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Chapter 1

Spongiform Encephalopathies: An Introduction to the Mysterious Etiological Agent

The spongiform encephalopathies or prion diseases affect both humans and animals (Table 1). They appear spontaneously only in humans, in small ruminants (sheep, goats and moufflon), and rarely in mule deer and elk (Table 1). However, the range of animal species that can be either accidentally or experimentally infected is large. At the time of writing, the disease has appeared in mink, cattle, other bovids and felines because of scrapie-contaminated food supplies. In humans, spongiform encephalopathies may occur with various clinical and neuropathological characteristics and consist of sporadic and familial Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker syndrome (GSS), fatal familial insomnia (FFI) and kuru. Except for kuru, which is confined to New Guineans living in a few of the valleys in the highlands of Papua-New Guinea and now disappearing, the diseases are found worldwide and distinguishing between them is not always possible. GSS, FFI and about 10% of CJD cases occur in families with an autosomal dominant pattern of inheritance, yet they are experimentally transmissible to laboratory animals. Scrapie of sheep and goats has been known to European veterinarians for more than two centuries (Parry, 1983) and until the mid-1960s was the only spongiform encephalopathy that was known to be caused by a transmissible agent.

Clinically, spongiform encephalopathies are characterised by behavioural abnormalities, which in humans always progress toward dementia and various neurological manifestations, including myoclonus, pyramidal, extrapyramidal, cerebellar or visual signs. The clinical course is inevitably fatal with a duration of 2–3 weeks to several months. The anatomical lesions are always limited to the central nervous system and are usually characterised by spongiform changes in the grey matter and variable degrees of neuronal loss and astrocytosis (Liberski and Budka, 1993; Bell and Ironside, 1993; Fraser, 1993). Occasionally, there are amyloid plaques of different morphology (Liberski *et al.*, 1993a) which are composed of the disease-specific, partially protease-resistant, amyloid protein PrP-res*. Most of the time, however, no amyloid deposition is observed under the

*This term (from Coughey *et al.*, 1990) is analogous to PrPsc which, however, assumes the unproven association of the protein with the infectious agent. On the contrary, PrP-res emphasises the partial resistance of the pathological protein to proteinase treatment which differentiates it operationally from the normal, proteinase sensitive protein (PrP-sen).

Table 1. Spongiform encephalopathies or prion diseases of man and animals

Natural host	Disease		Years of description/ transmission*
Man	Creutzfeldt-Jakob disease (CJD)		1920–21[1]/1968[2]
	Gerstmann-Sträussler-Scheinker syndrome (GSS)		1928[3]/1981[4]
	Fatal Familial Insomnia (FFI)		1986[5]/NT
Sheep	Scrapie		~1750[6]/1936[7]
Goat			1942[8]/1976[9]
Moufflon			1992[10]/NA
Mule deer	Chronic wasting disease (CWD)		1980[11]/1982[12]
Elk			1982[13]/NA
Accidential host		**Mode of transmission**	
Man	Kuru	Cannibalism	1957[14]/1966[15]
	Creutzfeldt–Jakob disease	Corneal transplantation	1974[16]
		Stereotactic EEG	1977[17]
		Neurosurgery	1980–82[18]
		Cadaveric human pituitary growth hormone	1985[19]
		Cadaveric dura mater graft	1987[20]
		Cadaveric human pituitary gonadotrophin hormone	1990[21]

Table 1. Continued

Natural host	Disease		Years of description/ transmission*
Sheep	Scrapie	Louping-ill vaccine	1946[22]
Farmed milk	Transmissible mink encephalopathy (TME)	Food borne (carcasses)	1965[23]/1967[24]
Domestic cattle	Bovine spongiform encephalopathy (BSE)	Food borne (bone and meat meal)	1987[25]/1988[26]
Nyala	Bovid spongiform encephalopathy	Food borne (bone and meat meal)	1988[27]/1993[28]
Gemsbok			1988[27]/NA
Eland			1990[29]/NA
Greater kudu			1990[30]/1993[28]
Arabian oryx			1990[30]/NA
Domestic cat	Feline spongiform encephalopathy (FSE)	Food borne (meat or pet food)	1990[31]/1993[28]
Puma			1992[32]/NA
Cheetah			1992[33]/NA

*NT, not yet successfully transmitted (Brown *et al.*, 1994b); NA, transmission not attempted; [1](Creutzfeldt, 1920, 1921; Jakob, 1921a, b, c, [2](Gibbs *et al.*, 1968), [3](Gerstmann, 1928; Gerstmann *et al.*, 1936), [4](Masters *et al.*, 1981), [5](Lugaresi *et al.*, 1986), [6](Parry, 1983), [7](Cuillè and Chelle, 1936), [8](Chelle, 1942), [9](Dickinson, 1976), [10](Wood *et al.*, 1992), [11](Williams and Young, 1980), [12](Williams *et al.*, 1982), [13](Williams and Young, 1982), [14](Gajdusek and Zigas, 1957), [15](Gajdusek *et al.*, 1966), [16](Duffy *et al.*, 1974), [17](Bernoulli *et al.*, 1977), [18](Foncin *et al.*, 1980; Will and Matthews, 1982), [19](Brown *et al.*, 1985; Gibbs *et al.*, 1985; Koch *et al.*, 1985; Powell Jackson *et al.*, 1985), [20](Prichard *et al.*, 1987), [21](Cochius *et al.*, 1990), [22](Gordon, 1946), [23](Hartsough and Burger, 1965), [24](Zlotnik and Barlow, 1967), [25](Wells *et al.*, 1987), [26](Fraser *et al.*, 1988), [27](Jeffrey and Wells, 1988), [28](Bruce, 1993), [29](Fleetwood and Furley, 1990), [30](Kirkwood *et al.*, 1990), [31](Wyatt *et al.*, 1990), [32](Willoughby *et al.*, 1992), [33](Peet and Curran, 1992).

microscope, yet the brain of affected individuals is loaded with PrP-res, so that these disorders are also referred to as "hidden amyloidoses" (Diringer, 1992). Since the accumulation of PrP-res precedes the histological lesions and the clinical appearance of the disease (Bolton *et al.*, 1991; Czub *et al.*, 1986; Xi *et al.*, 1992), its formation is the principal pathogenic mechanism of these disorders. PrP-res derives from a post-translational or, most likely, a conformational modification of a cellular 'normal' protein (PrP-sen*), but what is responsible for it and why the affected cell starts making the pathological protein is still unknown.

The spongiform encephalopathies resemble other neurodegenerative disorders, such as Alzheimer's disease, yet they are unique because of their transmissibility to experimental animals after an incubation period which may be as long as decades. They are caused by a transmissible agent whose nature, however, is still unknown and is now the subject of great controversy.

Of the many theories proposed, three of them are still feasible in light of the large amount of experimental and clinical data which have been collected in the last ten years (Fig. 1). The most provocative hypothesis considers the etiological agent to be composed of only a modified host protein and devoid of nucleic acid. Although it was proposed more than 25 years ago (Gibbons and Hunter, 1967; Griffith, 1967), nowadays this theory is mainly advocated, although with different prospects, by Stanley Prusiner and the Nobel laureate Carleton D. Gajdusek. Prusiner proposed the term 'prion' to indicate scrapie and related agents and to distinguish them from other known microorganisms, including viruses and viroids (Prusiner, 1982). Prion is the acronym for *pro*teinaceous *in*fectious particle and, although the presence of an as yet unidentified nucleic acid is not dismissed (Prusiner, 1993), it is considered to be entirely composed of the modified aggregate host protein PrP-res (Fig. 1A). Gajdusek embraced the protein only theory (Gajdusek, 1986), although from a different viewpoint and yet he continues to call these infectious agents viruses, meaning 'nothing more than invisible replicating parasites that required the energy and the informational systems of the host for their replication' (Gajdusek, 1993a).

Fig. 1. Simplified models for the replication of scrapie and related agents and for the formation of PrP-res according to the prion (A), virus (B), virino (C) and the unified theory of Weissmann (D). In the 'protein only' model (referred to as the prion hypothesis), PrP-res is the infectious agent which derives from the conformational change of PrP-sen. Step 1 is an extremely rare event when PrP-sen is not mutated (wild-type) but becomes more frequent, although still rare, when PrP-sen carries one of the mutations found in familial cases. Once the first PrP-res homodymer is produced (2) or is exogenously introduced (3) in the host, the conformational change from PrP-sen to PrP-res occurs at an exponential rate. In the virus hypothesis (B), PrP-sen is the viral receptor on the cell surface and its conformational change to PrP-res is driven by the virus. The virino is composed of an exogenous nucleic acid (black diamonds) surrounded by PrP-res (C). Here, it is speculated that the binding of the virino nucleic acid with PrP-sen is responsible for the conformational change from PrP-sen to PrP-res. In the unified theory of Weissmann, both the nucleic acid (coprion) and the protein (apoprion) of the infectious agent (holoprion) derive from the host and independently replicate in the cell. The apoprion (PrP-res) replicates as the prion. The coprion is responsible for the phenotypic properties which differentiate the various strains of scrapie and related agents.

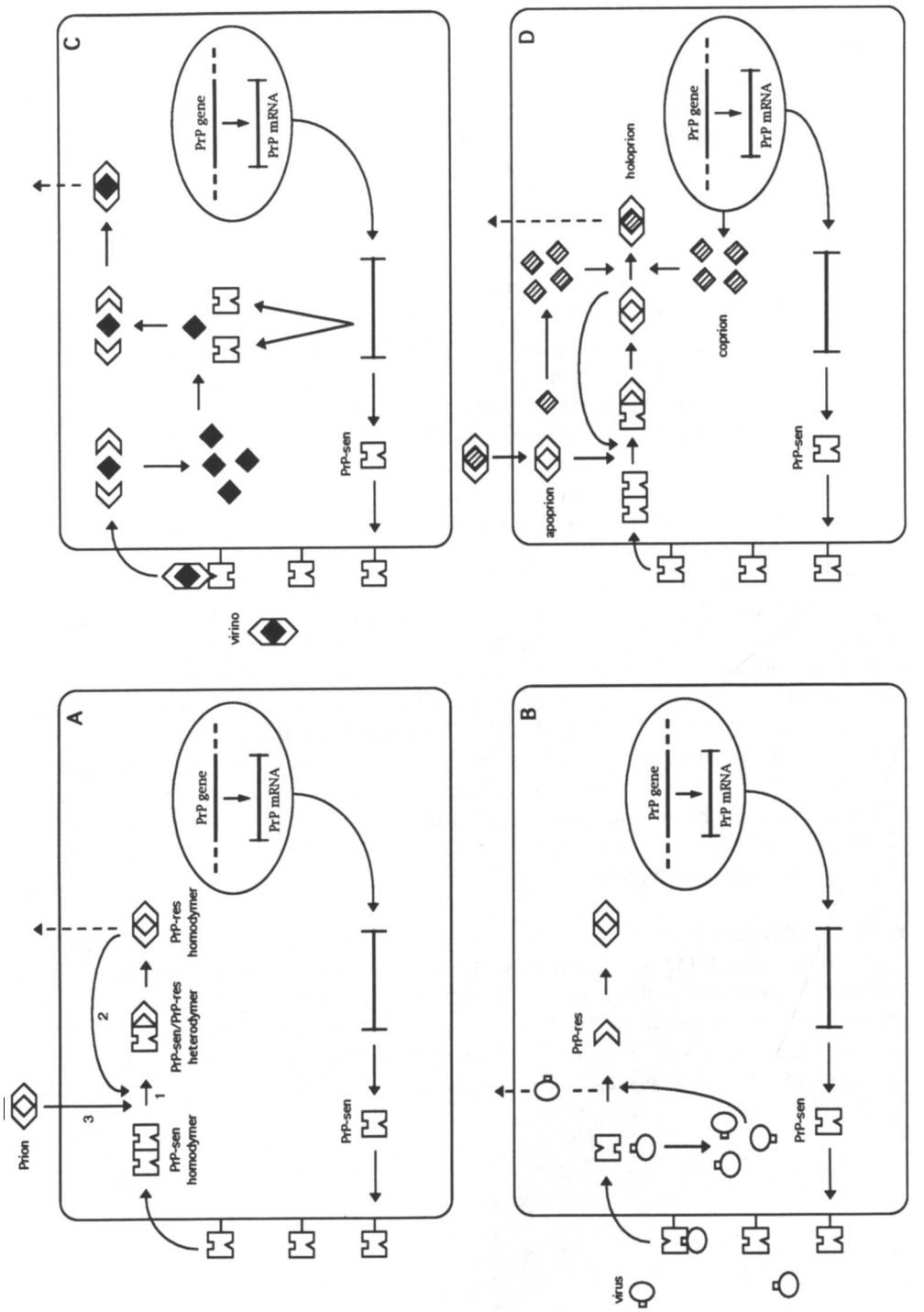

Fig. 1. Continued

A more conservative theory proposes that the infectious agent is a real virus (Aiken and Marsh, 1990; Rohwer, 1991; Diringer, 1992) (Fig. 1B) with bizarre biological and chemical-physical properties and therefore named 'unconventional virus' (Gajdusek, 1977). However, no nucleic acids nor endogenous proteins have ever been associated with infectivity (Diener *et al.*, 1982; Kellings *et al.*, 1992; Meyer *et al.*, 1991; Murdoch *et al.*, 1990; Oesch *et al.*, 1988; Sklaviadis *et al.*, 1990) and no immune response has ever been detected in the infected host (Brown, 1990b; Casaccia *et al.*, 1990; Berg, 1994) which implies that the virus-encoded protein(s) is not antigenic. The absence of specific anti-virus antibodies made the identification and purification of the putative virus unfeasible.

The third legitimate hypothesis was initially proposed by Dickinson and Outram (1979) who envisaged the infectious agent to be composed of an exogenous non-protein-coding nucleic acid surrounded by a host-tissue component (Dickinson and Outram, 1983), such as the prion protein (Dickinson and Outram, 1988; Kimberlin, 1990) (Fig. 1C). A possible variant of this hypothesis is that the nucleic acid derives from the host as well and that it is not required for infectivity (Weissmann, 1991) (Fig. 1D).

In the last ten years many excellent review articles have been devoted to the prion theory which, however, had given the impression that it was very well supported (DeArmond and Prusiner, 1993; Prusiner, 1982, 1987, 1993; Ridley and Baker, 1993). This theory gained credit and stimulated the imagination of many scientists regarding how a protein particle devoid of nucleic acids can replicate and induce different clinical and pathological entities in the same host. Stanley Prusiner must be credited for this challenging hypothesis that, if proved true, will open new avenues for the study of degenerative and infectious disorders. However, until then, it seems correct to me (and to other scientists as well) that other possible hypotheses on the nature of the scrapie and related agents, such as the 'virino' or the 'virus' ones, should not yet be discarded. However, none of these three major hypotheses on the nature of the infectious agent, taken alone, can entirely explain the different aspects of these disorders. The objective of this review is to analyse the clinical and epidemiological characteristics of spongiform encephalopathies and to interpret them in light of each theory.

To maintain objectivity throughout the manuscript, I decided to use generic or descriptive terms and refer to the hypothetical terminologies, such as 'prion', 'virino', 'virus' and related terms, only in regard to the relative specific hypothesis. Thus, the term 'agent' will be preferred to 'prion' or 'virino' or 'virus' to indicate the etiological particle causing scrapie and related disorders and 'spongiform encephalopathies' will be used instead of 'prion diseases' or 'virus-induced amyloid disorders' (Brown *et al.*, 1993).

Chapter 2

The Prion Protein and its Encoding Gene

In 1981, Merz and co-workers (Merz *et al.*, 1981) made the fundamental observation that detergent fractions of scrapie-infected brains were loaded with abnormal, disease-specific fibrils, which they called scrapie-associated fibrils (SAF; also called prion rods, Prusiner *et al.*, 1983) (see Fig. 2). The authors made the important observation that although SAF were amyloid-like fibrils, they were also present in scrapie-infected brains showing no amyloid-plaques at histology. SAF were subsequently observed in the brain of patients with CJD (Merz *et al.*1983a, 1984) and GSS (Merz *et al.*, 1983a,b), of sheep with natural scrapie (Merz *et al.*, 1984), of bovine with BSE (Hope *et al.*, 1988b) and of elk with chronic wasting disease (Guiroy *et al.*, 1993). Moreover, SAF were also found in the brains of animals with experimental spongiform encephalopathy

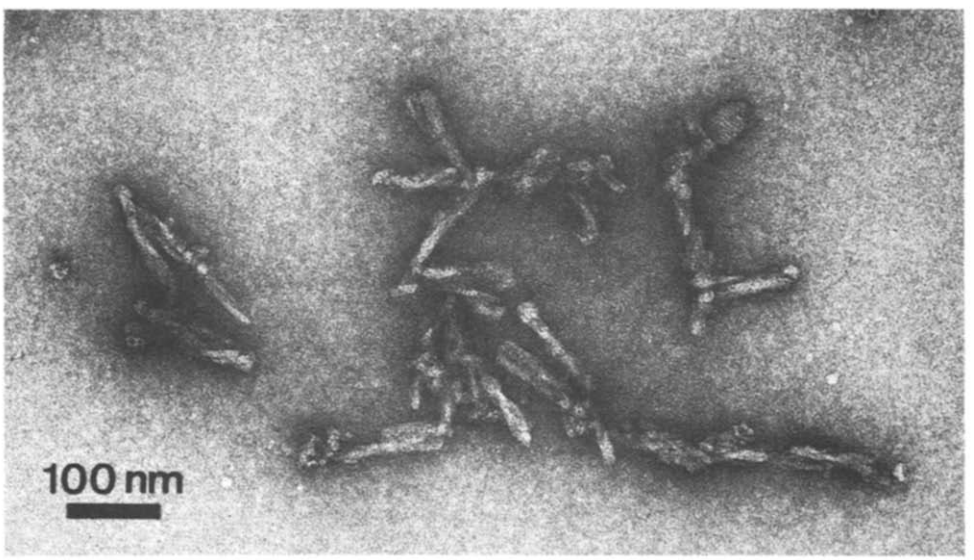

Fig. 2. Electron microscopy of scrapie-associated fibrils (SAF) from scrapie-infected hamster brain. By courtesy of Dr M. Özel, Robert Koch Institute, Berlin, Germany.

(Merz *et al.*, 1981, 1983a,b, 1984; Diringer *et al.*, 1983) but in none of the samples obtained from animals not inoculated or from patients with other neurological disorders including Alzheimer's disease (Merz *et al.*, 1983b). It was therefore immediately clear that SAF were a unique feature of spongiform encephalopathies and it was assumed that these fibrils might represent either the etiological agent of these diseases or a specific pathological product caused by the infectious agent (Diringer *et al.*, 1983; Merz *et al.*, 1984).

The major, if not the only, component of the SAF is the prion protein (Diringer *et al.*, 1983; Prusiner *et al.*, 1983). Originally, the protease resistance fragment of PrP-res (PrP27-30) was discovered in fractions of hamster brain enriched for scrapie infectivity, but not in uninfected brains (Bolton *et al.*, 1982; Prusiner *et al.*, 1982a). Although the possibility that PrP27-30 represented a pathological product of scrapie infection was not dismissed, this result encouraged the notion that PrP belonged to the infectious agent. This belief was further supported by the finding that antibodies raised against the hamster PrP-res (Bendheim *et al.*, 1984; Diringer *et al.*, 1984) immunostained PrP27-30 purified from the brains of CJD patients (Bockman *et al.*, 1985; Bode *et al.*, 1985; Manuelidis *et al.*, 1985; Brown *et al.*, 1986b) and of sheep with natural scrapie (Agrimi *et al.*, 1992) (Fig. 3). However, the determination of the N-terminal sequence of PrP27-30 (Prusiner *et al.*, 1984) led to the discovery that the gene encoding for PrP-res was a cellular gene (Chesebro *et al.*, 1985; Oesch *et al.*, 1985) and therefore also present in uninfected animals and that the amount of PrP mRNA was the same in

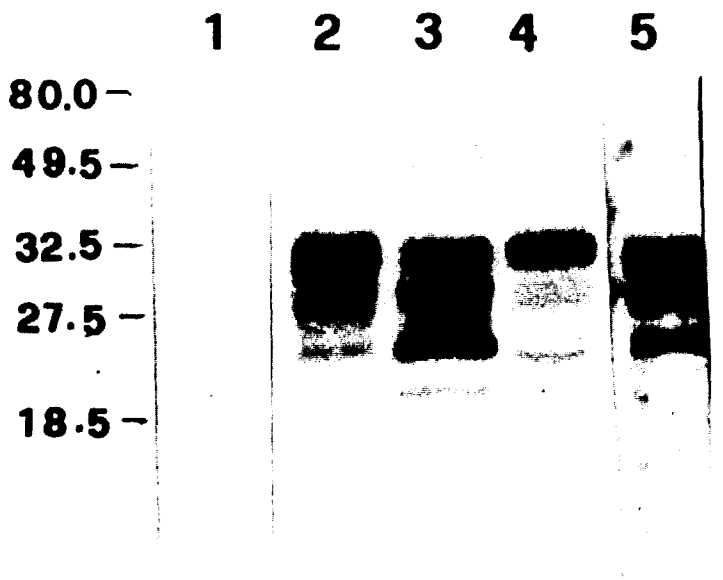

Fig. 3. Western blot of PrP27-30 in purified fraction of brains from hamster with experimental scrapie (lane 2), human with CJD (lane 3), cow with BSE (lane 4) and sheep with natural scrapie (lane 5). PrP27-30 was immunostained with rabbit polyclonal antibody against hamster PrP27-30. Standard molecular weights (lane 1).

the brains of scrapie-infected versus uninfected animals (Chesebro *et al.*, 1985; Oesch *et al.*, 1985; Caughey *et al.*, 1988). The immediate next step was the Western blot identification in uninfected animals of the normal equivalent of PrP-res which has a slightly larger molecular weight and a much greater sensitivity to protease treatment than PrP27-30 (Meyer *et al.*, 1986). This normal isoform was named PrPc (cellular) or PrP-sen (proteinase sensitive). It follows that PrP27-30 is a likely artefact of the purification procedure and that it is derived from a larger precursor following treatment with proteinase K. Omitting proteinase K in the purification procedure of PrP-res led to the realization that the precursor protein has an apparent molecular weight of 33–35 KDa which, under partial treatment with proteinase K, reproduces PrP27-30.

A brief account of the PrP gene and its transcription is given below (for a more detailed description of this subject see Basler *et al.*, 1986; Oesch *et al.*, 1991; Goldmann, 1993).

The gene

The PrP gene (named PRNP in humans, *prn-p* in mice and PrP gene in other species) has been sequenced in humans (Kretzschmar *et al.*, 1986b; Puckett *et al.*, 1991), ruminants (Goldmann *et al.*, 1990, 1991b; Poidinger *et al.*, 1993), rodents (Basler *et al.*, 1986; Locht *et al.*, 1986; Robakis *et al.*, 1986b; Westaway *et al.*, 1987; Lowenstein *et al.*, 1990; Gomi *et al.*, 1994) and in mink (Kretzschmar *et al.*, 1992b) (see Fig. 4). The gene is located on the short arm of chromosome 20 in humans (Robakis *et al.*, 1986a; Sparkes *et al.*, 1986) and on chromosome 2 in mice (Sparkes *et al.*, 1986). A homologous gene has been described in chicken (Gabriel *et al.*, 1992), but whether it has the same function as in mammals remains unknown. The supposed presence of PrP gene in invertebrates (Westaway and Prusiner, 1986) seems, at the moment, excluded (Iwasaki *et al.*, 1992).

The protein encoding region (ORF) and the 3′ untranslated mRNA region are located in a single exon in all the species (Fig. 5). The 5′ leader sequence of PrP mRNA is located on one (human, hamster) (Basler *et al.*, 1986; Puckett *et al.*, 1991) or two (sheep, mouse) (Büeler *et al.*, 1992; Goldmann, 1993) small 5′ exons which are separated from the ORF-containing exon by 1 or 2 introns of about 10–14 kb total size (Basler *et al.*, 1986). The promoter region contains no identifiable TATA box, is very rich in GC repeats (Basler *et al.*, 1986) and this feature makes the PrP gene nearer to the so-called 'house-keeping' or 'constitutive' genes, that is, genes that are expressed in all cells because they provide basic functions needed for sustenance of all cell types (Lewin, 1990). Indeed, the PrP mRNA is present in many tissues, including brain, spleen, lung, intestine and heart, in different cell types of neuronal and non-neuronal origins (Oesch *et al.*, 1985; Robakis *et al.*, 1986b; Brown H.R. *et al.*, 1990) and in many kinds of cell cultures (Caughey *et al.*, 1988). The amount of PrP mRNA is high in the CNS (Kretzschmar *et al.*, 1986a) and, outside the brain, varies considerably from tissue to tissue. Interestingly, no correlation has been found between PrP mRNA synthesis and the ability of tissues to replicate the scrapie agent (Robakis *et al.*, 1986b). PrP gene expression is detectable in mouse and rat embryos (Lieberburg, 1987; Manson *et al.*, 1992) and increases in the brain during development (McKinley *et al.*, 1987). Moreover, the finding that expression of the PrP gene is up-regulated by nerve growth factor

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                1           11          21          31          41          50
                |           |           |           |           |           |
Hum       1     ATGGCG**** **AACCTTGG CTGCTGGATG CTGGTTCTCT TTGTGGCCAC
SHa       1     ------**** **-------A- --A----C-- ----CA---- -------T-T
AHa       1     ------**** **-------A- --A----C-- ----CA---- -------T--
CHa       1     -----A**** **-------A- --A----C-- ----CA---- -------T--
Mo-a      1     ------**** **--------- --A----C-- ----CC---- -----A-T-T
Mo-b      1     ------**** **--------- --A----C-- ----CC---- -----A-T-T
Rat       1     ------**** **--------- --A----C-- ----CC---- -----A-T--
Mink      1     ----T-AAAA GCC--A-A-- -A-----C-C ---------- ----------
Sheep     1     ----T-AAAA GCC--A-A-- -A-T-----C ---------- ---------T
Bovine    1     ----T-AAAA GCC--A-A-- -A-T-----C ---------- ---------T
Kudu      1     ----T-AAAA GCC--A-A-- -A-T-----C -----C---- ---------T
Oryx      1     ----T-AAAA GCC--A-A-- -A-T-----C ---------- ---------T

                51          61          71          81          91          100
                |           |           |           |           |           |
Hum       45    ATGGAGTGAC CTGGGCCTCT GCAAGAAGCG CCCGAAGCCT ***GGAGGAT
SHa       51    G----C---T G-T------- ---------- G--A------ ***-----G-
AHa       51    G----C---T G-T------- ----A----- G--A------ ***-----G-
CHa       51    G----C---T G-T------- ---------- G--A------ ***-----G-
Mo-a      51    G----C---T G-C------- ----A----- G--A------ ***-----G-
Mo-b      51    G----C---T G-C------- ----A----- G--A------ ***-----G-
Rat       51    ---T-C---T G-T------- ----A----- G--A------ ***-----G-
Mink      51    ---------- A-T---T--- ---------- G--A------ GGA-----C-
Sheep     51    G--------- G--------- ---------- A--A--A--- GGC-------
Bovine    51    G--------- G--------- ---------- A--A--A--- GGA-------
Kudu      51    G--------- G---C----- ---------- A--A--A--- GGA-------
Oryx      51    G--------- G--------- ---------- A--A--A--- GGt-------

                101         111         121         131         141         150
                |           |           |           |           |           |
Hum       92    GGAACACTGG GGGCAGCCGA TACCCGGGGC AGGGCAGCCC TGGAGGCAAC
SHa       101   ---------- C--A------ -----T---- ---------- ----------
AHa       101   ---------- T--A------ -----T---- ---------- ----------
CHa       101   ---------- T--A------ -----T---- ---------- ----------
Mo-a      101   -------C-- T--A-----G --T--C---- ----A----- ----------
Mo-b      101   -------C-- T--A-----G --T--C---- ----A----- ----------
Rat       101   ---------- T--A-----G -----T---- ----A----- ----------
Mink      101   ---------- ---G------ -----A---- -------T-- ----------
Sheep     101   ---------- ---G------ --------A- -------T-- ----------
Bovine    101   ---------- ---G------ -----A--A- -------T-- ----------
Kudu      101   ---------- ---G------ --------A- -------T-- ----------
Oryx      101   ---------- ---A------ --------A- -------T-- ----------

                151         161         171         181         191         200
                |           |           |           |           |           |
Hum       142   CGCTACCCAC CTCAGGGCGG TGGTGGCTGG GGGCAGCCTC ATGGTGGTGG
SHa       151   --T------- -------T-- C--CACA--- -----A--C- ----------
AHa       151   --T------- -------T-- ---CACA--- -----A--C- ----------
CHa       151   --T------- -------T-- ---CAC---- -----A--C- ----------
Mo-a      151   --T------- -------T** *--CAC---- --------C- -C--------
Mo-b      151   --T------- -------T** *--CAC---- --------C- -C--------
Rat       151   --T------- -----A-T-- ----AC---- --------C- ----------
Mink      151   ---------- -C------T-- ---C------ --C-----C- -C--G-----
Sheep     151   -----T---- -------A-- G--------- --T-----C- ----A-----
Bovine    151   --T--T---- -------A-- A--------- --C------- ----A-----
Kudu      151   -----T---T -------A-- G--------- --T-----C- ----A-----
Oryx      151   -----T---- -------A-- G--------- --T-----C- ----A-----
```

Fig. 4. Continued

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                201         211         221         231         241         250
                |           |           |           |           |           |
Hum  192  CTGGGGGCAG CCTCATGGTG GTGGCTGGGG GCAGCCCCAT GGTGGTGGCT
SHa  201  ------A--- --C------- ---------- A--------- ----------
AHa  201  ------A--- --C------- ---------- A--A--T--- --------T-
CHa  201  A-----A--- --C------- ---------- A--A--T--- --------T-
Mo-a 198  ------A--A --C-----G- -CA------- A--A--T--- ------A-T-
Mo-b 198  ------A--A --C-----G- -CA------- A--A--T--- ------A-T-
Rat  201  ------A--A ---------- ---------- A--A--T--- ----------
Mink 201  ------A--- --C--C--G- ---------- T--------C --G-------
Sheep 201 ------C--A --------A- ---------- T--------- ----------
Bovine 201 ------C--- --------A- ---------- T--------- ----------
Kudu 201  ------C--- --C-----A- ---------- T--------- --------T-
Oryx 201  ------C--A --------A- ---------- T--------- ----------

                251         261         271         281         291         300
                |           |           |           |           |           |
Hum  242  GGGGACAGCC TCATGGTGGT GGC***TGGG GTCAAGGAGG TGGCACCCAC
SHa  251  ----T----- C--------- ---***---- ---------- ----------
AHa  251  ----T----- C--------- ---***---- ---------- ----------
CHa  251  ----T----- C--------- ---***---- ---------- ----------
Mo-a 248  ----T----- C-----C--- --A***---- -C-------- G--T-----T
Mo-b 248  ----T----- C-----C--- --A***---- -C-------- G--T-----T
Rat  251  ----T----- C-----C--G ---***---A ---------- G--T-----T
Mink 251  ---------- G--------C --TGGC---- -------T-- ---G-G----
Sheep 251 ---------- A--------- --AGGC---- -------T-- -***-G----
Bovine 251 ---------- A--------- --AGGC---- -------T-- -***------
Kudu 251  ---------- G--------- --AGGC---- -------T-- -***------
Oryx 251  ---------- A--------- --AGGC---- -------T-- -***------

                301         311         321         331         341         350
                |           |           |           |           |           |
Hum  289  AGTCAGTGGA ACAAGCCGAG TAAGCCAAAA ACCAACATGA AGCACATGGC
SHa  301  -A-------- -------C-- ---------- ---------- ----------
AHa  301  -A-------- -------C-A ---------- ----G----- ----T-----
CHa  301  -A-------- -------C-- ---------- ---------- ----TG----
Mo-a 298  -A-------- -------C-- C--A------ ------C-C- ----TG----
Mo-b 298  -A-------- -------C-- C--A------ ------T-C- ----TG----
Rat  301  -A-------- -------C-- C--------- ------C-C- ----TG----
Mink 301  G--------G G------C-- ------C--- ---------- ----TG----
Sheep 298 ---------- -------C-- ---------- ---------- ----TG----
Bovine 298 G----A---- ----A--C-- ---------- ---------- ----TG----
Kudu 298  G--------- -------C-- ---------- ---------- -A--TG----
Oryx 298  ---------- -------C-- ---------- ---------- ----TG----

                351         361         371         381         391         400
                |           |           |           |           |           |
Hum  339  TGGTGCTGCA GCAGCTGGGG CAGTGGTGGG GGGCCTTGGC GGCTACATGC
SHa  351  C--C-----T --G--A---- -C-------- ---------T ----------
AHa  351  A--------T --G------- ---------- ---------G ----------
CHa  351  A-----G--T --G------- -G-------- ---------G ----------
Mo-a 348  A--G-----G ---------- ----A----- ---------T ----------
Mo-b 348  A--G-----G ---------- ----A----- ---------T ----------
Rat  351  A--G-----C ---------- ----A----- ---------T ---------T
Mink 351  G--A--C--- -----C---- -G--C----- ------G--- ----------
Sheep 348 A--A-----T --------A- -------A-- ---------T ----------
Bovine 348 A--A-----T --------A- -------A-- ---------T ----------
Kudu 348  A--A-----T -----G--A- -------A-- ---------T ----------
Oryx 348  A--A-----T --------A- -------A-- ---------A ----------
```

Fig. 4. Continued

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                401         411         421         431         441         450
                |           |           |           |           |           |
Hum  389  TGGGAAGTGC CATGAGCAGG CCCATCATAC ATTTCGGCAG TGACTATGAG
SHa  401  ----G----- ---------- -----G--G- ----T----A -----GG---
AHa  401  ----G----- ----------A -----GC-C- ----T----A -----GG---
CHa  401  ----G--C-- ---------- -----GC-C- ----T----A -----GG---
Mo-a 398  ----G--C-- ---------- -----G--C- ----T----A C----GG---
Mo-b 398  ----G--C-- ---------- -----G--C- ----T----A C----GG---
Rat  401  ----G----- ---------- -----GC-C- ----T----A C----GG---
Mink 401  ----G--C-- ---------- ---C----T- ----T----A C---------
Sheep 398 ---------- ---------- --TC-T---- ----T----A ----------
Bovine 398 ---------- ---------- --TC-T---- ----T----- ----------
Kudu 398  ---------- ---------- --TC-T---- ----T----- ----------
Oryx 398  -------C-- ---------- --TC-T---- ----T----A ----------

                451         461         471         481         491         500
                |           |           |           |           |           |
Hum  439  GACCGTTACT ATCGTGAAAA CATGCACCGT TACCCCAACC AAGTGTACTA
SHa  451  -----C----- -C-------- ----A----C -----T---- -------T--
AHa  451  -----C----- -C-------- ----A----C -----T---- ----------
CHa  451  -----C----- -C-------- ----A----C -----T---- ----------
Mo-a 448  -----C----- -C-------- ----T----C -----T---- ----------
Mo-b 448  -----C----- -C-------- ----T----C -----T---- ----------
Rat  451  -----C----- -C--A----- ----T----- -----T---- ----------
Mink 451  -----C----- -C-----G-- ----T----C ---------- ----------
Sheep 448 ---------- ---------- ----T----- ---------- ----------
Bovine 448 ---------- ---------- ---------- ---------- ----------
Kudu 448  ---------- ---------- ----T----- ---------- ----------
Oryx 448  ---------- -C-------- ----T----- ---------- ----------

                501         511         521         531         541         550
                |           |           |           |           |           |
Hum  489  CAGGCCCATG GATGAGTACA GCAACCAGAA CAACTTTGTG CACGACTGCG
SHa  501  -C----AG-- --CC------ A--------- ---------- -----T--T-
AHa  501  -C----AG-- --CC------ A--------- ------C--- --------T-
CHa  501  -C----AG-- --CC------ A--------- ---------- --------T-
Mo-a 498  ------AG-- ---C------ ---------- ------C--- ----------
Mo-b 498  ------AG-- ---C------ ---------- ------C--- ----------
Rat  501  ------GG-- ---C------ ---------- ------C--- --------T-
Mink 501  --A---GG-- ---C------ ---------- ------C--- --T-------
Sheep 498 ---A--AG-- ---CG---T- -T-------- ---------- --T-----T-
Bovine 498 ------AG-- ---C----T- -T-------- ---------- --T-----T-
Kudu 498  ------AG-- ---C----T- -T-------- ---------- --T-----T-
Oryx 498  ---A--AG-- ---C----T- -T-------- ---------- --T-----T-

                551         561         571         581         591         600
                |           |           |           |           |           |
Hum  539  TCAATATCAC AATCAAGCAG CACACGGTCA CCACAACCAC CAAGGGGGAG
SHa  551  ----C----- C--------- -----A---- ----C----- ----------
AHa  551  ---------- G-----A--- --T--A---- ----C----- ----------
CHa  551  ---------- G--------- --T--A---- ----C----- ----------
Mo-a 548  ---------- C--------- ---------- ----C----- ----------
Mo-b 548  ---------- C---------G T---C----- ----------
Rat  551  ---------- C--------- --T--A---- ----C----- ----------
Mink 551  ----C----- GG-------- --------G- ----C----- ------C---
Sheep 548 ----C----- -G-------A -----A---- ----C----- ----------
Bovine 548 ----C----- -G-----G-A -----A---- ----C----- ----------
Kudu 548  ----C----- -G-------- -----A---- ----C----- ----------
Oryx 548  ----C----- -G-------A -----A---- ----C----- ----------
```

Fig. 4. Continued

```
                601         611         621         631         641         650
                |           |           |           |           |           |
Hum      589    AACTTCACCG  AGACCGACGT  TAAGATGATG  GAGCGCGTGG  TTGAGCAGAT
SHa      601    --------G-  --------A-  C-----A---  ----------  -G--------
AHa      601    --------G-  ----------  C---------  ----------  -G--------
CHa      601    --------G-  ----------  C---------  ----------  -G--------
Mo-a     598    ----------  -------T--  G---------  ----------  -G--------
Mo-b     598    ----------  -------T--  G---------  ----------  -G--------
Rat      601    --------G-  ----------  G---------  -----T----  -G--------
Mink     601    --------G-  --------A-  G-----C---  ----------  -G--------
Sheep    598    ----------  -A--T---A-  C-----A---  -----A----  -G-----A--
Bovine   598    ----------  -A--T---A-  C---------  -----A----  -G-----A--
Kudu     598    ----------  -A--T---A-  C---------  -----A----  -G-----A--
Oryx     598    ----------  -A--T---A-  C-----C---  -----A----  -G-----A--

                651         661         671         681         691         700
                |           |           |           |           |           |
Hum      639    GTGTATCACC  CAGTACGAGA  GGGAATCTCA  GGCCTATTAC  CAG***AGAG
SHa      651    -----C----  -----TC---  A---G--C--  ------C---  G-TGGA---A
AHa      651    ----G-----  -----TC---  A---G--C--  ------C--T  G-CGGA---A
CHa      651    ----G-----  -----TC---  A---G--C--  ------C---  G-CGGA---A
Mo-a     648    ---CG-----  ------C---  A---G--C--  ----------  G-CGGG---A
Mo-b     648    ---CG-----  ------C---  A---G--C--  ----------  G-CGGG---A
Rat      651    ---CG-----  -----TC---  A---G--C--  ----------  G-CGGG---A
Mink     651    ----G-----  ------C--C  -A--G--CG-  ---T--C---  ---***--G-
Sheep    648    ---C------  ------C---  -A-----C--  ---T------  --A***--G-
Bovine   648    ---C--T---  ------C---  -A-----C--  ---T------  --A***C---
Kudu     648    ---C------  ------C---  -A-----CG-  ---T------  --A***C---
Oryx     648    ---C------  ------C---  -A-----C--  ---T------  --A***----

                701         711         721         731         741         750
                |           |           |           |           |           |
Hum      686    GATCGAGC**  *ATGGTCCTC  TTCTCCTCTC  CACCTGTGAT  CCTCCTGATC
SHa      701    -G--C---**  *GC---G--G  --------C-  -T--------  ------C--T
AHa      701    ----C---**  *GC---G---  --------C-  -T--------  ------C--T
CHa      701    ----C---**  *GC---G---  ----------  -T--------  ------C--T
Mo-a     698    ----C---AG  C-CC--G--T  --------C-  -T-----C--  ------C---
Mo-b     698    ----C---AG  C-CC--G--T  --------C-  -T-----C--  ------C---
Rat      701    ----T---**  *GCC--G--T  --------C-  -T--------  ------C---
Mink     701    -GG-----**  *GCCA-----  -----GC-C-  -T--C-----  ------C---
Sheep    698    -GG-A--T**  *G--A-----  --T--T--C-  -T--------  ------C---
Bovine   698    -GG-A--T**  *G--A-----  -----T--C-  -T--------  ------C---
Kudu     698    -GG-A--T**  *G-CA-----  -----T--C-  -T--------  ------C---
Oryx     698    -GG-A--T**  *G--A-----  -----T--C-  -T--------  ------C---

                751         761         771
                |           |           |
Hum      733    TCTTTCCTCA  TCTTCCTGAT  AGTGGGATGA
SHa      751    --C--T----  ----------  G---------
AHa      751    --C-------  ----------  --------***
CHa      751    --C-------  ----------  ----------
Mo-a     748    --C-------  ----------  C---------
Mo-b     748    --C-------  ----------  C---------
Rat      751    --C-------  ----------  C---------
Mink     751    --AC-G----  -TC-------  --------***
Sheep    748    ----------  -T--T--C--  ---A----AG
Bovine   748    ----------  -T--T--C--  ---A----AG
Kudu     748    ----------  -T--T--C--  ---A----AG
Oryx     748    ----------  -T--T--C--  ---A----AG
```

Fig. 4. Alignment of the nucleotide sequences of the open reading frames (ORF) of the PrP gene from humans (Hum, from Kretzschmar *et al.*, 1986), hamsters (SHa, Syrian, from Basler *et al.*, 1986; AHa, Armenian and CHa, Chinese, from Lowenstein *et al.*, 1990) mice (Mo-a, allele a and Mo-b, allele b, most likely the *s7* and *p7* alleles of the sinc gene, respectively, from Westaway *et al.*, 1987), rats (from Gomi *et al.*, 1994), mink (from Kretzschmar *et al.*, 1992b), sheep (from Goldmann *et al.*, 1990), bovine (from Goldmann *et al.*, 1991b), great kudu and oryx (from Poidinger *et al.*, 1993).

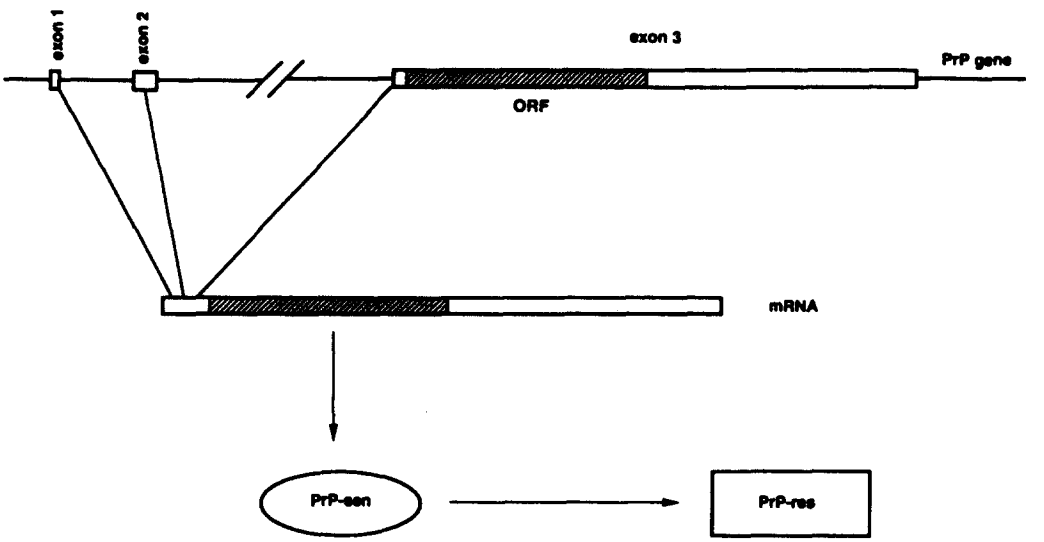

Fig. 5. Schematic representation of the PrP gene, its transcript (mRNA) and its product (PrP-sen). The entire open reading frame (ORF, shaded box) is located on a single exon in all the species. The 5′ leader sequence of the transcript is encoded either on exon 1 only (humans, hamsters) or exon 1 and 2 together (sheep, mice). PrP-sen is the product of the PrP gene both in normal and infected cells. In infected cells PrP-sen is then modified by an unknown mechanism in PrP-res.

(Mobley *et al.*, 1988; Wion *et al.*, 1988) and most likely by the HIV-Tat protein (Muller *et al.*, 1992), implies that regulatory elements may control the expression of this gene. Although these findings denote that the PrP gene is important for development and for normal cellular function, the ablation of this gene in mice does not apparently result in a modification of mice development, behaviour or immunological status (Büeler *et al.*, 1992).

The Protein

The predicted primary structures of PrP consist of 253 (human), 254 (rodents) 256 (ruminants) and 257 (mink) amino acids and have a calculated molecular weight of 27,700–29,000 prior to post-translational modifications (Fig. 6). The protein has a number of interesting features that are listed below.

The protein has a stretch of 22 (hamster sequence) hydrophobic residues at the N-terminal (signal peptide, in italics in Fig. 6) that target the protein to the endoplasmic reticulum and that are removed in the mature protein (Hope *et al.*, 1986; Bolton *et al.*, 1987; Turk *et al.*, 1988; Safar *et al.*, 1990). The N-terminal is the less conserved region of the protein except in mink and all ruminants where it shows a great homology.

A possible unknown post-translational modification of Arg residues in position 25 and 37 (hamster sequence) has been postulated (Hope *et al.*, 1986, 1988a; Bolton *et al.*,

1987; Turk *et al.*, 1988; Safar *et al.*, 1990; Stahl *et al.*, 1993) (in bold in Fig. 6). Five tandem repeats of 8/9 amino acids are present between residues 51/54 and 90/95 which are glycine-rich and very conserved among different species (underlined in Fig. 6). In humans, the deletion or insertion of one repeat has been reported in normal subjects (see Chapter 3). An extra octapeptide repeat has also been found in bovine without, however, influencing the susceptibility to BSE (Goldmann *et al.*, 1991b). Digestion of PrP-res with proteinase K removes about 60–70 (depending on the species) amino acids from the N-terminal of the mature protein (practically all the repeats are removed) yielding PrP27-30 (Oesch *et al.*, 1985; Meyer *et al.*, 1986; McKinley *et al.*, 1986; Bendheim *et al.*, 1988; Hope *et al.*, 1988a); this polypeptide aggregates into amyloid fibrils (Somerville *et al.*, 1989; Isomura *et al.*, 1991; McKinley *et al.*, 1991a) and is associated with infectivity (Diringer *et al.*, 1983; McKinley *et al.*, 1983a).

Both PrP-sen and PrP-res are N-glycosylated at 181^{Asn} and 197^{Asn} (hamster sequence, in bold in Fig. 6) (Bolton *et al.*, 1985; Multhaup *et al.*, 1985; Sklaviadis *et al.*, 1986; Haraguchi *et al.*, 1989) with heterogeneous, complex-type oligosaccharides (Endo *et al.*, 1989; Haraguchi *et al.*, 1989). This variability may account for the heterogeneous appearance of PrP during separation by electrophoresis (Ceroni *et al.*, 1990). These sugars are not essential for PrP-res formation (Rogers *et al.*, 1990; Taraboulos *et al.*, 1990).

Two cysteine residues, 179^{Cys} and 214^{Cys} (hamster sequence, in bold in Fig. 6), are covalently bonded in a disulfide linkage (Turk *et al.*, 1988). A C-terminal peptide is removed from both PrP-sen and PrP-res upon addition of a membrane anchor to 231^{Ser} (hamster sequence, in italics in Fig. 6) (Stahl *et al.*, 1987, 1990a, 1992; Baldwin *et al.*, 1990). However, only PrP-sen is released from the cell membrane by enzymatic treatment under non-denaturing conditions (Caughey *et al.*, 1990; Stahl *et al.*, 1990b; Safar *et al.*, 1991) implying that PrP-res accumulates inside the cell.

The current knowledge regarding the metabolism of PrP-sen and PrP-res derives from studies in neural cell cultures which are persistently infected with scrapie (Caughey *et al.*, 1989; Borchelt *et al.*, 1990, 1992; Taraboulos *et al.*, 1992; Chesebro *et al.*, 1993; Shyng *et al.*, 1993). As a glycoprotein, PrP synthesis starts in the endoplasmic reticulum and proceeds through the Golgi apparatus before reaching the surface of the cell where it is anchored to the cytoplasmic membrane by the glycoinositol-phospholipid moiety (Caughey *et al.*, 1989). Until this point, both PrP-sen and the precursor of PrP-res follow the same metabolic pattern described above (Caughey and Raymond, 1991; Borchelt *et al.*, 1992). However, while PrP-sen is then either released into the medium or rapidly metabolised (the half-life time is about 6 hr) via endocytosis by intracellular degradation in lysosomes (Borchelt *et al.*, 1990; Caughey *et al.*, 1989; Caughey and Raymond, 1991), the turnover of PrP-res is very slow or absent (Borchelt *et al.*, 1990; Caughey and Raymond, 1991) and it appears to accumulate in the lysosomes (Caughey and Raymond, 1991; Caughey *et al.*, 1991a; McKinley *et al.*, 1991b). These studies indicate that the formation of PrP-res occurs after the precursor has reached the cell surface.

	1				5					10					15					20					25
Hum	*Met*	*Ala*	*	*	*Asn*	*Leu*	*Gly*	*Cys*	*Trp*	*Met*	*Leu*	*Val*	*Leu*	*Phe*	*Val*	*Ala*	*Thr*	*Trp*	*Ser*	*Asp*	*Leu*	*Gly*	*Leu*	*Cys*	Lys
SHa	-	-	*	*	-	-	Ser	Tyr	-	Leu	-	Ala	-	-	-	-	Met	-	Thr	-	Val	-	-	-	-
AHa	-	-	*	*	-	-	Ser	Tyr	-	Leu	-	Ala	-	-	-	-	-	-	Thr	-	Val	-	-	-	-
CHa	-	-	*	*	-	-	Ser	Tyr	-	Leu	-	Ala	-	-	-	-	-	-	Thr	-	Val	-	-	-	-
Mo *a*	-	-	*	*	-	-	-	Tyr	-	Leu	-	Ala	-	-	-	Thr	Met	-	Thr	-	Val	-	-	-	-
Mo *b*	-	-	*	*	-	-	-	Tyr	-	Leu	-	Ala	-	-	-	Thr	Met	-	Thr	-	Val	-	-	-	-
Rat	-	-	*	*	-	-	-	Tyr	-	Leu	-	Ala	-	-	-	Thr	-	Cys	Thr	-	Val	-	-	-	-
Mink	-	Val	Lys	Ser	His	Ile	-	Ser	-	Leu	-	-	-	-	-	-	-	-	-	-	Ile	-	Phe	-	-
Sheep	-	Val	Lys	Ser	His	Ile	-	Ser	-	Ile	-	-	-	-	-	-	Met	-	-	-	Val	-	-	-	-
Bovine	-	Val	Lys	Ser	His	Ile	-	Ser	-	Ile	-	-	-	-	-	-	Met	-	-	-	Val	-	-	-	-
Kudu	-	Val	Lys	Ser	His	Ile	-	Ser	-	Ile	-	-	-	-	-	-	Met	-	-	-	Val	Ala	-	-	-
Oryx	-	Val	Lys	Ser	His	Ile	-	Ser	-	Ile	-	-	-	-	-	-	Met	-	-	-	Val	-	-	-	-

	26				30					35					40					45					50
Hum	Lys	Arg	Pro	Lys	Pro	*	Gly	Gly	Trp	Asn	Thr	Gly	Gly	Ser	Arg	Tyr	Pro	Gly	Gln	Gly	Ser	Pro	Gly	Gly	Asn
SHa	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AHa	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHa	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mo *a*	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mo *b*	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Rat	-	-	-	-	-	*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mink	-	-	-	-	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sheep	-	-	-	-	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Bovine	-	-	-	-	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kudu	-	-	-	-	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	Gly	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Fig. 6. Continued

	51			55				60				65				70						75
Hum	Arg	Tyr	50 Pro	Pro	Gln	Gly	Gly	55 Gly	Gly	Trp	Gly	Gln	60 Pro	His	Gly	Gly	65 Gly	Trp	Gly	Gln	Pro	His	70 Gly	Gly	Gly
SHa	-	-	50 -	-	-	-	-	55 -	Thr	-	-	-	60 -	-	-	-	-	65 -	-	-	-	-	70 -	-	-
AHa	-	-	-	-	-	-	-	-	Thr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHa	-	-	-	-	-	-	-	-	Thr	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mo *a*	-	-	50 -	-	-	-	-	*	55 Thr	-	-	-	-	60 -	-	-	-	-	65 -	-	-	-	-	70 -	Ser
b																									
Rat	-	-	50 -	-	-	Ser	-	55 -	Thr	-	-	-	60 -	-	-	-	-	65 -	-	-	-	-	70 -	-	-
Mink	-	-	-	-	55 -	-	-	-	-	60 -	-	-	-	-	65 -	-	-	-	-	70 -	-	-	-	75 -
Sheep	-	-	-	-	55 -	-	-	-	-	60 -	-	-	-	-	65 -	-	-	-	-	70 -	-	-	-	75 -
Bovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kudu	-	-	-	Ser	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	76			80				85				90				95						100
Hum	Trp	Gly	75 Gln	Pro	His	Gly	Gly	80 Gly	Trp	Gly	Gln	Pro	85 His	Gly	Gly	Gly	*	Trp	90 Gly	Gln	Gly	Gly	Gly	95 Thr	His
SHa	-	-	75 -	-	-	-	-	80 -	-	-	-	-	85 -	-	-	-	*	-	90 -	-	-	-	-	95 -	-
AHa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-
CHa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	*	-	-	-	-	-	-	-	-
Mo *a*	-	-	-	75 -	-	-	-	Ser	80 -	-	-	-	85 -	-	-	-	*	-	-	90 -	-	-	-	-	95 -
b																									
Rat	-	-	75 -	-	-	-	-	80 -	-	-	-	-	85 -	-	-	-	*	-	90 Ser	-	-	-	-	95 -	-
Mink	-	-	-	-	80 -	-	-	-	-	85 -	-	-	-	-	90 -	-	Gly	-	-	95 -	-	-	-	100 Ser	-
Sheep	-	-	-	-	80 -	-	-	-	-	85 -	-	-	-	-	90 -	-	Gly	-	-	95 -	-	-	*	Ser	-
Bovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Gly	-	-	-	-	-	*	-	-
Kudu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Gly	-	-	-	-	-	*	-	-
Oryx	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Gly	-	-	-	-	-	*	-	-

Fig. 6. Continued

	101					105				110				115				120				125
Hum	Ser	Gln	Trp	Asn	Lys	Pro	Ser	Lys	Thr	Asn	Met	Lys	His	Met	Ala	Gly	Ala	Ala	Ala	Gly	Ala	Val
				100		Leu						Leu						Val				
SHa	Asn	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AHa	Asn	-	-	-	-	-	Asn	-	-	-	Ser	-	-	-	-	-	-	-	-	-	-	-
CHa	Asn	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	-	-	-	-	-
					100					105				110				115				120
Mo *a*	Asn	-	-	-	-	-	-	-	-	-	Leu	-	-	Val	-	-	-	-	-	-	-	-
b											*Phe*											
				100						105				110				115				120
Rat	Asn	-	-	-	-	-	-	-	-	-	Leu	-	-	Val	-	-	-	-	-	-	-	-
				105						110				115				120				125
Mink	Gly	-	-	Gly	-	-	-	-	-	-	-	-	-	Val	-	-	-	-	-	-	-	-
	100					105				110				115				120				
Sheep	-	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	-	-	-	-	-
Bovine	Gly	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	-	-	-	-	-
Kudu	Gly	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	-	-	-	-	-
	126			130					135				140				145					150
Hum	Val	Gly	Gly	Leu	Gly	Gly	Tyr	Met	Leu	Gly	Ser	Ala	Met	Ser	Arg	Pro	Ile	Ile	His	Phe	Gly	Ser	Asp	Tyr	Glu
			125					*Val*	130					135				140				145		stop	
SHa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Met	Met	-	-	-	Asn	-	Trp	-
AHa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Met	Leu	-	-	-	Asn	-	Trp	-
CHa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Met	Leu	-	-	-	Asn	-	Trp	-
			125						130					135				140				145			
Mo *a*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Met	-	-	-	-	Asn	-	Trp	-
b																									
			125						130					135				140				145			
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Met	Leu	-	-	-	Asn	-	Trp	-
			130						135					140				145				150			
Mink	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Leu	-	-	-	-	Asn	-	-	-
	125			130					135					140				145							
Sheep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Leu	-	-	-	-	Asn	-	-	-
Bovine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Leu	-	-	-	-	-	-	-	-
Kudu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Leu	-	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Leu	-	-	-	-	Asn	-	-	-

Fig. 6. Continued

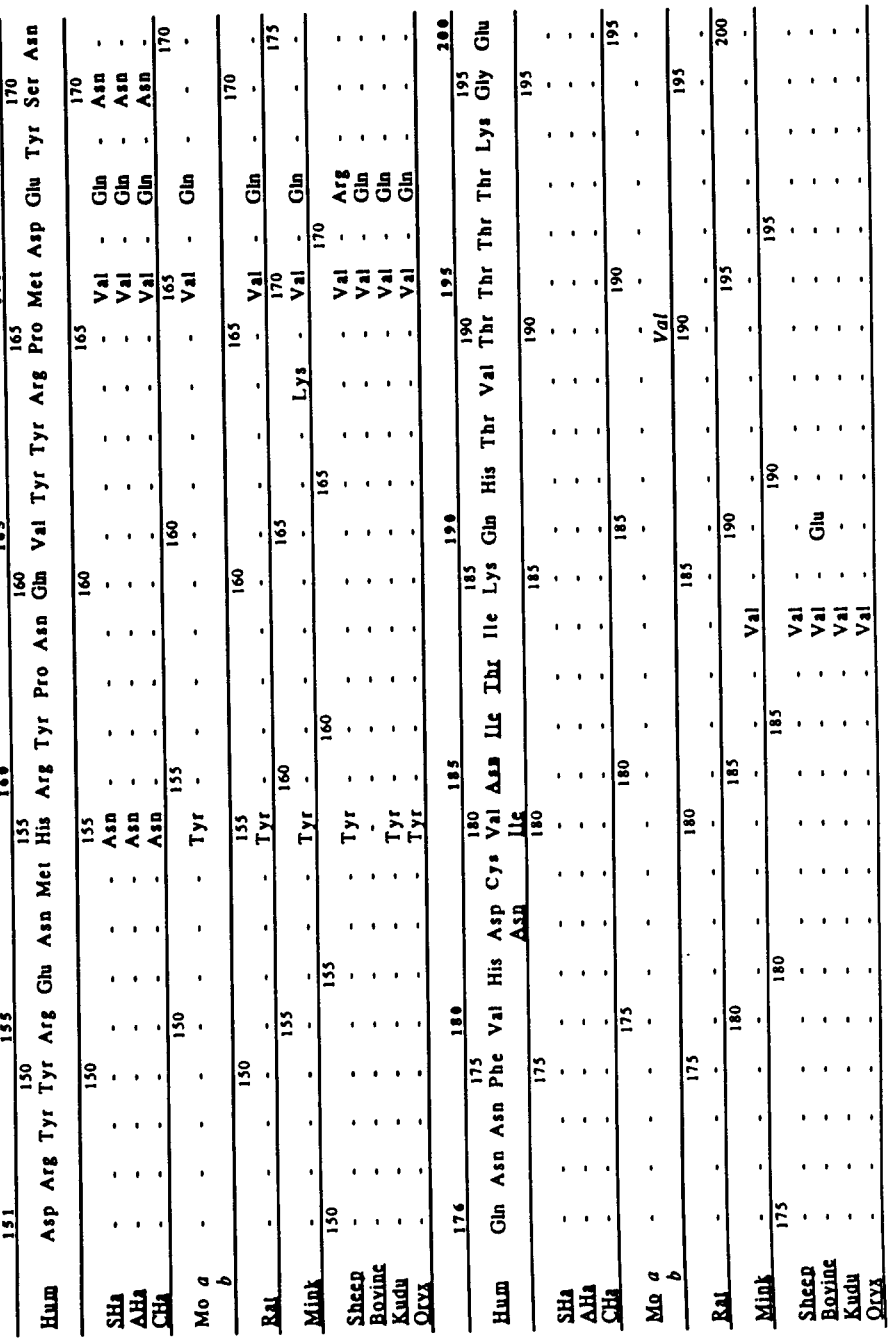

Fig. 6. Continued

	201				205				210				215				220				225
Hum	Asn	Phe Thr Ser	Glu Lys	Thr	Asp	Val	Lys	Met	Glu	Arg	Val	Val Ile	Glu	Gln	Met	Cys	Ile	Thr	Gln Arg	Tyr	Glu	Arg	Glu
			200					205				210					215					220	
SHa	-	-	-	-	-	Ile	-	Ile	-	-	-	-	-	-	-	-	Thr	-	-	-	Gln	Lys	-
AHa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	Gln	Lys	-
CHa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	Gln	Lys	-
			200					205				210					215					220	
Mo *a*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	Gln	Lys	-
b																							
			200					205				210					215					220	
Rat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Val	-	-	-	Gln	Lys	-
			205					210				215					220					225	
Mink	-	-	-	-	-	Met	-	Ile	-	-	-	-	-	-	-	-	Val	-	-	-	Gln	-	-
	200				205				210				215				220						
Sheep	-	-	-	-	-	Ile	-	Ile	-	-	-	-	-	-	-	-	-	-	-	-	Gln	-	-
Bovine	-	-	-	-	-	Ile	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Gln	-	-
Kudu	-	-	-	-	-	Ile	-	-	-	-	-	-	-	-	-	-	-	-	-	-	Gln	-	-
Oryx	-	-	-	-	-	Ile	-	Ile	-	-	-	-	-	-	-	-	-	-	-	-	Gln	-	-
		226		230					235				240				245					250	
Hum	Ser	Gln	Ala	Tyr	Tyr	Gln	*	Arg	Gly	Ser	*	*Met Val Leu*		*Phe Ser*	*Ser*	*Pro*	*Pro*	*Val*	*Ile*	*Leu*	*Leu*	*Ile*
				225					230			*Arg*										
				225					230				235				240					245	
SHa	-	-	-	-	-	Asp	Gly	-	Arg	-	*	Ala	-	-	-	-	-	-	-	-	-	-	-
AHa	-	-	-	-	-	Asp	Gly	-	Arg	-	*	-	-	-	-	-	-	-	-	-	-	-	-
CHa	-	-	-	-	-	Asp	Gly	-	Arg	-	*	-	-	-	-	-	-	-	-	-	-	-	-
				225					230				235				240					245	
Mo *a*	-	-	-	-	-	Asp	Gly	-	Arg	-	-	Ser Thr	-	-	-	-	-	-	-	-	-	-	-
b																							
				225					230				235				240					245	
Rat	-	-	-	-	-	Asp	Gly	-	Arg	-	-	*	-	-	-	-	-	-	-	-	-	-	-
				230					235				240				245						
Mink	-	-	-	-	-	*	-	-	Ala	-	*	Ala Ile	-	-	-	Pro	-	-	-	-	-	-	-
	225				230					235				240					245				
Sheep	-	-	-	-	-	*	-	-	Ala	-	*	Val Ile	-	-	-	-	-	-	-	-	-	-	-
Bovine	-	-	-	-	-	*	-	-	Ala	-	*	Val Ile	-	-	-	-	-	-	-	-	-	-	-
Kudu	-	Glu	-	-	-	*	-	-	Ala	-	*	Val Ile	-	-	-	-	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	*	-	-	Ala	-	*	Val Ile	-	-	-	-	-	-	-	-	-	-	-

Fig. 6. Continued

	251				255				
	245					250			253
Hum	*Ser*	*Phe*	*Leu*	*Ile*	*Phe*	*Leu*	*Ile*	*Val*	*Gly*
					250				254
SHa	-	-	-	-	-	-	Met	-	-
AHa	-	-	-	-	-	-	-	-	-
CHa	-	-	-	-	-	-	-	-	-
					250				254
Mo *a*	-	-	-	-	-	-	-	-	-
b									
					250				254
Rat	-	-	-	-	-	-	-	-	-
		250					255		257
Mink	-	Leu	-	-	Leu	-	-	-	-
			250					255	256
Sheep	-	-	-	-	-	-	-	-	-
Bovine	-	-	-	-	-	-	-	-	-
Kudu	-	-	-	-	-	-	-	-	-
Oryx	-	-	-	-	-	-	-	-	-

Fig. 6. Alignment of the predicted protein sequences of PrP-sen from humans and various other species (see legend of Fig. 4). The N- and C-termini amino acid sequences in italics are cleaved during post-translational processing. Amino acids in bold are post-translationally modified (see text for details) and the octapeptide repeats are underlined. Single amino acid substitutions observed in familial CJD patients are underlined. The polymorphism at codon 129 of the human PrP is in italics.

Chapter 3

The Heterogeneous Clinical Presentation of Spongiform Encephalopathies

There are many important and as yet unexplained points regarding spongiform encephalopathies that an outside reader should keep in mind while trying to make a correct judgement of why none of the different theories on the nature of the infectious agent are at the moment satisfactory. In this chapter, I shortly review the many facets of the clinical aspects and in the next chapter I will critically interpret them considering the available experimental data.

Natural Scrapie in Sheep

The first reports on scrapie gave credit to the observations of experienced shepherds who named the disease after the most important clinical signs seen in the syndrome (for a review see Palmer, 1959). In England its name is derived from the pronounced scratching and rubbing of the skin which is often reported as one of the first and most pronounced clinical signs of scrapie. Referring to the same symptom, some French writers termed the disease 'prurigo lombaire' because the itching and subsequent loss of wool often occurred in the region of the loins. On the other hand, among the many synonymous terms for scrapie, it was also referred to as 'La tremblante' (the trembles) in France, 'Rida' (ataxia or tremor) in Iceland and 'Traberkrankheit' (trotting disease) in Germany to emphasise the neurological signs: trembling of the head, tremors of the whole body and legs, resembling pronounced shivering, incoordination of the hind quarters with, in the early stages of the disease, a still normal movement of the forequarters which gives the animals a rather peculiar gait that resembles the trot and less often vertigo, paralysis, visual disturbances and epileptiform seizures.

Although both cutaneous and neurological symptoms are often present in the same animal (Stockman, 1926), the different names given to scrapie during the past two centuries reflect the presence of slight clinical variations which may be due to either genetic differences (e.g. in the prion protein gene) between hosts or strain differences in the agent (Dickinson, 1976).

Scrapie most likely occurs in every part of the world except in those countries (i.e., Australia, New Zealand and possibly Argentina) where a careful eradication program

for controlling the spread of the disease has been established. Although there are no data on the real incidence of the disease, it is conceivable that in affected flocks scrapie may kill 10–40% of animals or even the entire flock if shepherds did not immediately slaughter individual sheep at the earliest suspicious appearance of the disease. In experimental flocks of sheep where the natural disease was intentionally kept under no control, there was, in fact, a progressive decline in the age of death from natural scrapie which was most likely caused by an increased exposure to infection rather than a selection of a particularly virulent strain of scrapie or an increase in the frequency of susceptible genotypes among the sheep population (Foster and Dickinson, 1989).

The "Sporadic" Form of Human Spongiform Encephalopathies

In humans, as in natural scrapie, there are distinct clinical manifestations of the disease that, in the past, resulted in many synonymous terms for describing variants of what, in 1922, Spielmeyer (1922) called Creutzfeldt–Jakob disease. Although the original descriptions of Hans Creutzfeldt (Creutzfeldt, 1920, 1921) and at least two of the five cases of Alfons Jakob (Jakob, 1921a,b,c) do not fulfil the present day diagnostic criteria (Alemà and Bignami, 1959; Masters and Gajdusek, 1982), this eponym is presently used to describe most (about 99%) of the spongiform encephalopathies or prion diseases in humans.

The event that halted the sub-grouping of CJD occurred in 1968 when Gibbs, Gajdusek and their collaborators succeeded in the transmission of the disease to a chimpanzee (Gibbs *et al.*, 1968). Thirteen months after intracerebral and intravenous inoculation with a CJD brain homogenate, the primate developed a progressive fatal neurological disease characterised by behavioural abnormalities, ataxia of gait, intention tremor and intermittent jerking of the extremities. Microscopic examination of the brain showed marked status spongiosus of the cerebral grey matter, neuronal loss and proliferation and hypertrophy of astrocytes.

In the following years, the NIH and other laboratories from all over the world successfully transmitted many more cases of CJD to a variety of non-human primates (Gibbs and Gajdusek, 1973; Baker *et al.*, 1985; Brown *et al.*, 1994b), mice (Manuelidis *et al.*, 1978a; Tateishi *et al.*, 1979), rats (Tateishi *et al.*, 1979), guinea pigs (Manuelidis, 1975; Abbamondi *et al.*, 1983), hamsters (Manuelidis *et al.*, 1977) and cats (Gibbs and Gajdusek, 1973). The criterion of transmissibility became the unifying component of the many clinical and pathological variants of human spongiform encephalopathies and an essential element to distinguish these degenerative disorders of the central nervous system from other similar syndromes, such as Alzheimer's disease.

The analysis of more than 200 transmitted cases revealed that the clinical panorama of CJD has indeed many facets (Brown *et al.*1994b): males and females are equally affected, usually between the ages of 50 and 70, but the disease can affect people as young as 16 or over 80. In the majority of patients there are non-specific psychological prodromal symptoms of uncertain significance, followed by a rather gradual appearance of neurological deficits which may appear in the form of cognitive disturbances (i.e., memory loss, confusion or bizarre behaviour), cerebellar disturbances (ataxia, vertigo or nystagmus) or visual signs, or a combination of the above. However, about 15% of

patients experience a rapidly progressive or an abrupt onset of the disease which may resemble a cerebral vascular accident. After this initial phase, the disease inevitably progresses and all patients experience a severe dementia often coupled with myoclonus, cerebellar, visual and pyramidal or extra-pyramidal signs. Other neurological signs, however, may affect the patients as well and eventually they may have epileptic seizures, lower motor signs or pseudobulbar paralysis. In the majority of cases the duration of the illness is less than 6 months; more than 80% of patients die within 1 year from the onset of the disease (Will and Matthews, 1984; Brown *et al.*, 1986a, 1994b; Masullo *et al.*, 1988). However, exceptions to this rule are possible and some patients may last in a semi-vegetative state for more than 2 years (Brown *et al.*, 1984; Cutler *et al.*, 1984; Kitamoto and Tateishi, 1988).

Diagnostic tools

The occurrence of a rapidly progressive dementia associated with other neurological signs in 50–70 year-old patients makes the clinical diagnosis an easy task; otherwise the illness can be confused with many other neurological syndromes including Alzheimer's disease, Parkinson's disease with dementia and amyotrophic lateral sclerosis with dementia.

Of great help for the confirmation of the clinical diagnosis is the electroencephalogram (EEG) which often shows a disease-specific periodic activity of 1–2 cycles per second triphasic waves (Chiofalo *et al.*, 1980; Aguglia *et al.*, 1987; Brown, 1993a). Two dimensional gel electrophoresis of cerebrospinal fluid shows two abnormal proteins (*Mr* 26,000; p*I* 5.2 and *Mr* 29,000; p*I* 5.1) in all patients with CJD which are not found in other degenerative or infectious neurological disorders, except in herpes encephalitis (Harrington *et al.*, 1986). This test, although not easy to obtain in routine clinical laboratories, may be useful in the evaluation of patients with unclear progressive diagnosis (Blisard *et al.*, 1990). Brain CT-scan, magnetic resonance imaging (MRI) and positron emission tomography (PET), on the other hand, show no specific or reproducible patterns and are therefore of no help except for excluding alternative diagnoses.

Post-mortem diagnosis is based on routine histological examination of the brain; most of the cases present characteristic spongiform changes in the grey matter and, eventually, in the white matter (Masters and Richardson, 1978; Mizutani *et al.*, 1981; Brown *et al.*, 1993; Liberski *et al.*, 1993d), proliferation and hypertrophy of astrocytes which can often assume the aspect of gemystocytes (Liberski *et al.*, 1993b, c) and a variable degree of neuronal loss with a complete absence of inflammatory signs (see Figs 7 and 8). In 5–10% of cases, amyloid plaques are present which are immunostained by antisera anti-PrP (Doi-Yi *et al.*, 1991; Hashimoto *et al.*, 1992) (see Figs 9 and 10). The electron microscope gives no extra helpful information for routine diagnosis. On the contrary, the identification of PrP by Western blot is critical for the diagnosis of the disease (Bode *et al.*, 1985; Manuelidis *et al.*, 1985; Brown *et al.*, 1986b; Bockman *et al.*, 1987). Positive immunoblot detection of PrP in frozen brain material ranges from 85% to 100% of sporadic CJD patients. Moreover, it is possible to confirm the clinical diagnosis of CJD by Western blot detection of PrP purified from a small specimen of cerebral cortex such as can be obtained through biopsy (Xi *et al.*, 1994). Although the

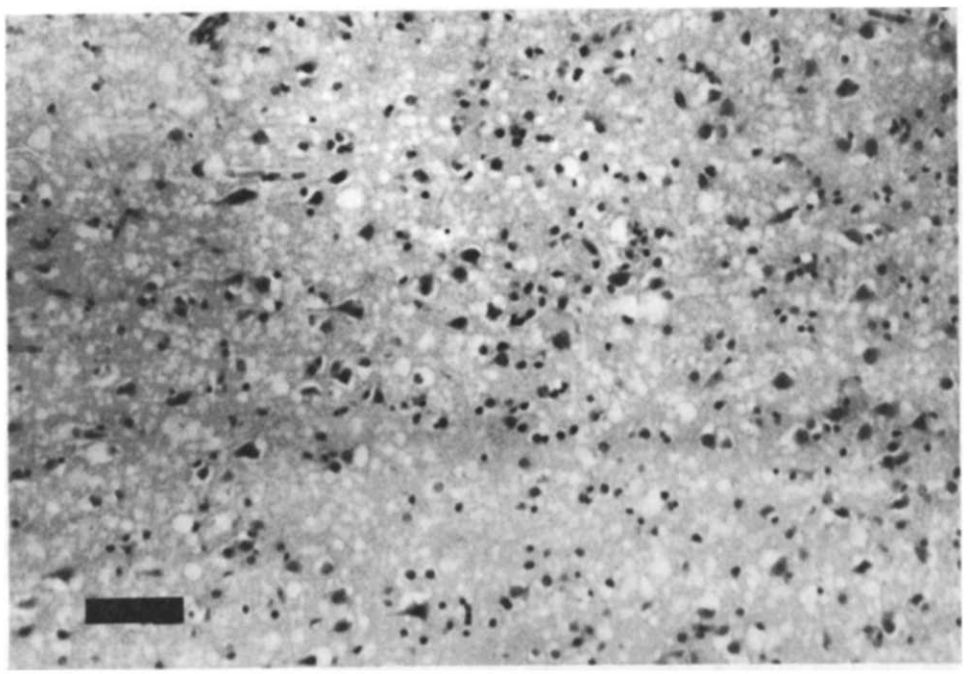

Fig. 7. Diffuse spongiform change in cerebral cortex of a sporadic case CJD (patient LA, referred by Dr G. Neri, S. Filippo Neri Hospital, Rome, Italy). Scale bar = 200 μm, hematoxylin-eosin. By courtesy of Prof. G. Macchi, Catholic University, Rome, Italy.

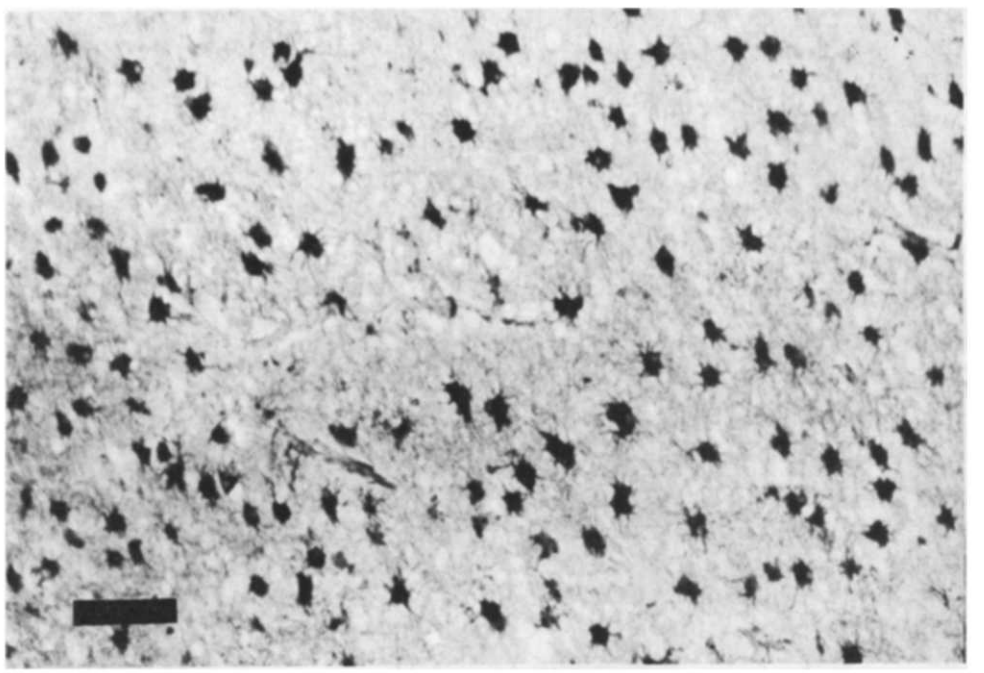

Fig. 8. Severe astrocytosis in cerebral cortex of a sporadic case CJD (patient LA, referred by Dr G. Neri, S. Filippo Neri Hospital, Rome, Itlay). Scale bar = 200 μm, Cajal gold sublimate. By courtesy of Prof. G. Macchi, Catholic University, Rome, Italy.

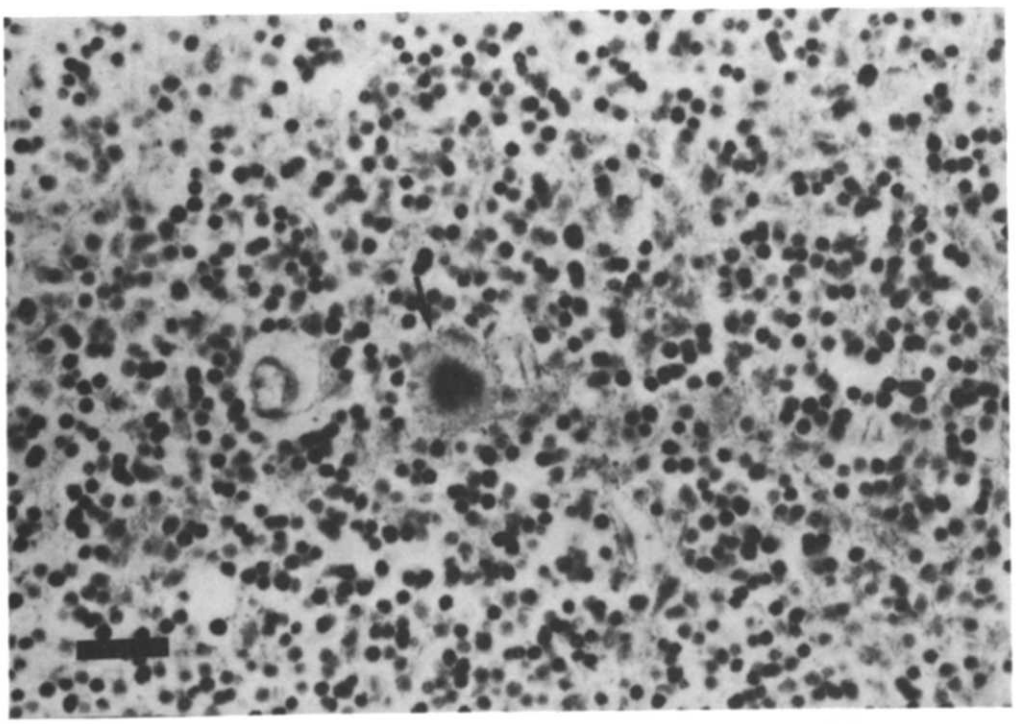

Fig. 9. Typical kuru plaque (arrow) in the granule cell layer of the cerebellum of a sporadic CJD case (patient CA, referred by Prof. M. Manfredi, University 'La Sapienza', Rome, Italy). Scale bar = 20 μm, Bielschowsky staining. By courtesy of Prof. G. Macchi, Catholic University, Rome, Italy.

use of cerebral biopsy for routine diagnosis of CJD is impractical since this procedure is associated with increased morbidity, there are instances where a precise diagnosis is important to determine the course of therapy or to provide a more accurate prognosis. This finding implies that open biopsy can be replaced by the less traumatic needle biopsy in the diagnosis of CJD.

Epidemiology and risk factors

CJD occurs world-wide with an incidence of about 1 case per 2 million people (Masters *et al.*, 1979; Brown *et al.*, 1987; Alperovitch *et al.*, 1994). The rarity of the disease, its long symptomless incubation period and the absence of any laboratory test for a pre-clinical diagnosis make the search for the possible source of contagion and for eventual risk factors a difficult task.

The higher incidence of CJD in urban areas of the Paris region (Brown *et al.*, 1979), the Boston metropolitan area (Masters *et al.*, 1979), Brooklyn and Staten Island in New York City (Farmer *et al.*, 1978), Santiago in Chile (Galvez *et al.*, 1980) and the province of Rome (Pocchiari and D'Alessandro, 1993), compared to the respective country as

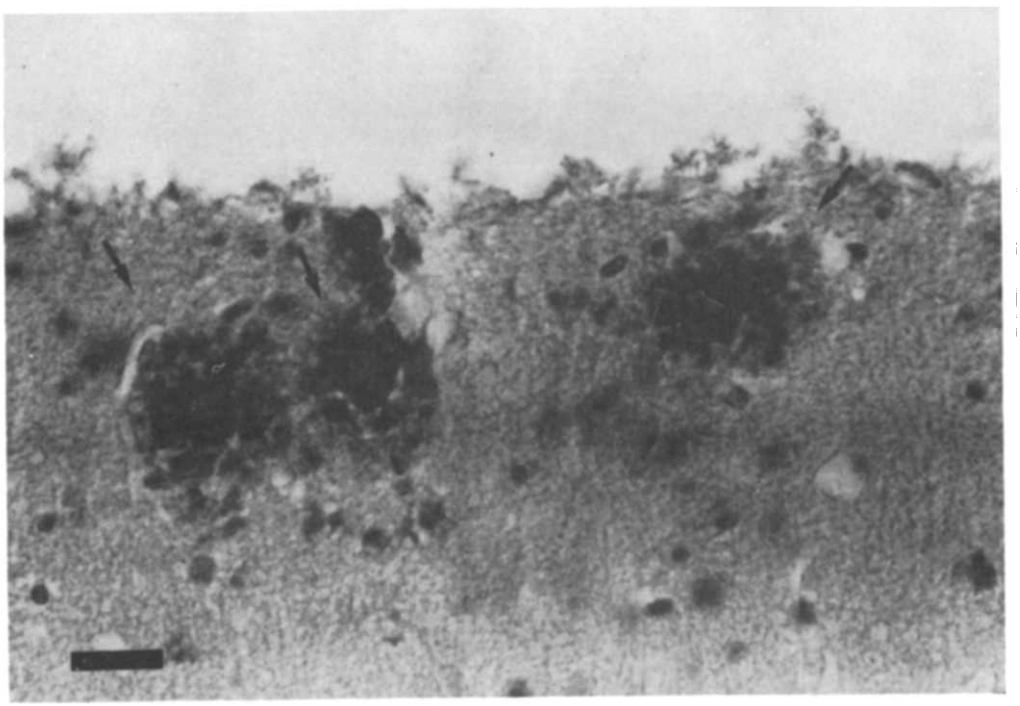

Fig. 10. Anti-PrP immunostaining of multicentric amyloid plaques (arrows) in the molecular cell layer of the cerebellum in a GSS case (referred by Prof. G.L. Lenzi, University 'La Sapienza', Rome, Italy). Scale bar = 20 μm. By courtesy of Prof. G. Macchi, Catholic University, Rome, Italy.

a whole, suggests that the incidence of CJD is related to population density and this implies a role of human-to-human infection in the transmission of sporadic CJD (Brown *et al.*, 1979). The spatial–temporal clustering of CJD that has been described (Kahana *et al.*, 1974; Mayer *et al.*, 1977; Masters *et al.*, 1979) confirms the inter-human transmission of the disease, although often it turns out to be familial aggregation of cases due to the not fully penetrant mutation of the PrP gene at the codon 200 (Goldfarb *et al.*, 1990a,b; Hsiao *et al.*, 1991a; Brown *et al.*, 1992a). The available epidemiological data, however, failed to show any contact between patients, but cross contamination by minor medical or dental procedure cannot be completely dismissed. The two reports of the concomitant (Jellinger *et al.*, 1972) or 3 years apart (Matthews, 1975) appearance of CJD in husband and wife also suggests a common exposure to an infectious agent years before, rather than a case-to-case transmission.

CJD has been described in health care workers (Miller, 1988; Sitwell *et al.*, 1988; Gorman *et al.*, 1992; Berger and Noble, 1993), but these cases are likely to be sporadic rather than iatrogenic CJD for two reasons: large epidemiological surveys have failed to link CJD with occupation (Brown *et al.*, 1979; Will and Matthews, 1984; Harries Jones *et al.*, 1988) and clinically they do not show the stereotypical symptomatology which is

usually observed in accidental transmission of CJD by peripheral routes (Brown, 1988c) (see below).

There is no epidemiological evidence that scrapie of sheep and goats is transmitted to humans (Bobowick *et al.*, 1973; Kondo and Kuroiwa, 1982; Harries Jones *et al.*, 1988), but this mode of transmission has been postulated several times (Alter *et al.*, 1977; Lo Russo *et al.*, 1980; Mitrova and Mayer, 1981; Davanipour *et al.*, 1985). Recently, two cases of CJD have occurred in British dairy farmers who had cases of BSE in their herds (Davies *et al.*, 1993; Sawcer *et al.*, 1993). Although these two cases still do not associate CJD and BSE on statistical grounds (Davies *et al.*, 1993), this theoretical risk will be monitored, in the following years, by the epidemiological surveillance of CJD incidence in Great Britain and in other European countries (Alperovitch *et al.*, 1994).

Thus, the only known mode of transmission remains the accidental infection from human-to-human which has occurred under several circumstances: after incomplete sterilization of contaminated surgical instruments (Bernoulli *et al.*, 1977), following cornea (Duffy *et al.*, 1974) or dura mater (Thadani *et al.*, 1988; Masullo *et al.*, 1989; Janssen and Schonberger, 1991; Miyashita *et al.*, 1991; Willison *et al.*, 1991; Pocchiari *et al.*, 1992; Esmonde *et al.*, 1993) transplantation from CJD affected donors, and, finally, after therapy with human-derived pituitary hormones extracted from CJD-infected glands (Brown, 1988a; Cochius *et al.*, 1990; Fradkin *et al.*, 1991).

The "Familial" Form of Human Spongiform Encephalopathies

Familial cases represent about 5–10% of CJD and are all linked to mutations of the PrP gene (see Table 2). Though at a lower rate than sporadic CJD, familial cases are also

Table 2. PRNP mutations

Mutations	Disease	Sequence			Aminoacid		
		wild	→	mutated	wild	→	mutated
102	GSS	CCG	→	CTG	Pro	→	Leu
105	GSS	CCA	→	CTA	Pro	→	Leu
117	GSS	GCA	→	GTG	Ala	→	Val
145	GSS (?)	TAT	→	TAG	Tyr	→	stop codon
178	CJD/FFI	GAC	→	AAC	Asp	→	Asn
180	CJD	GTC	→	ATC	Val	→	Ile
198	GSS	TTC	→	TCC	Phe	→	Ser
200	CJD	GAG	→	AAG	Glu	→	Lys
210	CJD	GTT	→	ATT	Val	→	Ile
217	GSS	CAG	→	CGG	Gln	→	Arg
232	CJD	ATG	→	AGG	Met	→	Arg
Inserts between codons 51 and 91	CJD	2, 5, 6, 7 extra 24 bp			2, 5, 6, 7 extra octapeptides		
Inserts between codons 51 and 91	GSS	8 extra 24 bp			8 extra octapeptides		

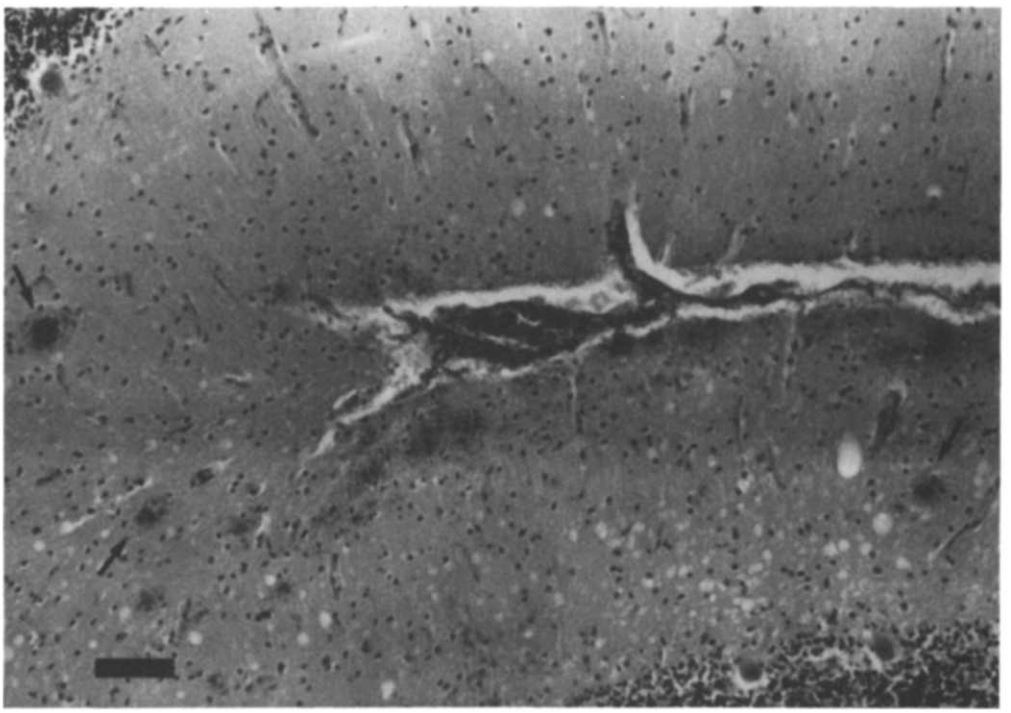

Fig. 11. Spongiform changes and multicentric amyloid plaques in the molecular cell layer of the cerebellum in a GSS case (referred by Prof. G. L. Lenzi, University 'La Sapienza', Rome, Italy). Scale bar = 200 μm, Periodic acid Schiff. By courtesy of Prof. G. Macchi, Catholic University, Rome, Italy.

transmissible to laboratory animals (Brown *et al.*, 1994b). Clinically, they resemble the sporadic form, but usually patients become ill at a younger age and the duration of the disease is longer than in sporadic CJD. In some families, moreover, the disease assumes the aspect of the Gerstmann–Sträussler–Scheinker syndrome, a chronic cerebellar ataxia of long duration (around 5 years, but with great variability) in which dementia, myoclonus and spinal cord or tract involvement occur frequently but not invariably and often late during the clinical course of the disease. Amyloid plaques, distributed widely throughout the brain, are always present and assume the characteristic aspect of a central dense core surrounded by smaller globules (see Fig. 11). Spongiform changes are common but not always present. Clinical variability in patients of the same family, hence bearing the same mutation on the PrP gene, is present as in sporadic cases, arguing that genetic background of the host is not the only factor that controls the manifestation of the disease. Some examples will be given for each of the known mutations of the PrP gene described in human spongiform encephalopathies.

Codon 102

The substitution of proline to leucine at codon 102^{Leu} of PRNP was the first point mutation described in human spongiform encephalopathy and is probably derived by

the deamidation of the methylated CpG triplet (Barker *et al.*, 1984), resulting in the conversion of a T (CTG) for C (CCG) (Hsiao *et al.*, 1989).

The recent finding that, in a large Italian family with 8 affected members in 3 generations bearing the codon 102^{Leu} mutation of the PrP gene, 3 patients showed severe dementia with cerebellar and extrapiramidal signs and a duration of illness of less than 1 year, while the other 4 patients developed a chronic cerebellar syndrome with moderate or no dementia and a clinical course of 2–4 years (Barbanti *et al.*, 1994), is an excellent example of clinical heterogeneity within a single family. Histology was performed on only one of the patients affected by the dementia-type, who showed a marked spongiosis with many GSS-like amyloid plaques (see Figs 10 and 11). The analysis of a polymorphism (Met/Val) at codon 129 in 6 of 8 patients of this family did not correlate with the clinical presentation of the disease: the Met/Met genotype was in 2 patients with dementia and in 1 with the chronic cerebellar syndrome and the Met/Val genotype was found in 1 demented patient and in 2 ataxic patients.

A similar clinical heterogeneity was previously described regarding the "JW" GSS family of British origin with the 102^{Leu} codon mutation (Hsiao *et al.*, 1989). In their superb review of GSS cases, Masters *et al.*, (1981) emphasise "the wide variety of clinical signs in this family, especially the presence or absence of dementia, myoclonus and spinal cord involvement" and the irregular and unpredictable occurrence of spongiform changes in affected members which did not correlate with clinical presentation. Transmission from brain homogenates of 3 affected members to monkeys or hamsters (Masters *et al.*, 1981; Baker *et al.*, 1985; Hsiao *et al.*, 1989) has been reported. Interestingly, at least 2 of the 3 transmitted cases had spongiform changes (to my knowledge, the histology of the third case has not been reported). Clinical manifestations and pathology are also variable in affected members of other GSS-codon 102^{Leu} families, such as the "Sch" family of German origin (Brown *et al.*, 1991) and the Italian family described by Kretzschmar and co-workers (Kretzschmar *et al.*, 1992a), where two patients were affected with cerebellar ataxia and one with clinical signs resembling amyotrophic lateral sclerosis. In other GSS families with codon 102^{Leu}, the kaleidoscopic clinical and pathological presentations are either not evident, as in the original family described by Gerstmann and co-workers, or not reported (Doh-ura *et al.*, 1990; Kretzschmar *et al.*, 1991; Goldhammer *et al.*, 1993).

Codon 117

Two other GSS families, one French Alsatian (Doh-ura *et al.*, 1989; Tateishi *et al.*, 1990; Tranchant *et al.*, 1992) and one American of German origin (Nochlin *et al.*, 1989; Hsiao *et al.*, 1991b), have been linked to the mis-sense change at codon 117 (GCA → GTG) which results in the substitution of alanine to valine (Doh-ura *et al.*, 1989; Tateishi *et al.*, 1990; Hsiao *et al.*, 1991b; Tranchant *et al.*, 1992). The C to T transition at the second letter of the triplet is a silent polymorphism found in about 10% of the population (Wu *et al.*, 1987). Although they bear the same mutation of PRNP, clinical and pathological features in affected members of the Alsatian family are distinct from those of the German origin family (Tranchant *et al.*, 1992). These cases have not yet been proved to be experimentally transmitted to laboratory animals (Tateishi *et al.*, 1990).

Codon 198, 217 and 105

Other point mutations in GSS patients are at codon 198^{Ser} (TTC → TCC, resulting in Phe → Ser), 217^{Arg} (CAG → CGG, Gln → Arg) and 105^{Leu} (CCA → CTA, Pro → Leu). The first mutation has been linked to the Indiana kindred variant of GSS (Dlouhy *et al.*, 1992) with about 70 affected family members in 6 generations, whose main clinical signs include progressive dementia, parkinsonian symptoms and cerebellar ataxia with a duration of illness ranging from 3 to more than 10 years (Farlow *et al.*, 1989). Pathologically, they are characterised by amyloid plaques which are immunolabelled with anti-PrP antibodies, consistent presence of neurofibrillary tangles and mild spongiform changes (Ghetti *et al.*, 1989). No other families carrying this mutation are presently known.

The 217^{Arg} point mutation has only been found in affected members of a Swedish family who had dementia, gait ataxia and a pathological picture similar to that observed in the Indiana family (Hsiao *et al.*, 1992a).

The third point mutation (codon 105^{Leu}) was observed in 6 patients belonging to 4 apparently unrelated Japanese families (Kitamoto *et al.*, 1993a, c; Yamada *et al.*, 1993). Clinically they manifested spastic gait disturbances, progressive dementia without cerebellar signs, myoclonus and periodic EEG. At histology, they revealed amyloid plaques, mostly in the cortex, neuronal loss, severe gliosis and no spongiosis.

In only one Japanese patient with unknown family history and with a slowly (21 years) progressive dementia as the only clinical sign, an amber mutation at the codon 145 (TAT → TAG, → Tyr stop codon) was identified. Pathology resembled Alzheimer's disease with no spongiosis but with amyloid plaques immunostained by anti-PrP antibodies (Kitamoto *et al.*, 1993b).

Codon 200

In familial Creutzfeldt–Jakob disease, the most frequent point mutation is at codon 200 of the PrP gene and consists of a G (GAG) to A (AAG) substitution in the first nucleotide of the triplet which results in a Glu to Lys substitution. The codon 200^{Lys} differs from the above reported mutations regarding the penetrance quotient of 0.56 (Goldfarb *et al.*, 1991b), which means that only about half of the mutated subjects will develop CJD during their life and that about three in four children of a 200^{Lys} mutated parent will eventually escape from the illness. Because of this, the disease may not develop in one, or even more, generations, giving the impression that mutation-positive patients are sporadic cases of CJD.

The belief that we are dealing with sporadic CJD patients is reinforced by the clinical presentation, the pathological findings and the high positive rate of experimental transmission to laboratory animals, which are practically indistinguishable from sporadic cases (Goldfarb *et al.*, 1991b). However, a recent report reveals a marked clinical heterogeneity in Jewish patients with the codon 200^{Lys} mutation (Chapman *et al.*, 1993). Moreover, there is one large American family with this mutation whose affected members show phenotypic features (i.e., supranuclear gaze palsy, no myoclonus and

periodic triphasic EEG) markedly different from other patients with codon 200^{Lys} mutation and from sporadic CJD (Bertoni *et al.*, 1992).

Families carrying the 200^{Lys} mutation are distributed in many countries; some investigators have asserted that this mutation originated in Spain and was then dispersed during the middle ages by the mass migration of Sephardic Jews expelled by the Inquisition authorities (Goldfarb *et al.*, 1991b). Others, on the contrary, suggest that the mutation has arisen independently with a deamidation mechanism similar to that described for the codon 102^{Lev} mutation (Prusiner, 1993). The finding of an identical mutation in a Japanese family (Inoue *et al.*, 1994) sustains this last hypothesis. The relatively high frequency of this point mutation was an important factor in the occurrence of geographic CJD clusters in rural Slovakia (Mayer *et al.*, 1977; Goldfarb *et al.*, 1990b), rural Chile (Brown *et al.*, 1992a) and in Libyan-born Jews living in Israel (Kahana *et al.*, 1974; Goldfarb *et al.*, 1990a; Hsiao *et al.*, 1991a; Zilber *et al.*, 1991; Gabizon *et al.*, 1993b).

Codon 210

Recently, a new G to A substitution at the first nucleotide of the 210 triplet (GTT → ATT, Val → Ile) was discovered in two sisters (family It-91) affected by a 'classic' CJD similar to that observed in 200^{Lys} mutation-positive individuals (Pocchiari *et al.*, 1993). Codon 210^{Ile} mutation was also found in four unrelated Italian (Pocchiari *et al.*, 1993) and one French patient (Ripoll *et al.*, 1993) with CJD whose first-degree relatives were unaffected. Moreover, the finding that the 210^{Ile} mutation was also present in 2 individuals of family It-91 who were still not affected at the ages of 81 and 82 suggests that this mutation has an incomplete penetrance, as observed for the 200^{Lys} mutation (Pocchiari *et al.*, 1993). It is interesting that 3 of the 6 patients with codon 210^{Ile} mutation dying of CJD at ages 49, 50 and 52 were methionine homozygous at codon 129, while the other three patients who died at ages 65, 68 and 70 carried in the non-mutated allele either a valine at codon 129 or a 24 bp deletion in the region encoding for the five octapeptide repeats. Moreover, the two non-affected subjects of family It-91 (81 and 82 years old with the 210^{Ile} mutation) also had the 24 bp deletion on the other allele (Pocchiari *et al.*, 1993). Thus, it could be speculated that this deletion may delay the appearance of the disease as it does heterozygosis at codon 129 polymorphism in familial CJD patients carrying either 144 bp insertion or codons 178^{Asn} and 198^{Ser} pathogenic mutations in the PRNP gene (Baker *et al.*, 1991; Dlouhy *et al.*, 1992; Goldfarb *et al.*, 1992b; Poulter *et al.*, 1992). It is noteworthy that an accelerated pathogenesis (early age at onset or shorter duration of the disease) has not been seen in familial CJD patients with codon 200^{Lys} mutations who are homozygous at the polymorphic 129 site (Gabizon *et al.*, 1993b) and this may be the only distinction between codon 200^{Lys} and codon 210^{Ile} mutations.

Codon 232 and 180

Clinical and pathological features resembling sporadic CJD were also reported in two patients from unrelated Japanese families bearing the codon 232^{Arg} mutation (ATG → AGG, Met → Arg) (Kitamoto *et al.*, 1993c). Interestingly, codon 232^{Arg} is in the C-terminus region of PrP that is replaced during post-translational processing by a

glycolipid anchor (Stahl *et al.*1990a) and therefore cannot influence the configuration of the mature protein. It is therefore likely that the substitution at codon 232 is a low frequency polymorphism rather than a pathogenic mutation.

Two other Japanese patients bore a mutation at codon 180^{Ile} (GTC → ATC, Val → Ile); one of them also had the 232^{Arg} substitution on the other allele (Kitamoto *et al.*, 1993c; Hitoshi *et al.*, 1993). They developed dementia, myoclonus, no periodic EEG and showed spongiosis but no amyloid plaques at histology.

Codon 178

The GAC (Asp) to AAC (Asn) substitution at codon 178 of PRNP results in even more complicated clinical and pathological patterns of CJD. The mutation was first identified in a large Finnish CJD family (Goldfarb *et al.*, 1991c) whose affected members showed typical clinical manifestations, except for an earlier onset and a longer duration of the illness and the absence of periodic EEG activity (Haltiae *et al.*, 1991). Subsequently, the codon 178^{Asn} mutation was discovered in several unrelated American families of European descent and in two French families (Fink *et al.*, 1991; Nieto *et al.*, 1991; Brown *et al.*, 1992b; Goldfarb *et al.*, 1992a). The phenotypic characteristics in affected members of these American families were similar to those described previously for the Finnish family, except for one case belonging to the French family "Wui" who developed the disease at the age of 57 and, besides the classic clinical features, showed periodic EEG activity. Transmission of disease to primates was also accomplished using brain tissue homogenates from 6 of 10 patients (Brown *et al.*, 1992b).

In 1992 the same mutation was linked to a novel prion disease (Medori *et al.*, 1992a) which was initially described in one Italian kindred by Lugaresi and his colleagues in 1986 (Lugaresi *et al.*, 1986) and later recognised in another unrelated Italian family (Medori *et al.*, 1992b), in 2 American and 1 French family (Petersen *et al.*, 1992). The affected members show, in association with the disease-specific clinical signs of ataxia, myoclonus and mental deterioration, an unusual loss of sleep, dysautonomia and endocrine disturbances. Although sleep disturbance has occasionally been reported in 'classical' cases of CJD (Nevin *et al.*, 1960) and in a Libyian patient with the codon 200^{Lys} mutation (Chapman *et al.*, 1993), the intensity of this feature justified the term 'fatal familial insomnia' (FFI) for describing this CJD variant. All FFI patients showed a marked atrophy of the anterior ventral and mediodorsal thalamic nuclei. FFI has not yet been transmitted to experimental animals, though the limited number of cases tested does not allow for any definite conclusion (Brown *et al.*, 1994b).

A possible explanation for these distinct phenotypes in families bearing the same mutation is that in FFI families the mutated codon 178^{Asn} carried methionine at codon 129 (129^{Met}) and in CJD families, valine (129^{Val}) (Goldfarb *et al.*, 1992b). However, a further American family of European/native American origin, with five affected members in four generations carrying the combination $178^{Asn}/129^{Met}$ plus a 24 bp deletion in the octapeptide coding region on the same allele of PRNP showed quite different clinical and pathological patterns: the clinical course resembles familial CJD rather than FFI (although 2 patients suffered from insomnia) and the histology

observation (done only in 1 patient) shows neuronal loss, severe astrocytosis and diffuse spongiosis with only mild changes in the anterior thalamus (Bosque *et al.*, 1992).

In CJD families with $178^{Asn}/129^{Val}$, codon 129 (Val/Val) homozygous patients show an earlier appearance of the disease and a shorter duration of the illness compared to codon 129 heterozygous (Val/Met) ones (Goldfarb *et al.*, 1992b). Controversial data have instead been reported on the pathogenetic importance of codon 129 in patients with FFI; some investigators found a shorter duration of the disease in homozygous (Met/Met) versus heterozygous (Met/Val) patients (Goldfarb *et al.*, 1992b), while others did not (Medori and Tritschler, 1993). Moreover, it also appears that the disease is fully penetrant in CJD families but not in FFI (Medori and Tritschler, 1993).

Insertions

Besides single point mutations of PRNP in families with CJD or GSS, 48 to 216 base pair insertions and 24 base pair deletions in the octapeptide repeats coding regions of the gene have been described. Insert mutations of different lengths have been linked to the development of familial CJD or GSS (Owen *et al.*, 1989; Goldfarb *et al.*, 1991a, 1993; Collinge *et al.*, 1992; Poulter *et al.*, 1992; Tateishi *et al.*, 1992; Duchen *et al.*, 1993). Except for the 7 extra octapeptide insert repeats which have been found in one American (Goldfarb *et al.*, 1991a) and one Japanese (Tateishi *et al.*, 1992) family, each of the other insertions (2, 5, 6 and 8 extra octapeptide repeats) has only been detected in a single family. Although, as a whole, the affected members with insert mutations show an early age at onset and a long duration of illness, they reveal a high degree of clinical and pathological heterogeneity. This marked variability was also observed within a single family as is well illustrated by the detailed study of the large English family carrying the 144 base pair gene insertion (Collinge *et al.*, 1992; Poulter *et al.*, 1992). The clinical phenotype varied from 'classical' CJD with a rapidly progressive dementia to that of Alzheimer-like disorders. This phenotypic variability was also observed at histology where the lesions ranged from severe spongiosis to GSS-type amyloid plaques or even no alterations.

Deletions

As is the case with insertions in the octapeptide coding region, a deletion in the same region might be expected to alter the protein conformation (Puckett *et al.*, 1991), thus enhancing the formation of PrP-res and the development of the disease. However, a deletion located downstream of codon 76 was recently identified in two out of 186 Italian control subjects, but in none of the sporadic CJD patients (Salvatore *et al.*, 1994). Similar deletions downstream codon 76 have been detected in normal control subjects (Laplanche *et al.*, 1990, 1991; Vnencak Jones and Phillips, 1992), in genomic HeLa and human brain cDNA libraries (Puckett *et al.*, 1991). However, deletions downstream of codon 76 have also been detected in a patient with unclassified dementia (Dietrich *et al.*, 1992), in two cases of iatrogenic CJD (Brown *et al.*, 1994a) and in a CJD patient with a codon 178^{Asn} mutation on the same allele (Bosque *et al.*, 1992); in these patients the deletion did not appear to influence the phenotypic expression of the disease. Different deletions located upstream of codon 76 were observed on the non-210-mutated allele of a familial CJD patient carrying a codon 210^{Ile} mutation (which probably delays the age

at onset of the disease (Pocchiari *et al.*, 1993)) in unaffected members of the same family and, in a homozygous state, in a 33-year-old woman with unclassified dementia (Masullo *et al.*, 1994). These findings indicate that deletion of a single repeat coding region is a low-frequency polymorphism, but its role, if any, in the manifestation of CJD has yet to be ascertained.

From the description of these familial cases it is evident that the development of spongiform encephalopathies in humans is linked to the genetic background of the host, although the marked clinical and histological variability found in patients bearing the same mutation of the PRNP argues that some other endogenous or exogenous factors are still missing.

Molecular Genetics in Sporadic CJD: The Codon 129 Polymorphism

What about sporadic CJD? Does it occur in individuals with genetic predisposition? The obvious place to search for genetic variation was the PRNP. No point or insert mutations have been discovered in sporadic CJD patients, but the genotype distribution at the polymorphic codon 129 significantly differs from control subjects.

This polymorphism results from the substitution of an A (ATG) to G (GTG) in the first position of codon 129 which corresponds to a valine from methionine change in the protein (Owen *et al.*, 1990a). The genotype distribution of codon 129 polymorphism in 4 Caucasian populations (British (Owen *et al.*, 1990b; Collinge *et al.*, 1991), French (Deslys *et al.*, 1994), American (Brown *et al.*, 1994a) and Italian (Salvatore *et al.*, 1994)) shows a similar pattern ($\chi^2 = 6.11$, $p = 0.4$); there are about an equal number of people carrying either the met/met or the met/val genotype and only 10–15% of them are homozygous for valine (see Table 3). In Japanese people (Doh-ura *et al.*,

Table 3. Codon 129 genotype distribution in CJD patients and control subjects

	Met/Met		Met/Val		Val/Val		Homoxygous	
	n	(%)	*n*	(%)	*n*	(%)	*n*	(%)
British controls[1]	39	(37)	54	(51)	13	(12)	52	(49)
French controls[2]	86	(42)	98	(47)	23	(11)	109	(53)
American controls[3]	45	(41)	56	(51)	9	(8)	54	(49)
Italian controls[4]	84	(45)	75	(40)	27	(15)	111	(60)
Caucasian controls	**254**	**(42)**	**283**	**(46)**	**72**	**(12)**	**326**	**(54)**
Japanese controls[5]	164	(92)	15	(8)	0	(0)	164	(92)
British sporadic CJD[6]	16	(76)	0	(0)	5	(24)	21	(100)
Italian sporadic CJD[4]	25	(81)	5	(16)	1	(3)	26	(84)
Japanese sporadic CJD[5]	16	(76)	4	(19)	1	(5)	17	(81)
British iatrogen CJD[1]	1	(14)	2	(29)	4	(57)	5	(71)
French iatrogen CJD[2]	13	(57)	0	(0)	10	(43)	23	(100)
Other iatrogen CJD[3]	20	(77)	2	(8)	4	(15)	24	(92)

[1](Collinge *et al.*, 1991); [2](Deslys *et al.*, 1994); [3](Brown *et al.*, 1994a); [4](Salvatore *et al.*, 1994); [5](Doh-ura *et al.*, 1991); [6](Palmer *et al.*, 1991).

1991), however, 92% of the population carry the met/met genotype, the rest are heterozygous and none of the 164 control subjects tested were valine homozygous. This distribution is obviously different from that of Caucasian populations (χ^2 = 106.7 (with Yates correction for continuity), $p < 0.0001$). Interestingly, although the allele frequencies at codon 129 between Caucasian populations and the Japanese people are sharply uneven (i.e., Met : Val 0.650 : 0.350 for Caucasians, 0.958 : 0.042 for Japanese), both their distributions follow the Hardy–Weinberg equilibrium (χ^2 = 0.257, $p < 0.1$; χ^2 = 0.342, $p < 0.1$, respectively).

In sporadic CJD, the genotype distribution of codon 129 differs from that of the respective control populations (χ^2 = 18.629, $p < 0.0001$; χ^2 =13.504, p = 0.0012; χ^2 = 11.272, p = 0.0036, for the British (Palmer *et al.*, 1991), Italian (Salvatore *et al.*, 1994) and Japanese (Doh-ura *et al.*, 1991) population, respectively), but the reason for this divergence varies from one group to the other (see Table 3). In the British study, patients with sporadic CJD are either homozygous in methionine or in valine and although the increase in homozygosity versus the control population is highly significant (χ^2 = 16.59 (with Yates correction for continuity), $p < 0.0001$), there is no excess of methionine or valine (χ^2 = 2.394, p = 0.12). In Italian CJD patients, there is a significant excess of homozygoses as well (c^2 = 5.683 (with Yates correction for continuity), p = 0.017), but the difference is exclusively related to an increase of the methionine allele over valine (χ^2 = 12.44, $p < 0.0004$). In contrast, Japanese CJD patients do not show any increase in homozygosity (χ^2 = 1.402, p = 0.24) with respect to control subjects, but there is an excess of valine to methionine (c^2 = 5.806, p = 0.016) which lengthens the clinical course of the disease in comparison with methionine patients (55.6 months versus 17.0) and also influences clinical and pathological characteristics (Doh-ura *et al.*, 1991). However, clinical heterogeneity between methionine and valine CJD-carriers was not observed in Italian CJD patients (Salvatore *et al.*, 1994).

These diversities may be due to the relatively small number of cases which, only by chance, show different statistical significance of one parameter over the other. However, the low incidence of CJD (about 1 case per 2 million people) compared to the large number of people (about 50% of the Caucasian population) carrying the homozygous genotype at codon 129 makes the theory of CJD predisposition in codon 129 homozygous individuals less tenable.

Accidental Cases in Humans and Animals

Uniformity of clinical signs in spongiform encephalopathies of humans and animals is, however, the rule when the disease is accidentally transmitted by peripheral injection of infectious material (Brown, 1988c). There are, unfortunately, several examples that support this view. In humans, iatrogenic cases due to therapy with cadaveric pituitary human growth hormone always show a primary cerebellar syndrome; mental deterioration, usually mild and gradually evolving, is a late event, if present and the characteristic periodicity in the EEG is rarely seen. These clinical manifestations resemble kuru, an exotic disease of the Fore-speaking tribes of New Guinea, which was also caused by peripheral injection of the infectious agent (Brown, 1993b). Kuru infection occurred during ritual endocannibalism practice either via the oral route, i.e.

eating close relatives as a rite of mourning, or through damaged skin and mucosae during the handling of internal organs (Gajdusek, 1977). The time lag between infection and the appearance of the disease was from several years to decades as recorded for CJD in growth hormone recipients (Brown, 1988b, c).

In iatrogenic cases of both central and peripheral origin, an excess of homozygosity at codon 129 (see Table 3), similar to that observed in sporadic cases, has been recently reported (Collinge *et al.*, 1991; Brown *et al.*, 1994a; Deslys *et al.*, 1994).

In animals, accidental transmission of scrapie was first recorded in Britain during the louping-ill eradication program which consisted of the injection of thousands of sheep with a vaccine prepared from brains of scrapie-infected animals (Gordon, 1946). Accidental transmission occurred several times in ranch-raised mink as a food-borne infection caused by scrapie or related infectious carcasses (Hartsough and Burger, 1965; Marsh *et al.*, 1991; Marsh and Bessen, 1993).

However, the most striking example is the recent epidemic of bovine spongiform encephalopathy in the U.K. which originated from the combination of several factors, the most important of which being the change in the method of production of meat and bone meal which led to an increased level of scrapie agent contamination in commercial foodstuffs and subsequently to the infection by oral route of the cattle population (Wilesmith *et al.*, 1988, 1991). Clinical signs in BSE are stereotypical and consist of changes in behaviour, apprehension, hyperaesthesia to touch and sound, abnormal posture and hind-limb ataxia. Frequently, there are muscular tremors and teeth grinding. Signs consistent with pruritus are not as common as in natural scrapie in sheep (Wilesmith *et al.*, 1992).

The reason for such a high frequency of accidental cases compared with the relatively low incidence of the disease is the strong and unusual resistance of these infectious agents to the most common disinfectants and microbial sterilization procedures (for a review on this subject, see Chesebro, 1990; Taylor, 1993). Scrapie and CJD agents are untouched by 70% alcohol, chloroform, ether and other similar compounds and only slightly or incompletely inactivated by 37% formalin, heat at 100°C for 1 hr or even 1 N sodium hydroxide treatment and autoclaving at 121°C for 1 or more hours (Brown *et al.*, 1982, 1986c, 1990; Kimberlin *et al.*, 1983; Walker *et al.*, 1983). The only reliable treatment for the sterilization of surgical instruments is two cycles of autoclaving at 134°C for 1 hr each (Taguchi *et al.*, 1991; Ernst and Race, 1993), although the wisest method would be the disposal of any medical device which has been in contact with CJD infected tissue. Great precautions should also be taken during the preparation of human/bovine-derived biological products for their use in humans or animal therapy or cosmetics (Pocchiari, 1991). Validation of the procedures with a suitable rodent-adapted strain of scrapie or CJD agent should always be performed to ensure the safety of the final product (Pocchiari *et al.*, 1988, 1991a; Di Martino *et al.*, 1992, 1993).

Therapy

Obviously, controlled clinical trials of potential anti-CJD drugs have been unfeasible because of the extreme rarity of the disease, and, therefore, the possible beneficial

effects of CJD therapy must be considered with caution (for a review on this subject see Brown, 1988b, 1990a). Among the several drugs tested in humans for attempting CJD therapy, amantadine, an anti-influenza drug known for its low toxicity and its capacity to cross the blood–brain barrier, has been reported to show some encouraging activity in early reports (Braham, 1971; Sanders and Dunn, 1973; Sanders, 1979; Terzano *et al.*, 1983). However, this beneficial effect has not been confirmed by other clinical (Goldhammer *et al.*, 1972; Herishanu, 1973; Ratcliffe *et al.*, 1975; Scully *et al.*, 1980; Neri *et al.*, 1984) or experimental studies (Cochran, 1971; Kimberlin and Walker, 1979b; Tateishi, 1981). The other drugs tested, except for isolated reports of stabilization of clinical course with methisoprinole and vidarabine (Furlow *et al.*, 1982; Villa *et al.*, 1982), did not show any beneficial effect.

The failure of CJD treatment has been attributed to late therapeutical intervention during the course of the disease when the biochemical and histological lesions in the brain have already occurred (Brown, 1990a; Pocchiari *et al.*, 1991b).

In experimental animal models, however, sulphated polyanions (Ehlers and Diringer, 1984; Ehlers *et al.*, 1984; Farquhar and Dickinson, 1986; Kimberlin and Walker, 1986b; Diringer and Ehlers, 1991; Ladogana *et al.*, 1992), Congo red (Ingrosso and Pocchiari, unpublished data) and the polyene antibiotic amphotericin B (Amyx *et al.*, 1984; Pocchiari *et al.*, 1987, 1989; Casaccia *et al.*, 1991) have given encouraging results. These drugs prolong or sometimes even prevent the appearance of the disease by delaying the formation of PrP-res and/or inhibiting scrapie replication (Kimberlin and Walker, 1986b; Diringer and Ehlers, 1991; Caughey and Race, 1992; Xi *et al.*, 1992; Caughey and Raymond, 1993; Caughey *et al.*, 1993; Gabizon *et al.*, 1993a). However, they are effective only when given either before or soon after the injection of the agent and are completely useless when administered at the appearance of clinical signs of disease (Pocchiari *et al.*, 1987). These data reveal that drug treatment during the clinical phase of spongiform encephalopathies does not have any rational basis, even if started during the very early stages of the illness. Earlier clinical diagnosis of these diseases would not help. However, these data suggest that young individuals at high risk for acquiring the disease, such as healthy relatives of patients affected with familial CJD or GSS who present a mutation in the gene coding for PrP might be candidates for an eventual preventive treatment. Other candidates for a preventive treatment might be recipients of human growth hormone (hGH) derived from cadaveric pituitaries (Pocchiari *et al.*, 1991b).

The recent discovery that mice have a 'normal' life without PrP-sen and that PrP-res derives from post-translational modification of PrP-sen, has suggested the inhibition of PrP-sen synthesis by specific antisense oligonucleotides as a possible therapeutical approach (Prusiner, 1992; Weissmann, 1994).

Chapter 4

Inside of the Theories

The clinical, pathological and molecular genetic features of spongiform encephalopathies (described in Chapter 3) lead to some speculation on the nature of the etiological agent and the pathogenetic mechanisms of the disease. These are more easily understood with the knowledge of experimental data from scrapie in rodents.

Creutzfeldt–Jakob disease apparently appears in three distinct manifestations: the sporadic, the 'infectious' (which includes kuru and iatrogenic cases) and the familiar form (Palmer and Collinge, 1993; Prusiner, 1993). In animals, the first two categories are clearly present; evidence of the 'familial' form of scrapie in sheep is obviously much more difficult to assess.

The first conundrum is how a disease which is experimentally transmissible to laboratory animals through the injection of tissue homogenates can, at the same time, be transmitted from one generation to the other by a genetic mechanism. There are no other examples in medicine.

The linkage of familial cases to point or insert mutations of PRNP (see Chapter 3 for details) weakens the hypothesis that affected members of these families developed the disease as a result of an exogenous infection. Similarly, sporadic and iatrogenic cases of CJD appear to be much more frequent in individuals carrying a homozygous genotype at codon 129 of PRNP (see Chapter 3), confirming that the genetic background of the host is important for the manifestation of the disease. However, clinical and pathological heterogeneity found in sporadic and familial CJD or GSS is not readily explained solely by the genetic background of the host. Furthermore, the finding that point mutations of PRNP at codon 200^{Lys} (Goldfarb *et al.*, 1991b) and 210^{Ile} (Pocchiari *et al.*, 1993) and perhaps at codon 178^{Asn} (Medori and Tritschler, 1993), are not completely penetrant, supports the hypothesis that some other factors are needed for the development of the disease. The same observation is pertinent for the supposed predisposition induced by the homozygosity at codon 129. In fact, more than 50% of normal individuals are homozygous at codon 129 and even assuming, though not true, that all cases of sporadic CJD appear in homozygous patients, the overall risk of a homozygous individual contracting the disease during his life is still less than 1 in 1000 people. Moreover, if homozygosity predisposes to CJD, then in the Japanese population the incidence

of the disease should be about double that found in eastern countries. Although such a relatively small increase of cases can only be detected by a careful co-ordination of national CJD surveillance programmes in Japan and in other countries, the available epidemiological data do not support these figures (Tsuji and Kuroiwa, 1983; Akai *et al.*, 1989). These data lessen the importance of codon 129 as an essential factor for controlling the disease.

Some investigators propose that the origin of sporadic and familial forms of the disease is a stochastic event which implies the transformation of the normal cellular PrP (PrP-sen) into the pathological isoform PrP-res (Prusiner, 1993).

Difference between PrP-sen and PrP-res

Although the primary structure of PrP-sen and PrP-res is identical and at the moment no apparent post-translational chemical modifications differentiate these isoforms (Stahl *et al.*, 1993), there is evidence that PrP-res presents an altered conformation consisting in the conversion of PrP-sen α-helices into β-sheets (Pan *et al.*, 1993). Fourier-transform infrared spectroscopy demonstrated that PrP-res has a reduction of α-helix content compared to PrP-sen (from 43% to 30%) while the β-sheet content increases from 3% in PrP-sen to 45% in PrP-res (Pan *et al.*, 1993). An even higher rate of β-sheets and a lower or absent α-helix content was determined in PrP27-30 (the N-terminal truncated PrP-res after limited proteolysis) (Caughey *et al.*, 1991b; Gasset *et al.*, 1993; Safar *et al.*, 1993). The prion hypothesis proposes that, in sporadic CJD, the initial conformational change from PrP-sen to PrP-res is a spontaneous but extremely rare event. This occurs through the formation of the PrP-sen/PrP-sen homodymer which subsequently and as a rare event, becomes PrP-sen/PrP-res and finally takes the stable and active 'infectious' form of PrP-res/PrP-res (see Fig. 1A). Once the first PrP-res is produced, this isoform induces an exponential cascade of conversions and the formation of PrP-res from newly synthesised PrP-sen does not stop until the death of the cell. Consequently, injection of the 'infectious' isoform in the host is responsible for the occurrence of iatrogenic cases, kuru, BSE, transmissible mink encephalopathy and experimentally induced spongiform encephalopathies in animals.

Proposed Mechanisms of PrP-res Formation

It has been suggested that the homodimer formation between two molecules of PrP-sen is facilitated when the proteins, synthesised by the two alleles of PRNP, share the same amino acid at codon 129 (Palmer *et al.*, 1991). This would explain why sporadic CJD occurs more frequently in patients carrying the homozygous rather than the heterozygous genotype at codon 129. This mechanism, however, would not apply to familial cases where, despite only one of the alleles being mutated, the risk of developing the disease is much higher in mutated heterozygous than in non-mutated homozygous individuals. Furthermore, affected individuals carrying the homozygous point mutation at codon 200^{Lys} did not show an accelerated pathogenesis with respect to mutated heterozygous patients (Hsiao *et al.*, 1991a; Chapman *et al.*, 1993; Gabizon *et al.*, 1993b).

Prusiner sustains that when PrP-sen carries one of the mutations described in Chapter 3, the spontaneous conformational change from PrP-sen to PrP-res is greatly facilitated (Prusiner, 1993). According to this hypothesis, a somatic mutation of the PrP gene in sporadic CJD patients may favour the transformation of PrP-sen into PrP-res. This theory is supported by the development of spontaneous CNS degeneration, indistinguishable from experimental murine scrapie, in transgenic mice following the introduction of the codon 101 point mutation (corresponding in mice to the GSS-related mutation at codon 102^{Leu}) into the PrP gene (Hsiao *et al.*, 1990). There is, however, some criticism of the conclusions drawn from this experiment and of the role played by PrP-sen and PrP-res in the development of the disease (see also Carp *et al.*, 1994). First, it is not clear whether the brains of 101-transgenic mice are infectious since transmission to hamsters but not to mice indicates the possibility of contamination with other agents (Hsiao *et al.*, 1992b) as also suggested by the lack of transmission of N2a cells expressing hamster PrP with 102^{Leu} (Chesebro *et al.*, 1993). Second, it can be argued that transgenic mice carrying multiple copies of a single gene cannot be compared to a 'natural' condition. The clinical signs occurring in transgenic mice may be related to the elevated amount of the protein rather then to the mutated isoform. Interestingly, despite the lesions resembling experimental scrapie observed in the CNS of these animals, low or no detectable PrP-res (examined by its intrinsic resistance to proteinase K treatment) was measured by Western blot (Hsiao *et al.*, 1990; Carp *et al.*, 1994). Moreover, the simple introduction of multiple copies of the wild-type PrP gene into mice produces neurological symptoms as well, confirming the supposition that the cellular protein itself is toxic when expressed at high concentrations (Westaway *et al.*, 1994).

A neurologic syndrome associated with extensive vacuolation in the brain has also been recently observed in transgenic mice expressing high levels of interleukin 6 (Campbell *et al.*, 1993) suggesting that neurons may respond in a similar manner to unrelated toxic factors. Likewise, constant exposure of primary rat hippocampal cultures to high concentrations of a peptide corresponding to residues 106–126 of the amino acid sequence of human PrP resulted in neuronal death (Forloni *et al.*, 1993). The neurotoxic mechanism of 106–126 peptide remains unclear. Its tendency to self-aggregate *in vitro* is not sufficient to explain the mechanism of cellular death. Two other peptides, corresponding to amino acid sequences 106–114 and 127–147 of human PrP, do not produce any or minimal toxic effect in neuronal cell culture, yet they have the same tendency of 106–126 peptides to form fibrils *in vitro* (Forloni *et al.*, 1993) (see Fig. 12). Thus, neuronal death may have resulted from the combination of several factors including the great amount of peptide fed to cells: respectively about a thousand or a hundred times the entire quantity of PrP-sen or PrP-res found in the brain of scrapie-infected hamsters.

Another point of comment contemplates the role of mutations and codon 129 polymorphism in the formation of PrP-res and amyloid fibrils. In codon 102^{Leu}- and codon 210^{Ile}-mutated individuals, PrP-res is composed mostly, if not only, of the mutated isoform (Doh-ura *et al.*, 1989; Maras *et al.*, 1994). However, in GSS patients with the codon 198^{Ser} mutation (Indiana kindred), the protein purified from amyloid plaques is composed of an 11 kDa fragment of PrP-res spanning residues 58 to about 150, which belongs to the mutated isoform, but does not include the region containing the amino

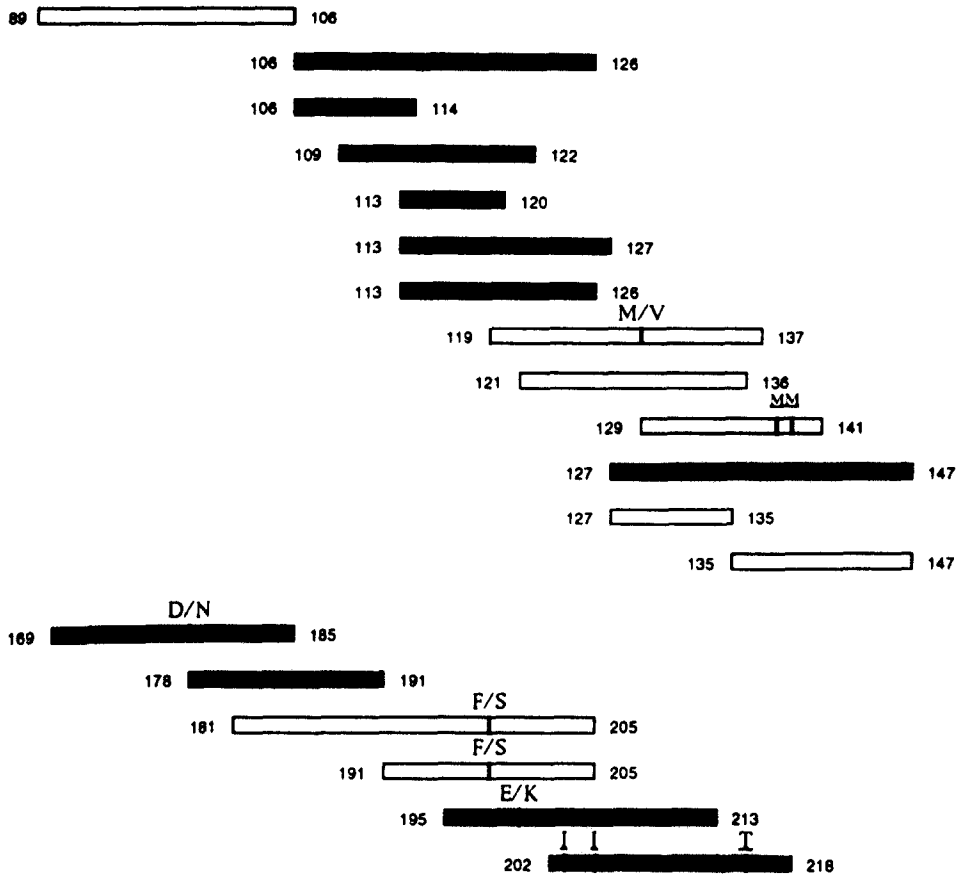

Fig. 12. Graphic representation of several peptides corresponding to different residues of the amino acid sequence of human PrP. Peptides 129–141 and 202–218 correspond to the hamster sequence and the letters (M, met; I, ile; T, thr) above the boxes indicate the amino acid differences in regards to the human sequence. The letters above the other boxes indicate the amino acid substitution at codon 129 (M, met; V, val), codon 178 (D, asp; N, asn), codon 198 (F, phe; S, ser) and codon 200 (E, glu; K, lys). Shaded boxes represent peptides which spontaneously produce fibrils *in vitro* and exhibit the tinctorial and optical features of amyloid. Data from Gasset *et al.*, 1992; Goldfarb *et al.*, 1993a; Tagliavini *et al.*, 1993.

acid substitution (Tagliavini *et al.*, 1991). Interestingly, both wild type and mutated peptides from the PrP region with the codon 198^{Ser} mutation (residues 191–205 and 181–205) were either barely or not at all fibrillogenic and do not exhibit any tinctorial and optical properties of *in situ* amyloid (Tagliavini *et al.*, 1993) (see Fig. 12). On the other hand, the peptide spanning residues 127–147 of PrP (outside the mutated region but within the 11 kDa fragment) spontaneously produce fibrils and exhibit the tinctorial and optical features of amyloid (Tagliavini *et al.*, 1993) (see Fig. 12). This finding suggests that codon 198^{Ser} mutation is not required for amyloid, nor most likely for PrP-res formation and that it differs from codon 178^{Asn} and codon 200^{Lys} mutations, in that the point mutation increases fibril formation and the amyloid-specific Congo red birefringence (Goldfarb *et al.*, 1993a).

Similar to peptides with codon 198, peptides spanning residues 119–137, either with methionine or valine in position 129, do not aggregate *in vitro* (Goldfarb *et al.*, 1993a). Furthermore, the expression of recombinant PrP with insertion of one, two, four or six octapeptide repeats besides the five present in wild-type PrP in scrapie-infected N2a cells does not change the efficiency of PrP-res formation (Rogers *et al.*, 1993). These data suggest that some mutations of the PrP gene may increase the susceptibility to the disease without, *per se*, enhancing the transformation from PrP-sen to PrP-res.

Considering the alternative hypothesis of viral infection, it can be speculated that the change from Phe to Ser at codon 198 or homozygosity at codon 129 may facilitate the binding of the virus to PrP-sen and that this interaction is responsible for the conformational change from PrP-sen to PrP-res (Diringer, 1992). In other words, the virus may interact with PrP-sen at two different sites, one responsible for the entry and the initiation of replication into the target cell (the 'replication' site, Fig. 13A) and the other for driving the conversion of PrP-sen into PrP-res (the 'conversion' site, Fig. 13A). In this scenario, some amino acid substitutions or insertions in PrP-sen may facilitate the replication of the agent, while others facilitate the conformational changes of the protein from α-helices into β-sheets.

PrP as the Receptor for the Infectious Agent

This conjecture is supported by the finding that treatment of scrapie-infected hamsters with the polyene antibiotic amphotericin B delays the accumulation in the brain of PrP-res without affecting scrapie replication both in the brain and spleen (Xi *et al.*, 1992). In this model, amphotericin B may preclude the binding of the agent to the 'conversion' site, i.e. no PrP-res formation, but not the 'replication' site (see Fig. 13B). This effect resulted in a delay of the appearance of scrapie clinical signs compared to infected controls, suggesting that the accumulation of PrP-res in the brain is more important than replication of the scrapie agent for the expression of disease (Xi *et al.*, 1992). Interestingly, the beneficial anti-scrapie effect of amphotericin B is limited to the 263K hamster-adapted strain (Pocchiari *et al.*, 1987, 1989; Casaccia *et al.*, 1991) and the murine strain C506 (Demaimay *et al.*, 1993). Amphotericin B treatment is ineffective with other hamster or mouse-adapted strains of scrapie (Carp, 1992; Xi *et al.*, 1992), suggesting that it interferes with strain-specific (that is, 263K and C506) components of the scrapie agent responsible for the modification of PrP-sen to PrP-res (Fig. 13C). In conclusion, it is possible that the binding of the agent to PrP-sen is strain-specific which means that different strains of scrapie, and most likely of human spongiform encephalopathies as well, recognise distinct epitopes on PrP-sen.

The Scrapie and CJD Strains: The Role of PrP

How can different strains of viruses be characterised if their structure and genome are unknown? Of course, not by searching their genoma for mutations, but by looking at their different phenotypic expression in the host. The suspicions on the existence of different strains started with the observation of clinical heterogeneity, the 'nervous' and 'itchy' forms (Stockman, 1926), in natural scrapie (see Chapter 3). This observation was expanded by the work of Pattison and Millson (1961b), who found that the intracerebral goat passage of scrapie brain material originating from the same source, i.e., SSBP/1 (scrapie sheep brain pool) after several passages through mostly Cheviot sheep (Wilson

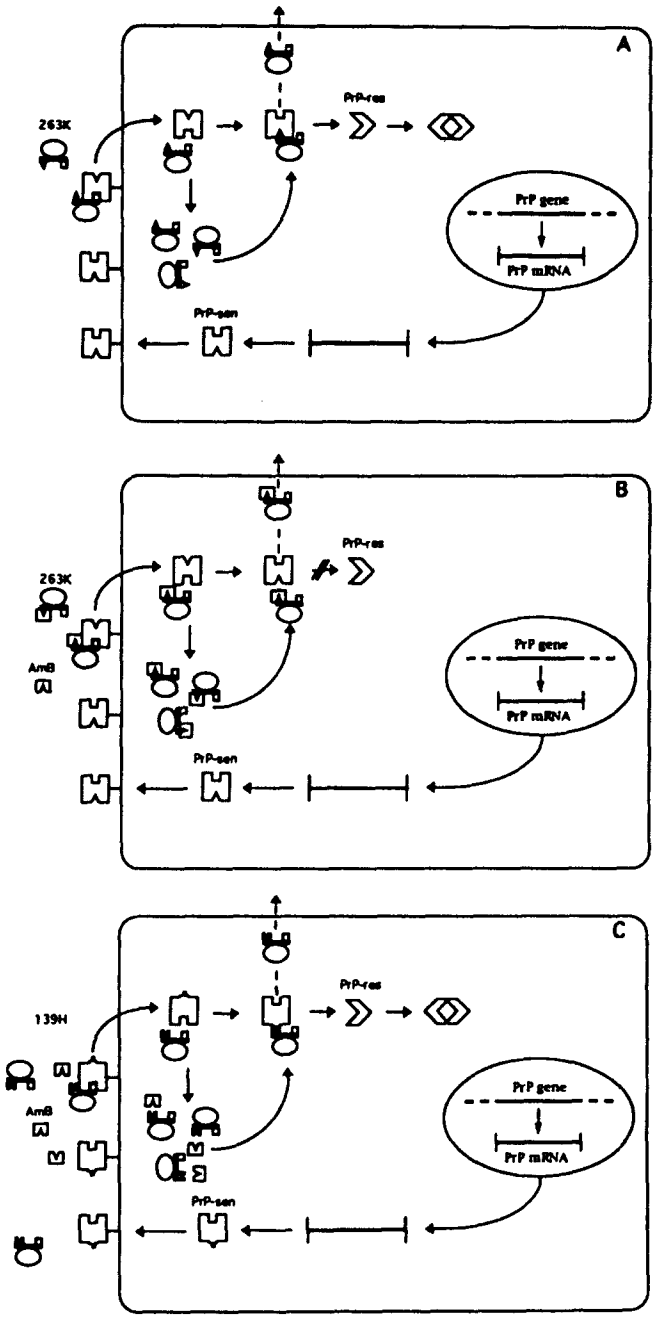

Fig. 13. Hypothetical mechanism of the antiscrapie strain-specific effect of amphotericin B (AmB). In A it is shown that the interaction between the strain 263K of scrapie and PrP-sen occurs at two different sites, one responsible for the entry and the initiation of replication in the target cell (the 'replication' site) and the other for driving the conversion of PrP-sen into PrP-res (the 'conversion' site). Amphotericin B precludes the binding of the 263K strain (B) but not of the 139H strain (C) of scrapie to the 'conversion' site.

et al., 1950), produced either the 'nervous' (later called the 'drowsy') or the 'scratching' form and that injection of brain homogenates prepared from 'drowsy' or 'scratching' animals produced respectively the 'drowsy' or the 'scratching' clinical syndromes in goats and sheep (Pattison, 1966). This was the first indication of the existence of different strains of scrapie which was followed by the identification of many scrapie strains in mice (Dickinson and Meikle, 1971; Fraser and Dickinson, 1973; Dickinson, 1976), sheep (Foster and Dickinson, 1988) and hamsters (Kimberlin and Walker, 1978b; Kimberlin *et al.*, 1989) and of CJD strains in mice (Mori *et al.*, 1989; Kitamoto *et al.*, 1990), hamsters (Manuelidis *et al.*, 1978b) and primates (Gibbs *et al.*, 1979) and transmissible mink encephalopathy in hamsters (Kimberlin *et al.*, 1986; Bessen and Marsh, 1992a,b). The distinction between strains is based on several phenotypic characteristics, some of which are strictly under the control of the scrapie agent while others depend upon the genetic control of both host and agent (for an exhaustive review, see Carp, 1992; Bruce, 1993).

The two most striking parameters used to differentiate between the strains are the incubation period between infection and clinical appearance of the disease (Dickinson and Meikle, 1971; Dickinson and Outram, 1988; Bruce *et al.*, 1991) and the distribution of spongiform changes in the brain (known as the lesion profile) (Fraser and Dickinson, 1968, 1973; Bruce and Fraser, 1982). In mice the host *sinc* (from *s*crapie *inc*ubation) gene (Dickinson *et al.*, 1968a) regulates, together with the scrapie agent, both these features. Although not formally proved, it is most likely that the *sinc* and PrP gene are one and the same (Carlson *et al.*, 1986; Hunter *et al.*, 1987; Westaway *et al.*, 1987). The *sinc* gene has two alleles, designated *s*7 and *p*7, which correspond to two amino acid differences at codon 108 (Leu → Phe) and codon 189 (The → Val) in the PrP sequence (Westaway *et al.*,1987). Experimental infection with the ME7 strain of scrapie produces a short incubation period in *s*7 homozygous mice, a prolonged incubation period in *p*7 homozygous mice and an intermediate one in heterozygous mice (*s*7*p*7). Other strains of scrapie, however, behave differently in relation to the *sinc* genotype. As an example, the 22A strain shows a short, intermediate and long incubation period respectively in *p*7 homozygous, *s*7 homozygous and heterozygous mice. Interestingly, both alleles appear dominant in ME7 infected mice (the incubation period in heterozygous mice lasts between those of *s*7 and *p*7 homozygous mice), while in 22A there is an over dominance of *p*7 in relation to *s*7 (the incubation period of heterozygous mice lasts longer than those of both homozygous mice). Each of the about 20 strains of murine scrapie has a characteristic and reproducible incubation period in the three *sinc* genotypes of mice supporting the view that this feature, in mice, is under the control of both the host and infectious agent (for a review see Bruce, 1993).

In sheep, the analog of *sinc* is the sip (*s*crapie *i*ncubation *p*eriod) gene (Dickinson *et al.*, 1968b; Dickinson and Outram, 1988); sip has two alleles, sA and pA, whose likely products correspond to PrP with a single amino acid difference at codon 136 (Hunter *et al.*, 1993; Laplanche *et al.*, 1993). Natural scrapie has been observed only in sheep carrying either the 136 Val/Val (sA/sA) or 136 Val/Ala (sA/pA) genotypes. The 136 Ala/Ala (pA/pA) genotype was never found in sheep with natural scrapie (Hunter *et al.*, 1993; Laplanche *et al.*, 1993). The sip gene also controls the susceptibility of sheep to subcutaneous injection of the scrapie agent. Both sA homozygous and heterozygous sheep develop the disease in about 500 days, whereas pA homozygous sheep fail to

contract the disease (Dickinson *et al.*, 1968b; Goldmann *et al.*, 1991a; Maciulis *et al.*, 1992). As a rule, we may conclude that in natural and in subcutaneous-induced scrapie the sA allele acts with full dominance and that sheep homozygous for the pA allele are resistant to natural infection. Although there is at least one scrapie isolate which differs from other strains in that the sip alleles act in the opposite way (Foster and Dickinson, 1988), it is possible to speculate that 'resistant' pA homozygous sheep harbour the infectious agent in peripheral organs, i.e., spleen and lymph nodes and that the entry into the CNS is somehow blocked by the pA isoform of PrP. Outside the CNS, PrP may bind the infectious agent and drive it to specific target areas of the brain (Bruce *et al.*, 1991; Hope and Baybutt, 1991; Scott *et al.*, 1992).

Combinations between strains and amino acid changes of PrP may facilitate the neuroinvasion of the agent following an early replication of scrapie in the CNS, may target the agent to different brain regions resulting in different lesion profiles and clinical manifestations, or may delay the access to the CNS and, consequently, the appearance of the disease during the life span of the host. This last event occurs in *s7* homozygous mice infected by the intraperitoneal route with low doses of 22A scrapie strain (the combination 22A/*s7s7* produces a long incubation period, see above). These mice do not develop the disease during their lifetime, but harbour infectivity in their spleen beginning from 300 days after inoculation (Dickinson *et al.*, 1975b). It is therefore possible that clinical and pathological heterogeneity in sporadic and familial forms of spongiform encephalopathies results from the combination of different strains of CJD with PrP polymorphisms or point and insert mutations.

The Importance of Neuroinvasion in the Development of Disease

Neuroinvasion is the key stage in the pathogenesis of scrapie and of other spongiform encephalopathies without which the disease never develops (Kimberlin and Walker, 1988). This explains why injection of the scrapie agent by intracerebral and intraspinal routes always produces an incubation period shorter than non-neural routes (Kimberlin *et al.*, 1987) and that the intraperitoneal route of infection is between 100 and 1000 times less efficient than the intracerebral route (Kimberlin and Walker, 1988; Pocchiari *et al.*, 1991b). In peripherally scrapie-infected mice, replication in the brain is always preceded by replication in the spleen, lymph nodes and other lymphoreticular tissues (Kimberlin and Walker, 1978a, 1979a). The spleen plays a key role in regulating the neuroinvasion of the agent and genetic asplenia (Dickinson and Fraser, 1972) or splenectomy (Fraser and Dickinson, 1970, 1978) performed before peripheral infection or soon afterwards lengthens the incubation period of the disease. In contrast, after intracerebral injection, splenectomy does not modify the timing of replication of the agent in the brain and this suggests that this route by-passes the extraneural stage of scrapie pathogenesis (Fraser and Dickinson, 1970). This explains the relatively shorter interval between accidental exposure of the CJD agent and appearance of clinical signs in centrally infected cases of CJD (1–2 years) compared with peripherally infected cases (several years to decades) (Brown, 1988c).

After peripheral injection, the scrapie agent moves from the spleen and lymph nodes through a retrograde axonal transport in autonomic nerve fibers to the spinal cord and from here, arrives at the 'clinical target areas' of the brain (Kimberlin and Walker, 1983). Alternatively, the scrapie agent may be taken up by carrier cells (most likely reticulo endothelial system cells) and spread to the 'clinical target areas' of the CNS

through the blood stream. This is suggested by a low but constant level of viremia after peripheral injection of scrapie in the pre-neural phase of infection (Diringer, 1984; Casaccia *et al.*, 1989). However, the lack of a viremic peak before the invasion of the CNS (Casaccia *et al.*, 1989) weakens the hypothesis of the spread of scrapie agent by this route. When the infectious agent has reached the brain, it replicates at an exponential rate until the appearance of the disease (Kimberlin, 1976; Moreau Dubois *et al.*, 1982; Kimberlin and Walker, 1986a; Pocchiari and Masullo, 1988). Each strain of scrapie and CJD produces histological lesions and PrP-res accumulation in specific brain regions and this may result, in humans, in the clinical and pathological heterogeneity described in Chapter 3.

In natural infection of scrapie in sheep and goats the spread of the agent follows a pattern similar to that described for the murine model (Hadlow *et al.*, 1980, 1982), although it is not yet unequivocally established which is the port of entry of the scrapie agent (Dickinson, 1976; Hourrigan *et al.*, 1979).

The Port of Entry in the Virus/Virino Hypothesis

The mechanism of natural transmission of spongiform encephalopathies according to the virus/virino hypothesis remains enigmatic. Most of our knowledge comes from field work in sheep, where scrapie disease is spread either from flock to flock by the movement of infected, but not necessarily sick animals, or by maternal transmission from infected ewe to lamb (Dickinson *et al.*, 1974; Dickinson, 1976; Hourrigan *et al.*, 1979). Whether maternal transmission in natural scrapie occurs before or shortly after birth remains a controversy (Foster *et al.*, 1992; Foote *et al.*, 1993). However, the finding that the progressive increase in scrapie incidence among lambs born from infected dams is related to the time that lambs spent with their mothers indicates that infection occurs after birth, most likely through ingestion of or scarification from contaminated foetal fluids or placenta (Pattison *et al.*, 1972, 1974). As in sheep, feeding or intragastric administration of scrapie or CJD infected tissues have also produced the disease in mice (Zlotnik and Rennie, 1962; Chandler, 1963; Kimberlin and Walker, 1989), hamsters (Prusiner *et al.*, 1985; Kimberlin and Walker, 1986a) and primates (Gibbs *et al.*, 1980). The supposed role of milk in establishing the infection contrasts with the failure to detect infectivity in mammary glands, colostrum and milk of scrapie-infected sheep and goats (Pattison and Millson, 1961a; Hadlow *et al.*, 1980, 1982; Hourrigan, 1990). This also supports the observation that kuru and CJD have never occurred in children whose only risk factor was their affected mothers (Prusiner *et al.*, 1982b; Brown *et al.*, 1987).

A possible source of infection for humans may well be natural scrapie in sheep or, in the near future, BSE. Humans could become infected through contaminated food or bovine-derived biological products and develop the disease decades after the initial infection (Dealler and Lacey, 1990). Although the available epidemiological data failed to show a relationship between scrapie and CJD (see Chapter 3), it is too early to forecast the impact of BSE on human health (Will *et al.*, 1992). The agent of BSE differs from those found in natural scrapie, remains stable after passage in other species (Fraser *et al.*, 1992a; Bruce, 1993) and is pathogenic via the oral route for many species including mice (Barlow and Middleton, 1990) and felines (Wyatt *et al.*, 1991). It

also causes the disease in pigs (Dawson *et al.*, 1990) and marmoset (Baker *et al.*, 1993), though by other routes.

The Species Barrier

However, the risk for humans of being infected by the scrapie or BSE agent may be minimal since the passage from one species to another usually results in a prolongation of the incubation period of the disease or in no disease at all. This phenomenon is referred to as the 'species barrier' and is governed by the interaction between the infectious agent and PrP-sen (Dickinson, 1976; Kimberlin, 1993). As we said, sometimes the 'species barrier' is absolute and the new animal species does not develop the disease. This is clearly illustrated by the failure to transmit the disease to mice by intracerebral injection of the 263K strain of hamster scrapie (Kimberlin *et al.*, 1989). This effect depends on the different sequences of PrP-sen in mice and hamsters. The construction of transgenic mice expressing the hamster PrP gene abrogates the 'species barrier' of 263K between these two species (Scott *et al.*, 1989; Prusiner *et al.*, 1990). Transgenic mice develop scrapie and the length of the incubation period is inversely proportional to the level of hamster PrP-sen (Prusiner *et al.*, 1990). Mouse and hamster PrP-sen differ in 16 amino acids, but only 5 (position 108, 111, 138, 154 and 169) seem necessary to regulate the 'species barrier' effect, as has been shown by transgenic mice expressing chimeric PrP genes (Scott *et al.*, 1993). Interestingly, none of them are in a region thought to be responsible for the formation of fibrils (see above and Fig. 12) arguing that substitution of these amino acids is most likely involved in the binding of 263K to the 'replication' site rather than to the 'conformational' site (see Fig. 13). Thus, PrP-sen may function as a cellular receptor molecule for spongiform encephalopathy agents and, if so, it does fit in with the observation that when the PrP gene is removed, no scrapie replication occurs (Büeler *et al.*, 1993).

The other phenomenon associated with crossing the species barrier is the selection of a strain from a mixture. This effect occurs when the inoculum contains more than one strain of agent, one of which replicates faster than the others in the new species (Dickinson, 1976). Occasionally, a mutant strain (e.g., the 263K strain of hamster scrapie) emerges during the passage from one species to another and is then selected because it has a better 'affinity' to the new host than the parental strain (Kimberlin *et al.*, 1989).

Thus, it is possible that human spongiform encephalopathy agents are widely diffuse in the population, but that the 'normal' conformation of PrP-sen does not allow the transfer of the infection from the periphery to the CNS and that, therefore, most people are infected, but only a very few develop clinical signs of the disease.

The finding that hamsters inoculated with blood of healthy people develop clinical and pathological signs of spongiform encephalopathies corroborates this hypothesis (Manuelidis and Manuelidis, 1993), but much more work is needed to confirm this result. Alternatively, it is feasible that man is normally infected by a strain of virus which is not pathogenic but that otherwise replicates in the lymphoreticular organs precluding the establishment of infection by other virulent strains. This agent competition presumes a limited number of replication sites in peripheral tissues which can be easily saturated by the non-pathogenic agent (Dickinson and Outram, 1979; Kimberlin and Walker, 1985). This hypothesis has been successfully tested in mice where, under rigorous

experimental procedure, the injection of a 'slow' strain of scrapie agent followed by a second injection of a 'quick' strain produces a total blockage of the second agent and its complete exclusion from participation in the disease (Dickinson *et al.*, 1972, 1975a; Dickinson and Outram, 1979).

The Theories

The most objective conclusion is that all the proposed theories have some degree of validity. The virus, the virino and the 'unified theory' proposed by Weissmann (1991), all agree that strain variability unequivocally proves the existence of a nucleic acid component of the infectious agent which, as in conventional viruses, may undergo mutations responsible for phenotypic variations. The problem with these theories is that no specific nucleic acid has yet been convincingly identified to copurify with infectivity (Manuelidis and Manuelidis, 1981; Duguid *et al.*, 1988; Oesch *et al.*, 1988; Murdoch *et al.*, 1990; Meyer *et al.*, 1991; Kellings *et al.*, 1992; Sklaviadis *et al.*, 1993). Moreover, chemical, enzymatic or physical treatments which usually inactivate or degrade nucleic acids have no effect whatsoever on the transmissible properties of the infectious agent (Alper *et al.*, 1966, 1978; McKinley *et al.*, 1983b; Bellinger Kawahara *et al.*, 1987a,b; Neary *et al.*, 1991). Possible reasons are that the amount of nucleic acid of the putative agent is too small to be detected with available techniques and that its tight bond to the protein protects it from chemical or physical inactivation. For the 'unified theory', then, the proposal that the nucleic acid comes from the host makes its identification even more difficult. Weakening the virus and virino hypotheses is also the fact that no convincing virus particles have ever been observed under the electron microscope (Vernon *et al.*, 1970; Narang, 1974, 1990; Bots *et al.*, 1971; Cho and Greig, 1975). However, the recent observation under the electron microscope by Özel and Diringer (1994) that pentagonal particles resembling virus structures are found close to SAF in fractions of scrapie-infected hamster brains may give new impulse to the 'virus' theory. These particles have a diameter of 10–12 nm, which is far smaller than the 18 nm diameter of the smallest known virus (porcine circo virus (Tischer *et al.*, 1982)). The lack of immune response in the infected host despite the high infectivity level found in lymphoreticular tissues also weakens the 'virus' hypothesis, unless the virus replicates in immunocompetent cells without causing their activation or dysfunction (Fraser *et al.*, 1992b). This is supported by the finding that scrapie agent replication in the spleen occurs mainly in non-dividing, radiation resistant cells which have been identified as follicular dendritic cells (Clarke and Kimberlin, 1984; Fraser and Farquhar, 1987; Kitamoto *et al.*, 1991; McBride *et al.*, 1992; Muramoto *et al.*, 1993).

Some investigators also claim that the other point that weakens the virus hypotheses is the apparent co-purification of infectivity with PrP-res (Bolton *et al.*, 1982; Prusiner *et al.*, 1982a; Diringer *et al.*, 1983; McKinley *et al.*, 1983a; Safar *et al.*, 1990). However, there is evidence that under definite experimental conditions this association is not maintained (Czub *et al.*, 1986, 1988; Manuelidis *et al.*, 1987; Xi *et al.*, 1992), arguing that all the proposed theories considering PrP as the only or an essential component of the infectious agent are unsuitable.

On the other hand, the 'prion' and the 'nucleation' theory of Gajdusek (1993a,b) have strong support not only where the other theories fail, but also in the linkage

between human PRNP mutations and the appearance of the disease (but see alternative explanations given earlier in this chapter). They, however, fail to explain the marked clinical and pathological heterogeneity observed in spongiform encephalopathies. The 'targeting theory' has been proposed to circumvent this difficulty (Hecker *et al.*, 1992). This theory sustains that each strain of scrapie (and of other related diseases) is derived from the 'replication' of PrP-res in different and strain-specific neuronal cells which produce PrP with distinct post-translational modifications, for example carbohydrate residues, that are retained in the formation of new and 'infectious' PrP-res. These strain-specific carbohydrate residues will then target PrP-res to the same subset of cells in the following transmission (see Fig. 14). This bizarre hypothesis is based on a personal interpretation that different strains of scrapie and CJD produce variable lesion profiles and PrP-res accumulation in the brain (see above).

Finally, it is unquestionable that PrP-sen is essential for the initiation of scrapie infection and that the change from PrP-sen to PrP-res is important for the development of clinical signs. However, this does not automatically mean that PrP-res is the etiological agent of spongiform encephalopathies. It is still possible that PrP-sen acts as the cellular receptor for these agents which are then responsible for the conformational change to PrP-res. Viral receptor means 'a host surface component that participates in virus binding and facilitates viral infection' (Haywood, 1994). Viruses must enter the host cell to replicate and this is accomplished through the binding of the virus to the cell surface receptor. Specific receptors have been defined for several viruses (Knipe, 1990; Haywood, 1994). Examples are the CD4 protein molecule for human immunodeficiency virus (HIV) (Dalgleish *et al.*, 1984; Klatzman *et al.*, 1984), the human membrane cofactor protein (CD46) for measles virus (Dörig *et al.*, 1993; Naniche *et al.*, 1993), the human poliovirus receptor (hPVR) for poliovirus (Mendelsohn *et al.*, 1989), the intercellular adhesion molecule 1 (ICAM-1) for rhinovirus (Greve *et al.*, 1989; Staunton *et al.*, 1989; Tomassini *et al.*, 1989), the C3d complement receptor (CR2) for Epstein–Barr virus (Fingeroth *et al.*, 1984; Numerow *et al.*, 1985) and a few others (Delmas *et al.*, 1992; Yeager *et al.*, 1992; Bates *et al.*, 1993; Haun *et al.*, 1993).

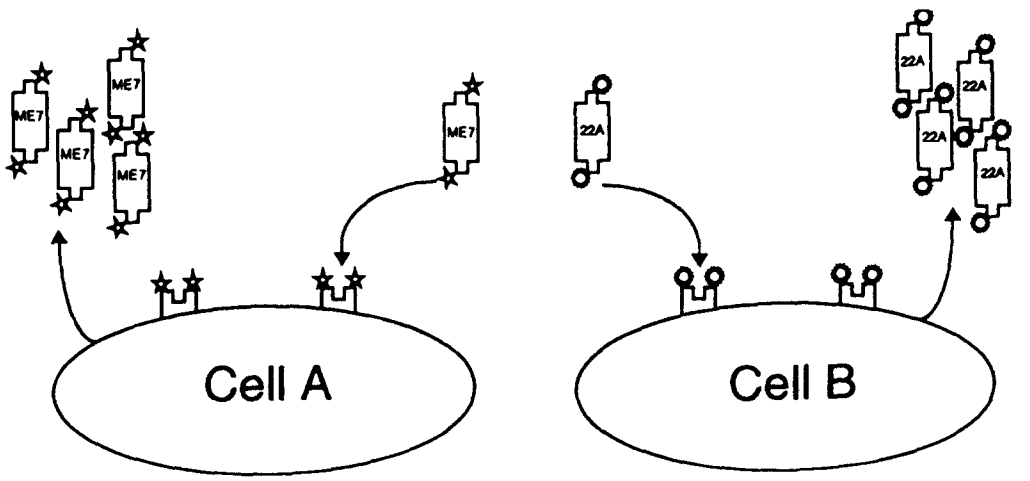

Fig. 14. Graphic representation of the 'targeting theory'. For details, see the text. ME7 and 22A represent two different strains of the scrapie agent. Stars and rosettes represent different sugar residues on the prion molecule.

PrP-sen is a good candidate for the scrapie cell receptor. Like receptors for conventional viruses (for a review see Lentz, 1990), PrP-sen is a sialoglycoprotein exposed to the cell surface (Bolton *et al.*, 1985). PrP-less transgenic mice do not allow for scrapie replication (Büeler *et al.*, 1993) similar to the absence of viral replication in cells which do not express the virus-specific receptor molecule (Maddon *et al.*, 1986; Mendelsohn *et al.*, 1989; Delmas *et al.*, 1992; Morrison and Racaniello, 1992; Yeager *et al.*, 1992; Dörig *et al.*, 1993; Haun *et al.*, 1993; Naniche *et al.*, 1993). However, recombinant expression of the virus-specific receptor confers infectivity by the cognate virus to otherwise non-permissive cell lines (Maddon *et al.*, 1986; Mendelsohn *et al.*, 1989; Delmas *et al.*, 1992; Morrison and Racaniello, 1992; Yeager *et al.*, 1992; Dörig *et al.*, 1993; Naniche *et al.*, 1993; Young *et al.*, 1993; Manchester *et al.*, 1994). In contrast to scrapie, mice are not susceptible to poliovirus infection simply because the murine homologue, Mph, of the poliovirus receptor, does not bind poliovirus even though it has an extensive sequence similarity to the extracellular domain of hPVR (Morrison and Racaniello, 1992; Morrison *et al.*, 1994). However, transgenic mice carrying the hPVR gene become permissive to poliovirus infection and show a paralytic disease which is clinically and pathologically similar to human poliomyelitis (Ren *et al.*, 1990; Koike *et al.*, 1991; Ren and Racaniello, 1992; Horie *et al.*, 1994). The inability of PrP-less transgenic mice to replicate the scrapie agent may, therefore, be simply attributed to the absence of the scrapie-specific receptor molecule which precludes the attachment of the scrapie particle to the host cell membrane. This is the initial stage in any viral infectious cycle.

Moreover, a single amino acid substitution (Ile to Leu at position 214) in the molecule expressed on *Mus dunni* tail fibroblast (MDTF) cells allows that protein to function as a receptor for the Moloney murine leukemia virus and renders those cells susceptible to infection (Eiden *et al.*, 1993). On the other hand, the substitution of one residue in the molecule of the human poliovirus receptor abolishes virus binding and virus replication (Morrison *et al.*, 1994). These findings suggest that a similar mechanism may occur in human spongiform encephalopathies where single amino acid substitutions may only render mutated individuals more susceptible to a widespread but otherwise low pathogenic infectious agent.

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