Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon

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Different forms of the bovine PrP gene have five or six copies of a short, G-C-rich element within the protein-coding exon

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Current models of the virus-like agents of scrapie and bovine spongiform encephalopathy (BSE) have to take into account that structural changes in a host-encoded protein (PrP protein) exhibit an effect on the time course of these diseases and the survival time of any man or animal exposed to these pathogens. We report here the sequence of different forms of the bovine PrP gene which contain either five or six copies of a short, G-C-rich element which encodes the octapeptide Pro-His-Gly-Gly-Gly-Trp-Gly-Gln or its longer variants Pro-Gln/His-Gly-Gly-Gly-Gly-Trp-Gly-Gln. Out of 12 cattle, we found eight animals homozygous for genes with six copies of the Gly-rich peptide (6:6), while four were heterozygous (6:5). Two confirmed cases of BSE occurred in (6:6) homozygous animals.

Bovine spongiform encephalopathy (BSE) is a transmissible disease (Fraser *et al.*, 1988; Dawson *et al.*, 1990; Barlow & Middleton, 1990) which produces neuropathological lesions in cattle similar to those seen in ovine scrapie (Wells *et al.*, 1987) and the rare human dementias Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler syndrome (GSS) (Beck & Daniel, 1987). A cellular membrane protein (PrP) has a key role in the transmission and development of these diseases. This protein accumulates in the brain and other tissues during the protracted time course of these diseases and, in a disease-specific, protease-resistant isoform (SAF-PrP), has been purified by subcellular fractionation of scrapie, BSE or CJD-affected brain (Merz *et al.*, 1981, 1983; Diringer *et al.*, 1983; Hope *et al.*, 1988; McKinley *et al.*, 1983; Bolton *et al.*, 1982; Prusiner *et al.*, 1984).

Allelic variants in the protein coding regions of the ovine, murine and hamster PrP genes are linked to the relative survival time of these species following experimental exposure (inoculation) to strains or isolates of scrapie, BSE or CJD (Dickinson *et al.*, 1990; Mohri & Tateishi, 1989; Kingsbury *et al.*, 1983; Carp *et al.*, 1987; Westaway *et al.*, 1987; Lowenstein *et al.*, 1990; Goldmann *et al.*, 1990; Hunter *et al.*, 1989) and recent transgenic mouse experiments have elegantly confirmed the dependence of cross-species transmission efficiency on the PrP genotype of donor and recipient animals (Scott *et al.*, 1989).

The incidence of an ataxic form of GSS has been linked to a Leu for Pro change at codon 102 of the PrP gene (Hsiao *et al.*, 1989; Doh-ura *et al.*, 1989). Similarly, the incidence of familial CJD has been linked to a Lys for Glu substitution at codon 200 (Goldgaber *et al.*, 1989; Goldfarb *et al.*, 1990) or the presence of six extra Gly-rich octapeptide sequences in addition to the five normally seen in the N-terminal region of the human PrP protein (Owen *et al.*, 1990; Collinge *et al.*, 1989). These data and the occurrence of BSE have rekindled debate on the aetiology and persistence of these spongiform encephalopathies (Parry, 1979; Kimberlin, 1979; Westaway & Prusiner, 1990) and have led us to search for mutations of the bovine PrP gene which might predispose cattle to BSE. We report here the sequence of alleles of the bovine PrP gene which encode either five or six copies of the Gly-rich repeat peptide. Out of 12 cattle, we found eight animals homozygous for genes with six copies of the repeat peptide (6:6), while four were heterozygous (6:5). Two confirmed cases of BSE occurred in (6:6) homozygous animals.

Bovine DNA was purified from peripheral leukocytes sampled from nine Holstein/Friesian cows, a Holstein/Friesian bull, a BSE-affected Friesian bull and a BSE-affected Hereford bull. BSE was diagnosed on the basis of clinical signs and neuropathological lesions (Wells *et al.*, 1987). Oligonucleotide primers complementary to sequences of the ovine PrP gene (Goldmann *et al.*, 1990) were synthesized for use in the polymerase chain reaction (PCR) (Saiki *et al.*, 1985) and were used to amplify segments of bovine DNA. The PCR products were examined in ethidium bromide-stained agarose gels. In four cases, two bands of 495 bp and 471 bp were consistently visualized, but in the remaining eight

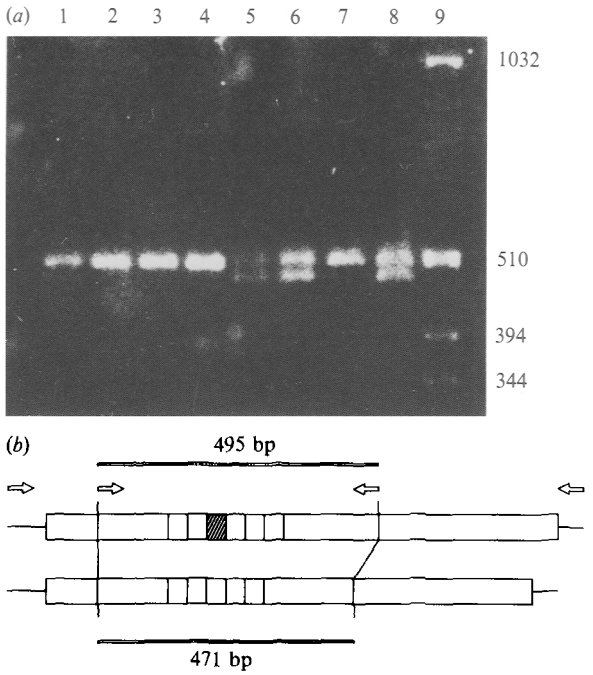

Fig. 1. (*a*) Ethidium bromide-stained agarose gel of PCR-amplified bovine DNA fragments. PCR-amplified bovine DNA fragments of 495 bp and/or 471 bp were produced using the oligonucleotides as shown in Fig. 1(*b*). Lanes 1 and 2 contain fragments obtained from BSE-affected cattle DNA, lanes 3 to 8 contain fragments obtained from unaffected cattle DNA and lane 9 contains DNA size standards (bp) (BRL). (*b*) Relative positions of PCR primer sequences and the fragments of PCR-amplified bovine DNA. The arrows indicate the positions of PCR primers and the direction of DNA amplification. The open reading frame of the bovine PrP gene is indicated by the large box, which includes the five or six octapeptide segments as smaller boxes. The extra segment (R3) found in some cattle DNA samples is shown cross-hatched.

(*a*)

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1   ATGGTGAAAAGCCACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGCCAT
      M  V  K  S  H  I  G  S  W  I  L  V  L  F  V  A  M
    GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT
      W  S  D  V  G  L  C  K  K  R  P  K  P  G  G  G  W
101 GGAACACTGGGGGGAGCCGATACCCAGGACAGGGCAGTCCTGGAGGCAAC
      N  T  G  G  S  R  Y  P  G  Q  G  S  P  G  G  N
    CGTTATCCACCTCAGGGAGGGGGTGGCTGGGGTCAGCCCCATGGAGGTGG
      R  Y  P  P  Q  G  G  G  G  W  G  Q  P  H  G  G  G
201 CTGGGGCCAGCCTCATGGAGGTGGCTGGGGCCAGCCTCATGGAGGTGGCT
      W  G  Q  P  H  G  G  G  W  G  Q  P  H  G  G  G  W
    GGGGTCAGCCCCATGGTGGTGGCTGGGGACAGCCACATGGTGGTGGAGGC
      G  Q  P  H  G  G  G  W  G  Q  P  H  G  G  G  G
301 TGGGGTCAAGGTGGTACCCACGGTCAATGGAACAAACCCAGTAAGCCAAA
      W  G  Q  G  G  T  H  G  Q  W  N  K  P  S  K  P  K
    AACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGGTAG
      T  N  M  K  H  V  A  G  A  A  A  A  G  A  V  V  G
401 GGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTTATA
      G  L  G  G  Y  M  L  G  S  A  M  S  R  P  L  I
    CATTTTGGCAGTGACTATGAGGACCGTTACTATCGTGAAAACATGCACCG
      H  F  G  S  D  Y  E  D  R  Y  Y  R  E  N  M  H  R
501 TTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAGTAACCAGA
      Y  P  N  Q  V  Y  Y  R  P  V  D  Q  Y  S  N  Q  N
    ACAACTTTGTGCATGACTGTGTCAACATCACAGTCAAGGAACACACAGTC
      N  F  V  H  D  C  V  N  I  T  V  K  E  H  T  V
601 ACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGATGAT
      T  T  T  K  G  E  N  F  T  E  T  D  I  K  M  M
    GGAGCGAGTGGTGGAGCAAATGTGCATTACCCAGTACCAGAGAGAATCCC
      E  R  V  V  E  Q  M  C  I  T  Q  Y  Q  R  E  S  Q
701 AGGCTTATTACCAACGAGGGGCAAGTGTGATCCTCTTCTCTTCCCCTCCT
      A  Y  Y  Q  R  G  A  S  V  I  L  F  S  S  P  P
    GTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG
      V  I  L  L  I  S  F  L  I  F  L  I  V  G  *
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(*b*)

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160-186 [R1] CCTCAGGGAGGGGGTGGCTGGGGTCAG
187-210 [R2] CCCCATGGA---GGTGGCTGGGGCCAG
211-234 [R3] CCTCATGGA---GGTGGCTGGGGCCAG
235-258 [R4] CCTCATGGA---GGTGGCTGGGGTCAG
259-282 [R5] CCCCATGGT---GGTGGCTGGGGACAG
283-309 [R6] CCACATGGTGGTGGAGGCTGGGGTCAA
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Pro His Gly Gly Gly Gly Trp Gly Gln

Fig. 2. (*a*) Nucleotide and amino acid sequences of the protein-coding region of the bovine PrP gene. (*b*) Nucleotide sequences of the octapeptide-encoding segments of the bovine PrP gene. The numbers on the left refer to the nucleotide positions of these segments in the sequence of Fig. 2(*a*). Note that in R1 His is replaced by Gln.

samples only the 495 bp band was seen (Fig. 1*a*). The location of the PCR primers indicated that these alleles differed in the protein-coding region of the bovine PrP gene (Fig. 1*b*). Direct analysis of the chromosomal copies of the PrP gene by vertical electrophoresis of digested genomic DNA (*Sau*3AI) in 2% agarose gels and Southern blot analysis provided PCR-independent verification of this PrP gene polymorphism (data not shown). The frequency of these PrP alleles in the U.K. national herd and their possible linkage to the incidence of BSE is under investigation.

To determine the exact nature of this sequence variation, isolated PrP-specific DNA fragments were either amplified asymmetrically to produce single-stranded copies which could be sequenced directly (Engelke *et al.*, 1988) or were cloned and sequenced (Sambrook *et al.*, 1989). The difference in these alleles lies in the number of Gly-rich peptides encoded by the 24 or 27 nucleotide (nt), G-C-rich elements on this gene (Fig. 2*a*, *b*). One allele has six copies of these elements (R1, R2, R3, R4, R5, R6) whereas another has five (R1, R2, R4, R5, R6). The five-copy allele has the same arrangement of the 27 nt (R1, R6) and 24 nt sequence elements (R2, R4, R5) as the sheep PrP; the six-copy allele contains a new 24 nt element (R3). Within a gene, each 24 nt element is unique since, although they all encode the octapeptide Pro-His-Gly-Gly-Gly-Trp-Gly-Gln, they also contain silent base changes which distinguish each repeat from its neighbours (Fig. 2*b*). Apart from rare human alleles in two pedigrees of CJD (Owen *et al*, 1990; Collinge *et al.*, 1989), genes from humans and all other species sequenced to date encode PrP proteins containing five copies of this sequence (Westaway *et al.*, 1987; Goldmann *et al.*, 1990; Oesch *et al.*, 1985; Lowenstein *et al.*, 1990; Locht *et al.*, 1986; Liao *et al.*, 1987; Kretzschmar *et al.*, 1986). If the (5:5) homozygote is regarded as producing the original form of bovine PrP, then comparision of the sequences of the

five- and six-copy variants suggests that the mutant allele arose by insertion (by duplication and mutation) of the R3 element, nt 211 to 234 (Fig. 2*b*).

Sequence analysis from three independent animals, one (6:5) heterozygote and two (6:6) homozygotes determined only one further polymorphism (a silent C to T transition) within the protein-coding region at nt 576. The T in this position deletes a *Hin*dII site, creating an 8 kb instead of a 0·6 kb genomic restriction fragment, which so far has only been found in the allele with six octapeptide-coding elements. However, this restriction fragment length polymorphism is not informative as there is also a six-copy allele with the *Hin*dII site. The full amino acid sequence is given in Fig. 2(*a*). (The DNA sequence is deposited in the EMBL sequence data library, accession number X55882.)

Direct amino acid sequencing of the BSE fibril protein (Hope *et al.*, 1988) indicates an amino-terminal sequence of Lys-Lys-Arg. . . (codons 25 to 27) which is identical to that in other species but which has an extra Gly residue in the cow and sheep sequences between codons 31 to 32 (Goldmann *et al.*, 1990). Residues 1 to 24 are cleaved from the protein during its biosynthesis and act as a signal peptide. The amino acid sequences of the five- and six-copy variants were otherwise identical, and this highly conserved protein showed only six or seven differences from ovine PrP while retaining other structural features previously identified. The allelic forms of bovine PrP and rare human alleles found in two pedigrees of familial CJD differ in the number of Pro-His/Gln-Gly-Gly/(X)-Gly-Gly-Trp-Gly-Gln sequences they contain in the N-terminal region of the protein. The functional importance of this domain is evidenced by strong conservation of the number and sequence of this structural motif between the PrP proteins from different species. Similar Pro, Gly-rich segments are found in other proteins where they have been predicted to fold into a coiled-spring structure. For example, collagen forms flexible, fibrillar structures whose formation and subsequent polymerization into supramolecular bundles appears to be mediated by such Pro, Gly-rich repetitive sequences. Partial deletion or point mutations of the collagen Gly-X-Y motif lead in man to the autosomal dominant disease osteogenesis imperfecta type II, which can be mimicked in transgenic mice (Stacey *et al.*, 1988). Insertion of one or more extra copies of the Pro, Gly motif into the PrP protein might produce similar interference with its normal function, enhancing host susceptibility to environmental factors. Indeed, mutations in different regions of the PrP protein have been linked to the survival time of rodents and sheep exposed to scrapie (Westaway *et al.*, 1987; Lowenstein *et al.*, 1990; Goldmann *et al.*, 1990; Hunter *et al.*, 1989) or to the incidence of GSS or familial CJD (Hsiao *et al.*, 1989; Doh-ura *et al.*, 1989; Goldgaber *et al.*, 1989; Goldfarb *et al.*, 1990).

The question of whether these data indicate that BSE is a genetic disease of cattle or a genetic predisposition to infection cannot yet be answered but, in this context, epidemiological analysis of BSE is overwhelmingly in favour of a food-borne source of disease, with the exposure of cattle to a scrapie-like agent in feedstuffs containing ruminant-derived protein (Wilesmith *et al.*, 1988). Current transgenic studies (Scott *et al.*, 1989) will help define the molecular mechanism(s) through which amino acid changes in the PrP protein might influence the incidence and course of disease following exposure to scrapie or BSE agents and aid an assessment of the risk of inter-species exchange of these diseases.

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