Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie

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Scrapie is a fatal neurodegenerative disease of sheep that belongs to the group of prion diseases found in humans and animals. The host encoded prion protein (PrP) plays a central role in the disease process. In the PrP genes of man, mice and sheep, polymorphisms have been found that are associated with disease susceptibility and pathogenesis. We have used denaturing gradient gel electrophoresis (DGGE) to detect polymorphisms in the sheep PrP gene. In addition to the already described polymorphisms at codons 136, 154 and 171, we identified a hitherto unknown G → T transition at codon 171. This transition is responsible for a glutamine to histidine substitution. An arginine to glutamine substitution at this position has been described previously. DGGE allowed us to identify five different combinations of these polymorphisms within the PrP gene representing five allelic variants, which were cloned and sequenced. Based on the triplet sequences present at codons 136, 154 and 171 these allelic variants were designated PrPvRq, PrPARR, PrPAIH, PrPAIH and PrPAIH. To determine the association of these allelic variants with natural scrapie, we screened 34 scrapie affected and 91 healthy control sheep of the Texel breed for the presence of these allelic variants. In these two groups, the five variants gave rise to 13 different genotypes. The distribution of the allelic variants among both groups showed marked differences. The PrPvRq variant was present with high frequency in scrapie affected sheep, whereas the PrPAIH variant was almost exclusively present in the healthy group. Two other variants, PrPAIH and PrPAHR, were found in both groups with equal frequencies. The data obtained suggest modulation of disease susceptibility in these Texel sheep by at least five different PrP allelic variants, with the PrPvRq and PrPAIH alleles acting in a dominant, but opposite fashion over the PrPAIH and PrPAHR alleles. The frequency of the PrPAIH variant was too low to draw any conclusions.

Introduction

Scrapie is a fatal neurodegenerative disease of sheep and goats and is the archetype of a group of disorders known as transmissible spongiform encephalopathies or prion diseases. Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS) and fatal familial insomnia (FFI) of man and bovine spongiform encephalopathy (BSE) are also members of this group. Prion diseases can manifest as infectious, sporadic and dominantly inherited disorders (Prusiner, 1991). An abnormal, protease resistant isoform of the host encoded prion protein (PrP), designated PrPsc in scrapie, plays a central role in the pathogenesis of these diseases. PrPsc is derived from the cellular prion protein (PrPc) by a post-translational process, probably a conformational change (Stahl & Prusiner 1991). Infectious prions are composed largely, if not entirely, of PrPsc molecules (Prusiner et al., 1982).

The aetiology of sheep scrapie has been debated for many years. Parry (1962) concluded that natural scrapie is a genetic disorder, caused by an autosomal recessive gene, which just happens to be experimentally transmissible. Later, maternal and lateral contagious transmission of the disease was reported, indicating that natural scrapie is an infectious disorder (Dickinson et al., 1965, 1974; Brotherston et al., 1968). The current view is that scrapie is an infectious disease where host genetic factors play a central role. Dickinson & Outram (1988) showed that the susceptibility of sheep for scrapie is controlled by a single gene, designated the Sip gene. Recent experimental data suggest that Sip and the PrP gene are linked, or even are one and the same gene (Hunter et al., 1989, 1991). As with Sip, the corresponding Sinc or Prn-i gene of mice seems to be identical to the mouse PrP gene (Carlson et al., 1986, Hunter et al., 1987).
In the PrP gene of man, mice and sheep several mutations and polymorphisms have been found that are associated with prion diseases. In man at least ten missense, one nonsense, and seven insert mutations segregate with the disease. (Prusiner, 1993). In addition, it has been suggested that a common polymorphism at codon 129 of the human PrP gene is associated with susceptibility to iatrogenic and sporadic cases of CJD (Collinge et al., 1991; Palmer et al., 1991). This polymorphism also determines the disease phenotype in familial CJD and FFI patients who have a PrP mutation at codon 178. When the allele with the 178 mutation encodes a methionine at position 129, patients develop FFI. When the allele with the 178 mutation encodes a valine at position 129, patients develop CJD (Goldfarb et al., 1992). Thus manifestation of disease characteristics may depend on the presence of specific combinations of mutations or polymorphisms within the PrP open reading frame.

In the PrP gene of sheep four polymorphisms have been described (Goldmann et al., 1990, 1991a; Laplanche et al., 1993). A polymorphism at codon 136 is associated with scrapie susceptibility in both experimental (Goldmann et al., 1991a; Maciulis et al., 1992) and natural scrapie (Laplanche et al., 1993; Hunter et al., 1993). A polymorphism at codon 171 has recently been investigated with respect to experimental scrapie in the Cheviot breed (Goldmann et al., 1994) and with respect to natural scrapie in the Suffolk breed (Westaway et al., 1994). Two other polymorphisms, at codons 112 and 154, are rare and have not been associated with any disease phenotype.

The objectives of the study reported here were first, to identify polymorphisms in the PrP gene of sheep of the Texel breed; second, to identify and characterize the various allelic variants of the gene, representing different combinations of these polymorphisms; third, to investigate the association of these PrP allelic variants with natural scrapie; and fourth, to assess the feasibility of using these variants as genetic markers for scrapie susceptibility. To this end we screened a group of scrapie affected sheep and a group of healthy control sheep. We first analysed the polymorphisms at the codons 136 and 154 of sheep, then we applied denaturing gradient gel electrophoresis (DGGE) for the combined detection of these polymorphisms and the analysis of PrP variant genotypes present among the investigated sheep. DGGE also allowed us to search for hitherto unknown polymorphisms in the sheep PrP gene.

**Methods**

**Sheep.** All sheep were of the Texel breed. Scrapie-affected sheep (n = 34, from 18 different flocks; aged between 2 to 5 years) were collected throughout the Netherlands from August 1991 to October 1992. All scrapie cases were confirmed by histology and immunocytochemistry (L. J. M. van Keulen et al., 1995). Age matched control sheep (n = 91), obtained from 15 different flocks in the Netherlands, were collected in the same period. These flocks were all free from scrapie for the last 5 years. About 4% to 8% of the Dutch flocks suffer from scrapie, with an incidence of 1 in 100 sheep/year (Schreuder et al., 1993). From all sheep blood samples were collected in EDTA vacutainers (Venoject, Terumo Europe) and frozen at −20 °C until further use.

**DNA amplification.** High-molecular-mass DNA was isolated from blood as described by Sambrook et al. (1989). The PrP open reading frame, which resides on a single exon (Goldmann et al., 1990), was amplified by PCR. Amplification reactions were done in 50 μl reaction volumes containing 100 ng genomic DNA, 25 mM-Tris-HCl pH 8.7, 2 mM-MgCl2, 0.005% gelatine, 200 μM-dNTPs, 1 μM of primer set p8-p9 or 100 μM of primer set p78-p79 and 1 unit of Taq DNA polymerase (Perkin Elmer Cetus). The sequences of the oligonucleotide primers are indicated in Table 1, the positions of the primers in Fig. 2. The amplification reactions were performed in a preheated Perkin Elmer Cetus DNA Thermal Cycler (80 °C) for 35 cycles of 1 min at 92 °C, 1.5 min at 58 °C and 1.5 min at 72 °C.

**Detection of polymorphisms at codons 136, 154 and 171.** Polymorphisms at codon 136 and 154 were detected by RFLP analysis as described by Hunter et al. (1993) except that RcaI instead of BspHI was used. DNA was amplified with primer set p8-p9 and digested without prior purification of the PCR products. In each reaction pUC 19 plasmid DNA was included as an internal control for complete digestion. The C → A transition at codon 171 was detected by allele specific amplification (ASA) with the primer sets p112-p79 and p113-p79. Sheep genomic DNA was first amplified with the primers p78 and p79 using the conditions as described above. Subsequently the DNA was diluted 10 times. A second semi-nested PCR was done in 50 μl containing 5 μl of the diluted DNA, 25 mM-Tris-HCl, pH 8.7, 1.5 mM-MgCl2, 0.005% gelatine, 200 μM-dNTPs, 100 μM of primer set p112-p79 or primer set p113-p79, and 1 unit of Taq DNA polymerase. The reaction was done in a preheated Thermal Cycler (80 °C) for 30 cycles of 1 min at 94 °C, 2 min at 50 °C and 3 min at 72 °C. The ASA products were analysed on a 2% agarose gel.

**DGGE analysis.** The melting domains in the open reading frame of the sheep PrP gene were determined by the MELT87 program, kindly provided by L. Lerman (Lerman & Silverstein, 1987). Sheep genomic DNA was first amplified with the primer set p8-p9 as described above and 15 μl was digested with the restriction enzyme HindIII 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 (v/v), gel length = 17 cm) in 40 mM-Tris-acetate, 1 mM-EDTA, pH 7.4. Electrophoresis was done at 50 V for 24 h at 60 °C in 40 mM-Tris-acetate, 1 mM-EDTA, pH 7.4 in an electrophoresis tank as described by Myers et al. (1987). 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). Amplification reactions were done in 50 μl containing 100 ng genomic DNA, 10 mM-KCl, 10 mM-(NH4)2SO4, 20 mM-Tris-HCl, pH 8.8, 1.5 mM-MgSO4, 0.1% Triton X-100, 200 μM-dNTPs, 500 nm of primer set p78-p79 and 1 unit of Vent DNA polymerase. The amplification reactions were performed in a Perkin Elmer Cetus DNA Thermal Cycler for 35 cycles...
Table 1. Oligonucleotide primers for amplification and sequencing of the open reading frame of the sheep PrP gene

<table>
<thead>
<tr>
<th>Primer*</th>
<th>Position†</th>
<th>Restriction site‡</th>
<th>Sequence (5' → 3')§</th>
</tr>
</thead>
<tbody>
<tr>
<td>p8 (+)</td>
<td>72-91</td>
<td>HpaI</td>
<td>CAGGTTAACGATGGTAAGAGCCACATGG</td>
</tr>
<tr>
<td>p9 (−)</td>
<td>842-818</td>
<td>EcoRI</td>
<td>GGAATTCTATCTACTATGAGAAAAATGAGG</td>
</tr>
<tr>
<td>p78 (+)</td>
<td>22-41</td>
<td>EcoRI</td>
<td>AGTGAAATTCTACGGTGGCCATTGTGCTG</td>
</tr>
<tr>
<td>p79 (−)</td>
<td>915-896</td>
<td>XbaI</td>
<td>ACCCTTAGGGGCTGAGTACACCTC</td>
</tr>
<tr>
<td>p60 (−)</td>
<td>531-512</td>
<td>−</td>
<td>GATAGTAAACGGTCCTCAG</td>
</tr>
<tr>
<td>p61 (+)</td>
<td>398-417</td>
<td>−</td>
<td>AACCAACATGAACATGGG</td>
</tr>
<tr>
<td>p112 (+)</td>
<td>567-583</td>
<td>−</td>
<td>TACAGACCAGTGGATCA</td>
</tr>
</tbody>
</table>

* Primer orientation: +, sense, −, antisense. Relative positions of the oligonucleotide primers in the PrP gene are indicated in Fig. 2.
† Numbers are according to the sequence of the sheep PrP gene, published by Goldmann et al. (1990).
‡ Restriction sites were introduced for cloning purposes and are indicated in italics in the sequence.
§ Residues indicated in italics in p112 and p113 represent allele specific bases for ASA.

of 1 min at 92°C, 1·5 min at 58°C, 1·5 min at 72°C. Amplified fragments were inserted into the EcoRI and XbaI site of pGEM-7 (Promega). Clones harbouring different allelic variants of the PrP gene were distinguished from each other by DGGE analysis as described above.

Sequencing of cloned material was done using the chain termination reaction described by Sanger et al. (1977). At least two independent clones of each allelic variant were sequenced to exclude PCR artefacts. Sequencing was performed using primers p60, p61, p78 and p79 (Table 1). Direct sequencing of PCR material was performed as described by Lichtenauer-Kaligis et al. (1993).

Statistical analysis. Results were analysed using the χ² test of association by comparing genotype frequencies in the scrapie affected sheep and the healthy control sheep.

Results

Polymorphisms at codons 136 and 154 and their association with natural scrapie

The frequencies of codon 136 and codon 154 polymorphisms among scrapie affected and healthy control sheep are summarized in Table 2. With respect to codon 136, there was a significant difference between the two groups. In the scrapie affected group 91% of all individuals carried at least one allele sensitive for RcaI at codon 136, whereas only 11% of the healthy control sheep carried this allele. Thus in the Texel breed codon 136 valine is associated with a high incidence of natural scrapie (χ² = 73·18, P < 0·0001). This suggests that sheep with at least one codon 136 valine allele are more susceptible to the disease than sheep that are homozygous for codon 136 alanine. With respect to codon 154, no significant differences were found between the two groups. The codon 154 allele sensitive to RcaI was present at a low frequency in the control group (8%) and was absent in the scrapie affected group.

Polymorphism at codon 171 and its association with natural scrapie

Goldmann et al. (1990) reported the presence of a polymorphism at codon 171 (G → A transition) resulting in an arginine to glutamine substitution in the PrP protein. We determined the frequencies of the codon 171 polymorphism in both groups of sheep by ASA. First we searched for sheep heterozygous for codon 171 by partial sequencing of their PCR amplified PrP open reading frames. Among the first three sheep analysed, one sheep was heterozygous and carried both the codon for glutamine (CAG) and for arginine (CGG) at this position. Both alleles were cloned, and the cloned DNA was used to determine the proper conditions that allowed ASA with the primer sets p112–p79 and p113–p79. Using similar conditions on PCR amplified DNA instead of cloned DNA we were able to characterize the codon 171 polymorphism in all sheep. Note that discrimination between the two codons is based on differences in the second base of each codon. An example of the ASA analysis is shown in Fig. 1. The results for both groups of sheep are summarized in Table 2. Only one sheep (3%) in the scrapie affected group carried a codon 171 arginine (CGG) allele, whereas in the control group this allele was found in more than 50% of the population. This indicates a clear association of the codon 171 arginine allele with a low incidence of natural scrapie (χ² = 26·77, P < 0·0001), suggesting that sheep with at least one codon 171 arginine allele are less susceptible to natural scrapie.

Setting up DGGE analysis

The results obtained so far allowed us to conclude that disease incidence in Texel sheep is associated with at least two polymorphic sites in the PrP open reading frame.
Table 2. Genotype analysis of PrP codons 136, 154 and 171 in scrapie affected and healthy control sheep

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Scrapie (n = 34)</th>
<th>Control (n = 91)</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td><strong>Codon 136</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RcaI Sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ + GTC/GTC → V/V</td>
<td>3</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ - GTC/GAC → V/A</td>
<td>28</td>
<td>82</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>- - GAC/GAC → A/A</td>
<td>3</td>
<td>9</td>
<td>81</td>
<td>89</td>
</tr>
<tr>
<td><strong>Codon 154</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RcaI Sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ + CAT/CAT → H/H</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>+ - CAT/CGT → H/R</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>- - CGT/CGT → R/R</td>
<td>34</td>
<td>100</td>
<td>83</td>
<td>91</td>
</tr>
<tr>
<td><strong>Codon 171</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ASA Sequence</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ - CGG/CGG → R/R</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>17</td>
</tr>
<tr>
<td>+ + CGG/CAG → R/Q§</td>
<td>1</td>
<td>3</td>
<td>34</td>
<td>37</td>
</tr>
<tr>
<td>- + CAG/CAG → Q/Q§</td>
<td>33</td>
<td>97</td>
<td>42</td>
<td>46</td>
</tr>
</tbody>
</table>

* Polymorphism determined by RcaI digestion: + + indicates digestion of both alleles; + - indicates digestion of one allele (heterozygote); - - indicates no digestion at the specified codon. Expected codon sequences and corresponding amino acids are indicated. Nucleotides recognized by the enzyme RcaI are indicated in italics.

† Yates corrected.

‡ Polymorphism determined by ASA: + - indicates the presence of a PCR product with primer p112; + + indicates the presence of a PCR product with primers p112 and p113 (heterozygote); - + indicates the presence of a PCR product with primer p113. Expected codon sequences and corresponding amino acids are indicated. Nucleotides recognized by one of the two specific primers are indicated in italics.

§ DGGE revealed that another codon 171 polymorphism (CAT → H) also contributed to the primer 113 product; see below.

Fig. 1. Detection of the codon 171 polymorphism by ASA. Sheep DNA amplified with primer set p78–p79 was diluted 10⁵ times and subjected to ASA with primer set p112–p79 (every first lane of a set) or primer set p113–p79 (every second lane of a set) as described in Methods. Lane sets 1–17, PCR amplified DNA from 17 different sheep; lane sets QQ and RR, PCR amplified DNA from cloned alleles harbouring a CAG triplet (QQ) or a CGG triplet (RR) at codon 171; lane set QR, PCR amplified DNA from a mixture of the two cloned alleles.

Whether the manifestation of disease characteristics depends on the presence of specific combinations of these polymorphisms within the PrP open reading frame remained unclear. In addition, we did not know whether polymorphisms other than those at codons 136, 154 and 171 were present in the sheep under investigation. We applied DGGE to investigate these possibilities. Conditions for the combined analysis of polymorphisms in the region of the PrP gene that include codons 136, 154 and 171 were determined with the help of the melt-map shown in Fig. 2, and further established empirically. The conditions were tested and further improved with DNA
PrP allelic variants and natural scrapie

Fig. 2. Melt-map of the PrP open reading frame. The plot shows the temperature, calculated by the computer program MELT87, at which each base is distributed with 50% probability between either the helical or the melted states (Tm). Melt-domains A (76.2°C) and B (72.1°C), polymorphic codons 136, 154 and 171, restriction endonuclease site HinfI, and the oligonucleotide primers used in this study are indicated. The proteinase resistant core of the PrP\textsuperscript{res} protein (PrP 27–30) is indicated in grey.

Fig. 3. DGGE analysis of PCR amplified DNA. Lanes 2–6, DGGE patterns obtained from five cloned and sequenced allelic variants. Lanes 8–20, DGGE patterns obtained from 13 different sheep, representing all 13 genotypes found in 34 scrapie affected sheep and 91 healthy control sheep. Lanes 1, 7 and 21 are control lanes that contain four combined allelic variants which were not denatured and renatured before loading. The haplotypes of these allelic variants (designations are explained in the text) are indicated on the right and on the left.

obtained from sheep heterozygous for the codons 136, 154 or 171. Optimal results were obtained when we used HinfI digested PCR products amplified with primer set p8–p9 (Fig. 2), in combination with the DGGE conditions as described in Methods.

Cloning of five PrP allelic variants and identification of a novel polymorphism

Based on the differences in the DGGE patterns, we could identify five different allelic variants of the sheep PrP gene during the course of this study. We cloned and sequenced all variants to identify the base changes responsible for the characteristic migration of each allelic variant in the gel (Fig. 3, lanes 2–6). The first variant (Fig. 3, lane 2) was designated PrP\textsuperscript{ARR}, based on the triplet sequences present at codons 136, 154 and 171. The second and third variants (Fig. 3, lanes 3 and 4) could not be distinguished from each other by DGGE under the conditions used, but differed at codon 136 as determined by RcaI digestion. Sequence analysis revealed that the C → T transition at codon 136 was the only difference between the two cloned variants. Apparently, homoduplex bands of variants with polymorphisms in domain A (Fig. 2) are not distinguished under the conditions that are optimal for the detection of polymorphisms in domain B. According to the triplet sequences present at codons 136, 154 and 171, the two variants were designated PrP\textsuperscript{ARQ} and PrP\textsuperscript{VRQ}. A fourth variant, initially indistinguishable from the PrP\textsuperscript{ARQ} variant by RcaI digestion and ASA, showed a clear difference in electrophoretic mobility (compare Fig. 3, lanes 3 and 5). Sequence analysis revealed a hitherto unknown polymorphism at the third base of codon 171.
G

C

A G C T A G C T

171R
171Q
171H

Fig. 4. Sequence analysis of three different PrP allelic variants demonstrating the existence of three different triplet sequences at codon 171.

The G → T transition found at this position results in a glutamine to histidine substitution in the PrP protein. This means that three different triplet sequences of codon 171 are known now, CGG coding for arginine, CAG coding for glutamine and CAT coding for histidine (Fig. 4). According to the triplet sequences present at codons 136, 154 and 171 this variant was designated PrPARRH. It should be noted that ASA did not allow us to discriminate between CAG and CAT at codon 171, since ASA, as it is used here, only detects G → A transitions at the second base of the triplet. Fortunately, this was not detrimental to our conclusion that codon 171 arginine is associated with low scrapie incidence. Two silent mutations at codon 231 (A → C) and codon 237 (C → G) were found as well in the PrPARRH variant. These mutations are located outside the fragment analysed by DGGE and could therefore not be responsible for the change in electrophoretic mobility of this fragment. The fifth allelic variant (Fig. 3, lane 6) was designated PrPAQ. Apart from the already known G → A transition at codon 154, no other polymorphisms were found in this variant. The polymorphism at codon 112, a T → C transition responsible for a methionine to threonine substitution as has been detected by Laplanche et al. (1993) in a single flock of Ile-de-France sheep, was not present in either of the five variants.

Identification of PrP genotypes by DGGE analysis and their association with natural scrapie

To identify the PrP variant genotypes in the scrapie affected and healthy control group, we analysed all sheep (n = 125) by DGGE. We found 12 different DGGE patterns (Fig. 3, lanes 8–20), all of which could be reproduced by mixing two of the five cloned and well defined allelic variants (data not shown). It should be noted that genotypes that differed at codon 136 could not be distinguished from each other by comparison of their homoduplex bands, as has been shown above for the cloned variants PrPARR and PrPVQR. In heterozygotes, however, they could be distinguished from each other by comparison of the heteroduplex bands [compare for instance Fig. 3, lane 11 (PrPARRH/PrPVQR) and lane 12 (PrPARRH/PrPARRQ)]. Therefore, all samples showing only one homoduplex band in the gel were mixed with another variant, thereby inducing heteroduplex bands. In this way we could identify polymorphisms in domains A and B simultaneously and discriminate further between the genotypes PrPARRQ/PrPARRQ and PrPARRQ/PrPVQR (data not shown). Taking this into account, the results indicate that the 12 different DGGE patterns represent a total of 13 different PrP variant genotypes in the investigated sheep.

The frequencies of the various genotypes in the scrapie affected and the healthy control group are summarized in Table 3. The PrPVQR variant was present in 91% of all scrapie cases and in only 10% of the healthy sheep. The PrPVQR homozygotes were found exclusively in the group of scrapie affected sheep. Therefore we can conclude that the PrPVQR allelic variant is associated with a high incidence of natural scrapie (Table 3; χ² = 73.18, P < 0.0001). The PrPARRR variant was present in 50% of all healthy control sheep and in only 3% (one sheep) of the scrapie affected sheep. The PrPARRR homozygotes were found exclusively in the group of healthy sheep. The PrPARRR variant is thus associated with a low incidence of natural scrapie (Table 3; χ² = 26.77, P < 0.0001). PrPARRQ and PrPARRR did not show association with disease incidence (PrPARRQ, χ² = 2.33, P = 0.1113; PrPARRH, χ² = 5.22, P = 0.0735). Both these variants were almost equally distributed in the scrapie affected and healthy control group. The occurrence of the PrPAQ variant in both groups was too low to draw any conclusions.
PrP ARQ and prpA~R~ PrP A~R~ genotypes were found only in the healthy control group, whereas the prpVRQ/prpAaQ and prpV"Q/PrP A~r~ genotypes were found in the scrapie affected group at a much higher frequency (79%) compared to the healthy control group (6%). The PrP vRQ increased susceptibility and PrP AR~ associated with reduced susceptibility or resistance. Since the prpA~r V natural scrapie. The data as presented in Table 3 suggest disease susceptibility, with PrP vRQ associated with an increased incidence of natural scrapie, and that codon 171 arginine is associated with a low incidence of natural scrapie in this breed.

Combined detection of the polymorphisms at codons 136, 154 and 171 by RcaI digestion and ASA, revealed that codon 136 valine is associated with a high incidence of natural scrapie, and that codon 171 arginine is associated with a low incidence of natural scrapie in this breed.

Table 3. Frequencies of PrP genotypes and allelic variants in scrapie affected and healthy control sheep

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Scrapie* No.</th>
<th>Control* No.</th>
<th>χ²</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrPVQR/PrPARR</td>
<td>12</td>
<td>4</td>
<td>73.18 &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>PrPAAR/PrPARR</td>
<td>15</td>
<td>2</td>
<td>26.77 &lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>PrPAAR/PrPARR</td>
<td>15</td>
<td>23</td>
<td>5.22 0.0735</td>
<td></td>
</tr>
<tr>
<td>PrPAAR/PrPARR</td>
<td>15</td>
<td>23</td>
<td>5.22 0.0735</td>
<td></td>
</tr>
</tbody>
</table>

Allelic variant

PrPVQR 34 50 10 5 73.18 < 0.0001
PrPAAR 11 1 60 33 26.77 < 0.0001
PrPAAR 18 26 68 37 2.33 0.3113
PrPAAR 15 22 36 20 5.22 0.0735
PrPAAR 0 0 8 4 1.89 0.1687†

* Scrapie affected sheep, n = 34 (68 chromosomes); healthy control sheep, n = 91 (182 chromosomes).
† Calculated by comparing homo- and heterozygotes in the scrapie affected and healthy control group (XX, XY and YY, where X = the given allelic variant and Y = all other possible variants).
‡ Yates corrected.

Discussion

We have screened scrapie affected and healthy control sheep of the Texel breed for the presence of polymorphisms in the PrP open reading frame. The first analysis, the individual detection of polymorphisms at codons 136, 154 and 171 by RcaI digestion and ASA, revealed that codon 136 valine is associated with a high incidence of natural scrapie, and that codon 171 arginine is associated with a low incidence of natural scrapie in this breed.

Combined detection of the polymorphisms at codons 136, 154 and 171 of the sheep PrP gene by DGGE resulted in the identification of five allelic variants of this gene and revealed that the PrPVQR allele is associated with a high incidence of natural scrapie, and that the PrPARR allele is associated with a low incidence of natural scrapie in this breed.

The data as presented in Table 3 suggest that PrPVQR and PrPARR are antagonists in determining disease susceptibility, with PrPVQR associated with an increased susceptibility and PrPARR associated with reduced susceptibility or resistance. Since the PrPARR/PrPVQR and PrPARR/PrPARR genotypes were found only in the healthy control group, whereas the PrPVQR/PrPARR and PrPVQR/PrPARR genotypes were found in the scrapie affected group at a much higher frequency (79%) compared to the healthy control group (6%), the PrPVQR and PrPARR alleles seem to be dominant over the PrPARR and PrPARR alleles. This might also explain the presence of the PrPVQR/PrPARR genotype in both groups with equal frequencies.

Three scrapie cases were found among the 13 sheep homozygous for the PrPARR allelic variant. In PrPARR/PrPARR sheep the contaminated environment (infectious dose) or the scrapie source might play a more decisive role in inducing the disease.

DGGE not only allowed us to identify specific combinations of polymorphisms within the PrP open reading frame, it also allowed us to look for hitherto unknown polymorphisms. In the 125 sheep samples we discovered a G → T transition at the third base of codon 171 that results in a glutamine to histidine substitution in the PrP protein. We did not find the codon 112 polymorphism as has been reported by LaPlanche et al. (1993) in an Ile-de-France flock. Since all the 125 PCR amplified sheep PrP genes appeared to have the same length (data not shown), we have no evidence for the presence of octapeptide repeat inserts in the PrP protein of these sheep as has been reported for the human and bovine PrP (Owen et al., 1989, 1991, Goldmann et al., 1991b).

Almost all known base substitutions in the PrP genes of man, mice and sheep that are associated with prion diseases and have been identified to date are found in the region of the PrP open reading frame coding for the protease resistant core of the prion protein (PrP 27–30, Fig. 2). We were able to simultaneously screen polymorphisms in domain A (±codons 141–223) as well as in domain A (±codons 105–140) covering almost 90% of this region coding for PrP 27–30. Detection of polymorphisms in domain A was achieved using either native or artificial heterozygotes, since differences in DGGE banding patterns were only seen in the heteroduplex bands.

Due to the high conservation of the PrP gene in the different species, the melt-maps of the different PrP open reading frames are very similar. Consequently, the conditions used here for DGGE analysis of mutations or polymorphisms in the PrP gene can be adapted easily for species other than sheep.

Studies on experimental scrapie with Cheviot sheep selected for low and high scrapie susceptibility have shown that codon 136 valine is associated with a high susceptibility to experimental challenge (Goldmann et al., 1991a; Maciulis et al., 1992). The association of codon 136 valine with natural scrapie was confirmed by LaPlanche et al. (1993) for Ile-de-France and Romanov sheep and by Hunter et al. (1993) for Swaledales. The results described in this paper confirm these findings. The association of the codon 136 alanine allele with low susceptibility or resistance as predicted in these reports...
PrP AR~ alleles. These latter two were not associated with detection of the different polymorphisms in the sheep needs refinement, however. As a result of the combined detection of the different polymorphisms in the sheep PrP open reading frame, we identified four different allelic variants with codon 136 alanine. All four variants showed different characteristics. The PrP ARR allele was associated with a low incidence of natural scrapie and was suggested to be dominant over the PrP ARR and PrP ARR alleles. These latter two were not associated with either high or low incidence of the disease. This indicates that detection of codon 136 alanine is not sufficient to draw conclusions with regard to scrapie susceptibility. Recently, Westaway et al. (1994) demonstrated the importance of codon 171 in this regard. They showed that in Suffolk sheep homozygosity for codon 171 glutamine is associated with scrapie susceptibility. This is consistent with our findings as shown in Table 2. Our ASA data, however, were confused by the presence of the codon 171 histidine variant which was not distinguished from codon 171 glutamine by ASA, but could be distinguished by DGGE. We do not know whether the codon 171 histidine variant is present in the Suffolk breed, but it is possible that the data presented by Westaway et al. and obtained by allele specific oligonucleotide hybridization are confused in a similar way.

Whether the association of the PrP ARR and the PrP ARR variants with high and low disease susceptibility and the suggested relative dominance of these variants accounts for all scrapie sources (‘strains’) or whether this association is reversed for certain strains as has been demonstrated in mice needs to be addressed in future experiments. Experimental challenge of sheep of different genotypes with different scrapie isolates would be very helpful. The recent data presented by Goldmann et al. (1994) are very interesting in this regard. They showed that all sheep homozygous for codon 136 alanine and either homo- or heterozygous for codon 171 arginine were resistant to experimental challenge with scrapie isolates from A-group (SSBP/1) and C-group (CH1641) agents and to a BSE isolate which reacts very similar to CH1641. They also demonstrated that all homozygotes or heterozygotes for codon 136 valine not having a codon 171 arginine allele became affected after experimental challenge with either SSBP/1 or the BSE isolate. These results are all consistent with our findings in natural scrapie and indicate that our observations have a more common basis. In the same study, Goldmann et al. demonstrated that sheep heterozygous at both codon 136 (valine/alanine) and codon 171 (glutamine/arginine) were very susceptible to SSBP/1 but were resistant to challenge with the BSE isolate. These sheep are probably comparable with our PrP ARR/PrP ARR sheep, which were found in both the healthy and the affected group with equal frequencies. This marked difference in susceptibility indicates that in these sheep disease development might be restricted to the A-group agents. However, since a BSE isolate was used, the species barrier might have played a role here as well.

The role of the PrP ARR allele might be important in scrapie eradication programmes. It is not clear from this study whether the low scrapie incidence in sheep carrying the PrP ARR allele is due to a relative resistance to the infectious agent or that disease development is retarded. Also we cannot exclude that sheep homozygous for the PrP ARR allele might be able to replicate the agent to high titre levels without clinical signs and can serve as a reservoir for infection in flockmates and other species. Further research is needed to address this question. Our results suggest, however, that a positive selection for the PrP ARR allele in breeding programmes might help to control natural scrapie.

The same holds for the PrP ARR allele. Are sheep with the PrP ARR allele just highly susceptible to the scrapie agent, or are they able to develop the disease spontaneously as has been suggested for inherited prion diseases in man (Hsiao et al., 1990). In either case, a negative selection against the PrP ARR allele could be helpful in scrapie eradication.

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