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Transgenetic Investigations of Prion Diseases of Humans and Animals

Stanley B. Prusiner

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Transgenetic investigations of prion diseases of humans and animals

STANLEY B. PRUSINER

Departments of Neurology and of Biochemistry and Biophysics, University of California, San Francisco, California 94143, U.S.A.

SUMMARY

Prions cause transmissible and genetic neurodegenerative diseases. Infectious prion particles are composed largely, if not entirely, of an abnormal isoform of the prion protein (PrP^{Sc}), which is encoded by a chromosomal gene. Although the PrP gene is single copy, transgenic mice with both alleles of the PrP gene ablated develop normally. A post-translational process, as yet unidentified, converts the cellular prion protein (PrP^{C}) into PrP^{Sc}. Scrapie incubation times, neuropathology and prion synthesis in transgenic mice are controlled by the PrP gene. Mutations in the PrP gene are genetically linked to development of neurodegeneration. Transgenic mice expressing mutant PrP spontaneously develop neurological dysfunction and spongiform neuropathology. Investigations of prion diseases using transgenesis promise to yield much new information about these once enigmatic disorders.

1. INTRODUCTION

The prion diseases are a group of neurodegenerative disorders of animals and humans. These diseases are transmissible under some circumstances to experimental animals by inoculation. Unlike other transmissible disorders, the prion diseases can also be caused by mutations in the prion protein, PrP, which is encoded by a chromosomal gene. Four diseases of animals and four of humans are caused by prions (table 1). Scrapie of sheep and goats is the prototypic prion disease. Mink encephalopathy, chronic wasting disease and bovine spongiform encephalopathy (BSE) are all thought to occur after the consumption of prion-infected foodstuffs. Similarly, kuru of the New Guinea Fore people is thought to have resulted from the consumption of brains from dying relatives during ritualistic cannibalism (Alpers 1979; Gajdusek 1977). Creutzfeldt–Jakob disease (CJD) occurs primarily as a sporadic disorder (Masters *et al.* 1981*b*) but iatrogenic CJD is thought to result from the accidental inoculation of patients with prions (Fradkin *et al.* 1991; Gibbs *et al.* 1985). Familial CJD, Gerstmann–Sträussler–Scheinker syndrome (GSS) and fatal familial insomnia are all dominantly inherited prion diseases which have been shown to be caused by mutations in the PrP gene (Brown *et al.* 1991; Collinge *et al.* 1989; Hsiao & Prusiner 1990; Medori *et al.* 1992*b*).

For more than a century, scrapie was considered an enigmatic disorder of sheep and goats, the etiology of which was unknown (M'Gowan 1914; Parry 1983). By 1938, experimental transfer of scrapie from one sheep to another began to suggest an infectious etiology (Cuillé & Chelle 1939). Meanwhile, observations that the genetic backgrounds of flocks profoundly influence their susceptibility to scrapie raised the possibility that scapie might be an inherited disorder (Gordon 1966). These opposing views sparked many controversial encounters (Dickinson *et al.* 1965; Parry 1962) and foreshadowed a series of equally bitter arguments about the possible structure of the transmissible scrapie agent (Pattison 1988).

Over the past decade, a growing body of experimental data has begun to provide a coherent yet unprecedented picture of the novel infectious pathogens or prions causing scrapie (Prusiner 1982, 1991). Whereas inherited, transmissible and sporadic prion diseases of humans are now well documented, the situation with natural prion diseases of animals is less clear. Progress in understanding the human prion diseases has its roots in their transmission to animals (Masters *et al.* 1979, 1981*a*) and the discovery of the prion protein (PrP) (Bolton *et al.* 1982; Prusiner 1982) followed by the molecular cloning of the PrP gene (Chesebro *et al.*, 1985; Oesch *et al.*, 1985; Prusiner *et al.* 1984).

As molecular, biological and genetic analyses of both the human and animal prion diseases have advanced, the biochemistry of the prion protein has continued to pose both methodological and conceptual problems. For example, transmissible prions are composed largely, if not entirely, of an abnormal isoform of cellular PrP designated PrP^{Sc} (Gabizon & Prusiner 1990; Prusiner 1991). Although PrP^{Sc} is synthesized from cellular PrP (PrP^{C}) by a post-translational process (Basler *et al.* 1986; Borchelt *et al.* 1990, 1992; Caughey & Raymond 1991), the precise nature of this protein transformation remains unknown. Whether the conversion of PrP^{C} to PrP^{Sc} involves an as yet unidentified chemical modification,

Table 1. *Prion diseases*[1]

disease	natural host
scrapie	sheep and goats
transmissible mink encephalopathy (TME)	mink
chronic wasting disease (CWD)	mule deer and elk
bovine spongiform encephalopathy (BSE)	cattle
kuru	humans – fore[2]
Creutzfeldt–Jakob disease (CJD)	humans
Gerstmann–Sträussler–Scheinker syndrome (GSS)	humans
fatal familial insomnia (FFI)	humans

[1] Alternative terminologies include slow virus infections, subacute transmissible spongiform encephalopathies, and unconventional slow virus diseases (Gajdusek 1977).

[2] Kuru is confined to the Fore tribe and surrounding tribes in the highlands of Papua New Guinea.

perhaps labile under the conditions of analysis, or whether it only involves a conformational change (Stahl *et al.* 1992b) remains to be established.

To date, it has not been possible to synthesize PrP^{Sc} in cell-free systems (Raeber *et al.* 1992), but studies of this insoluble protein in cultured cells have yielded information about the subcellular site of its synthesis and deposition (Borchelt *et al.* 1992; McKinley *et al.* 1991*b*). In the brains of animals and humans dying of prion diseases, PrP^{Sc} is found in the neuropil (Taraboulos *et al.* 1992*a*) and sometimes in the extracellular space as discrete accumulations called plaques (DeArmond *et al.* 1985; Kitamoto *et al.* 1986). These PrP plaques were first described as amyloid deposits because they exhibited a green-gold birefringence after staining with Congo red dye when viewed by polarization microscopy (Kiatzo *et al.* 1959). When present, PrP amyloid plaques are diagnostic of prion diseases. Rod-shaped polymers of PrP with the properties of amyloid can be generated by limited protease digestion of PrP^{Sc} in the presence of detergent (McKinley *et al.* 1991*a*; Prusiner *et al.* 1983).

The function of PrP^{C} is unknown but PrP^{C} molecules appear to be unnecessary because mice homologous for disruption of the PrP gene develop normally and are healthy for more than 9 months (Büeler *et al.* 1992). These results argue that scrapie and the other prion diseases do not result from an inhibition of PrP^{C} function caused by PrP^{Sc}, but rather the accumulation of PrP^{Sc} interferes with some as yet undefined cellular process.

2. THE PRION PROTEIN

Once it was established that scrapie prion infectivity depended upon protein (Prusiner *et al.* 1981), the search for a scrapie-specific protein intensified. Although the insolubility of scrapie infectivity made purification problematic, we took advantage of this property, along with its relative resistance to degradation by proteases, 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 (PrP) with an apparent molecular mass of 27–30 kDa, or PrP 27–30 (McKinley *et al.* 1983*a*).

Subsequent studies showed that PrP 27–30 is derived from a larger protein of molecular mass 33–35 kDa, designated PrP^{Sc} (Meyer *et al.* 1986; Oesch *et al.* 1985). At the same time it was 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). The function of PrP^{C} is unknown, although it has been suggested that a PrP-like molecule from chickens may have acetylcholine receptor-inducing activity (Harris *et al.* 1991). Furthermore, PrP^{C} does not seem to be essential, at least in young mice, as disruption of the PrP gene has not caused any detectable abnormalities in the nervous, musculoskeletal or lymphoreticular systems at 9 months of age (Büeler *et al.* 1992). Perhaps the absence of PrP^{C} will result in abnormalities later in life, as is the case for the p53 tumor suppressor protein where young animals lacking p53 are normal but as they age neoplasms develop (Donehower *et al.* 1992).

3. STRUCTURE, ORGANIZATION AND EXPRESSION OF THE PrP GENE

The entire open reading frame (ORF) of all known mammalian and avian PrP genes is contained within a single exon (figure 1) (Basler *et al.* 1986; Gabriel *et al.* 1992; Hsiao *et al.* 1989*a*; Puckett *et al.* 1991; Westaway *et al.* 1989, 1991). This feature of the PrP gene eliminates the possibility that PrP^{Sc} arises from

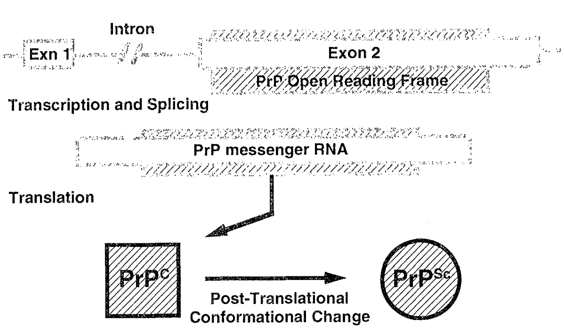

Figure 1. Structure and organization of the chromosomal prion protein gene. In all mammals examined, the entire open reading frame (ORF) is contained within a single exon. The 5′ untranslated region of the PrP mRNA is derived from either one or two additional exons (Basler *et al.* 1986; Puckett *et al.* 1991; Westaway *et al.* submitted. Only one PrP mRNA has been detected. PrP^{Sc} is thought to be derived from PrP^{C} by a post-translational process (Basler *et al.* 1986; Borchelt *et al.* 1990, 1992; Caughey & Raymond 1991; Taraboulos *et al.* 1992*b*). The amino acid sequence of PrP^{Sc} is identical to that predicted from the translated sequence of the DNA encoding the PrP gene (Basler *et al.* 1986; Stahl *et al.* 1992*b*) and no unique post-translational chemical modifications have been identified that might distinguish PrP^{Sc} from PrP^{C}. Thus, it seems likely that PrP^{C} undergoes a conformational change as it is converted to PrP^{Sc}.

alternative RNA splicing (Basler *et al.* 1986; Westaway *et al.* 1987, 1991); however, mechanisms such as RNA editing or protein splicing remain a possibility (Blum *et al.* 1990; Kane *et al.* 1990). The two exons of the Syrian hamster (SHa) PrP gene are separated by a 10 kilobase (kb) intron: exon 1 encodes a portion of the 5′ untranslated leader sequence, whereas exon 2 encodes the ORF and 3′ untranslated region (Basler *et al.* 1986). The mouse (Mo) PrP gene is comprised of three exons, with exon 3 analogous to exon 2 of the hamster (Westaway *et al.* 1991). The promoters of both the SHa and MoPrP genes contain copies of G–C rich repeats 3 and 2, respectively, but 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).

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*a*).

PrP^{C} expression in brain was defined by standard immunohistochemistry (DeArmond *et al.* 1987) and by histoblotting (Taraboulos *et al.* 1992*a*) (figure 2). Immunostaining of PrP^{C} in the SHa brain was most

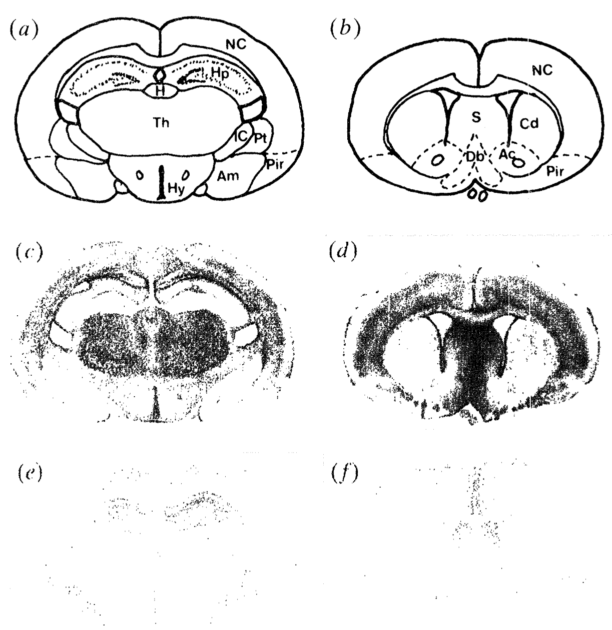

Figure 2. Histoblots of Syrian hamster brain immunostained for PrP^{C} or PrP^{Sc}. Coronal sections through the hippocampus-thalamus (*a,c,e*) and the septum-caudate (*b,d,f*). Brain sections of a Syrian hamster (*c,d*) clinically ill after inoculation with Sc237 prions and (*e,f*) an uninfected, control animal. Immunostaining for (*c,d*) PrP^{Sc} and (*e,f*) PrP^{C}. Ac, nucleus accumbens; Am, amygdala; Cd, caudate nucleus; Db, diagonal band of Broca; H, habenula; Hp, hippocampus; Hy, hypothalmus; IC, internal capsule; NC, neocortex; Th, thalamus; Pir, piriform cortex; Pt, putamen; S, septal nuclei. Reproduced from Taraboulos *et al.* (*Proc. natn. Acad. Sci. U.S.A.*, 1992*a*).

intense in the stratum radiatum and stratum oriens of the CA1 region of the hippocampus and was virtually absent from the granule cell layer of the dentate gyrus and the pyramidal cell layer throughout Ammon's horn. PrP^{Sc} staining was minimal in these regions which were intensely stained for PrP^{C}. A similar relation between PrP^{C} and PrP^{Sc} was found in the amygdala. In contrast, PrP^{Sc} accumulated in the medial habenular nucleus, the medial septal nuclei and the diagonal band of Broca; these areas were virtually devoid of PrP^{C}. In the white matter, bundles of myelinated axons contained PrP^{Sc} but were devoid of PrP^{C}. These findings suggest that prions are transported along axons in agreement with earlier findings where scrapie infectivity was found to migrate in a pattern consistent with retrograde transport (Fraser & Dickinson 1985; Jendroska *et al.* 1991; Kimberlin *et al.* 1983). Although the rate of PrP^{Sc} synthesis appears to be a function of the level of PrP^{C} expression in transgenic (Tg) mice, the level to which PrP^{Sc} accumulates appears to be independent of PrP^{C} concentration (Prusiner *et al.* 1990).

4. POST-TRANSLATIONAL SYNTHESIS OF PrP^{Sc}

Metabolic labelling studies of scrapie-infected cultured cells have shown that PrP^{C} is synthesized and degraded rapidly whereas PrP^{Sc} is synthesized slowly by an as yet undefined post-translational process (figure 1) (Borchelt *et al.* 1990, 1992; Caughey *et al.* 1989; Caughey & Raymond, 1991). 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; Endo *et al.* 1989; Haraguchi *et al.* 1989; Manuelidis *et al.* 1985; Rogers *et al.* 1990). PrP^{C} is presumably transported within secretory vesicles to the external cell surface where it is anchored by a glycosyl phosphatidylinositol (GPI) moiety (Baldwin *et al.* 1990; Safar *et al.* 1990; Stahl *et al.* 1987, 1990*a,b*). In contrast, PrP^{Sc} accumulates primarily within cells where it is deposited in cytoplasmic vesicles, many of which appear to be secondary lysosomes (Borchelt *et al.* 1992; Butler *et al.* 1988; Caughey *et al.* 1991; McKinley *et al.* 1991*b*; Taraboulos *et al.* 1992*b*; Taraboulos *et al.* 1990*b*).

Whether PrP^{C} is the substrate for PrP^{Sc} formation or whether 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} (Borchelt *et al.* 1992; Caughey & Raymond 1991; Taraboulos *et al.* 1992*b*). Interestingly, the GPI anchors of both PrP^{C} and PrP^{Sc}, which presumably

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feature in directing the subcellular trafficking of these molecules, are sialylated (Stahl *et al.* 1992*a*). It is unknown whether sialylation of the GPI anchor participates in some aspect of PrP^{Sc} formation.

Although most of the difference in mass of PrP 27–30 predicted from the amino acid sequence and that observed after post-translational modification is due to complex-type oligosaccharides, these sugar chains are not required for the synthesis of protease-resistant PrP in scrapie-infected cultured cells based on experiments with the Asn-linked glycosylation inhibitor tunicamycin and on site-directed mutagenesis studies (Taraboulos *et al.* 1990*a*). Whether unglycosylated PrP^{Sc} is associated with scrapie prion infectivity remains to be established, but experiments with transgenic mice may resolve this issue.

Cell-free translation studies have demonstrated two forms of PrP: a transmembrane form which spans the bilayer twice at the transmembrane (TM) and amphipathic helix (AH) domains, and a secretory form (Bazan *et al.* 1987; Hay *et al.* 1987*a*,*b*; Lopez *et al.* 1990; Yost *et al.* 1990). The stop transfer effector (STE) domain controls the topogenesis of PrP. That PrP contains both a TM domain and a GPI anchor poses a topological conundrum. It seems likely that membrane-dependent events feature in the synthesis of PrP^{Sc}, especially as brefeldin A, which selectively destroys the Golgi stacks (Doms *et al.* 1989; Lippincott-Schwartz *et al.* 1989), prevents PrP^{Sc} synthesis in scrapie-infected cultured cells (Taraboulos *et al.* 1992*b*). For many years, the association of scrapie infectivity with membrane fractions has been appreciated (Gibbons & Hunter 1967; Griffith 1967; Millson *et al.* 1971); indeed, hydrophobic interactions are thought to account for many of the physical properties displayed by infectious prion particles (Gabizon *et al.* 1987; Prusiner *et al.* 1978, 1980).

5. PRION DISEASES OF SHEEP AND CATTLE

Even though scrapie was recognized as a distinct disorder of sheep with respect to its clinical manifestations as early as 1738, the disease remained enigmatic even with respect to its pathology for more than two centuries. Some veterinarians thought that scrapie was a disease of muscle caused by parasites, whereas others thought that it was a dystrophic process. 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.

While the transmissibility of scrapie became well established, the spread of 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; Parry 1983). He considered its transmission by inoculation of importance primarily for laboratory studies and communicable infection of little consequence in nature. Scrapie is widely recognized as a naturally transmissible disease of sheep and goats, and it has been argued that host genetics only modulates susceptibility to an endemic infectious agent (Dickinson *et al.* 1965).

Studies of PrP genes (*Prn-p*) in mice have revealed that short or long incubation times occur before scrapie. A genetic linkage has been demonstrated between a *Prn-p* restriction fragment length polymorphism 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 (Carlson *et al.* 1988; Hunter *et al.* 1987; Race *et al.* 1990). The incubation time gene for experimental scrapie in Cheviot sheep called *Sip* is said to be linked to a PrP gene restriction fragment length polymorphism (Hunter *et al.* 1989), a situation perhaps analogous to *Prn-i* and *Sinc* in mice. *Sinc* was first described by Dickinson and colleagues over 20 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 198 (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 ($Prn\text{-}p^a \times Prn\text{-}p^b$) F1 mice which exhibit long incubation times that are dominant (Carlson *et al.* 1986, 1988; Dickinson *et al.* 1968; Hunter *et al.* 1987; Race *et al.* 1990).

Since 1986 more than 70 000 cattle have been killed with BSE in Great Britain (Dealler & Lacey 1990; Wilesmith & Wells 1991; Wilesmith *et al.* 1988, 1992*a*,*b*). Neither the cause of BSE, often referred to as 'mad cow disease' nor methods of controlling the spread of this disorder are known. Many investigators contend that BSE resulted from the feeding of dietary protein supplements derived from rendered scrapie-infected sheep offal to cattle, a practice banned since 1988. Curiously, the majority of BSE cases have occurred in herds with a single affected animal within a herd; several cases of BSE in a single herd are infrequent (Dealler & Lacey 1990; Wilesmith & Wells 1991; Wilesmith *et al.* 1988). Whether the distribution of BSE cases within herds will change as the epidemic progresses and BSE will disappear with the cessation of feeding rendered meat and bone meal are uncertain.

6. HUMAN PRION DISEASES

The discovery of human prion diseases came from the recognition that the neuropathology of the cerebellar disorder kuru, which is confined to natives in the Fore region of New Guinea (Gajdusek 1977; Gajdusek *et al.* 1966), was similar to that of scrapie. Once the most common cause of death among women and children, kuru has almost disappeared with the cessation of ritualistic cannibalism (Alpers 1987). These findings suggest that kuru was transmitted orally. Of note are recent cases of kuru which have occurred in people exposed to prions more than three decades ago (Prusiner *et al.* 1982). Spongiform degeneration in kuru prompted Hadlow (1959) to suggest that transmission studies in apes be done. The success of those studies (Gajdusek *et al.* 1966) was followed by the transmission of CJD to apes (Gibbs *et al.* 1968) based on the earlier recognition that the neuropathological changes in kuru were similar to those found in CJD (Klatzo *et al.* 1959). In 1920, Creutzfeldt reported the case of a 23-year-old women who died of a neurodegenerative disease, and the following year Jakob reported five cases (Jakob 1921*a*,*b*,*c*). Ironically, some investigators doubt that Creutzfeldt described the disease that now bears his name (Richardson 1977).

In humans, a genetic basis of the condition was first thought to have a role in CJD with the recognition that ~10% of cases are familial (Gajdusek 1977; Masters *et al.* 1981*b*). Like sheep scrapie, the relative contributions of genetic and infectious etiologies in the human prion diseases remained puzzling. The discovery of the PrP gene raised the possibility that mutation might feature in the hereditary human prion diseases. A point mutation at codon 102 (P→L) was shown to be linked genetically to development of Gerstmann–Straussler–Scheinker disease (GSS) with a LOD score exceeding 3 (figure 3) (Hsiao *et al.* 1989*a*). This mutation may be caused by the deamination of a methylated CpG in a germline PrP gene resulting in the substitution of a T for C. The codon 102 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*a*,*b*).

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

For many years, the unusually high incidence of CJD among Israeli Jews of Libyan origin was thought to be caused by the consumption of lightly cooked sheep brain or eyeballs (Alter & Kahana 1976; Herzberg *et al.* 1974; Kahana *et al.* 1974; Neugut *et al.* 1979). Recent studies have shown that some Libyan

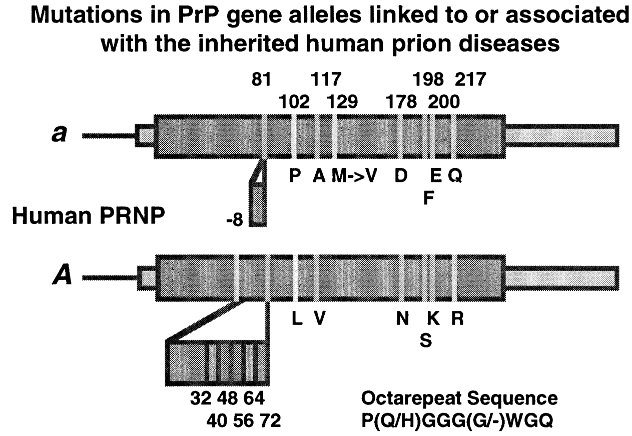

Figure 3. Human prion protein gene. The open reading frame (ORF) is denoted by the large grey rectangles and the exon by the smaller rectangles. Codon numbers are indicated above the amino acid sequence. Human PrP wild-type polymorphisms are shown in the upper rectangle denoted 'a' whereas mutations linked to or associated with prion diseases are depicted in the lower rectangle denoted 'A'. The wild-type human PrP gene contains five octarepeats [P(Q/H)GGG(G/−)WGQ] from codons 51 to 91 (Kretzschmar *et al.* 1986*b*). Deletion of a single octarepeat at codon 81 or 82 is not associated with prion disease (Laplanche *et al.* 1990; Puckett *et al.* 1991; Vnencak-Jones & Phillips 1992). Whether such a deletion alters the phenotypic characteristics of a prion disease is unknown, but homozygosity for Met or Val at codon 129 appears to increase susceptibility to sporadic CJD (Palmer *et al.* 1991). Octarepeat inserts of 32, 40, 48, 56, 64, and 72 amino acids at codons 67, 75 or 83 have been found and are either genetically linked to or associated with familial CJD (Collinge *et al.* 1989, 1990; Crow *et al.* 1990; Goldfarb *et al.* 1990*c*, 1991*a*; Owen *et al.* 1989, 1990; J. Collinge & M. S. Palmer, unpublished data). Point mutations at codons 102 (Pro→Leu), 117 (Ala→Val), and 198 (Phe→Ser) are found in patients with GSS (Doh-ura *et al.* 1989; Goldfarb *et al.* 1990*a*,*c*,*d*; Goldgaber *et al.* 1989; Hsiao *et al.* 1989*a*,*b*, 1991*b*; Hsiao & Prusiner 1990; Tateishi *et al.* 1990). There are common polymorphisms at codons 117 (Ala→Ala) and 129 (Met→Val). Point mutations at codons 178 (Asp→Asn) and 200 (Glu→Lys) are found in patients with familial CJD (Gabizon *et al.* 1991; Goldfarb *et al.* 1990*b*, 1991*c*; Hsiao *et al.* 1991*a*). Point mutations at codons 198 (Phe→Ser) and 217 (Gln→Arg) are found in patients with GSS who have PrP amyloid plaques and neurofibrillary tangles (Dlouhy *et al.* 1992; Hsiao *et al.* 1992). Single letter code for amino acids is as follows: A, Ala; D, Asp; E, Glu; F, Phe; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; and V, Val.

and Tunisian Jews in families with CJD have a PrP gene point mutation at codon 200 resulting in a E→K substitution (Gabizon *et al.* 1991; Goldfarb *et al.* 1990; Hsiao *et al.* 1991*a*). One patient was homozygous for the mutation, but her clinical presentation was similar to that of heterozygotes (Hsiao *et al.* 1991*a*), suggesting that familial prion diseases are true autosomal dominant disorders like Huntington's disease (Wexler *et al.* 1987). The codon 200 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). Some investigators have argued that the codon 200 mutation originated in a Sephardic Jew whose descendants migrated from Spain and Portugal at the time of the inquisition (Goldfarb *et al.* 1991*b*). It is more likely that the codon 200 mutation has arisen independently multiple times by the deamidation of a methylation CpG as described above the codon 102 mutation (Hsiao *et al.* 1989*a*, 1991*a*). In support of this hypothesis are historical records of Libyan and Tunisian Jews showing that they are descended from Jews living on the island of Jerba where Jews first settled around 500 BC and not from Sephardim (Udovitch & Valensi 1984).

Many families with CJD have been found to have point mutations at codon 178 (Brown *et al.* 1992; Fink *et al.* 1991; Goldfarb *et al.* 1991*c*, 1992; Haltia *et al.* 1991). In these patients, as well as those with the codon 200 mutation, PrP amyloid plaques are rare; the neuropathological 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 codon 178 mutation (Medori *et al.* 1992*a*,*b*). 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 codon 178 mutation or only a subset present with sleep disturbances. 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*b*).

Other point mutations at codons 117, 198 and 217 also segregate with inherited prion diseases (Doh-ura *et al.* 1989; Hsiao *et al.* 1991*b*, 1992). 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; Giaccone *et al.* 1990; Nochlin *et al.* 1989). Patients with the codon 198 mutation have numerous neurofibrillary tangles that stain with antibodies to τ. They have amyloid plaques (Farlow *et al.* 1989; Ghetti *et al.* 1989; Giaccone *et al.* 1990; Nochlin *et al.* 1989) 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.

At PrP codon 129, an amino acid (Met–Val) polymorphism (figure 3) has been identified (Owen *et al.* 1990). Patients with CJD after treatment with human pituitary growth hormone (Buchanan *et al.* 1991; Fradkin *et al.* 1991) or gonadotrophin have a significant preponderance of the Val allele (Collinge *et al.* 1991) compared with the general population. Sporadic CJD patients were found to be homozygous for the Met or Val allele at codon 129 but were rarely heterozygous (Palmer *et al.* 1991). The finding was interpreted (Hardy 1991; Palmer *et al.* 1991) as being consistent with the hypothesis for the existence of PrP^{c}/PrP^{Sc} heterodimers and that these forms feature in the replication of prions (Prusiner 1991; Prusiner *et al.* 1990; see § 10).

7. SPONTANEOUS NEURODEGENERATION IN TRANSGENIC MICE: ATTEMPTS TO DEMONSTRATE *DE NOVO* SYNTHESIS OF PRIONS

Transgenic modifications have been used to investigate the control of onset of infections, prion synthesis and neuropathology. When the codon 102 point mutation was introduced into MoPrP in transgenic (Tg) mice, spontaneous central nervous system (CNS) degeneration occurred, characterized by clinical signs indistinguishable from experimental murine scrapie and neuropathology consisting of widespread spongiform morphology and astrocytic gliosis (Hsiao *et al.* 1990). By inference, these results suggest that PrP mutations cause GSS and familial CJD. It is unclear whether low levels of protease-resistant PrP in the brains of Tg mice with the GSS mutation is PrP^{Sc}or residual PrP^{c}. Undetectable or low levels of PrP^{Sc} in the brains of these Tg mice are consistent with the results of transmission experiments that suggest low titres of infectious prions. Brain extracts transmit CNS degeneration to inoculated recipients, and the *de novo* synthesis of prions has been demonstrated by serial passage from one Tg (GSSMoPrP) mouse that developed spontaneous neurodegeneration (Hsiao *et al.* 1991*c*). If these observations can be supported by additional studies with similar results and the possibility of contamination eliminated, then it can be argued that prions are devoid of foreign nucleic acid, in accord with many studies that use other experimental approaches (Bellinger-Kawahara *et al.* 1987*a*,*b*; Diedrich *et al.* 1987; Diener *et al.* 1982; Duguid *et al.* 1988; Gabizon *et al.* 1988; Kellings *et al.* 1992; McKinley *et al.* 1983*b*; Meyer *et al.* 1991; Neary *et al.* 1991; Oesch *et al.* 1988).

One view of the PrP gene mutations has been that they render individuals susceptible to a common 'virus' (Aiken & Marsh 1990; Chesebro *et al.* 1985; Kimberlin 1990). In this scenario, the putative scrapie virus is thought to persist within a worldwide reservoir of humans, animals or insects without causing detectable illness. Yet 1 in 10^6 individuals develop sporadic CJD and die from a lethal 'infection' while $\sim 100\%$ of people with PrP point mutations or inserts appear eventually to develop neurologic dysfunction. That germline mutations found in the PrP genes of patients and at-risk individuals are the cause of familial prion diseases is supported by experiments with Tg(GSS MoPrP) mice described above (Hsiao & Prusiner 1990; Hsiao *et al.* 1991*c*; Weissmann 1991*b*). The Tg mouse studies also argue that sporadic CJD might arise from the spontaneous conversion of PrP^{C} to PrP^{CJD} due to either a somatic mutation of the PrP gene or a rare event involving modification of wild-type PrP^{C} (Prusiner 1991).

8. SPECIES BARRIERS IN THE TRANSMISSION OF PRION DISEASES

Passage of prions between species is a stochastic process characterized by prolonged incubation times (Pattison 1965, 1966; Pattison & Jones 1967). 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.

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 (figure 4*a*). 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 (figure 4*b*) (Prusiner *et al.* 1990; Scott *et al.* 1989). 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 (figure 4*b*,*c*) (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 (figure 4*d*). 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 (figure 4*e*). Conversely, inoculation of Tg(SHaPrP) mice with SHa prions led to only the synthesis of SHa prions (figure 4*f*). 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 (figure 4*g*). 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 (figure 4*h*).

9. PRION DIVERSITY

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 (Bruce *et al.* 1989; Dickinson *et al.* 1968; Fraser & Dickinson, 1973; Hecker *et al.* 1992). The mechanism by which isolate-specific information is carried by prions remains enigmatic; 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). To date, no such polynucleotide has been identified by a wide variety of techniques 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 (Weissman 1991*a*). This accessory cellular RNA is postulated to induce its own synthesis upon transmission from one host to another.

Two additional hypotheses not involving a nucleic acid have been offered 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 conformational alone remains to be established, but no candidate chemical modifications have been identified. Structural studies of the GPI anchors of two SHa isolates have failed to reveal any differences; interestingly, about 40% of the anchor glycans have sialic acid residues (Stal *et al.* 1992*a*). A portion of the PrP^{C} GPI anchors also has sialic acid residues; PrP is the first protein found to have sialic acid residues attached to GPI anchors.

Although the structures of Asn-linked carbohydrates have been analysed for PrP^{Sc} of one isolate (Endo *et al.* 1989), no data are available for PrP^{Sc} of other isolates or PrP^{C}. The great diversity of Asn-linked carbohydrates makes them candidates for isolate-specific information but there is no precedent for Asn-linked carbohydrates instructing the synthesis of more of the same compounds. In recent studies, we found that distinct isolates produce different, reproducible patterns of PrP^{Sc} accumulation (Hecker *et al.* 1992). These findings have given rise to the hypothesis that PrP^{Sc} synthesis occurs in particular sets of cells for a given distinct prion isolate. Whether different Asn-linked carbohydrates function to target PrP^{Sc} of a distinct isolate to a particular set of cells where the same Asn-linked carbohydrates will be coupled to PrP^{C} before its conversion to PrP^{Sc} remains to be established. 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*a*). Whether SHa scrapie prions can be synthesized in Tg mice expressing SHaPrP with mutated Asn-linked glycosylation concensus sites and the properties exhibited by distinct isolates is currently under investigation. Of note, two different isolates from mink dying of trans-

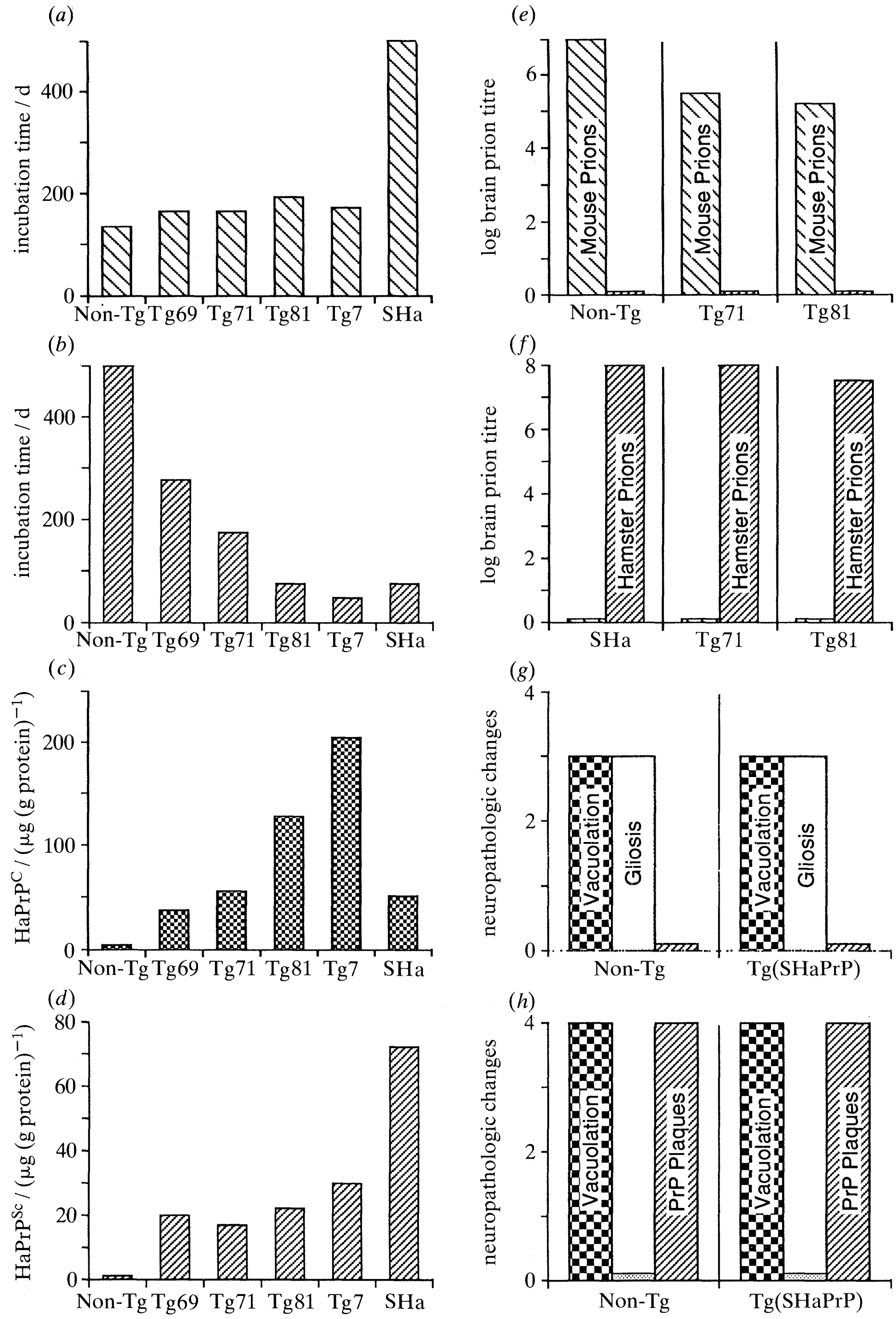

Figure 4. Transgenic mice expressing Syrian hamster (SHa) prion protein exhibit species-specific scrapie incubation times, infectious prion synthesis and neuropathology (Prusiner *et al.* 1990). (*a*) Scrapie incubation times in non-transgenic mice (Non-Tg) and four lines of transgenic mice expressing SHaPrP and Syrian hamsters inoculated intracerebrally with $\sim 10^6$ ID_{50} units of Chandler Mo prions serially passaged in Swiss mice. The four lines of transgenic mice have different numbers of transgene copies: Tg69 and Tg71 mice have two to four copies of the SHaPrP transgene, whereas Tg81 have 30 to 50 copies and Tg7 mice have > 60. Incubation times are number of days from inoculation to onset of neurologic dysfunction. (*b*) Scrapie incubation times in mice and hamsters inoculated with $\sim 10^7$ ID_{50} units of Sc237 prions serially passaged in Syrian hamsters and as described in (*a*). (*c*) Brain SHaPrP^{C} in transgenic mice and hamsters. SHaPrP^{C} levels were quantitated by an enzyme-linked immunoassay. (*d*) Brain SHaPrP^{Sc} in transgenic mice and hamsters. Animals were killed after exhibiting clinical signs of scrapie. SHaPrP^{Sc} levels were determined by immunoassay. (*e*) Prion titres in brains of clinically ill animals after inoculation with Mo prions. Brain extracts from Non-Tg, Tg71, and Tg81 mice were bioassayed for prions in mice (left) and hamsters (right). (*d*) Prion titres in brains of clinically ill animals after inoculation with SHa prions. Brain extracts from Syrian hamsters as well as Tg71 and Tg81 mice were bioassayed for prions in mice (left) and hamsters (right). (*g*) Neuropathology in Non-Tg mice and Tg(SHaPrP) mice with clinical signs of scrapie after inoculation with Mo prions. Vacuolation in grey (left) and white (centre) matter; PrP amyloid plaques (right). Vacuolation score: 0 = none, 1 = rare, 2 = modest, 3 = moderate, 4 = intense. (*h*) Neuropathology in Syrian hamsters and transgenic mice inoculated with SHa prions. Degree of vacuolation and frequency of PrP amyloid plaques as described in (*g*). Adapted from Prusiner (*Science, Wash.* **252**, 1515–1522, 1991).

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missible mink encephalopathy exhibit different sensitivities of PrP^{Sc} to proteolytic digestion, supporting the suggestion that isolate-specific information might be carried by PrP^{Sc} (Bessen & Marsh 1992*a,b*; Marsh *et al.* 1991).

10. PRION REPLICATION

Many experimental studies argue persuasively that prions are devoid of nucleic acid, yet the complete structure of the prion particle, as well as the mechanism by which prions multiply, remains to be established. 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 (Aiken *et al.* 1990; Akowitz *et al.* 1990; Bellinger-Kawahara *et al.* 1987*a,b*; Diedrich *et al.* 1987; Diener *et al.* 1982; Duguid *et al.* 1988; Gabizon *et al.* 1988; Kellings *et al.* 1992; McKinley *et al.* 1983*b*; Meyer *et al.* 1991; Murdoch *et al.* 1990; Neary *et al.* 1991; Oesch *et al.* 1988), 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, 1992; Caughey & Raymond 1991).

Let us consider the remote possibility that prions do contain an as yet undetected polynucleotide, then, presumably, prion replication would involve a virus-like strategy. The putative scrapie-specific nucleic acid would act as a template for its own synthesis using cellular polymerases. By an as yet undefined mechanism, the putative scrapie-specific nucleic acid would stimulate the conversion of PrP^{C} to PrP^{Sc}. Although this putative scrapie-specific nucleic acid would provide a plausible explanation for prion diversity, it would require that this nucleotide sequence be able to discriminate between SHaPrP and MoPrP in Tg(SHaPrP) mice. In addition, the putative scrapie-specific nucleic acid would have to be ubiquitous to explain how sporadic CJD occurs with an incidence of 1 in 10^6 (Brown 1980; Masters *et al.* 1978) all over the planet whereas virtually all people carrying PrP gene mutations develop prion disease.

A more likely scenario is that prions do not contain a scrapie-specific nucleic acid; rather, they are composed entirely of PrP^{Sc} molecules. If this is the case, then the species barrier for prion transmission, the results with Tg(SHaPrP) mice, and infectious prions in the brains of patients with inherited prion diseases can be more readily explained. If prions are composed entirely of PrP^{Sc}, then replication must involve the interaction of nascent PrP^{C} or a precursor with PrP^{Sc} (Prusiner 1991; Prusiner *et al.* 1990). Although there are no physical data demonstrating the existence of PrP^{C}/PrP^{Sc} heterodimers, it is difficult to explain the results obtained with Tg(SHaPrP) mice in studies of prion replication. Moreover, other studies have shown that patients homologous for the Met–Val polymorphism at codon 129 are predisposed to sporadic CJD whereas those with heterozygous alleles at codon 129 are relatively protected (Palmer *et al.* 1991). These findings have been interpreted as being consistent with the hypothesis that prion replication is most efficient when the primary structures of PrP^{C} and PrP^{Sc} are the same. As noted above, although the PrP^{Sc} model is consistent with all of the experimental data, it continues to be problematic with respect to explaining the molecular basis of multiple distinct scrapie prion isolates or 'strains'.

The formal possibility remains that prions contain a second component which is not a nucleic acid. A small polypeptide, a polysaccharide, a lipid-glycan or a phospholipid–sterol complex are all possibilities, but there is no evidence for any of these molecules as prion components.

Some investigators have suggested that scrapie agent multiplication proceeds through a crystalization process involving PrP amyloid formation (Gajdusek 1988, 1990; Gajdusek & Gibbs 1990). Against this hypothesis is the absence or rarity of amyloid plaques in many prion diseases, as well as the inability to identify any amyloid-like polymers in cultured cells chronically synthesizing prions (McKinley *et al.* 1991*a*; Prusiner *et al.* 1990). Purified infectious preparations isolated from scrapie-infected hamster brains exist as amorphous aggregates; only if PrP^{Sc} is exposed to detergents and limited proteolysis, does it then polymerize into prion rods exhibiting the ultrastructural and tinctorial features of amyloid (McKinley *et al.* 1991*a*). Furthermore, dispersion of prion rods into detergent–lipid–protein complexes results in a 10- to 100-fold increase in scrapie titre and no rods could be identified in these fractions by electron microscopy (Gabizon *et al.* 1987).

11. CONCLUDING REMARKS

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, 12 different mutations in 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. 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 suggest that prion diseases should be considered pseudoinfections because the particles transmiting 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 using experimental protocols adapted from virology, we continue to use terms such as infection, incubation period, transmissibility and end-point titration in studies of prion diseases.

It seems likely that the principles learned from the study of prion diseases will be applicable to elucidating the causes of more common neurodegenerative diseases. Such disorders include Alzheimer's disease, amyotrophic lateral sclerosis and Parkinson's disease. Because people at risk for inherited prion diseases can now be identified decades before neurologic dysfunction is evident, the development of an effective therapy is imperative. If PrP^{C} can be diminished in humans without deleterious effects, as is the case for *Prn-p*$^{0/0}$ mice (Büeler *et al.* 1992), then reducing the level of PrP mRNA with antisence oligonucleotides might prove an effective therapeutic approach delaying the onset of CNS symptoms and signs.

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. It seems likely that learning how prions multiply and cause disease may open up new vistas into many areas of disease-related research.

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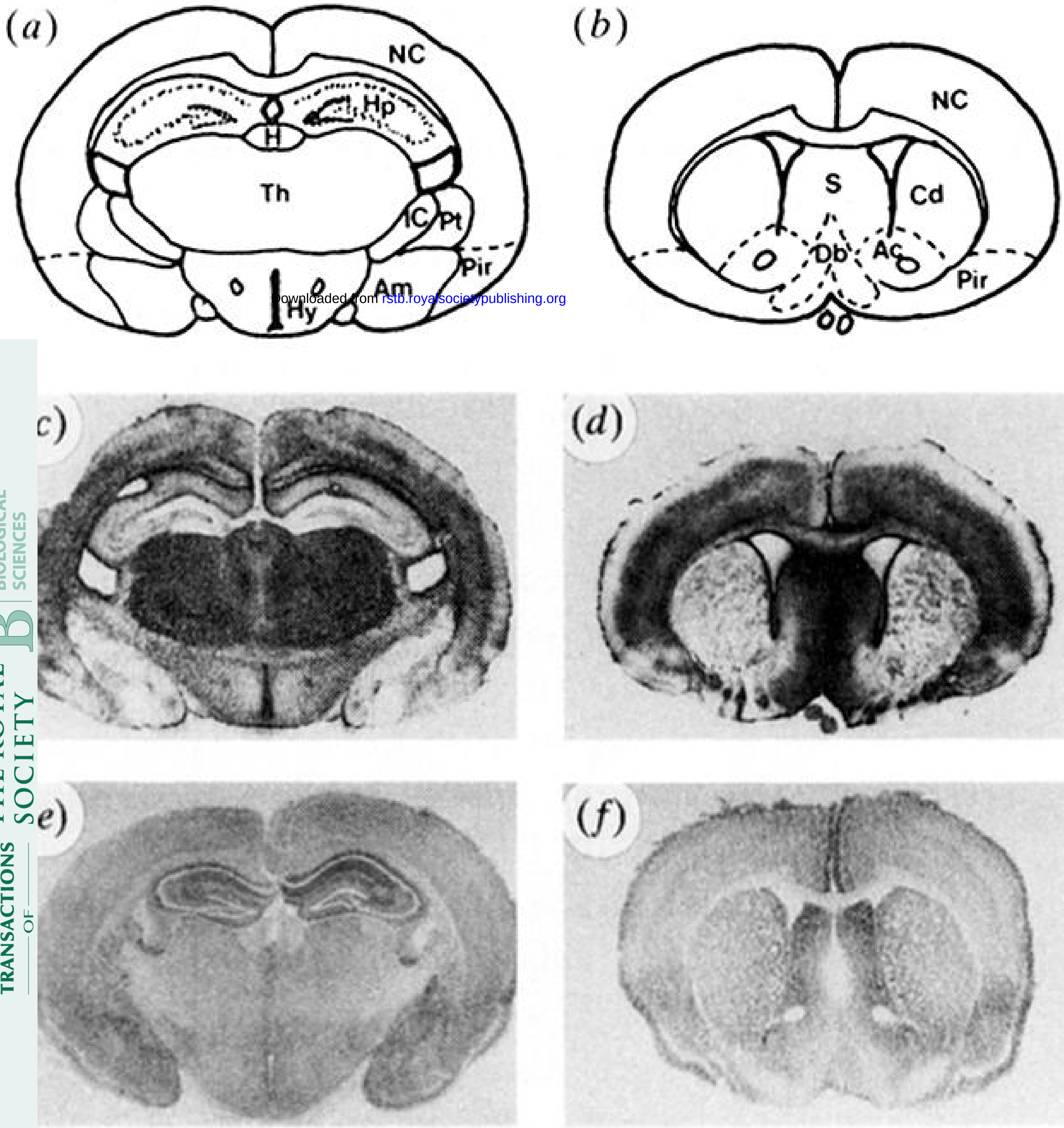
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES



igure 2. Histoblots of Syrian hamster brain immunostained r PrP^{C} or PrP^{Sc}. Coronal sections through the hippocam-us-thalamus (*a*,*c*,*e*) and the septum-caudate (*b*,*d*,*f*). Brain ctions of a Syrian hamster (*c*,*d*) clinically ill after inocula-on with Sc237 prions and (*e*,*f*) an uninfected, control nimal. Immunostaining for (*c*,*d*) PrP^{Sc} and (*e*,*f*) PrP^{C}. Ac, ucleus accumbens; Am, amygdala; Cd, caudate nucleus; b, diagonal band of Broca; H, habenula; Hp, hippocam-us; Hy, hypothalmus; IC, internal capsule; NC, neocortex; h, thalamus; Pir, piriform cortex; Pt, putamen; S, septal uclei. Reproduced from Taraboulos *et al.* (*Proc. natn. Acad. ci. U.S.A.*, 1992*a*).

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