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SUMMARY

Scrapie was thought for many years to be caused by a virus. Enriching fractions from Syrian hamster (SHa) brain for scrapie infectivity led to the discovery of the prion protein (PrP). To date, no scrapie-specific nucleic acid has been found. As well as scrapie, prion diseases include bovine spongiform encephalopathy (BSE) of cattle, as well as Creutzfeldt–Jakob disease (CJD) and Gerstmann–Sträussler–Scheinker syndrome (GSS) of humans. Transgenic (Tg) mice expressing both SHa and mouse (Mo) PrP genes were used to probe the molecular basis of the species barrier and the mechanism of scrapie prion replication. The prion inoculum was found to dictate which prions are synthesized *de novo*, even though the cells express both PrP genes. Discovery of mutations in the PrP genes of humans with GSS and familial CJD established that prion diseases are both genetic and infectious. Tg mice expressing MoPrP with the GSS point mutation spontaneously develop neurologic dysfunction, spongiform degeneration and astrocytic gliosis. Inoculation of brain extracts prepared from these Tg(MoPrP-P101L) mice produced neurodegeneration in many of the recipient animals after prolonged incubation times. These and other results suggest that prions are devoid of foreign nucleic acid and are thus different from viruses and viroids. Studies on the structure of PrP^{Sc} and PrP^{C} suggest that the difference is conformational. Whether one or more putative α-helices in PrP^{C} are converted into β-sheets during synthesis of PrP^{Sc} is unknown. Distinct prion isolates or 'strains' exhibit different patterns of PrP^{Sc} accumulation which are independent of incubation times. Whether variations in PrP^{Sc} conformation are responsible for prion diversity remains to be established. Prion studies have given new insights into the etiologies of infectious, sporadic and inherited degenerative diseases.

1. INTRODUCTION

Investigations of scrapie in animals and related human diseases provide a fascinating saga in biomedical research that has yielded many unprecedented findings. For more than 25 years, two uncommon human diseases and several animal disorders including scrapie were labelled transmissible encephalopathies, spongiform encephalopathies or slow virus diseases (Gajdusek 1977, 1985; Sigurdsson 1954). These fatal illnesses were often transmissible to experimental animals after a prolonged incubation period, and some features of the transmissible pathogen resembled those of viruses. Yet early attempts to characterize the infectious pathogen causing scrapie of sheep and goats produced results which suggested that these transmissible agents differed from both viruses and viroids (Alper *et al.* 1966, 1967; Hunter 1972).

An investigation into the etiology of scrapie followed the vaccination of sheep for looping ill virus with formalin-treated extracts of ovine lymphoid tissue unknowingly contaminated with scrapie prions (Gordon 1946). Two years later, more than 1500 sheep developed scrapie from this vaccine. Although the transmissibility of experimental scrapie became well established, the spread of natural scrapie within and among flocks of sheep remained puzzling. Parry argued that host genes were responsible for the development of scrapie in sheep. He was convinced that natural scrapie is a genetic disease which could be eradicated by proper breeding protocols (Parry 1962, 1982). He considered its transmission by inoculation of importance, primarily for laboratory studies, and communicable infection of little consequence in nature. Other investigators viewed natural scrapie as an infectious disease, and argued that host genetics only modulates susceptibility to an endemic infectious agent (Dickinson *et al.* 1965).

Some remarkable discoveries in the past three decades have led to the molecular and genetic characterization of the transmissible pathogen causing scrapie in animals and a quartet of human illnesses: kuru, Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease (GSS) and fatal familial insomnia (FFI) (table 1). To distinguish this infectious pathogen from viruses and viroids, the term 'prion' was introduced to emphasize its proteinaceous and infectious nature (Prusiner 1982). An abnormal isoform of the prion protein (PrP), PrP^{Sc}, is the only known component of the prion (Prusiner 1991). PrP is encoded by a gene on the short arm of chromosome 20 in humans (Sparkes *et al.* 1986). PrP^{Sc} differs physically from the normal, cellular isoform PrP^{C} by its high β-sheet content, its insolubility in detergents, its

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Table 1. *Human prion diseases*

disease	etiology
kuru	infection
Creutzfeldt–Jakob disease	
iatrogenic	infection
sporadic	unknown
familial	PrP mutation
Gerstmann–Sträussler–Scheinker disease	PrP mutation
fatal familial insomnia	PrP mutation

propensity to aggregate, and its relative resistance to proteolysis (Oesch *et al.* 1985; Meyer *et al.* 1986; Pan *et al.* 1993).

Accumulation of PrP^{Sc} in the brain has been found in most of the human prion diseases. The presence of PrP^{Sc} implicates prions in the pathogenesis of these diseases. However, in rare patients (Brown *et al.* 1992; Medori *et al.* 1992) and some transgenic (Tg) 174 mice which appear to have low or undetectable amounts of PrP^{Sc}, neurodegeneration appears, at least in part, to be caused by abnormal metabolism of mutant PrP (Hsiao *et al.* 1990). In these cases, horizontal transmission of neurodegeneration from such patients to experimental animals may not be demonstrable (Tateishi *et al.* 1992) but has been demonstrated for some mice expressing wild-type or mutant PrP transgenes (Westaway *et al.* 1994*a*) (K. K. Hsiao, D. Groth, S.-L. Yang, H. Serban, D. Rapp, D. Foster, M. Scott, M. Torchia, S. J. DeArmond & S. B. Prusiner, unpublished results). Whether it will be useful to distinguish between those prion diseases in which transmission can be demonstrated and those in which it cannot with current animal models remains to be established (Prusiner & Hsiao 1994). As our knowledge of the prion diseases increases, and more is learned about the molecular and genetic characteristics of prion proteins, these disorders will undoubtedly undergo modification with respect to their classification. Indeed, the discovery of PrP and the identification of pathogenic PrP gene mutations have already forced us to view these illnesses from perspectives not previously imagined.

2. DEVELOPMENT OF THE PRION CONCEPT

Once an effective protocol was developed for preparation of partly purified fractions of scrapie agent from hamster brain, it became possible to demonstrate that those procedures which modify or hydrolyse proteins produce a diminution in scrapie infectivity (Prusiner 1982; Prusiner *et al.* 1981). At the same time, tests done in search of a scrapie-specific nucleic acid were unable to demonstrate any dependence of infectivity on a polynucleotide (Prusiner 1982), in agreement with earlier studies reporting the extreme resistance of infectivity to ultraviolet irradiation at 254 nm (Alper *et al.* 1967).

Based on these findings, the term 'prion' was introduced to distinguish the proteinaceous infectious particles that cause scrapie, CJD, GSS and kuru from both viroids and viruses (Prusiner 1982). Hypotheses for the structure of the infectious prion particle included: (i) proteins surrounding a nucleic acid encoding them (a virus); (ii) proteins associated with a small polynucleotide; and (iii) proteins devoid of nucleic acid (Prusiner 1982). Mechanisms postulated

Table 2. *Evidence that PrP^{Sc} is a major and essential component of the infectious prion*

1. Copurification of PrP 27–30 and scrapie infectivity by biochemical methods. Concentration of PrP 27–30 is proportional to prion titer (Bolton *et al.* 1982; Prusiner *et al.* 1982; McKinley *et al.* 1983*a*; Hope *et al.* 1986; Turk *et al.* 1988; Safar *et al.* 1990*b*; Jendroska *et al.* 1991).
2. Kinetics of proteolytic digestion of PrP 27–30 and infectivity are similar (Bolton *et al.* 1982; Prusiner *et al.* 1982; McKinley *et al.* 1983*a*).
3. Copurification of PrP^{Sc} and infectivity by immunoaffinity chromatography. α-PrP antisera neutralization of infectivity (Gabizon *et al.* 1988*b*; Gabizon & Prusiner 1990).
4. PrP^{Sc} detected only in clones of cultured cells producing infectivity (Butler *et al.* 1988; Taraboulos *et al.* 1990*b*; McKinley *et al.* 1991*b*).
5. PrP amyloid plaques are specific for prion diseases of animals and humans (Bendheim *et al.* 1984; DeArmond *et al.* 1985; Kitamoto *et al.* 1986; Roberts *et al.* 1988). Deposition of PrP amyloid is controlled, at least in part, by the PrP sequence (Prusiner *et al.* 1990).
6. PrP^{Sc} (or PrP^{CJD}) is specific for prion diseases of animals and humans (Bockman *et al.* 1985; Brown *et al.* 1986; Serban *et al.* 1990).
7. Genetic linkage between MoPrP gene and scrapie incubation times (Carlson *et al.* 1986, 1988; Hunter *et al.* 1987; Race *et al.* 1990). PrP gene of mice with long incubation times encodes amino acid substitutions at codons 108 and 189, compared with mice with short or intermediate incubation times (Westaway *et al.* 1987).
8. The level of SHaPrP transgene expression and the primary structure of PrP^{Sc} in the inoculum govern the 'species barrier', scrapie incubation times, neuropathology, and prion synthesis in mice (Scott *et al.* 1989; Prusiner *et al.* 1990).
9. Genetic linkage between PrP gene point mutations at codons 102, 178, 198 or 200 and the development of inherited prion diseases in humans was demonstrated (Hsiao *et al.* 1989; Dlouhy *et al.* 1992; Petersen *et al.* 1992; Gabizon *et al.* 1993). Genetic linkage was also established between the mutation insert of six additional octarepeats and familial CJD (Poulter *et al.* 1992).
10. Mice expressing MoPrP transgenes with the point mutation of GSS spontaneously develop neurologic dysfunction, spongiform brain degeneration, and astrocytic gliosis (Hsiao *et al.* 1990).
11. Ablation of the PrP gene in mice prevents scrapie and propagation of prions after intracerebral inoculation of prions (Büeler *et al.* 1993; Prusiner *et al.* 1993*b*).
12. Mice expressing chimeric Mo/SHaPrP transgenes produce "artificial" prions with novel properties (Scott *et al.* 1993).

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for the replication of infectious prion particles ranged from those used by viruses to the synthesis of polypeptides in the absence of nucleic acid template to post-translational modifications of cellular proteins. Subsequent discoveries have narrowed the hypotheses for both prion structure and the mechanism of replication.

Considerable evidence has accumulated over the past decade supporting the prion hypothesis (Prusiner 1991). Furthermore, the replication of prions and their mode of pathogenesis also appear to be without precedent. After a decade of severe criticism and serious doubt, the prion concept is now enjoying considerable acceptance.

3. DISCOVERY OF THE PRION PROTEIN

After it was established that scrapie prion infectivity in partly purified fractions depended upon protein (Prusiner *et al.* 1981), the search for a scrapie-specific protein intensified. Although the insolubility of scrapie infectivity made purification problematic, this property and the relative resistance to degradation by proteases were used to extend the degree of purification. Radio-iodination of partly purified fractions revealed a protein unique to preparations from scrapie-infected brains (Bolton *et al.* 1982; Prusiner *et al.* 1982). This protein was later named 'prion protein' and abbreviated PrP with an apparent molecular mass of 27–30 kDa, or PrP 27–30 (McKinley *et al.* 1983*a*). The existence of this protein was rapidly confirmed (Diringer *et al.* 1983).

Subsequent studies showed that PrP 27–30 is derived from a larger protein of molecular mass 33–35 kDa, designated PrP^{Sc} (Oesch *et al.* 1985; Meyer *et al.* 1986). It was also found that the brains of normal and scrapie-infected hamsters express similar levels of PrP mRNA and a protease-sensitive prion protein designated PrP^{C} (Oesch *et al.* 1985). PrP^{C}, or a subset of PrP molecules, are the substrate for PrP^{Sc}. Many lines of evidence suggest that PrP^{Sc} is an essential component of the infectious prion particle (table 2); all attempts to find a second component of the prion particle have so far been unsuccessful.

Results from many experimental and clinical studies show that prions are composed largely, if not entirely, of PrP^{Sc} molecules. Although some investigators contend that PrP^{Sc} is merely a pathologic product of scrapie infection, and that PrP^{Sc} coincidentally purifies with the 'scrapie virus' (Brain & Diringer 1985; Aiken *et al.* 1989, 1990; Sklaviadis *et al.* 1989, 1990), there are few data to support this view. No infective fractions containing less than one PrP^{Sc} molecule per ID_{50} unit have been found; such a result would show that PrP^{Sc} is not required for infectivity. Some investigators report that PrP^{Sc} accumulation in hamsters occurs after the synthesis of many infective units (Czub *et al.* 1986, 1988), but these results have been refuted (Jendroska *et al.* 1991). The discrepancy appears to be due to comparisons of infectivity in crude homogenates with PrP^{Sc} concentrations measured in purified fractions. In another study, the investigators claimed to have dissociated scrapie infectivity from PrP 27–30 in brains of Syrian hamsters treated with amphotercin B and inoculated with the 263K isolate, but not if they were inoculated with the 139H isolate; also, no dissociation was seen with mice inoculated with Me7 prions (Xi *et al.* 1992). No confirmation of these studies has yet been published.

The discovery of PrP 27–30 in fractions enriched for scrapie infectivity was accompanied by the identification of rod-shaped particles (Prusiner *et al.* 1982, 1983). The rods are ultrastructurally indistinguishable from many purified amyloids, and display the tinctorial properties of amyloids (Prusiner *et al.* 1983). These findings were followed by the demonstration that amyloid plaques in prion diseases contain PrP, as determined by immunoreactivity and amino acid sequencing (Bendheim *et al.* 1984; DeArmond *et al.* 1985; Kitamoto *et al.* 1986; Roberts *et al.* 1988; Tagliavini *et al.* 1991). Some investigators believe that scrapie-associated fibrils are synonymous with the prion rods and are composed of PrP, even though these fibrils can be distinguished ultrastructurally and tinctorially from amyloid polymers (Merz *et al.* 1989, 1984).

The formation of prion rods requires limited proteolysis in the presence of detergent (McKinley *et al.* 1991*a*). Thus the prion rods in fractions enriched for scrapie infectivity are largely, if not entirely, artefacts of the purification protocol. Solubilization of PrP 27–30 into liposomes with retention of infectivity (Gabizon *et al.* 1987) demonstrated that large PrP polymers are not required for infectivity, and allowed the immunoaffinity copurification of PrP^{Sc} and infectivity (Gabizon *et al.* 1988*b*; Gabizon & Prusiner 1990).

4. SEARCH FOR A SCRAPIE-SPECIFIC NUCLEIC ACID

Based upon the resistance of the scrapie agent to both ultraviolet and ionizing radiation (Alper *et al.* 1966, 1967), the possibility was raised that the scrapie agent might contain a small polynucleotide similar in size and properties to viroids of plants (Diener 1972). Subsequently, evidence for a putative DNA-like viroid was published (Malone *et al.* 1979), but the findings could not be confirmed (Prusiner *et al.* 1980), and the properties of the scrapie agent were found to be antithetical to those of viroids (Diener *et al.* 1982). As well as ultraviolet irradiation, reagents specifically modifying or damaging nucleic acids, such as nucleases, psoralens, hydroxylamine and Zn^{2+} ions, were found not to alter scrapie infectivity in homogenates (Prusiner 1982), microsomal fractions (Prusiner 1982), purified prion rod preparations or detergent–lipid–protein complexes (McKinley *et al.* 1983*b*; Bellinger–Kawahara *et al.* 1987*a*,*b*, 1988; Gabizon *et al.* 1988*a*; Neary *et al.* 1991).

Attempts to find a scrapie-specific polynucleotide by using physical techniques such as polyacrylamide gel electrophoresis were as unsuccessful as molecular cloning approaches. Substractive hybridization studies identified several cellular genes, the expression of which is increased in scrapie, but no unique sequence could be identified (Weitgrefe *et al.* 1985; Diedrich

et al. 1987; Duguid *et al.* 1988). Extensively purified fractions were analysed for a scrapie-specific nucleic acid by using a specifically developed technique designated return refocusing gel electrophoresis, but none was found (Meyer *et al.* 1991). These studies suggest that if such a molecule exists then its size is 80 nt or less (Kellings *et al.* 1992; Riesner *et al.* 1992). Attempts to use these highly enriched fractions to identify a scrapie-specific nucleic acid by molecular cloning were also unsuccessful (Oesch *et al.* 1988).

Despite these studies, some investigators continue to champion the idea that scrapie is caused by a 'virus' (Chesebro 1992; Kimberlin 1990). A few argue that the scrapie virus is similar to a retrovirus (Sklaviadis *et al.* 1989, 1993), and others argue that the scrapie virus induces amyloid deposition in the brain (Braig & Diringer 1985; Diringer 1992). Others argue that scrapie is caused by a larger pathogen, similar to spiroplasma bacterium (Bastian 1979, 1993), and still others contend that elongated protein polymers covered by DNA are the etiologic agents in scrapie (Narang *et al.* 1988; Narang 1992). DNA molecules, such as the D-loop DNA of mitochondria, have also been suggested as the cause of scrapie (Aiken *et al.* 1989).

5. PrP GENE STRUCTURE, ORGANIZATION AND EXPRESSION

The entire open-reading frame (ORF) of all known mammalian and avian PrP genes is contained within a single exon (Basler *et al.* 1986; Westaway *et al.* 1987; Hsiao *et al.* 1989; Gabriel *et al.* 1992). This feature of the PrP gene eliminates the possibility that PrP^{Sc} arises from alternative RNA splicing (Basler *et al.* 1986; Westaway *et al.* 1987, 1991). The two exons of the Syrian hamster (SHa) PrP gene are separated by a 10 kb intron: exon 1 encodes a portion of the 5′ untranslated leader sequence, while exon 2 encodes the ORF and 3′ untranslated region (Basler *et al.* 1986). The mouse (Mo) and sheep PrP gene is composed of three exons, with exon 3 analogous to exon 2 of the hamster (Westaway *et al.* 1991, 1994*b*). The promoters of both the SHa and MoPrP genes contain multiple copies of G–C rich repeats, and are devoid of TATA boxes. These G–C nonamers represent a motif which may function as a canonical binding site for the transcription factor Sp1 (McKnight & Tjian 1986).

Mapping PrP genes to the short arm of human chromosome 20 and the homologous region of Mo chromosome 2 argues for the existence of PrP genes before the speciation of mammals (Sparkes *et al.* 1986). Hybridization studies demonstrated <0.002 PrP gene sequences per ID_{50} unit in purified prion fractions, suggesting that a gene encoding PrP^{Sc} is not a component of the infectious prion particle (Oesch *et al.* 1985). This is a major feature which distinguishes prions from viruses, including those retroviruses that carry cellular oncogenes, and from satellite viruses that derive their coat proteins from other viruses previously infecting plant cells.

Although PrP mRNA is constitutively expressed in the brains of adult animals (Chesebro *et al.* 1985; Oesch *et al.* 1985), it is highly regulated during development. In the septum, levels of PrP mRNA and choline acetyltransferase were found to increase in parallel during development (Mobley *et al.* 1988). In other brain regions, PrP gene expression occurred at an earlier age. *In situ* hybridization studies show that the highest levels of PrP mRNA are found in neurons (Kretzschmar *et al.* 1986).

6. EXPERIMENTAL SCRAPIE

For many years, studies of experimental scrapie were performed exclusively with sheep and goats. The disease was first transmitted by intraocular inoculation (Cuillé & Chelle 1939), and later by intracerebral, oral, subcutaneous, intramuscular and intravenous injections of brain extracts from sheep developing scrapie. Incubation periods of 1–3 years were common, and often many of the inoculated animals failed to develop disease (Dickinson & Stamp 1969; Hadlow *et al.* 1980; Hadlow *et al.* 1982). Different breeds of sheep exhibited markedly different susceptibilities to scrapie prions inoculated subcutaneously, suggesting that the genetic background might influence host permissiveness (Gordon 1966).

A crucial methodologic advance in experimental studies of scrapie was created by the demonstration that scrapie could be transmitted to mice (Chandler 1961). Endpoint titrations using mice were done to determine the titres of prions in particular samples. In addition, pathogenesis experiments directed at elucidating factors governing incubation times and neuropathological lesions were done (Eklund *et al.* 1967; Dickinson *et al.* 1968; Fraser & Dickinson 1968).

Studies of PrP genes (*Prn-p*) in mice with short and long incubation times demonstrated genetic linkage between a *Prn-p* restriction fragment length polymorphism (RFLP) and a gene modulating incubation times (*Prn-i*) (Carlson *et al.* 1986). Other investigators have confirmed the genetic linkage, and one group has shown that the incubation time gene *Sinc* is also linked to PrP (Hunter *et al.* 1987; Race *et al.* 1990). *Sinc* was first described by Dickinson and colleagues over 25 years ago (Dickinson *et al.* 1968); whether the genes for PrP, *Prn-i* and *Sinc* are all congruent remains to be established. The PrP sequences of NZW ($Prn\text{-}p^a$) and I/Ln ($Prn\text{-}p^b$) mice with short and long scrapie incubation times, respectively, differ at codons 108 (L→F) and 189 (T→V) (Westaway *et al.* 1987). Although these amino acid substitutions argue for the congruency of *Prn-p* and *Prn-i*, experiments with $Prn\text{-}p^a$ mice expressing $Prn\text{-}p^b$ transgenes demonstrated a paradoxical shortening of incubation times (Westaway *et al.* 1991) instead of a prolongation as predicted from ($Prn\text{-}p^a \times Prn\text{-}p^b$) F_1 mice which exhibit long incubation times that are dominant (Dickinson *et al.* 1968; Carlson *et al.* 1986). Whether this paradoxical shortening of scrapie incubation times in Tg ($Prn\text{-}p^b$) mice results from high levels of PrP^C-B expression remains to be established (Westaway *et al.* 1991).

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7. HUMAN PRION DISEASES

The human prion diseases are manifest as infectious, inherited and sporadic disorders, and are often referred to as kuru, CJD, GSS and FFI, depending upon the clinical and neuropathological findings (table 1). Infectious forms of prion diseases result from the horizontal transmission of the infectious prions, as occurs in iatrogenic CJD and kuru. Inherited forms, notably GSS, familial CJD and FFI comprise 10–15% of all cases of prion disease. A mutation in the ORF or protein-coding region of the PrP gene has been found in all reported kindreds with inherited human prion disease. Sporadic forms of prion disease comprise most cases of CJD and possibly some cases of GSS (Masters *et al.* 1978). How prions arise in patients with sporadic forms is unknown, but hypotheses include horizontal transmission from humans or animals (Gajdusek 1977), somatic mutation of the PrP gene ORF, and spontaneous conversion of PrP^{C} into PrP^{Sc} (Prusiner 1989; Hsiao *et al.* 1991*a*). Numerous attempts to establish an infectious link between sporadic CJD and a pre-existing prion disease in animals or humans have been unrewarding (Malmgren *et al.* 1979; Harries-Jones *et al.* 1988; Cousens *et al.* 1990).

Genetics were first thought to have a role in CJD with the recognition that *ca.* 10% of cases are familial (Meggendorfer 1930; Stender 1930; Davison & Rabiner 1940; Jacob *et al.* 1950; Friede & DeJong 1964; Rosenthal *et al.* 1976; Masters *et al.* 1979, 1981*a*,*b*; in contrast, most cases of GSS are familial (Gerstmann *et al.* 1936). Like sheep scrapie, the relative contributions of genetic and infectious etiologies in the human prion diseases remained puzzling.

The discovery of the PrP gene and its linkage to scrapie incubation times in mice (Carlson *et al.* 1986) raised the possibility that mutation might feature in the hereditary human prion diseases. A proline (P)→leucine (L) mutation at codon 102 was shown to be linked genetically to development of GSS with a lod score exceeding 3 (figure 1) (Hsiao *et al.* 1989). This mutation may be due to the deamination of a methylated CpG in a germline PrP gene resulting in the substitution of a thymine (T) for cytosine (C). The P102L mutation has been found in ten different families in nine different countries, including the original GSS family (Doh-ura *et al.* 1989; Goldgaber *et al.* 1989; Kretzschmar *et al.* 1991, 1992).

An insert of 144 base pairs (b.p.) at codon 53 containing six octarepeats were described in patients with CJD from four families all residing in southern England (figure 1) (Collinge *et al.* 1992; Owen *et al.* 1989; Poulter *et al.* 1992). This mutation must have arisen through a complex series of events as the human PrP gene contains only five octrarepeats, so a single recombination event could not have created the insert. Genealogic investigations have shown that all four families are related, suggestive of a single founder born more than two centuries ago. The lod score for this extended pedigree exceeds 11. Studies from several laboratories have demonstrated that two, four, five, six, seven, eight or nine octarepeats in addition to the normal five are found in individuals with inherited CJD (Owen *et al.* 1989, 1992; Goldfarb *et al.* 1991*a*; Brown 1992), whereas deletion of one octarepeat has been identified without the neurologic disease (Lapanche *et al.* 1990; Vnencak-Jones & Phillips 1992; Palmer *et al.* 1993).

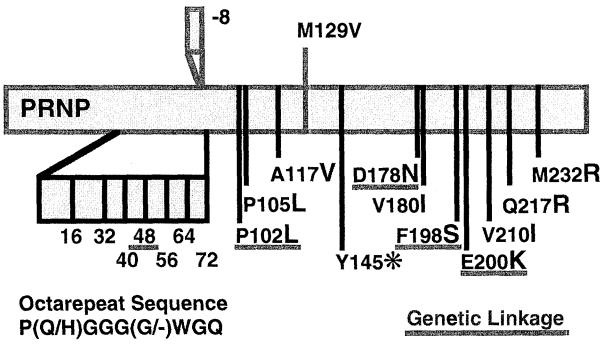

Figure 1. Human prion protein gene (PRNP). The open reading frame (ORF) is denoted by the large grey rectangle. Human PRNP wild-type polymorphisms are shown above the rectangle, whereas mutations that segregate with the inherited prion diseases are depicted below. The wild-type human PrP gene contains five octarepeats [P(Q/H)-GGG(G/-)WGQ] from codons 51–91. Deletion of a single octarepeat at codon 81 or 82 is not associated with prion disease. There are common polymorphisms at codons 117 (Ala→Ala) and 129 (Met→Val); homozygosity for Met or Val at codon 129 appears to increase susceptibility to sporadic CJD. Octarepeat inserts of 16, 32, 40, 48, 56, 64, and 72 amino acids at codons 67, 75 or 83 are designated by small rectangles below the ORF. These inserts segregate with familial CJD, and significant genetic linkage has been demonstrated where sufficient specimens from family members are available. Point mutations are designated by the wild-type amino acid preceding the codon number and the mutant residue follows, e.g. P102L. These point mutations segregate with the inherited prion diseases, and significant genetic linkage (underlined mutations) has been demonstrated where sufficient specimens from family members are available. Reprinted from Prusiner (1993).

For many years the unusually high incidence of CJD among Israeli Jews of Libyan origin was thought to be due to the consumption of lightly cooked sheep brain or eyeballs (Kahana *et al.* 1974). Recent studies have shown that some Libyan and Tunisian Jews in families with CJD have a PrP gene point mutation at codon 200 resulting in a glutamate (E)→lysine (K) substitution (Goldfarb *et al.* 1990; Hsiao *et al.* 1991*a*). One patient was homozygous for the E200K mutation, but her clinical presentation was similar to that of heterozygotes (Hsiao *et al.* 1991*a*), which suggests that familial prion diseases are true autosomal dominant disorders. The E200K mutation has also been found in Slovaks originating from Orava in North Central Czechoslovakia (Goldfarb *et al.* 1990), in a cluster of familial cases in Chile (Goldfarb *et al.* 1991*b*) and in a large German family living in the United States (Bertoni *et al.* 1992).

Many families with CJD have been found to have a point mutation at codon 178 resulting in an aspartic

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acid (D)→asparagine (N) substitution (Fink *et al.* 1991; Goldfarb *et al.* 1991*c*). In these patients, as well as those with the E200K mutation, PrP amyloid plaques are rare; the neuropathologic changes generally consist of widespread spongiform degeneration. Recently a new prion disease which presents with insomnia has been described in three Italian families with the D178N mutation (Medori *et al.* 1992). The neuropathology in these patients with fatal familial insomnia is restricted to selected nuclei of the thalamus. It is unclear whether all patients with the D178N mutation or only a subset present with sleep disturbances. It has been proposed that the allele with the D178N mutation encodes a methionine at position 129 in fatal familial insomnia, whereas a valine is encoded at position 129 in familial CJD (Goldfarb *et al.* 1992). The discovery that fatal familial insomnia is an inherited prion disease clearly widens the clinical spectrum of these disorders and raises the possibility that many other degenerative diseases of unknown etiology may be caused by prions (Johnson 1992; Medori *et al.* 1992).

A valine (V)→isoleucine (I) mutation at PrP codon 210 produces CJD with classic symptoms and signs (Pocchiari *et al.* 1993; Ripoll *et al.* 1993). It appears that this V210I mutation is also incompletely penetrant.

Other point mutations at codons 105, 117, 145, 198, 217 and possibly 232 also segregate with inherited prion diseases (Doh-ura *et al.* 1989; Hsiao *et al.* 1991*b*, 1992; Brown 1992; Tranchant *et al.* 1992; Kitamoto *et al.* 1993*a*,*b*). Patients with a dementing or telencephalic form of GSS have a mutation at codon 117. These patients, as well as some in other families, were once thought to have familial Alzheimer's disease, but are now known to have prion diseases on the basis of PrP immunostaining of amyloid plaques and PrP gene mutations (Farlow *et al.* 1989; Ghetti *et al.* 1989; Nochlin *et al.* 1989; Giaccone *et al.* 1990). Patients with the codon 198 mutation have numerous neurofibrillary tangles that stain with antibodies to τ and have amyloid plaques (Farlow *et al.* 1989; Ghetti *et al.* 1989; Nochlin *et al.* 1989; Giaccone *et al.* 1990) that are composed largely of a PrP fragment extending from residues 58 to 150 (Tagliavini *et al.* 1991). A genetic linkage study of this family produced a lod score exceeding 6 (Dlouhy *et al.* 1992). The neuropathology of two patients of Swedish ancestry with the codon 217 mutation (Ikeda *et al.* 1991) was similar to that of patients with the codon 198 mutation.

Patients with GSS who have a substitution of leucine for proline at PrP codon 105 have been reported (Kitamoto *et al.* 1993*b*). One patient with a prolonged neurologic illness spanning almost two decades with PrP amyloid plaques was found to have an amber mutation of the PrP gene resulting in a stop codon at residue 145 (Kitamoto *et al.* 1993*a*). Staining of the plaques with anti-PrP peptide antisera suggested that they might be composed exclusively of the truncated PrP molecules. That a PrP peptide ending at residue 145 polymerizes to amyloid filaments is to be expected as an earlier study noted above showed that the major PrP peptide in plaques from patients with the F198S mutation was a 11 kDa PrP peptide beginning at codon 58 and ending at *ca.* 150 (Tagliavini *et al.* 1991). Furthermore, synthetic PrP peptides adjacent to and including residues 109–122 readily polymerize into rod-shaped structures with the tinctorial properties of amyloid (Gasset *et al.* 1992; Come *et al.* 1993; Forloni *et al.* 1993; Goldfarb *et al.* 1993).

8. SYNTHESIS OF PrP^{C} AND PrP^{Sc}

Metabolic labelling studies of scrapie-infected cultured cells have shown that PrP^{Sc} is synthesized slowly by a post-translational process (Borchelt *et al.* 1990, 1992; Caughey & Raymond 1991) in contrast to PrP^{C} which is synthesized and degraded rapidly (Caughey *et al.* 1989). These observations are consistent with earlier findings showing that PrP^{Sc} accumulates in the brains of scrapie-infected animals, while PrP mRNA levels remain unchanged (Oesch *et al.* 1985). Furthermore, the structure and organization of the PrP gene made it likely that PrP^{Sc} is formed during a post-translational event (Basler *et al.* 1986).

Both PrP isoforms appear to transit through the Golgi apparatus where their Asn-linked oligosaccharides are modified and sialylated (Bolton *et al.* 1985; Manuelidis *et al.* 1985; Endo *et al.* 1989; Haraguchi *et al.* 1989; Rogers *et al.* 1990). PrP^{C} is presumably transported within secretory vesicles to the external cell surface where it is anchored by a glycosyl phosphatylinositol (GPI) moiety (Stahl *et al.* 1987, 1992; Safar *et al.* 1990*a*). In contrast, PrP^{Sc} accumulates primarily within cells where it is deposited in cytoplasmic vesicles, many of which appear to be secondary lysosomes (Taraboulos *et al.* 1990*b*, 1992*b*; Caughey *et al.* 1991*a*; McKinley *et al.* 1991*b*; Borchelt *et al.* 1992).

Whether PrP^{C} is the substrate for PrP^{Sc} formation or a restricted subset of PrP molecules are precursors for PrP^{Sc} remains to be established. Several experimental results suggest that PrP molecules destined to become PrP^{Sc} exit to the cell surface, as does PrP^{C} (Stahl *et al.* 1987), before their conversion into PrP^{Sc} (Caughey & Raymond 1991; Borchelt *et al.* 1992; Taraboulos *et al.* 1992*b*). Interestingly, the GPI anchors of both PrP^{C} and PrP^{Sc}, which presumably feature in directing the subcellular trafficking of these molecules, are sialylated (Stahl *et al.* 1992). The reentry of PrP^{C} into cells appears to occur through the caveolae (Anderson *et al.* 1992).

Although most of the difference in the mass of PrP 27–30 predicted from the amino acid sequence and that observed after post-translational modification is due to complex-type oligosaccharides (Haraguchi *et al.* 1989), these sugar chains are not required for PrP^{Sc} synthesis in scrapie-infected cultured cells based on experiments with the Asn-linked glycosylation inhibitor tunicamycin and site-directed mutagenesis studies (Taraboulos *et al.* 1990*a*).

9. TRANSGENETICS AND GENE TARGETING

The passage of prions between species is a stochastic process characterized by prolonged incubation times

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(Pattison 1965). Prions synthesized *de novo* reflect the sequence of the host PrP gene and not that of the PrP^{Sc} molecules in the inoculum (Bockman *et al.* 1987). On subsequent passage in a homologous host, the incubation time shortens to that recorded for all subsequent passages, and it becomes a non-stochastic process. The species barrier concept is of practical importance in assessing the risk for humans of developing CJD after consumption of scrapie-infected lamb or BSE beef (Prusiner *et al.* 1993*a*; Wilesmith *et al.* 1992).

To test the hypothesis that differences in PrP gene sequences might be responsible for the species barrier, Tg mice expressing SHaPrP were constructed (Prusiner *et al.* 1990; Scott *et al.* 1989). The PrP genes of Syrian hamsters and mice encode proteins differing at 16 positions. Incubation times in four lines of Tg(SHaPrP) mice inoculated with Mo prions were prolonged compared with those observed for non-Tg, control mice. Inoculation of Tg(SHaPrP) mice with SHa prions demonstrated abrogation of the species barrier, resulting in abbreviated incubation times due to a non-stochastic process (Scott *et al.* 1989; Prusiner *et al.* 1990). The length of the incubation time after inoculation with SHa prions was inversely proportional to the level of $SHaPrP^{C}$ in the brains of Tg(SHaPrP) mice (Prusiner *et al.* 1990). $SHaPrP^{Sc}$ levels in the brains of clinically ill mice were similar in all four Tg(SHaPrP) lines inoculated with SHa prions. Bioassays of brain extracts from clinically ill Tg(SHaPrP) mice inoculated with Mo prions revealed that only Mo prions but no SHa prions were produced. Conversely, inoculation of Tg(SHaPrP) mice with SHa prions led to only the synthesis of SHa prions. Thus, the *de novo* synthesis of prions is species specific and reflects the genetic origin of the inoculated prions. Similarly, the neuropathology of Tg(SHaPrP) mice is determined by the genetic origin of prion inoculum. Mo prions injected into Tg(SHaPrP) mice produced a neuropathology characteristic of mice with scrapie. A moderate degree of vacuolation in both the grey and white matter was found, but amyloid plaques were rarely detected. Inoculation of Tg(SHaPrP) mice with SHa prions produced intense vacuolation of the grey matter, sparing of the white matter and numerous SHaPrP amyloid plaques characteristic of Syrian hamsters with scrapie.

During transgenetic studies, we discovered that uninoculated older mice with high-copy numbers of wild-type transgenes derives from Syrian hamsters, sheep and PrP-B mice spontaneously developed truncal ataxia, hind-limb paralysis and tremors (Westaway *et al.* 1994*a*). These Tg mice exhibited a profound necrotizing myopathy involving skeletal muscle, a demyelinating polyneuropathy and focal vacuolation of the central nervous system (CNS). Development of disease was dependent on transgene dosage. For example, $Tg(SHaPrP^{+/+})7$ mice homozygous for the SHaPrP transgene array regularly developed disease between 400 d and 600 d of age; hemizygous $Tg(SHaPrP^{+/0})7$ mice also developed disease, but after >650 d.

Attempts to demonstrate PrP^{Sc} in either muscle or brain were unsuccessful, but transmission of disease with brain extracts from $Tg(SHaPrP^{+/+})7$ mice inoculated into Syrian hamsters did occur. These Syrian hamsters had PrP^{Sc} as detected by immunoblotting and spongiform degeneration (D. Groth & S. B. Prusiner, unpublished data). Serial passage with brain extracts from these animals to recipients was observed. *De novo* synthesis of prions in $Tg(SHaPrP^{+/+})7$ mice overexpressing wild-type $SHaPrP^{C}$ provides support for the hypothesis that sporadic CJD does not result from infection but rather is a consequence of the spontaneous, although rare, conversion of PrP^{C} into PrP^{Sc}. Alternatively, a somatic mutation in which mutant $SHaPrP^{C}$ is spontaneously converted into PrP^{Sc}, as in the inherited prion diseases, could also explain sporadic CJD. These findings, as well as those described below for Tg(MoPrP-P101L) mice, suggest that prions are devoid of foreign nucleic acid, in accord with many earlier studies that use other experimental approaches reviewed above.

Transgenic mice expressing chimeric PrP genes derived from SHa and Mo PrP genes were constructed (Scott *et al.* 1992). One SHa/MoPrP gene, designated MH2M PrP, contains five amino acid substitutions encoded by SHaPrP, and another construct designated MHM2 PrP has two substitutions. Tg(MH2M PrP) mice were susceptible to both SHa or Mo prions, whereas three lines expressing MHM2 PrP were resistant to SHa prions (Scott *et al.* 1993). The brains of Tg(MH2M PrP) mice dying of scrapie contained chimeric PrP^{Sc} and prions with an artificial host range favouring propagation in mice which express the corresponding chimeric PrP, and were also transmissible, at reduced efficiency, to non-Tg mice and hamsters. These findings provide genetic evidence for homophilic interactions between PrP^{Sc} in the inoculum and PrP^{C} synthesized by the host.

Ablation of the PrP gene in Tg ($Prn\text{-}p^{0/0}$) mice has, unexpectedly, not affected the development of these animals (Büeler *et al.* 1992). In fact, they are healthy at almost 2 years of age. $Prn\text{-}p^{0/0}$ mice are resistant to prions and do not propagate scrapie infectivity (Büeler *et al.* 1993; Prusiner *et al.* 1993*b*). $Prn\text{-}p^{0/0}$ mice crossed with Tg(SHaPrP) mice were rendered susceptible to SHa prions but remained resistant to Mo prions (Büeler *et al.* 1993; Prusiner *et al.* 1993*b*). As the absence of PrP^{C} expression does not provoke disease, it is likely that scrapie and other prion diseases are a consequence of PrP^{Sc} accumulation rather than an inhibition of PrP^{C} function (Büeler *et al.* 1992).

Mice heterozygous ($Prn\text{-}p^{0/+}$) for ablation of the PrP gene had prolonged incubation times when inoculated with Mo prions (Prusiner *et al.* 1993*b*). The $Prn\text{-}p^{0/+}$ mice developed signs of neurologic dysfunction at 400–460 d after inoculation. These findings are in accord with studies on Tg(SHaPrP) mice where increased SHaPrP expression was accompanied by diminished incubation times (Prusiner *et al.* 1990).

Because $Prn\text{-}p^{0/0}$ do not express PrP^{C}, we reasoned that they might more readily produce anti-PrP antibodies. $Prn\text{-}p^{0/0}$ mice immunized with Mo or SHa

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prion rods produced anti-PrP antisera which bound Mo, SHa and human PrP (Prusiner *et al.* 1993*b*). These findings contrast with earlier studies in which anti-MoPrP antibodies could not be produced in mice, presumably because the mice had been rendered tolerant by the presence of $MoPrP^{C}$ (Barry & Prusiner 1986; Kascsak *et al.* 1987; Rogers *et al.* 1991). That Prn-$p^{0/0}$ mice readily produce anti-PrP antibodies is consistent with the hypothesis that the lack of an immune response in prion diseases is due to the fact that PrP^{C} and PrP^{Sc} share many epitopes. Whether Prn-$p^{0/0}$ mice produce anti-PrP antibodies that specifically recognize conformational-dependent epitopes present on PrP^{Sc} but absent from PrP^{C} remains to be determined.

The codon 102 point mutation found in GSS patients was introduced into the MoPrP gene, and Tg(MoPrP-P101L)H mice were created expressing high (H) levels of the mutant transgene product. The Tg(MoPrP-P101L)H mice spontaneously developed CNS degeneration, characterized by clinical signs indistinguishable from experimental murine scrapie and neuropathology consisting of widespread spongiform morphology and astrocytic gliosis (Hsiao *et al.* 1990) and PrP amyloid plaques. By inference, these results contend that PrP gene mutations cause GSS, familial CJD and FFI.

Although brain extracts prepared from Tg(MoPrP-P101L)H mice transmitted CNS degeneration to some inoculated recipients, little or no PrP^{Sc} was detected by immunoassays after limited proteolysis. Many Tg(MoPrP-P101L)L mice expressing low (L) levels of the mutant transgene product, and some Syrian hamsters, developed CNS degeneration between 115 d and 600 d after inoculation, but inoculated CD-1 Swiss mice remained well (K. K. Hsiao, D. Groth, S.-L. Yang, H. Serban, D. Rapp, D. Foster, M. Scott, M. Torchia, S. J. DeArmond & S. B. Prusiner, unpublished results). Undetectable or low levels of PrP^{Sc} in the brains of these Tg(MoPrP-P101L)H mice are consistent with the results of these transmission experiments which suggest low titres of infectious prions. Although no PrP^{Sc} was detected in the brains of inoculated Tg(MoPrP-P101L)L mice exhibiting neurologic dysfunction by immunoassays after limited proteolysis, PrP amyloid plaques, as well as spongiform degeneration, were frequently found. We propose that the neurodegeneration found in inoculated Tg(MoPrP-P101L)L mice results from a modification of mutant PrP which is initiated by mutant PrP^{Sc} present in the brain extracts prepared from ill Tg(MoPrP-P101L)H mice. In support of this explanation are the findings in some inherited human prion diseases where neither protease-resistant PrP (Brown *et al.* 1992; Medori *et al.* 1992) nor transmission to experimental animals could be demonstrated (Tateishi *et al.* 1992). Furthermore, transmission of disease from Tg(MoPrP-P101L)H mice to Tg(MoPrP-P101L)L mice but not to Swiss mice is consistent with earlier findings which demonstrate that homotypic interactions between PrP^{C} and PrP^{Sc} feature in the formation of PrP^{Sc} as described above.

10. PROPAGATION OF PRIONS

Although the search for a scrapie-specific nucleic acid continues to be unrewarding, some investigators steadfastly cling to the notion that this putative polynucleotide drives prion replication. If prions are found to contain a scrapie-specific nucleic acid, then such a molecule would be expected to direct scrapie agent replication using a strategy similar to that used by viruses. In the absence of any chemical or physical evidence for a scrapie-specific polynucleotide, it seems reasonable to consider some alternative mechanisms that might feature in prion biosynthesis. The multiplication of prion infectivity is an exponential process in which the post-translational conversion of PrP^{C} or a precursor to PrP^{Sc} appears to be obligatory (Borchelt *et al.* 1990).

As illustrated, PrP^{Sc} appears to combine with PrP^{C} to form a PrP^{C}/PrP^{Sc} complex which is subsequently transformed into two molecules of PrP^{Sc}. In the next cycle, two PrP^{Sc} molecules combine with two PrP^{C} molecules giving rise to two complexes that dissociate to combine with four PrP^{C} molecules creating an exponential process. Studies with Tg(SHaPrP) mice suggest that prion synthesis involves 'replication', not merely 'amplification' (Prusiner *et al.* 1990). Assuming prion biosynthesis simply involves amplification of post-translationally altered PrP molecules, we might expect Tg(SHaPrP) mice to produce both SHa and Mo prions after inoculation with either prion, as these mice produce both SHa and $MoPrP^{C}$. Yet Tg(SHaPrP) mice synthesize only those prions present in the inoculum. These results suggest that the incoming prion and PrP^{Sc} interact with the homotypic PrP^{C} substrate to replicate more of the same prions.

Additional evidence in support of the proposed model for prion propagation comes from Tg(Mo/SHaPrP) mice expressing chimeric $Mo/SHaPrP^{C}$ (Scott *et al.* 1993). The chimeric Mo/SHaPrP gene was constructed by substituting the SHaPrP sequence for MoPrP from codon 94 to 188; within this domain, there are five amino acid substitutions which distinguish Mo from SHaPrP. When inoculated with either Mo or SHa prions, these Tg(Mo/SHaPrP) mice develop scrapie after *ca.* 140 d. The chimeric Tg mice produce $Mo/SHaPrP^{Sc}$ and Mo/SHa prions and inoculation with SHa prions and probably Mo prions as well. Evidence for chimeric Mo/SHa prions comes from the development of scrapie in Tg(Mo/SHaPrP) mice *ca.* 70 d after inoculation with brain extracts from Tg(Mo/SHaPrP) mice containing the chimeric prions.

As studies of PrP^{Sc} failed to reveal a candidate post-translational chemical modification that might distinguish it from PrP^{C} (Stahl *et al.* 1993), we considered the possibility that these two PrP isoforms may differ only in their conformations. To assess this possibility, the secondary structures of PrP^{C} and PrP^{Sc} were determined (Pan *et al.* 1993). Fourier transform infrared (FTIR) spectroscopy demonstrated that PrP^{C} has a high α-helix and low β-sheet content, findings that were confirmed by circular dichroism measurements (Pan *et al.* 1993). In contrast, the β-sheet

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content of PrP^{Sc} was more than 40% and the α-helix 30%, as measured by FTIR. The N-terminally truncated PrP^{Sc} derived by limited proteolysis and designated PrP 27–30 showed an even higher β-sheet and a lower α-helix content than was found for PrP^{Sc} (Caughey *et al.* 1991*b*; Gasset *et al.* 1993). Although these findings suggest that the conversion of α-helices into β-sheets underlies the formation of PrP^{Sc}, we cannot eliminate the possibility that an undetected chemical modification of a small fraction of PrP^{Sc} initiates this process.

Structure prediction studies of $SHaPrP^{C}$ and $SHaPrP^{Sc}$ (residues 23–231) were done by using a neural network algorithm (Kneller *et al.* 1990; Presnell *et al.* 1993). Class-dependent (α/α,α/β,β/β) and naive predictions were made. The α/α class contains proteins which are composed largely of α-helices. Similarly, β/β class contains proteins that are mostly β-sheets. Interestingly, the four putative α-helical domains of PrP (Gasset *et al.* 1992) showed both strong α-helix preference in the α/α class prediction and strong β-sheet preference in the β/β class prediction. These results are consistent with the hypothesis that these domains undergo conformational changes from α-helices to β-sheets during the formation of PrP^{Sc}. Further support for this hypothesis comes from structural investigations of synthetic PrP peptides.

Three of the four peptides corresponding to the four putative α-helical domains of PrP^{C} formed amyloid polymers with high β-sheet content when dispersed into water but formed α-helices in hexafluoroisopropanol (Gasset *et al.* 1992). Furthermore, denaturation of PrP 27–30 under conditions which reduced scrapie infectivity resulted in a concomitant diminution of β-sheet content (Gasset *et al.* 1993). Thus it seems likely that both the conversion of PrP^{C} to PrP^{Sc} and the propagation of infectious prion particles involves a structural transition in which α-helical domains acquire β-sheets.

In humans carrying point mutations or inserts in their PrP genes, mutant PrP^{C} molecules might spontaneously convert into PrP^{Sc}. Although the initial stochastic event may be inefficient, once it happens the process becomes autocatalytic. The proposed mechanism is consistent with individuals having germline mutations who do not develop CNS dysfunction for decades, and with studies on Tg(MoPrP-P101L)H mice that spontaneously develop CNS degeneration (Hsiao *et al.* 1990). Whether all GSS and familial CJD cases contain infectious prions or some represent inborn errors of PrP metabolism in which neither PrP^{Sc} nor prion infectivity accumulates is unknown; however, transmission of inherited human prion diseases to animals is less frequent than for sporadic CJD (Tateishi *et al.* 1992). It seems likely that mutant PrP^{C} molecules alone can also produce CNS degeneration.

11. STRAINS AND PRION DIVERSITY

The diversity of scrapie prions was first appreciated in goats inoculated with 'hyper' and 'drowsy' isolates (Pattison & Millson 1961). Subsequently, studies in mice demonstrated the existence of many scrapie 'strains' (Dickinson & Fraser 1979; Bruce & Dickinson 1987; Kimberlin *et al.* 1987; Dickinson & Outram 1988), which continues to pose a fascinating conundrum. What is the macromolecule that carries the information required for each strain to manifest a unique set of biological properties if it is not a nucleic acid?

There is good evidence for multiple 'strains' or distinct isolates of prions as defined by specific incubation times, distribution of vacuolar lesions and patterns of PrP^{Sc} accumulation (Dickinson *et al.* 1968; Fraser & Dickinson 1973; Bruce *et al.* 1989; Hecker *et al.* 1992). Incubation times have been used to distinguish strains inoculated into sheep, goats, mice and hamsters. Recent studies have shown that the incubation time is not characteristic for a particular strain but rather it depends on the host as demonstrated in studies with Tg(SHaPrP) mice (DeArmond *et al.* 1993; Prusiner *et al.* 1993*b*) and mice expressing chimeric Mo/SHaPrP transgenes (Scott *et al.* 1993). For example, three SHa prion 'strains' passaged in Syrian hamsters (Kimberlin *et al.* 1987, 1989) have profoundly different incubation times, depending upon the host in which they were passaged.

Each 'strain' was found to have a unique lesion profile as determined by counting vacuoles in various regions of the brain (Fraser & Dickinson 1973) which were subsequently shown to be the result of PrP^{Sc} accumulation (Hecker *et al.* 1992; DeArmond *et al.* 1993).

With the development of a new procedure for *in situ* detection of PrP^{Sc} designated histoblotting (Taraboulos *et al.* 1992*a*), it became possible to determine whether or not 'strains' produce different, reproducible patterns of PrP^{Sc} accumulation (Hecker *et al.* 1992; DeArmond *et al.* 1993). Microdissection of individual brain regions has shown that those regions with intense vacuolation have high levels of PrP 27–30 (Casaccia-Bonnefil *et al.* 1993). These findings have given rise to the hypothesis that PrP^{Sc} synthesis occurs in specific populations of cells for a given distinct prion isolate.

Isolation of scrapie 'strains' in mice uses extracts prepared from scrapied sheep (Dickinson *et al.* 1968). Cloning of new 'strains' was done by limiting dilution in mice. Although many strains were isolated, most of the studies used only a few strains, and passaging was limited. For example, Me7 prions were passaged in C57BL mice (Dickinson & Fraser 1969), which were later shown to have the MoPrP-A allele, but 22a and 87V were passaged in VM mice, which have the MoPrP-B allele. The A and B alleles of MoPrP differ at codons 108 and 189 (Westaway *et al.* 1987); propagation of the Me7 strain was much more rapid in the 'A' mouse than the 'B' mouse and vice versa for 22a and 87V in the 'B' mouse (Dickinson & Meikle 1971; Bruce *et al.* 1976). In other words, propagation of a particular strain was restricted by the PrP sequence in the host. It is noteworthy that a number of new 'strains' have been isolated by passage of murine isolates into hamsters (Kimberlin *et al.* 1987, 1989) where the PrP genes differ at 16 positions (Basler *et al.* 1986; Locht *et al.* 1986).

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Although 'mutation' of scrapie isolates or 'strains' was reported, virtually nothing is known about the molecules that conspire in this process. Low dilution of 87A was reported to give rise to 7D prions, and passage at high dilution preserved the 87A properties (Bruce & Dickinson 1979). Dickinson thought that it was important to prepare inocula from the smallest regions of individual brains to minimize contamination with other strains or mutants.

The construction of Tg(MH2MPrP) mice that are susceptible to both mouse and hamster prions has provided a new tool for the study of strains. The Tg(MH2MPrP) mice produce artificial prions which infect Syrian hamsters as well as non-Tg and Tg(MH2MPrP) mice (Scott *et al.* 1993).

The mechanism by which isolate-specific information is carried by prions remains unknown; indeed, explaining the molecular basis of prion diversity seems to be a formidable challenge. For many years some investigators argued that scrapie is caused by a virus-like particle which contains a scrapie-specific nucleic acid that encodes the information expressed by each isolate (Bruce & Dickinson 1987). No such polynucleotide has yet been identified by the wide variety of techniques used including measurements of the nucleic acids in purified preparations. An alternative hypothesis has been suggested, where PrP^{Sc} alone is capable of transmitting disease but the characteristics of PrP^{Sc} might be modified by a cellular RNA (Weissmann 1991). This accessory cellular RNA is postulated to induce its own synthesis upon transmission from one host to another, but there is no experimental evidence to support its existence.

Two additional hypotheses not involving a nucleic acid have been suggested to explain distinct prion isolates: a non-nucleic acid second component might create prion diversity, or post-translational modification of PrP^{Sc} might be responsible for the different properties of distinct prion isolates (Prusiner 1991). Whether the PrP^{Sc} modification is chemical or only conformational remains to be established, but no candidate chemical modifications have been identified (Stahl *et al.* 1993). Structural studies of GPI anchors of two SHa isolates have failed to reveal any differences; interestingly, about 40% of the anchor glycans have sialic acid residues (Stahl *et al.* 1992). A portion of the PrP^{C} GPI anchors also have sialic acid residues; PrP is the first protein found to have sialic acid residues attached to GPI anchors.

The finding that the pattern of PrP^{Sc} accumulation in the CNS is characteristic for a particular strain offers a mechanism for the propagation of distinct prion isolates (Hecker *et al.* 1992). In this model, a different set of cells would propagate each isolate. Whether different Asn-linked carbohydrates (CHOs) function to target PrP^{Sc} of a distinct isolate to a particular set of cells expressing specific surface lectins which function as receptors remains to be established. These surface lectins would bind the same Asn-linked CHOs that are covalently attached to PrP^{C} during its synthesis and remain bound during the conversion of PrP^{C} into PrP^{Sc}. The great diversity of Asn-linked CHOs makes them potential candidates for carrying isolate-specific information (Prusiner 1989). Even though this hypothesis is attractive, it must be noted that PrP^{Sc} synthesis in scrapie-infected cells occurs in the presence of tunicamycin, which inhibits Asn-linked glycosylation, and with PrP molecules mutated at the Asn-linked glycosylation concensus sites (Taraboulos *et al.* 1990*b*). Although the structures of Asn-linked CHOs have been analysed for PrP^{Sc} of one isolate (Endo *et al.* 1989), no data are available for PrP^{Sc} of other isolates of PrP^{C}. The large number of Asn-linked CHOs found attached to the PrP 27–30 of Sc237 prions purified from Syrian hamster would seem to make the argument for Asn-linked CHOs being responsible for strain variation less likely, but experimental data addressing this point are still needed.

Another possibility to explain the region-specific distribution of PrP^{Sc} in brain observed in each strain might involve the formation of a complex between PrP^{Sc} and as yet undetected peptide or protein of cellular origin. Such a complex would bind cell-specific receptors facilitating the entry of PrP^{Sc} into those cells. Against this hypothesis is the finding that the properties of SHa(Sc237) and Mo(RML) prions do not change upon passage through the spleen. Furthermore, no auxillary proteins have been found to purify with PrP^{Sc}. In favour of such a hypothesis is the fact that receptors for proteins are numerous and could provide the specificity required.

Alternatively, explaining the problem of multiple distinct prion isolates might be accommodated by multiple PrP^{Sc} conformers that act as templates for the folding of *de novo* synthesized PrP^{Sc} molecules during prion 'replication'. A conformer corresponding to a specific strain would need to bind to a particular PrP receptor which would either facilitate its entry into cells for the conversion of PrP^{C} into PrP^{Sc} in those particular cells. Although all these proposals are rather unorthodox, they are consistent with observations generated from Tg(SHaPrP)Mo studies contending that PrP^{Sc} in the inoculum binds to homotypic PrP^{C} to form an intermediate in the propagation of prions (Prusiner *et al.* 1990). Whether foldases, chaperonins or other types of molecules feature in the conversion of the PrP^{C}/PrP^{Sc} complex into two molecules of PrP^{Sc} is unknown. The molecular mass of a PrP^{Sc} homodimer is consistent with the ionizing radiation target size of $55\,000 \pm 9000$ Da, as determined for infectious prion particles independent of their polymeric form (Bellinger-Kawahara *et al.* 1988). Of note, two different isolates from mink dying of transmissible mink encephalopathy exhibit different sensitivities of PrP^{Sc} to proteolytic digestion, supporting the suggestion that isolate-specific information might be carried by PrP^{Sc} (Marsh *et al.* 1991; Bessen & Marsh, 1992*a*,*b*).

12. A PERSPECTIVE

The study of prions has taken several unexpected directions over the past few years. The discovery that prion diseases in humans are uniquely both genetic and infectious has greatly strengthened and extended the prion concept. To date, 18 different mutations in

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the human PrP gene all resulting in non-conservative substitutions have been found to be either linked genetically to, or segregate with, the inherited prion diseases (figure 1). Yet the transmissible prion particle is composed largely, if not entirely, of an abnormal isoform of the prion protein designated PrP^{Sc} (Prusiner 1991). These findings argue that prion diseases should be considered pseudoinfectious because the particles transmitting disease appear to be devoid of a foreign nucleic acid, and thus differ from all known microorganisms as well as viruses and viroids. Because much information, especially about scrapie of rodents, has been derived by using experimental protocols adapted from virology, we continue to use terms such as infection, incubation period, transmissibility and endpoint titration in studies of prion disease.

Transgenic mice expressing foreign or mutant PrP genes now allow virtually all facets of prion diseases to be studied, and have created a framework for future investigations. Furthermore, the structure and organization of the PrP gene suggested that PrP^{Sc} is derived from PrP^{C} or a precursor by a post-translational process. Studies with scrapie-infected cultured cells have provided much evidence that the conversion of PrP^{C} to PrP^{SC} is a post-translational process that probably occurs in the endocytic pathway. The molecular mechanism of PrP^{Sc} formation remains to be elucidated, but chemical and physical studies have shown that the conformations of PrP^{C} and PrP^{Sc} are profoundly different.

The study of prion biology and diseases seems to be a new and emerging area of biomedical investigation. Although prion biology has its roots in virology, neurology and neuropathology, its relations to the disciplines of molecular and cell biology, as well as protein chemistry, have become evident only recently. Certainly, it is likely that learning how prions multiply and cause disease will open up new vistas in boichemistry and genetics.

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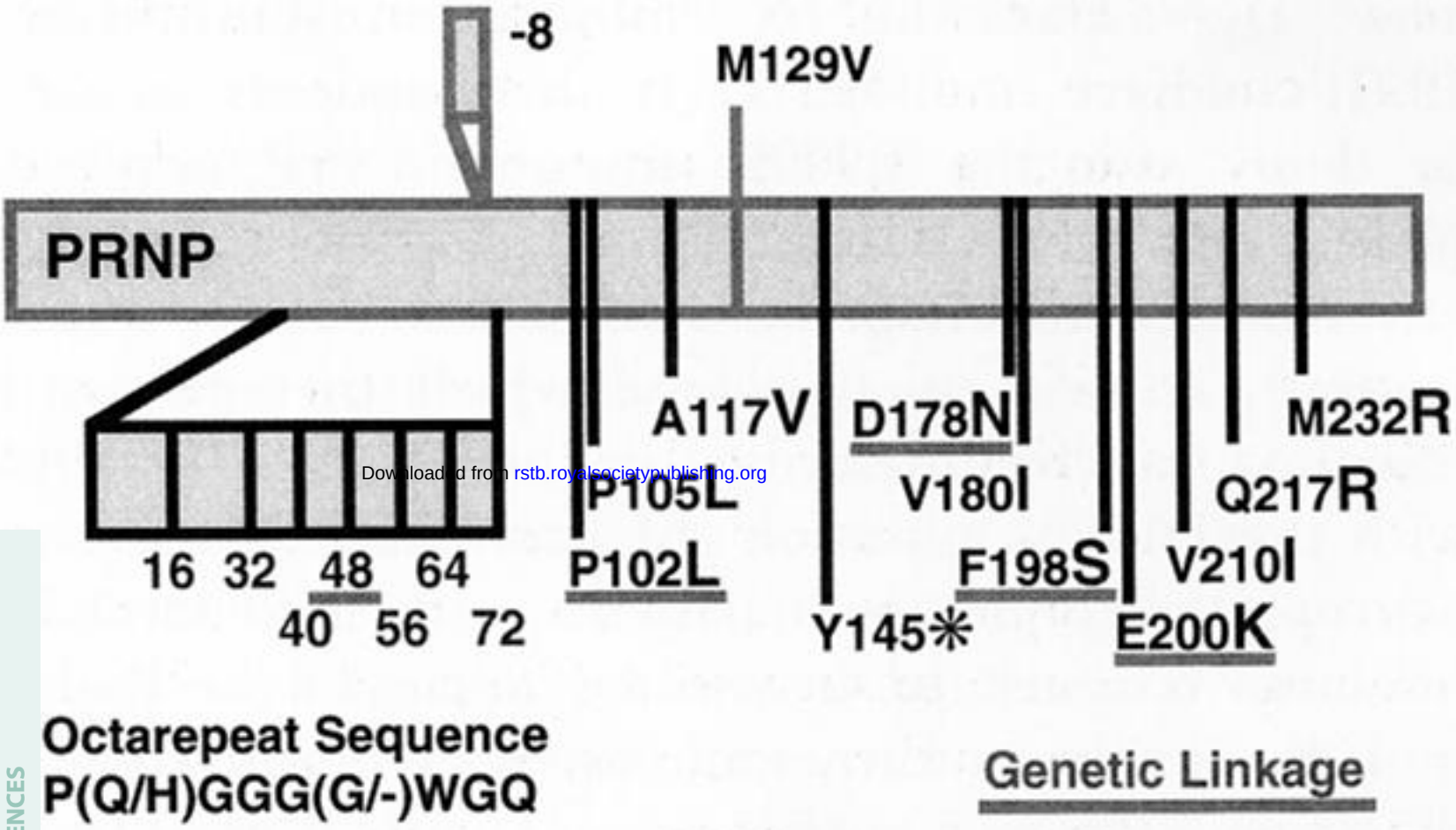

gure 1. Human prion protein gene (PRNP). The open ading frame (ORF) is denoted by the large grey rectangle. uman PRNP wild-type polymorphisms are shown above e rectangle, whereas mutations that segregate with the herited prion diseases are depicted below. The wild-type uman PrP gene contains five octarepeats [P(Q/H)-GG(G/-)WGQ] from codons 51–91. Deletion of a single tarepeat at codon 81 or 82 is not associated with prion sease. There are common polymorphisms at codons 117 la→Ala) and 129 (Met→Val); homozygosity for Met or al at codon 129 appears to increase susceptibility to oradic CJD. Octarepeat inserts of 16, 32, 40, 48, 56, 64, and amino acids at codons 67, 75 or 83 are designated by all rectangles below the ORF. These inserts segregate with milial CJD, and significant genetic linkage has been monstrated where sufficient specimens from family embers are available. Point mutations are designated by e wild-type amino acid preceding the codon number and e mutant residue follows, e.g. P102L. These point utations segregate with the inherited prion diseases, and gnificant genetic linkage (underlined mutations) has been monstrated where sufficient specimens from family embers are available. Reprinted from Prusiner (1993).