Different scrapie-associated fibril proteins (PrP) are encoded by lines of sheep selected for different alleles of the \textit{Sip} gene

Wilfred Goldmann,* Nora Hunter, Grace Benson, James D. Foster and James Hope

Institute for Animal Health, AFRC & MRC Neuropathogenesis Unit, Ogston Building, West Mains Road, Edinburgh EH9 3JF, U.K.

The incubation period of scrapie in sheep is controlled by the \textit{Sip} gene which has two alleles (sA and pA). Following experimental challenge with SSBP/1 scrapie, a short incubation period is conferred by the partially dominant sA allele. Restriction fragment length polymorphisms of the scrapie-associated fibril protein (PrP) gene are associated with the \textit{Sip} alleles. By sequencing the protein coding region of the PrP gene in Cheviot sheep selected for differing \textit{Sip} genotypes, we have found four PrP protein variants which differ at three positions: amino acid 112 (Ala/Val), amino acid 130 (Arg/His) and amino acid 147 (Arg/Gln). The Val 112 variant can be distinguished at the DNA level by an \textit{RspXI} restriction site which is not present in the Ala 112 form. Val 112 appears to be linked to a short incubation period of experimentally induced scrapie in the Cheviot sheep and therefore with the \textit{Sip} sA allele. These results provide new evidence that the PrP protein may be a product of the \textit{Sip} locus.

Introduction

Scrapie is a transmissible, degenerative disease of the central nervous system which occurs naturally in sheep and goats. Infectivity copurifies with a partially protease-resistant form of a host neuronal membrane glycoprotein (scrapie-associated fibril protein, PrP) (Diringer et al., 1983; Bolton et al., 1982) of \textit{M}, 33K to 35K. Infection-specific PrP was originally discovered in the form of scrapie-associated fibrils (Merz et al., 1981, 1983) and is a hallmark of the molecular pathology of all scrapie-like diseases. To date, PrP protein sequences of eight species have been predicted from gene analysis (Oesch et al., 1985; Loch et al., 1986; Kretzschmar et al., 1986; Liao et al., 1987; Lowenstein et al., 1990; Goldmann et al., 1990, 1991) with at least another 10 species-specific PrP variants detected by screening DNA of various inbred mouse strains, cattle herds and the human population (Westaway et al., 1987; Owen et al., 1990; Laplanche et al., 1990; Goldgaber et al., 1989; Goldfard et al., 1991; Hsiao et al., 1989; Doh-ura et al., 1989).

Although the effect of sequence variation on PrP structure and its normal function is unknown, expression of allelic variants of PrP have been linked to the incidence and the incubation period of disease in humans and mice. In addition, studies on transgenic animals have shown that extra copies of PrP genes can affect the incubation period and the incidence of disease (Prusiner et al., 1990; Hsiao et al., 1990). It has been suggested that PrP is the protein product of the gene that controls scrapie replication and timing of disease symptoms. However, this has not been proven beyond doubt; there are reports of individual recombinant mice (Carlson et al., 1988; Race et al., 1990) and one transgenic study may indicate the presence of two genes at the PrP locus (Westaway et al., 1991).

In a selected flock of Cheviot sheep [Neuropathogenesis Unit (NPU) Cheviot sheep], the incubation period of experimentally induced scrapie (SSBP/1; Dickinson et al., 1976) was controlled by the alleles of the \textit{Sip} gene, sA and pA (Dickinson & Outram, 1988). Negative-line sheep are \textit{Sip} pApA and will survive a subcutaneous (s.c.) injection of SSBP/1. Using the intracerebral route, negative-line sheep may succumb with long incubation periods (800 to 1000 days) but a proportion will survive. Positive-line sheep are \textit{Sip} sAsA or sApA (as sA is partially dominant) and develop SSBP/1 scrapie in 150 to 400 days depending on the genotype and the route of infection.

If PrP and \textit{Sip} are congruent, sequence differences might be expected between the PrP genes in sheep differing in their \textit{Sip} genotype. The ovine PrP gene has already been partially sequenced following cloning of DNA from a Suffolk sheep, and two variants differing at codon 171 (Gln or Arg) were identified. It was not possible to link this polymorphism directly to the scrapie response phenotype (or the \textit{Sip} genotype) because the
DNA-donor sheep was unavailable for experimental scrapie challenge. However, this PrP protein polymorphism was linked to a non-coding region EcoRI restriction fragment length polymorphism (RFLP), similar to one associated with Sip in NPU Cheviot sheep (Hunter et al., 1989) and to natural scrapie (Hunter et al., 1991).

In this study we have analysed the coding region of the PrP gene from NPU Cheviot sheep to search for amino acid differences in PrP which may be associated with the alleles of the Sip gene.

Methods

**Sheep.** The animals selected for PrP gene analysis were NPU Cheviots from the Institute for Animal Health, Neuropathogenesis Unit Edinburgh, U.K.

**DNA preparation and Southern analysis.** High M, DNA was made from blood, liver or brain tissue using the Applied Biosystems Nucleic Acid Extractor (340A) which uses a modification of the method of Blin & Stafford (1976). Digestion, electrophoresis and Southern transfer in the analysis of EcoRI and HindIII polymorphisms were carried out as described in Hunter et al. (1987) except that the probe was derived from the sheep PrP genomic clone, pNPU42, a Sau3A subclone of pSc23.4 (Goldmann et al., 1990). Modifications for the analysis of genomic DNA with RspXl were as follows: 10 μg digested DNA was separated on vertical 15 x 15 x 0.4 cm agarose gels (1 %; in 0.09 M-Tris-borate pH 8.0, 2 mM-EDTA, 0.1 μg/ml ethidium bromide) for 2.5 h at 50 mA. PrP gene from NPU Cheviot sheep to search for amino acid differences in PrP which may be associated with the alleles of the Sip gene.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>EcoRI</th>
<th>HindIII</th>
<th>RspXl</th>
</tr>
</thead>
<tbody>
<tr>
<td>prp-a*</td>
<td>e1</td>
<td>h1</td>
<td>r1</td>
</tr>
<tr>
<td>prp-b</td>
<td>e1</td>
<td>h2</td>
<td>r4</td>
</tr>
<tr>
<td>prp-c-1</td>
<td>e3</td>
<td>h1</td>
<td>r1</td>
</tr>
<tr>
<td>prp-c-2</td>
<td>e3</td>
<td>h1</td>
<td>r2, r3</td>
</tr>
<tr>
<td>prp-d</td>
<td>e3</td>
<td>h2</td>
<td>ND</td>
</tr>
<tr>
<td>prp-e</td>
<td>e2</td>
<td>h2</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Haplotype found in the Suffolk sheep described by Goldmann et al. (1990) but not found so far in NPU Cheviots.
† ND, Not done

**Results**

**RFLP analysis of the sheep PrP gene: description of fragments and haplotypes**

There are three EcoRI and HindIII fragments containing the PrP open reading frame (ORF) in NPU Cheviot sheep: e1, 6.8 kb; e2 5.2 kb; e3 4.0 kb (previously reported as 4.4 kb Hunter et al., 1989, 1991). With HindIII, two fragments have been found: h1, 5.0 kb and h2, 3.4 kb (Table 1 and Fig. 1a). Using these two restriction enzymes, 108 sheep from the NPU Cheviot flock have been analysed and have provided the following genotype frequencies.

Of the 108 sheep, all 31 negative-line animals (putative Sip pApA) exhibited the same genotype, e3e3/h1h1. The 77 positive-line animals (putative Sip sAsA or sApA) were heterogeneous, with 30% genotype e1e1/h2h2 and 61% genotype e1e3/h1h2. None of the positive-line animals was e3e3/h1h1. There is a clear association of e1 and h2 with Sip sA, and e3 and h1 with Sip pA. Fragment e2 has not yet been unambiguously associated with either Sip allele due to its low frequency (2%) in

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**Table 1. Sheep PrP gene RFLP haplotypes**

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>EcoRI</th>
<th>HindIII</th>
<th>RspXl</th>
</tr>
</thead>
<tbody>
<tr>
<td>prp-a*</td>
<td>e1</td>
<td>h1</td>
<td>r1</td>
</tr>
<tr>
<td>prp-b</td>
<td>e1</td>
<td>h2</td>
<td>r4</td>
</tr>
<tr>
<td>prp-c-1</td>
<td>e3</td>
<td>h1</td>
<td>r1</td>
</tr>
<tr>
<td>prp-c-2</td>
<td>e3</td>
<td>h1</td>
<td>r2, r3</td>
</tr>
<tr>
<td>prp-d</td>
<td>e3</td>
<td>h2</td>
<td>ND</td>
</tr>
<tr>
<td>prp-e</td>
<td>e2</td>
<td>h2</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Haplotype found in the Suffolk sheep described by Goldmann et al. (1990) but not found so far in NPU Cheviots.
† ND, Not done
Sip gene and PrP proteins in scrapie

Fig. 1. Restriction mapping of the ovine PrP gene. (a) Map of 11 kb of the PrP gene from NPU Cheviot sheep. The EcoRI and HindIII polymorphic sites are highlighted in black. The open box is the PrP protein coding region. (b) Map of 1.5 kb region of (a) showing RspXI sites 1 to 4 in/near the PrP coding region (open box). Polymorphic sites are highlighted in black, with the fragments r2 to r4 between RspXI sites 3/4, 1/3 and 1/2, respectively. Arrows indicate the position, direction and identification of the oligonucleotides used for in vitro amplification by PCR. The position of pNPU42 used as a probe in Southern analysis is indicated. (c) Southern blot analysis of four NPU Cheviot sheep DNA samples digested with RspXI. Lane 1, negative-line (Sip sAsA, PrP genotype prp-c-1/prp-c-2) sheep showing fragment r3 (0.6 kb); lanes 3 and 4, positive-line (Sip sAsA, prp-b/prp-b) sheep showing fragment r4 (0.55 kb); lane 2, positive-line (Sip sApA, prp-b/prp-c-2) sheep with both r3 and r4. Size markers are shown on the right (kb). A fragment of 0.9 kb (r2) is visible after a longer exposure only in lanes 1 and 2. The band at 1.5 kb is probably due to partial digestion.

NPU Cheviot sheep and lack of incubation period data. These results confirm and extend previous studies on this flock (Hunter et al., 1989, 1991).

(ii) RspXI
A third enzyme, RspXI, was shown to have polymorphic restriction fragments associated with the PrP gene (described in detail below). RspXI digestion of ovine genomic DNA generated the following restriction fragments which were detected using a PrP ORF probe: r1, >10 kb; r2, 0.9 kb; r3 0.6 kb and r4, 0.55 kb (Table 1). The locations of the restriction sites generating fragments r2 to r4 are shown in Fig. 1(b).

(iii) Haplotype
The restriction sites of the three endonucleases EcoRI, HindIII and RspXI in the PrP gene occur in many combinations (creating different PrP gene haplotypes), but in the NPU Cheviots, only five haplotypes (prp-b to prp-e) have been found so far, as summarized in Table 1. In the NPU Cheviots, fragment e1 appeared to be linked to h2 and r4 (prp-b), whereas e3 was linked to h1, r1, r2 and r3 (prp-c-1 and prp-c-2) except in two animals where it occurred in combination with h2 (prp-d). The ratio of prp-c-1 to prp-c-2 in the negative-line sheep is approximately 3:1, but only a small number of animals have been analysed for this difference so far. An association between e2 and h2 can be inferred from four positive-line sheep. The haplotype prp-a, found originally in a Suffolk sheep (Goldmann et al., 1990) has not yet been found in NPU Cheviots.

The sheep PrP gene protein coding region

(i) Sequencing of four NPU Cheviot PrP genes
Based on the EcoRI and HindIII RFLP analysis and scrapie incubation period data (Foster & Hunter, 1991), PrP-specific DNA amplification (oligonucleotide pair 284/285, see Fig. 1b) and PrP sequence analysis were initially performed on the following four sheep: sheep 1 and 2 (positive-line, PrP genotype elel/h2h2), incubation periods following s.c. injection with SSBP/1 scrapie were 156 and 175 days respectively (presumed Sip sAsA); sheep 3 and 4 (negative-line, PrP genotype e3e3/h1h1), survivors of s.c. injection with SSBP/1 for 1095 and 1825 days (presumed Sip sApA). Sequencing the entire PrP coding regions from these animals revealed only two differences between the two positive-line (1 and 2) and the two negative-line (3 and 4) sheep. These were at nucleotide 478 (T in sheep 1 and 2, C in sheep 3 and 4) and nucleotide 583 (A in sheep 1 and 2, G
in sheep 3 and 4). Fig. 2 shows details of the sequencing gels at these position in Sip sAsA and Sip pApA sheep and also in a Sip sApA heterozygote.

Both nucleotide differences lead to amino acid differences (codons 136 and 171) in the predicted PrP proteins. (The numbering system and description of protein variants is described in Methods.) The codon 171 difference (Gln 147 to Arg 147) has been described before (Goldmann et al., 1990) but the codon 136 difference (Ala 112 to Val 112) is novel.

These results suggest that Sip sAsA and Sip pApA Cheviots have only two amino acid differences in their predicted PrP proteins: Ala 112/Arg 147 in Sip pApA and Val 112/Gln 147 in Sip sAsA. In order to confirm this, the analysis was extended to include a larger number of sheep and in this way other PrP variants were found (see below).

(ii) RspXI analysis of NPU Cheviot sheep DNA
The C/T transition in nucleotide 478 created a new polymorphic restriction site for RspXI, a restriction enzyme which recognizes TCATGA (Val 112) but not C_CATGA (Ala 112). PrP-specific hybridization of RspXI-digested genomic DNA from Sip sAsA Cheviots resulted in visualization of a 0.55 kb fragment (r4) (Fig. 1 c, lanes 3 and 4), whereas most of the pApA Cheviots show a >10 kb fragment (r1) (not shown).

Further analysis of Cheviot sheep from the negative line (Sip pApA, e[e3]/h[11]) revealed another PrP haplotype, which exhibited a G/A transition in nucleotide 532 thereby creating a second polymorphic RspXI site in the PrP ORF. This site is just 54 nucleotides downstream of the first polymorphic RspXI site (and generates r3, 0.6 kb; Fig. 1 c, lane 1), but is associated with Sip pA. The mutation is predicted to result in an amino acid change at codon 154 (Arg 130 to His 130). His 130 was found in a PrP protein variant with Ala 112 and Gin 147 (PrP^AH; see summary Table 4).

To evaluate linkage of the Val/Ala 112 and His/Arg 130 polymorphisms to the Sip genotype, we used the two RspXI RFLPs as markers for the differences. The restriction sites were named RspXI-112 and RspXI-130 and digestion of the DNA at these sites identifies Val 112 and His 130, respectively. The linkage analysis is dependent on the detection of a very small difference (50 bp) between the two sites so we performed the RFLP analysis on PCR-amplified and purified DNA fragments (PCR oligonucleotide pair 331/138, Fig. 1 b). The results are shown in Table 2.

DNAs from eight positive-line Cheviots, characterized as Sip sAsA by incubation time and/or RFLP analysis, were entirely digested at RspXI-112 suggesting that both PrP alleles encode Val 112. Another 16 positive-line Cheviots, including six sheep predicted to
Table 2. RspXI RFLP analysis of the PrP gene in NPU Cheviot sheep

<table>
<thead>
<tr>
<th>Sheep line and putative Sip genotype</th>
<th>Restriction sites present (one or both alleles)</th>
<th>RspXI-112*</th>
<th>RspXI-130*</th>
<th>PstI†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive line (24‡)</td>
<td></td>
<td>Both</td>
<td>One</td>
<td>Both</td>
</tr>
<tr>
<td>sAsA or sApA</td>
<td></td>
<td>8</td>
<td>16</td>
<td>0 1</td>
</tr>
<tr>
<td>Negative line (11‡)</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0 3</td>
</tr>
</tbody>
</table>

* Presence of RspXI-112 site indicates PrP variant with Val 112 not Ala 112; presence of RspXI-130 site indicates PrP variant with His 130 not Arg 130.
† Control digestion.
‡ Number of sheep tested.

Table 3. NPU Cheviot sheep PrP protein sequence polymorphisms and association with the Sip gene

<table>
<thead>
<tr>
<th>Putative Sip genotype*</th>
<th>PrP protein genotype</th>
<th>PrP variants: amino acid position†</th>
</tr>
</thead>
<tbody>
<tr>
<td>sAsA (n* = 5)</td>
<td>PrP-b</td>
<td>112†</td>
</tr>
<tr>
<td>sApA (n = 6)</td>
<td>PrP-b</td>
<td>130†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>147†</td>
</tr>
</tbody>
</table>

* Sip genotype based on incubation period of SSBP/1 scrapie (s.c. injection) and EcoRI/HindIII RFLP analysis. The incubation period for five Sip sAsA sheep was 168 ± 14 (days ± s.d.), for six Sip sApA sheep was 309 ± 32 days, and four Sip pApA sheep had survived for 1095, 1825, > 760 and > 790 days (the last two are still alive) at the time of writing.
† Amino acids 112 and 130 were detected using RspXI DNA digestion and 147 was detected by DNA sequencing.
‡ n, Number of sheep.

be Sip sApA from incubation period data, consistently showed approximately half of their DNA digested at RspXI-112, suggesting that only one allele encodes Val 112. The same analyses performed on 11 negative-line Cheviots (presumed to be Sip pApA) were totally negative for RspXI-112 (both alleles encode Ala 112) but three of them were positive for RspXI-130 digestion (indicating His 130).

(iii) Analysis of association of PrP protein variants Gln/Arg 147 with alleles of Sip
As there is no restriction enzyme to distinguish between PrP gene alleles encoding Gln 147 and Arg 147, sheep DNAs were partially sequenced at this region. The results of this analysis at the three polymorphic positions in sheep challenged with SSBP/1 scrapie are summarized in Table 3. Arg 147 has so far been found only with Ala 112 giving a possible linkage with Sip pA; however, Gln 147 was found in association with both Val 112 and Ala 112, and heterozygous sheep (putative Sip sApA) encoding the protein variants PrpARR or PrpAHQ paired with PrpVRQ exhibited a significant difference in their scrapie incubation period. Gln/Arg 147 therefore seems less informative than the Val/Ala 112 polymorphism in NPU Cheviot sheep.

Discussion
Although non-coding region RFLP analysis of the PrP gene is a powerful predictor of disease susceptibility to both experimental and natural scrapie (Hunter et al., 1991), PrP protein polymorphisms may also provide information about mechanisms involved in disease. Our study of the protein coding region of PrP genes in NPU Cheviot sheep of various Sip genotypes has revealed four PrP protein variants, two of which are novel, and suggests that substitutions at amino acid 112 are associated with survival time differences following experimental challenge with scrapie source SSBP/1. Val 112 is associated with a short incubation period and with Sip sA, the dominant allele, and Ala 112 with a longer incubation period and with Sip pA. An incubation period slightly longer in putative Sip sApA than in putative Sip sAsA has been noted recently (Foster & Hunter, 1991) showing linkage with sheep PrP gene EcoRI and HindIII RFLP genotypes and we have demonstrated that the genotype analysis with RspXI, which in effect detects Val 112, shows the same linkage. Using the PrP protein variant terminology of Table 4, homozygous sheep (PrpVRQ/PrpVRQ or PrpAHQ) have a mean incubation period of 168 days, and heterozygous sheep (PrpVRQ/PrpARR or PrpAHQ) have a mean incubation period of 309 days.

In an assessment of the effect of the polymorphism reported in Goldmann et al. (1990), an association of Gln 147 with Sip sA and Arg 147 with Sip pA can be inferred from our data, as most of the negative-line sheep (Sip pApA) appear to encode PrpARR. However, PrpARR is encoded in other breeds with low susceptibility (N. Hunter, W. Goldmann & J. Hope, unpublished results), and heterozygous (putative Sip sApA) Cheviot
sheep encoding either PrPARR or PrPAPQ exhibited no significant difference in their incubation period.

The PrP variants we have detected may have been selected by SSBP/1 challenge of the sheep such that this scrapie isolate differentiates sheep carrying PrPVRQ from those with PrPAPQ, PrPARR, and PrPAPQ, and associates three PrP protein variants with negative-line sheep (which are all genetically classified Sip pApA). However, Dickinson et al. (1968), pointed out the variability of incubation times in NPU Cheviot negative-line sheep after intracerebral injection with SSBP/1 and suggested the possibility of subgroups in sheep with low susceptibility. Direct scrapie challenge of sheep with known PrP protein variants will be required to find whether our protein subgroups match Dickinson’s incubation period subgroups. In mice, allelic forms of the PrP gene have been linked to the selection of different strains of scrapie from natural isolates (Carlson et al., 1989; Bruce et al., 1991). It will be of great interest to see whether passage of scrapie in sheep expressing the different forms of PrP may have been linked to disease in humans; the hatched box indicates the stop transfer effector sequence of Yost et al. (1990).

**References**


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