h-sff		Scrapie<->h-saf		h-sff<->h-saf	
Variant	χ^2	P	χ^2	P	χ^2	P
PrP^{VRQ}	73.18	<0.0001	46.16	<0.0001	5.70	0.0170
PrP^{ARR}	26.77	<0.0001	33.36	<0.0001	4.29	0.1169
PrP^{ARQ}	2.33	0.3113	2.32	0.3139	4.08	0.1303
PrP^{ARH}	5.22	0.0735	3.55	0.1697	0.43	0.8065
PrP^{AHQ}	1.89	0.1687	3.89	0.1427	1.10	0.5755

Table 2. χ^2 test of association by comparing PrP genotype frequencies in the scrapie-affected sheep, the healthy control sheep derived from scrapie-free flocks (h-sff), and the healthy control sheep derived from scrapie-affected flocks (h-saf) of the Texel breed. Calculated by comparing homo- and heterozygotes in the scrapie-affected and healthy control groups (XX,XY and YY, where X= the given allelic variant and Y= all other possible variants).

The PrP^{ARR} variant was present in only 3% (one sheep) of the scrapie-affected sheep, in 54% of the healthy sheep derived from scrapie-free flocks, and in 61% of the healthy sheep derived from scrapie-affected flocks. The PrP^{ARR} homozygotes were found exclusively in the groups of the healthy sheep. We concluded that, at least in the Texel breed, the PrP^{ARR} variant is associated with a low incidence of natural scrapie (Table 2, χ^2= 26.77, P< 0.0001

for scrapie vs healthy sheep derived from scrapie-free flocks and χ^2= 33.36, P< 0.0001 for scrapie vs healthy sheep derived from scrapie-affected flocks). As the PrP^{ARQ} variant, the PrP^{ARH} did not show any significant association with disease incidence (Table 2). Since the PrP^{ARR}/PrP^{ARQ} and PrP^{ARR}/PrP^{ARH} genotypes were only present in the healthy groups and the PrP^{VRQ}/PrP^{ARQ} and PrP^{VRQ}/PrP^{ARH} genotypes preferentially in the scrapie-affected group, the PrP^{VRQ} and PrP^{ARR} alleles seem to be dominant over the PrP^{ARQ} and PrP^{ARH} alleles. The occurrence of the PrP^{AHQ} variant in the three groups was to low to draw any conclusions.

Table 3. *PrP genotypes of scrapie-affected sheep in other breeds than the Texel breed.*

Genotype	n	%
PrP^{VRQ}/PrP^{VRQ}	8	23
PrP^{VRQ}/PrP^{ARQ}	9	26
PrP^{VRQ}/PrP^{ARH}	6	17
$PrP^{VRQ}/PrP^{ARQQ211}$	1	3
PrP^{ARQ}/PrP^{ARQ}	11	31

In the 35 scrapie-affected sheep of seven other breeds we found 5 different genotypes. Of these sheep 69% was homozygous or heterozygous for PrP^{VRQ} and none possessed the PrP^{ARR} allele (Table 3). This strengthens our conclusion that, also in other breeds, the PrP^{VRQ} allele is associated with a high incidence of scrapie and the PrP^{ARR} allele with a low incidence of scrapie. Similar to the Texel breed, the scrapie-affected sheep of the other breeds that did not possess a PrP^{VRQ} allele, were homozygous for the PrP^{ARQ} allele (Table 3). The occurrence of the $PrP^{ARQQ211}$ variant was to low to draw any conclusions.

Discussion

In this paper we present evidence that specific combinations of polymorphisms within the PrP gene of sheep, rather than single polymorphisms, are associated with the incidence of scrapie. We have used DGGE analysis to screen 245 sheep for the presence of various PrP alleles. We used 210 sheep of the Texel breed, 34 were scrapie-affected, 91

were healthy and derived from scrapie-free flocks, and 85 were healthy and derived from scrapie-affected flocks. In addition we used 35 scrapie-affected sheep of seven other breeds. We found six different allelic variants with polymorphisms at codons 136, 154, 171, and/or 211. Based on the triplet sequences present at codons 136, 154, and 171 the allelic variants were designated PrP^{VRQ}, PrP^{ARR}, PrP^{ARQ}, PrP^{ARH}, PrP^{AHQ}, and $PrP^{ARQQ211}$. With these six allelic variants we found a total of 15 different PrP genotypes. The PrP^{VRQ} allele is associated with a high incidence of natural scrapie and the PrP^{ARR} allele with a low incidence. The PrP^{VRQ} and PrP^{ARR} alleles are antagonists in determining disease susceptibility and seem to be dominant over the PrP^{ARQ} and PrP^{ARH} alleles. Whether sheep with the PrP^{VRQ} allele investigated in this study are just highly susceptible to the scrapie agent, or whether they have the potential to develop the disease spontaneously similar to the inherited prion diseases (24) can not be said from these experiments.

All scrapie-affected sheep that did not possess a PrP^{VRQ} allele were homozygous for PrP^{ARQ}. Texel sheep homozygous for PrP^{ARQ} were however also present in the healthy control groups. In addition we found no association between the PrP^{ARQ} allele and disease incidence (Table 2). Obviously in PrP^{ARQ}/PrP^{ARQ} sheep other factors, for example the contaminated environment (infectious dose) or the scrapie source, play a more decisive role in inducing the disease than in sheep with a PrP^{VRQ} allele. This is consistent with recent conclusions of Westaway et al. (17). They found that scrapie-affected Suffolk sheep from the U.S. were homozygous A at codon position 136 and homozygous Q at codon position 171. However they found this genotype also with a high frequency among phenotypically normal sheep of at least 4 years old. In accordance with our conclusions these authors indicated that the high percentage of healthy sheep with 136 A/A and 171 Q/Q formally excludes that this genotype itself, which probably resembles the PrP^{ARQ}/PrP^{ARQ} genotype described here, causes disease.

As indicated above the PrP^{VRQ} and PrP^{ARR} alleles are antagonists in determining disease susceptibility with the PrP^{VRQ} allele associated with a high incidence of natural scrapie and PrP^{ARR} with a low incidence. PrP^{VRQ}/PrP^{ARR} heterozygotes were found in 3% of the scrapie-affected animals, in 4% of the healthy sheep from scrapie-free flocks, but in 13% of the healthy sheep from scrapie-affected flocks. We concluded that, at least in the scrapie-affected flocks, the PrP^{ARR} allele protects PrP^{VRQ}/PrP^{ARR} sheep from disease onset. This is consistent with the finding that the 171 R allele seems to be very rare among sheep affected by natural scrapie (13, 17). Laplache and coworkers (13) showed that 3 Ile-de-France sheep with a 136 A/V, 154 R/R, 171 R/Q genotype (which is probably similar to the PrP^{VRQ}/PrP^{ARR} genotype) became scrapie-affected with a very late clinical onset of disease at 7 years of age. Apparently also in these sheep the PrP^{ARR} allele delays clinical

manifestation of the disease. In this respect it is interesting to note that we found PrP^{Sc} depositions in the brain, spleen, tonsil, and lymph nodes in 54 of the 55 scrapie-affected sheep that were investigated (van Keulen *et al*., manuscript in preparation). In only one sheep we found PrP^{Sc} depositions in the brain but not in lymphoid tissue. This particular sheep turned out to be the animal with the PrP^{VRQ}/PrP^{ARR} genotype. Whether there is any correlation between this lack of PrP^{Sc} accumulation in lymphoid tissue and the relative protection attributed to the PrP^{ARR} allele remains to be established.

Studies on experimental scrapie with Cheviot sheep have shown that codon 136 V is associated with a high susceptibility to experimental infection (12, 15). The association of codon 136 V with natural scrapie was confirmed for Ile de France and Romanov sheep (13) and for Swaledales (16). The data described here confirm these findings. The association of codon 136 A with low susceptibility as predicted in several reports needs however refinement. The data presented here clearly indicate that only the PrP^{ARR} allele is associated with a low incidence of natural scrapie and not the PrP^{ARQ} and PrP^{ARH} alleles. Therefore the detection of codon 136 A alone is not sufficient to predict scrapie resistance or susceptibility.

From the experiments described here, it cannot be concluded that the association of the PrP^{VRQ} and the PrP^{ARR} alleles with high and low scrapie susceptibility accounts for all scrapie "strains". The data suggest that the association at least accounts for most of the "strains" (if present) that circulate in the Netherlands. In addition recent data of Goldmann et al. (14) indicate that our observations have a more general significance. They showed that all sheep homozygous for codon 136 A and either homo- or heterozygous for codon 171 R are resistant to experimental challenge with scrapie isolates from the A-group (SSBP/1) and the C-group (CH1641), and to a BSE isolate. They also showed that all homozygotes or heterozygotes for codon 136 V that do not have a codon 171 R allele, became affected after experimental challenge with SSBP/1 and the BSE isolate. These results are completely consistent with the findings presented here. In contrast, the same study indicated that sheep, heterozygous for codons 136 (V/A) ánd 171 (Q/R) were very susceptible to experimental infection with SSBP/1 but not with the BSE isolate. These sheep are probably comparable with the PrP^{VRQ}/PrP^{ARR} sheep described here. In these sheep disease development might be restricted to the A-group agents, although the species barrier for the BSE isolate could have been the reason for the observed resistance as well.

The data presented in this paper suggest that a positive selection for the PrP^{ARR} allele and/or a negative selection for the PrP^{VRQ} allele in breeding programs could help to control natural scrapie. We can not exclude yet whether such breeding programs will result in the selection and circulation of other scrapie "strains". We also do not know whether

sheep with the PrP^{ARR} allele are able to replicate the scrapie agent without showing clinical signs of disease and therefore can serve as a reservoir for infectious agent. Further research is needed to address these questions.

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CHAPTER 3

PrP Genotype Contributes to Determining Survival Times of Sheep with Natural Scrapie

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Summary

Several allelic variants of the sheep PrP gene are associated with scrapie susceptibility. However, it is not known whether and to what extent the PrP genotype contributes in determining survival times of scrapie sheep. We therefore determined the PrP genotype and lifespans of over fifty Flemish and Swifter sheep within a single scrapie affected flock. Eightythree percent of the scrapie sheep were homozygous for the PrP^{VQ} allele (polymorphic amino acids at codons 136 and 171 are indicated) and these sheep died from scrapie at a mean age of 25 months. In sheep heterozygous for PrP^{VQ} development of scrapie was delayed or did not occur. Sheep with at least one PrP^{AR} allele, including PrP^{VQ}/PrP^{AR} sheep, did not develop scrapie. No scrapie sheep were found without a PrP^{VQ} allele. We concluded that the PrP genotype contributes in determining survival times of sheep with natural scrapie. Additional we describe two novel sheep PrP allelic variants.

Introduction

Scrapie is a fatal neurodegenerative disease that occurs naturally in sheep and goats. It is the archetype of a group of disorders known as spongiform encephalophaties or prion diseases. Prion diseases manifest as infectious, sporadic, and/or inherited disorders (Prusiner, 1991) and are characterized by the accumulation of a protease-resistant isoform of the host-encoded prion protein (PrP) in the brain of affected animals and humans. Although the exact nature of the agent remains unknown, the agent is thought to be composed largely if not entirely of these PrP molecules (Prusiner, 1982). Others however, consider it as a virus-like agent by which the outcome of the disease is influenced by host genetic factors (Dickinson *et al.*, 1965, 1974).

Several polymorphisms in the gene for PrP have been shown to be associated with incidence, susceptibility and pathology of the disease (Prusiner, 1991, 1993). For the PrP gene of sheep six polymorphisms have been described (Goldmann *et al.*, 1990, 1991; Laplanche *et al.*, 1993; Belt *et al.*, 1995*a*, *b*). In sheep from the Texel and Flemish breeds we previously identified 16 different PrP genotypes containing six different allelic variants of the PrP gene (Belt *et al.*, 1995*a*, *b*). Here we analyzed the PrP genotypes of over 50 sheep of the Swifter and Flemish breed which resided within a single scrapie affected flock and found five novel PrP genotypes.

Materials and methods

DNA was extracted from blood samples using Chelex 100^{TM} (Walsh *et al.*, 1991). Amplification reactions were essentially performed as described by Belt *et al.* (1995*b*) in 50 :l containing 40 µl Chelex extracted DNA and the primers p8 (5' CAGGTTAACGAT-GGTGAAAAGCCACATAGG 3') and p143 (5' CTGGGATTCTCTCTGGTACT 3'). The amplified DNA was analyzed by DGGE and DNA that produced hitherto unknown DGGE patterns was further analyzed by cloning and sequencing (Belt *et al*., 1995*a,b*).

Results

Four of the five new patterns, all four obtained from Swifter sheep, shared a common hitherto unknown homoduplex band, three of which are shown in Figure 1 (lanes 4, 5 and 7). Cloning and sequencing revealed a hitherto unidentified polymorphism at codon 141 of the PrP gene: a leucine (L) to phenylalanine (F) substitution. The fifth DGGE pattern, obtained from a Flemish sheep, showed homoduplex bands similar to the codon 136 V or A variants

but their heteroduplex bands differed (compare Figure 1, lanes 2 and 3; and 6, 8 and 9). After cloning of both alleles and DGGE analysis of a mixture of each allele with the PrP^{AR} allele (if not indicated otherwise only polymorphic amino acids at codon 136 and 171 are indicated) it became clear that this particular genotype consisted of a PrP^{VQ} allele and a novel allele (Figure 1 lanes 6 and 9) with a polymorphism at codon 137: a methionine (M) to threonine (T) substitution. We recently found the latter variant also among Texel sheep. This brings the total number of known allelic variants of the sheep PrP gene to nine (Table 1). All polymorphisms seem to be mutually exclusive.

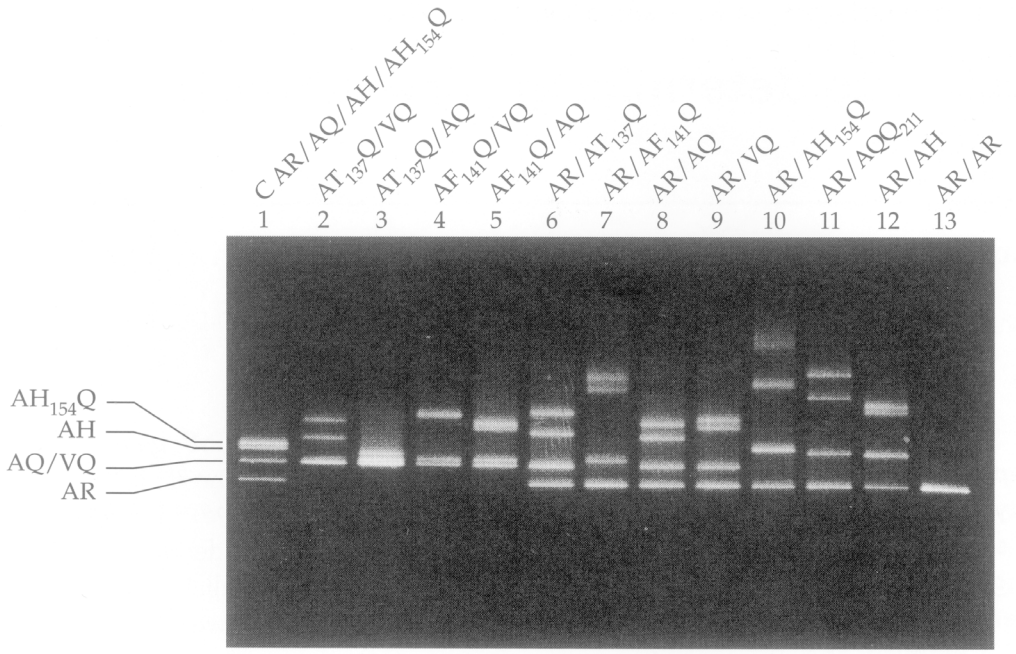

Figure 1. DGGE analysis of PCR amplified DNA. Lanes 2-13, DGGE patterns obtained from cloned and sequenced allelic variants. Lanes 2-7, DGGE patterns of genotypes containing the two novel polymorphisms at positions 137 and 141. Lane 1 is a control lane that contains four combined allelic variants which were not denatured and renatured before loading. The haplotypes of these allelic variants are indicated on the left (designations are explained in the text).

Polymorphisms at codons 112, 137, 141, 154, and 211 are rare and have not been significantly associated with any disease phenotype in natural or experimental scrapie. However, the polymorphisms at codons 136 and 171 have been significantly associated with scrapie susceptibility in several breeds. The PrP^{VQ} allele is associated with high susceptibility to natural and experimental scrapie (Laplanche *et al.*, 1993; Hunter *et al.*, 1993; Belt *et al.*, 1995*a*; Goldmann *et al.*, 1991; Maciulis *et al.*, 1992) and the PrP^{AR} allele is associated with low susceptibility to natural and experimental scrapie (Westaway *et al.*, 1994; Clouscard *et al.*, 1995; Belt *et al.*, 1995*a*; Goldmann *et al.*, 1994). Intriguing differences have been

Table 1. *PrP allelic variants of sheep.*

Allelic variant*	Polymorphic amino acid positions						
	112	**136**	**137**	**141**	**154**	**171**	**211**
PrP^{AQ} (wildtype)	M	A	M	L	R	Q	R
PrP^{T112AQ} °	**T**	A	M	L	R	Q	R
PrP^{VQ}	M	**V**	M	L	R	Q	R
PrP^{AT137Q} •	M	A	**T**	L	R	Q	R
PrP^{AF141Q} •	M	A	M	**F**	R	Q	R
PrP^{AH154Q}	M	A	M	L	**H**	Q	R
PrP^{AH}	M	A	M	L	R	**H**	R
PrP^{AR}	M	A	M	L	R	**R**	R
PrP^{AQQ211}	M	A	M	L	R	Q	**Q**

* If not indicated separately only significantly associated polymorphic amino acids at codons 136 and 171 are indicated.

° This polymorphism was not found in our studies.

• Novel polymorphism described in this study.

observed in the contribution of each of the 136 and 171 polymorphisms to scrapie susceptibility in various outbreaks of scrapie in different breeds, or flocks in different countries (Hunter *et al.*, 1993, 1994; Laplanche *et al.*, 1993; Belt *et al.*, 1995*a*, *b*; Westaway *et al.*, 1994; Ikeda *et al.*, 1995). It is not known yet whether and to what extent the PrP genotype contributes in determining the length of the survival time of sheep with natural scrapie. Survival time is defined as the time between birth and appearance of clear clinical signs of scrapie.

In this study we determined the PrP genotype and the survival time of sheep from the Flemish or Swifter breed present within a single scrapie affected flock. Initially we analyzed the genotypes of 18 sheep (12 Flemish and 6 Swifter sheep) that died from natural scrapie between 1992 and early 1995. The genotype was determined by DGGE analysis and 15 out of the 18 scrapie sheep (83%) were homozygous for the PrP^{VQ} allele. The survival times of these animals were within a very narrow window with a mean age at death of 25 months (Table 2). Three of the 18 scrapie sheep were heterozygous for the PrP^{VQ} allele and the age at death of these animals was considerably longer than 25 months. No scrapie sheep were found that did not posses a PrP^{VQ} allele. All except one of the homozygous PrP^{VQ} sheep died in the range of 23-29 months after birth. The one exception died from scrapie at an age of 16 months. Her mother and grandmother also died from scrapie. It could be that in this case maternal transmission played a major role. However, a PrP^{VQ} heterozygous twin sister of the latter sheep was still alive and healthy at the age of 47 months. Other observations also

suggest that the parental scrapie status does not play a major role in determining the survival time of scrapie sheep. For example three PrP^{VQ}/PrP^{VQ} sheep derived from healthy mothers died from scrapie at a similar age as two other PrP^{VQ}/PrP^{VQ} sheep derived from a mother that died from scrapie herself.

Table 2. *PrP genotypes, age at death or age at removal from the flock and allele frequencies of scrapie-affected and healthy sheep born in 1990.*

Marker	no. healthy	no. scrapie	age **at death** or age at removal from flock* (months)
Genotype			
PrP^{VQ}/PrP^{VQ}	-	3 (15)#	**26, 26, 27 (25.3±1.8)[1]**
PrP^{VQ}/PrP^{AQ}	-	2 (2)#	**35, 35**
$PrP^{VQ}/PrP^{AF_{141}Q}$	-	1 (1)#	**59**
$PrP^{VQ}/PrP^{AH_{154}Q}$	1	-	45
$PrP^{VQ}/PrP^{AT_{137}Q}$	1	-	45
PrP^{VQ}/PrP^{AR}	17	-	60, 39, 43, 48, 38, 50, 40, 31, >76 (9)•
PrP^{AR}/PrP^{AR}	6	-	39, 40, 40, 29, 46, >76 (1)•
PrP^{AR}/PrP^{AQ}	1	-	>76 (1)•
$PrP^{AR}/PrP^{AF_{141}Q}$	1	-	31
$PrP^{AR}/PrP^{AH_{154}Q}$	3	-	56, 40, >76 (1)•
PrP^{AR}/PrP^{AH}	3	-	31, 45, >76 (1)•
$PrP^{AF_{141}Q}/PrP^{AQ}$	4	-	40, >76 (3)•
$PrP^{AF_{141}Q}/PrP^{AH_{154}Q}$	2	-	40, >76 (1)•
$PrP^{AH_{154}Q}/PrP^{AQ}$	1	-	>76 (1)•
Allele			
PrP^{VQ}	19 (23.8%)	9 (75.0%)	
PrP^{AR}	37 (46.2%)	-	
PrP^{AQ}	6 (7.5%)	2 (16.7%)	
$PrP^{AF_{141}Q}$	7 (8.8%)	1 (8.3%)	
$PrP^{AT_{137}Q}$	1 (1.2%)	-	
$PrP^{AH_{154}Q}$	7 (8.8%)	-	
PrP^{AH}	3 (3.8%)	-	

* Several sheep were sold, died from another disease or were slaughtered, scrapie negative, confirmed by histology or immunohistochemistry.

° Number of scrapie sheep born between 1989 and 1992.

[1] Mean ± SD age at death of sheep born between 1989 and 1992.

• >76 (x) means that this number of sheep (x) is still alive and under observation.

In this flock 461 sheep were born in march 1990: 210 rams and 251 ewes (63% Flemish and 37% Swifter). The first year 338 lambs were sold and 110 ewes and 13 rams were kept for breeding purposes. In 1992 another 77 animals of this group were removed from the flock and 46 ewes were left. Since selection of the animals was done on basis of the litter sizes, we regarded the 46 sheep (67% Flemish and 33% Swifter) representative for all 461 sheep born in 1990. This group is of particular interest because all these sheep lived under the same environmental conditions and it seems logical to assume that the infective scrapie dose from the environment was more or less the same for all these animals. The PrP genotypes of these 46 ewes were determined by DGGE and are shown in Table 2. From the given allele frequencies it is clear that the frequencies of the PrP^{VQ} and PrP^{AR} alleles in this flock are rather high. Precise these two alleles have significantly been associated with scrapie susceptibility. In a previous study we concluded that both alleles seem to act in a (co-) dominant but opposite fashion (Belt *et al.*, 1995*a*).

The PrP genotypes of the 46 sheep and their age at death or the age at removal from the flock are indicated in Table 2. In addition to the three homozygous PrP^{VQ} sheep that died from scrapie 26-27 months after birth, three other animals born in 1990 died from scrapie. Two had the PrP^{VQ}/PrP^{AQ} genotype and died 35 months after birth and one had the PrP^{VQ}/PrP^{AF141Q} genotype and died 59 months after birth. Of the sheep that did not develop clinical signs of scrapie during the observation period 19 were heterozygous for the PrP^{VQ} allele. Two of these with the genotype PrP^{VQ}/PrP^{AH154Q} and PrP^{VQ}/PrP^{AT137Q} left the flock after 45 months. The other 17 sheep possessed both the susceptible PrP^{VQ} allele as well as the resistant PrP^{AR} allele. Nine of these PrP^{VQ}/PrP^{AR} sheep are still present in the flock and were still healthy at the age of 76 months. Three of the PrP^{VQ}/PrP^{AR} sheep produced PrP^{VQ}/PrP^{VQ} lambs that in the meantime died from scrapie at ages of 24, 24, and 27 months. Of the 21 sheep that did not possess a PrP^{VQ} allele none showed clinical signs of scrapie.

Discussion

From this study we concluded that in this flock homozygosity for PrP^{VQ} predominates in the scrapie affected sheep and that the PrP genotype is more important for the manifestation of scrapie than the parental scrapie status. In addition the data and other published data strongly suggest that PrP^{VQ}/PrP^{VQ} sheep are at highest risk of scrapie. In sheep with at least one PrP^{VQ} allele, the scrapie susceptibility and the length of the survival times seem to be dependent on the identity of the second PrP allele; with the second allele PrP^{VQ} linked to the shortest survival time, PrP^{AQ} linked to an intermediate survival time, and PrP^{AR} linked to a "survival time" that spans beyond the lifetime of a sheep. Nothing can be said yet

with respect to the effect of other alleles since the numbers of sheep with PrP^{AF141Q}, PrP^{AT137Q}, and PrP^{AH154Q} alleles in combination with the PrP^{VQ} allele were too low to draw any conclusion.

The data presented here are consistent with data obtained by others in similar studies performed with other breeds or flocks in different countries. In most of these studies however only polymorphisms at one specific position in the PrP gene were determined instead of the simultaneous detection of all polymorphisms present in a single allele. In addition in most of these studies one compared life spans of sheep that were not born in the same year and that lived under different environmental conditions. Very recently Hunter *et al.* (1995) presented comparable data obtained from a closed flock of NPU Cheviot sheep.

From published data and the data presented here it is tempting to speculate that each PrP allelic variant provides its own specific contribution in a homozygous or heterozygous setting to the timing of scrapie onset and scrapie development. Therefore it could be hypothesized that the PrP^{VQ}, PrP^{AQ} (PrP^{AF141Q}), and PrP^{AR} variants are linked to an increasing survival time. The PrP^{AF141Q}, PrP^{AH154Q}, and PrP^{AT137Q} alleles are than probably linked to survival times longer than that for PrP^{VQ} and shorter or equal to that of PrP^{AR}.

This hypothesis is consistent with the observation that in all breeds PrP^{AR} is associated with the longest survival time. In fact only one PrP^{AR}/PrP^{AR} sheep has been documented to be scrapie positive (Ikeda *et al.*, 1995). Also for Lacaune and Romanov sheep, data have been published indicating that the susceptibility of sheep homozygous or heterozygous for PrP^{VQ} is more pronounced than in sheep homozygous for PrP^{AQ} as shown by higher disease incidence in this group and the shorter survival times (Clouscard *et al.*, 1995). In Suffolk sheep, the PrP^{VQ} allele has not been associated with high disease incidence since this allele is very rare in this breed (Westaway *et al.*, 1994; Hunter *et al.*, 1994). Recently however, Ikeda *et al.* (1995) described 7 Suffolk sheep of unknown age that possessed the PrP^{VQ} allele. Although these sheep were apparently "healthy", the lymph nodes and spleens of 4 animals were found to be positive for PrP^{Sc} and one of the four was confirmed to be scrapie infected by mouse bioassay. It was predicted by the authors that the PrP^{VQ} allele confers susceptibility to scrapie in Suffolk sheep as well.

In the outbreak of natural scrapie described in this study, but also in others, the primary risk factor associated with scrapie is unambiguously the PrP genotype. Vertical transmission seems to be of minor or no importance. Nevertheless natural scrapie is a transmissible disease. By which mechanisms transmission of scrapie under field conditions occurs, remains to be established. It is also of importance to define whether different natural scrapie sources exist that behave differently in sheep with particular PrP genotypes as has been found with experimentally induced scrapie after intracerebral and subcutaneous

inoculation with SSBP/1-like (A-group) and CH1641-like (C-group) agents (Goldmann *et al.*, 1994; Foster & Dickinson, 1988). This information will be required for a successful selection of sheep with low susceptibility in an attempt to control or eradicate scrapie.

In conclusion, from this "within flock" study we concluded that the PrP genotype strongly determines the susceptibility of sheep to scrapie and the survival time of sheep in scrapie affected flocks. Although all family lines related to scrapie sheep were removed from the flock under study, persistence of scrapie was probably due to the high frequency of the susceptible PrP^{VQ} allele in clinically healthy PrP^{VQ}/PrP^{AR} sheep. These apparently resistant sheep help to maintain susceptibility of the flock because of a continuous supply of PrP^{VQ} alleles. Current breeding programs in this flock are now assisted by PrP genotyping. The future will learn whether this will decrease the incidence of scrapie.

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CHAPTER 4

PrP Genotype Frequencies of the Most Dominant Sheep Breed in a Country Free From Scrapie

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Summary

Polymorphisms within the prion protein (PrP) gene are associated with scrapie susceptibility. We analysed the PrP genes of 140 sheep of the most dominant breed, Romney Marsh, in New Zealand, a country free from scrapie. We found PrP alleles that are associated with a high susceptibility to scrapie. Sheep with these PrP genotypes would probably succumb to scrapie when born and raised in a scrapie endemic environment. These findings correspond to those obtained in minor breeds from New Zealand. We conclude that scrapie development not only depends on host genetic factors but also requires exogenous factors. Our findings demonstrate the effectiveness of the measures taken by New Zealand to maintain free from scrapie.

Introduction

In several animal species and in humans, polymorphisms within the open reading frame of the prion protein (PrP) gene are associated with the incidence and pathology of transmissible spongiform encephalopathy (12). In the PrP gene of sheep the alanine (A) to valine (V) polymorphisms at codon 136, the glutamine (Q) to arginine (R) at codon 171, and to a minor extent the R to histidine (H) at codon 154 contribute to the susceptibility of sheep for scrapie (14). The polymorphisms at codons 112, 137, 141, 171 (Q to H), and 211 are rare and have not been associated yet with a scrapie phenotype. In sheep breeds that carry the PrP^{VRQ} allelic variant (amino acids at codons 136, 154, and 171 are indicated in superscript), this allele is associated with a high susceptibility to scrapie. The PrP^{ARR} allelic variant is associated with resistance in all breeds investigated so far (1 - 3, 6 - 8, 10). In breeds in which the PrP^{VRQ} allele is rare or absent, for instance the Suffolk breed, the wildtype PrP^{ARQ} allele is associated with susceptibility to scrapie (5, 15). In breeds that contain the PrP^{VRQ} allele, the PrP^{VRQ}/PrP^{VRQ} sheep are at greatest risk from scrapie followed by the PrP^{VRQ}/PrP^{ARQ} sheep, at least in Britain and Europe. In breeds in which the PrP^{VRQ} allele is rare or absent, the PrP^{ARQ}/PrP^{ARQ} sheep are at greatest risk. In sheep with the latter homozygous PrP genotypes survival times of less than 14 months have been recorded (L. van Keulen, pers. comm).

Previously it has been debated that scrapie is a genetic disease arising spontaneously from sheep with certain PrP alleles in the absence of any infectious agent (11, 13). Observations on the transmission of scrapie in embryo transfer experiments however indicated that scrapie is not purely a genetic disease (4). In addition, sheep with PrP genotypes at greatest risk from scrapie have been found in Cheviot and Suffolk sheep from Australia and/or New Zealand (9). Since these countries are free from scrapie for decades and scrapie has become a notifiable disease in New Zealand since 1955, it is likely that scrapie is not a spontaneous genetic disease linked to the PrP gene alone, although development of the disease is strongly influenced by PrP.

Here we extended the PrP genotyping work on sheep from countries free from scrapie and we determined the PrP allele- and genotype frequencies of the most dominant sheep breed in New Zealand since 1900, the Romney Marsh. This breed was first imported from Britain into New Zealand in 1852. It was used to develop two new breeds, the Perendale and the Coopworth. The Romney Marsh and these two new breeds form the bases of the entire New Zealand sheep flock, which consists of about 46 million sheep, 58% Romney Marsh, 17% Perendale and Coopworth, and 25% other breeds. No new Romney Marsh sheep were imported into New Zealand since the beginning of this century. We

collected blood samples of random animals less than two years of age from 14 different flocks, scattered all over the country. These farms normally breed their own replacement animals and only purchase rams. The sampled flocks are likely representative for the entire Romney Marsh flock.

Materials and methods

After DNA extraction from the blood by standard procedures, PrP genes were amplified by PCR for 35 cycles (1 min. 94^{o}C, 1 min. 58^{o}C, and 1 min. 72^{o}C). The primers that were used, anneal to sheep PrP DNA at nucleotide positions 71-86, 5'-caggttaacg-atggtgaaaagccacatagg-3', and 730-749, 5'-ctgggattctctctggtact-3' (numbering according to 5). PCR amplified products were analysed by denaturing gradient gel electrophoresis (DGGE) as described before (1, 2). DGGE analysis not only identifies known PrP alleles but also allows to look for hitherto unknown allelic variants.

Results and discussion

The analysis revealed the presence of five different PrP alleles in the New Zealand Romney Marsh breed, including alleles linked to the highest scrapie susceptibilities (Table 1). The PrP^{VRQ} allele, linked to the highest susceptibility, was present at a frequency of 2.5%. This is much lower than the 18% frequency of the PrP^{VRQ} allele found in Cheviots from New Zealand (9). The PrP^{ARR} allele was present at a frequency of 47,1% which is slightly higher than the frequency of the wildtype PrP^{ARQ} allele (40,7%). In this respect the Romney Marsh breed resembles the Cheviot breed in New Zealand and the Suffolk breed in the UK. Striking is the absence of PrP^{ARH} and the relatively high frequencies of $PrP^{AF141RQ}$. The latter allele has only be described for UK-Cheviot and NL-Swifter sheep and is present in these breeds at a low frequency. The PrP^{AHQ} allele, probably linked to an increased scrapie resistance, is also present with a relatively high frequency in the New Zealand Romney Marsh. The alleles were not totally equally distributed over the different flocks (Table 2), although flock VIII had an unusually high PrP^{VRQ} frequency of 15%, flock X had a relatively high frequency of PrP^{AHQ}, and flocks XII and XIV were high in $PrP^{AF141RQ}$.

Table 1. *PrP allele frequencies.*

PrP allele	Total	Freq. (%)
ARQ (wt)	114	40.71
VRQ	7	2.50
ARR	132	47.14
AHQ	20	7.14
$AF_{141}RQ$	7	2.50
Total	280	100

Table 2. *PrP allele frequencies per flock.*

	Flock (n=20/flock)														Average per flock
PrP allele	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	(±var)
ARQ (wt)	8	9	8	10	10	10	13	6	8	6	7	12	3	4	8.1±7.97
VRQ	1	0	0	0	1	1	0	3	0	1	0	0	0	0	0.5±0.73
ARR	10	10	11	8	7	9	4	10	10	8	11	6	15	13	9.4±7.80
AHQ	0	1	1	2	2	0	3	1	1	5	1	0	2	1	1.4±1.80
$AF_{141}RQ$	1	0	0	0	0	0	0	0	1	0	1	2	0	2	0.5±0.58
Total	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20

In countries were scrapie is endemic, sheep with a PrP^{VRQ}/PrP^{VRQ} genotype are at greatest risk from scrapie. No such sheep were found among the 140 tested Romney Marsh sheep (Table 3). This is not surprising, since the frequency of this genotype is expected to be only 0.06% (one per 1666 animals) based on the assumption of a random distribution of the available PrP alleles. In the less than two year old sheep of flock VIII, however, the calculated frequency of the PrP^{VRQ}/PrP^{VRQ} genotype is 2.25% (one per 44 animals). In general, the calculated genotype frequencies are in agreement with the observed frequencies (Table 3). These data strongly indicate that sheep at greatest risk from scrapie should be present among the New Zealand Romney Marsh breed.

Table 3. *PrP genotype frequencies.*

PrP genotype	Total	Freq. (%)	Calc.Freq[1] (%)
ARQ/ARQ	21	15.0	16.6
ARR/ARR	32	22.9	22.2
AHQ/AHQ	1	0.7	0.5
VRQ/ARQ	4	2.9	2.0
VRQ/ARR	3	2.1	2.4
ARQ/ARR	54	38.6	38.4
ARQ/AF_{141}RQ	5	3.6	2.0
ARQ/AHQ	9	6.4	5.8
ARR/AHQ	9	6.4	6.7
ARR/AF_{141}RQ	2	1.4	2.4
Other	0	0	1.0
Total	140	100	100

[1] Calculated on basis of a random distribution of the PrP alleles shown in Table 1.

Table 4. *PrP genotype frequencies per flock.*

	Flock (n=10/flock)														Average per flock (±var)
PrP genotype	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	
ARQ/ARQ	1	1	1	3	2	3	4	1	2	1	0	2	0	0	1.5±1.50
ARR/ARR	3	2	2	3	1	2	0	2	3	3	2	0	5	4	2.3±1.91
AHQ/AHQ	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0.1±0.07
VRQ/ARQ	1	0	0	0	1	0	0	1	0	1	0	0	0	0	0.3±0.22
VRQ/ARR	0	0	0	0	0	1	0	2	0	0	0	0	0	0	0.2±0.34
ARQ/ARR	4	6	6	2	4	4	3	3	3	1	6	6	3	3	3.9±2.59
ARQ/AF_{141}RQ	1	0	0	0	0	0	0	0	1	0	1	2	0	0	0.4±0.40
ARQ/AHQ	0	1	0	2	1	0	2	0	0	2	0	0	0	1	0.6±0.40
ARR/AHQ	0	0	1	0	1	0	1	1	1	1	1	0	2	0	0.6±0.71
ARR/AF_{141}RQ	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0.1±0.29
Total	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10.0

The most predominant PrP genotypes present in this breed are PrP^{ARQ}/PrP^{ARR} (38,6%), PrP^{ARR}/PrP^{ARR} (22,9%) and PrP^{ARQ}/PrP^{ARQ} (16,6%). These PrP genotypes are distributed almost equally over the 14 flocks (Table 4). However, in flock VII we found a rather high frequency of PrP^{ARQ}/PrP^{ARQ} genotypes and no PrP^{ARR}/PrP^{ARR} genotypes, whereas the PrP^{ARQ}/PrP^{ARR} genotype was present at an expected frequency. A similar unusual distribution of these three alleles is seen in flock XII.

In summary, the most dominant sheep breed in New Zealand, the Romney Marsh, contains PrP alleles which are associated with a high susceptibility to scrapie. Although sheep with a PrP^{VRQ}/PrP^{VRQ} genotype, which are at greatest risk from scrapie, were not found among the 140 tested animals, they should be present in the population at a frequency of about one per 1666 animals. Three of the 140 tested animals (2,9%) contain the PrP^{VRQ}/PrP^{ARQ} genotype. In an environment where scrapie is endemic, such animals would also succumb to scrapie.

Note added in proof: After submission of this paper similar data were reported for Merino sheep of Australia and Poll Dorsetts of New Zealand (Hunter N. and Cairns D. (1998). J. Gen. Virol. 79, 2079-2082).

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CHAPTER 5

Scrapie Susceptibility-linked Polymorphisms Modulate the *in vitro* Conversion of Sheep Prion Protein to Protease-resistant Forms

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Abstract

Prion protein diseases are natural transmissible neurodegenerative disorders in man and animals. They are characterized by the accumulation of a protease-resistant isoform (PrP^{Sc}) of the host-encoded prion protein (PrP^{C}) mainly in the central nervous system. Polymorphisms in the PrP gene are linked to differences in susceptibility for prion protein diseases. The mechanisms underlying these effects are still unknown. Here we describe studies of the influence of sheep PrP polymorphisms on the conversion of PrP^{C} into protease resistant forms. In a cell-free system, sheep PrP^{Sc} induced the conversion of sheep PrP^{C} into protease-resistant PrP (PrP-res) similar or identical to PrP^{Sc}. Polymorphisms present in either PrP^{C} or PrP^{Sc} had dramatic effects on the cell-free conversion efficiencies. The PrP variant which is associated with a high susceptibility to scrapie and short survival times of scrapie affected sheep was efficiently converted into PrP-res. The 'wildtype' PrP variant which is associated with a neutral effect on susceptibility and intermediate survival times, was converted with intermediate efficiency. The PrP variant which is associated with scrapie resistance and long survival times was poorly converted. Thus the *in vitro* conversion characteristics of the various sheep PrP variants reflect their linkage with scrapie susceptibility and survival times of scrapie affected sheep. The modulating effect of the polymorphisms in PrP^{C} and PrP^{Sc} on the cell-free conversion characteristics suggests that besides the species barrier, 'polymorphism barriers' play a significant role in the transmissibility of prion protein diseases.

Introduction

Prion protein diseases such as Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker syndrome (GSS), bovine spongiform encephalopathy (BSE) and scrapie manifest as infectious, sporadic, and/or inherited disorders (1). They are characterized by the accumulation of an abnormal isoform (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{C}) mainly in the central nervous system of mammals. This protease-resistant PrP^{Sc} arises from protease-sensitive PrP^{C} by a posttranslational process (2, 3) involving profound conformational changes of mainly alpha-helical (PrP^{C}) into beta-sheeted (PrP^{Sc}) structure (4, 5). The prion agent has been proposed to be composed largely if not entirely of these PrP^{Sc} molecules (6, 7).

Several PrP polymorphisms of man have been associated with incidence, susceptibility and pathology of the disease (1, 8). For sheep eight mutually exclusive PrP polymorphisms have been described (9-15) resulting in nine different allelic variants. The allelic variants with polymorphisms at codons 112, 137, 141, 154, or 211 are rare and have not been significantly associated with any disease phenotype yet. In contrast, the PrP^{VQ} allele (only polymorphic amino acids at positions 136 and 171 are indicated by single letter code) is associated with high susceptibility to scrapie and short survival times of scrapie affected sheep (9-12, 15-18), whereas the PrP^{AR} allele is associated with resistance or incubation times that span beyond the lifetime of sheep (9-12, 16, 17). In breeds where PrP^{VQ} is rare, e.g. the Suffolk breed, the 'wildtype' PrP^{AQ} allele is associated with susceptibility to scrapie, although with a low or incomplete penetrance (18, 19). The mechanisms by which the different PrP allelic variants contribute to differences in scrapie susceptibility and survival time, are not yet understood. However, it is possible that the various PrP^{C} variants differ in their conversion kinetics into PrP^{Sc}. Such differences may be due to differences in expression levels, differences in co- or post-translational modifications, or differences in conformational structures of the various PrP variants.

Recent *in vitro* studies have demonstrated that in a cell-free system hamster PrP^{C} can be converted into protease-resistant forms which are at least similar if not identical to PrP^{Sc} without the synthesis of new macromolecules (20). Further biochemical studies with this cell-free system have shown that there is strain and species specificity in the PrP^{C}-PrP^{Sc} interaction that could account for the observed differences between prion strains and the barriers to interspecies transmission of prion agents respectively (21, 22). Species specificity *in vitro* was determined by specific amino acids between positions 113 and 188 of the hamster/mouse PrP sequence (22). Species specificity between human and mouse, as

determined *in vivo* using transgenic mice carrying chimeric human/mouse PrP genes, seems to be dependent on amino acid substitutions between positions 97 and 167 (23). In a reciprocal manner using murine scrapie infected neuroblastoma cells, the conversion of mouse PrP^{C} into PrP^{Sc} could be blocked by a single hamster-specific amino acid at position 138 of the murine PrP sequence (24). *In vivo* studies with transgenic mice carrying chimeric human/mouse PrP genes with single amino acid mismatches at position 109, 129, or 200 demonstrated that single amino acid substitutions in PrP can lead to an altered susceptibility to prions (25). In addition, transmission of human CJD and FFI to human transgenic mice also indicated that polymorphisms in the PrP-gene may lead to distinct prion protein properties (26). All these findings indicate that polymorphisms in the PrP gene might lead to differences in the PrP^{C}-PrP^{Sc} interaction and/or conversion of PrP^{C} into PrP^{Sc}.

In the present study, we explore whether sheep PrP^{C} can be converted *in vitro* to protease-resistant forms using a cell-free system. In addition we investigated whether the various sheep PrP allelic variants have different cell-free conversion characteristics and whether these characteristics reflect the observed differences in sheep scrapie susceptibility and the observed differences in survival times of scrapie-affected sheep *in vivo*.

Materials and methods

Cell-lines and PrP constructs. The ShPrP allelic variants PrP^{VQ}, PrP^{AQ}, and PrP^{AR} were cloned and analysed as described previously (10). PrP open reading frames were subcloned between the β-globin intron and β-globin polyadenylation sequences downstream the hCMV promoter of expression vector pECV7, a derivative of expression vector pECV6 (27) in which the RSV promoter has been substituted for the hCMV promoter. Mouse neuroblastoma cells (N2a-cells; Hubrechts laboratory, Utrecht, The Netherlands) were stably transfected with these constructs by electroporation (28), hygromycine-B (500 μg/ml) resistant single-cell clones were isolated, and high PrP expressing clones were selected by immunoperoxidase monolayer assay using the anti-peptide antibody R521-7 (peptide corresponds to amino acids 94-105 of the ShPrP sequence) (29). These cell-lines were used for the isolation of the various $ShPrP^{C}$ variants.

Isolation of ^{35}S-PrP^{C}. Cells expressing the different PrP variants were radiolabelled as initially described (30) using 1 mCi [^{35}S]methionine-[^{35}S]cysteine TRANS^{35}S-label (ICN Pharmaceuticals Inc.) per 70-80% confluent 25 cm^2 flask and labelled ^{35}S-proteins were methanol precipitated from detergent cell lysates and subsequently sonicated in 0.7 ml DLPC buffer (0.05 M Tris-HCl, pH 8.2, 0.15 M NaCl, 2 % (w/v) N-lauryl sarcosine, 0.4 % (w/v) lecithin (from soybean))

containing protease inhibitors (25 μg/ml Pefabloc SC, 0.7 μg/ml pepstatin, 0.5 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 mM EDTA). ^{35}S-PrP^{C} was immunoprecipitated using the R521-7 antibody (1:100) and 10 μl of 50 % (v/v) protein-A sepharose beads per μl antibody. Non-glycosylated ^{35}S-PrP^{C} was obtained by radiolabelling in the presence of 15 μg/ml tunicamycin-D. PrP^{C} was finally eluted 20 minutes at room temperature from complexes of antibody and protein-A beads in 0.1 M acetic acid, pH 2.8 containing protease inhibitors (Raymond and Caughey, pers. comm.). Eluates were stored on ice until further use.

Isolation of PrP^{Sc}. Proteinase-K treated PrP^{Sc} was isolated from brains of genotyped sheep (30, 31) using sarkosyl homogenization, ultracentrifugation, and PK digestion. After pelleting trough a 20% sucrose cushion the pellet was sonicated in 400 μl 0.1 % sulfobetaine (SB 3-14) in Tris buffered saline and stored in small portions at 4 °C. Quantification (silver staining and Western blotting) of the PrP^{Sc} revealed that the PrP$^{Sc(VQ/VQ)}$ and the PrP$^{Sc(AQ/AQ)}$ isolates contained about 135μg PrP^{Sc} per 24 g equivalent of brain. Both isolates were further diluted to a final concentration of 0.325μg/μl and were prior to use briefly sonicated using a cuphorn sonicator.

Cell-free conversion reaction. PrP^{Sc} isolates in siliconized tubes were partially or more complete denatured for 2.5 h at 37°C in 2.5 M or 6 M GdnHCl respectively. Aliquots of denatured PrP^{Sc} (3.3 μg) and 25 kcpm purified ^{35}S-PrP^{C} (≈5-10 ng) were mixed and further diluted to a volume of 35 μl at 1 M GdnHCl in conversion buffer (50 mM sodium-citrate, pH 6.0, 5 mM cetylpyridinium chloride, 1 % N-lauryl sarcosine, and protease inhibitors). Conversion reactions were performed for 2 or 5 days at 37°C and the reaction mixtures were subsequently diluted to 100 μl in 50 mM sodium-citrate pH 6.0 and digested with 35 μg/ml proteinase K at 37°C for 1 h. Thereafter PK inhibitor (Pefabloc SC; Boerhinger Mannheim) was added and all proteins were precipitated with 4 volumes of methanol at -20°C using 20 μg thyroglobulin as a carrier protein. Precipitated proteins were boiled and briefly sonicated in Laemmli sample buffer with 4 M urea and 1/10 volume was stored separately to be analysed by Western blotting. All samples were separated by 15 % SDS-PAGE, the gels were fixed and subsequently enhanced using Amplify (Amersham), ^{35}S-labelled proteins were visualized on X-ray film, and integrated intensities of bands were measured using the Intelligent Quantifier (Bio Image). Comparable results were obtained between different sets of conversions and only data are shown of representative experiments.

Western blotting. Western blotting was performed by standard methods on nitrocellulose membranes and protease-resistant PrP was visualized using (1:1000) R521-7 antibody and (1:1000) alkaline phosphatase-Goat anti-Rabbit IgG (Zymed Laboratories Inc.).

Results

Expression of ShPrP^{C} in cell-lines. Plasmid constructs encoding the wildtype ShPrP^{AQ}, the ShPrP^{VQ}, and the ShPrP^{AR} allelic variants were used to generate stably transfected N2a-cells. Single-cell clones that showed intensive and equal staining with the R521-7 antibody in an immunoperoxidase monolayer assay were selected for further use. These PrP^{C} expressing clones contained about 4-6 random integrated copies of the expression vector (data not shown) and almost equal amounts of the various radiolabelled PrP^{C} variants could be isolated. The various PrP^{C} variants were labelled with ^{35}S-met/cys in the presence or absence of tunicamycin-D and analyzed by radioimmunoprecipitation, using the R521-7 anti-peptide antibody. In the absence of tunicamycin, the PrP proteins were glycosylated and showed predominant bands with molecular weights of 38-39 kDa and 32-33 kDa in contrast to the 26-27 kDa unglycosylated form of PrP as shown in the tunicamycin-D treated sample (compare Fig. 1, lanes 1 and 2). PrP^{Sc} and PrP^{C} isolated from

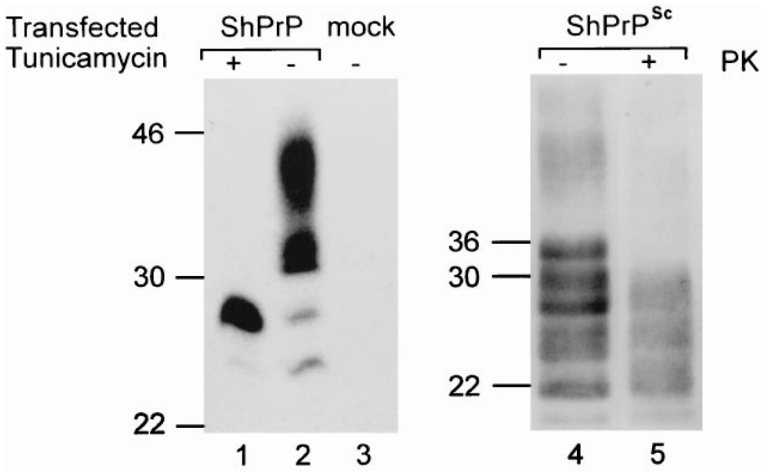

Figure 1: Migration of PrP^{C} and PrP^{Sc}. Radioimmunoprecipitation of PrP^{C} from stably transfected N2a-cells with pECV7 (mock, lane 3) or with pECV7-PrP (lanes 1 and 2). Proteins were radiolabelled in the presence (lane 1) or absence (lanes 2 and 3) of tunicamycin D. A Western blot of sheep brain PrP^{Sc} before and after PK treatment is shown in lane 4 and 5. Lane 4 in fact shows the migration of PrP^{Sc+C} (27-35 kDa) including the naturally N-terminally truncated forms of PrP^{Sc} (21-29 kDa)). Molecular mass markers (kDa) are indicated at the left.

sheep brain normally have molecular weights of 35 kDa, 31 kDa, and 27 kDa (Fig. 1, lane 4). Since the molecular weight of the unglycosylated PrP produced by the N2a cell-line is similar to the molecular weight of the unglycosylated PrP from sheep brain (compare Fig. 1, lanes 1 and 4), we concluded that the N2a cell-lines produced overglycosylated or less-

sialated PrP^C. The R521 antibody used to isolate the PrP^C variants is specific for sheep PrP and did not precipitate the endogenous mouse PrP (Fig. 1, lane 3). This eliminated the possibility of interference by mouse PrP^C in the sheep conversion reactions. The three different variants of PrP^C: PrP^{C_AQ}, PrP^{C_VQ}, and PrP^{C_AR} expressed in neuro-2A cells did not show notable differences in posttranslational modifications and all three PrP^C variants could be reduced to a single band of 27 kDa by inhibiting glycosylation with tunicamycin D (compare Fig. 2, lanes 1-3 with Fig. 3a, lanes 1-3).

Conversion of sheep PrP^C to protease resistant forms. In order to define the most optimal partial renaturation conditions of the $ShPrP^{Sc}$ for successful conversions, we first pretreated $ShPrP^{Sc}$ under various GdnHCl conditions. By measuring PK resistant PrP^{Sc} on Western blots we found >95 % denaturation and >95 % renaturation of at least the R521 epitope (amino acid residues 94-105) if denatured in 2.5 M GdnHCl for 2-24 hr at 37°C and subsequently renatured for 5 days in 0.75-1.0 M GdnHCl at 37°C.

To explore whether $ShPrP^C$ could be converted to protease-resistant forms in a cell-free system, as shown for hamster and mouse PrP^C by Kocisko *et al.* (20, 22), ^{35}S-$ShPrP^{CVQ}$ was incubated at 37 °C for 5 days (1 M GdnHCl) with partially denatured (>2.5 h in 2.5 M GdnHCl at 37°C) $ShPrP^{Sc(VQ/VQ)}$ and ^{35}S-$ShPrP^{CAQ}$ was incubated under the same conditions with partially denatured $ShPrP^{Sc(AQ/AQ)}$. After PK digestion, PK-resistant ^{35}S-ShPrP bands were detectable in both conversion reactions (Fig. 2, lanes 4 and 8).

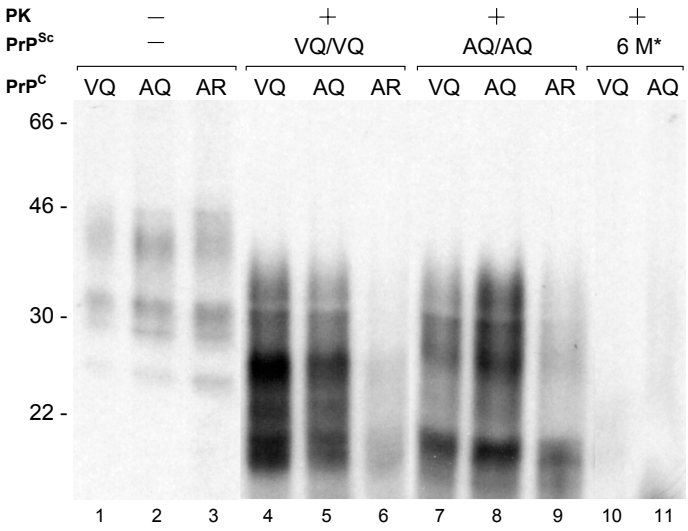

Figure 2: Cell-free conversion of ^{35}S-PrP^C into PK-resistant forms. PrP^{Sc} from sheep with different PrP genotypes were pretreated in 2.5 M GdnHCl, except for reactions in lanes 10 and 11 in which PrP^{Sc} was denatured in 6 M GdnHCl. After incubation for 5 days at 37°C (1 M GdnHCl), the samples were PK digested and analyzed by 15% SDS-PAGE. Molecular mass markers (kDa) are indicated at the left.

$ShPrP^{Sc}$ more completely denatured with 6 M GdnHCl induced very little conversion to PK-resistant forms in similar reactions (Fig. 2, lanes 10 and 11). Although the gels revealed a smear rather than discrete bands, predominant ^{35}S labelled PK-resistant bands with molecular weights of 32-33 kDa, 26-27 kDa, and 20-21 kDa were detectable, indicating a downward shift in molecular weight by PK digestion of about 6 kDa as expected for bonafide PrP-res products (compare Fig. 1, lanes 4 and 5).

Polymorphisms modulate the cell-free conversion of PrP^C to protease resistant forms. Although the amounts of conversion products are not easy to quantify from a smear of ^{35}S labelled PK-resistant PrP, it is obvious from Figure 2 that if using different allelic forms of PrP^C (PrP^{C_VQ}, PrP^{C_AQ}, or PrP^{C_AR}) or different PrP^{Sc} isolates ($PrP^{Sc(VQ/VQ)}$ or $PrP^{Sc(AQ/AQ)}$), different amounts of PrP-res are formed. For example in the $ShPrP^{Sc(VQ/VQ)}$-induced reactions the PrP^{C_VQ} to PrP-res conversion was the most efficient one, the PrP^{C_AQ} to PrP-res conversion was intermediate, and the PrP^{C_AR} to PrP-res conversion was poor (Fig. 2, lanes 4-6). In the $PrP^{Sc(AQ/AQ)}$ induced reactions, the three PrP^C allelic variants also converted with different efficiencies into PrP-res (Fig. 2, lanes 7-9).

In order to be able to quantify the conversion products more accurately and to address if N-linked glycosylation plays a role in determining the differences in conversion efficiency between the three PrP^C variants, we repeated the above experiments with unglycosylated PrP^C variants which were radiolabelled in the presence of tunicamycin D to obtain more discrete and quantifiable PrP bands (Fig. 3a, lanes 1-3). From the hamster cell-free conversions it was already known that the unglycosylated form of hamster PrP^C converted more efficiently into protease resistant forms than the glycosylated form of hamster PrP^C (20, 22). Mock transfected cell-lines did not show discrete labelled material indicating the absence of endogenous mouse PrP^C in the preparations (Fig. 3a, lane 4). The radiolabelled PrP^C products did not convert into protease-resistant products when incubated for 5 days under conversion conditions without PrP^{Sc} (Fig. 3a, lanes 5-8). However, incubation of non-glycosylated PrP^{C_VQ}, PrP^{C_AQ}, or PrP^{C_AR} under conversion conditions for 2 or 5 days with either $PrP^{Sc(VQ/VQ)}$ or $PrP^{Sc(AQ/AQ)}$ resulted in discrete and readily quantifiable protease-resistant bands of predominantly 20-21 kDa (Fig. 3a, lanes 9-11, 13-15, 17-19, and 21-23). As expected, the material from the mock transfected cells did not produce such PK resistant bands (Fig. 3a, lanes 12, 16, 20, and 24). The downward shift in molecular weight by PK digestion was about 6 kDa, which is equal to the downward shift found for the converted glycosylated PrP^C variants (Fig. 2) and PrP^{Sc} isolated from sheep brain (Fig. 1, lanes 4 and 5). The bar diagram (Fig. 3b) shows the percentages of PrP^C

which are converted into PrP-res by quantification of the 20-21 kDa PK resistant conversion products and comparison with the 27 kDa input PrP^{C}. For quantification of the conversions with glycosylated PrP^{C} the region between 21 and 33 kDa of the conversion products (Fig. 2) was used (only to give a relative indication of these conversion efficiencies). PrP^{VQ} which is associated with high susceptibility to scrapie and short survival times in scrapie affected sheep is overall the allelic form of PrP^{C} that is most efficiently converted to PrP-res (Fig. 3b, bars 1, 2, 10, and 11). The homologous conversions with this allelic form (Fig. 3b, bars 1 and 2) seem more efficient than the heterologous conversions (Fig. 3b, bars 10 and 11). PrP^{AQ} which is associated with intermediate susceptibility to scrapie (with an incomplete penetrance) and with intermediate survival times in scrapie affected sheep is less efficiently converted to PrP-res (Fig. 3b, bars 7, 8, 16, and 17). PrP^{AR} which is associated with resistance to scrapie and with incubation times that span beyond the lifetime of sheep is poorly converted to PrP-res (Fig. 3b, bars 4, 5, 13, and 14).

The conversion data obtained with the different allelic forms of PrP^{Sc} revealed that not only the polymorphisms in PrP^{C} determine the conversion efficiencies. Differences in conversion efficiencies were also obtained using PrP^{Sc} isolates from sheep with different PrP genotypes (Fig. 3b, bars 1-9 and 10-18). The $PrP^{Sc(VQ/VQ)}$ induced conversion with homologous PrP^{C_VQ} was the most efficient reaction in which about 35 % of the initial PrP^{C} was converted into 20-21 kDa PrP-res (Fig. 3b, bars 1 and 2). The $PrP^{Sc(VQ/VQ)}$ induced conversion with heterologous PrP^{C_AQ} resulted in an intermediate (17-24 %) conversion into PrP-res (Fig. 3b, bars 7 and 8). In contrast, the $PrP^{Sc(AQ/AQ)}$ induced conversions with either heterologous PrP^{C_VQ} or homologous PrP^{C_AQ} resulted in almost equal intermediate conversion efficiencies (Fig. 3b, bars 10, 11, 16, and 17). PrP^{C_AR} was poorly converted into PrP-res by both PrP^{Sc} isolates (Fig. 3b, bars 4, 5, 11, and 14).

The intriguing efficiency differences between the conversions of non-glycosylated PrP^{C_VQ}, PrP^{C_AQ}, and PrP^{C_AR} were consistent with the relative efficiency differences observed with (the inaccurate quantifiable) glycosylated forms of PrP^{C} (compare Fig. 3b, bars 2, 5, 8, 11, 14, and 17 with bars 3, 6, 9, 12, 15, and 18). Therefore glycosylation of PrP^{C} seems to be of minor or no importance in determining the differences in conversion efficiencies between the various PrP^{C} variants.

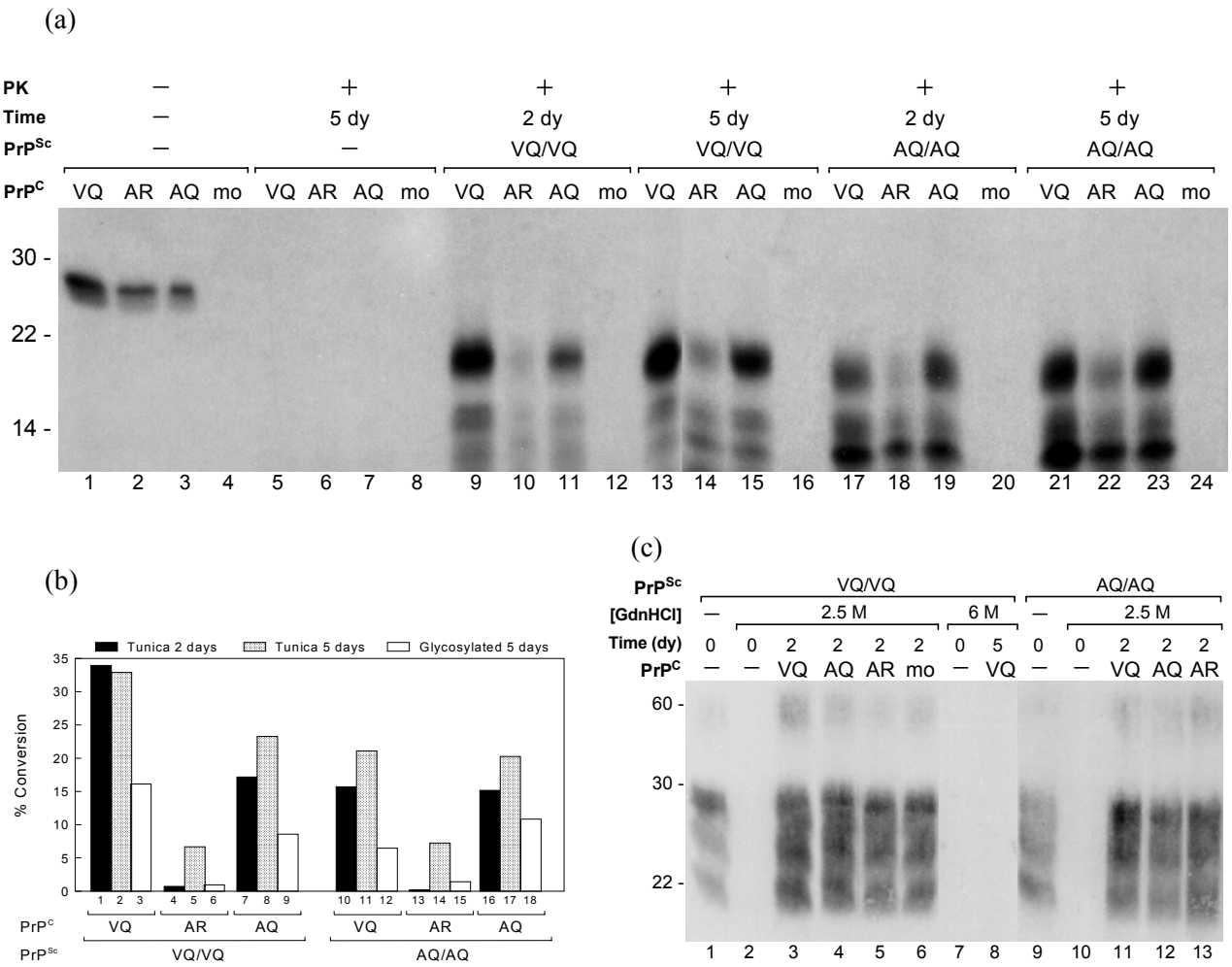

Figure 3: **(a)** Cell-free conversion of non-glycosylated ^{35}S-PrP^{C} into protease-resistant forms. PrP^{Sc} from sheep with different PrP genotypes were pretreated in 2.5 M GdnHCl. The conversions were started by adding the different allelic forms of ^{35}S-PrP^{C} or adding controls (mo) and dilution to 1 M GdnHCl. Reactions were incubated for 2 or 5 days at 37°C and subsequently PK digested (except lanes 1-4). Lanes 1-4 (starting material) contain approximately 10 % of the input material for conversions shown in lanes 5-24. Samples were analyzed by 15% SDS-PAGE. Molecular mass markers (kDa) are indicated at the left. **(b)** Percent conversion of the different allelic forms of PrP^{C} into PrP-res using different ShPrP^{Sc} isolates. Conversion percentages from figure 3a were determined by deviding the integrated intensities of bands at 21 kDa (lanes 5-24) by the integrated intensities of bands at 27 kDa (lanes 1-4) and multiplication by 10 (starting material=10%). To give an indication of conversion efficiencies in conversions with glycosylated PrP^{C}, the bands between 27-39 kDa (Fig. 2, lanes 1-3) and between 21-33 kDa (Fig. 2, lanes 10-15) were also 'quantified'. The different types of conversions using either non-glycosylated PrP^{C} or glycosylated PrP^{C}, as well as the length of the incubation time in days (dy) and the allelic forms of PrP^{Sc} and PrP^{C} are indicated. **(c)** Immunoblot analysis using the R521-7 antibody after PK digestion of 1/10 volume of the conversion reactions shown in Figure 3a. Indicated are the different allelic forms of PrP^{Sc} and PrP^{C}, the renaturation time, and the concentration (M) of GdnHCl used to pretreat the PrP^{Sc}. Untreated PrP^{Sc} (starting material) is shown in lanes 1 and 9. Molecular mass markers (kDa) are indicated at the left.

To conclude that the differences in amounts of conversion products are only the result of the polymorphisms present in PrP, it is important to have as close to identical concentrations of PrP^{Sc} and PrP^{C} in each reaction. All conversions with the same PrP^{C} variant have identical PrP^{C} concentrations on basis of protein content, since we aliquoted equal volumes from one batch into the different conversion reactions. All conversions with different PrP^{C} variants have equal amounts of PrP^{C} on basis of radiolabel and equal immunoperoxidase monolayer staining of the PrP^{C} expressing cells. The content of PrP^{Sc} in each conversion reaction as well as the rate of unfolding/refolding was compared by Western blotting 1/10 volume of each conversion reaction (Fig. 3c). This blot shows that the PrP^{Sc} isolates contained about the same quantity of protease-resistant PrP (Fig. 3c, lanes 1 and 9). At least the R521 epitope of PrP^{Sc} became PK sensitive after pretreatment in 2.5 M GdnHCl (Fig. 3c, lanes 2 and 10) and recovered PK resistance after 2 days of renaturation (Fig. 3c, lanes 3-6 and 11-13). Renaturation in the presence of different allelic forms of PrP^{C} did not detectably inhibit the refolding of PrP-res (Fig. 3c, lanes 3-5 and 11-13). The denaturation of PrP^{Sc} with 6 M GdnHCl was not reversible (Fig. 3c, lane 7 and 8). We concluded that, since the amounts of PrP^{Sc} and PrP^{C} of the different allelic forms were similar in each conversion, differences in quantity of conversion products were probably solely an effect of the primary ShPrP amino acid sequence.

Discussion

In this paper we report, for the first time, the cell-free conversion of sheep PrP^{C} into protease-resistant forms similar or identical to $ShPrP^{Sc}$. In addition we report that polymorphisms which are associated with differences in scrapie susceptibility and differences in survival times of scrapie affected sheep, also account for comparable differences in cell-free conversion efficiencies. This suggests that the PrP conversion kinetics are directly related to scrapie susceptibility and the length of survival times of sheep affected by natural scrapie. Since their is a good correlation between *in vitro* cell-free conversion data and *in vivo* scrapie susceptibility data thus far (9-12, 16, 17), this assay may be useful for determining the relative susceptibility of individual allelic forms of PrP to different prion sources and/or the relative transmissibility of these prion sources.

The efficiency of the cell-free conversion reaction was strongly dependent on both the type of PrP^{C} variant and on the source of PrP^{Sc} used to induce the conversion. The PrP^{C_VQ} variant, which is associated with high susceptibility and short survival times of scrapie affected sheep, was very efficiently converted into protease-resistant forms. The

'wildtype' PrP^{C_AQ} variant, which is associated with a neutral effect on susceptibility and intermediate survival times, was converted into protease-resistant forms with intermediate efficiency. The PrP^{C_AR} variant, which is associated with resistance and long survival times, was poorly converted into protease-resistant forms. Although in some breeds, i.e. Suffolk and Romanov, PrP^{AQ} is associated with an incomplete penetrance to scrapie susceptibility, probably due to the low incidence of PrP^{VQ} (16, 19, 32), PrP^{VQ} carriers of these breeds still have the shortest scrapie survival time (16, 32). Another point of interest is the finding that PrP^{C_AR} can be converted, although with a very low efficiency, into protease-resistant forms suggesting the possibility of scrapie agent 'replication' in PrP^{AR} carrying sheep as has been described by Ikeda *et al.* (32).

Not only the primary PrP^{C} sequence was found to determine the conversion characteristics but also the primary amino acid sequence of PrP^{Sc}. $PrP^{Sc(VQ/VQ)}$ converted PrP^{C_VQ}, PrP^{C_AQ}, and PrP^{C_AR} with decreasing efficiencies. In contrast, $PrP^{Sc(AQ/AQ)}$ converted PrP^{C_VQ} almost as efficiently as the PrP^{C_AQ} variant. The PrP^{C_AR} variant was poorly converted by both PrP^{Sc} isolates. This suggests that scrapie susceptibility is not only determined by the PrP genotype of the acceptor animal but also by the PrP genotype of the animal that produced the infectious PrP^{Sc}. This is consistent with the finding that the SSBP/1 scrapie isolate obtained from PrP^{VQ} NPU-Cheviot sheep is best transmitted to PrP^{VQ} sheep (12, 17). It is also consistent with the striking behaviour of the CH1641 scrapie isolate, which was primarily isolated from a 'positive line' (mainly PrP^{VQ} carrying) NPU Cheviot sheep, when passaged in 'positive-line' or 'negative-line' (non-PrP^{VQ}) Cheviot sheep. The first (primary) intracerebral passage of this 'positive-line' material to 'positive-line' Cheviot sheep resulted in short incubation times. Passage of the primary CH1641 isolate into 'negative-line' Cheviot sheep resulted in longer incubation times (33) probably due to polymorphism barriers. If the 'negative-line' passaged isolates were subsequently passaged in 'negative-line' Cheviot sheep the incubation times in this line of sheep decreased (17, 33). A subsequent passage from these 'negative-line' to 'positive-line' Cheviot sheep increased the incubation times dramatically (17, 33) again probably due to the 'polymorphism barrier'.

Modification of scrapie isolate properties were also found in mice scrapie transmission experiments in which the properties of PrP^{Sc} could be modified by passage of scrapie isolates through mice with different PrP^{C} amino acid sequences (34). Further support is derived by the transmission of human CJD or GSS to mice expressing chimeric mouse/human PrP transgenes carrying specific mutations. Mice carrying the Glu to Lys mutation at position 200 (E200K) were resistant to human prions from a patient with GSS

carrying a Pro to Leu mutation at position 102 (P102L) but were susceptible to prions from familial CJD patients harbouring the E200K mutation. However, mice carrying the mouse/human transgene with the P102L mutation were susceptible to GSS prions (24).

Interestingly, a homogenate of BSE, of which the primary amino acid sequence (at the polymorphic amino acid positions of sheep PrP) is best comparable with the sheep PrP^{AQ} genotype, gives the shortest incubation times in PrP^{AQ} sheep if inoculated by the intracerebral route. If inoculated via the longer oral route however, PrP^{VQ} sheep have the shortest incubation time (17). Probably inoculation via the oral route, compared to inoculation by the intracerebral route, extends the incubation time long enough to 'overcome' the polymorphism barrier and subsequently allows the agent to spread more quickly using PrP^{C_VQ} instead of PrP^{C_AQ}.

Preliminary data from cell-free conversion experiments with the three PrP^{C} variants using PrP^{Sc} isolated from a $PrP^{VQ/AQ}$ sheep suggests that this PrP^{Sc} isolate mainly consists of PrP^{Sc_VQ} since this $PrP^{Sc(VQ/AQ)}$ isolate converted PrP^{C_VQ} at least three times as efficiently as PrP^{C_AQ} into protease-resistant forms (Fig. 4). This again is consistent with the finding that PrP^{C_VQ} is more readily converted into PrP-res than PrP^{C_AQ}. Thus in sheep containing the mutant PrP^{VQ} allele, it is likely that the PrP^{C_VQ} variant will be the preferred converted variant, similar to what has been found for the mutant human PrP allele in GSS (35). Consequently, after infection of flocks of sheep having the PrP^{VQ} allele, the 'agent pool' would be predicted to become enriched for PrP^{VQ}.

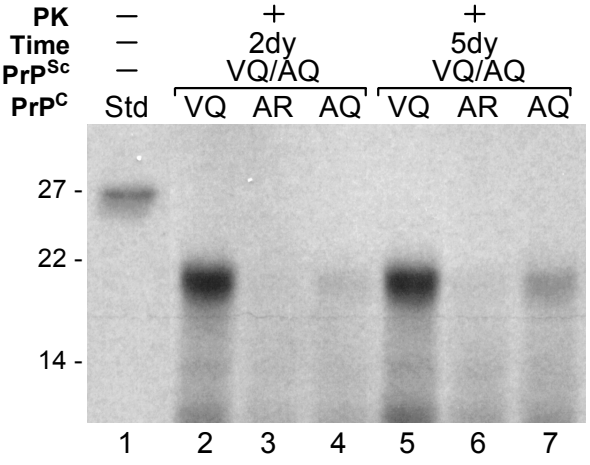

Figure 4: Cell-free conversion of non-glycosylated ^{35}S-PrP^{C} into protease-resistant forms. PrP^{Sc} from a heterozygous sheep was pre-treated in 2.5 M GdnHCl. Reactions were incubated for 2 or 5 days at 37°C (1M GdnHCl) and subsequently PK digested (except lane 1). Lane 1 contains approximately 2-5 % of the input material (Std) for conversions shown in lanes 2-7. Samples were analyzed by 15% SDS-PAGE. Molecular mass markers (kDa) are indicated at the left.

This study shows that the cell-free system is an excellent system to 'measure' the relative transmissibility of a prion source to animals or humans with known PrP genotypes. Although the mechanism by which PrP^{C} is converted into PrP^{Sc} and the mechanism by which polymorphisms in PrP modulate the conversion efficiency is not yet clear, studies with the cell-free conversion reaction (36) and small synthetic PrP peptides (37) are consistent with a nucleated polymerization mechanism (38, 39). The conversion of PrP^{C} to PrP^{Sc} involves a transition from a state that is predominantly alpha-helical to one that is largely beta sheet (4, 5, 40). PrP^{C} may rapidly interchange between these two conformations in its normal monomeric state but only be stabilized and accumulated in the beta sheet conformation by binding to a preformed PrP^{Sc} polymer (37, 38, 41). Alternatively, the transition to the PrP^{Sc} conformation may only be induced (catalized) upon direct binding of PrP^{C} to the PrP^{Sc} polymer. PrP polymorphisms may influence the equilibrium between the alpha-helical and beta sheet conformations in PrP^{C} and/or the ease with which PrP^{Sc} induces PrP^{C} to switch to the beta sheet conformation. Polymorphisms which destabilize the alpha helical conformation of PrP^{C} would be expected to have these effects.

In this study we have tested the cell-free conversion of three (PrP^{VQ}, PrP^{AQ}, and PrP^{AR}) of the nine different PrP variants found in sheep, including the two allelic variants which are associated with the extremes in susceptibility to scrapie (highly susceptible or resistant). From the other six allelic variants: PrP^{T112AQ}, PrP^{AT137Q}, PrP^{AF141Q}, PrP^{AH154Q}, PrP^{AH}, and PrP^{AQQ211}, it is not known whether they are significantly associated with susceptibility to natural or experimental scrapie in sheep. Using the recently published high resolution NMR structure of the mouse PrP^{C} domain containing residues 121-232 together with 'Novotny' secondary structure predictions, it might be possible to rationalize the effects of certain of the sheep PrP polymorphisms on PrP^{C} conformation. At least two other polymorphisms in the sheep PrP gene could be 'associated', by these predictions, with scrapie susceptibility. The PrP^{AT137Q} variant could be grouped with the PrP^{VQ} variant, since both give a prediction of more beta-sheeted structure and a change in hydrophobicity in the loop between β-sheet-1 and α-helix-1 which may indicate helix breaking or hydrophobic core destabilizing properties as found in theoretical studies of the Ala to Val mutation at position 117 in the human PrP sequence (42). The PrP^{AH154Q} variant is protective against scrapie and no scrapie affected sheep with this genotype have been found (10, 12, 15, 32). This variant could be grouped with the PrP^{AR} variant, since both involve a charge inversion compared to the wildtype PrP^{AQ} variant. The latter two polymorphisms are located in the loops between α-helix-1 and β-sheet-2, and between β-sheet-1 and α-helix-3 respectively, and may influence the stabilization of the hydrophobic core or the dipolar character of PrP^{C}. The other four alleles did not show differences in 'Novotny' secondary structure predictions

other than the PrP^{AQ} variant and therefore may probably be grouped with this variant. Additional cell-free conversion data with all known sheep PrP^{C} variants may enable us in the near future to determine more exactly the relative scrapie susceptibility between sheep having different PrP alleles.

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CHAPTER 6

Susceptibility of Sheep for Scrapie as Assessed by *in vitro* Conversion of Nine Natural Occurring Variants of PrP.

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Abstract

Polymorphisms in the prion protein gene are associated with different phenotypic expressions of transmissible spongiform encephalopathies in animals and humans. In sheep at least 8 different mutually exclusive polymorphisms are present in PrP. Here we determined the efficiency of the *in vitro* formation of protease-resistant PrP of these sheep PrP allelic variants in order to gauge the relative susceptibility of sheep for scrapie. No detectable spontaneous protease-resistant PrP formation occurred under the used cell-free conditions. All nine PrP^{C} variants had distinct conversion efficiencies induced by PrP^{Sc} isolated from sheep with three different homozygous PrP genotypes. In general, PrP allelic variants with polymorphisms at either codons 136 (Ala to Val) or 141 (Leu to Phe), and wildtype sheep PrP^{C} converted with highest efficiency into protease-resistant forms which indicates a linkage with a high susceptibility of sheep for scrapie. PrP^{C} variants with polymorphisms at either codons 171 (Gln to Arg), 154 (Arg to His), and to a minor extent at codon 112 (Met to Thr) converted with low efficiency into protease-resistant isoforms. This indicates a linkage of these alleles with a reduced susceptibility or resistance for scrapie. In addition, PrP^{Sc} with the codon 171 (Gln to His) polymorphism is the first ever described variant which induced higher conversion efficiencies with heterologous rather than with homologous PrP variants. The results of this study strengthen our views on polymorphism-barriers and have further implications for scrapie control programs by breeding strategies.

Introduction

Scrapie is a fatal and infectious neurodegenerating disease occurring in sheep and goats. The disease belongs to the group of transmissible spongiform encephalopathies (TSEs) or prion diseases found in humans and animals. Creutzfeldt-Jacob disease (CJD), Gerstmann-Straussler-Scheinker syndrome (GSS) and fatal familial insomnia (FFI) of man and bovine spongiform encephalopathy (BSE) in cattle also belong to this group. Prion diseases are characterised by the accumulation of an abnormal protease-resistant isoform (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^{C}) in tissues of the central nervous system. Although the exact origin and nature of the causative agent remains unknown, it is thought to transmit and replicate by protein only (Griffith, 1967). PrP^{Sc} molecules form the major, if not the only, component of the agent transmitting the disease (Prusiner, 1982).

Several polymorphisms in the open reading frame (ORF) of PrP are associated with different phenotypic expressions of prion diseases such as incubation period, pathology and clinical signs. In PrP of sheep eight mutually exclusive amino acid polymorphisms at positions 112 (Met to Thr), 136 (Ala to Val), 137 (Met to Thr), 141 (Leu to Phe), 154 (Arg to His), 171 (Gln to Arg or Gln to His), and 211 (Arg to Gln) respectively have been described (see Table 1) (Belt *et al*., 1995, Belt *et al*., 1996, Bossers *et al*., 1996, Goldmann *et al*., 1991, Goldmann *et al*., 1990, Hunter *et al*., 1996, Laplanche *et al*., 1993).

Table 1. *Allelic variants of sheep PrP.*

Variant designation		Polymorphic amino acid residues						
Allele[a]	Protein	**112**	**136**	**137**	**141**	**154**	**171**	**211**
PrP^{ARQ}	PrP^{C_wt} / PrP^{Sc_wt}	M	A	M	L	R	Q	R
$PrP^{T112ARQ}$	PrP^{C_112T}	**T**	A	M	L	R	Q	R
PrP^{VRQ}	PrP^{C_136V} / PrP^{Sc_136V}	M	**V**	M	L	R	Q	R
$PrP^{AT137RQ}$	PrP^{C_137T}	M	A	**T**	L	R	Q	R
$PrP^{AF141RQ}$	PrP^{C_141F}	M	A	M	**F**	R	Q	R
PrP^{AHQ}	PrP^{C_154H}	M	A	M	L	**H**	Q	R
PrP^{ARR}	PrP^{C_171R}	M	A	M	L	R	**R**	R
PrP^{ARH}	PrP^{C_171H} / PrP^{Sc_171H}	M	A	M	L	R	**H**	R
$PrP^{ARQQ211}$	PrP^{C_211Q}	M	A	M	L	R	Q	**Q**

[a] Unless indicated otherwise, only polymorphic amino acids at codons 136, 154, and 171 are given.

The allelic variant PrP^{VRQ} (amino acids at positions 136 (Val), 154 (Arg) and 171 (Gln) are indicated in superscript by single letter amino acid codes, polymorphisms at other codons are indicated separately), is significantly associated with a high susceptibility to scrapie and short survival times of scrapie-affected sheep in many different breeds (Bossers *et al*., 1996, Clouscard *et al*., 1995, Goldmann *et al*., 1994, Hunter *et al*., 1996, Hunter *et al*., 1994, Laplanche *et al*., 1993). In contrast, the allelic variant PrP^{ARR} is significantly associated with resistance to natural and experimental infections with scrapie and BSE in probably all sheep breeds (Belt *et al*., 1995, Bossers *et al*., 1996, Clouscard *et al*., 1995, Goldmann *et al*., 1994, Goldmann *et al*., 1996, Hunter *et al*., 1996). In breeds where the PrP^{VRQ} allele is rare or absent (for instance the Suffolk breed) the 'wildtype' PrP^{ARQ} allelic variant is associated with increased scrapie susceptibility, but with a lower penetrance than found for the PrP^{VRQ} allele (Hunter *et al*., 1994, Westaway *et al*., 1994). Little is known yet with regards to the association of the allelic variants $PrP^{T112ARQ}$, $PrP^{AT137RQ}$, PrP^{AHQ}, PrP^{ARH}, and $PrP^{ARQQ211}$ with susceptibility for scrapie.

The mechanisms by which the different allelic variants contribute to the susceptibility for scrapie susceptibility are not completely understood. From genetic studies it can be deduced however that natural occurring variants of PrP, including those associated to a high risk for scrapie, probably can not induce the spontaneous development of scrapie in sheep (Bossers *et al*., 1999, Hunter & Cairns, 1998, Hunter *et al*., 1997) as has been found for inherited prion diseases. Furthermore it has been shown that the scrapie-susceptibility linked polymorphisms at codon 136 and 171 can modulate the efficiency of the cell-free conversion of sheep PrP^{C} into protease-resistant forms (PrP-res) induced by PrP^{Sc} (Bossers *et al*., 1997). In this cell-free system, sheep PrP^{C_136V} and PrP^{C_wt} are efficiently converted into PrP-res by their homologous PrP^{Sc} (PrP^{Sc_136V} and PrP^{Sc_wt} respectively). In contrast PrP^{C_171R} is poorly converted into PrP-res by PrP^{Sc} (Bossers *et al*., 1997, Raymond *et al*., 1997). Also other biological aspects of TSE diseases such as species-barriers, polymorphism-barriers, and the non-genetic propagation of prion strain phenotypes are reflected in the specificities of PrP conversion reactions in this cell-free system (Bessen *et al*., 1995, Bossers *et al*., 1997, Kocisko *et al*., 1995, Raymond *et al*., 1997). Therefore the system may be used to gauge the potential *in vivo* transmissibility of TSEs and to prognosticate the susceptibility of hosts for TSEs (Bessen *et al*., 1995, Kocisko *et al*., 1995, Raymond *et al*., 1997).

In the present study we used this cell-free system to determine the relative conversion efficiencies of the various sheep PrP allelic variants by PrP^{Sc} isolated from sheep with different PrP genotypes. Based on the observed conversion efficiencies we tried to gauge the linkage between the nine natural occurring variants of PrP and the

susceptibility of sheep for scrapie and to a minor extent we tried to gauge the transmissibility of scrapie between sheep. This relative scrapie susceptibility profile may allow the further development and/or justification of specific sheep breeding programs. It may also provide insight in structurally important residues involved in modulation of the conversion efficiencies of PrP^{C} into PrP^{Sc} and it may reveal important sites in the PrP molecule to target in the development of potential therapeutics. The effect of each of the eight polymorphisms on the cell-free conversion efficiencies and the *in vivo* predicted modulation of scrapie phenotype will be discussed.

Materials and methods

PrP constructs and expression. The sheep PrP allelic variants PrP^{ARQ}, PrP^{VRQ}, PrP^{ARR} variants were cloned, expressed and characterised as described before (Bossers *et al*., 1997). The full ORF's of the sheep allelic variants $PrP^{AT137RQ}$, $PrP^{AF141RQ}$, PrP^{AHQ}, PrP^{ARH}, $PrP^{ARQQ211}$ were cloned by PCR amplification and proof-read by DNA sequencing essentially as described by Bossers *et al.* 1996. Sheep PrP allelic variant $PrP^{T112ARQ}$, which was not readily available, was constructed by site-directed mutagenesis using standard methods. After subcloning all the different sheep PrP allelic variants (EMBL accession numbers AJ000679 to AJ000681 and AJ000734 to AJ000739) to eukaryotic expression vector pECV7 (Bossers *et al*., 1997), the PrP constructs were transfected to eukaryotic cells as described (Bossers *et al*., 1997). Single cell clones that expressed each PrP allelic variant were analysed for expression by immunoperoxidase monolayer assay (IPMA) and radio-immuno precipitation (Bossers *et al*., 1997). Best and equal PrP^{C} expressing single cell clones were selected and/or stored for further labelling experiments. To monitor the stability and validity of the expressed sheep PrP sequences, the genomically integrated sequences were proof-read by PCR amplification and subsequent analysed on denaturing gradient gel electrophoresis (DGGE) after several rounds of subculturing the single cell clones (Bossers *et al*., 1996).

Radiolabelling and purification PrP^{C}. Single cell clones, that expressed the different sheep PrP^{C} variants, were subcultured two days before labelling and labelled at near confluency as described (Bossers *et al*., 1997, Caughey *et al*., 1995). Briefly, cells were starved for 30-60 minutes in culture medium containing only 1/10 of the normal concentration of methionine and 7.5 µg/ml tunicamycin-D. After labelling for 90-120 minutes at 37°C using at least 1mCi of [^{35}S]methionine/[^{35}S]cysteine Tran^{35}S-label (ICN) per 25 cm^2 flask, the cells were lysed (0.5% Triton X-100; 0.5% Nadeoxycholate, 5mM Tris-Cl pH 7.4 (4°C); 150mM NaCl; 5mM EDTA) on ice in the presence of protease

inhibitors (Pefabloc SC, leupeptine, pepstatin and aprotinin). Proteins were precipitated from detergent lysates by 4 volumes of methanol at -20°C and subsequently sonicated in DLPC buffer (0.05M Tris-HCl pH 8.2; 0.15M NaCl; 2%w/v N-lauryl sarcosine; 0.4% w/v lecithin) containing the same protease inhibitor cocktail. ^{35}S-PrP^{C} was immunopurified using antibody R521-7 that specifically reacts with sheep PrP^{C} (Bossers *et al.*, 1997). Immunecomplexes were collected using protein-A sepharose and bound ^{35}S-labelled proteins were eluted in 0.1M acetic acid pH 2.8. If necessary, eluants were concentrated by vacuum evaporation and reconstituted in 50mM citrate buffer pH 6.0 (G.J. Raymond; personal communication). Yield of radiolabelled PrP^{C} was measured using a micro-β counter and eluates were stored on ice until further use.

PrP^{Sc} purification and analysis. PrP^{Sc} was isolated from brain tissue (cerebrum) of five different PrP$^{ARQ/ARQ}$ sheep, from four different PrP$^{VRQ/VRQ}$ sheep, and from one PrP$^{ARH/ARH}$ sheep. All suffered from natural scrapie confirmed by immunohistochemistry. PrP^{Sc} was purified in the absence of protease inhibitors by ultracentrifugational pelleting from sarkosyl homogenated brains (Bossers *et al.*, 1997, Caughey *et al.*, 1991). After pelleting trough a 20% sucrose cushion the pellet was sonicated in phosphate buffered saline (PBS) containing 1% sulfobetaine (SB 3-14) and stored in portions at 4°C. After protease-K (PK) digestion PrP-res was quantified by SDS-PAGE followed by SYPRO-orange (Molecular Probes) staining and measuring direct fluorescence with the STORM-840 (Molecular Dynamics). In addition, the relative amounts of PrP^{Sc} were quantitated by Western blotting using the antibody R521-7, ECF fluorescence substrate (Amersham-Pharmacia biotech) and the STORM-840. All isolates contained about 2-6 µg PrP-res per gram of brain. PrP-res yields from PrP$^{ARQ/ARQ}$ sheep brain (3-6 µg/g) were almost always higher than yields from PrP$^{VRQ/VRQ}$ sheep brain (2-4 µg/g). PrP-res concentrations were equalised by further dilution in PBS containing 1% SB 3-14. Isolates of PrP^{Sc} were stored at 4°C and briefly sonicated prior to use.

Cell-free conversion. At least 0.8 µg of PrP-res per conversion reaction in siliconized tubes was partially denatured at a final concentration of 2.5M GdnHCl (using 8-9M GdnHCl in 50mM NaCitrate) for at least 3 hours 37°C. The partially denatured PrP-res was subsequently added to a conversion mix containing ^{35}S-labelled PrP^{C} and conversion buffer to give a final concentration of ≤1M GdnHCl, 50mM NaCitrate pH 6.0, 1% N-Lauryl sarcosine and 5mM cetyl pyridinium chloride in a total volume of 34 µl or less. Reactions were mixed regularly and incubated for 1-4 days at 37°C. After incubation, each reaction volume was raised to 100 µl by Tris-NaCl (50mM Tris-Cl pH 7.5; 150mM NaCl) and split 1:10. The major fraction was digested with 35 µg/ml PK for 1hour at 37°C and

thereafter PK was inactivated by adding Pefabloc SC. The proteins in both fractions, minus and plus PK, were precipitated with methanol in the presence of 20 µg thyroglobulin as a carrier. Pellets were sonicated and boiled in 4% β-mercapthoethanol and 4M Urea containing Laemmli sample buffer and analysed by 14% SDS-PAGE (Novex). Percentage of conversion was determined by measuring radiolabel in specific regions of the gels (input PrP^{C} M_r 25-27 kDa, conversion products M_r 20-22 kDa) using for the initial experiments exposures of X-ray film and for later experiments phosphor imaging (STORM-840). Conversion % = conversion signal / start signal x 10% x 100/90. At least two or more independent conversion reactions were performed from each different PrP variant. Normalisation was performed to pronounce the relative conversion efficiencies within each set of PrP^{Sc}.

Results

The nine different natural occurring sheep PrP^{C} variants could be readily detected after labelling, immuno precipitation, and SDS-PAGE by phosphor imaging (Figure 1 lanes 1-9) or by Western blotting. A uniform product with a molecular weight of about 26 kDa was present in all lanes indicating that, apart from the single amino acid changes, no differences could be observed with regard to processing and/or partial endogenous proteolysis. In addition, also at the cell culture level no (microscopic) differences could be detected between the nine different variants in biosynthesis and localisation of PrP^{C} using immunoperoxidase monolayer assays (IPMA). The nine radio labelled PrP^{C} variants revealed no PrP-res formation after incubation under cell-free conversion conditions without addition of exogenous PrP^{Sc} (Figure 1 lanes 10-18). This indicates that under these *in vitro* conditions, no detectable spontaneous formation of PrP-res occurs, which coincides with the requirement of exogenous agent for natural scrapie development (Bossers *et al.*, 1999, Hunter & Cairns, 1998, Hunter *et al.*, 1997). In contrast, if partially denatured PrP^{Sc} is added to the conversion reaction, readily detectable PrP-res formation occurs with the specific shift in molecular weight of about 6 kDa (Figure 1 lanes 19-27).

Three different PrP^{Sc} preparations, i.e. PrP^{Sc_wt}, PrP^{Sc_136V}, and PrP^{Sc_171H} respectively, could be obtained from natural scrapie cases homozygous for PrP. Of several independent isolates, about 1 µg of PrP^{Sc} was partially denatured and subsequently renatured under conversion conditions in the presence of one of each of the nine different ^{35}S labelled PrP^{C} molecules. After PK digestion, SDS-PAGE, and phosphor-imaging it became clear that different amounts of PrP-res could be observed (Figure 1 lanes 19-27). These sets of nine conversion experiments were repeated several times using PrP^{Sc}

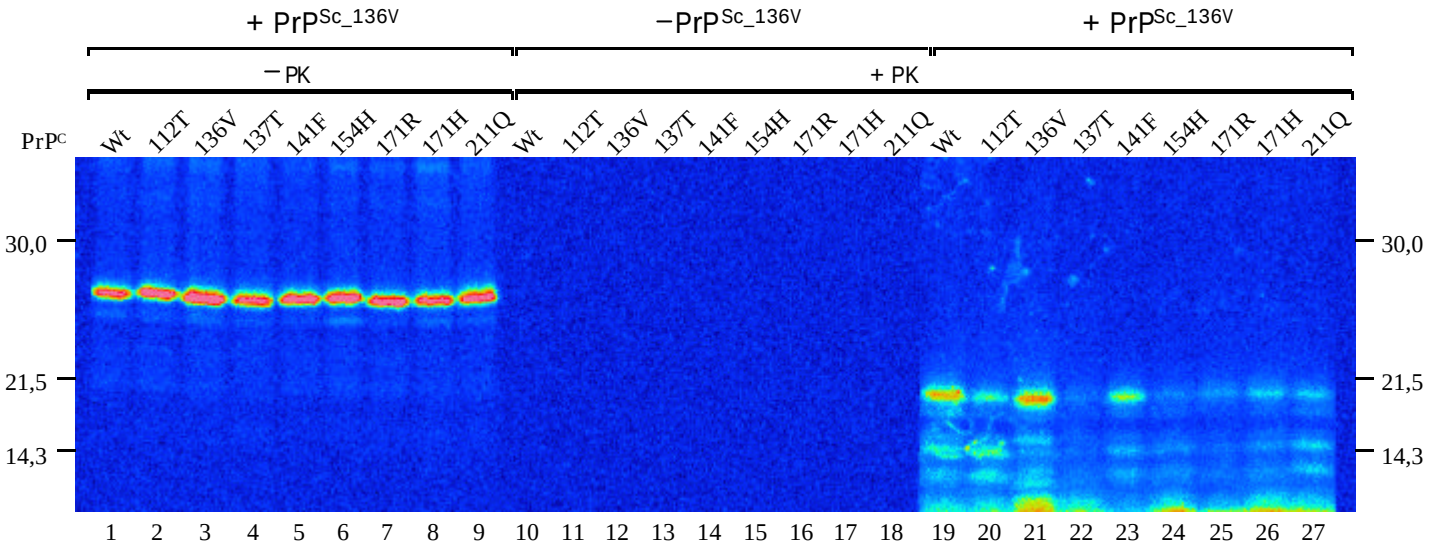

Figure 1: Example phosphor-image of an SDS-PAGE (indicated in pseudo intensity staining) showing cell-free conversion reactions of nine different sheep PrP^{C} variants by sheep PrP^{Sc_136V} into protease-K resistant forms. Lanes 1-9 show approximately 1/10 of the input of PrP^{C} in the various conversion reactions. Incubation of these variants under cell-free conditions revealed no spontaneous protease resistant PrP formation after PK digestion (lanes 10-18). Different amounts of protease-resistant PrP were formed however, induced by addition of partially denatured exogenous PrP^{Sc} (lanes 19-17). PK digestion removes about 6 kDa from the N-terminal part of PrP when converted into protease resistant forms (compare lanes 1-9 with lanes 19-27). Molecular mass markers (kDa) are indicated.

preparations isolated from different sheep brains, except for PrP^{Sc_171H} of which only one homozygous scrapie case was available. From the data it is obvious that the conversion efficiencies between sets vary enormously (Figure 2). However, the relative conversion efficiencies of the nine allelic variants induced by either PrP^{Sc_wt}, PrP^{Sc_136V}, or PrP^{Sc_171H} in multiple and independent experiments were very similar. For example, in all PrP^{Sc_136V} induced conversion sets, the PrP^{C_136V} allelic variant was always converted with highest efficiency, PrP^{C_wt} with reduced efficiency, and PrP^{C_171R} almost not. Therefore we normalised the data for each set of nine conversion experiments to the homologous conversion reaction, usually the strongest conversion reaction (Figure 3).

Within each conversion set remarkable differences in the convertibility of PrP^{C} into PrP-res are visible. In each PrP^{Sc} set a trend in decreasing convertibility can be observed. The order of this trend in the three different sets is summarised in Table 2. From these three

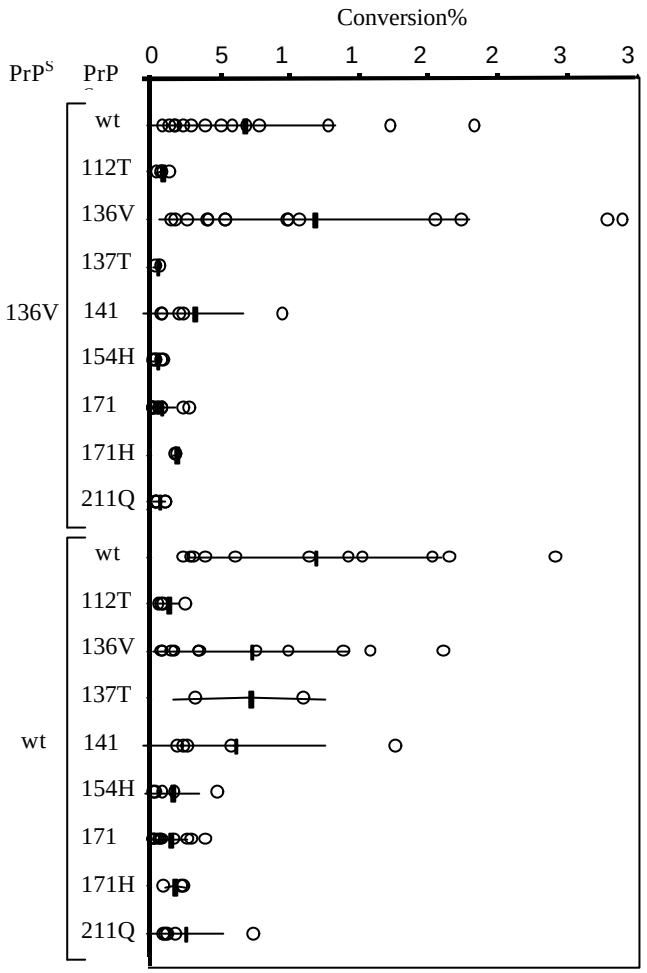

Figure 2. Scatter graph of individual percentages of PrP^{C} conversion into protease-resistant forms (open circles). The nine different sheep PrP^{C} variants convert with different efficiencies by either PrP^{Sc_136V} or PrP^{Sc_wt}. Mean conversion percentages (±s.d.) are indicated.

different PrP^{Sc} sets a consensus was created in which the rare allelic variants $PrP^{AT137RQ}$ and $PrP^{ARQQ211}$ were excluded (Table 2). This consensus indicates the relative order in which these allelic variants are predicted to be linked to susceptibility for scrapie. The most efficient converting variants by each of the three types of PrP^{Sc} are PrP^{C_136V}, PrP^{C_wt}, and PrP^{C_141F} while PrP^{C_112T}, PrP^{C_154H}, and PrP^{C_171R} converted with low efficiency. The variants PrP^{C_171H} and PrP^{C_211Q} converted with intermediate efficiency. Some remarkable differences between the first two PrP^{Sc} sets exist (compare Figure 3a with 3b). One observed difference is the switch in convertibility of PrP^{C_136V} and PrP^{C_wt} with homologous and heterologous PrP^{Sc} (Figure 3a and b, bars 1 and 3). Another point of interest is the relative high conversion rate of PrP^{C_141F} which has similar conversion properties as PrP^{C_wt} (Figure 3a and b, bars 1 and 5). The PrP^{C_137T} variant behaves like PrP^{C_136V} in PrP^{Sc_wt} and PrP^{Sc_171H} induced reactions (Figure 3b and c, bars 3 and 4) while this variant is inconvertible by PrP^{Sc_136V} (Figure 3a bar 4).

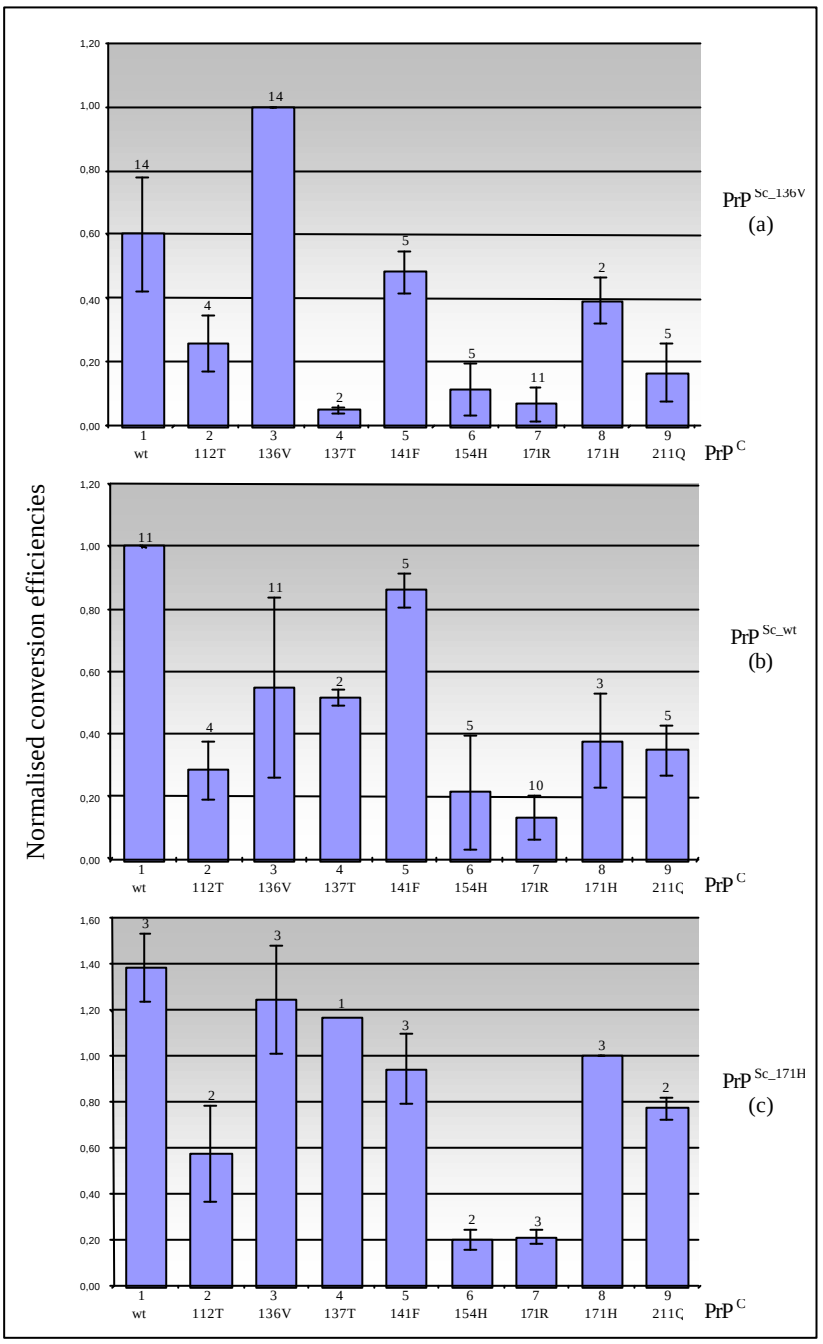

Figure 3. Sets of conversions (a+b+c), defined as all nine different PrP^{C} variants incubated with one type of PrP^{Sc}, were normalised to the homologous conversion reactions. Mean normalised conversion efficiencies (±s.d.), and the number of independent conversion reactions of each PrP^{C} variant are indicated.

The smaller data set of the convertibility of various PrP^{C} variants by PrP^{Sc_171H} shows also a shift in the relative conversion efficiencies of the nine PrP variants. Due to the limited availability of PrP^{Sc_171H}, only 1-3 data points could be generated for each PrP^{C} variant. The results show however that the heterologous variants PrP^{C_wt}, PrP^{C_136V}, and PrP^{C_137T} have higher conversion efficiencies with PrP^{Sc_171H} than the homologous

PrP^{C_171H} variant itself (compare Figure 3 bars 1, 3, and 4 with bar 8 within each panel). More importantly, the convertibility of the PrP^{C_154H} and PrP^{C_171R} variants in the PrP^{Sc_171H} set remained low (Figure 3c bars 6 and 7).

We concluded from these data that in general the PrP^{VRQ}, PrP^{ARQ}, or $PrP^{AF141RQ}$ alleles and to lesser extent the PrP^{ARH} allele are linked to highest susceptibility for scrapie. In addition, the PrP^{ARR} allele, the PrP^{AHQ} and to minor extent the $PrP^{T112ARQ}$ alleles contribute to resistance of sheep for scrapie.

Table 2. *Relative conversion efficiencies of the various sheep PrP^{C} variants by each PrP^{Sc}.*

Type	Conversion efficiency [a] High ⟶ Low								
PrP^{Sc_136V}	136V	wt	141F	171H	112T	211Q	154H	171R	137T
PrP^{Sc_wt}	wt	141F	136V	137T	171H	211Q	112T	154H	171R
PrP^{Sc_171H}	wt	136V	137T	171H	141F	211Q	112T	154H	171R
Consensus [b]	136V / wt / 141F				171H		112T	154H / 171R	

[a] PrP^{C} allelic variants are indicated by their single amino acid polymorphism and ranked within each type of PrP^{Sc} in order of decreased conversion efficiency.

[b] Virtually absent PrP allelic variants *in vivo*; $PrP^{AT137RQ}$ and $PrP^{ARQQ211}$ were excluded.

Discussion

In a previous study (Bossers *et al.*, 1997) we demonstrated that the conversion efficiencies of three sheep PrP variants (PrP^{C_136V}, PrP^{C_wt}, and PrP^{C_171R}) reflected their linkage with scrapie susceptibility. In this study we measured the conversion efficiencies of nine different PrP allelic variants with three different types of PrP^{Sc}. Based on these results we tried to gauge the linkage of PrP alleles with relative susceptibility for scrapie. Of these nine allelic variants, those with polymorphisms at either codon 112, 137, 141, 154, 171, or 211 respectively have not been significantly associated with particular scrapie phenotypes yet.

The nine different natural variants of sheep PrP^{C}, incubated with different types of PrP^{Sc}, converted into PrP-res *in vitro* with different efficiencies. The most efficient converting PrP^{C} variants were the variants PrP^{C_136V}, PrP^{C_wt}, and PrP^{C_141F}. The conversion

efficiencies of the variants PrP^{C_154H}, PrP^{C_171R}, and to minor extent PrP^{C_112T} were consistently low in all reactions induced with the different types of PrP^{Sc}. From the perspective of breeding strategies of great interest are the low-convertible variants PrP^{C_154H} and PrP^{C_171R} for positive selection and the variants PrP^{C_136V}, PrP^{C_wt}, and PrP^{C_141F} for negative selection. The presence of several PrP variants in the same flock which are linked to resistance may turn out to be more safe in scrapie-control programs by breeding strategies and it may contribute to maintain a more genetically diverse sheep population.

The conversion efficiencies of the allelic variants PrP^{C_136V}, PrP^{C_wt}, and PrP^{C_171R} are consistent with the earlier *in vitro* observations of a smaller conversion data set (Bossers *et al.*1997). The codon 112 polymorphism Met to Thr reduces the convertibility of PrP compared to PrP^{C_wt} in conversion reactions induced by different types of PrP^{Sc}. This reduced convertibility is consistent with observations that this allelic variant in homozygous form is only found in healthy sheep (at low frequency). Only a few heterozygous $PrP^{VRQ}/PrP^{T112ARQ}$, or $PrP^{ARQ}/PrP^{T112ARQ}$ scrapie sheep have been described of the Ile-de-France or Japanese Suffolk breeds (Ikeda *et al*., 1995, Laplanche *et al*., 1993). In these cases the other alleles are probably dominant and result in scrapie development. In the theoretical three dimensional structure of sheep PrP, this polymorphism is located in the highly flexible N-terminal region (Figure 4). The larger side chain of Met, the polarity of Thr, or the shorter hydrogen-bond between amino acids at codons 110 and 112 may influence the stability of PrP^{C} and/or the interaction with PrP^{Sc} since both side chains are directed towards the potential interaction site.

The allelic variant PrP^{C_137T} demonstrates conversion properties similar to PrP^{C_136V} in PrP^{Sc_wt} and PrP^{Sc_171H} induced conversion reactions. In PrP^{Sc_136V} induced conversion reactions however, the codon 137 polymorphism results in inconvertibility. Probably the neighbouring polymorphisms at codons 136 and 137 interfere with the potential interaction site between PrP^{C} and PrP^{Sc} (Figure 4). No *in vivo* data of this allelic variant are available since this allele is rare (only one healthy sheep has been found having this allele (Bossers *et al*., 1996)). This allele is therefore irrelevant for breeding strategies and excluded from the consensus linkage with scrapie susceptibility in Table 2.

The polymorphism Phe at codon 141 (PrP^{C_141F}) had minor reduction effects on the convertibility into PrP-res and this variant reacted in the same order of magnitude as PrP^{C_wt}. This seems to be consistent with observations that sheep having the $PrP^{VRQ}/PrP^{AF141RQ}$ genotype have somewhat longer survival times than sheep having the PrP^{VRQ}/PrP^{ARQ} genotype, while both have longer survival times than PrP^{VRQ}/PrP^{VRQ} sheep in a flock of Swifter sheep where PrP^{VRQ} is linked to highest scrapie susceptibility and shortest survival times (Bossers *et al.*, 1996). Based on the *in vitro* conversion efficiencies

the $PrP^{AF141RQ}$ allele is expected to be associated with high susceptibility in flocks where PrP^{ARQ} is associated with highest scrapie susceptibility. Also from amino acid sequence comparisons between different species, this L141F polymorphism is expected to be less important since L141M in hamster and mouse, L141V in brush-tailed possum, and L141I in human and gorilla are found without any linkage to TSEs which indicates that some degree of amino acid freedom at this position of the prion protein is allowed. However, the neighbouring polymorphism in goat PrP at codon 142 (Ile to Met) is associated with decreased susceptibility to experimental infections with scrapie and BSE (Goldmann *et al.*, 1996) suggesting that the loop between β-sheet 1 and α-helix 1 (Figure 4) is not completely unimportant.

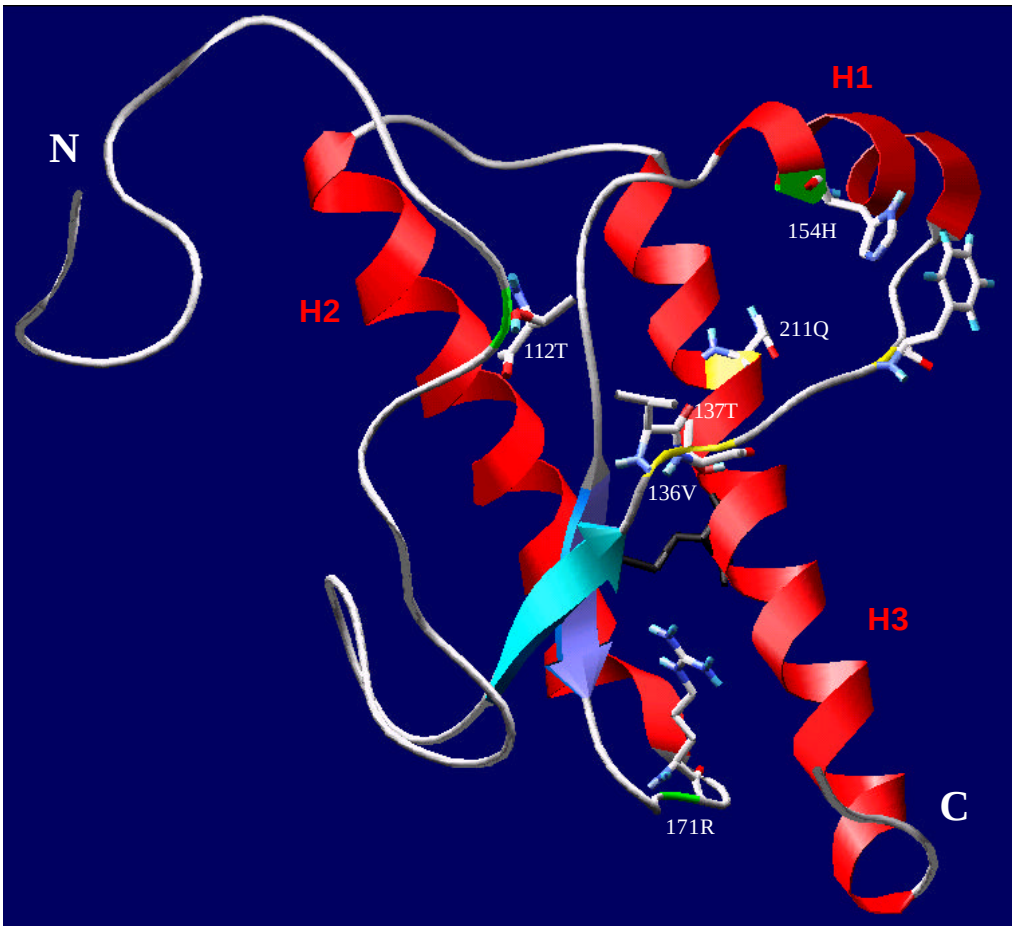

Figure 4. Three dimensional representation of all polymorphic residues in sheep prion protein. Sheep PrP fragment (amino acids 93-234) was modelled using SwissModel (Peitsch, 1996) and the three dimensional structures (PDB) of recombinant hamster PrP (2PrP) and mouse PrP (1AG2) as templates. The N-terminus (N), C-terminus (C), α-helices 1-3, and the separate polymorphic positions are indicated.

The observed low convertibility of the PrP^{C_154H} variant confirms previous speculations that this variant might also induce a certain degree of resistance to scrapie development. However, to our knowledge only one natural case of scrapie in a PrP^{AHQ}/PrP^{AHQ} sheep has so far been found in the UK in a flock where PrP^{ARQ} is associated with highest susceptibility (Dawson *et al.*, pers. comm.). This observation and the data of experimental challenge of sheep with the PrP^{AHQ} allele, show that this allele does not induce such an absolute resistance to TSEs as the PrP^{ARR} allele (Foster *et al*., 1996, Somerville *et al*., 1997). This seems to be consistent with the slightly increased *in vitro* conversion efficiencies of the PrP^{C_154H} variant compared to the PrP^{C_171R} variant *in vitro*. After *intracerebral* infections with CH1641-scrapie (predominant PrP^{Sc_wt}) or BSE of sheep with the PrP^{AHQ}/PrP^{AHQ} genotype, but not with the PrP^{ARR}/PrP^{ARR} genotype, developed scrapie with some unusual features (Somerville *et al*., 1997). The ten fold reduction in convertibility of PrP^{C_154H} compared to PrP^{C_136V} in the PrP^{Sc_136V} induced reactions and the five fold reduction in convertibility of this PrP^{C_154H} variant compared to PrP^{C_wt} in the PrP^{Sc_wt} induced reactions, suggests that *in vivo* the PrP^{AHQ} allele may induce relative more resistance in flocks were PrP^{VRQ} is associated with highest scrapie susceptibility rather than in flocks where PrP^{ARQ} is associated with highest scrapie susceptibility. The codon 154 polymorphism is located in α-helix 1 (Figure 4) and its charged side-chain may interfere with the potential nucleation-site between the two smaller β-sheets towards α-helix one. In addition, this polymorphism induces a comparable charge inversion as found in the resistant PrP^{C_171R} variant indicating that both polymorphisms may destabilise the hydrophobic core and/or the dipolar character of PrP by a similar mechanism.

The allelic variant PrP^{C_171H} has a reduced convertibility compared to PrP^{C_wt} in conversion reactions induced by all three PrP^{Sc} types (Figure 3a, b, and c bar 1 and 8). The codon 171 Gln to His polymorphism is mainly found in Texel and some smaller cross-breeds. Several scrapie sheep have been described with the PrP^{VRQ}/PrP^{ARH} genotype (Belt *et al*., 1996) and thus far only a single scrapie PrP^{ARH}/PrP^{ARH} sheep of the Dutch Zwartbles breed has been found (unpublished results). Based on the *in vitro* conversion data and observations that this allele in homozygous PrP^{ARH}/PrP^{ARH} setting is almost absent in sheep with scrapie, it might be that the PrP^{ARH} allele introduces a certain degree of resistance to scrapie at least in a homozygous setting, but has a more neutral role in heterozygous PrP^{ARH} genotypes.

The codon 211 polymorphism (Arg to Gln), located inside α-helix 3 (Figure 4), reduces convertibility compared to the PrP^{C_wt} variant. Polymorphisms at almost the same location in human PrP are all associated with spontaneous CJD or GSS (polymorphic codons 211 Arg to His, 213 Val to Ile, 215 Gln to Pro, or 220 Gln to Arg using sheep PrP

numbering) (Hsiao *et al*., 1992, Mastrianni *et al*., 1996, Ripoll *et al*., 1993, Young *et al*., 1998). Under the used cell-free conditions however, no detectable spontaneous PrP-res formation occurred in the PrP^{C_211Q} reaction (Figure 1 lane 18).Although only a single sheep with this $PrP^{ARQQ211}$ allele has been found (Belt *et al*., 1996), this polymorphism is found as a 'common' polymorphism in goats (Schatzl *et al.* unpublished results) and its introduction in sheep therefore might actually have its origin within goats. Since goats and sheep have identical 'wildtype' PrP sequences, it is predicted that this polymorphism is associated with reduced susceptibility in goats as well.

Only three different types of PrP^{Sc} could be isolated from sheep homozygous for PrP^{ARQ}, PrP^{VRQ} and PrP^{ARH} with natural scrapie. Since PrP^{Sc} could not be obtained of sheep homozygous for one of the other six PrP genotypes, no *in vitro* conversion data could be generated of these PrP^{Sc} types. Nevertheless, PrP^{Sc} from other homozygous types of PrP^{Sc} like PrP^{Sc_141F} or PrP^{Sc_154H} are of great interest. Conversion reactions using for instance PrP^{Sc_154H} may elucidate whether allelic variants linked to resistance remain inconvertible even when induced using homologous PrP^{Sc}. Such conversion reactions are important for the future justification of scrapie-control programs which introduce TSE resistant PrP genotypes. The set of nine conversion reactions induced by PrP^{Sc_171H} indicate that the most efficient reaction is not always the homologous one (Figure 3c).

Since only one scrapie sheep with the PrP^{ARH}/PrP^{ARH} genotype was found, limited PrP^{Sc} was available for *in vitro* conversions. Although the data set using PrP^{Sc_171H} is limited, some interesting observations could be made. The relative conversion efficiencies of the various PrP^{C} variants by PrP^{Sc_171H} reflected most of the features of reactions induced with the PrP^{Sc_wt} or PrP^{Sc_136V}. For instance, the reduced convertibility of the PrP^{C_154H} and PrP^{C_171R} variants, the reduced convertibility of the PrP^{C_112T} variant, the comparable character of PrP^{C_141F} and PrP^{C_wt}, and the overall high convertibility of the PrP^{C_wt} and PrP^{C_136V} variants. A rather unusual feature however, is the relative high convertibility of PrP^{C_wt}, PrP^{C_136V}, and to lesser extend PrP^{C_141F} compared to the homologous PrP^{C_171H} variant (Figure 3c bars 1, 3, 5, and 8). This PrP^{Sc_171H} variant seems to be the first ever described variant of PrP^{Sc} inducing higher conversion efficiencies in heterologous PrP^{C} variants rather than in homologous variants. This conversion set even more pronounces that some polymorphic PrP^{C} variants are more prone to convert than others, independent of PrP^{Sc} type.

Some conversion reactions showed a large variation in conversion efficiency between independent PrP^{Sc} isolates (Figure 3a bar 1, 3b bar 3 and 6). This variation might be linked to PrP^{Sc} isolate quality and/or it might be linked to the PrP allele which in that particular flock is associated with high scrapie susceptibility. For example, PrP^{Sc_wt} isolated

from PrP^{ARQ}/PrP^{ARQ} sheep residing in a flock of sheep in which the PrP^{VRQ} allele is associated with high scrapie susceptibility might be different from PrP^{Sc_wt} isolated from sheep in a flock in which the PrP^{ARQ} allele is associated with highest scrapie susceptibility. These structural differences between PrP^{Sc} isolates may cause shifted conversion profiles between the various PrP^{C} variants. For instance, PrP^{Sc_wt} from a PrP^{VRQ} background might convert PrP^{C_136V} relatively better and PrP^{C_wt} relatively less than PrP^{Sc_wt} from a PrP^{ARQ} background. This might indicate that, if scrapie develops in sheep which resides in a flock in which a different PrP allele is linked to highest risk of scrapie, the scrapie agent has overcome the polymorphism-barrier but may not be completely adapted yet to the PrP genotype of the new host and therewith the isolated PrP^{Sc} might still be carrying *"PrP^{VRQ} information"*. Adaptation effects in strains when transmitted to different species have been theoretically predicted by numerical integration to require at least two or three passages for stabilisation (Kellershohn & Laurent, 1998). Data of the isolation, transmission and characterisation of the CH1641-scrapie isolate, indicates that these adaptation effects may also occur *in vivo* (Foster & Dickinson, 1988, Goldmann *et al*., 1994). If these adaptation effects between the different PrP genotypes *in vivo* indeed occur, that even more stresses the importance to know the PrP alleles in the direct environment of sheep before assessing their risk for scrapie development.

All the different sheep PrP polymorphisms are located (except codon 211) in, or their side chains are directed to, the potential interaction site between PrP^{C} and PrP^{Sc} (Figure 4). This interface, which has been predicted to be located between the two smaller β-sheets towards α-helix one, might interact very specific in the process of 'self' recognition between PrP^{Sc} and PrP^{C} (PrP^{C_136V} is most efficiently converted by PrP^{Sc_136V} while PrP^{C_wt} is most efficiently converted by PrP^{Sc_wt}). The polymorphism at codon 136 (Ala or Val) which influences convertibility or polymorphisms like Pro to Leu in human PrP at codon 102 (Telling *et al*., 1996), may modulate the interaction ('recognition') between PrP^{C} and PrP^{Sc}. Such a recognition-site might be a potential location in PrP to interfere with PrP^{Sc} formation or TSE replication by targeting antibodies or synthetic peptides.

In summary, the current studies revealed a clear effect of different polymorphisms in sheep PrP^{C} and to a lesser extent in sheep PrP^{Sc} on the *in vitro* convertibility of PrP^{C} into protease-resistant isoforms. The conversion data indicate that sheep with the PrP^{VRQ}, PrP^{ARQ}, and the $PrP^{AF141RQ}$ alleles are at high risk for scrapie development. The alleles PrP^{AHQ}, PrP^{ARR}, and to minor extent $PrP^{T112ARQ}$ seem to be linked to resistance to scrapie. Therefore these alleles seem to be most suitable to introduce genetic resistance to scrapie by breeding strategies using positive selection for PrP^{ARR} and PrP^{AHQ} and negative

selection for PrP^{VRQ}, PrP^{ARQ}, and $PrP^{AF141RQ}$. It should be mentioned however, that *in vivo* other factors such as dose, strain of TSE, route of infection, and the efficiency of its delivery are likely to be important in determining scrapie susceptibility and transmissibility. Future studies including BSE, sheep passaged BSE or PrP^{Sc} of other species may reveal which animals are most prone to carry or become infected with a cross-species TSE infection.

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CHAPTER 7

Molecular Assessment of the Potential Transmissibilities of BSE and scrapie to humans.

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Abstract

More than a million cattle infected with bovine spongiform encephalopathy (BSE) may have entered the human food chain[1] and fears BSE might transmit to man were raised when atypical cases of Creutzfeldt-Jakob disease (CJD), a human transmissible spongiform encephalopathy (TSE), emerged in the UK[2,3]. In BSE and other TSE diseases, the conversion of the protease-sensitive, host prion protein (PrP-sen) to a protease-resistant isoform (PrP-res) is a key event in pathogenesis[4-7]. Important biological aspects of TSE diseases are reflected in the specificities of in vitro PrP conversion reactions[8-12]. Here we show a correlation between in vitro conversion efficiencies and known transmissibilities of BSE, sheep scrapie and CJD. On this basis, we used this in vitro system to gauge the potential transmissibility of scrapie and BSE to humans. We found limited conversion of human PrP-sen to PrP-res driven by both scrapie (PrP^{Sc})- and BSE (PrP^{BSE})-associated PrP-res. The efficiencies of these heterologous conversion reactions were similar but much lower than those of relevant homologous conversions. Thus the inherent ability of these BSE and scrapie infectious agents to affect humans following equivalent exposure may be finite but similarly low.

Methods

Purification and analysis of PrP-res.

PrP-res preparations were purified as described[21] and stored at 4 ^{o}C in 1%-sulphobetaine 3-14 in phosphate-buffered saline (PBS: 20mM sodium phosphate and 130mM NaCl, pH 7.4)[17]. Two or more independant isolates of each type of PrP-res were tested. Yields of PrP-res were assayed by immunoblotting using R505, a polyclonal antiserum raised against a peptide epitope which, N-terminal amino-acid apart, is common to all these species (sheep peptide 100-111; provided by Dr. J. Langeveld, Lelystad, The Netherlands)[22]: haPrP^{Sc} from Syrian golden hamsters infected with the 263K strain, ~ 50 ug/g; moPrP^{Sc} from VM/Dk mice infected with the 87V strain, ~ 10 ug/g; ov-PrP$^{Sc(VQ)}$ from natural scrapie cases in Cheviot sheep provided by Mr J. Foster, IAH-NPU, Edinburgh, UK), ~1-5 ug/g; PrP^{BSE} from brainstem of BSE-affected cattle (provided by MAFF VI Centre, Thirsk, UK), ~1 ug/g; PrP^{CJD} from cases of new variant or sporadic CJD (~1 ug/g).

Labelling, purification and analysis of ^{35}S-PrP-sen.

^{35}S-PrP-sen proteins were radiolabelled and purified using PrP-specific antisera as described[17] except that they were eluted from the protein A-Sepharose beads using 0.1M acetic acid at 22^{o}C for 30 min and stored at 4^{o}C. To prepare aglycosylated ^{35}S-PrP-sen, preincubation and labelling of cells were done in the presence of tunicamycin (2.5 ug/mL). Bovine adrenocortical cells (SBAC, ATCC CRL-1796) were used to obtain radiolabelled boPrP-sen which was immunoprecipitated using a rabbit polyclonal PrP antiserum (R35) raised against a synthetic peptide sequence (residues 93-107) of bovine PrP (provided by Dr R. Race, RML, Montana). Labelled huPrP-M and huPrP-V were produced from molecular clones expressed in human neuroblastoma cells and immunoprecipitated using the 3F4 monoclonal antibody (3F4-mAb)[23,24]. Endogenous moPrP-sen from mouse neuroblastoma (N2a) cells was harvested using R30 serum. Recombinant mo-3F4 PrP-sen[9] expressed in N2a cells was immunoprecipitated with the 3F4-mAb. ^{35}S-PrP-sen expressed from ov-PrP clones encoding three of the sheep PrP genotypes[11]were expressed in N2a cells and immunoprecipitated with R521, a sheep-specific polyclonal antiserum against sheep PrP peptide 94-105 provided by Dr J. Langeveld[22]. Hamster ^{35}S-PrP-sen proteins were immunoprecipitated from mouse fibroblast cells expressing recombinant haGPI- PrP-sen or the full, unmodified haPrP-sen[9].

Cell-free conversion reactions

To begin a conversion reaction[25], the PrP-res was sonicated, mixed with GdnHCl to a final concentration of 2.0M, 2.5M or 6M, typically in an 8 uL volume and incubated at 37^{o}C for 1-3hr. Approximately equal amounts of each ^{35}S-PrP-sen tested were lyophylised and reconstituted in conversion buffer (final composition in the conversion mix: 50 mM- sodium citrate, pH 6.0, 5 mM-cetyl pyridinium chloride, 1.25%-(w/v)-N-lauryl sarcosinate). Pre-treated PrP-res (in GdnHCl) and 8M-GdnHCl (to give a final concentration of 1 or 1.5M) were added into the ^{35}S-PrP-sen solution (final volume 16-24 uL) and incubated at 37^{o}C for 3 days. The amount of different PrP-res proteins used in any single experiment was kept constant at either ~0.5 or 0.8 ug. Using these amounts, the system was at or near saturation with PrP-res as the higher amount did not significantly increase the degree or efficiency of conversion (data not shown).

Following incubation, each reaction was split 1:10 and the major fraction digested with 100 ug/mL PK in Tris-saline (50 mM-TrisHCl, pH 8; 130 mM-NaCl) for 1 hour at 37^{o}C. Pefabloc (2 mM) and thyroglobulin (20 ug) were added to each fraction (+ or -PK) and the proteins precipitated by methanol. Per cent conversions were determined by quantitating the proportion of radioactivity within a specific Mr range in each fraction (+PK and -PK) by phosphor autoradiographic imager analysis of SDS-PAGE gels. Aglycosylated ^{35}S-PrP-sen was quantitated within a Mr range of ~24-26 kDa while the glycoforms produced in non-tunicamycin-treated cells were scanned over an Mr of ~24-38 kDa. PK-resistant products of conversions using aglycosylated ^{35}S-PrP-sen were quantitated within a Mr range of ~18-20 kDa while the variably glycosylated products of conversions using ^{35}S-PrP-sen labelled without tunicamycin were quantitated over a Mr range of ~18-30kDa. The significance of differences in percent conversion of PrP-sen/PrP-res pairs were calculated using the Mann-Whitney U-test.

Results and discussion

Experimental transmission of BSE to mice[13], cattle[14] and two sheep PrP genotypes [$A^{136}Q^{171}$ (ov-AQ) and $V^{136}Q^{171}$ (ov-VQ)] [15] has been reported but hamsters (Foster & Hope, unpublished) and at least one PrP genotype of sheep [$A^{136}R^{171}$ (ov-AR)][16] appear resistant to clinical disease. To test for a correlation between *in vitro* cell-free conversion and *in vivo* transmission, we included types of ^{35}S-PrP-sen from each susceptible or resistant group of species in PrP^{BSE} -driven conversion experiments. PK-resistant ^{35}S-labelled conversion products that were the characteristic 6-7 kDa smaller than the ^{35}S-PrP-sen substrate[8-12,17] were generated in the reactions with both glycosylated and aglycosyl ^{35}S-PrP-sen molecules from the BSE-susceptible, but not BSE- resistant, hosts (Fig 1). For example, the 25-kDa aglycosyl bovine and sheep (ov-AQ and ov-VQ) ^{35}S-PrP-sen proteins generated 18 kDa ^{35}S-PrP-res bands (Figure 1, lanes 1,6,7,13,15,16), while the aglycosyl hamster and ov-AR gave no PK-resistant product (Figure 1, lane 17,20,21). Thus, the efficiency of PrP^{BSE}-induced conversion of PrP-sen of a given host correlated with the *in vivo* transmissibility of BSE to that host. This correlation encouraged us to look for an *in vitro* indication of the transmissibility of BSE to humans using a PrP^{BSE}/huPrP-sen conversion assay.

Wild-type human PrP has two common allelic forms encoding either methionine (huPrP-M) or valine (huPrP-V) at codon 129[18]. Hence we tested both types of huPrP-sen in conversion experiments. PrP^{BSE} converted the ~25 kDa aglycosyl forms of ^{35}S-huPrP-M and ^{35}S-huPrP-V to ~18 kDa PK-resistant forms compatible with the PK-resistant core of PrP found in the human diseases (Figure 1, lanes 2,3, 4, 14; Figure 2, lane 8). When more glycosylated ^{35}S-huPrP-M was used, the PK-resistant conversion product was still largely restricted to an ~18 kDa band, suggesting preferential conversion of the 25 kDa aglycosylated form of ^{35}S-huPrP-M (Figure 1, lanes 9). Little or no spontaneous formation of PK-resistant PrP was observed in the absence of PrP-res (Figure 1, bottom panel). The ^{35}S-huPrP-V labels were converted by PrP^{BSE} ~3-fold less efficiently than the ^{35}S-huPrP-M labels (Figure 1, lanes 2-5, 9, 10; Fig. 2, 8,9; Fig 4; $p = 0.0025$). To date, new variant CJD has only been found in patients homozygous for methionine at codon 129[2] and, although it may be premature to speculate based on these conversion data, such genotypic selection may result from a more efficient conversion of huPrP-M than huPrP-V by PrP^{BSE}.

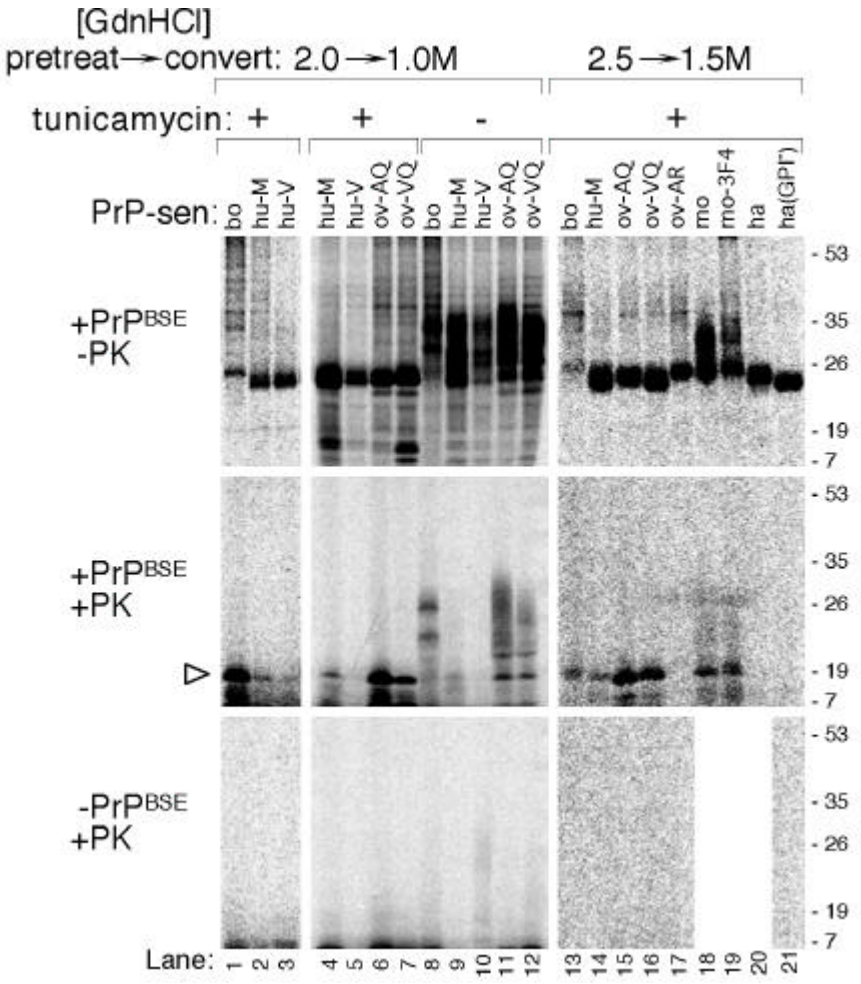

Figure 1. *Phosphor autoradiographic analysis of SDS-PAGE gels of cell-free conversion products formed on incubation of PrP^{BSE} with ^{35}S-PrP-sen from different species.* The molecular size range of ^{35}S-radiolabelled PrP proteins following incubation with PrP^{BSE} is seen before (-PK) or after (+PK) digestion with proteinase K in the top two panels of images (+PrP^{BSE}, -PK and +PrP^{BSE},+PK). The effect of omitting PrP^{BSE} from the incubation is shown in the bottom panels (-PrP^{BSE}, + PK). The migration of molecular weight markers are shown in kDa on the right. The ~18-kDa PK-resistant ^{35}S-PrP product is marked by the arrowhead. Equal amounts of PrP^{BSE} (~0.5 ug) were used in each reaction with an approximately equivalent amount of radioactive PrP-sen (~25-40 kcpm/reaction; apart from boPrP-sen reactions where ~10 kcpm were used due to inefficient labeling in the bovine cells). The reaction sample was divided for analysis ~1:10 (-PK:+PK) between the gels shown in the top two panels (and also between the top and bottom panels). Results are shown using differing concentrations of GdnHCl in the pretreatment and conversion phases of the reaction (see Methods). Conversions using ^{35}S-PrP-sen molecules labelled in the presence (+) or absence (-) of tunicamycin are shown. The relative intensities of bands in the +PrP^{BSE},+PK panel need not reflect the efficiency of conversion as the specific activities of each label are not always the same; the percentage of label converted to proteinase-K resistant fragments (summarized in Fig. 4) is a better indicator of this efficiency than absolute intensities of the PK-resistant bands in the +PK, +PrP^{BSE} panel.

To help gauge the efficiency of these various PrP^{BSE}-induced reactions, we compared these data with the proportion of labelled huPrP-sen (M or V) converted by a similar amount of human PrP^{CJD}. PrP^{CJD-M} isolated from cases of either new variant CJD (Figure 2, 4) or sporadic CJD (not shown) were used in these experiments: both preparations were from patients homozygous for wild-type PrP with methionine at codon 129. PrP^{CJD-M} converted the 25 kDa ^{35}S-huPrP-M and ^{35}S-huPrP-V lacking N-glycans to an ~18 kDa PK-resistant form compatible with the PK-resistant core of PrP^{CJD} (Fig 2, lanes 5,6). Using predominantly glycosylated forms of ^{35}S-huPrP-sen, we saw that the PrP^{CJD} again preferentially generated the same 18 kDa, presumably aglycosyl, conversion

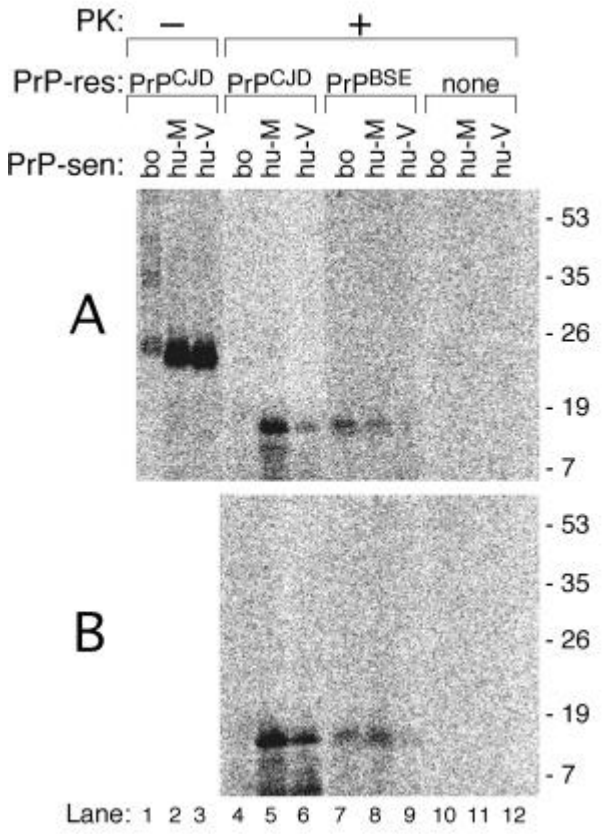

Figure 2. *Comparison of cell-free conversions induced by PrP^{BSE} and PrP^{CJD}.*
Bovine (bo) and human (hu-M, hu-V) ^{35}S-PrP-sen proteins were labelled in the presence of tunicamycin (A : - PK, left panel) and incubated either alone (none) or with equivalent amounts (~0.5 ug) of PrP^{BSE} or human PrP^{CJD-M} before processing with or without PK treatment. The conversion reaction samples were split 1:10 between the -PK and +PK gel lanes. Phosphor autoradiographic images are shown of the labelled proteins from conversions using different concentrations of GdnHCl in the pretreatment and conversion phases of the reaction (see Methods): A, pre-treatment in 2M-GdnHCl followed by conversion in 1M-GdnHCl; B, pre-treatment in 2.5M-GdnHCl, conversion in 1.5M-GdnHCl.

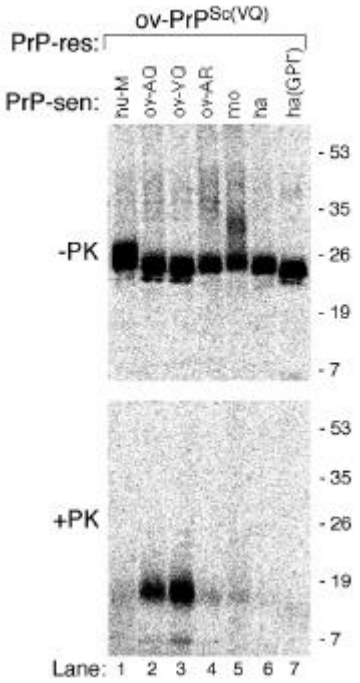

Figure 3. *Cell-free conversions induced by sheep PrP^{Sc}*
Human (hu-M), mouse (mo), hamster (ha and ha-GPI-) and three allelic forms of sheep (ov-AQ, ov-VQ and ov-AR) ^{35}S-PrP-sen proteins were labelled in the presence of tunicamycin (- PK). These aglycosyl PrP-sen molecules were incubated with equivalent amounts (~0.5 ug) of ov-$PrP^{Sc(VQ)}$ and processed as described in Methods. The reaction samples were split 1:10 (-PK:+PK) for analysis on the gels.

product, although some generation of the larger glycosylated conversion products was also observed (not shown). The ^{35}S-huPrP-M label was converted 3-fold more efficiently than ^{35}S-huPrP-V by PrP^{CJD-M} (Figure 2, lanes 5,6; Figure 4; P=0.0025), similar to what we observed in conversions with PrP^{BSE} (Figs 1 and 4).

The efficiency of cross-species conversion of huPrP-sen of either genotype by PrP^{BSE} was significantly less than the efficiencies observed in either of the homologous conversion reactions: huPrP-M or -V with PrP^{CJD-M} (P = 0.0005 or 0.005, respectively) or boPrP-sen with PrP^{BSE} (P = 0.0005) (Figure 1, lanes 1-5, 8-10, 13,14; Figure 2, lanes 5-9; Figure 4). On average, PrP^{BSE} converted boPrP-sen 30-fold more than huPrP-V (P=0.0005) and 10-fold greater than huPrP-M (P=0.0005). However, this low level of conversion was significantly higher than that seen on incubation of PrP^{BSE} with PrP-sen from hamsters (P= 0.005) or sheep homozygous for the AR genotype (P = 0.025) - hosts shown *in vivo* to be resistant to BSE (Figure 1, lanes 17,20,21). To put this into perspective, we evaluated the efficiency of conversion of huPrP with PrP^{Sc} from scrapie-infected sheep - a source of agent of no measurable risk to the human population.

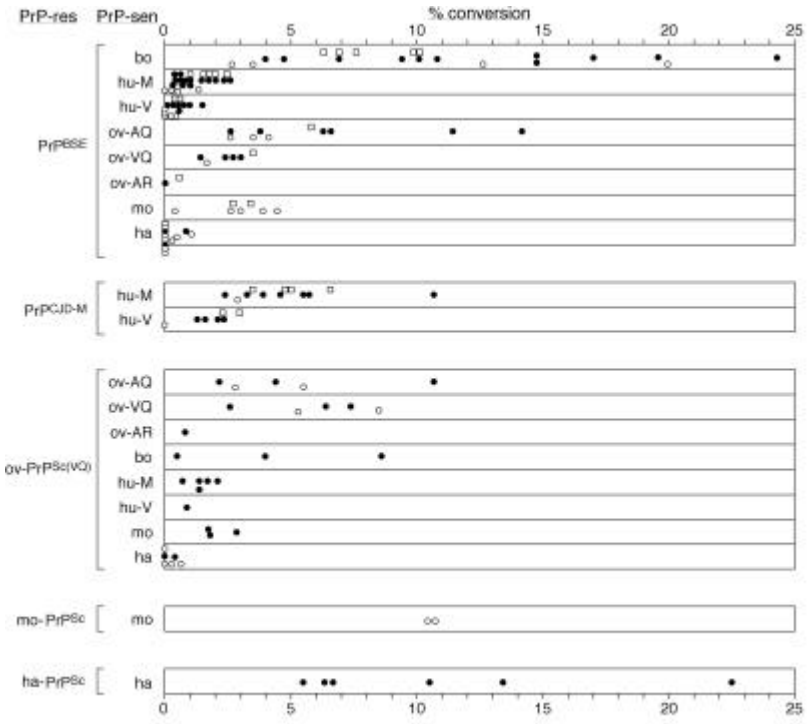

Figure 4a. *Percentages of ^{35}S-PrP-sen proteins converted to PK-resistant forms by incubation with PrP^{CJD}, PrP^{BSE}, sheep, mouse or hamster $PrP^{Sc.}$ Scatter graph of percentage conversion of PrP-sen to PK-resistant PrP forms in multiple, independent experiments.* Each symbol represents percent conversions observed in independent conversion reactions using the designated PrP-res incubated with glycosylated (open circles) or unglycosylated (filled circles) ^{35}S-PrP-sen with a pretreatment/conversion protocol of 2M -> 1M-GdnHCl or unglycosylated ^{35}S-PrP-sen with the 2.5M -> 1.5 M-GdnHCl protocol (squares).

In conversions driven by ov-$PrP^{Sc(VQ)}$, the conversion efficiency of ^{35}S-huPrP-M (Fig. 3, lane 1) ranked with that of mouse (lane 5) and sheep ov-AR (lane 4) proteins; no conversion of haPrP-sen was observed (lanes 6,7). In contrast, much higher conversion efficiencies were observed with the ov-VQ and ov-AQ PrP-sen alleles. Thus, the conversion efficiencies of the different aglycosyl ov-PrP-sen alleles by ov-$PrP^{Sc(VQ)}$ correlated with their sequence homology at codon 171 : ov-VQ, ov-AQ >>> ov-AR (Figure 3, 4)[11]. Interestingly, these relative conversion efficiencies correlated with the fact that while animals of VQ/VQ, AQ/VQ or AQ/AQ genotypes can develop natural scrapie (depending on breed) and can be experimentally induced to develop clinical scrapie (depending on agent strain) and BSE, animals with at least one copy of the AR allele appear to have enhanced survival following exposure to either natural or experimental disease[16]. Also, the lack of conversion of hamster PrP-sen by ov-$PrP^{Sc(VQ)}$ correlates with the lack of documented transmission of the sheep scrapie agent directly to hamsters (Fig 4).

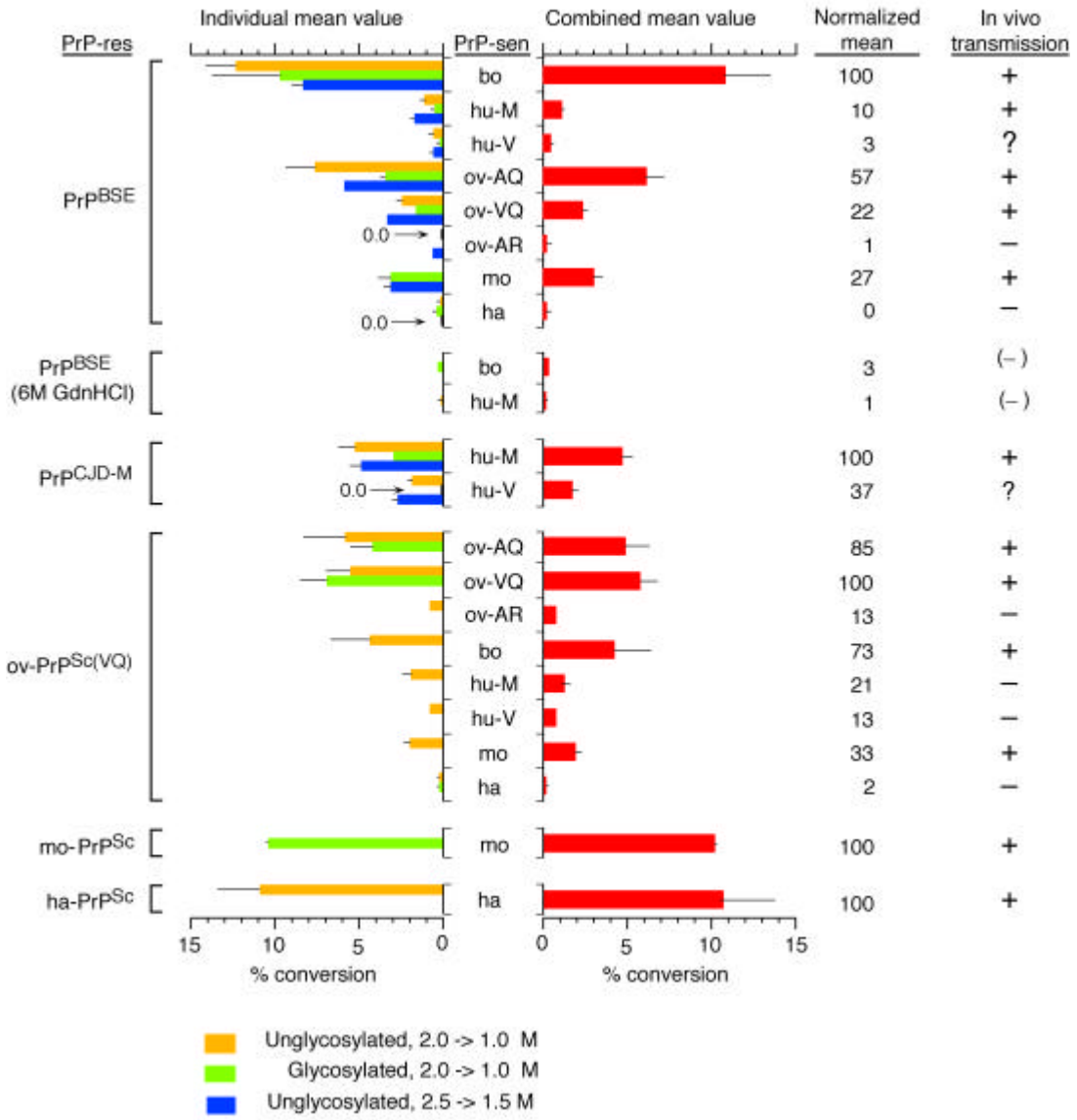

Figure 4b. *Bar graph of percentage conversion of PrP-sen to PK-resistant PrP forms.* The mean, with standard error of the mean (SEM) of per cent conversions for each PrP-res/PrP-sen pair is shown calculated for the two GdnHCl pretreatment/conversion protocols and types of PrP-sen differentiated in Figure 4a by the filled circles, open circles and squares (left scale) or summarised by combining the data from all experiments (right scale). The absence of a bar on the left means that no data were collected under those conditions except for the cases where the percent conversions were negligible; such cases are shown by 0.0. In addition to the results of Figure 4a, this graph also shows the negligible conversion produced using 6M-GdnHCl-pretreated PrP^{BSE}. Relative mean percentage conversions have been normalized against the mean of the homologous conversions induced by each type of PrP-res, with the latter set at 100%. Question marks acknowledge that it is likely, but not certain, that the human new variant CJD cases in huPrP-M129 homozygotes are due to BSE infections and that, although no new variant CJD cases have been reported in huPrP-V129 carriers, it is possible that all codon-129 genotypes may eventually be affected. Minus signs in brackets refer to data indicating that 6M GdnHCl treatment inactivates hamster scrapie infectivity[26], although this has not been demonstrated specifically for BSE infectivity.

In summary, the most efficient conversion reactions were observed between homologous PrP-sen and PrP-res molecules just as TSE transmissions are usually most efficient between hosts of homologous PrP genotype (Fig 4). In contrast, little or no conversion was observed with nonhomologous PrP combinations associated with a lack of transmission in vivo. Although overall conversion efficiencies varied and were influenced by the pretreatment and conversion GdnHCl concentrations, the optimal conversion conditions for each species fell within the range shown in Figs 1 and 2. Furthermore, variations of these conditions, the amount of PrP-res, or the glycosylation state of PrP-sen did not seem to affect the relative efficiencies of conversion of the various PrP-sen molecules (Fig 4). Comparison of the amino-acid sequences of human, sheep, bovine and other PrP molecules strongly implicated the loop structures of the protein[19] as critical regions affecting the conversion of PrP-sen to PrP-res and for maintenance of at least part of the barrier to transmission of TSE's between species.

This study provides further evidence for a correlation between the efficiency of *in vitro* conversions and the transmissibilities of TSE diseases. It extends the basis for using the cell-free conversion reaction as an *in vitro* indicator of the transmission barrier to novel species. We must emphasize that the cell-free assay is a test only of the molecular compatibility between PrP-res and PrP-sen of different sequences and other factors such as the dose, strain and route of infection, the stability of the infectivity in the host, and the efficiency of its delivery to the central nervous system are all likely to be critical factors *in vivo*. Nonetheless, some degree of molecular compatibility is likely to be essential in the transmission of TSE diseases especially given the remarkable correlation between PrP conversion efficiencies and transmissibilities (Fig 4). The conversion experiments with ^{35}S-huPrP-sen showed that the relative converting activity of PrP-res depends on its species of origin : $PrP^{CJD\text{-}M} > PrP^{Sc}$, PrP^{BSE}. The relatively low efficiency of conversion by PrP^{Sc} and PrP^{BSE} may explain at a molecular level the observed failure to transmit BSE to transgenic mice expressing human PrP[20] and the lack of an epidemiological link of CJD to sheep scrapie. Though it is premature to draw firm conclusions from these experiments on the likelihood of BSE passing to humans, the results suggest that BSE would be no more inherently transmissible to humans than sheep scrapie.

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CHAPTER 8

Summary and General Discussion

Summary and General Discussion

At the time this research started, it was known that polymorphisms in the PrP gene can have an effect on the manifestation of prion diseases. In addition there were indications that the susceptibility of hosts for prion diseases is, at least for a part, genetically determined. It was even hypothesised that scrapie in sheep was a pure genetic disorder. The polymorphisms in PrP involved in the genetically determined susceptibility for prion disease were, however, not well determined. Furthermore, the molecular mechanisms underlying the differences in the susceptibility of hosts for prion diseases were not completely understood. With regard to the transmissibility of prion diseases it was known that transmissions between hosts of the same species were in general more efficient than transmissions between hosts of different species. There were also indications that PrP plays a role in determining this 'species-barrier'. However, the PrP amino-acid residues involved in this process were not yet identified. The mechanisms underlying the differences in transmissibility were also poorly understood. This knowledge was, however, urgently needed a) to understand the pathogenic mechanism of these unconventional diseases, and b) to assess the risks of these diseases for animal and public health. Finally for these diseases, rapid and reliable methods were necessary to assess these risks of susceptibility and transmissibility (or the level of the species barrier).

The research described in this thesis was carried out in order to find answers to the above questions, to understand the (molecular) mechanisms involved in the transmissibility of prion diseases, and in order to determine the susceptibility of hosts for these diseases. Furthermore, the research described in this thesis:

1) contributed to the development of a scrapie control program for the sheep population not necessarily restricted to The Netherlands by increasing the frequency of PrP alleles associated with resistance or decreased susceptibility for scrapie;
2) provided the scientific justification of such scrapie control programs;
3) resulted in the development of a rapid and reliable *in vitro* system (cell-free) to assess, at the molecular level, the susceptibility of hosts for prion diseases and to assess the transmissibility of prion diseases among and between species;
4) contributed to an assessment of the susceptibility of sheep for scrapie and the potential transmissibility of BSE and scrapie to other species/humans;
5) provided insight in structurally important amino acid residues for determining prion transmissibility and susceptibility, which may prove useful for the development of inhibition strategies of TSE replication.

First experiments were set up to identify and characterise polymorphisms in the sheep PrP gene. Next we tried to find and associate these polymorphisms with susceptibility to natural scrapie and with differences in survival times of scrapie infected sheep. In addition we investigated whether some of the polymorphisms in PrP (allelic variants) could be associated with spontaneous or genetic forms of scrapie development. Subsequently we tried to understand the molecular mechanisms by which these polymorphisms modulate scrapie susceptibility and transmissibility. For this purpose an *in vitro* (cell-free) system for the conversion of normal prion protein into abnormal isoforms of PrP was used. Finally, this cell-free system was exploited to:

1) predict the linkage between nine sheep PrP alleles and the susceptibility for scrapie;
2) to gauge the transmissibilities of BSE and scrapie to other species, including human;
3) to determine the effects of amino acid changes in prion protein on the conversion efficiency of normal prion protein into abnormal isoforms.

For determining polymorphisms in the sheep PrP gene tools like DGGE and ASA were developed to 'routinely' screen sheep for PrP allelic variants. The DGGE technique also allowed the identification of hitherto unidentified polymorphisms. Case-control and ‘within flock’ studies demonstrated the association of certain polymorphisms in sheep PrP with susceptibility for natural scrapie and differences in survival times of sheep affected with scrapie. PrP genotyping studies in a country free from scrapie showed that presence of scrapie agent is a prerequisite for scrapie development, even in sheep with PrP genotypes associated with a high susceptibility to scrapie. To study the effects of specific polymorphisms in PrP on the cell-free conversion efficiencies of normal PrP into abnormal isoforms we developed:

1) techniques to express PrP *in vitro* and purify polymorphic variants of sheep PrP^{C};
2) techniques for PrP^{Sc} isolation and characterisation;
3) techniques and methods for determination of PrP conversion efficiencies *in vitro*.

The research described in this thesis has lead to a better understanding of the phenotypic expression of certain polymorphisms in the sheep PrP gene. Based on *in vivo* and *in vitro* data, at least three sheep PrP allelic variants could be associated with high scrapie susceptibility and short survival times. Another three PrP allelic variants could be associated, on basis of these data, with reduced susceptibility or even resistance to scrapie. Initially these findings had a direct spin-off to smaller breeding advice programs in which we tried to reduce the frequency of the allelic variant of PrP associated with highest scrapie susceptibility and to increase the frequency of the PrP allelic variant associated with

resistance. Subsequently, a similar approach was applied in a national-wide scrapie control program.

The *in vitro* studies described in this thesis show that natural occurring polymorphisms in the sheep PrP gene have a direct effect on the ability of PrP^{C} to become converted into protease resistant forms of PrP by incubation with exogenous PrP^{Sc}. Apparently this difference in the convertibility of PrP^{C} variants is an important molecular mechanism involved in susceptibility and transmissibility. Furthermore, we were able to use the *in vitro* conversion system to assess the relative scrapie susceptibility of sheep with PrP variants which are rare or which previously couldn't be significantly associated with scrapie susceptibility *in vivo*. These results provided a further justification for the current strategy in scrapie control programs by selected breeding. Finally, we assessed, in a large international collaborative study, the potential transmissibilities of BSE and sheep scrapie to humans on a molecular level.

The results of the *in vitro* studies described here underline the biological relevance of the cell-free conversion reaction. Therefore the system could be used, and can be used in the future, to predict *in vivo* displayed phenotypes of TSEs like susceptibility, transmissibility, and differences in survival times. The system will enable to find and to study other parameters important in TSE transmission within and between species. Without such a cell-free system these parameters could otherwise only be measured by time consuming, expensive, and animal unfriendly experimental transmissions. These studies helped to get insight into the molecular mechanisms involved in TSE susceptibility and transmissibility. This knowledge will certainly stimulate other investigations on TSE polymorphism- or species-barriers and eventually the development of therapeutic strategies interfering with the conversion of prion protein, i.e. TSE replication.

The next paragraphs summarise and discuss the studies as described in the various chapters (2-7) thereby trying to avoid repetition of already extensively discussed topics in each chapter. At the end a brief description of ongoing and future studies is given. Where applicable, some important topics for future research are highlighted.

Chapter 2, *PrP allelic variants associated with natural scrapie.* This study was initiated to find an association between allelic variants of sheep PrP and the susceptibility for natural scrapie. The study was a case-control study of 'age matched' sheep (2-5 years of age) mainly from the Texel breed. The samples were collected throughout The Netherlands from 15 scrapie-free flocks (no clinical scrapie diagnosed for the last five years) and from scrapie-affected flocks (scrapie positive sheep from 18 flocks and healthy sheep from 13 flocks). PrP genotyping revealed a significant association of the PrP^{VRQ} allelic variant

(polymorphic codon positions 136, 154, and 171 are indicated) with increased scrapie susceptibility. The PrP^{ARR} allelic variant was significantly associated with resistance against scrapie. All scrapie affected sheep had at least one PrP^{VRQ} allele or had the homozygous PrP^{ARQ}/PrP^{ARQ} genotype. The frequency of the PrP^{VRQ} allele was lower in the healthy sheep from scrapie free flocks than in healthy sheep from scrapie-affected flocks. Sheep with the PrP^{ARR} allele were all healthy, except for one. The one scrapie positive PrP^{ARR} carrier had the PrP^{VRQ}/PrP^{ARR} genotype but the majority of sheep having the PrP^{VRQ}/PrP^{ARR} genotype were healthy. Healthy sheep from scrapie affected flocks also had a higher frequency of this codominant PrP^{VRQ}/PrP^{ARR} genotype compared to healthy sheep from scrapie free flocks. The frequency of alleles PrP^{AHQ}, PrP^{ARH}, and $PrP^{ARQQ211}$ were too low to show a significant association with scrapie susceptibility. However, it was concluded that the PrP^{ARH} allele is likely to have a neutral effect on scrapie susceptibility while the PrP^{AHQ} allele seems to induce a certain degree of resistance. Scrapie occurrence in seven other breeds than the Texel breed revealed a similar association of PrP^{VRQ} with increased susceptibility for scrapie indicating that this study has implications not only restricted to the Texel breed. Current sheep breeding programs in different European countries will demonstrate whether a decrease in the PrP^{VRQ} allele frequency and for instance an increase in the PrP^{ARR} allele frequency will lead to a reduced scrapie incidence. In this context it should be noticed that in theory one day a specific strain of scrapie might evolve which can cause scrapie in sheep with ‘resistant’ PrP genotypes. The gaps in our knowledge with regard to the nature of the TSE agent and the mechanism by which strain specific properties are inherited, further contribute to this uncertainty. To gauge the risks of the evolution of such 'new' scrapie strains, a better understanding of the molecular mechanisms involved in TSE susceptibility and transmissibility is necessary.

Chapter 3, *PrP genotype contributes to determining survival times of sheep with natural scrapie.* This study was initiated to evaluate whether specific PrP alleles or PrP genotypes contribute to differences in survival times of sheep with natural scrapie. Therefore we analysed scrapie incidence and PrP genotypes in a flock suffering several subsequent years from scrapie. In the observation period the shortest recorded survival times declined over several years until a steady level of about 2 years of age. From this flock a representative group of sheep were analysed which were all born in the same year (1990). The sheep in this group were expected to be exposed to a similar dose of scrapie agent at equivalent times. Several PrP genotypes could be associated with differences in survival times and all scrapie affected animals had at least one PrP^{VRQ} allele. While PrP^{VRQ}/PrP^{VRQ} sheep were at greatest risk, they also had the shortest survival times of about

2 years of age. Sheep heterozygous for PrP^{VRQ} had extended survival times dependent on the second PrP allele. If the second allele was the wildtype PrP^{ARQ} allele or $PrP^{AF141RQ}$ the survival times increased to 35 or 59 months respectively, while sheep having PrP^{AHQ} or PrP^{ARR} as the second allele did not develop scrapie. Sheep carrying the PrP^{ARR} allele were found to be resistant (or the incubation time exceeded their normal life span).

This within flock study revealed the clear association of particular PrP genotypes with scrapie susceptibility and with differences in survival times. In this flock the modulating effect of polymorphisms in PrP on scrapie development was supposed to be determined by the nature of the second allele in a heterozygous setting next to the PrP^{VRQ} allele. In breeds or flocks were the PrP^{VRQ} is absent or rare and scrapie is associated with the PrP^{ARQ} allele, the same situation is expected to occur for alleles in heterozygous settings next to the PrP^{ARQ} allele. In classical breeding strategies, where rams from family lines suffering from clinical scrapie are excluded for breeding, healthy sheep with a PrP^{VRQ}/PrP^{ARR} genotype may be inadvertently selected for breeding purposes. Such rams are responsible for the continuous supply of susceptible PrP^{VRQ} alleles to the flock. This is the most probable explanation for the long lasting scrapie problem in the flock under study. Subsequent breeding advice within this flock showed a shift in PrP allele frequency from high PrP^{VRQ} and high PrP^{ARR} to a lower frequency of PrP^{VRQ} and an even higher frequency of PrP^{ARR}. The scrapie incidence in this flock now is declining which advocates the nation-wide implementation of such control programs (work in progress; personal observations). For an efficient execution of the scrapie-control program, our laboratory developed a high throughput PrP genotyping technique for large scale PrP genotyping on blood samples of rams selected for breeding (Harders *et al.*, unpublished results).

Chapter 4, *PrP genotype frequencies of the most dominant sheep breed in a country free from scrapie.* Since it has been debated that scrapie might be solely a genetic disease arising spontaneously from sheep with certain PrP alleles in the absence of any infectious agent (Parry *et al.* 1962; Ridley and Baker, 1996), PrP genotyping studies were performed in a country free from scrapie. From New Zealand we genotyped 140 sheep from 14 different flocks of the Romney Marsh breed. The Romney Marsh breed is the most dominant sheep breed of New Zealand (about 75% of the sheep are from the Romney Marsh or thereof derived breeds like Perendale and Coopworth). Alleles associated with high susceptibility to scrapie, PrP^{VRQ} and to a lesser extent PrP^{ARQ}, were present. The frequency of PrP^{VRQ} was low (2.5% of the total alleles) and no homozygous PrP^{VRQ}/PrP^{VRQ} sheep were found. This was not surprising since the expected frequency of this genotype is only 0.06% (one per 1666 animals). However, the PrP^{VRQ}/PrP^{ARQ} genotype was found

(almost 3%) and these sheep would normally also succumb to natural scrapie in a scrapie endemic environment.

This study indicated that the measures as taken by New Zealand may keep a country free from endemic scrapie. The chosen 'scrapie-free' strategies, which are not based on breeding strategies, are manageable in isolated regions like New Zealand but probably not in the mainland of Europe. Therefore breeding strategies seem to be preferred for scrapie-control programs in the European countries. Although PrP genotypes associated with high scrapie susceptibility are present in sheep from New Zealand, the possibility exists that other genetic factors in these sheep prevent development of natural scrapie. This hypothesis can easily be challenged by the import of young sheep with a particular PrP genotype from New Zealand into a scrapie endemic environment and study whether they develop natural scrapie. In addition these sheep may be challenged experimentally with scrapie. Such experiments are currently in progress in the UK.

Chapter 5, *Scrapie susceptibility-linked polymorphisms modulate the in vitro conversion of sheep prion protein to protease-resistant forms.* This study was initiated to explore whether sheep prion proteins could be converted into protease-resistant isoforms by other means than *in vivo* infection with TSE agent. Secondly, we studied whether the PrP allelic variants, linked to differences in scrapie susceptibility and survival times, have different *in vitro* conversion characteristics. We readily demonstrated the formation of protease-resistant isoforms of sheep PrP using a cell-free system. Subsequently we analysed the efficiency by which three different PrP^{C} variants of sheep were converted into protease-resistant forms after incubation with PrP^{Sc}. We found that polymorphisms in either PrP^{C} or PrP^{Sc} had dramatic effects on the cell-free conversion efficiencies. These efficiencies correlated well with the known association with natural scrapie susceptibility. PrP^{C_VRQ} was efficiently converted into protease-resistant forms, PrP^{C_ARQ} was intermediately converted into protease-resistant PrP, while PrP^{C_ARR} was almost not converted into protease-resistant PrP. Polymorphisms in PrP^{Sc} had an additional effect on the convertibility of the various PrP^{C} variants. PrP^{Sc_VRQ} converted PrP^{C_VRQ} most efficient, PrP^{C_ARQ} intermediate, and PrP^{C_ARR} almost not. PrP^{Sc_ARQ} converted PrP^{C_ARQ} best, PrP^{C_VRQ} somewhat less, and PrP^{C_ARR} almost not. These observations give an explanation for the linkage between polymorphisms in PrP and the differences in scrapie susceptibility. It is likely that after an TSE infection some PrP^{C} allelic variants are more prone to be converted into protease-resistant isoforms by PrP^{Sc} *in vivo*, comparable to what has been observed *in vitro*. We concluded that besides the species-barrier, polymorphism-barriers play a significant role in

determining the susceptibility of hosts for TSEs and the transmissibility of TSEs to other individuals.

The studies described in this chapter indicate that scrapie susceptibility and transmissibility is largely 'encrypted' in the amino acid sequence of PrP^{C} and PrP^{Sc} itself. It is not yet known however, whether specific strains of natural scrapie prefer particular sheep PrP genotypes or whether the strain specific properties have an effect on the efficiency of the interaction between PrP^{C} and PrP^{Sc} and the subsequent conversion of PrP^{C} into protease-resistant isoforms. Therefore it is necessary to understand the direct molecular interactions between different variants of prion proteins and PrP^{Sc} associated with different scrapie strains. The cell-free system seems to be a suitable experimental tool to study these

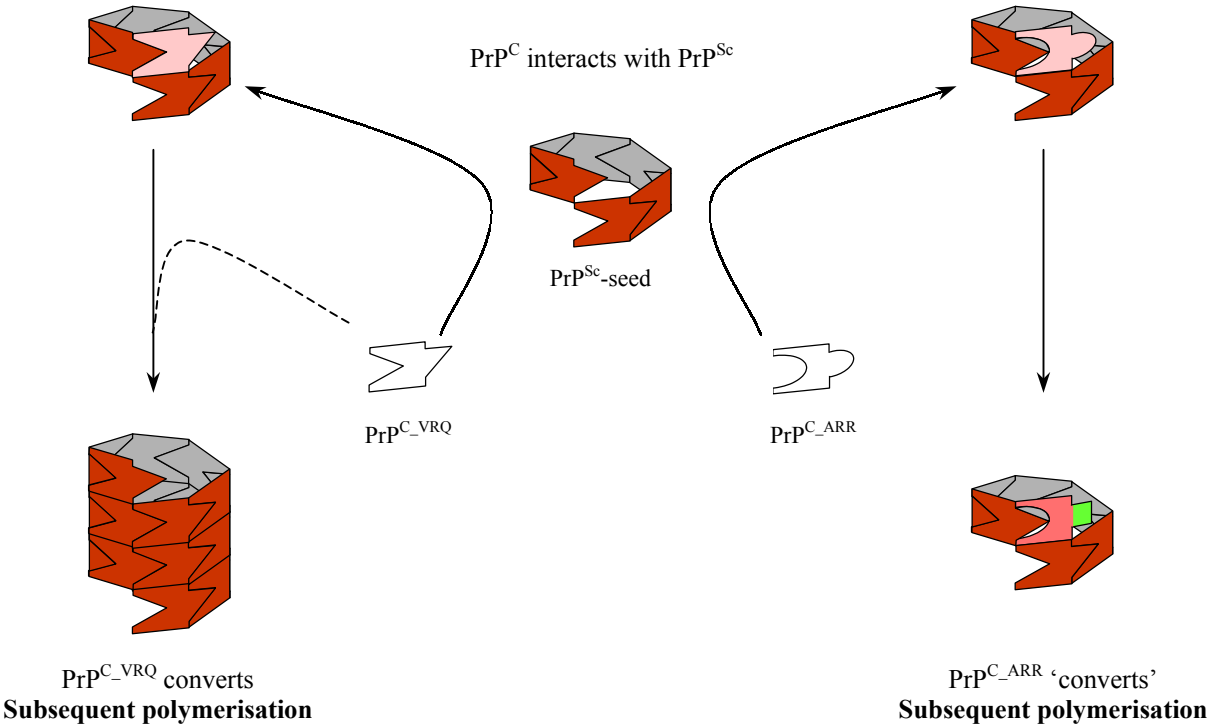

Figure 1 (hypothetical). The protease-resistant PrP^{C_ARR} molecule which is associated with resistance to scrapie, binds to the PrP^{Sc} seed ('infectious particle') and is subsequently 'converted' (to a certain degree, if at all) into protease-resistant forms. The bound (not necessarily converted) PrP^{C_ARR} molecule subsequently blocks the resulting nucleation-site and thereby inhibits the further 'replication' of TSE agent. In contrast, PrP^{C_VRQ} readily binds and converts allowing the subsequent polymerisation (replication).

interactions. Such an experimental approach may, for instance, also be used to: a) determine whether the resistant effect of a polymorphism is caused by a disturbed interaction between PrP^{C} and PrP^{Sc} (affinity switch), b) whether the mutant protein itself is difficult to convert from PrP^{C} into the PrP^{Sc} isoform, or c) whether the resistant protein converts from PrP^{C} into

'PrP^{Sc}' but thereafter blocks the resultant nucleation site which is necessary for subsequent polymerisation and replication of the TSE agent (Figure 1).

Studies measuring the affinity between particular PrP^{C} variants and PrP^{Sc} types, by for instance surface plasmon resonance (SPR) technology or capillary electrophoresis, may elucidate whether specific polymorphisms disturb this protein-protein interaction or whether the polymorphisms disturb the actual conversion of PrP^{C} into PrP^{Sc}. Natural or artificial PrP^{C} variants that are resistant to conversion or block further prion replication may provide the basis for the development of therapeutics for prion diseases.

Chapter 6, *Susceptibility of sheep for scrapie as assessed by in vitro conversion of nine natural occurring variants of PrP.* Based on the study described in chapter 5, we tried to further explore the effects of eight natural occurring polymorphisms in sheep PrP on the susceptibility of hosts and the transmissibility of scrapie between sheep by studying their cell-free conversion efficiencies. None of the nine PrP^{C} allelic variants showed detectable spontaneous protease-resistant PrP formation under the used cell-free conditions. All nine PrP^{C} variants had distinct convertibilities induced by PrP^{Sc} isolated from sheep with three different PrP genotypes. From these three different PrP^{Sc} sets a consensus was created in which the rare allelic variants $PrP^{AT137RQ}$ and $PrP^{ARQQ211}$ were excluded (of these two allelic variants only a single sheep has been found). In this consensus: PrP^{C_136V}/PrP^{C_wt}/PrP^{C_141F} >> PrP^{C_171H} > PrP^{C_112T} >> PrP^{C_154H} > PrP^{C_171R}, the variants are predicted to be linked to a descending susceptibility of sheep for scrapie.

For introducing genetically encoded TSE resistance in a particular flock or country, the PrP^{ARR} and PrP^{AHQ} alleles seem to be suitable for positive selection, while the variants PrP^{VRQ}, PrP^{ARQ}, and $PrP^{AF141RQ}$ seem suitable for negative selection. The presence of several PrP variants in the same flocks, which are linked to resistance, may contribute to maintain a more genetically diverse sheep population. In addition, such PrP genetically diverse populations (having both PrP^{ARR} and PrP^{AHQ} alleles) may also prevent future problems which might occur from a homogenous PrP^{ARR} sheep population. For example, a potentially to PrP^{ARR} adapting TSE strain has to overcome high polymorphism barriers when transmitted between PrP^{AHQ} and PrP^{ARR} sheep. The use of two PrP variants in the same flock, which are linked to resistance, may turn out to be more safe in scrapie-control programs by breeding strategies.

Some conversion reactions showed a large variation in conversion efficiency between independent PrP^{Sc} isolates (i.e. PrP^{C_136V} and PrP^{C_wt} induced by either PrP^{Sc_136V} or PrP^{Sc_wt}). This variation might be linked to PrP^{Sc} isolate quality and/or it might be linked to the PrP allele which in that particular flock is associated with high scrapie susceptibility.

For example, PrP^{Sc_wt} isolated from PrP^{ARQ}/PrP^{ARQ} sheep residing in a flock of sheep in which the PrP^{VRQ} allele is associated with high scrapie susceptibility might be different from PrP^{Sc_wt} isolated from sheep in a flock in which the PrP^{ARQ} allele is associated with highest scrapie susceptibility. These structural differences between PrP^{Sc} isolates may cause shifted conversion profiles between the various PrP^{C} variants. For instance, PrP^{Sc_wt} from a PrP^{VRQ} background might convert PrP^{C_136V} relatively better and PrP^{C_wt} relatively less than PrP^{Sc_wt} from a PrP^{ARQ} background. These observations bring up the following hypothesis: *if scrapie develops in sheep with a PrP genotype different from the PrP genotype linked to scrapie in that flock, the scrapie agent has overcome the polymorphism-barrier but may not be completely adapted yet to the (PrP genotype of the) new host and therewith the isolated* PrP^{Sc} *might still carry "*PrP^{VRQ} *information".* Adaptation effects in strains when transmitted to different species have been theoretically predicted by numerical integration (Kellershohn and Laurent, 1998). Data of the isolation, transmission and characterisation of the CH1641-scrapie isolate (predominant PrP^{Sc_wt}), indicates that these adaptation effects indeed may occur *in vivo* (Foster and Dickinson, 1988; Goldmann *et al.,* 1994). In these transmissions the primary CH1641 isolate, isolated from a 'positive line' (mainly PrP^{VRQ} carrying) NPU Cheviot sheep, showed that *intra cerebral* passage of this 'positive-line' material to 'negative-line' Cheviot sheep (non-PrP^{VRQ}) resulted in long incubation periods (Foster and Dickinson, 1988) due to the polymorphism-barrier. If subsequently these 'negative-line' passaged isolates was serial passaged in 'negative-line' Cheviot sheep, the incubation times in this line of sheep decreased in two serial passages and stabilised (Goldmann *et al.*,1994; Foster and Dickinson, 1988).

If these adaptation effects between the different PrP genotypes *in vivo* indeed occur, that stresses even more the importance to know the PrP alleles in the direct environment of sheep before assessing the risk for particular sheep to develop scrapie. *In vitro* conversions using PrP^{Sc_wt} of PrP^{ARQ}/PrP^{ARQ} scrapie sheep residing in flocks in which the PrP^{VRQ} allele is primarily associated with highest scrapie susceptibility or using PrP^{Sc_wt} from sheep residing in flocks in which PrP^{ARQ} is primarily associated with highest scrapie susceptibility, may elucidate whether *in vivo* such adaptations take place and in which order of magnitude.

Chapter 7, *Molecular assessment of the potential transmissibilities of BSE and scrapie to humans.* This chapter describes our efforts to assess on a molecular level the potential transmissibilities of BSE and scrapie to humans. In a large international collaborative study, we therefore compared the relative convertibility of two different human PrP^{C} variants by BSE and scrapie, the latter being normally at no detectable risk to

humans. First we demonstrated that the cell-free system displayed conversion properties that reflected the known transmissibilities of BSE, scrapie and CJD *in vivo*. Therefore the conversion efficiencies of various PrPs of different species, induced by different PrP^{Sc} isolates (PrP^{TSE}) were compared with their known transmission efficiencies *in vivo*.

The results revealed a clear correlation between conversion efficiencies and *in vivo* susceptibility and transmissibility. Expected effects of polymorphisms in for instance human PrP^{C} (codon 129 Met results in shorter incubation times while 129 Val results in longer incubation times) and sheep PrP (codon 136 (Ala or Val) and 171 (Arg or Gln)) were observed. Conversion experiments using BSE or scrapie PrP^{Sc} and human PrP^{C} showed that the convertibility of human PrP^{C} by either BSE or sheep scrapie was finite but similarly low. These results were consistent with experiments in which transgenic mice carrying human PrP transgenes were inoculated with BSE (Collinge *et al.* 1995). After BSE inoculation these transgenic mice developed TSE after 500 days. Inoculation with CJD however, resulted in short incubation periods of about 196 days. Although the transgenic mice carried the human PrP^{C} transgene (codon 129 Val) linked to longer incubation periods, these experiments showed a clear but not absolute species-barrier for transmission of BSE to humans. Unfortunately these results were not validated by using sheep scrapie, which is at no detectable risk for humans, as a negative control. Several studies (Will *et al.* 1996; Bruce *et al.* 1997; Collinge *et al.* 1996) have indicated now, that the agents for BSE and human nvCJD posses similar properties which indicates a common source for these diseases. The species barrier between cattle and human as measured *in vitro* and in transgenic mice seems to be rather high but finite. The *in vitro* conversions also suggest that human PrP^{C} can be converted by PrP^{Sc} from sheep in the same order of magnitude as BSE. Based on epidemiological observations however no linkage between scrapie and human TSEs has been demonstrated. It will be interesting to find out whether transgenic mice carrying human PrP transgenes are susceptible for infection with sheep scrapie and whether the transmission efficiency is at equal level with BSE. In this regard it is noteworthy to mention that some cases of CJD have been described that may be linked to hormone or vaccine (rabies) preparations derived from scrapie infected sheep brains (Arya, 1991a, 1991b, 1996; Hill and Arya, 1990). Perhaps we should reconsider whether the epidemiological assumption that sheep scrapie is being of no harm to humans is correct.

Ongoing and future studies. While finishing the manuscript of this thesis, several other studies are in progress or are nearly completed. An example of such an ongoing study is the molecular assessment of the susceptibility of pigs for prion diseases like BSE and sheep scrapie. This study was initiated because it has been demonstrated that pigs can be infected with BSE intracerebrally, although with a low efficiency (Dawson *et al.* 1990). This study was also initiated because in several countries pigs are still legally fed with meat-and-bonemeal including the remains of cattle and sheep. We therefore tried to assess the transmissibilities of BSE and sheep scrapie to pigs, using a similar *in vitro* approach as described for humans in chapter 7. We isolated and characterised the PrP-ORFs of pigs of several different breeds. All different PrP-ORFs were subsequently transformed into eukaryotic cells. With the aid of antibodies, immunoreactive with swine PrP^{C}, we were able to isolate radiolabelled PrP^{C} from transformed eukaryotic cells. With these PrP^{C} preparations we are now in the process of performing series of *in vitro* conversion experiments. A major drawback in the interpretation of the results of these experiments, is the unavailability of positive control PrP^{Sc} preparations, i.e. PrP^{Sc} derived from pigs suffering from TSE. Since experimental infections of pigs with BSE are carried out again on at least two different locations in Europe, it should be possible to get access to this material in the near future (Rogers *et al.*, personal communication).

Secondly, more fundamental studies have been undertaken to better understand the modulating effects of certain amino acid polymorphisms on the conversion characteristics of prion proteins. These studies might reveal PrP^{C} variants extremely resistant to conversion, variants with lowered or increased affinity for PrP^{Sc}, or variants that very easily convert from PrP^{C} into protease-resistant isoforms. Such variants are useful for a better understanding of the molecular interaction between PrP^{C} and PrP^{Sc} and the subsequent conformational changes that occur in these proteins. The availability of such variants may also be of help for the development of sensitive conversion assays allowing the detection of low amounts of PrP^{Sc} for example in crude samples or other biological preparations (vaccines). Another line of future research might be the development of therapeutics based on natural or artificial variants of PrP that have the property to inhibit or block PrP^{Sc} formation as outlined before.

In the meantime a large set of artificial sheep PrP^{C} variants were engineered which are expected to have different effects on PrP conversion efficiencies. This set includes PrP variants with disturbed N-linked glycosylation site(s), broken disulphide bonds by cysteine modifications, proline substitutions, variants corresponding to polymorphisms linked to phenotypic expressions of TSEs in humans, and other variants expected to be important for conversion. After expression of the different artificial PrP^{C} variants in eukaryotic cells,

differences in protein translocation were readily detectable by immuno-peroxidase monolayer assay. Some mutant proteins tended to form granule-like staining patterns, others showed strong perinuclear staining, a further group of mutants showed intense staining of cytoplasmatic sections without perinuclear staining. These differences in immuno-staining indicated the presence of aberrantly folded or processed forms of prion protein. Some mutants were unexpectedly disturbed in N-linked glycosylation while others showed no signs of aberrant glycosylation, processing or cellular translocation. Experiments to study the behaviour of these proteins with regard to PK resistance, spontaneous aggregation and convertibility in the cell-free system are currently in progress.

In summary, the work described in this thesis clearly shows the importance of specific polymorphism in the PrP gene on susceptibility of hosts and transmissibility of prion diseases. Several polymorphisms in the sheep PrP gene could be significantly associated by *in vivo* studies with scrapie susceptibility and differences in scrapie survival times. *In vitro* studies showed that the observed differences in TSE susceptibility are almost certainly caused by the differences in convertibility of the PrP protein variants. The *in vitro* studies also indicate that not only species-barriers, but also polymorphism-barriers play a significant role in the transmissibility of TSEs. The cell-free system proliferated itself as a quick and rather accurate biological relevant tool to determine inter- and intra-species transmissibilities of various TSEs. We therefore used the cell-free system to a) gauge the relative susceptibility of sheep having different PrP genotypes, and b) to assess the relative transmissibilities of BSE and sheep scrapie humans. The *in vivo* and *in vitro* studies described in this thesis resulted thus far in a solid fundament for understanding TSE susceptibility and transmissibility at the molecular level. At present this knowledge is used already to prevent and control the archetype of TSEs, i.e. scrapie in sheep. It is hoped that this knowledge will also contribute (in the future) to prevent and control other TSE diseases including those of man.

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Samenvatting

Samenvatting

Prionziekten, ofwel overdraagbare spongieuze encephalopathieën, zijn neuro-degeneratieve aandoeningen die voorkomen bij mensen en dieren. In het algemeen worden prionziekten veroorzaakt door een infectie met zogenaamde prionen. De ziekte kan echter ook op andere manieren ontstaan. De bekendste prionziekten zijn BSE ('gekke-koeienziekte') bij runderen, scrapie bij schapen en de ziekte van Creutzfeldt-Jacob (CJD) bij mensen. Prionziekten verlopen progressief en zijn altijd fataal. Karakteristiek voor prionziekten is de degeneratie van neuronen in het centrale zenuwstelsel hetgeen sponsvorming van het weefsel tot gevolg heeft. Deze degeneratie en sponsvorming gaat gepaard met de ophoping van een verkeerd gevouwen vorm van een eiwit van de gastheer, het prion eiwit (PrP). Dit verkeerd gevouwen prion eiwit (PrP^{Sc}) ontstaat door een verandering van de ruimtelijke structuur van het normale prion eiwit (PrP^{C}). De structuurverandering van PrP^{C} naar PrP^{Sc} heeft onder andere tot gevolg dat het eiwit niet meer kan worden afgebroken door eiwit-splitsende enzymen. Hierdoor hoopt het zich op in het centrale zenuwstelsel. De huidige gedachtegang is dat PrP^{Sc} het infectieuze prion deeltje is. Het staat in ieder geval vast dat PrP^{Sc} de belangrijkste component is van prionen. De resultaten van tal van experimenten wijzen erop dat deze prionen, of PrP^{Sc} moleculen, vermenigvuldigen zonder dat daarvoor prion-specifiek DNA of RNA, dragers van erfelijke informatie, nodig is. Het infectieuze PrP^{Sc} eiwit is namelijk in staat om normaal PrP^{C} om te vormen tot nieuwe infectieuze PrP^{Sc} moleculen.

Bij de aanvang van dit onderzoek was het bekend dat veranderingen in het PrP gen van de gastheer, ook wel polymorfismen genoemd, een effect kunnen hebben op de manier waarop prionziekten tot uitdrukking komen (fenotype). Tevens waren er aanwijzingen dat de gevoeligheid van gastheren voor prionziekten genetisch bepaald wordt. Er werd door sommigen zelfs verondersteld dat een prionziekte zoals scrapie bij schapen een genetische ziekte is. Het was echter niet bekend welke polymorfismen in het PrP gen betrokken zijn bij de wijze waarop prionziekten tot uitdrukking komen. Tevens was weinig of niets bekend over de moleculaire mechanismen die ten grondslag liggen aan de verschillen in de gevoeligheid van gastheren voor prionziekten.

Aanvankelijk was ook bekend dat de overdracht van prionziekten tussen gastheren van dezelfde soort efficiënter verloopt dan de overdracht tussen gastheren van verschillende soorten. Tevens waren er aanwijzingen dat het PrP gen een belangrijke rol speelt bij het bepalen van de sterkte van deze soort-barrière. Over de mechanismen die ten grondslag liggen aan de verschillen in overdraagbaarheid, was echter nog weinig bekend. Toch was er grote behoefte aan deze kennis, ten eerste om de ziekteprocessen van deze onconventionele

aandoeningen beter te kunnen doorgronden, en ten tweede om de risico's die mensen en dieren lopen om deze ziekten te krijgen beter te kunnen bepalen. Om de hiaten in deze kennis op te kunnen vullen, waren snelle en betrouwbare methoden nodig waarmee de gevoeligheid van gastheren en de overdraagbaarheid van prionziekten konden worden gemeten. Het onderzoek dat in dit proefschrift beschreven wordt, werd uitgevoerd om een bijdrage te leveren aan het opvullen van bovenstaande kennisleemtes en om de moleculaire mechanismen op te helderen die ten grondslag liggen aan de gevoeligheid van gastheren voor prionziekten en de overdraagbaarheid van deze aandoeningen.

Allereerst werden experimenten uitgevoerd om polymorfismen in het PrP gen van schapen op te sporen. Hiervoor werden experimentele procedures zoals DGGE en ASA ontwikkeld. Met de DGGE techniek kunnen PrP genen met verschillende polymorfismen (allel varianten) van elkaar onderscheiden worden. Deze techniek stelde ons ook in staat om onbekende PrP allel varianten te identificeren. Hierna werd onderzocht of deze PrP varianten iets te maken hebben met de gevoeligheid van schapen voor natuurlijke scrapie of met verschillen in overlevingstijden van schapen met scrapie. Hiervoor zijn 'case-control' studies en studies binnen één koppel schapen uitgevoerd. Deze studies hebben duidelijk aangetoond dat er een verband bestaat tussen het voorkomen van bepaalde PrP varianten en de gevoeligheid van schapen voor scrapie en met de overlevingstijden van schapen met scrapie. Daarna is onderzocht of enkele van deze PrP varianten mogelijk samenhangen met spontane of genetische vormen van scrapie. Hiervoor werd het PrP genotype bepaald van schapen uit een land dat geheel vrij is van scrapie, namelijk Nieuw-Zeeland. Dit onderzoek heeft duidelijk aangetoond dat de aanwezigheid van scrapie agens een voorwaarde is voor de ontwikkeling van scrapie.

Vervolgens werd geprobeerd de moleculaire mechanismen op te helderen die ten grondslag liggen aan scrapie gevoeligheid en overdraagbaarheid van scrapie. Hiervoor werd een *in vitro* (celvrij) systeem gebruikt waarin de omvorming van normaal PrP naar abnormaal PrP gemeten kan worden. Om dit systeem te kunnen gebruiken moesten eerst technieken ontwikkeld worden voor de *in vitro* expressie en zuivering van PrP^{C} varianten en de isolatie van PrP^{Sc} uit hersenen van schapen met scrapie. Uiteindelijk werd het celvrije systeem ook gebruikt om:

1) de gevoeligheid van schapen voor scrapie te voorspellen op basis van hun PrP genotype;
2) de transmissie van BSE en scrapie naar andere soorten te bepalen, inclusief de mogelijke overdraagbaarheid naar de mens;
3) de effecten van aminozuur veranderingen te bestuderen op de omzettings-efficiëntie van normaal PrP naar abnormaal PrP.

Het onderzoek beschreven in dit proefschrift heeft onder andere geleid tot een beter inzicht in de effecten die verschillende polymorfismen in het PrP gen van schapen hebben op de ontwikkeling en het fenotype van scrapie. Gebaseerd op *in vivo* en *in vitro* data, konden tenminste drie verschillende PrP allel varianten van het schaap worden geassocieerd met hoge gevoeligheid voor scrapie en met korte overlevingstijden. Drie andere PrP allel varianten konden worden geassocieerd met een gereduceerde scrapie gevoeligheid of zelfs met resistentie tegen scrapie. In eerste instantie hadden deze resultaten een directe spin-off naar kleinschalige fokprogramma's. Hierbij wordt geprobeerd de incidentie van scrapie te reduceren door in een schapenpopulatie de frequentie van die PrP varianten te verlagen, die geassocieerd zijn met een hoge gevoeligheid voor scrapie. De frequentie van 'resistente' PrP allelen neemt hierbij toe. Momenteel wordt eenzelfde aanpak toegepast in een landelijk scrapie bestrijdingsprogramma.

De *in vitro* studies die in dit proefschrift beschreven zijn, hebben aangetoond dat polymorfismen in schapen-PrP een direct effect hebben op het vermogen van normaal PrP om omgezet te worden in abnormaal PrP. Tevens werd er een verband gevonden tussen de efficiëntie van deze omzetting en de associatie van de desbetreffende PrP varianten met de gevoeligheid voor scrapie. Het is daarom aannemelijk dat de verschillen in de gevoeligheid van gastheren voor scrapie worden veroorzaakt door verschillen in de PrP conversie kinetiek. Met andere woorden, modulatie van de PrP conversie efficiëntie is een belangrijk mechanisme dat ten grondslag ligt aan de verschillen in de gevoeligheid voor prionziekten. Hierdoor werd het mogelijk om het *in vitro* systeem te gebruiken om de koppeling tussen alle natuurlijke schapen PrP varianten en de gevoeligheid voor scrapie vast te stellen. De resultaten van de *in vitro* studies gaven een verdere wetenschappelijke onderbouwing van de scrapie bestrijdingsprogramma's die gebaseerd zijn op selectief fokken. Uiteindelijk werd het *in vitro* conversie systeem ook gebruikt om, in een groot internationaal samenwerkingsverband, de mogelijke overdraagbaarheid van BSE en scrapie naar andere soorten, inclusief de mens, te bepalen. Op basis van de *in vitro* conversie resultaten werd geconcludeerd dat mogelijke transmissies van BSE en scrapie naar de mens van een vergelijkbaar maar zeer laag niveau zijn.

Het beschreven onderzoek toont aan dat polymorfismen in PrP genen, niet alleen van schapen, een belangrijke rol spelen bij het bepalen van de gevoeligheid van gastheren voor prionziekten en de overdraagbaarheid van deze aandoeningen. Deze verschillen in gevoeligheid worden voor een belangrijk deel veroorzaakt door verschillen in de converteerbaarheid van PrP. De *in vitro* studies hebben ook laten zien dat naast de soort-barrières, polymorfisme-barrière's een belangrijke rol spelen bij de overdraagbaarheid van prionziekten. De resultaten van de *in vitro* studies benadrukken de biologische

relevantie van de celvrije conversiereactie. Daarom is dit systeem geschikt om voorspellingen te doen over een aantal eigenschappen van mogelijk alle prionziekten zoals gevoeligheid, overdraagbaarheid en verschillen in incubatietijd. Het *in vitro* systeem lijkt ook geschikt om eventueel andere aspecten van prionziekten te bestuderen, zoals bijvoorbeeld het effect van potentiële therapeutica. Zonder dit *in vitro* systeem zou dit alleen maar kunnen door middel van tijdrovende, dure en dieronvriendelijke proeven.

De *in vivo* en *in vitro* studies beschreven in dit proefschrift hebben een significante bijdrage geleverd aan onze kennis over de gevoeligheid van gastheren voor prionziekten en de overdraagbaarheid van deze aandoeningen op moleculair niveau. Het is te verwachten dat deze kennis verder prion onderzoek naar polymorfisme- en soort-barrières zal stimuleren, waaruit mogelijk therapeutische strategieën geformuleerd kunnen worden om te interfereren met prion replicatie. Op dit moment wordt de opgebouwde kennis reeds toegepast om scrapie bij schapen te bestrijden. Het is te hopen dat deze kennis in de toekomst ook zal bijdragen aan preventie en controle van andere prionziekten, inclusief die van de mens.

Curriculum Vitae

List of Publications

Curriculum vitae

Alex Bossers was born on February 22nd, 1970 in Rotterdam, The Netherlands. In 1986 he finished secondary school in Almelo. After ‘finishing’ three years of education in laboratory engineering (clinical chemistry) at the HTS-SVL in Hengelo, he attended higher education in laboratory engineering (medical biochemistry) at the Enschede Polytechnic. He graduated and got his BSc degree in 1993. He worked for an additional period on baculovirus expression at the department of Molecular Biology from the former Central Veterinary Institute in Lelystad. After military service he joined in 1994 the BSE/Scrapie project at the Institute for Animal Science and Health, Lelystad. Meanwhile he attended professional education at the graduate school Infection and Immunity of the University of Utrecht, The Netherlands.

List of Publications

Peter B.G.M. Belt, **Alex Bossers**, Bram E.C. Schreuder, and Mari A. Smits. (1996). PrP allelic variants associated with natural scrapie. In: *Bovine Spongiform Encephalopathy; The BSE dilemma* ed. Gibbs, C.J. (Springer, New York), pp. 294-305.

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G.J. Raymond, J. Hope, D.A. Kocisko, S.A. Priola, L.D. Raymond, **A. Bossers**, J. Ironside, R.G. Will, S.G. Chen, R.B. Petersen, P. Gambetti, R. Rubenstein, M.A. Smits, P.T. Lansbury Jr, and B. Caughey. (1997). Molecular assessment of the potential transmissibilities of BSE and scrapie to humans. *Nature* **388**:285-288.

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Alex!