PRION BIOLOGY AND DISEASES

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Prion Biology and Diseases

PRION BIOLOGY AND DISEASES

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The saga of prions truly represents the triumph of scientific investigation over prejudice.

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Preface

Studies of prions seem to be rapidly expanding. The widespread recognition that prions exist and that they are different from all other infectious pathogens has aroused considerable interest among scientists. The possibility that bovine prions have spread from cattle with bovine spongiform encephalopathy (BSE) to humans in Great Britain and other parts of Europe continues to worry physicians, public health officials, and politicians. Despite the fact that molecular investigations of prions only began in 1982 with the first report of the prion protein PrP 27-30, knowledge of prions has increased steadily. With the isolation of PrP 27-30, the application of modern molecular biological approaches, immunologic techniques, and structural biological studies became possible.

This book was assembled with the hope of stimulating young investigators to enter the field of prion biology. We hope that it will also provide an authoritative source for more senior investigators who wish to pursue investigations on prions or who find themselves having to lecture to students on the subject. Because of the size constraints for this book imposed by the publisher, there are many investigators who have made important contributions to prion biology but could not be asked to write chapters: To these individuals, the editor apologizes. Instead, a representative group of authors, who have contributed substantially to the study of prions over the past decade, were selected to participate in this undertaking.

The five chapters at the beginning of this book were written to provide a proper introduction for those not already schooled in prion biology. These chapters give an overview of prion biology, record the development of the prion concept, describe bioassays for prions, explain the basic principles of prion replication, and outline some methods for the study of prions. The remaining chapters of this book cover an array of diverse topics

ranging from the cell biology of prions to transgenic and gene-targeted mice to inherited and infectious prion diseases of humans to bovine spongiform encephalopathy. In addition, scrapie of sheep, the experimental neuropathology of genetically engineered mice, biocontainment and biosafety issues in prion disease, as well as studies of mammalian prion strains and fungal prions, are discussed. Because the literature on prions has grown to be immense, interested readers are urged to consult papers published in refereed journals that are readily retrievable through Medline searches.

The saga of prions over the last 50 years records an extraordinary chapter in the history of biology and medicine. The difficulties that have marked acceptance of the prion concept are not unique. Skepticism has been the rule in science rather than the exception when new ideas are first introduced. Only the steady accumulation of data has served to change the thinking of most, but not all, skeptical scholars. That a few vocal skeptics remain seems to be a frequent and perhaps constant feature of paradigm shifts in science. Certainly, studies of prions are no exception, but fortunately, the science of prion biology has clearly triumphed over prejudice.

Stanley B. Prusiner

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Lastly, the editor expresses a special thanks to all the authors, without whose dedication and labors this book could not have been assembled. The devotion of these scientists to producing a very special monograph is greatly appreciated.

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An Introduction to Prion Biology and Diseases

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The history of prions is a fascinating saga in the annals of biomedical science. For nearly five decades with no clue as to the cause, physicians watched patients with a central nervous system (CNS) degeneration called Creutzfeldt-Jakob disease (CJD) die, often within a few months of its onset (Creutzfeldt 1920; Jakob 1921; Kirschbaum 1968). CJD destroys the brain while the body remains unaware of this process. No febrile response, no leucocytosis or pleocytosis, and no humoral immune response is mounted in reaction to this devastating disease. Despite its recognition as a distinct clinical entity, CJD remained a rare disease; first, it was the province of neuropsychiatrists and, later, neurologists and neuropathologists. Although multiple cases of CJD were recognized in families quite early (Kirschbaum 1924; Meggendorfer 1930; Stender 1930; Davison and Rabiner 1940; Jacob et al. 1950; Friede and DeJong 1964; Rosenthal et al. 1976; Masters et al. 1979, 1981a,b), this observation did little to advance understanding of the disorder.

The unraveling of the etiology of CJD is a wonderful story that has many threads, each representing a distinct piece of the puzzle. An important observation was made by Igor Klatzo in 1959, when he recognized that the neuropathology of kuru resembled that of CJD (Klatzo et al. 1959); the same year, William Hadlow suggested that kuru, a disease of New Guinea highlanders, was similar to scrapie, a hypothesis also based on light microscopic similarities (Hadlow 1959). However, Hadlow's insight was much more profound because he suggested that kuru is a transmissible disease like scrapie and that demonstration of the infectivity of kuru could be accomplished using chimpanzees because they are so

closely related to humans. He also noted that many months or years might be required before clinically recognizable disease would be seen in these inoculated nonhuman primates. Moreover, he argued that brain tissue from patients dying of kuru should be homogenized and injected intra-cerebrally into the chimpanzees, as was often done in studies of sheep scrapie.

At the time Hadlow's hypothesis was set forth, scrapie was considered to be caused by a "slow virus." The term "slow virus" had been coined by Bjorn Sigurdsson in 1954 based on his studies in Iceland on scrapie and visna of sheep (Sigurdsson 1954). Although Hadlow suggested that kuru, like scrapie, was caused by a slow virus, he did not perform the experiments required to demonstrate this phenomenon (Hadlow 1959, 1995). In fact, seven years were to pass before the transmissibility of kuru was established by passaging the disease to chimpanzees (Gajdusek et al. 1966), and in 1968, the transmission of CJD to chimpanzees after intracerebral inoculation was reported (Gibbs et al. 1968).

An early clue to unusual properties of the scrapie agent emerged from studies of 18,000 sheep that were inadvertently inoculated with the scrapie agent or "slow virus." These animals had been vaccinated against louping ill virus with a formalin-treated suspension of ovine brain and spleen that, as was subsequently shown, had been contaminated with the scrapie agent (Gordon 1946). Three different batches of vaccines were administered, and two years later, 1500 sheep developed scrapie. These findings demonstrated that the scrapie agent is resistant to inactivation by formalin, unlike most viruses, which are readily inactivated by such treatment.

The unusual biological properties of the scrapie agent were no less puzzling than the disease process itself, for the infectious agent caused a devastating degeneration of the CNS in the absence of an inflammatory response (Zlotnik 1962; Beck et al. 1964). Although the immune system remained intact, its surveillance system was unaware of a raging infection. The infectious agent that causes scrapie, now generally referred to as a "prion," achieved status as a scientific curiosity when its extreme resistance to ionizing and ultraviolet irradiation was discovered (Alper et al. 1966, 1967; Latarjet et al. 1970). Later, similar resistance to inactivation by UV and ionizing radiation was reported for the CJD agent (Gibbs et al. 1978). Tikvah Alper's radiation resistance data on the scrapie agent evoked a torrent of hypotheses concerning its composition. Suggestions as to the nature of the scrapie agent ranged from small DNA viruses to membrane fragments to polysaccharides to proteins, the last of which eventually proved to be correct (Pattison 1965; Gibbons and Hunter 1967; Griffith 1967; Pattison and Jones 1967; Hunter et al. 1968; Field et al. 1969; Hunter 1972; see Chapter 2).

Because the scrapie agent had been passaged from sheep into mice (Chandler 1961), scrapie was the most amenable of these diseases to study experimentally. Although the agents causing scrapie, CJD, and kuru were for many years thought to be different slow viruses, we now know that prions cause all these disorders and that this distinction was artificial.

Studies of prions have wide implications ranging from basic principles of protein conformation to the development of effective therapies for prion diseases (Prusiner 1998). In this chapter, the structural biology of prion proteins, as well as the genetics and molecular neurology of prion diseases, is introduced. How information is enciphered within the infectious prion particle is described.

PRION BIOLOGY AND DISEASES

Our current understanding of the prion particles that cause scrapie, CJD, and related diseases in mammals is described in this book (Table 1). Prions are unprecedented infectious pathogens that cause a group of invariably fatal, neurodegenerative diseases by an entirely novel mechanism (Prusiner 1998). Prion diseases may present as genetic, infectious, or sporadic disorders, all of which involve modification of the prion protein (PrP). CJD generally presents as a progressive dementia whereas scrapie of sheep and bovine spongiform encephalopathy (BSE) are generally manifest as ataxic illnesses (Wells et al. 1987).

Prions have also been reported in yeast and other fungi. These fascinating studies have greatly expanded our thinking about the role of prions in biology (Chapter 6).

Prions

Prions are defined as proteinaceous infectious particles that are devoid of nucleic acid and seem to be composed exclusively of a modified isoform of the prion protein designated PrP^{Sc} (Table 2). The normal, cellular PrP denoted PrP^{C} is converted into PrP^{Sc} through a process whereby some of its α-helical structure is converted into β-sheet (Pan et al. 1993). This structural transition is accompanied by profound changes in the physicochemical properties of the PrP. Whereas PrP^{C} is soluble in nondenaturing detergents, PrP^{Sc} is not (Meyer et al. 1986; Chapter 5). PrP^{C} is readily digested by proteases whereas PrP^{Sc} is partially resistant (Oesch et al. 1985). The species of a particular prion is determined by the sequence of the chromosomal PrP gene of the mammal in which it last replicated.

Table 1 The prion diseases

	Disease	Host	Mechanism of pathogenesis
A.	Kuru	Fore people	infection through ritualistic cannibalism
	Iatrogenic CJD	humans	infection from prion-contaminated HGH, dura mater grafts, etc.
	Variant CJD	humans	infection from bovine prions?
	Familial CJD	humans	germ-line mutations in PRNP
	GSS	humans	germ-line mutations in PRNP
	FFI	humans	germ-line mutation in PRNP (D178N, M129)
	Sporadic CJD	humans	somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
	FSI	humans	somatic mutation or spontaneous conversion of PrP^{C} into PrP^{Sc}?
B.	Scrapie	sheep	infection in genetically susceptible sheep
	BSE	cattle	infection with prion-contaminated MBM
	TME	mink	infection with prions from sheep or cattle
	CWD	mule deer, elk	unknown
	FSE	cats	infection with prion-contaminated bovine tissues or MBM
	Exotic ungulate encephalopathy	greater kudu, nyala, oryx	infection with prion-contaminated MBM

Abbreviations: (BSE) bovine spongiform encephalopathy; (CJD) Creutzfeldt-Jakob disease; (sCJD) sporadic CJD; (fCJD) familial CJD; (iCJD) iatrogenic CJD; (vCJD) (new) variant CJD; (CWD) chronic wasting disease; (FFI) fatal familial insomnia; (FSE) feline spongiform encephalopathy; (FSI) fatal sporadic insomnia; (GSS) Gerstmann-Sträussler-Scheinker disease; (HGH) human growth hormone; (MBM) meat and bone meal; (TME) transmissible mink encephalopathy.

Human PrP is encoded by a gene on the short arm of chromosome 20, and all other mammals examined also have a single-copy gene encoding PrP (Sparkes et al. 1986; Schätzl et al. 1995). In contrast to pathogens with a nucleic acid genome, prions encipher strain-specific properties in the tertiary structure of PrP^{Sc}. Transgenetic studies argue that PrP^{Sc} acts as a template upon which PrP^{C} is refolded into a nascent PrP^{Sc} molecule and that this process is facilitated by another protein or complex of proteins.

Although both the primary structure and posttranslational chemical modifications determine the tertiary structure of PrP^{C}, the conformation of PrP^{C} is modified by PrP^{Sc} as it is refolded into a nascent molecule of PrP^{Sc} (Telling et al. 1996b). The hallmark common to all of the prion dis-

eases, whether sporadic, dominantly inherited, or acquired by infection, is that they involve the aberrant metabolism of PrP (Prusiner 1991).

Prions Are Distinct from Viruses

Like viruses, prions are infectious because they stimulate a process by which more of the pathogen is produced. As prions or viruses accumulate in an infected host, they eventually cause disease. Both prions and viruses exist in different varieties or subtypes that are called strains, but many features of prion structure and replication distinguish them from viruses and all other known infectious pathogens.

Prions differ from viruses and viroids because they lack a nucleic acid genome that directs the synthesis of their progeny. Prions are composed of an abnormal isoform of a cellular protein, whereas most viral proteins are encoded by a viral genome, and viroids are devoid of protein.

Prions can exist in multiple molecular forms, whereas viruses exist in a single form with a distinct ultrastructural morphology. Prions have no constant structure, in marked contrast to viruses. Prion infectivity has been detected in fractions containing particles with an extremely wide range of sizes (Kimberlin et al. 1971; Prusiner et al. 1978b; Diringer and Kimberlin 1983). Initially, the small size of prions as determined by ionizing radiation inactivation studies (Alper et al. 1966) was confusing, because scrapie infectivity was clearly associated with larger particles (Rohwer 1984). Eventually, aggregation due to hydrophobic interactions between PrP^{Sc} molecules was found to be responsible for such anomalous behavior (Prusiner et al. 1978b).

Prions are nonimmunogenic, in contrast to viruses that almost always provoke an immune response. Prions do not elicit an immune response because the host has been rendered tolerant to PrP^{Sc} by PrP^{C} (Prusiner et al. 1993a; Williamson et al. 1996). In contrast, the foreign proteins of viruses that are encoded by the viral genome often elicit a profound immune response. Thus, it seems unlikely that a useful strategy for preventing or treating prion diseases will be vaccination, which has been so effective in preventing many viral illnesses.

The phenomenon of prion strains has posed a profound conundrum with respect to how prions might be composed of only host-encoded PrP^{Sc} molecules and yet exhibit diversity. An enlarging body of data argues that strains of prions are enciphered in the conformation of PrP^{Sc}. In contrast, strains of viruses and viroids have distinct nucleic acid sequences that produce pathogens with different properties. Many investigators argued for a nucleic acid genome within the infectious prion particle while oth-

ers contended for a small noncoding polynucleotide of either foreign or cellular origin (Dickinson et al. 1968; Kimberlin 1982, 1990; Dickinson and Outram 1988; Bruce et al. 1991; Weissmann 1991b). However, no nucleic acid was found despite intensive searches using a wide variety of techniques and approaches (Kellings et al. 1992, 1994). On the basis of a wealth of evidence, it is reasonable to assert that such a nucleic acid has not been found because it does not exist.

That many of the prion diseases were discovered prior to our current understanding of prion biology has created confusion in an environment where decisions of great economic and political consequence and possibly public health import are being made. For example, scrapie of sheep and bovine spongiform encephalopathy (BSE) have different names; yet, they are the same disease in two different species. Both scrapie and BSE are prion diseases that differ from each other in only two respects: First, the sheep PrP sequence differs from that of cattle at 7 or 8 positions out of 270 amino acids (Goldmann et al. 1990a, 1991b), giving rise to different PrP^{Sc} molecules in each; and second, some aspects of each disease are determined by the particular prion strain that infects a given host.

Understanding prion strains and the "species barrier" is of paramount importance with respect to the BSE epidemic in Britain in which more than 170,000 cattle have died over the past decade (Wells and Wilesmith 1995; Chapters 11 and 17). Brain extracts from eight cattle with BSE all gave the same patterns of incubation times and vacuolation of the neuropil when inoculated into a variety of inbred mice (Bruce et al. 1994; Bruce 1996). Incubation times and profiles of neuronal vacuolation have been used for three decades to study prion strains. Brain extracts prepared from three domestic cats, one nyala, and one kudu, all of which died with a neurologic illness, produced incubation times and lesion profiles indistinguishable from those found in the BSE cattle. Cats and exotic ungulates such as the kudu presumably developed prion disease from eating food containing bovine prions (Jeffrey and Wells 1988; Wyatt et al. 1991; Kirkwood et al. 1993).

NOMENCLATURE

A listing of the different prion diseases is given in Table 1. Although the prions that cause TME and BSE are referred to as TME prions and BSE prions, this may be unjustified, because both are thought to originate from the oral consumption of scrapie prions in sheep-derived foodstuffs and because many lines of evidence argue that the only difference among the various prions is the sequence of PrP, which is dictated by the host and not

the prion itself. The human prions present a similar semantic conundrum. Transmission of human prions to laboratory animals produces prions carrying PrP molecules with sequences dictated by the PrP gene of the host, not that of the inoculum.

To simplify the terminology, the generic term PrP^{Sc} is suggested in place of such terms as PrP^{CJD}, PrP^{BSE}, and PrP^{res} (Prusiner et al. 1998b). To distinguish PrP^{Sc} found in humans or cattle from that found in other animals, $HuPrP^{Sc}$ or $BoPrP^{Sc}$ is suggested instead of PrP^{CJD} or PrP^{BSE}, respectively (Table 2). Once human prions, and thus $HuPrP^{Sc}$ molecules, have been passaged into animals, the prions and PrP^{Sc} are no longer of the human species unless they were formed in an animal expressing a HuPrP transgene.

The "Sc" superscript of PrP^{Sc} was initially derived from the term scrapie because scrapie was the prototypic prion disease. Since all of the known prion diseases (Table 1) of mammals involve aberrant metabolism of PrP similar to that observed in scrapie, the Sc superscript is suggested for all abnormal, pathogenic PrP isoforms (Prusiner et al. 1998b). In this context, the Sc superscript is used to designate the scrapie-like or disease-

Table 2 Glossary of prion terminology

Term	Description
Prion	a *pro*teinaceous *in*fectious particle that lacks nucleic acid; composed largely, if not entirely, of PrP^{Sc} molecules
PrP^{Sc}	abnormal, pathogenic isoform of the prion protein that causes sickness; the only identifiable macromolecule in purified preparations of prions
PrP^{C}	cellular isoform of the prion protein
PrP 27-30	digestion of PrP^{Sc} with proteinase K generates PrP 27-30 by hydrolysis of the amino terminus
PRNP	human PrP gene located on chromosome 20
Prnp	mouse PrP gene located on syntenic chromosome 2; controls the length of the prion incubation time and is congruent with the incubation time genes *Sinc* and *Prn-i*. PrP-deficient ($Prnp^{0/0}$) mice are resistant to prions
PrP amyloid	fibril of PrP fragments derived from PrP^{Sc} by proteolysis; plaques containing PrP amyloid are found in the brains of some mammals with prion disease
Prion rod	an amyloid polymer composed of PrP 27-30 molecules; created by detergent extraction and limited proteolysis of PrP^{Sc}
Protein X	hypothetical macromolecule thought to act like a molecular chaperone in facilitating the conversion of PrP^{C} into PrP^{Sc}

causing isoform of PrP; for those who desire a more general derivation, Sc can equally well be derived from the term "prion sickness" (Table 2).

In the case of mutant PrPs, the mutation and any important polymorphism can be denoted in parentheses following the particular PrP isoform. For example in FFI, the pathogenic PrP isoform would be referred to as PrP^{Sc} or $HuPrP^{Sc}$; alternatively, if it were important to identify the mutation, then it would be written as $HuPrP^{Sc}$(D178N, M129) (Table 3). The term PrP^{res} or PrP-res is derived from the protease-*res*istance of PrP^{Sc}, but protease-resistance, insolubility, and high β-sheet content should be considered only as surrogate markers of PrP^{Sc} since one or more of these may not always be present. Whether PrP^{res} is useful in denoting PrP molecules that have been subjected to procedures that modify their resistance to proteolysis but have not been demonstrated to convey infectivity or cause disease remains debatable.

The term PrP* has been used in two different ways. First, it has been used to identify a fraction of PrP^{Sc} molecules that are infectious (Weissmann 1991a). Such a designation is thought to be useful since there are ~10^5 PrP^{Sc} molecules per infectious unit (Prusiner et al. 1982a, 1983). Second, PrP* has been used to designate a metastable intermediate of PrP^{C} that is bound to protein X (Cohen et al. 1994). It is noteworthy that neither a subset of biologically active PrP^{Sc} molecules nor a metastable intermediate of PrP^{C} has been identified, to date.

In mice, the PrP gene denoted *Prnp* is now known to be identical with two genes denoted *Sinc* and *Prn-i* that were known to control the length of the incubation time in mice inoculated with prions (Carlson et al. 1994; Moore et al. 1998). These findings permit a welcome simplification. A

Table 3 Examples of human PrP gene mutations found in the inherited prion diseases

Inherited prion disease	PrP gene mutation
Gerstmann-Sträussler-Scheinker disease	PrP P102L*
Gerstmann-Sträussler-Scheinker disease	PrP A117V
Familial Creutzfeldt-Jakob disease	PrP D178N, V129
Fatal familial insomnia	PrP D178N, M129*
Gerstmann-Sträussler-Scheinker disease	PrP F198S*
Familial Creutzfeldt-Jakob disease	PrP E200K*
Gerstmann-Sträussler-Scheinker disease	PrP Q217R
Familial Creutzfeldt-Jakob disease	PrP octarepeat insert*

*Signifies genetic linkage between the mutation and the inherited prion disease (Hsiao et al. 1989; Dlouhy et al. 1992; Petersen et al. 1992; Poulter et al. 1992; Gabizon et al. 1993).

gene designated *Pid-1* on mouse chromosome 17 also appears to influence experimental CJD and scrapie incubation times, but information on this locus is limited.

Distinguishing among CJD, GSS, and FFI has grown increasingly difficult with the recognition that fCJD, GSS, and FFI are autosomal dominant diseases caused by mutations in the human PrP gene, PRNP (Table 3). Initially, it was thought that a specific PRNP mutation was associated with a particular clinico-neuropathologic phenotype, but an increasing number of exceptions are being recognized. Multiple examples of variations in the clinico-neuropathologic phenotype within a single family where all affected members carry the same PRNP mutation have been recorded. Most patients with a PRNP mutation at codon 102 present with ataxia and have PrP amyloid plaques; such patients are generally given the diagnosis of GSS, but some individuals within these families present with dementia, a clinical characteristic that is usually associated with CJD. One suggestion is to label these inherited disorders as "prion disease" followed by the mutation in parentheses, and another is to use the terms fCJD and GSS followed by the mutation. In the case of FFI, describing the D178N mutation and M129 polymorphism seems unnecessary since this is the only known mutation-polymorphism combination that gives the FFI phenotype.

DISCOVERY OF THE PRION PROTEIN

The discovery of the prion protein transformed research on scrapie and related diseases (Bolton et al. 1982; Prusiner et al. 1982a). It provided a molecular marker that was subsequently shown to be specific for these illnesses and identified the major, and possibly the only, component of the prion particle. The protease-resistant fragment of the scrapie isoform of the prion protein, designated PrP 27-30, was discovered by enriching fractions from Syrian hamster (SHa) brain for scrapie infectivity (Bolton et al. 1982; Prusiner et al. 1982a). PrP 27-30 has an apparent molecular weight (M_r) of 27–30 kD (Fig. 1).

Over a 20-year period from 1960 to 1980, there were many unsuccessful attempts to purify the scrapie agent or to identify a biochemical marker that copurified (G.D. Hunter et al. 1963, 1969, 1971; Hunter and Millson 1964, 1967; Kimberlin et al. 1971; Millson et al. 1971, 1976; Marsh et al. 1974, 1978, 1980; Siakotos et al. 1976; Gibbs and Gajdusek 1978; Millson and Manning 1979). Studies on the sedimentation properties of scrapie infectivity in mouse spleens and brains suggested that hydrophobic interactions were responsible for the non-ideal physical

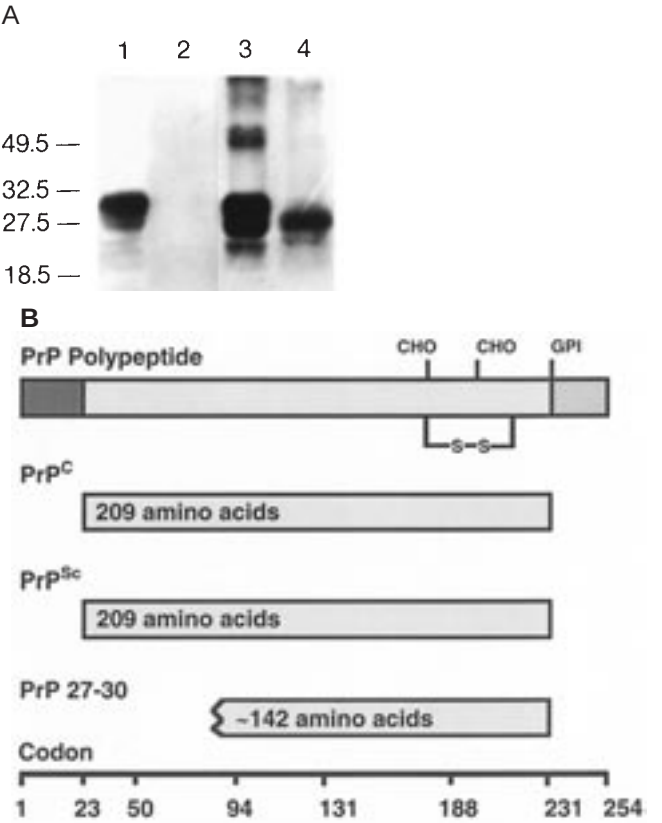

Figure 1 Prion protein isoforms. (*A*) Western immunoblot of brain homogenates from uninfected (lanes *1* and *2*) and prion-infected (lanes *3* and *4*) Syrian hamsters. Samples in lanes *2* and *4* were digested with 50 μg/ml proteinase K for 30 min at 37ºC. PrP^{C} in lanes 2 and 4 was completely hydrolyzed under these conditions, whereas approximately 67 amino acids were digested from the amino terminus of PrP^{Sc} to generate PrP 27-30. After polyacrylamide gel electrophoresis (PAGE) and electrotransfer, the blot was developed with anti-PrP R073 polyclonal rabbit antiserum. Molecular size markers are in kD. (*B*) Bar diagram of SHaPrP which consists of 254 amino acids. After processing of the amino and carboxyl termini, both PrP^{C} and PrP^{Sc} consist of 209 residues. After limited proteolysis, the amino terminus of PrP^{Sc} is truncated to form PrP 27-30, which is composed of approximately 142 amino acids.

behavior of the scrapie particle (Prusiner 1978; Prusiner et al. 1978a). Indeed, the scrapie agent presented a biochemical nightmare: Infectivity was spread from one end to the other of a sucrose gradient and from the

void volume to fractions eluting at 5–10 times the included volume of chromatographic columns. Such results demanded new approaches and better assays. Only the development of improved bioassays allowed purification of the infectious pathogen that causes scrapie and CJD (Prusiner et al. 1980, 1982b).

Enriching fractions from the brains of scrapie-infected Syrian hamsters for infectivity yielded a single protein, PrP 27-30, as noted above. PrP 27-30 was later found to be the protease-resistant core of PrP^{Sc} (Prusiner et al. 1984; Basler et al. 1986). Copurification of PrP 27-30 and scrapie infectivity demand that the physicochemical properties as well as antigenicity of these two entities be similar (Gabizon et al. 1988; Chapter 2). The results of a wide array of inactivation experiments demonstrated the similarities in the properties of PrP 27-30 and scrapie infectivity (McKinley et al. 1983; Prusiner et al. 1983). To explain these findings in terms of the virus hypothesis, it is necessary to postulate either a virus that has a coat protein that is highly homologous with PrP or a virus that binds tightly to PrP^{Sc}. In either case, the PrP-like coat proteins or the PrP^{Sc}/virus complexes must display properties indistinguishable from PrP^{Sc} alone (Chapter 2). The inability to inactivate preparations highly enriched for scrapie infectivity by procedures that modify nucleic acids was interpreted as evidence against the existence of a scrapie-specific nucleic acid (Alper et al. 1967; Prusiner 1982). To explain the findings in terms of a virus, one must argue that PrP^{Sc} or an as yet undetected PrP-like protein of viral origin protects the viral genome from inactivation.

Determination of the Amino-terminal Sequence of PrP 27-30

The molecular biology and genetics of prions began with the purification of PrP 27-30 significantly to allow determination of its amino-terminal amino acid sequence (Prusiner et al. 1984). Multiple signals in each cycle of the Edman degradation suggested that either multiple proteins were present in these "purified fractions" or a single protein with a ragged amino terminus was present. When the signals in each cycle were grouped according to their intensities of strong, intermediate, and weak, it became clear that a single protein with a ragged amino terminus was being sequenced (Fig. 2). Determination of a single, unique sequence for the amino terminus of PrP 27-30 permitted the synthesis of isocoding mixtures of oligonucleotides that were subsequently used to identify incomplete PrP cDNA clones from hamster (Oesch et al. 1985) and mouse (Chesebro et al. 1985). cDNA clones encoding the entire open

Relative Amount	Amino Acid Sequence*
1	G-Q-G-G-G-T-H-N-Q-W-N-K-P-S-K
0.4	X-X-X-T-H-N-X-W-X-K-P
0.2	X-X-P-W-X-Q-X-X-X-T-H-X-Q-W

*Single-letter amino acid code. X = amino acid not determined at that cycle.

Figure 2 Interpreted amino acid sequence of the amino terminus of PrP 27-30. The "ragged ends" of PrP 27-30 are shown (Prusiner et al. 1984).

reading frames (ORF) of SHa and Mo PrP were eventually recovered (Basler et al. 1986; Locht et al. 1986).

PRION PROTEIN ISOFORMS

PrP mRNA levels are similar in normal, uninfected and scrapie-infected tissues (Chesebro et al. 1985). This finding produced skepticism about whether or not PrP 27-30 was related to the infectious prion particle. Nevertheless, the search for a protein encoded by the PrP mRNA revealed a protease-sensitive protein that is soluble in nondenaturing detergents, designated PrP^{C} (Oesch et al. 1985; Meyer et al. 1986).

PrP^{C} and PrP^{Sc} have the same covalent structure, and each consists of 209 amino acids in Syrian hamsters (Fig. 1). The amino-terminal sequencing, the deduced amino acid sequences from PrP cDNA, and immunoblotting studies argue that PrP 27-30 is a truncated protein of about 142 residues that is derived from PrP^{Sc} by limited proteolysis of the amino terminus (Prusiner et al. 1984; Oesch et al. 1985; Basler et al. 1986; Locht et al. 1986; Meyer et al. 1986).

In general, ~10^5 PrP^{Sc} molecules correspond to one ID_{50} unit of prions using the most sensitive bioassay (Prusiner et al. 1982a, 1983). PrP^{Sc} is probably best defined as the abnormal isoform of the prion protein that stimulates conversion of PrP^{C} into nascent PrP^{Sc}, accumulates, and causes disease. Although resistance to limited proteolysis has proved to be a convenient tool for detecting PrP^{Sc}, not all PrP^{Sc} molecules possess protease resistance (Hsiao et al. 1994; Telling et al. 1996a). Some investigators equate protease-resistance with PrP^{Sc} and this erroneous view has been compounded by the use of the term "PrP-res" (Caughey et al. 1990).

Although insolubility as well as protease-resistance was used in initial studies to differentiate PrP^{Sc} from PrP^{C} (Meyer et al. 1986), subsequent

investigations showed that these properties are only surrogate markers, as are high β-sheet content and polymerization into amyloid (Prusiner et al. 1983; Caughey et al. 1991b; McKinley et al. 1991; Gasset et al. 1993; Safar et al. 1993b; Muramoto et al. 1996; Riesner et al. 1996). When these surrogate markers are present they are useful, but their absence does not establish the lack of prion infectivity. PrP^{Sc} is usually not detected by Western immunoblotting if less than 10^5 ID_{50} units/ml of prions are present in a sample (Lasmézas et al. 1996a); furthermore, PrP^{Sc} from different species may exhibit different degrees of protease resistance.

In our experience, the method of sample preparation from scrapie-infected brain also influences the sensitivity of PrP^{Sc} immunodetection, in part because PrP^{Sc} is not uniformly distributed in the brain (DeArmond et al. 1987; Taraboulos et al. 1992). Some experiments in which PrP^{Sc} detection proved problematic in partially purified preparations (Czub et al. 1986; Xi et al. 1992) were repeated with crude homogenates where PrP^{Sc} was readily measured (Jendroska et al. 1991; McKenzie et al. 1994).

Cell Biology of PrP^{Sc} Formation

In scrapie-infected cells, PrP^{C} molecules destined to become PrP^{Sc} exit to the cell surface prior to conversion into PrP^{Sc} (Stahl et al. 1987; Borchelt et al. 1990; Caughey and Raymond 1991; Chapter 9). Like other GPI-anchored proteins, PrP^{C} appears to re-enter the cell through a subcellular compartment bounded by cholesterol-rich, detergent-insoluble membranes, which might be caveolae or early endosomes (Taraboulos et al. 1995; Vey et al. 1996; Kaneko et al. 1997a;). Within this cholesterol-rich, nonacidic compartment, GPI-anchored PrP^{C} can be either converted into PrP^{Sc} or partially degraded (Taraboulos et al. 1995). Subsequently, PrP^{Sc} is trimmed at the amino terminus in an acidic compartment in scrapie-infected cultured cells to form PrP 27-30 (Caughey et al. 1991a). In contrast, amino-terminal trimming of PrP^{Sc} is minimal in brain, where little PrP 27-30 is found (McKinley et al. 1991).

RODENT MODELS OF PRION DISEASE

Mice and hamsters are commonly used in experimental studies of prion disease (Chapters 4 and 14). The shortest incubation times are achieved with intracerebral inoculation of homologous prions; under these conditions all of the animals develop prion disease within a narrow interval for a particular dose. The term homologous prions indicates that the PrP gene of the donor in which the prion was previously passaged has the same sequence as that of the recipient. When the PrP sequence of the donor dif-

fers from that of the recipient, the incubation time is prolonged, the length of the incubation becomes quite variable, and often, many inoculated animals do not develop disease (Carlson et al. 1989; Telling et al. 1994, 1995; Tateishi et al. 1996). This phenomenon is often called the prion "species barrier" (Pattison 1965).

PrP GENE STRUCTURE AND ORGANIZATION

The entire open reading frame (ORF) of all known mammalian and avian PrP genes resides within a single exon (Basler et al. 1986; Westaway et al. 1987; Hsiao et al. 1989; Gabriel et al. 1992), which 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). Recently, a low-abundance SHaPrP mRNA containing an additional small exon in the 5′ untranslated region was discovered that is encoded by the SHaPrP gene (Li and Bolton 1997). The mouse (Mo), sheep, and rat PrP genes contain three exons with exon 3 analogous to exon 2 of the hamster (Saeki et al. 1996; Westaway et al. 1994a; Westaway et al. 1991; Westaway et al. 1994c). 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 that may function as a canonical binding site for the transcription factor Sp1 (McKnight and Tjian 1986). Mapping of PrP genes to the short arm of Hu chromosome 20 and to the homologous region of Mo chromosome 2 argues for the existence of PrP genes prior to the speciation of mammals (Robakis et al. 1986; Sparkes et al. 1986).

Expression of the PrP Gene

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. 1986a).

Since no antibodies are currently available that clearly distinguish PrP^{C} from PrP^{Sc} and vice versa, PrP^{C} is generally measured in tissues from uninfected control animals where no PrP^{Sc} is found. Whether a

recently isolated α-PrP IgM monoclonal antibody reacts exclusively with PrP^{Sc} remains to be confirmed (Korth et al. 1997). With the possible exception of this α-PrP IgM monoclonal antibody, PrP^{Sc} must be measured in tissues of infected animals after PrP^{C} has been hydrolyzed by digestion with a proteolytic enzyme. PrP^{C} expression in brain was defined by standard immunohistochemistry (DeArmond et al. 1987) and by histoblotting in the brains of uninfected controls (Taraboulos et al. 1992). Immunostaining of PrP^{C} in the SHa brain was most 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 the regions that were intensely stained for PrP^{C}. A similar relationship 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, which is consistent with earlier findings in which scrapie infectivity migrated in a pattern consistent with retrograde transport (Kimberlin et al. 1983; Fraser and Dickinson 1985; Jendroska et al. 1991). Although the rate of PrP^{Sc} synthesis appears to be a function of the level of PrP^{C} expression in Tg mice, the level to which PrP^{Sc} accumulates appears to be independent of PrP^{C} concentration (Prusiner et al. 1990).

Overexpression of wtPrP Transgenes

Mice were constructed expressing different levels of the wild-type (wt) SHaPrP transgene (Chapter 8). Inoculation of these Tg(SHaPrP) mice with SHa prions demonstrated abrogation of the species barrier resulting in abbreviated incubation times due to a nonstochastic process (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). 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 the synthesis of only SHa prions.

During transgenetic studies, we discovered that uninoculated older mice harboring high copy numbers of wtPrP transgenes derived from Syrian hamsters, sheep, and *Prnp*^{b} mice spontaneously developed truncal ataxia, hind-limb paralysis, and tremors (Westaway et al. 1994a). These

Tg mice exhibited a profound necrotizing myopathy involving skeletal muscle, a demyelinating polyneuropathy, and focal vacuolation of the 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 and 600 days of age, whereas hemizygous Tg(SHaPrP$^{+/0}$)7 mice also developed disease, but after >650 days. Whether overexpression of wtPrP^{C} or a somatic mutant PrP^{C} is responsible for this neuromyopathy remains to be established.

PrP Gene Dosage Controls the Scrapie Incubation Time

Incubation times have been used to isolate prion strains inoculated into sheep, goats, mice, and hamsters (Dickinson et al. 1968). A major determinant of scrapie incubation periods in mice is a gene initially denoted as *Sinc*. Once molecular clones of the PrP gene, denoted *Prnp* in mice, became available, a study was performed to determine if control of the length of the scrapie incubation time was genetically linked to the PrP gene. Because the availability of VM mice with prolonged incubation times that were used to define *Sinc* was restricted, we used I/LnJ mice (Kingsbury et al. 1983) in our crosses and provisionally labeled the incubation time locus *Prn-i*. Indeed, *Prn-i* was found to be either congruent with or closely linked to *Prnp* (Carlson et al. 1986).

Although the amino acid substitutions in PrP that distinguish *Prnp^{a}* from *Prnp^{b}* mice argued for the congruency of *Prnp* and *Prn-i* (Westaway et al. 1987), experiments with Prnp^{a} mice expressing Prnp^{b} transgenes demonstrated a "paradoxical" shortening of incubation times (Westaway et al. 1991). We had predicted that these Tg mice would exhibit a prolongation of the incubation time after inoculation with RML prions based on (*Prnp^{a}* × *Prnp^{b}*) F_1 mice, which do exhibit long incubation times. We described those findings as paradoxical shortening because we and other workers had believed for many years that long incubation times are dominant traits (Dickinson et al. 1968; Carlson et al. 1986). From studies of congenic and transgenic mice expressing different numbers of the *a* and *b* alleles of *Prnp*, we learned that these findings were not paradoxical; indeed, they result from increased PrP gene dosage (Carlson et al. 1994). When the RML isolate was inoculated into congenic and transgenic mice, increasing the number of copies of the *a* allele was found to be the major determinant in reducing the incubation time; however, increasing the number of copies of the *b* allele also reduced the incubation time, but not to the same extent as that seen with the *a* allele. Recently, gene targeting

studies have established that the *Prnp* gene controls the incubation time and as such is congruent with both *Prn-i* and *Sinc* (Moore et al. 1998).

PrP-deficient Mice

Ablation of the PrP gene ($Prnp^{0/0}$) in mice did not affect development of these animals (Büeler et al. 1992; Manson et al. 1994; Chapter 7). In fact, they generally remain healthy at almost 2 years except in one report in which the Purkinje cell loss was accompanied by ataxia beginning about 70 weeks of age (Sakaguchi et al. 1996). Although brain slices from $Prnp^{0/0}$ mice were reported to show altered synaptic behavior (Collinge et al. 1994; Whittington et al. 1995), these results could not be confirmed by other investigators (Herms et al. 1995; Lledo et al. 1996).

$Prnp^{0/0}$ mice are resistant to prions (Büeler et al. 1993; Prusiner et al. 1993a). $Prnp^{0/0}$ mice were sacrificed 5, 60, 120, and 315 days after inoculation with RML prions, and brain extracts were bioassayed in CD-1 Swiss mice. Except for residual infectivity from the inoculum detected at 5 days after inoculation, no infectivity was detected in the brains of $Prnp^{0/0}$ mice (Prusiner et al. 1993a). One group of investigators found that $Prnp^{0/0}$ mice inoculated with mouse-passaged scrapie prions and sacrificed 20 weeks later had $10^{3.6}$ ID_{50} units/ml of homogenate by bioassay (Büeler et al. 1993). Another group also found measurable titers of prions many weeks after inoculation of $Prnp^{0/0}$ mice with mouse-passaged CJD prions (Sakaguchi et al. 1995; Chapter 7). Some investigators have argued that these data imply that prion infectivity replicates in the absence of PrP gene expression (Chesebro and Caughey 1993; Caughey and Chesebro 1997; Lasmézas et al. 1997).

$Prnp^{0/0}$ 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. 1993a). Since 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). Such an interpretation is consistent with the dominant inheritance of familial prion diseases.

Mice heterozygous ($Prnp^{0/+}$) for ablation of the PrP gene had prolonged incubation times when inoculated with Mo prions and developed signs of neurologic dysfunction at 400–460 days after inoculation (Prusiner et al. 1993a; Büeler et al. 1994). These findings are in accord with studies on Tg(SHaPrP) mice in which increased SHaPrP expression was accompanied by diminished incubation times (Prusiner et al. 1990).

Since *Prnp*$^{0/0}$ mice do not express PrP^{C}, we reasoned that they might more readily produce α-PrP antibodies. Prnp$^{0/0}$ mice immunized with Mo or SHa prion rods produced α-PrP antisera that bound Mo, SHa, and Hu PrP (Prusiner et al. 1993a; Williamson et al. 1996). These findings contrast with earlier studies in which α-MoPrP antibodies could not be produced in mice, presumably because the mice had been rendered tolerant by the presence of MoPrP^{C} (Barry and Prusiner 1986; Kascsak et al. 1987; Rogers et al. 1991). That *Prnp*$^{0/0}$ mice readily produce α-PrP antibodies is consistent with the hypothesis that the lack of an immune response in prion diseases is because PrP^{C} and PrP^{Sc} share many epitopes.

SPECIES VARIATIONS IN THE PrP SEQUENCE

PrP is posttranslationally processed to remove a 22-amino acid amino-terminal signal peptide. PrP contains two conserved disulfide-bonded cysteines and a sequence that marks for the addition of a glycophosphatidyl inositol (GPI) anchor. Twenty-three residues are removed from the carboxyl terminus during the addition of this GPI moiety, which anchors the protein to the cell membrane (Stahl et al. 1990). Contributing to the mass of the protein are two asparagine side chains linked to large oligosaccharides with multiple structures that have been shown to be complex and diverse (Endo et al. 1989). Although many species variants of PrP have now been sequenced (Schätzl et al. 1995), only the chicken sequence has been found to be greatly different from the human (Harris et al. 1989; Gabriel et al. 1992). The alignment of the translated sequences from more than 40 PrP genes shows a striking degree of conservation between the mammalian sequences and is suggestive of the retention of some important function through evolution (Fig. 3A). Cross-species conservation of PrP sequences makes it difficult to draw conclusions about the functional importance of many of the individual residues in the protein.

Amino-terminal Sequence Repeats

The amino-terminal domain of mammalian PrP contains five copies of a P(H/Q)GGG(G)WGQ octarepeat sequence, occasionally more, as in the case of one sequenced bovine allele that has six copies (Goldmann et al. 1991b; Prusiner et al. 1993b). These repeats are remarkably conserved between species, which implies a functionally important role. The chicken sequence contains a different repeat, PGYP(H/Q)N (Harris et al. 1989; Gabriel et al. 1992). Although insertions of extra repeats have been found

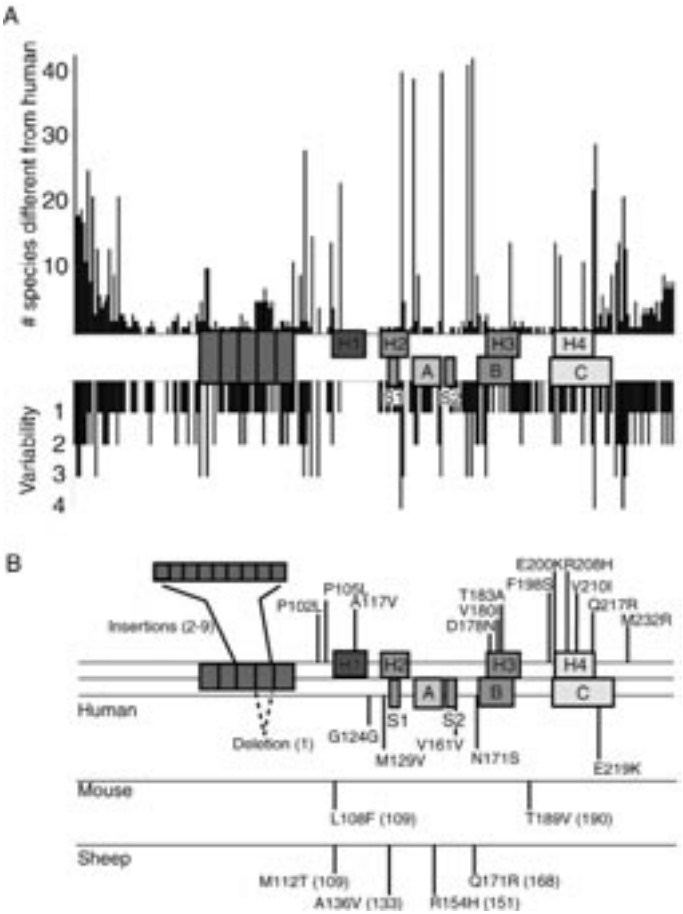

Figure 3 Species variations and mutations of the prion protein gene. (*A*) Species variations. The x-axis represents the human PrP sequence, with the five octarepeats and H1-H4 regions of putative secondary structure shown as well as the three α-helices A, B, and C and the two β-strands S1 and S2 as determined by NMR. The precise residues corresponding to each region of secondary structure are given in Fig. 5. Vertical bars above the axis indicate the number of species that differ from the human sequence at each position. Below the axis, the length of the bars indicates the number of alternative amino acids at each position in the alignment. (*B*) Mutations causing inherited human prion disease and polymorphisms in human, mouse, and sheep. Above the line of the human sequence are mutations that cause prion disease. Below the lines are polymorphisms, some but not all of which are known to influence the onset as well as the phenotype of disease. Data were compiled by Paul Bamborough and Fred E. Cohen. (Reprinted, with permission, from Prusiner 1997 [copyright AAAS].)

in patients with familial disease, naturally occurring deletions of single octarepeats do not appear to cause disease, and deletion of all these repeats does not prevent PrP^{C} from undergoing a conformational transition into PrP^{Sc} (Rogers et al. 1993; Fischer et al. 1996).

Conserved Alanine–Glycine Region

The other region of notable conservation is in the sequence following the carboxyl terminus of the last octarepeat. Here an unusual glycine- and alanine-rich region from A113 to Y128 is found (Fig. 3A). Although no differences between species have been found in this part of the sequence, a single point mutation A117V is linked to GSS (Hsiao et al. 1991a). The conservation of structure suggests an important role in the function of PrP^{C}; in addition, this region is likely to be important in the conversion of PrP^{C} into PrP^{Sc}.

STRUCTURES OF PrP ISOFORMS

Mass spectrometry and gas phase sequencing were used to search for posttranslational chemical modifications that might explain the differences in the properties of PrP^{C} and PrP^{Sc}. No modifications differentiating PrP^{C} from PrP^{Sc} were found (Stahl et al. 1993). These observations forced consideration of the possibility that a conformational change distinguishes the two PrP isoforms.

Molecular Modeling of PrP Isoforms

In comparing the amino acid sequences of 11 mammalian and 1 avian prion proteins, structure prediction algorithms identified four regions of putative secondary structure. Using a neural network algorithm, these four regions were predicted to be α-helices and were designated H1, H2, H3, and H4 (Gasset et al. 1992; Huang et al. 1994). When synthetic peptides corresponding to each of these domains were produced, the H1, H3, and H4 peptides readily adopted a β-sheet conformation in aqueous buffer (Gasset et al. 1992). Once we learned that PrP^{C} has a high α-helical content as described below, three-dimensional models of PrP^{C} and PrP^{Sc} were developed (Huang et al. 1994, 1995; Fig. 4).

Optical Spectroscopy of PrP Isoforms

After PrP^{C} and PrP^{Sc} were purified using nondenaturing procedures, they were examined by Fourier transform infrared (FTIR) spectroscopy and

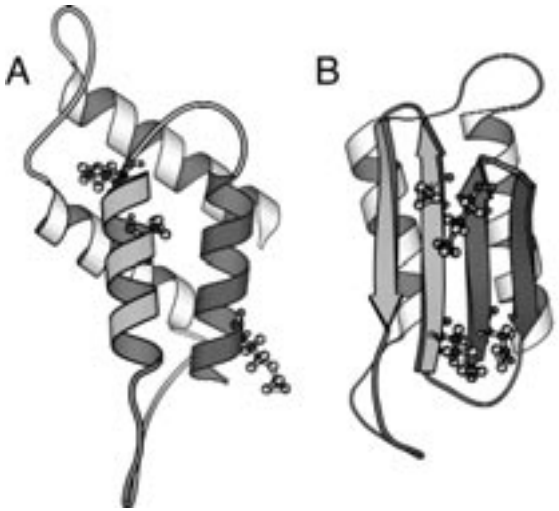

Figure 4 Models for the tertiary structures of PrP^{Sc} and PrP^{C}. (*A*) Proposed three-dimensional structure of PrP^{C} (Huang et al. 1994). Helix 1 is shown in red and helix 2 is in green. We believe that helices 1 and 2 are converted into β-sheet structure during the formation of PrP^{Sc}. The H_2-H_3 loop corresponding to the S_{2b}-H_3 loop in PrP^{Sc} is shown in yellow. Four residues (Asn-108, Met-112, Met-129, and Ala-133) implicated in the species barrier as noted above are shown in the ball and stick model. (*B*) Proposed three-dimensional structure of PrP^{Sc} (Huang et al. 1995). This structure was chosen from the six penultimate models of PrP^{Sc} because it appeared to correlate best with genetic data on residues involved in species barrier. It contains a 4-strand mixed β-sheet with two α-helices packed against one face of the sheet. Strands 1a and 1b (in *red*) correspond to the helix 1 in PrP^{C}, and strands 2a and 2b (in *green*) correspond to the helix 2. Helices 3 and 4 in this model remain unchanged from the PrP^{C} model (Huang et al. 1994). Four residues (Asn-108, Met-112, Met-129, and Ala-133) implicated in the species barrier (Schätzl et al. 1995) are shown in the ball and stick model. They cluster on the solvent-accessible surface of the β-sheet, which might provide a plausible interface for the PrP^{Sc}-PrP^{C} interaction. The S_{2b}-H_3 loop connecting the β-sheet and helix 3 is implicated in the species barrier and is shown in *yellow*. This conformationally flexible loop could come into contact with PrP^{C} during the formation of the PrP^{Sc}-PrP^{C} complex. Therefore, the specific molecular recognition during prion replication might involve both the β-sheet as the primary binding site and the S_{2b}-H_3 loop as an additional site for interaction.

circular dichroism (CD). These studies demonstrated that PrP^{C} has a high α-helix content (42%) and little β-sheet (3%) (Pan et al. 1993). In contrast, the β-sheet content of PrP^{Sc} was 43% and the α-helix 30%, as mea-

sured by FTIR (Pan et al. 1993) and CD spectroscopy (Safar et al. 1993a). Like PrP^{Sc}, the amino-terminally truncated PrP 27-30 has a high β-sheet content (Caughey et al. 1991b; Gasset et al. 1993), which is consistent with the earlier finding that PrP 27-30 polymerizes into amyloid fibrils (Prusiner et al. 1983). Denaturation of PrP 27-30 under conditions that reduced scrapie infectivity resulted in a concomitant diminution of β-sheet content (Gasset et al. 1993; Safar et al. 1993b).

We and other workers have suggested that the conversion of PrP^{C} into PrP^{Sc} may proceed through an intermediate designated PrP* (Jarrett and Lansbury 1993; Cohen et al. 1994). Possible intermediates in the refolding of PrP 27-30 from a denatured state have been identified by fluorescence and CD spectroscopy (Safar et al. 1994). In experiments demonstrating the binding of PrP^{C} to PrP^{Sc}, PrP^{C} was partially denatured in guanidine (Gdn) HCl into a form that probably corresponds to PrP*. In these studies, ^{35}S-labeled PrP^{C} was mixed with a large excess of purified PrP^{Sc} and the resistance of labeled PrP^{C} to limited proteolysis was measured (Kocisko et al. 1994). Whether the conformation of PrP^{C} was altered or the molecule was simply protected from proteolysis by binding to PrP^{Sc} could not be established. Using a similar protocol, synthetic PrP peptides were also found to produce protease-resistant PrP^{C} (Kaneko et al. 1995, 1997b).

NMR Structural Studies of Recombinant PrP

Nuclear magnetic resonance (NMR) studies have provided evidence supporting some and arguing against other aspects of the molecular models described above. Chemical shift measurements of a 56-mer synthetic peptide spanning H1 and H2 showed that the H1 region could form an α-helix in the presence of 2.8% SDS (Zhang et al. 1995). When the NMR structure of a 111-residue fragment of mouse (Mo) PrP, denoted MoPrP(121-231), expressed in *E. coli* was solved, it was found to have H3 and H4 as predicted but there was no evidence for H1 and H2; instead an α-helix in the loop between H2 and H3 was found (Riek et al. 1996). Moreover, this α-helix is flanked by a small two-stranded, antiparallel β-sheet (Riek et al. 1996). Subsequently, the NMR structure of recombinant (r) PrP(90-231) corresponding to the sequence of PrP 27-30 in an α-helical form of rPrP that appears to resemble PrP^{C} was solved (Fig. 5) (Mehlhorn et al. 1996; James et al. 1997; Zhang et al. 1997). The structure of rPrP comprises three α-helices and a small two-stranded, antiparallel β-sheet, but the additional residues of rPrP have profound consequences for the structure of

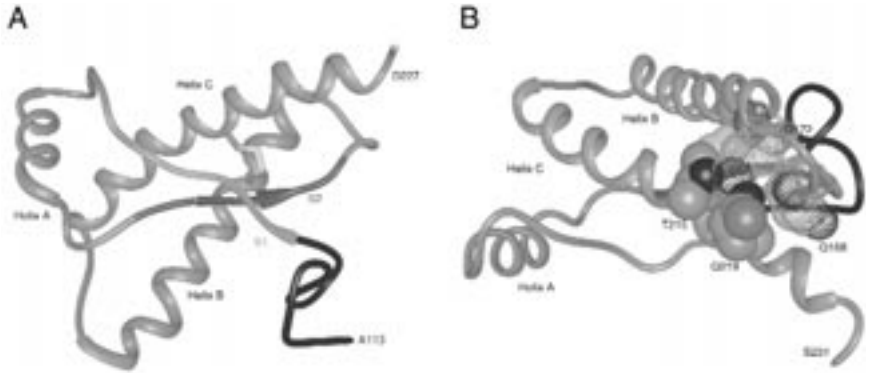

Figure 5 NMR structure of a recombinant prion protein. (*A*) Ribbon diagram of the NMR structure of Syrian hamster (SHa) recombinant (r) PrP(90-231). Presumably, the structure of the α-helical form of rPrP(90-231) resembles that of PrP^{C}. rPrP(90-231) is viewed from the interface where PrP^{Sc} is thought to bind to PrP^{C}. The color scheme is: α-helices A (residues 144–157), B (172–193), and C (200–227) in *pink*; disulfide between Cys-179 and Cys-214 in *yellow*; conserved hydrophobic region composed of residues 113–126 in *red*; loops in *gray*; residues 129–134 in *green* encompassing strand S1 and residues 159–165 in *blue* encompassing strand S2; the arrows span residues 129–131 and 161–163, as these show a closer resemblance to β-sheet (James et al. 1997). (*B*) NMR structure of rPrP(90-231) is viewed from the interface where protein X is thought to bind to PrP^{C}. Protein X appears to bind to the side chains of residues 165–171 that form a loop and to residues on the surface of helix C that lie between the disulfide bond and the carboxyl terminus (Kaneko et al. 1997c). (Reprinted, with permission, from Prusiner et al. 1998a [copyright Cell Press].)

PrP throughout the entire protein (James et al. 1997). Although identifiable secondary structure is largely lacking within the amino terminus of rPrP, residues 112–125 form a conserved hydrophobic region that packs against the β-sheet. The amino terminus of rPrP also seems to confer stability within the carboxyl terminus; a more compact structure with order in a loop (residues 165–171) was found that was unstructured in MoPrP(121-231) as well as substantial lengthening of the two carboxy-terminal helices denoted B and C. NMR spectra reveal multiple signals for some nuclei indicating that the amino terminus and spatially proximate regions assume multiple discrete conformers. This conformational heterogeneity may be the basis for transformation of PrP^{C} into PrP^{Sc}.

Although some features of the structure of rPrP are similar to those reported earlier for the smaller recombinant MoPrP containing residues

121–231 (Riek et al. 1996), the additional residues of rPrP have profound consequences for the structure of PrP throughout the entire protein (James et al. 1997). For example, the loop (165-171) at the amino terminus of helix B is well defined in rPrP(90-231) but is disordered in MoPrP(121-231); in addition, helix C is composed of residues 200–227 in rPrP(90-231) but extends only from 200 to 217 in MoPrP(121-231) (Chapter 5). The loop and the carboxy-terminal portion of helix C are particularly important because they form a discontinuous epitope to which protein X binds (Fig. 5B). Protein X is thought to feature in the conversion of PrP^{C} into PrP^{Sc} by acting like a molecular chaperone in facilitating the unfolding of PrP^{C} and its refolding into nascent PrP^{Sc} (Telling et al. 1995; Kaneko et al. 1997c). Another difference between the structures of rPrP and MoPrP (121-231) lies in the proximity of residues 178 and 129. The side chains of these residues determine the phenotypes of two inherited human prion diseases (Goldfarb et al. 1992). In rPrP, residue 178 lies within helix B and is located opposite residue 129 with strand S2 partially intervening. Such an arrangement raises the possibility that the D178N mutation destabilizes PrP by partially unraveling helix B and that the conformation of PrP^{Sc} is modulated by the side chain of residue 129. The particular conformation adopted by mutant PrP^{Sc} might determine in which regions of the CNS PrP^{Sc} is deposited and, thus, be responsible for whether patients present with insomnia or dementia (Goldfarb et al. 1992). When residue 129 is a valine, patients present with a dementing illness called familial CJD. In MoPrP(121-231), residues 178 and 129 are quite distant from each other; in fact, residue 178 does not even form part of helix B. Whether the differences between the two recombinant PrP fragments are due to (1) their different lengths, (2) species-specific differences in sequences, or (3) the conditions used for solving the structures remains to be determined (Chapter 5).

Recent NMR studies of full-length MoPrP(23-231) and SHaPrP(29-231) have shown that the amino termini are highly flexible and lack identifiable secondary structure under the experimental conditions employed (Donne et al. 1997; Riek et al. 1997). Studies of SHaPrP(29-231) indicate transient interactions between the carboxy-terminal end of helix B and the highly flexible, amino-terminal random-coil containing the octarepeats (residues 29–125) (Donne et al. 1997); such interactions were not reported for MoPrP(23-231) (Riek et al. 1997). Tertiary structure of the amino terminus is of considerable interest, since it is within this region of PrP that a profound conformational change occurs during the formation of PrP^{Sc} as described below (Peretz et al. 1997).

Prion Species Barrier and Protein X

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-infected beef (Cousens et al. 1997). The passage of prions between species is usually a stochastic process involving a randomly determined sequence of events that results in prolonged incubation times during the first passage into a new host (Chapter 8) (Pattison 1965). On subsequent passage in a homologous host, the incubation time shortens to that recorded for all subsequent passages. As noted previously, this prolongation of the incubation time and the apparent resistance of some animals to the inoculated prions from another species is often called the prion "species barrier" (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).

Attempts to abrogate the prion species barrier between humans and mice by using an approach similar to that described for the abrogation of the species barrier between Syrian hamsters and mice (Scott et al. 1989) were initially unsuccessful. Mice expressing HuPrP transgenes did not develop signs of CNS dysfunction more rapidly or frequently than non-Tg controls (Telling et al. 1994). Success in breaking the species barrier between humans and mice came with mice expressing chimeric PrP transgenes derived from Hu and Mo PrP. Hu PrP differs from Mo PrP at 28 of 254 positions (Kretzschmar et al. 1986b), whereas chimeric MHu2MPrP differs at 9 residues. The mice expressing the MHu2M transgene develop disease after injection with human prions and exhibit abbreviated incubation times of ~200 days (Telling et al. 1994). In these initial studies the chimeric MHu2M transgene encoded a methionine at codon 129, and all of the patients were homozygous for methionine at this residue.

From Tg(SHaPrP) mouse studies, prion propagation is thought to involve the formation of a complex between PrP^{Sc} and the homotypic substrate PrP^{C} (Prusiner et al. 1990). Propagation of prions may require the participation of other proteins, such as chaperones, which might be involved in catalyzing the conformational changes that feature in the formation of PrP^{Sc} (Pan et al. 1993). Notably, efficient transmission of HuCJD prions to Tg(HuPrP)/$Prnp^{0/0}$ mice was obtained when the endogenous MoPrP gene was inactivated, suggesting that $MoPrP^{C}$ competes with $HuPrP^{C}$ for binding to a cellular component (Telling et al. 1995). In contrast, the sensitivity of Tg(MHu2M) mice to HuCJD prions was only minimally affected by the expression of $MoPrP^{C}$. One explanation for the difference in susceptibility

of Tg(MHu2M) and Tg(HuPrP) mice to Hu prions in mice may be that mouse chaperones catalyzing the refolding of PrP^{C} into PrP^{Sc} can readily interact with the $MHu2MPrP^{C}/HuPrP^{CJD}$ complex but not with $HuPrP^{C}/HuPrP^{CJD}$. The identification of protein X is an important avenue of research since isolation of this protein or complex of proteins would presumably facilitate studies of PrP^{Sc} formation (Yehiely et al. 1997).

Because the conversion of PrP^{C} into PrP^{Sc} involves a profound conformational change, the participation of one or more molecular chaperones seems likely; certainly, protein X may function as a chaperone. Scrapie-infected cells in culture display marked differences in the induction of heat-shock proteins (Tatzelt et al. 1995, 1996), and Hsp70 mRNA has been reported to increase in scrapie of mice (Kenward et al. 1994). By two-hybrid analysis in yeast, PrP has been shown to interact with Bcl-2 and Hsp60 (Edenhofer et al. 1996; Kurschner and Morgan 1996). Although these studies are suggestive, a molecular chaperone involved in prion formation in mammalian cells has not yet been identified.

SPECTRUM OF HUMAN PRION DISEASES

The human prion diseases present as infectious, genetic, and sporadic disorders (Prusiner 1989). This unprecedented spectrum of disease presentations demanded a new mechanism; prions provide a conceptual framework within which this remarkably diverse spectrum can be accommodated.

Human prion disease should be considered in any patient who develops a progressive subacute or chronic decline in cognitive or motor function. Typically, adults between 40 and 80 years of age are affected (Roos et al. 1973). The young age of more than 30 people who have died of variant (v) CJD in Britain and France has raised the possibility that these individuals were infected with bovine prions that contaminated beef products (Chapter 12) (Chazot et al. 1996; Will et al. 1996; Cousens et al. 1997). More than 90 young adults have also been diagnosed with iatrogenic CJD 4 to 30 years after receiving human growth hormone (HGH) or gonadotropin derived from cadaveric pituitaries (Koch et al. 1985; Public Health Service 1997). The longest incubation periods (20–30 years) are similar to those associated with more recent cases of kuru (Gajdusek et al. 1977; Klitzman et al. 1984).

In most patients with CJD and possibly some with Gerstmann-Sträussler-Schienker disease (GSS) (Masters et al. 1978), mutations of the PrP gene are not found. How prions arise in patients with sporadic forms is unknown; 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.

1991b). Numerous attempts to establish an infectious link between sporadic CJD and a preexisting prion disease in animals or humans have been unrewarding (Malmgren et al. 1979; Cousens et al. 1990).

Heritable Prion Diseases

Most cases of CJD are sporadic, probably the result of somatic mutation of the PrP gene or the spontaneous conversion of PrP^{C} into PrP^{Sc}. The recognition that 10–15% of CJD cases are familial led to the suspicion that genetics plays a role in this disease (Meggendorfer 1930; Masters et al. 1981a). As with sheep scrapie, the relative contributions of genetic and infectious etiologies in the human prion diseases remained puzzling. Twenty different mutations of the PrP gene have been shown to segregate with the heritable human prion diseases (Fig. 3B) (Chapter 13). Five of these mutations have been genetically linked to the inherited human prion diseases (Table 3). Virtually all cases of GSS and FFI appear to be caused by germ-line mutations in the PrP gene. The brains of humans dying of inherited prion disease contain infectious prion particles that have been transmitted to experimental animals.

GSS and Genetic Linkage

The discovery that GSS, which was known to be a familial disease, could be transmitted to apes and monkeys was first reported when many still thought that scrapie, CJD, and related disorders were caused by viruses (Masters et al. 1981a). Only the discovery that a proline (P)→leucine (L) mutation at codon 102 of the human PrP gene was genetically linked to GSS permitted the unprecedented conclusion that prion disease can have both genetic and infectious etiologies (Hsiao et al. 1989; Prusiner 1989). In that study, the codon-102 mutation was linked to development of GSS with a logarithm of the odds (LOD) score exceeding 3, demonstrating a tight association between the altered genotype and the disease phenotype (Fig. 3B). This mutation may be caused by the deamination of a methylated CpG in a germ-line PrP gene, which results in the substitution of a thymine (T) for cytosine (C). This mutation has been found in many families in numerous countries including the original GSS family (Doh-ura et al. 1989; Goldgaber et al. 1989; Kretzschmar et al. 1991).

fCJD Caused by Octarepeat Inserts

An insert of 144 bp containing six octarepeats at codon 53, in addition to the five that are normally present, was described in patients with CJD

from four families residing in southern England (Owen et al. 1989; Poulter et al. 1992). Genealogic investigations have shown that all four families are related, arguing for 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 inserts of two, four, five, six, seven, eight, or nine octarepeats in addition to the normal five are found in individuals with inherited CJD (Fig. 3B) (Owen et al. 1989; Goldfarb et al. 1991b).

fCJD in Libyan Jews

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). Molecular genetic investigations revealed that Libyan and Tunisian Jews with fCJD have a PrP gene point mutation at codon 200 resulting in a glutamic acid→lysine substitution (Fig. 3B) (Goldfarb et al. 1990b; Hsiao et al. 1991b). The E200K mutation has been genetically linked to the mutation with a LOD score exceeding 3 (Gabizon et al. 1993), and the same mutation has also been found in patients from Orava in North Central Slovakia (Goldfarb et al. 1990b), in a cluster of familial cases in Chile (Goldfarb et al. 1991c), and in a large German family living in the United States (Bertoni et al. 1992).

Most patients are heterozygous for the mutation and thus express both mutant and wild-type PrP^{C}. In the brains of patients who die of fCJD(E200K), the mutant PrP^{Sc} is both insoluble and protease-resistant, whereas much of wild-type PrP differs from both PrP^{C} and PrP^{Sc} in that it is insoluble but readily digested by proteases. Whether this form of PrP is an intermediate in the conversion of PrP^{C} into PrP^{Sc} remains to be established (Gabizon et al. 1996).

Penetrance of fCJD

Life table analyses of carriers harboring the codon 200 mutation exhibit complete penetrance (Chapman et al. 1994; Spudich et al. 1995). In other words, if the carriers live long enough, they will all eventually develop prion disease. Some investigators have argued that the inherited prion diseases are not fully penetrant, and thus an environmental factor such as the ubiquitous "scrapie virus" is required for illness to be manifest, but as reviewed above, no viral pathogen has been found in prion disease (Goldfarb et al. 1990a,b).

Fatal Familial Insomnia

Studies of inherited human prion diseases demonstrate that changing a single polymorphic residue at position 129 in addition to the D178N pathogenic mutation alters the clinical and neuropathologic phenotype. The D178N mutation combined with a methionine encoded at position 129 results in a prion disease called fatal familial insomnia (FFI) (Goldfarb et al. 1992; Medori et al. 1992). In this disease, adults generally over age 50 present with a progressive sleep disorder and usually die within about a year (Lugaresi et al. 1986). In their brains, deposition of PrP^{Sc} is confined largely within the anteroventral and the dorsal medial nuclei of the thalamus. The D178N mutation has been linked to the development of FFI with a LOD score exceeding 5 (Petersen et al. 1992). More than 30 families worldwide with FFI have been recorded (Gambetti et al. 1995). In contrast, the same D178N mutation with a valine encoded at position 129 produces fCJD in which the patients present with dementia and widespread deposition of PrP^{Sc} is found postmortem (Goldfarb et al. 1991a). The first family to be recognized with CJD has been found to carry the D178N mutation (Meggendorfer 1930; Kretzschmar et al. 1995).

Human PrP Gene Polymorphisms

At PrP codon 129, an amino acid polymorphism for the methionine→valine has been identified (Fig. 3B) (Owen et al. 1990). This polymorphism appears able to influence prion disease expression not only in inherited forms, but also in iatrogenic and sporadic forms of prion disease (Goldfarb et al. 1992; Palmer et al. 1991; Collinge and Palmer 1997). A second polymorphism resulting in an amino acid substitution at codon 219 (Glu→Lys) has been reported to occur with a frequency of about 12% in the Japanese population but not in Caucasians (Furukawa et al. 1995; Kitamoto and Tateishi 1994). Lysine at 219 appears to protect against CJD by binding to protein X and preventing the conversion of PrP^{C} with a glutamic acid at 219 from being converted into PrP^{C} (K. Kaneko et al. 1997c). A third polymorphism results in an amino acid substitution at codon 171 (Asn→Ser) (Fink et al. 1994), which lies adjacent to the protein X binding site. This polymorphism has been found in Caucasians but it has not been studied extensively and it is not known to influence the binding of PrP^{C} to protein X (Kaneko et al. 1997c; Chapter 9). A fourth polymorphism is the deletion of a single octarepeat (24 bp) that has been found in 2.5% of Caucasians (Laplanche et al. 1990;

Vnencak-Jones and Phillips 1992; Cervenáková et al. 1996). In another study of more than 700 individuals, this single octarepeat was found in 1.0% of the population (Palmer et al. 1993).

Studies of Caucasian patients with sCJD have shown that most are homozygous for methionine or valine at codon 129 (Palmer et al. 1991). This contrasts with the general population, in which frequencies for the codon 129 polymorphism in Caucasians are 12% V/V, 37% M/M, and 51% M/V (Collinge et al. 1991). In contrast, the frequency of the valine allele in the Japanese population is much lower (Doh-ura et al. 1991; Miyazono et al. 1992) and heterozygosity at codon 129 (M/V) is more frequent (18%) in CJD patients than in the general population, where the polymorphism frequencies are 0% V/V, 92% M/M, and 8% M/V (Tateishi and Kitamoto 1993).

Although no specific mutations have been identified in the PrP gene of patients with sporadic CJD (Goldfarb et al. 1990c), homozygosity at codon 129 in sCJD (Palmer et al. 1991) is consistent with the results of Tg mouse studies. The finding that homozygosity at codon 129 predisposes to sCJD supports a model of prion production which favors PrP interactions between homologous proteins, as appears to occur in Tg mice expressing SHaPrP inoculated with either hamster prions or mouse prions (Scott et al. 1989; Prusiner et al. 1990; Prusiner 1991), as well as in Tg mice expressing a chimeric SHa/Mo PrP transgene inoculated with "artificial" prions (Scott et al. 1993).

Variant Creutzfeldt-Jakob Disease

Studies of the prion diseases have taken on new significance with the recent reports of more than 20 cases of an atypical new variant Creutzfeldt-Jakob disease (vCJD) in teenagers and young adults (Bateman et al. 1995; Britton et al. 1995; Chazot et al. 1996; Will et al. 1996; Chapters 12 and 17). To date, all of these cases have been reported from Britain, with the exception of one case from France. It now seems possible that bovine prions passed to humans through the consumption of tainted beef products (Bruce et al. 1997; Hill et al. 1997). How many cases of vCJD caused by bovine prions will occur in the years ahead is unknown (Cousens et al. 1997). Until more time passes, we shall be unable to assess the magnitude of this problem. These tragic cases have generated a continuing discourse concerning mad cows, prions, and the safety of human and animal food supplies throughout the world. Untangling politics and economics from the science of prions seems to have been difficult in disputes between Great Britain and other European countries over the safety of beef and lamb products.

DE NOVO GENERATION OF PRIONS

Perhaps the most widely held view of the initial transmissions of prions from the brains of patients who died of GSS or fCJD to apes and monkeys was that these individuals carried a mutant gene that rendered them susceptible to the "CJD virus" (Masters et al. 1981a). Once mutations in the PrP gene of such patients were discovered (Hsiao et al. 1989), these transmissions could be reinterpreted in terms of the prion concept: Patients carrying a pathogenic mutation of their PrP gene generate PrP^{Sc} and thus prion infectivity de novo (Prusiner 1989). Indeed, the prion concept provided, for the first time, an intellectual framework within which to explain how a single disease process can be manifest as genetic, infectious, and sporadic illnesses.

Introduction of the codon 102 point mutation found in GSS patients into the MoPrP gene resulted in Tg(MoPrP-P101L) mice that developed CNS degeneration indistinguishable from experimental murine scrapie with neuropathology consisting of widespread spongiform morphology, astrocytic gliosis, and PrP amyloid plaques (Hsiao et al. 1994; Telling et al. 1996a). Brain extracts prepared from spontaneously ill Tg(MoPrP-P101L) mice transmitted CNS degeneration to Tg196 mice (Hsiao et al. 1994; Telling et al. 1996a). The Tg196 mice express low levels of the mutant transgene MoPrP-P101L and do not develop spontaneous disease, whereas the Tg(MoPrP-P101L) mice expressing high levels of the mutant transgene product do develop CNS degeneration spontaneously. These studies, as well as transmission of prions from patients who died of GSS to apes and monkeys (Masters et al. 1981a) and to Tg(MHu2M-P102L) mice (Telling et al. 1995), argue persuasively that prions are generated de novo by mutations in PrP. In contrast to most species-specific variations in the PrP sequence, all of the known point mutations in PrP occur either within or adjacent to regions of putative secondary structure in PrP and, as such, appear to destabilize the structure of PrP (Huang et al. 1994; Zhang et al. 1995; Riek et al. 1996).

Why mutations of the PrP gene that produce seemingly unstable PrP^{C} molecules require many decades in humans to be manifest as CNS dysfunction is unknown. In Tg(MoPrP-P101L) mice, the level of expression of the mutant transgene is inversely related to the age of disease onset. In addition, the presence of the wild-type MoPrP gene slows the onset of disease and diminishes the severity of the neuropathological changes.

STRAINS OF PRIONS

Scrapied goats with two different syndromes, one in which the goats became hyperactive and the other in which they became drowsy, raised

the possibility that strains of prions might exist (Pattison and Millson 1961; Chapter 8). Subsequent studies with mice documented the existence of multiple strains in mice through careful measurements of incubation times and the distribution of vacuoles in the CNS (Dickinson et al. 1968; Fraser and Dickinson 1973). Two different groups of prion strains were identified using two strains of mice, C57Bl and VM: One group typified by Me7 prions exhibited short incubation times in C57Bl mice and long ones in VM mice. The other group of strains, including 22A and 87V, showed inverse behavior with respect to the length of the incubation time. Particularly puzzling was the finding that long incubation times were a dominant trait for either group of prion strains as evidenced by studies in F1(C57BL x VM) mice.

Molecular genetic studies later showed genetic linkage of control of incubation times to the PrP gene using NZW and Iln mice (Carlson et al. 1986) that later proved to be analogous in the C57BL and VM mice (Hunter et al. 1987). The PrPs of NZW and Iln mice differ at residues 108 and 189 and were termed PrP-A and PrP-B, respectively (Westaway et al. 1987). By using transgenic mice expressing different levels of PrP^{C}-A and PrP^{C}-B, it was shown that long incubation times in F_1 mice were not a dominant trait but rather were due to a gene dosage effect (Carlson et al. 1994).

Selective Neuronal Targeting

With the development of a new procedure for in situ detection of PrP^{Sc}, designated histoblotting (Taraboulos et al. 1992), it became possible to localize and to quantify PrP^{Sc} as well as to determine whether or not "strains" produce different, reproducible patterns of PrP^{Sc} accumulation. The patterns of PrP^{Sc} accumulation were found to be different for each prion strain when the genotype of the host was held constant (Hecker et al. 1992; DeArmond et al. 1993). This finding was in accord with earlier studies showing that spongiform degeneration is strain-specific (Fraser and Dickinson 1973) since PrP^{Sc} accumulation precedes vacuolation. Because a single prion strain produced many different patterns when inoculated into mice expressing various PrP transgenes, we concluded that the pattern of PrP^{Sc} deposition is a manifestation of the particular strain but not related to its propagation (DeArmond et al. 1997). The results of studies of three prion strains prepared from three brain regions and spleens of inbred mice support this contention (Carp et al. 1997).

Although studies with both mice and Syrian hamsters established that each strain has a specific signature as defined by a specific pattern of PrP^{Sc} accumulation in the brain (Hecker et al. 1992; DeArmond et al. 1993; Carlson et al. 1994), comparisons must be done on an isogenic background (Scott et al. 1993; Hsiao et al. 1994). When a single strain is inoculated into mice expressing different PrP genes, variations in the patterns of PrP^{Sc} accumulation were found to be as great as those seen between two strains (DeArmond et al. 1997). On the basis of the initial studies that were performed in animals of a single genotype, we suggested that PrP^{Sc} synthesis occurs in specific populations of cells for a given distinct prion isolate (Prusiner 1989; Hecker et al. 1992).

Isolation of New Strains

Further evidence implicating PrP in the phenomenon of prion strains comes from studies on the transmission of strains from one species to another. Such studies were especially revealing when mice expressing chimeric SHa/MoPrP transgenes were used. It was shown that new strains pathogenic for Syrian hamsters could be obtained from prion strains which had been previously cloned by limiting dilution in mice (Dickinson et al. 1969; Scott et al. 1997a). Both the generation and propagation of prion strains seem to be results of interactions between PrP^{Sc} and PrP^{C}. Additionally, strains once thought to be distinct that were isolated from different breeds of scrapied sheep were shown to have indistinguishable properties. Such findings argue for the convergence of some strains and raise the issue of the limits of prion diversity.

Although other comparisons of prion strains had not revealed any biochemical or physical differences in PrP^{Sc} (Hecker et al. 1992), a difference in PrP^{Sc} related to strains was found when two prion strains were isolated from mink with transmissible encephalopathy (Bessen and Marsh 1992). One strain (HY) produced hyperactivity in Syrian hamsters and the other (DY) was manifest as a drowsy syndrome like the scrapie strains first seen in goats (Bessen and Marsh 1992). PrP^{Sc} produced by the DY prions showed diminished resistance to proteinase K digestion and truncation of the amino terminus compared to PrP^{Sc} produced by HY and many other strains of prions (Bessen and Marsh 1994), providing evidence for the hypothesis that different strains might represent different conformers of PrP^{Sc} (Prusiner 1991). This altered sensitivity to protease displayed by the DY strain in vivo was demonstrated in vitro when partially denatured, radiolabeled PrP^{C} was bound to PrP^{Sc} (Bessen et al.

1995). Notably, it was not possible to demonstrate the de novo synthesis of infectious prions using this system.

Enciphering Diversity through Protein Conformation

Although the notion that PrP^{Sc} tertiary structure might encrypt the information for each strain is consistent with all of the foregoing experimental data, such a hypothesis was received with little enthusiasm. Only with the transmission of two different inherited human prion diseases to mice expressing chimeric Hu/Mo PrP transgenes has firm evidence emerged supporting this concept. In fatal familial insomnia (FFI), the protease-resistant fragment of PrP^{Sc} after deglycosylation has an M_r of 19 kD, whereas that from other inherited and sporadic prion diseases is 21 kD (Monari et al. 1994; Parchi et al. 1996). Extracts from the brains of FFI patients transmitted disease to mice expressing a chimeric MHu2M PrP gene about 200 days after inoculation and induced formation of the 19-kD PrP^{Sc}, whereas fCJD (E200K) and sCJD produced the 21-kD PrP^{Sc} in these mice (Telling et al. 1996b). These findings argue that PrP^{Sc} acts as a template for the conversion of PrP^{C} into nascent PrP^{Sc}. Imparting the size of the protease-resistant fragment of PrP^{Sc} through conformational templating provides a mechanism for both the generation and propagation of prion strains.

A highly sensitive, conformation-dependent immunoassay (CDI) that discriminated PrP^{Sc} molecules among eight different prion strains propagated in Syrian hamsters was developed (Safar et al. 1998). This immunoassay for PrP^{Sc} does not depend on the protease resistance of PrP^{Sc} to distinguish it from PrP^{C} but instead utilizes antibodies that react with epitopes of PrP that are exposed in PrP^{C} but become buried in PrP^{Sc}. The CDI measures the increase in immunoreactivity that occurs when the cryptic epitopes are exposed by denaturation with GdnHCl. The assay is extremely sensitive because the antibody is labeled with europium (Eu), which can be measured by time-resolved fluorescence (TRF).

In a plot of the ratio of antibody binding to denatured/native PrP, graphed as a function of the concentration of PrP^{Sc}, each strain occupied a unique position, indicative of a particular PrP^{Sc} conformation (Safar et al. 1998). This conclusion was supported by a unique pattern of equilibrium unfolding of PrP^{Sc} found with each strain. When the incubation times of these eight strains were plotted as a function of the concentration of either PrP^{Sc} or PrP 27-30, no relationship could be discerned. Incubation times were also plotted as a function of the ratio of denatured/native PrP and, again, no correlation could be found.

Table 4 Influence of prion species and strain on transmission from Syrian hamsters to hamsters and mice

		Incubation time [days ± S.E.M.] (n/n_0)			
Inoculum	Recipient	Sc237		139H	
SHa→SHa	SHa	77 ± 1	(48/48)	167 ± 1	(94/94)
SHa→SHa	non-Tg mice	>700	(0/9)	499 ± 15	(11/11)
SHa→SHa	Tg(SHaPrP)81 mice	75 ± 2	(22/22)	110 ± 2	(19/19)

The *species* of prion is *encoded* by the primary structure of PrP^{Sc}, and the *strain* of prion is *enciphered* by the tertiary structure of PrP^{Sc}. We recognize that the primary structure, as well as posttranslational chemical modifications, determines the tertiary structure of PrP^{C}, but we argue that the conformation of PrP^{C} is modified by PrP^{Sc} as it is refolded into a nascent molecule of PrP^{Sc} (Telling et al. 1996b).

In contrast to the lack of any correlation with incubation times noted above, an excellent correlation was found when the proteinase-K-sensitive fraction of PrP^{Sc} ([Prp^{Sc}]–[PrP 27-30]) was plotted as a function of the incubation time for all eight prion strains (Safar et al. 1998). The proteinase-K-sensitive fraction of PrP^{Sc} can be considered a surrogate for PrP^{Sc} clearance. Since the binding of PrP^{C} or a metastable intermediate PrP* to protein X seems to be the rate-limiting step in prion replication (Kaneko et al. 1997c), it follows that the different incubation times of various prion strains should arise predominantly from distinct rates of PrP^{Sc} clearance rather than from the different rates of PrP^{Sc} formation. In accord with the excellent correlation between proteinase-K-sensitive PrP^{Sc} and incubation times, prion strains that seem to be readily cleared have prolonged incubation times, whereas those that are poorly cleared display abbreviated incubation periods. However, it is important to recognize that protein K sensitivity is an imperfect model for in vivo clearance and that only one strain with a long incubation time has been studied.

Interplay between the Species and Strains of Prions

The recent advances described above in our understanding of the role of the primary and tertiary structures of PrP in the transmission of disease have given new insights into the pathogenesis of the prion diseases. The amino acid sequence of PrP encodes the species of the prion (Table 4) (Scott et al. 1989; Telling et al. 1995), and the prion derives its PrP^{Sc} sequence from the last mammal in which it was passaged (Scott et al. 1997a). Whereas the primary structure of PrP is likely to be the most

important or even sole determinant of the tertiary structure of PrP^{C}, existing PrP^{Sc} seems to function as a template in determining the tertiary structure of nascent PrP^{Sc} molecules as they are formed from PrP^{C} (Prusiner 1991; Cohen et al. 1994). In turn, prion diversity appears to be enciphered in the conformation of PrP^{Sc}, and prion strains may represent different conformers of PrP^{Sc} (Bessen and Marsh 1994; Telling et al. 1996b; Scott et al. 1997a).

Although the foregoing scenario seems to be unprecedented in biology, considerable experimental data now support these concepts. However, it is not yet known whether multiple conformers of PrP^{C} also exist that serve as precursors for selective conversion into different PrP^{Sc}. In this light, it is useful to consider another phenomenon that is not yet understood: the selective targeting of neuronal populations in the CNS of the host mammal. Recent data suggest that variations in glycosylation of the asparagine-linked sugar chains may influence the rate at which a particular PrP^{C} molecule is converted into PrP^{Sc} (DeArmond et al. 1997). Since asparagine-linked oligosaccharides are known to modify the conformation of some proteins (Otvos et al. 1991; O'Connor and Imperiali 1996), it seemed reasonable to assume that variations in complex type sugars may alter the size of the energy barrier that must be traversed during formation of PrP^{Sc}. If this is the case, then regional variations in oligosaccharide structure in the CNS could account for selective targeting, i.e., formation of PrP^{Sc} in particular areas of the brain. Such a mechanism could also explain the variations in the ratio of the various PrP^{Sc} glycoforms observed by some investigators (Collinge et al. 1996). However, such a mechanism, while accounting for specific patterns of PrP^{Sc} distribution, does not seem to influence to any measurable degree the properties of the resulting PrP^{Sc} molecule. In fact, molecular modeling and NMR structural studies may provide an explanation for such phenomena since the asparagine-linked oligosaccharides appear to be on the face of PrP opposite that where PrP^{C} and PrP^{Sc} are expected to interact during the formation of nascent PrP^{Sc} (Huang et al. 1994, 1995; Zhang et al. 1995; Riek et al. 1996).

PRION DISEASES OF ANIMALS

The prion diseases of animals include scrapie of sheep and goats, bovine spongiform encephalopathy, transmissible mink encephalopathy, chronic wasting disease of mule deer and elk, feline spongiform encephalopathy, and exotic ungulate encephalopathy (Chapters 10 and 11) (Table 1).

Sheep and Cattle PrP Gene Polymorphisms

Parry argued that host genes were responsible for the development of scrapie in sheep. He was convinced that natural scrapie is a genetic disease that could be eradicated by proper breeding protocols (Parry 1962, 1983). 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).

In sheep, polymorphisms at codons 136, 154, and 171 of the PrP gene that produce amino acid substitutions have been studied with respect to the occurrence of scrapie (Fig. 3B) (Goldmann et al. 1990a,b; Laplanche et al. 1993; Clousard et al. 1995). Studies of natural scrapie in the US have shown that ~85% of the afflicted sheep are of the Suffolk breed. Only those Suffolk sheep homozygous for glutamine (Q) at codon 171 developed scrapie, although healthy controls with QQ, QR, and RR genotypes were also found (Hunter et al. 1993, 1997a,b; Goldmann et al. 1994; Westaway et al. 1994b; Belt et al. 1995; Clousard et al. 1995; Ikeda et al. 1995; O'Rourke et al. 1997). These results argue that susceptibility in Suffolk sheep is governed by the PrP codon 171 polymorphism. In Cheviot sheep, the PrP codon 171 polymorphism has a profound influence on susceptibility to scrapie, as in the Suffolk breed, and codon 136 seems to play a less pronounced role (Goldmann et al. 1991a; Hunter et al. 1991).

In contrast to sheep, different breeds of cattle have no specific PrP polymorphisms. The only polymorphism recorded in cattle is a variation in the number of octarepeats: most cattle, like humans, have five octarepeats but some have six (Goldmann et al. 1991b; Prusiner et al. 1993b); however, the presence of six octarepeats does not seem to be overrepresented in BSE (Goldmann et al. 1991b; Prusiner et al. 1993b; Hunter et al. 1994).

Bovine Spongiform Encephalopathy

Prion strains and the species barrier are of paramount importance in understanding the BSE epidemic in Britain, in which it is estimated that almost one million cattle were infected with prions (Anderson et al. 1996; Nathanson et al. 1997). The mean incubation time for BSE is about 5 years. Therefore, most cattle did not manifest disease since they were slaughtered between 2 and 3 years of age (Stekel et al. 1996). Nevertheless, more than 175,000 cattle, primarily dairy cows, have died of BSE over the past decade (Anderson et al. 1996). BSE is a massive common source epidemic caused

by meat and bone meal (MBM) fed primarily to dairy cows (Wilesmith et al. 1991; Nathanson et al. 1997). The MBM was prepared from the offal of sheep, cattle, pigs, and chickens as a high-protein nutritional supplement. In the late 1970s, the hydrocarbon-solvent extraction method used in the rendering of offal began to be abandoned, resulting in MBM with a much higher fat content (Wilesmith et al. 1991). It is now thought that this change in the rendering process allowed scrapie prions from sheep to survive rendering and to be passed into cattle. Alternatively, bovine prions were present at low levels prior to modification of the rendering process and with the processing change survived in sufficient numbers to initiate the BSE epidemic when inoculated back into cattle orally through MBM. Against the latter hypothesis is the widespread geographical distribution throughout England of the initial 17 cases of BSE, which occurred almost simultaneously (Wilesmith 1991; Kimberlin 1996; Nathanson et al. 1997). Furthermore, there is no evidence of a preexisting prion disease of cattle, either in Great Britain or elsewhere.

Origin of BSE Prions?

The origin of the bovine prions causing BSE cannot be determined by examining the amino acid sequence of PrP^{Sc} in cattle with BSE, since the PrP^{Sc} in these animals has the bovine sequence whether the initial prions in MBM came from cattle or sheep. The bovine PrP sequence differs from that of sheep at seven or eight positions (Goldmann et al. 1990a; 1991b; Prusiner et al. 1993b). In contrast to the many PrP polymorphisms found in sheep, only one PrP polymorphism has been found in cattle. Although most bovine PrP alleles encode five octarepeats, some encode six. PrP alleles encoding six octarepeats do not seem to be overrepresented in BSE, as noted above (Fig. 3B) (Hunter et al. 1994).

Brain extracts from BSE cattle cause disease in cattle, sheep, mice, pigs, and mink after intracerebral inoculation (Fraser et al. 1988; Dawson et al. 1990a,b; Bruce et al. 1993, 1997), but prions in brain extracts from sheep with scrapie fed to cattle produced illness substantially different from BSE (Robinson et al. 1995). However, no exhaustive effort has been made to test different strains of sheep prions or to examine the disease following bovine-to-bovine passage. The annual incidence of sheep with scrapie in Britain over the past two decades has remained relatively low (J. Wilesmith, unpubl.). In July 1988, the practice of feeding MBM to sheep and cattle was banned. Recent statistical analysis argues that the epidemic is now disappearing as a result of this ruminant feed ban

(Anderson et al. 1996), reminiscent of the disappearance of kuru in the Fore people of New Guinea (Gajdusek 1977; Alpers 1987).

Monitoring Cattle for BSE Prions

Although many plans have been offered for the culling of older cattle in order to minimize the spread of BSE (Anderson et al. 1996), it seems more important to monitor the frequency of prion disease in cattle as they are slaughtered for human consumption. No reliable, specific test for prion disease in live animals is available, but immunoassays for PrP^{Sc} in the brain stems of cattle might provide a reasonable approach to establishing the incidence of subclinical BSE in cattle entering the human food chain (Hope et al. 1988; Serban et al. 1990; Taraboulos et al. 1992; Prusiner et al. 1993b; Grathwohl et al. 1997; Korth et al. 1997). Determining how early in the incubation period PrP^{Sc} can be detected by immunological methods is now possible, since a reliable bioassay has been created by expressing the BoPrP gene in Tg mice (Scott et al. 1997b). Prior to development of Tg(BoPrP)$Prnp^{0/0}$ mice, non-Tg mice inoculated intracerebrally with BSE brain extracts required more than 300 days to develop disease (Taylor 1991; Fraser et al. 1992; Bruce et al. 1997; Lasmézas et al. 1997). Depending on the titer of the inoculum, the structures of PrP^{C} and PrP^{Sc}, and the structure of protein X, the number of inoculated animals developing disease can vary over a wide range. Some investigators have stated that transmission of BSE to mice is quite variable with incubation periods exceeding one year (Lasmézas et al. 1997), and others report low prion titers in BSE brain homogenates (Taylor 1991; Fraser et al. 1992) compared to rodent brain scrapie (Hunter et al. 1963; Eklund et al. 1967; Kimberlin and Walker 1977; Prusiner et al. 1982b).

Have Bovine Prions Been Transmitted to Humans?

In 1994, the first cases of CJD in teenagers and young adults that were eventually labeled new variant (v) CJD occurred in Great Britain (Will et al. 1996). In addition to the young age of these cases (Bateman et al. 1995; Britton et al. 1995), the brains of these patients showed numerous PrP amyloid plaques surrounded by a halo of intense spongiform degeneration (Ironside 1997). These unusual neuropathologic changes have not been seen in CJD cases in the United States, Australia, or Japan (CDC 1996; Ironside 1997). Both macaque monkeys and marmosets developed

neurologic disease several years after inoculation with bovine prions (Baker et al. 1993), but only the macaques exhibited numerous PrP plaques similar to those found in vCJD (Lasmézas et al. 1996b; R. Ridley and H. Baker, unpubl.).

The restricted geographical occurrence and chronology of vCJD have raised the possibility that BSE prions have been transmitted to humans. That only about 30 vCJD cases have been recorded and the incidence has remained relatively constant make establishing the origin of vCJD difficult. No set of dietary habits distinguishes vCJD patients from apparently healthy people. Moreover, there is no explanation for the predilection of vCJD for teenagers and young adults. Why have older individuals not developed vCJD-based neuropathologic criteria? It is noteworthy that epidemiological studies over the past three decades have failed to find evidence for transmission of sheep prions to humans (Malmgren et al. 1979; Brown et al. 1987; Harries-Jones et al. 1988; Cousens et al. 1990). Attempts to predict the future number of cases of vCJD, assuming exposure to bovine prions prior to the offal ban, have been uninformative because so few cases of vCJD have occurred (Collinge et al. 1995; Cousens et al. 1997; Raymond et al. 1997). Are we at the beginning of a human prion disease epidemic in Britain like those seen for BSE and kuru, or will the number of vCJD cases remain small as seen with iCJD caused by cadaveric HGH (Billette de Villemeur et al. 1996; Public Health Service 1997)?

Strain of BSE Prions

Was a particular conformation of bovine PrP^{Sc} selected for heat-resistance during the rendering process and then reselected multiple times as cattle infected by ingesting prion-contaminated MBM were slaughtered and their offal rendered into more MBM? Recent studies of PrP^{Sc} from brains of patients who died of vCJD show a pattern of PrP glycoforms different from those found for sCJD or iCJD (Collinge et al. 1996; Hill et al. 1997). However, the utility of measuring PrP glycoforms is questionable in trying to relate BSE to vCJD (Parchi et al. 1997; Somerville et al. 1997) because PrP^{Sc} is formed after the protein is glycosylated (Borchelt et al. 1990; Caughey and Raymond 1991) and enzymatic deglycosylation of PrP^{Sc} requires denaturation (Endo et al. 1989; Haraguchi et al. 1989). Alternatively, it may be possible to establish a relationship between the conformations of PrP^{Sc} from cattle with BSE and those from humans with vCJD by using Tg mice, as was done for strains generated in the brains of patients with FFI or fCJD (Telling et al. 1996b; Scott et al. 1997b). A rela-

tionship between vCJD and BSE has been suggested based on the finding of similar incubation times in non-Tg RIII mice of about 310 days after inoculation with Hu or Bo prions (Bruce et al. 1997).

FUNGAL PRIONS

Although prions were originally defined in the context of an infectious pathogen (Prusiner 1982), it is now becoming widely accepted that prions are elements that impart and propagate variability through multiple conformers of a normal cellular protein (Chapter 6). It is likely that such a mechanism would not be restricted to a single class of transmissible pathogens. Indeed, it is probable that this original definition will need to be extended to encompass other situations where a similar mechanism of information transfer occurs. Two notable prion-like determinants, [URE3] and [PSI], have been described in yeast (Wickner 1994), and another prion-like determinant has been reported in other fungi (Deleu et al. 1993; Coustou et al. 1997).

The [URE3] Determinant

In considering what properties a yeast prion-like determinant would possess, Wickner and colleagues (Wickner et al. 1995) have proposed a series of useful criteria: (1) they would behave as non-Mendelian genetic elements, (2) the associated phenotype would be reversible, (3) a maintenance gene encoding the normal protein would manifest as a related, Mendelian genetic element, (4) overproduction of the maintenance gene product would increase the generation of the non-Mendelian element, and (5) defective, interfering replicons would not be evident. Two non-Mendelian genetic determinants that fulfill these criteria, [URE3] and [PSI], were first described over 25 years ago. The ure2 and [URE3] mutations were isolated by their ability to utilize ureidosuccinate in the medium, thereby overcoming a defect in uracil biosynthesis caused by mutations in aspartate transcarbamylase (Ura2p) (Lacroute 1971). Although the behavior of the ure2 mutations was entirely consistent with a normal chromosomal locus, when [URE3] strains were mated with wild-type strains, an irregular segregation pattern was observed (Lacroute 1971; Aigle and Lacroute 1975). Subsequently, it was shown by cytoduction that [URE3] could be transferred in the absence of nuclear fusion, confirming their non-Mendelian nature, and that [URE3] can be cured by growth of cells on rich medium containing 5 mM guanidine HCl (GdnHCl) (Wickner et al. 1995). Significantly, the cured strains could

then be used to generate further [URE3] mutants, arguing strongly against the participation of a nucleic acid genome, and thus satisfying two of the aforementioned criteria expected of a prion-like determinant. Notably, the ure2 mutations are recessive and display the same phenotype as [URE3] mutants, and a series of genetic arguments showed clearly that the URE2 chromosomal gene is necessary for propagation of the [URE3] phenotype (Wickner 1994). However, since the phenotype of ure2 mutants is the same as that observed in the presence of [URE3], it seems most likely that URE2 encodes the normal, active form of the protein. Conversion to the abnormal, inactive form leads to the [URE3] state (Wickner 1994). Following the introduction of the URE2 gene on a high-copy plasmid, an increase of 50- to 100-fold was observed in the frequency with which [URE3] mutants were obtained (Wickner 1994). This is entirely expected for a prion mechanism, since the stochastic event that gives rise to the abnormal conformer will increase in frequency in cells in which the normal "precursor" is overproduced. A similar mechanism may lead to spontaneous prion disease in transgenic mice overexpressing PrP (Westaway et al. 1994c).

The [PSI] Determinant

Another non-Mendelian genetic element in yeast called the [PSI] factor exaggerates the effect of a weak chromosomal ochre suppressor, SUQ5 (Cox 1965). Subsequent studies showed that the action of [PSI] was more general, affecting other weak ochre suppressors (Broach et al. 1981), and that strong ochre suppressors become lethal in the presence of [PSI] (Cox 1965), probably due to an intolerably low frequency of correct translational termination. [PSI] also affects the efficiency of suppression of UGA and UAG codons by the aminoglycoside antibiotics (Palmer et al. 1979).

Many lines of evidence suggest that [PSI] is an abnormal, prion-like conformer of the Sup35 protein (Sup35p) (for reviews, see Lindquist et al. 1995; Wickner et al. 1995; Tuite and Lindquist 1996). Like [URE3], [PSI] can be cured by growth on 5 mM GdnHCl (Tuite et al. 1981), as well as hyperosmotic media (Singh et al. 1979). Other characteristics of [PSI] mirror those of [URE3]: The [PSI] phenotype is the same as that of the omnipotent suppressor mutations sup35 and sup45 (Hawthorne and Mortimer 1968), and overproduction of Sup35p leads to a 100-fold increase in the frequency of occurrence of [PSI] (Chernoff et al. 1993). In addition to these similarities to [URE3], the influence of [PSI] upon protein synthesis in vitro provides further evidence for a prion-like mode of propagation (Tuite et al. 1987). When the efficiency of translational readthrough by extracts of yeast cells in the presence of added suppressor

tRNAs in vitro was assessed, it was found that substantial readthrough occurred only when the extracts were prepared using strains that contained [PSI] (Tuite et al. 1987).

Yeast Prion Domains

[PSI] and [URE3] share another important characteristic. In both cases the "functional" determinants have been mapped to the carboxy-terminal region of the protein, distinct from the "prion" domain, which comprises the amino-terminal 65 and 114 residues of Ure2p (Masison and Wickner 1995) and Sup35p, respectively (Doel et al. 1994; Ter-Avanesyan et al. 1994; Derkatch et al. 1996). Although neither of the prion domains displays any sequence identity to each other or to PrP, the amino-terminal regions of Sup35p and mammalian PrPs both contain short repeated sequence elements: PQGGYQQYN in Sup35p and PHGGGWGQ in PrP (Tuite and Lindquist 1996). Interestingly, when the prion domains of both Sup35 and Ure2p are expressed in *E. coli* and purified, they polymerize spontaneously into amyloid-like fibrils (Glover et al. 1997; King et al. 1997). Although polymerization of the prion domains of both Sup35 and Ure2p is unrelated to [PSI] and [URE3], it may be relevant to the mechanism by which yeast undergo transformation into the [PSI] or [URE3] states.

Dependence of Yeast Prions on Molecular Chaperones

The intrinsic power of the yeast genetic system has provided striking evidence for the involvement of chaperones in the propagation of yeast [PSI] "prions." A genetic screen for factors that suppress the [PSI] phenotype resulted in the isolation of a single suppressor plasmid, which was found to contain the chaperone Hsp104 (Chernoff et al. 1995). Furthermore, propagation of [PSI] was eliminated by either overproduction or absence of Hsp104, and treatment of cells with guanidine or UV light led to induction of Hsp104 (Chernoff et al. 1995; Patino et al. 1996). The significance of Hsp104 is unclear, since there are no published data to indicate that [URE3] utilizes Hsp104; furthermore, overexpression of Sup35 at high levels can induce [PSI] in the absence of Hsp104 (Glover et al. 1997).

Differences between the Yeast and Mammalian Prions

Although the preceding arguments provide an intriguing case for the existence of prion-like elements in yeast, it is essential to state that no bio-

chemical data exist that show that the basis for the yeast prion phenomena is a change in the conformation of a protein. Perhaps most importantly, the putative prion state is proposed to be functionally inert in the case of both [PSI] and [URE3] and produces the same phenotype as inactivation of the maintenance gene. In contrast, prion diseases in mammals cannot be explained simply by the loss of function of PrP, since ablation of the PrP gene had no detectable deleterious effect (Büeler et al. 1992). Furthermore, the existence of distinct prion "strains" described elsewhere in this book argues that PrP^{Sc} may be both conformationally and functionally diverse.

PRION DISEASES ARE DISORDERS OF PROTEIN CONFORMATION

The study of prions has taken several unexpected directions over the past three decades. The discovery that prion diseases in humans are uniquely both genetic and infectious has greatly strengthened and extended the prion concept. To date, 20 different mutations in the human PrP gene all resulting in nonconservative substitutions have been found to either be linked genetically to, or segregate with, the inherited prion diseases (Fig. 3B). Yet, the transmissible prion particle is composed largely, if not entirely, of an abnormal isoform of the prion protein designated PrP^{Sc} (Prusiner 1991).

Understanding how PrP^{C} unfolds and refolds into PrP^{Sc} will be of paramount importance in transferring advances in the prion diseases to studies of other degenerative illnesses. The mechanism by which PrP^{Sc} is formed must involve a templating process where existing PrP^{Sc} directs the refolding of PrP^{C} into a nascent PrP^{Sc} with the same conformation. Undoubtedly, molecular chaperones of some type participate in a process that appears to be associated with caveolae-like domains of the cell. Studies of prion-like proteins in yeast may prove particularly helpful in dissecting some of the events that feature in PrP^{Sc} formation (Wickner 1994).

PREVENTION AND THERAPEUTICS FOR PRION DISEASES

As our understanding of prion propagation increases, it should be possible to design effective therapeutics. Because people at risk for inherited prion diseases can now be identified decades before neurologic dysfunction is evident, the development of an effective therapy for these fully penetrant disorders is imperative (Chapman et al. 1994; Spudich et al. 1995). Although we have no way of predicting the number of individuals who may develop neurologic dysfunction from bovine prions in the future

(Cousens et al. 1997), seeking an effective therapy now seems most prudent. Interfering with the conversion of PrP^{C} into PrP^{Sc} would seem to be the most attractive therapeutic target (Cohen et al. 1994). Reasonable strategies are either stabilizing the structure of PrP^{C} by binding a drug or modifying the action of protein X, which might function as a molecular chaperone (Fig. 5B). Whether it is more efficacious to design a drug that binds to PrP^{C} at the protein X binding site or one that mimics the structure of PrP^{C} with basic polymorphic residues that seem to prevent scrapie and CJD remains to be determined. Since PrP^{Sc} formation seems limited to caveolae-like domains (Gorodinsky and Harris 1995; Taraboulos et al. 1995; Vey et al. 1996; Kaneko et al. 1997a; Naslavsky et al. 1997), drugs designed to inhibit this process need not penetrate the cytosol of cells, but they do need to be able to enter the CNS. Alternatively, drugs that destabilize the structure of PrP^{Sc} might also prove useful.

The production of domestic animals that do not replicate prions may also prove to be a practical way to prevent prion disease. Sheep encoding the R/R polymorphism at position 171 seem resistant to scrapie (Hunter et al. 1993, 1997a,b; Goldmann et al. 1994; Westaway et al. 1994b; Belt et al. 1995; Clousard et al. 1995; Ikeda et al. 1995; O'Rourke et al. 1997); presumably, this was the genetic basis of Parry's scrapie eradication program in Great Britain 30 years ago (Parry 1962,1983). A more effective approach using dominant negatives for producing prion-resistant domestic animals, including sheep and cattle, is probably the expression of PrP transgenes encoding K219 or R171, or possibly both basic residues (Fig. 5B). Such an approach can be readily evaluated in Tg mice and, once shown to be effective, it can be instituted by artificial insemination of sperm from males homozygous for the transgene. Less practical is the production of PrP-deficient cattle and sheep. Although such animals would not be susceptible to prion disease (Büeler et al. 1993; Prusiner et al. 1993a), they might suffer some deleterious effects from ablation of the PrP gene (Collinge et al. 1994; Lledo et al. 1996; Sakaguchi et al. 1996; Tobler et al. 1996).

PRINCIPLES OF PRION BIOLOGY

Many principles of prion replication are clearly unprecedented in biology. As such, it not surprising that some of these principles have not been readily embraced. Although prion replication resembles viral replication superficially, the underlying principles are quite different. For example, in prion replication, the substrate is a host-encoded protein, PrP^{C}, which undergoes modification to form PrP^{Sc}, the only known component of the

infectious prion particle. In contrast, viruses carry a DNA or RNA genome that is copied and directs the synthesis of most, if not all, of the viral proteins. The mature virion consists of a nucleic acid genome surrounded by a protein coat, whereas a prion appears to be composed of a dimer of PrP^{Sc}.

When viruses pass from one species to another, they often replicate without any structural modification, whereas prions undergo a profound change. The prion adopts a new PrP sequence which is encoded by the PrP gene of the current host. That change in amino acid sequence can result in a restriction of transmission for some species while making the new prion permissive for others. In viruses, the different properties exhibited by distinct strains are encoded in the viral genome, whereas in prions, strain-specific properties seem to be enciphered in the conformation of PrP^{Sc}.

Implications for Common Neurodegenerative Diseases

Understanding how PrP^{C} unfolds and refolds into PrP^{Sc} may also open new approaches to deciphering the causes of and to developing effective therapies for some common neurodegenerative diseases including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS). Whether therapies designed to prevent the conversion of PrP^{C} into PrP^{Sc} will be effective in these more common neurodegenerative diseases is unknown. Alternatively, developing a therapy for the prion diseases might provide a blueprint for designing somewhat different drugs for these common disorders. Like the inherited prion diseases, an important subset of Alzheimer's disease and ALS are caused by mutations that result in nonconservative amino acid substitutions in proteins expressed in the CNS.

As the information about prions continues to expand, our understanding of how prions replicate and cause disease will undoubtedly evolve. It is important to add that many of the basic principles of prion biology, as set forth in succeeding chapters, are becoming well understood.

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2

Development of the Prion Concept

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The prion concept developed in the aftermath of many unsuccessful attempts to decipher the nature of the scrapie agent (Prusiner 1998). In some respects, the early development of the prion concept mirrors the story of DNA (Avery et al. 1944; Stanley 1970; McCarty 1985). Prior to the acceptance of DNA as the genetic material (Hershey and Chase 1952; Watson and Crick 1953), many scientists asserted that the DNA preparations were contaminated with protein, the true genetic material (Mirsky and Pollister 1946). For more than half a century, many biologists had thought that genes were made of protein and that proteins were reproduced as replicas of themselves (Haurowitz 1950; Stanley 1935). The prejudices of these scientists were similar in some ways to those of investigators who have disputed the prion concept. However, the scientists who attacked the hypothesis that genes are composed of DNA had no well-proven alternative; they had only a set of feelings derived from poorly substantiated data sets that genes are made of protein. In contrast, those who attacked the hypothesis that the prion is composed only of protein had more than 30 years of cumulative evidence showing that genetic information in all organisms on our planet is encoded in DNA. Studies of viruses and eventually viroids extended this concept to these small infectious pathogens (Diener 1979) and showed that genes could also be composed of RNA (Fraenkel-Conrat and Williams 1955; Gierer and Schramm 1956).

It is with this background that investigators working on scrapie began to unravel the curious and often puzzling properties of this infectious pathogen. The resistance of the scrapie agent to inactivation by formalin and heat treatments (Gordon 1946), which were commonly used to produce vaccines against viral illnesses, was an important clue that the scrapie agent might be different from viruses, but it came at a time before the structure

of viruses was understood. Later, this resistance was dismissed as an interesting observation but of little importance since some viruses could be shown to survive such treatments; indeed, this was not an unreasonable viewpoint. Two decades were to pass before reports of the extreme radioresistance of the scrapie agent to inactivation again trumpeted the puzzling nature of this infectious pathogen (Alper et al. 1966, 1967).

WHAT MIGHT HAVE BEEN: AN ALTERNATIVE PATH OF DISCOVERY

By 1930, the high incidence of familial (f) Creutzfeldt-Jakob disease (CJD) in some families was known (Fig. 1) (Meggendorfer 1930; Stender 1930). Almost 60 years were to pass before the significance of this finding could be appreciated (Masters et al. 1981; Hsiao et al. 1989; Prusiner 1989). CJD remained a curious, rare neurodegenerative disease of unknown etiology throughout this period of three score years (Kirschbaum 1968). Only with transmission of prion disease to apes by the inoculation of brain extracts prepared from patients who died of CJD did the story begin to unravel (Gibbs et al. 1968).

Once CJD was shown to be a transmissible disease, relatively little attention was paid to the familial form of the disease, since most cases were not found in families. It is interesting to speculate how the course of scientific investigation might have proceeded had transmission studies not been performed until after the molecular genetic lesion had been identified. Had the PrP gene been identified in families with prion disease

Figure 1 Pedigree of the Backer family with familial Creutzfeldt-Jakob disease (CJD) (Kirschbaum 1968). Sixty years after the first report that CJD could be a familial disease, the D178N mutation of the PrP gene was shown to be the cause (Kretzschmar et al. 1995). (Reprinted from Kirschbaum 1968.)

before brain extracts were shown to be transmissible, the prion concept, which readily explains how a single disease can have a genetic or infectious etiology, might have been greeted with much less skepticism (Prusiner 1995).

Within the scenario of finding mutations in the PrP gene prior to learning that prion diseases are transmissible, it seems likely that investigators would have focused their efforts on explaining how a mutant gene product might stimulate modification of the wild-type protein after inoculation into a susceptible host (Prusiner 1998). The modified wild-type protein would in turn stimulate production of more of its modified self. Less likely would have been the postulate that a mutant protein unrelated to immune defenses would render the host more susceptible to an infectious pathogen with a foreign genome, such as a virus, bacterium, or fungus.

TRANSMISSION OF SCRAPIE TO MICE

The transmission of the scrapie agent to mice (Chandler 1961) made possible a series of radiobiological studies (Alper et al. 1966, 1967). With reports of the extreme resistance of the scrapie agent to inactivation by UV and ionizing radiation came a flurry of hypotheses to explain these curious observations. In some cases, these postulates ignored the lessons learned from the studies of DNA while others tried to accommodate them in rather obtuse but sometimes clever ways.

As the number of hypotheses about the molecular nature of the scrapie agent began to exceed the number of laboratories working on this problem (Table 1), the need for new experimental approaches became evident. Much of the available data on the properties of the scrapie agent had been gathered using brain homogenates prepared from mice with clinical signs of scrapie. These mice had been inoculated 4–5 months earlier with the scrapie agent, which had originated in sheep but had been passaged multiple times in mice (Chandler 1963; Eklund et al. 1963). Once an experiment was completed on these homogenates, an additional 12 months were required to perform an endpoint titration in mice. Typically, 60 mice were required to determine the titer of a single sample. These slow, tedious, and expensive experiments discouraged systematic investigation.

Sedimentation Studies

Although many studies had been performed on the physicochemical nature of the scrapie agent using the mouse endpoint titration system (Hunter 1972), few systematic investigations had been performed on the

Table 1 Hypothetical structures proposed for the scrapie agent

1.	Sarcosporidia-like parasite
2.	"Filterable" virus
3.	Small DNA virus
4.	Replicating protein
5.	Replicating abnormal polysaccharide with membrane
6.	DNA subvirus controlled by a transmissible linkage substance
7.	Provirus consisting of recessive genes generating RNA particles
8.	Naked nucleic acid similar to plant viroids
9.	Unconventional virus
10.	Aggregated conventional virus with unusual properties
11.	Replicating polysaccharide
12.	Nucleoprotein complex
13.	Nucleic acid surrounded by a polysaccharide coat
14.	Spiroplasma-like organism
15.	Multicomponent system with one component quite small
16.	Membrane-bound DNA
17.	Virino (viroid-like DNA complexed with host proteins)
18.	Filamentous animal virus (SAF)
19.	Aluminum-silicate amyloid complex
20.	Computer virus
21.	Amyloid-inducing virus
22.	Complex of apo- and co-prions (unified theory)
23.	Nemavirus (SAF surrounded by DNA)
24.	Retrovirus

fundamental characteristics of the infectious scrapie particle. In fact, 12 years after introduction of the mouse bioassay, there were no extensive data on the sedimentation behavior of the scrapie particle. Since differential centrifugation is frequently a very useful initial step in the purification of many macromolecules, some knowledge of the sedimentation properties of the scrapie agent under defined conditions seemed mandatory (Prusiner 1978). To perform such studies, Swiss mice were inoculated intracerebrally with the Chandler isolate of scrapie prions, and the mice were sacrificed about 30 and 150 days later when the titers in their spleens and brains, respectively, were at maximum levels (Prusiner et al. 1977, 1978a). The two tissues were homogenized, extracted with detergent, and centrifuged at increasing speeds and for increasing amounts of time. The disappearance of scrapie infectivity was measured in supernatant fractions by endpoint titration, which required one year to score as described above. Thus, a single set of experiments required 18 months to obtain results. This long time frame severely retarded progress.

Probing the Molecular Structure of Prions

The incubation time assay transformed investigations on the development of effective purification schemes for enriching fractions for scrapie infectivity (Prusiner et al. 1980b, 1982b). It provided a quantitative means to assess whether or not those fractions were enriched for scrapie infectivity. Such studies rather rapidly led to the development of a protocol for separating scrapie infectivity from almost all proteins and nucleic acids. With a ~100-fold purification of infectivity relative to protein, >98% of the proteins and polynucleotides were eliminated, which permitted more reliable probing of the constituents of these enriched fractions than was previously possible with crude preparations. As reproducible data accumulated that indicated scrapie infectivity could be reduced by procedures that hydrolyze or modify proteins but was resistant to procedures that alter nucleic acids, a family of hypotheses about the molecular architecture of the scrapie agent began to emerge (Prusiner 1982). These data established for the first time that a particular macromolecule was required for infectivity and that this macromolecule was a protein. These experimental findings also extended the earlier observations on the resistance of scrapie infectivity to UV irradiation at 250 nm: (1) Four procedures based on physical principles independent of UV radiation damage were used to probe for a nucleic acid and (2) demonstration of a protein requirement provided a reference macromolecule. No longer could the scrapie agent be considered phlogiston or linoleum!

Once the requirement for a protein was established, it was possible to revisit the long list of hypothetical structures that had been proposed for the scrapie agent (Table 1) and to eliminate carbohydrates, lipids, and nucleic acids as the infective elements within a scrapie agent devoid of protein (Prusiner 1982). No longer could structures such as a viroid-like nucleic acid, a replicating polysaccharide, or a small polynucleotide surrounded by a carbohydrate be entertained as reasonable candidates to explain the seemingly enigmatic properties of the scrapie agent (Prusiner 1982). However, the family of hypotheses that remained was still large and required a continued consideration of all possibilities in which a protein was a critical element. Thus, the prion concept evolved from this family of hypotheses in which an infectious protein was only one of several possibilities. With the accumulation of experimental data on the molecular properties of the prion, it became possible to discard an increasing number of hypothetical structures. In prion research as well as in many other areas of scientific investigation, a single hypothesis has all too often been championed at the expense of a reasoned approach that involves

continuing to entertain a series of complex arguments until one or more can be discarded on the basis of experimental data (Chamberlin 1890).

RADIOBIOLOGY OF SCRAPIE

The experimental transmission of scrapie from sheep to mice (Chandler 1961) gave investigators a more convenient laboratory model, which yielded considerable information on the nature of the unusual infectious pathogen that causes scrapie (Alper et al. 1966, 1967, 1978; Gibbons and Hunter 1967; Pattison and Jones 1967; Millson et al. 1971). Yet progress was slow because quantification of infectivity in a single sample required holding 60 mice for one year before accurate scoring could be accomplished, as noted above (Chandler 1961).

Resistance to UV Radiation

The extreme resistance of the scrapie agent to both ionizing and UV irradiation suggested this infectious pathogen was quite different from all known viruses. The D_{37} value for irradiation at 254 nm was 42,000 J/m^2, which argued that the target was unlikely to be a nucleic acid. Irradiation at different wavelengths of UV light showed that scrapie infectivity was equally resistant at 250 and 280 nm (Alper et al. 1967). Because proteins in general and aldolase in particular (Setlow and Doyle 1957) are more sensitive to UV irradiation at 280 nm than at 250 nm, Alper and her colleagues concluded that the scrapie agent was unlikely to contain protein (Alper et al. 1967). The sensitivity of proteins to inactivation at 280 nm is usually attributed to the destruction of amino acids with aromatic side chains. Later, the scrapie agent was found to be six times more sensitive to inactivation at 237 nm than at 250 nm or 280 nm (Fig. 2) (Latarjet et al. 1970). This finding served to reinforce the arguments that the scrapie agent contains neither a protein nor a nucleic acid. Although the data were not sufficiently precise to eliminate protein as a candidate, a polysaccharide or polynucleotide composed of numerous modified nucleosides seemed more likely. Ironically, the inactivation spectrum of trypsin, which was published in the same paper with the data on aldolase (Setlow and Doyle 1957), was not recognized to be similar to that of the scrapie agent. The increased sensitivity of trypsin to inactivation by UV irradiation at 237 nm relative to 250 nm and 280 nm is due to the modification of cysteine residues (Setlow and Doyle 1957).

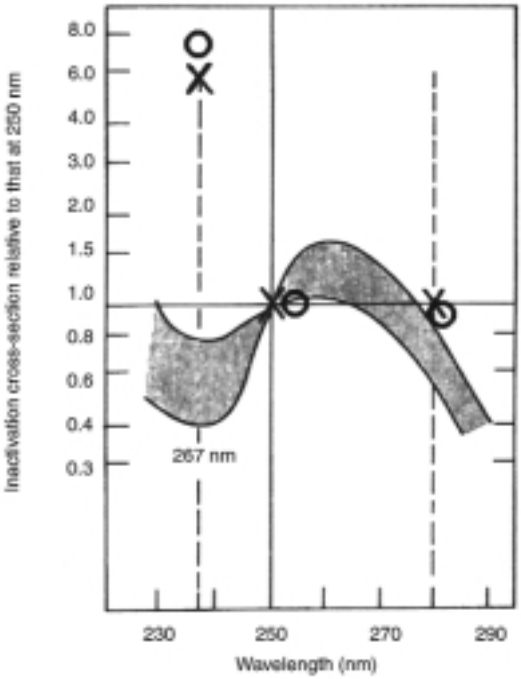

Figure 2 Relative resistance of scrapie prions to inactivation by UV light at three wavelengths. (X) Titer of mouse scrapie prions; (O) enzymatic activity of trypsin. Data adapted from Setlow and Doyle (1957) and Latarjet et al. (1970). Comparison of prions and trypsin performed by R. Setlow (pers. comm.).

Target Size

Inactivation by ionizing radiation gave a target size of about 150,000 D (Alper et al. 1966), which was later revised to 55,000 D (Fig. 3) (Bellinger-Kawahara et al. 1988). The earlier data argued that the scrapie agent was as small as viroids and prompted speculation that a viroid might be the cause of scrapie (Diener 1972). Later, when purified preparations of prions became available, their properties were compared with those of viroids. The properties of prions and viroids were found to be antithetical—consistent with the notion the viroids are composed of polynucleotides and prions are proteins (Table 2) (Diener et al. 1982). Because inactivation by ionizing radiation of viruses had given spurious results due to repair of double-stranded genomes, the size of the putative scrapie virus was thought to be substantially larger than 150,000 D (Rohwer 1984a, 1986). Once a protein was thought to be the most likely target of ionizing radiation, such arguments faded (Bellinger-Kawahara et al. 1988).

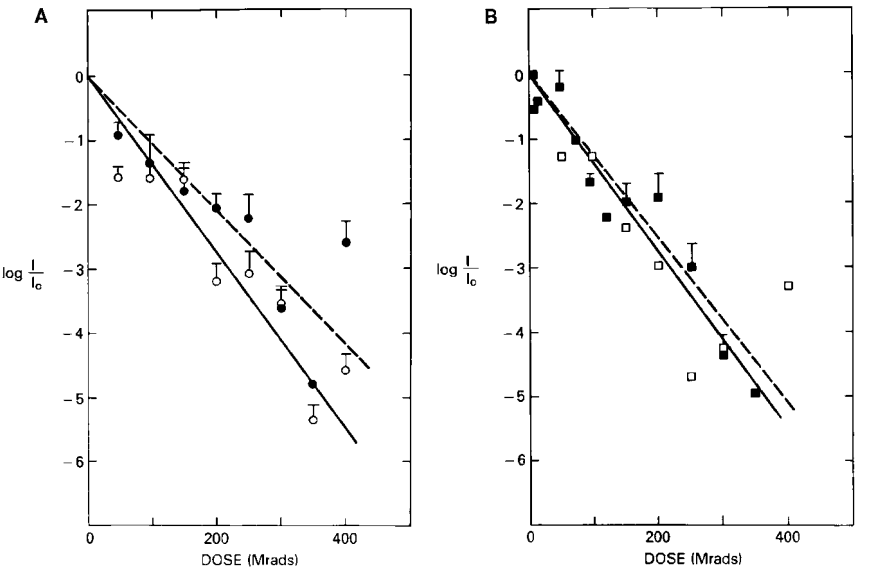

Figure 3 Resistance of scrapie prions to inactivation by ionizing radiation. (*A*) Microsomes (*filled circles*) isolated from scrapie-infected hamster brains were compared to detergent-extracted microsomes (*open circles*). Microsomes were isolated by a series of differential centrifugations as previously described. Two percent sodium dodecyl sarcosinate was added to preparations and incubated for 90 min at room temperature prior to freezing. (*B*) Amyloid rods (*filled squares*) containing PrP 27-30 purified from scrapie-infected hamster brains were dissociated into liposomes (*open squares*). The rods and liposomes were prepared as described previously (Prusiner et al. 1983; Gabizon et al. 1987). All samples were frozen in ethanol dry ice baths prior to storage at –70ºC. The samples were irradiated with 13 MV electrons at –135º. Controls receiving no irradiation were subjected to the same protocol. (Reprinted, with permission, from Bellinger-Kawahara et al. 1988.)

HYPOTHESES ABOUT THE NATURE OF THE SCRAPIE AGENT

A fascinating array of structural hypotheses (Table 1) was offered to explain the unusual features first of the disease and later of the infectious agent; speculation was enhanced by the extreme resistance of the scrapie agent to both ionizing and UV irradiation. Among the earliest hypotheses was the notion that scrapie was a disease of muscle caused by the parasite *Sarcosporidia* (M'Gowan 1914; M'Fadyean 1918). With the successful transmission of scrapie to animals, the hypothesis that scrapie is caused by a "filterable" virus became popular (Cuillé and Chelle 1939; Wilson et al. 1950). With the radiobiological findings of Alper and her colleagues

Table 2 Stabilities of prions and viroids after chemical and enzymatic treatment

Chemical treatment	Concentration	PSTV[a]	Scrapie agent
Et_2PC	10–20 mM	–	+
NH_4OH	0.1–0.5 M	+	–
Psoralen (AMT)	10–500 µg/ml	+	–
Phenol	saturated	–	+
SDS	1–10%	–	+
Zn^{++}	2 mM	+	–
Urea	3–8 mM	–	+
Alkali	pH 10	(–)	+
KSCN	1 M	–	+
RNase A	0.1–100 µg/ml	+	–
DNase	100 µg/ml	–	–
Proteinase K	100 µg/ml	–	+
Trypsin	100 µg/ml	–	+

"+" indicates sensitivity.

[a]Potato spindle tuber viroid.

described above, a myriad of hypotheses on the chemical nature of the scrapie agent emerged. Among the hypothetical structures proposed were a small DNA virus (Kimberlin and Hunter 1967), a replicating protein (Griffith 1967; Pattison and Jones 1967; Lewin 1972, 1981), a replicating abnormal polysaccharide with membranes (Gibbons and Hunter 1967; Hunter et al. 1968), a DNA subvirus controlled by a transmissible linkage substance (Adams and Field 1968; Adams 1970), a provirus consisting of recessive genes generating RNA particles (Parry 1962, 1969), and a naked nucleic acid similar to plant viroids (Diener 1972). As already noted, subsequent investigations showed the viroid suggestion to be incorrect (Diener et al. 1982), and many other studies argue that the scrapie agent is composed only of a protein that adopts an abnormal conformation (Pan et al. 1993; Stahl et al. 1993; Telling et al. 1996a), as previously proposed (Griffith 1967).

Unconventional Viruses and More

The term "unconventional virus" was proposed, but no structural details were ever given with respect to how these unconventional virions differ from the conventional viral particles (Gajdusek 1977). Some investigators have suggested that the term unconventional virus obscured the ignorance that continued to shroud the molecular nature of the infectious pathogens that cause scrapie and CJD (Pattison 1988). Other suggestions included

an aggregated conventional virus with unusual properties (Rohwer and Gajdusek 1980), a replicating polysaccharide (Field 1967), a nucleoprotein complex (Latarjet et al. 1970), a nucleic acid surrounded by a polysaccharide coat (Adams and Caspary 1967; Narang 1974; Siakotos et al. 1979), a spiroplasma-like organism (Bastian 1979; Humphery-Smith et al. 1992), a multicomponent system with one component quite small (Hunter et al. 1973; Somerville et al. 1976), membrane-bound DNA (Marsh et al. 1978), and a virino (viroid-like DNA complexed with host proteins) (Dickinson and Outram 1979). Small spherical particles about 10 nm in diameter were found in fractions said to be enriched for infectivity; these particles were thought to represent the smallest possible virus but were later shown to be ferritin.

BIOASSAYS FOR PRIONS

Experimental transmission of scrapie to mice allowed many more samples to be analyzed than was previously possible with sheep or goats, but the 1- to 2-year intervals between designing experiments and obtaining results discouraged sequential studies in which the results of one set of experiments were used as a foundation to the next. The measurement of scrapie infectivity by titration required 60 animals to evaluate a single sample and one year to establish the endpoint (Chandler 1961). Furthermore, the large number of mice needed to quantify a single sample prevented large experiments in which many studies were performed in parallel. Such problems often encouraged premature publication of experimental data, which forced investigators to defend their flawed studies.

Incubation Time Assays

The identification of an inoculum that produced scrapie in the golden Syrian hamster in about 70 days after intracerebral inoculation was the basis for an important advance (Marsh and Kimberlin 1975; Kimberlin and Walker 1977; Chapters 3 and 4). In earlier studies, Syrian hamsters had been inoculated with prions, but serial passage with short incubation times was not reported (Zlotnik 1963). Using the Syrian hamster, an incubation time assay was developed that accelerated research by nearly a factor of 100 (Prusiner et al. 1980b, 1982b). Development of the incubation time bioassay reduced the time required to measure prions in samples with high titers by a factor of about six: only 70 days were required instead of the 360 days previously needed. Equally important, 4 animals

could be used in place of the 60 that were required for endpoint titrations, making possible a large number of parallel experiments.

Notably, earlier attempts using mice to develop more economical bioassays that related the titer to incubation times in mice were unsuccessful (Eklund et al. 1963; Hunter and Millson 1964). Some investigators used incubation times to characterize different "strains" of scrapie agent while others determined the kinetics of prion replication in rodents (Dickinson and Meikle 1969; Dickinson et al. 1969; Kimberlin and Walker 1978b, 1979), but they refrained from trying to establish quantitative bioassays for prions based on incubation times despite the successful application of such an approach for the measurement of picornaviruses and other viruses three decades earlier (Gard 1940).

PURIFICATION OF SCRAPIE INFECTIVITY

Over two decades, many investigators attempted to purify the scrapie agent but with relatively little success (Hunter et al. 1963, 1969, 1971; Hunter and Millson 1964, 1967; Kimberlin et al. 1971; Millson et al. 1971, 1976; Marsh et al. 1974, 1978, 1980; Siakotos et al. 1976; Gibbs and Gajdusek 1978; Millson and Manning 1979). The slow, cumbersome, and tedious bioassays in sheep and later in mice greatly limited the number of samples that could be analyzed. Little progress was made with sheep and goats because of the very limited numbers of samples and incubation times that exceeded 18 months (Hunter 1972; Pattison 1988).

Hydrophobic Interactions

Although endpoint titrations in mice had revealed some properties of the scrapie agent, development of an effective purification protocol was difficult because the interval between execution of the experiment and the availability of the results was nearly a year (Siakotos et al. 1976; Prusiner et al. 1984; Prusiner 1988). The resistance of scrapie infectivity to nondenaturing detergents, nucleases, proteases, and glycosidases was determined by using endpoint titrations in mice (Hunter and Millson 1964, 1967; Hunter et al. 1969; Millson et al. 1976). Attempts to purify infectivity were complicated by the apparent size and charge heterogeneity of scrapie infectivity, which was interpreted to be a consequence of hydrophobic interactions (Prusiner et al. 1978a,c). Studies on the sedimentation properties of scrapie infectivity in mouse spleens and brains suggested that hydrophobic interactions were responsible for the non-

ideal physical behavior of the scrapie particle (Prusiner et al. 1977, 1978a; Prusiner 1978). Indeed, the scrapie agent presented a biochemical nightmare: Infectivity was spread from one end to the other of a sucrose gradient and from the void volume to fractions eluting at 5–10 times the included volume of chromatographic columns. Such results demanded new approaches and better assays. Only the development of improved bioassays allowed purification of the infectious pathogen that causes scrapie and CJD (Prusiner et al. 1980b, 1982b).

Fractions Enriched for Scrapie Infectivity

The transmission of scrapie to Syrian hamsters by an inoculum previously passaged in rats produced disease in about 70 days (Marsh and Kimberlin 1975). These shorter incubation times coupled with the development of an improved bioassay permitted much more rapid quantification of specimens (Prusiner et al. 1980b, 1982b). This methodological advance made possible the development of protocols for the significant enrichment of scrapie infectivity using a series of detergent extractions, limited digestions with proteases and nucleases, and differential centrifugation (Prusiner et al. 1980b) followed first by agarose gel electrophoresis (Prusiner et al. 1980a) and later by sucrose gradient centrifugation (Prusiner et al. 1982a, 1983).

THE PRION CONCEPT

For many years, the prion diseases were thought to be caused by slow-acting viruses, as described above. These diseases were often referred to as slow virus diseases, transmissible spongiform encephalopathies (TSE), or unconventional viral diseases (Sigurdsson 1954; Gajdusek 1977, 1985). Considerable effort was expended searching for the "scrapie virus"; yet none was found either with respect to the discovery of a virus-like particle or a genome composed of RNA or DNA. This situation posed an interesting conundrum that generated many hypotheses, as noted above.

A Protein Component

Once an effective protocol was developed for preparation of partially purified fractions of scrapie agent from hamster brain, it became possible to demonstrate that those procedures that modify or hydrolyze proteins diminish scrapie infectivity (Table 2) (Prusiner et al. 1981; Prusiner

1982). 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 that reported the extreme resistance of infectivity to UV irradiation (Alper et al. 1967; Latarjet et al. 1970).

On the basis on these findings, it seemed likely that the infectious pathogen of scrapie was neither a virus nor a viroid. For this reason the term "prion" was introduced to distinguish the *pro*teinaceous *in*fectious particles that cause scrapie, CJD, and kuru from both viroids and viruses (Prusiner 1982). Prions were defined as "proteinaceous infectious particles that resist inactivation by procedures that modify nucleic acids." Hypotheses for the structure of the infectious prion particle included: (1) proteins surrounding a nucleic acid encoding them (a virus), (2) proteins associated with a small polynucleotide, and (3) proteins devoid of nucleic acid (Prusiner 1982). Mechanisms postulated for the replication of infectious prion particles ranged from those used by viruses to the synthesis of polypeptides in the absence of a nucleic acid template, to the activation of transcription of cellular genes, and to the posttranslational modifications of cellular proteins. Subsequent discoveries were used to narrow the hypotheses that could explain both the structure of the prion and the mechanism of replication.

A Family of Hypotheses

The prion hypothesis was not meant to champion a single entity as the structural explanation for the scrapie agent, but rather to embody a family of hypotheses (Chamberlin 1890) in which a protein was required for infectivity (Prusiner 1982). Almost immediately upon introduction of the term prion, some investigators redefined prion to signify an infectious particle composed exclusively of protein (Kimberlin 1982). The term "virino" was resuscitated to signify a particle composed of protein and a noncoding small nucleic acid similar to a viroid. Later, a few investigators preferred the term "protein-only hypothesis" to describe one possibility that had been postulated for the prion, an infectious particle composed exclusively of protein (Bolton and Bendheim 1988; Weissmann 1991).

Considerable evidence has accumulated over the past 15 years in support of the prion concept and in particular, the hypothesis that prions are composed entirely of protein (Prusiner 1991, 1997, 1998). Not only is the prion particle without precedent, but so are its mechanism of replication and mode of pathogenesis. A prudent, working definition of a prion is a

"proteinaceous infectious particle that lacks nucleic acid." Perhaps this is an overly cautious and conservative definition, since we now understand much about the mechanism of prion diversity, and a second molecule is not required to explain prion strains (Prusiner 1997, 1998).

Attempts to Falsify the Prion Hypothesis

All attempts to falsify the prion hypothesis over the past 15 years have failed. Experiments to separate scrapie infectivity from protein and more specifically from PrP^{Sc} have been unsuccessful (McKinley et al. 1983a). No preparations of prions containing less than one PrP^{Sc} molecule per ID_{50} unit have been reported and no replication of prions in PrP-deficient ($Prnp^{0/0}$) mice was found, as described below. Other investigators have attempted to identify a scrapie-specific nucleic acid but none has been found (Kellings et al. 1992; Manuelidis et al. 1995; Chapter 15).

Convergence of Data Supporting the Prion Concept

A remarkable convergence of experimental data accumulated over the last two decades convincingly argues that prions are composed of PrP^{Sc} molecules and that, unlike all other infectious pathogens, they are devoid of nucleic acid (Table 3). The copurification of scrapie infectivity and PrP^{Sc} was the first evidence that prions contained this macromolecule (Prusiner et al. 1982a, 1983), and implicit in this finding is that the molecular properties of prions and PrP^{Sc} are very similar if not identical (Bolton et al. 1984). For example, both PrP^{Sc} and prion infectivity exhibited similar degradation kinetics by prolonged proteolysis (McKinley et al. 1983a). A variety of studies were performed attempting to separate scrapie prion infectivity from PrP^{Sc}, but no conditions were identified under which this could be accomplished.

The specificity of PrP^{Sc} for the prion diseases of humans and animals was a critical finding, since it distinguished PrP^{Sc} from a variety of macromolecules that are expressed in response to CNS injury. That a fragment of PrP^{Sc} formed amyloid in vitro (Prusiner et al. 1983), and was later shown to be a major component of amyloid plaques in the brains of animals and humans with prion disease, was still another important line of evidence implicating PrP^{Sc} as the causative agent.

Once the amino-terminal sequence of PrP 27-30 was determined, recovery of cognate cDNAs encoding PrP was possible. With PrP cDNAs, genetic linkage between the PrP gene polymorphisms and the length of the scrapie incubation time was established. Subsequently, mutations in

Table 3 Arguments for prions being composed largely, if not entirely, of PrP^{Sc} molecules and devoid of nucleic acid

1. PrP^{Sc} and scrapie infectivity copurify using biochemical and immunologic procedures.
2. The unusual properties of PrP^{Sc} mimic those of prions. Many different procedures that modify or hydrolyze PrP^{Sc} inactivate prions.
3. Levels of PrP^{Sc} are directly proportional to prion titers. Non-denatured PrP^{Sc} has not been separated from scrapie infectivity.
4. No evidence for a virus-like particle or a nucleic acid genome.
5. Accumulation of PrP^{Sc} invariably associated with the pathology of prion diseases including PrP amyloid plaques that are pathognomonic.
6. PrP gene mutations are genetically linked to inherited prion disease and cause formation of PrP^{Sc}.
7. Overexpression of PrP^{C} increases the rate of PrP^{Sc} formation, which shortens the incubation time. Knockout of the PrP gene eliminates the substrate necessary for PrP^{Sc} formation and prevents both prion disease and prion replication.
8. Species variations in the PrP sequence are responsible, at least in part, for the species barrier that is found when prions are passaged from one host to another.
9. PrP^{Sc} preferentially binds to homologous PrP^{C}, resulting in formation of nascent PrP^{Sc} and prion infectivity.
10. Chimeric and partially deleted PrP genes change susceptibility to prions from different species and support production of artificial prions with novel properties that are not found in nature.
11. Prion diversity is enciphered within the conformation of PrP^{Sc}. Strains can be generated by passage through hosts with different PrP genes. Prion strains are maintained by PrP^{C}/PrP^{Sc} interactions.
12. Human prions from fCJD(E200K) and FFI patients impart different properties to chimeric MHu2M PrP in transgenic mice, which provides a mechanism for strain propagation.

the human PrP gene resulting in nonconservative amino acid substitutions were shown to be genetically linked to inherited prion diseases such as Gerstmann-Sträussler-Scheinker disease (GSS) and fCJD.

The dispersion of purified PrP amyloid rods into liposomes without a loss of scrapie infectivity demonstrated that polymers of PrP 27-30 were not required for infectivity, in agreement with the small radiation target size of the prion. The dispersion of PrP^{Sc} into liposomes permitted immunoaffinity purification of PrP^{Sc} with a concomitant enrichment of prion infectivity by a procedure that was independent of the scheme initially used to purify scrapie infectivity. Anti-PrP antibodies were also

shown to neutralize scrapie infectivity when PrP^{Sc} was dispersed into liposomes.

Mutant PrP transgenes expressed in mice were shown to cause spontaneous neurodegeneration that is indistinguishable from experimental scrapie in mice. In studies on the transmission of prions from one species to another, the species-specific differences in the sequences of PrP were found to be pivotal. Additionally, the length of the incubation time was found to be inversely proportional to the level of PrP^{C} expression. By abolishing PrP expression through genetic ablation, $Prnp^{0/0}$ mice were shown to be resistant to experimental prion disease. When $Prnp^{0/0}$ mice inoculated with prions were sacrificed at various intervals after inoculation, bioassay of their brains revealed no evidence of prion replication.

The demonstration that prion diversity is enciphered in the conformation of PrP^{Sc} has removed the last legitimate argument for a scrapie-specific polynucleotide encased within a virus-like particle. Different strains of prions were generated de novo in patients with PrP gene mutations and propagated to mice expressing a chimeric Hu/Mo PrP transgene. Extracts from the brains of fatal familial insomnia (FFI) and fCJD(E200K) patients transmitted disease to mice expressing a chimeric MHu2M PrP gene about 200 days after inoculation. The FFI inoculum induced formation of a 19-kD PrP^{Sc} as measured by SDS-PAGE after limited proteolysis and removal of asparagine-linked carbohydrates, whereas fCJD(E200K) produced a 21-kD PrP^{Sc} in mice expressing the same transgene (Telling et al. 1996b). On second passage, Tg(MHu2M) mice inoculated with FFI prions showed an incubation time of about 130 days and a 19-kD PrP^{Sc}, while those inoculated with fCJD(E200K) prions exhibited an incubation time of about 170 days and a 21-kD PrP^{Sc} (Prusiner 1997). The experimental data demonstrate that $MHu2MPrP^{Sc}$ can exist in two different conformations based on the sizes of the protease-resistant fragments; yet, the amino acid sequence of $MHu2MPrP^{Sc}$ is invariant. The results of these studies argue that PrP^{Sc} acts as a template for the conversion of PrP^{C} into nascent PrP^{Sc}. Imparting the size of the protease-resistant fragment of PrP^{Sc} through conformational templating provides a mechanism for both the generation and propagation of prion strains.

SEARCH FOR A SCRAPIE-SPECIFIC NUCLEIC ACID

Although the search for a scrapie-specific nucleic acid has been intense, thorough, and comprehensive, it has been unrewarding. The challenge to find a scrapie-specific polynucleotide was initiated by investigators who

found that scrapie agent infectivity is highly resistant to UV and ionizing radiation (Alper et al. 1966, 1967, 1978). Their results prompted speculation that the scrapie pathogen might be devoid of nucleic acid—a postulate initially dismissed by many scientists. Although some investigators have argued that the interpretation of these data was flawed (Rohwer 1984a,b, 1986, 1991), they and others have failed to demonstrate the putative scrapie nucleic acid (Manuelidis and Fritch 1996; Chesebro 1998).

Selective Inactivation Studies

On the basis of the resistance of the scrapie agent to both UV and ionizing radiation, 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. 1978, 1979; Marsh et al. 1978), but the findings could not be confirmed (Prusiner et al. 1980a) and the properties of the scrapie agent were found to be incompatible with those of viroids (Diener et al. 1982). Besides UV irradiation, reagents that specifically modify or damage nucleic acids, such as nucleases, psoralens, hydroxylamine, and Zn^{++} ions, were found not to alter scrapie infectivity in homogenates (Table 2) (Prusiner 1982), microsomal fractions (Prusiner 1982), purified prion rod preparations, or detergent/lipid/protein complexes (McKinley et al. 1983b; Bellinger-Kawahara et al. 1987a,b, 1988; Gabizon et al. 1988b; Neary et al. 1991).

Physical and Molecular Cloning Studies

Attempts to find a scrapie-specific polynucleotide using physical techniques such as polyacrylamide gel electrophoresis were as unsuccessful as molecular cloning approaches. Subtractive hybridization studies identified several cellular genes with increased expression 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 analyzed for a scrapie-specific nucleic acid by use of a specially developed technique designated return refocusing gel electrophoresis, but none was found (Meyer et al. 1991). These studies argue that if such a molecule exists, its size is 80 nucleotides or less; larger nucleic acids were excluded as components essential for infectivity (Kellings et al. 1992; Riesner et al. 1992). Data from UV inactivation studies of scrapie prions argue that if prions contain a scrapie-specific nucleic acid, then a single-stranded molecule cannot exceed 6 bases in length, and a double-stranded mole-

cule can contain up to 40 base pairs (Bellinger-Kawahara et al. 1987a). This larger estimate for a double-stranded polynucleotide accounted for the possibility of repair of UV-damaged molecules after injection into rodents for bioassay. 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" (Kimberlin 1990; Chesebro 1992, 1998; Manuelidis and Fritch 1996). A few argue that the scrapie virus is similar to a retrovirus (Manuelidis and Manuelidis 1989; Sklaviadis et al. 1989, 1990, 1993, 1992; Akowitz et al. 1990; Murdoch et al. 1990), while others argue that the scrapie virus induces amyloid deposition in brain (Diringer 1991, 1992; Braig and Diringer 1985). Others argue that scrapie is caused by a larger pathogen similar to the spiroplasma bacterium (Bastian 1979, 1993). Still others contend that elongated protein polymers covered by DNA are the etiologic agents in scrapie (Narang 1992a,b; Narang et al. 1987a,b, Narang et al. 1988). DNA molecules like the D-loop DNA of mitochondria have also been suggested as the cause of scrapie (Aiken et al. 1989, 1990).

The search for a component other than PrP^{Sc} within the prion particle has focused largely on a nucleic acid because some properties of prions are similar to those of viruses, and a polynucleotide would most readily explain different isolates or "strains" of infectivity (Kimberlin and Walker 1978a; Dickinson and Fraser 1979; Bruce and Dickinson 1987; Dickinson and Outram 1988). Specific scrapie isolates characterized by distinct incubation times retain this property when repeatedly passaged in mice or hamsters (Kimberlin and Walker 1978a; Dickinson and Fraser 1979; Bruce and Dickinson 1987; Dickinson and Outram 1988). Since prion diversity has been shown to be enciphered within the conformation of PrP^{Sc}, the biological argument for a scrapie-specific polynucleotide has diminished (Bessen and Marsh 1994; Telling et al. 1996b; Chapter 8), and its existence seems unlikely.

THE ELUSIVE SCRAPIE "VIRUS" IS A PRION

Many of the foregoing experimental results indicate that if the scrapie "virus" exists, then PrP^{Sc} must be an integral component of this virus (Table 4). Such a scenario demands that PrP^{C} must function as a receptor for this elusive virus; furthermore, pathologic mutations in PrP^{C} that cause inherited prion diseases enhance the binding of the virus. Similarly, knockout of the PrP gene in mice prevents the scrapie virus from binding

Table 4 Arguments to support the contention that the scrapie agent is a virus

1. Virus is tightly bound to PrP^{Sc} or possesses a coat protein that shares antigenic sites and physical properties with PrP^{Sc}, explaining copurification.
2. Structural properties of virus same as PrP^{Sc}; procedures that modify PrP^{Sc} inactivate the virus.
3. Viral genome is protected from procedures modifying nucleic acid by PrP^{Sc}.
4. No nucleic acid >50 nt found in purified preparations: it has unusual properties and might be small, and thus is difficult to detect.
5. Virus hidden by PrP^{Sc} and thus not found.
6. PrP amyloid plaques and spongiform degeneration result from production of PrP^{Sc} induced by the virus.
7. Virus uses PrP^{C} as a receptor; virus has higher affinity for mutant than wild-type receptor.
8. At least two different ubiquitous viruses must exist.
9. Species specificity and artificial prions with new host range demand PrP^{C} or PrP^{Sc} be tightly bound to the virus.
10. PrP^{Sc} is a cofactor for the virus that is necessary for infectivity.

to its receptor PrP^{C}, which is not expressed, thus explaining the resistance of these mice to experimental prion disease.

Furthermore, a scrapie "virus" must mimic PrP^{Sc} in its properties, and since its putative genome does not contain a PrP-like gene, it must acquire PrP from the cell. With each species that the virus invades, it must acquire a new PrP sequence as well as be capable of incorporating artificial PrP molecules formed from the mixing of MoPrP sequences with those of SHa or Hu PrP (Scott et al. 1993; Telling et al. 1994). To circumvent these arguments, it could be hypothesized that the genetic code used by the PrP gene differs so greatly from that found in the cell that a PrP cDNA probe failed to detect it in highly purified preparations. If this were the case, then such a genome would also be expected to encode some specialized proteins required for replication as well as some unique tRNAs required for assembly of the PrP-like protein. However, both UV and ionizing radiation inactivation studies, as well as physical studies, have eliminated the possibility of a large nucleic acid hiding within purified preparations of prions (Bellinger-Kawahara et al. 1987a,b, 1988; Kellings et al. 1992). Additionally, no protein other than PrP^{Sc} (or PrP 27-30) has been found in purified preparations of prions.

Numerous attempts to falsify the prion hypothesis over the past 15 years have failed. Such studies have tried unsuccessfully to separate scrapie infectivity from protein and, more specifically, from PrP^{Sc}. Other

investigations have attempted to identify a scrapie-specific nucleic acid, but none has been found (Manuelidis et al. 1995). Only oligonucleotides of less than 50 bases were found at a concentration of one molecule per ID_{50} unit in prion preparations highly enriched for scrapie infectivity (Kellings et al. 1992, 1994). These small nucleic acids were of variable length and thought to be degradation by-products generated during purification of prions.

The copurification of PrP^{Sc} (or PrP 27-30) and scrapie infectivity demands that the physicochemical properties as well as antigenicity of these two entities be similar (Table 3) (Gabizon et al. 1988a). The results of a wide array of inactivation experiments demonstrated the similarities in the properties of PrP 27-30 and scrapie infectivity (McKinley et al. 1983a; Prusiner et al. 1983, 1993a; Bolton et al. 1984; Riesner et al. 1996). To explain these findings in terms of the virus hypothesis, it is necessary to postulate either a virus that has a coat protein that is highly homologous with PrP or a virus that binds tightly to PrP^{Sc}. In either case, the PrP-like coat protein or the PrP^{Sc}/virus complex must display properties indistinguishable from PrP^{Sc} alone (Table 4). The inability to inactivate preparations highly enriched for scrapie infectivity by procedures that modify nucleic acids was interpreted as evidence against the existence of a scrapie-specific nucleic acid (Alper et al. 1967; Prusiner 1982). To explain the findings in terms of a virus, one must argue that PrP^{Sc} or an as-yet-undetected PrP-like protein of viral origin protects the viral genome from inactivation. The notion that the putative scrapie virus encodes a PrP-like protein was refuted by nucleic acid hybridization studies using a PrP cDNA probe. Less than 0.002 nucleic acid molecules encoding PrP per ID_{50} unit were found in purified preparations of SHa prions (Oesch et al. 1985).

When nucleic acids were repeatedly measured in preparations exhaustively treated with nucleases under conditions where prion infectivity was maintained, only oligonucleotides of 80 bases or less were found in sufficient quantity to serve as a viral genome (Kellings et al. 1992, 1994). Failure to find a bona fide genome is attributed to the unusual properties of the putative viral nucleic acid or technical incompetence on the part of the investigators who were unable to find it (Manuelidis et al. 1995). Similarly, the inability to find virus-like particles in purified preparations of PrP^{Sc} is attributed to these particles being hidden, although tobacco mosaic viruses could be detected when one virion was added per ID_{50} unit of scrapie prions (Gabizon et al. 1987). Prion rods, which are composed largely of PrP 27-30 molecules and sometimes referred to as scrapie-associated fibrils (SAF), were thought by some

investigators to represent the first example of a filamentous animal virus (Merz et al. 1984). Since the rods proved to be an artifact of prion purification and not required for scrapie infectivity (Gabizon et al. 1987; McKinley et al. 1991), this argument faded. However, the rods proved to be indistinguishable from many purified amyloids, which predicted that amyloid plaques in prion diseases would be composed of PrP (Prusiner et al. 1983). The idea that scrapie prions could be composed of amyloid protein was truly heretical when it was introduced. To accommodate the accumulation of PrP^{Sc} in prion diseases, it was hypothesized that the virus induces the formation of PrP amyloid (Diringer 1991).

When PrP gene mutations were discovered to cause familial prion diseases (Hsiao et al. 1989), it was postulated that PrP^{C} is a receptor for the ubiquitous scrapie virus that binds more tightly to mutant than to wild-type PrP^{C} (Kimberlin 1990; Chesebro 1998). A similar hypothesis was proposed to explain why the length of the scrapie incubation time was found to be inversely proportional to the level of PrP expression in Tg mice and why $Prnp^{0/0}$ mice are resistant to scrapie (Chesebro and Caughey 1993): The higher the level of PrP expression, the faster the spread of the putative virus, which results in shorter incubation times; conversely, $Prnp^{0/0}$ mice lack the receptor required for spread of the virus. Recent studies on the transmission of mutant prions from FFI and fCJD(E200K) to Tg(MHu2M) mice, which results in the formation of two different PrP^{Sc} molecules (Telling et al. 1996b), has forced a corollary to the ubiquitous virus postulate. To accommodate this result, at least two different viruses must reside worldwide, each of which binds to a different mutant $HuPrP^{C}$ and each of which induces a different MHu2M PrP^{Sc} conformer when transferred to Tg mice. Alternatively, one ubiquitous virus could acquire different mutant PrP^{Sc} molecules corresponding to FFI or fCJD(E200K) and then induce different MHu2M PrP^{Sc} conformers upon transmission to Tg mice. To explain the species barrier as well as the production of artificial prions from chimeric or mutant PrP transgenes (Scott et al. 1993), PrP^{Sc} molecules must be incorporated into the viruses. In the case of mice expressing chimeric PrP transgenes, artificial prions are produced with host ranges not previously found in nature.

No preparations of purified prions containing less than one PrP^{Sc} molecule per ID_{50} unit have been reported (Akowitz et al. 1994). That PrP^{C} is required for prion replication was surmised from the large body of evidence showing the PrP^{Sc} is a major component of the infectious prion particle (Prusiner 1991, 1992). That view was confirmed by experiments with $Prnp^{0/0}$ mice (Büeler et al. 1992). These mice were found to be resistant to prion disease and not to replicate prions. In two initial stud-

ies, no evidence of prion disease could be found many months after inoculation of $Prnp^{0/0}$ mice with RML (Rocky Mountain Laboratory) prions (Büeler et al. 1993; Prusiner et al. 1993b). In one of these studies, no evidence for prion replication was found in $Prnp^{0/0}$ mice (Prusiner et al. 1993b), but in the other, $Prnp^{0/0}$ mice sacrificed 20 weeks after inoculation were found to have $10^{3.6}$ ID_{50} units/ml of homogenate by bioassay (Büeler et al. 1993). This result could not be confirmed by the authors in a subsequent study (Sailer et al. 1994) but has been ascribed to contamination, residual inoculum, and even as evidence for scrapie infectivity propagation in the absence of PrP. The latter interpretation has been cited as evidence for the mythical "scrapie virus" (Chesebro and Caughey 1993; Manuelidis et al. 1995; Lasmézas et al. 1997). Two additional reports on the resistance of $Prnp^{0/0}$ mice to prion infection have been published (Manson et al. 1994; Sakaguchi et al. 1995).

In summary, no single hypothesis involving a virus can explain the findings summarized above (Table 3); instead, a series of ad hoc hypotheses, many of which can be refuted by available data, must be constructed to accommodate the currently available data (Table 4). Despite so much evidence to the contrary, some investigators persist with the belief that scrapie is caused by a virus (Kimberlin 1990; Rohwer 1991; Xi et al. 1992; Chesebro and Caughey 1993; Akowitz et al. 1994; Özel and Diringer 1994; Manuelidis et al. 1995; Narang 1996; Caughey and Chesebro 1997; Lasmézas et al. 1997). Yet, we are left with only one logical conclusion from this debate: That is, the 50-year quest for the scrapie virus (Gordon 1946) has failed because it does not exist!

MINIPRIONS

Each region of proposed secondary structure in the four-helix bundle model of PrP^{C} (Huang et al. 1994) was systematically deleted, and the mutant constructs were expressed in scrapie-infected neuroblastoma (ScN2a) cells and Tg mice (Muramoto et al. 1996, 1997). Deletion of any of the four putative helical regions prevented PrP^{Sc} formation, whereas deletion of the amino-terminal region containing residues 23–89 did not affect the yield of PrP^{Sc}. In addition to the 67 residues at the amino terminus, 36 residues from position 141 to 176 could be deleted without altering PrP^{Sc} formation. The resulting PrP molecule of 106 amino acids was designated PrP106. In this mutant PrP, helix A as well as the S2 β-strand was removed. When PrP106 was expressed in ScN2a cells, $PrP^{Sc}106$ was soluble in 1% Sarkosyl. Whether the structure of $PrP^{Sc}106$ can be more readily determined than that of full-length PrP^{Sc} remains uncertain.

Transgene-specified Susceptibility

Tg(MHM2PrP106)Prnp$^{0/0}$ mice that expressed PrP106 developed neurological dysfunction about 300 days after inoculation with RML prions previously passaged in CD-1 Swiss mice (Supattapone et al., in press). The resulting prions containing PrP^{Sc}106 produced CNS disease in about 66 days upon subsequent passage in Tg(MHM2PrP106)Prnp$^{0/0}$ mice (Table 5). Besides widespread spongiform degeneration and PrP deposits, the pyramidal cells of the hippocampus comprising the CA-1, CA-2, and CA-3 fields disappeared in Tg(MHM2PrP106)Prnp$^{0/0}$ mice inoculated with prions containing PrP^{Sc}106. In no previous study of Tg mice have we seen similar neuropathologic lesions. The Tg(MoPrP-A) mice overexpressing MoPrP are resistant to RML106 miniprions but are highly susceptible to RML prions. These mice require more than 400 days to produce illness after inoculation with miniprions but develop disease in about 50 days when inoculated with RML prions containing full-length MoPrP^{Sc}.

Smaller Prions and Mythical Viruses

The unique incubation times and neuropathology in Tg mice caused by miniprions are difficult to reconcile with the notion that scrapie is caused by an as-yet-unidentified virus. When the mutant or wild-type PrP^{C} of the host matched PrP^{Sc} in the inoculum, the mice were highly susceptible (Table 5). However, when there was a mismatch between PrP^{C} and PrP^{Sc}, the mice were resistant to the prions. This principle of homologous PrP interactions, which underlies the species barrier, is recapitulated in studies of PrP106 where the amino acid sequence has been drastically changed by deleting nearly 50% of the residues. Indeed, the unique properties of the miniprions provide another persuasive argument that prions are infectious proteins.

PrP^{Sc} IS SPECIFIC FOR PRION DISEASE

With the discovery of PrP 27-30 and production of antiserum (Bendheim et al. 1984), brain tissues from humans and animals with putative prion

Table 5 Susceptibility and resistance of transgenic mice to artificial miniprions

	Incubation time [days ± S.E.M.] (n/n_o)			
Host	RML106 miniprions		RML prions	
Tg(PrP106)Prnp$^{0/0}$ mice	66 ± 3	(10/10)	300 ± 2	(9/10)
Tg(MoPrP-A) mice	>450	(0/11)	50 ± 2	(16/16)

Data from Carlson et al. (1994) and Supattapone et al. (in press).

diseases were examined for the presence of this protein. In each case, PrP 27-30 was found, while it was absent in other neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Bockman et al. 1985, 1987; Manuelidis et al. 1985; Brown et al. 1986). The specificity of PrP^{Sc} for prion disease is an important feature of this protein and is consistent with the postulated role of PrP^{Sc} in both the transmission and pathogenesis of these illnesses (Prusiner 1987).

The accumulation of PrP^{Sc} contrasts markedly with that of glial fibrillary acid protein (GFAP) in prion disease. In scrapie, GFAP mRNA and protein levels rise as the disease progresses (Manuelidis et al. 1987), but this is neither specific nor necessary for either the transmission or pathogenesis of disease (Gomi et al. 1995; Tatzelt et al. 1996).

A protein designated 14-3-3 seems to increase in the cerebrospinal fluid (CSF) of many patients dying of CJD, but this protein is not specific for CJD (Harrington et al. 1986; Hsich et al. 1996). Increased CSF levels of 14-3-3 have been found in the CSF of patients with herpes simplex encephalitis, multi-infarct dementia, and stroke. Rather, 14-3-3 is a stress protein, levels of which increase nonspecifically in CNS injury.

No macromolecule has been found in tissues of patients dying of the prion diseases that is specific for these encephalopathies except PrP^{Sc}. In searches for a scrapie-specific nucleic acid, cDNAs complementary to mRNAs encoding other proteins with increased expression in prion disease have been identified (Duguid et al. 1988, 1989; Diedrich et al. 1993). Yet, none of the proteins have been found to be specific for prion disease.

Copurification of Prion Infectivity and PrP^{Sc}

Data from several studies suggested that scrapie infectivity might depend on protein (Hunter et al. 1969; Millson et al. 1976; Cho 1980, 1983), whereas other studies had demonstrated that infectivity was resistant to protease digestion (Marsh et al. 1978). Only after an effective protocol was developed for enriching fractions approximately 100-fold for scrapie infectivity with respect to cellular protein (Prusiner et al. 1980a,b) could the dependence of scrapie infectivity on protein be established (Prusiner et al. 1981). Studies with partially purified fractions prepared from SHa brain showed loss of infectivity as a function of the concentration of protease and the time of digestion; these results demonstrated that a polypeptide is required for propagation of the infectious scrapie pathogen (Prusiner et al. 1981).

Once the dependence of prion infectivity on protein was clear, the search for a scrapie-specific protein intensified. Although the insolubil-

ity of scrapie infectivity made purification problematic, we took advantage of this property as well as its relative resistance to degradation by proteases to extend the degree of purification (Prusiner et al. 1980b, 1981). In subcellular fractions from hamster brain enriched for scrapie infectivity, a protease-resistant polypeptide of 27–30 kD, later designated PrP 27-30, was identified and which was absent from controls (Bolton et al. 1982; Prusiner et al. 1982a; McKinley et al. 1983a). Radioiodination of partially purified fractions revealed a protein unique to preparations from scrapie-infected brains (Bolton et al. 1982; Prusiner et al. 1982a). The existence of this protein was rapidly confirmed (Diringer et al. 1983).

Prions Contain PrP^{Sc}

There is much evidence to argue that PrP^{Sc} is an essential component of the infectious prion particle (Table 3). Attempts to find a second component of the prion particle have been unsuccessful to date; indeed, many lines of investigation have converged to contend that prions are composed largely, if not entirely, of PrP^{Sc} molecules. Although some investigators think that PrP^{Sc} is merely a pathologic product of scrapie infection and that PrP^{Sc} coincidentally purifies with the "scrapie virus" (Braig and Diringer 1985; Aiken et al. 1989, 1990; Manuelidis and Manuelidis 1989; Sklaviadis et al. 1989, 1990; Akowitz et al. 1990; Murdoch et al. 1990), such views are not supported by the data. No infective fractions containing <1 PrP^{Sc} molecule per ID_{50} unit have been found, which argues that PrP^{Sc} is 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). In another study, the kinetics of PrP^{Sc} and infectivity production in mice inoculated with mouse-passaged CJD prions were similar in brain but were thought to be different in salivary gland (Sakaguchi et al. 1993). The discrepancies between PrP^{Sc} and infectivity levels in the above studies appear to be due to comparisons of infectivity in crude homogenates with PrP^{Sc} concentrations in purified fractions. Other investigators claim to have dissociated scrapie infectivity from PrP 27-30 in brains of Syrian hamsters treated with amphotericin 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 139a prions (Xi et al. 1992). A subsequent study refuted the dissociation of PrP^{Sc} and infectivity in SHa inoculated with 263K prions and treated with amphotericin (McKenzie et al. 1994).

The covalent structure of PrP^{Sc} remains uncertain because purified fractions contain about 10^5 PrP 27-30 molecules per ID_{50} unit, the infectious dose at which 50% of the animals develop scrapie (Bolton et al. 1982; Prusiner et al. 1982a; McKinley et al. 1983a). If <1% of the PrP^{Sc} molecules contained an amino acid substitution or posttranslational modification that conferred scrapie infectivity, our methods would not detect such a change (Stahl et al. 1993).

PrP AMYLOID AND ULTRASTRUCTURE

In preparations highly enriched for scrapie infectivity and containing only PrP 27-30 by silver staining of gels after SDS-PAGE, numerous rod-shaped particles were seen by electron microscopy after negative staining (Fig. 4) (Prusiner et al. 1983). Each of the rods was slightly different, in contrast to viruses which exhibit extremely uniform structures (Williams 1954). These irregular rods, composed largely if not entirely of PrP 27-30, were indistinguishable morphologically from many other purified amyloids (Cohen et al. 1982). Studies of the prion rods with Congo red dye demonstrated that the rods also fulfilled the tinctorial criteria for amyloid (Prusiner et al. 1983), and immunostaining later showed that PrP is a major component of amyloid plaques in some animals and humans with prion disease (DeArmond et al. 1985; Kitamoto et al. 1986; Roberts et al. 1986). Subsequently, it was recognized that the prion rods were not required for scrapie infectivity (Gabizon et al. 1987). Furthermore, the rods were shown to be an artifact of purification during which limited proteolysis of PrP^{Sc} generated PrP 27-30, which polymerized spontaneously in the presence of detergent (Fig. 4) (McKinley et al. 1991).

The idea that scrapie prions were composed of an amyloidogenic protein was truly heretical when it was introduced (Prusiner et al. 1983). Since the prevailing view at the time was that scrapie is caused by an atypical virus, many argued that amyloid proteins are mammalian polypeptides and not viral proteins!

Scrapie-associated Fibrils

In crude extracts prepared from brain tissue of rodents with scrapie, as well as humans with CJD, fibrillar structures composed of two or four helically wound subfilaments were found (Merz et al. 1981, 1983a). The crossing of these subfilaments occurred at specific intervals and the distinctive ultrastructure of these fibers, designated scrapie-associated fibrils

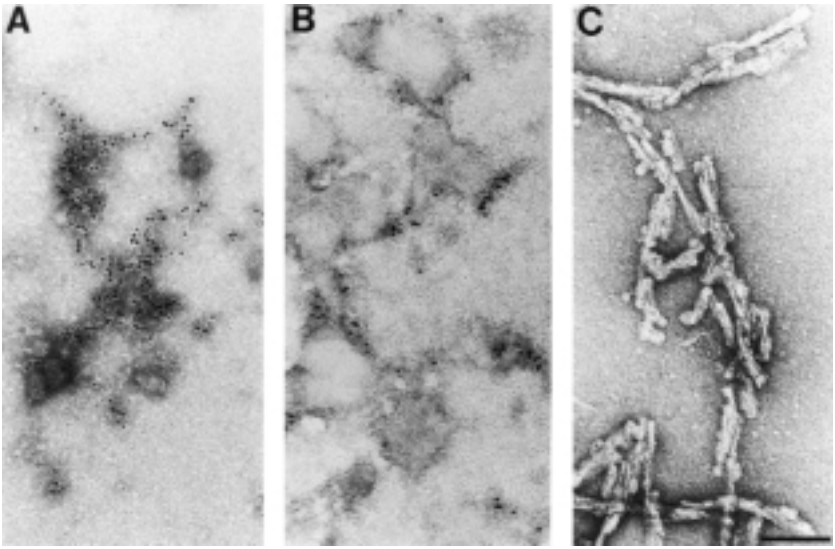

Figure 4 Electron micrographs of negatively stained and immunogold-labeled prion proteins: (*A*) PrP^{C} and (*B*) PrP^{Sc}. Neither PrP^{C} nor PrP^{Sc} forms recognizable, ordered polymers. (*C*) Prion rods composed of PrP 27-30 were negatively stained. The prion rods are indistinguishable from many purified amyloids. Bar, 100 nm. (Reprinted, with permission, from Prusiner 1998 [copyright The Nobel Foundation].)

(SAF), permitted them to be distinguished from both intermediate filaments and amyloids (Merz et al. 1983b). The regular substructure of SAF prompted some investigators to propose that these particles might be the first example of a filamentous animal virus and that this virus causes scrapie (Merz et al. 1984).

Some investigators have argued that SAF are synonymous with prion rods (Diringer et al. 1983; Merz et al. 1987; Somerville et al. 1989; Kimberlin 1990), although morphologic and tinctorial features of these fibrils clearly differentiated them from amyloid and, as such, from the prion rods (Merz et al. 1981, 1983b). After the argument for a filamentous animal virus causing scrapie faded, in order to explain the accumulation of PrP^{Sc} in prion diseases, it was hypothesized that a virus induces the formation of PrP amyloid (Diringer 1991). The term scrapie-associated fibrils has been inappropriately used as a synonym for prion rods which has prompted the conclusion that SAF are composed of PrP (Diener 1987).

Ultrastructural Studies in Search of a Virus

For many years, investigators searched for virus-like particles in brain sections from scrapie-infected sheep and rodents as well as from humans who had died of CJD. Despite the small target size of the infectious pathogen, as determined by inactivation by ionizing radiation (Alper et al. 1966), there were many candidate structures reported (Bouteille et al. 1965; Vernon et al. 1970; Lamar et al. 1974; Jeffrey et al. 1992; Kato et al. 1992). Among these were tubulo-vesicular structures within postsynaptic evaginations that seemed to be composed of arrays of spherical particles (David-Ferreira et al. 1968; Baringer and Prusiner 1978; Baringer et al. 1981; Liberski et al. 1990, 1992). These particles were relatively infrequent and could not be found in the brains of Syrian hamsters with clinical signs of scrapie (Baringer et al. 1983). Other structures such as filamentous particles composed of protein internally and DNA on the exterior have also been reported (Narang 1992a,b,c, 1996; Narang et al. 1987a, 1987b, 1988), but these findings have not been confirmed (Bountiff et al. 1996). In addition to the lack of evidence for a virus of any shape, no compelling data have been offered in support of the idea that prion diseases are caused by a filamentous bacterium called a spiroplasma (Bastian 1993).

AND MORE HYPOTHESES

After the prion concept was introduced and data from many lines of investigation were found to support it, additional hypotheses on the nature of the scrapie agent were proposed, perhaps in part because the properties of the prion were unprecedented. Setting forth the prion concept probably also functioned as a challenge that intensified research on the scrapie pathogen. SAFs that were readily distinguished from amyloid by distinct morphology were proposed to be a filamentous animal virus (Merz et al. 1984). To explain the phenomenon of prion strains, the virino was resuscitated first in the form of a particle containing a foreign, viroid-like nucleic acid surrounded by PrP^{Sc} (Kimberlin 1982, 1990; Dickinson and Outram 1988) and more recently in the form of a small cellular nucleic acid called a coprion that modifies the properties of PrP^{Sc}, labeled the apoprion (Weissmann 1991). No evidence for any polynucleotide that modifies the biological properties of PrP^{Sc} has been forthcoming. Infectious amyloid was proposed as the cause of scrapie, CJD, and kuru; PrP was renamed the scrapie amyloid protein and in its infectious form it was said to be complexed with aluminum-silicate (Gajdusek 1988). These

infectious amyloids were likened to computer viruses on the premise that the term virus can be applied to any infectious entity (Gajdusek 1988), but use of the word virus in this context was as unhelpful as the term unconventional virus noted above. Another proposal is that of the amyloid-inducing virus which is responsible for the conversion of PrP^{C} into PrP^{Sc} (or SAF protein as it is sometimes called). Evidence for the existence of this virus is ~10-nm spherical particles in purified preparations of PrP 27-30. Recent studies show that partially denatured PrP 27-30 can form such spheres that are devoid of infectivity (Riesner et al. 1996). The filamentous animal virus composed of SAF was proposed to have its genome of DNA surround the protein even though the scrapie agent is highly resistant to DNase digestion; this viral-like entity was called a nemavirus. Some investigators continue to speculate that scrapie and CJD are caused by a retrovirus, for which they have presented no credible evidence.

PRESCIENT SPECULATION

Pattison suggested that the scrapie agent might be a basic protein, based on the results of his attempts to purify the infectious pathogen and those of Alper and her colleagues (Alper et al. 1966, 1967; Pattison and Jones 1967). That speculation was amplified by Griffith, who offered three possible mechanisms as to how a protein might mimic an infectious pathogen (Griffith 1967). First, he proposed that a gene G might be silent normally but its expression could be induced by its own product, protein S. If protein S were the scrapie agent, then it would induce the expression of gene G. Spontaneous disease could be explained if gene G were not fully repressed, and scrapie-resistant sheep could be explained by a mutation in gene G. A different strain could be explained by two silent genes G_1 and G_2 with products S_1 and S_2. Second, he proposed that a protein may exist in two stable conformations α and α', where α' is the normal conformation. In this postulate, the dimer α_2 is the scrapie agent, which acts as template for converting α into a conformation that forms another α_2 dimer. Spontaneous disease could be explained in terms of the equilibrium between α and α_2. An alternate strain might be due to different isozymic subunits of the protein denoted β' that can form mixed polymers such as $\alpha_2\beta$. Third, he postulated that an antigen (A) induces antibody A′ and that A and A′ are identical, but the apparent absence of an immunologic component in the disease process made him less enthusiastic about this proposal.

Some aspects of Griffith's first and second proposals were truly predictive. In agreement with his first proposal, we now know that changes in the amino acid sequence of PrP can render humans or animals resistant to prion disease, whereas other mutations can produce inherited forms of the disease that are fully penetrant. In disagreement, the PrP gene is not silent normally; instead, it is constitutively expressed in adults. Several other features of prions are in line with his second proposal in which he suggests a conformational change in subunits of a multimeric protein could explain the infectious process. PrP^{Sc} and PrP^{C} represent α and α' in his nomenclature and the dimer α_2 is the prion; this agrees with the ionizing radiation target analysis, which argues that the infectious prion particle has a molecular weight of 55,000 D, which is probably a dimer of PrP^{Sc}. The notion that PrP^{Sc} acts as a template to direct the formation of nascent PrP^{Sc} also is in agreement with Griffith's second proposal. What is especially interesting about Griffith's second proposal is that he seemed unencumbered by the proposition emerging at the time that the primary structure of a protein dictates a single tertiary structure under physiologic conditions (Anfinsen 1973). That prions violate this unitary relationship between primary and tertiary structure was genuinely unexpected (Pan et al. 1993). Even more surprising has been the finding that PrP^{Sc} can adopt more than one conformation and that this is the explanation for strains of prions (Telling et al. 1996b). In both his first and second proposals, Griffith attempted to explain strains by alternative amino acid sequences and, presumably, he would have invoked a similar explanation for his third proposal.

Were Griffith's proposals of value in deciphering the prion problem? The answer is probably no, but his truly prescient speculations serve to enrich the history of the field. In contrast, Tikvah Alper's radiation inactivation data placed some important constraints on the physical features of the infectious pathogen of scrapie (Alper et al. 1966, 1967). Interestingly, many investigators ignored her findings and a few continue to do so although the results have been confirmed and greatly extended (Bellinger-Kawahara et al. 1987a,b, 1988).

FUNGAL PRIONS

Although prions were originally defined in the context of an infectious pathogen (Prusiner 1982), it is now becoming widely accepted that prions are elements that impart and propagate conformational variability. Such a mechanism must surely not be restricted to a single class of transmissible pathogens. Indeed, it is likely that the original definition will need to be

extended to encompass other examples where a similar mechanism of information transfer occurs.

Two notable prion-like determinants, [URE3] and [PSI], have already been described in yeast and one in another fungus denoted [Het-s*] (Chapter 6) (Wickner 1994; Chernoff et al. 1995; Coustou et al. 1997; Glover et al. 1997; King et al. 1997; Paushkin et al. 1997). Studies of candidate prion proteins in yeast may prove particularly helpful in the dissection of some of the events that feature in PrP^{Sc} formation. Interestingly, different strains of yeast prions have been identified (Derkatch et al. 1996). Conversion to the prion-like [PSI] state in yeast requires the molecular chaperone Hsp104; however, no homolog of Hsp104 has been found in mammals (Chernoff et al. 1995; Patino et al. 1996). The amino-terminal prion domains of Ure2p and Sup35 that are responsible for the [URE3] and [PSI] phenotypes in yeast have been identified. In contrast to PrP, which is a GPI-anchored membrane protein, both Ure2p and Sup35 are cytosolic proteins (Wickner 1997). When the prion domains of these yeast proteins were expressed in *E. coli*, the proteins were found to polymerize into fibrils with properties similar to those of PrP and other amyloids (Glover et al. 1997; King et al. 1997; Paushkin et al. 1997).

Whether prions explain some other examples of acquired inheritance in lower organisms is unclear (Sonneborn 1948; Landman 1991). For example, studies on the inheritance of positional order and cellular handedness on the surface of small organisms have demonstrated the epigenetic nature of these phenomena, but the mechanism remains unclear (Beisson and Sonneborn 1965; Frankel 1990).

LOOKING TO THE FUTURE

Although the study of prions has taken several unexpected directions over the past three decades, a rather novel and fascinating story of prion biology is emerging. Investigations of prions have elucidated a previously unknown mechanism of disease in humans and animals. While learning the details of the structures of PrPs and deciphering the mechanism of PrP^{C} transformation into PrP^{Sc} will be important, the fundamental principles of prion biology have become reasonably clear. Although some investigators prefer to view the composition of the infectious prion particle as unresolved (Aguzzi and Weissmann 1997; Chesebro 1998), such a perspective denies an enlarging body of data, none of which refutes the prion concept (Prusiner 1988). Moreover, the discovery of prion-like phenomena mediated by proteins unrelated to PrP in yeast and other fungi serve not only to strengthen the prion concept, but also to widen it (Wickner 1997).

Hallmark of Prion Diseases

The hallmark of all prion diseases—whether sporadic, dominantly inherited, or acquired by infection—is that they involve the aberrant metabolism and resulting accumulation of the prion protein (Prusiner 1991, 1997, 1998). The conversion of PrP^{C} into PrP^{Sc} involves a conformational change whereby the α-helical content diminishes and the amount of β-sheet increases (Pan et al. 1993). These findings provide a reasonable mechanism to explain the conundrum presented by the three different manifestations of prion disease.

Understanding how PrP^{C} unfolds and refolds into PrP^{Sc} will be of paramount importance in transferring advances in the prion diseases to studies of other degenerative illnesses. The mechanism by which PrP^{Sc} is formed must involve a templating process whereby existing PrP^{Sc} directs the refolding of PrP^{C} into a nascent PrP^{Sc} with the same conformation. Not only will a knowledge of PrP^{Sc} formation help in the rational design of drugs that interrupt the pathogenesis of prion diseases, but it may also open new approaches to deciphering the causes of, and to developing effective therapies for, the more common neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. Indeed, the expanding list of prion diseases and their novel modes of transmission and pathogenesis, as well as the unprecedented mechanisms of prion propagation and information transfer, indicate that much more attention to these fatal disorders of protein conformation is urgently needed.

Prions may have even wider implications than those noted for the common neurodegenerative diseases. If we think of prion diseases as disorders of protein conformation and do not require the diseases to be transmissible, then what we have learned from the study of prions may reach far beyond these common illnesses.

Multiple Conformers

The discovery that proteins may have multiple biologically active conformations may prove no less important than the implications of prions for diseases. How many different tertiary structures can PrP^{Sc} adopt? This query not only addresses the issue of the limits of prion diversity, but also applies to proteins as they normally function within the cell or act to affect homeostasis in multicellular organisms. The expanding list of chaperones that assist the folding and unfolding of proteins promises much new knowledge about this process. For example, it is now clear that pro-

proteases can carry their own chaperone activity where the *pro* portion of the protein functions as a chaperone in *cis* to guide the folding of the proteolytically active portion before it is cleaved (Shinde et al. 1997). Such a mechanism might well feature in the maturation of polypeptide hormones. Interestingly, mutation of the chaperone portion of prosubtilisin resulted in the folding of a subtilisin protease with properties different from the one folded by the wild-type chaperone. Such chaperones have also been shown to work in *trans* (Shinde et al. 1997). In addition to transient metabolic regulation within the cell and hormonal regulation of multicellular organisms, it is not unreasonable to suggest that polymerization of proteins into multimeric structures such as intermediate filaments might be controlled at least in part by alternative conformations of proteins. Such regulation of multimeric protein assemblies might occur either in the proteins that form the polymers or in the proteins that function to facilitate the polymerization process. Additionally, apoptosis during development and throughout adult life might also be regulated, at least in part, by alternative tertiary structures of proteins.

Shifting the Debate

The debate about prions and the diseases that they cause has now shifted to such issues as how many biological processes are controlled by changes in protein conformation (Prusiner 1998). Although the extreme radiation-resistance of the scrapie infectivity suggested that the pathogen causing this disease and related illnesses would be different from viruses, viroids, and bacteria (Alper et al. 1966, 1967), few thought that alternative protein conformations might even remotely feature in the pathogenesis of the prion diseases (Griffith 1967). Indeed, an unprecedented mechanism of disease has been revealed where an aberrant conformational change in a protein is propagated. The discovery of prions and their eventual acceptance by the community of scholars represents a triumph of the scientific process over prejudice. The future of this new and emerging area of biology should prove even more interesting and productive as a multitude of unpredicted discoveries emerge.

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3

Bioassays of Prions

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Prior to the experimental transmission of scrapie to inoculated goats with tissue extracts prepared from scrapied sheep (Cuillé and Chelle 1939), no experimental studies of the disease were possible. Despite the cumbersome bioassays in sheep, some limited studies were possible. For example, the susceptibility of 24 different breeds of sheep was measured after subcutaneous inoculation with brain extract prepared from a scrapied sheep (Gordon 1946). The resistance of the scrapie agent to inactivation by formalin and heat was also shown by using bioassays in sheep (Pattison and Millson 1960); moreover, the first evidence for strains of prions was accumulated with goats that presented with two different clinical syndromes (Pattison and Millson 1961b). One set of goats was described as "drowsy" due to the lethargy manifested during the clinical phase of scrapie, and the other was called "hyper" because these animals were highly irritable and easily aroused.

One of the most distinctive and remarkable features of slow infections is their prolonged incubation periods, during which the host is free of recognizable clinical dysfunction. The onset of clinical illness marks the end of the incubation period and the beginning of a relatively short, progressive course, which ends with death. Although prolonged incubation periods are fascinating phenomena, they have been the biggest impediment to scrapie research. Since animals remain healthy throughout the incubation period, investigators must wait until signs of clinical illness appear before assigning a positive score. In early studies of scrapie using sheep, incubation periods of 1–3 years were required.

Goats seemed to be a better host than sheep. Although the incubation periods were not substantially shorter, goats develop scrapie more consistently than sheep. In Table 1, data from an endpoint titration of the

Table 1 Endpoint titrations in goats

Inoculum[a]	Log dilution	Number[b]	Incubation period (months)
Scratching	1	2/2	11, 12
	2	2/2	9, 22
	3	2/2	11, 12
	4	0/2	
	5	1/3	14
	6	0/3	
	7	1/3	15
	8	1/3	11
Drowsy	1	2/2	8, 8
	2	2/2	10, 11
	3	2/2	9, 11
	4	1/2	22
	5	0/3	
	6	1/3	11
	7	0/3	
	8	0/3	

Data from Pattison (1966).

[a]Inocula prepared from pools of three goat brains. The goats had either the scratching or drowsy forms of scrapie.

[b]Number with scrapie over the number tested.

scrapie agent in goats are recorded (Pattison 1966). As shown, an entire herd of goats was required to quantify the concentration of prions in a single sample.

TRANSMISSION OF SCRAPIE TO RODENTS

An important milestone in the history of prion research was the experimental transmission of scrapie from sheep to mice about 18 months after intracerebral inoculation of brain extracts (Chandler 1961). On second passage, the incubation periods shortened to 4–5 months and remained constant on subsequent passages. The demonstration that scrapie could be transmitted to a small laboratory rodent made possible many new experimental studies that were previously not possible in sheep or goats.

Endpoint Titrations

Studies with rodents ushered in a new era of scrapie research. Although still slow, tedious, and expensive, endpoint titrations of scrapie prions in mice represented an important advance. Typical endpoint titrations in mice require 60 mice and 1 year to determine the titer of a single sample.

Each 10-fold dilution of a titration is generally inoculated into 6 mice; each mouse receives 30 μl inoculated intracerebrally. At about 130 days after inoculation, mice inoculated with the highest dose (a 10-fold dilution of a 10% brain homogenate from a mouse with scrapie) develop clinical signs of neurologic dysfunction. Despite the large number of mice and the extremely long time interval, much was learned about the physicochemical properties of the scrapie agent and the pathogenesis of the illness (Hunter 1972; Kimberlin 1976; Dickinson and Fraser 1977; Hadlow et al. 1979). Yet the year-long intervals between designing experiments and obtaining results discouraged sequential studies. Furthermore, the large number of mice needed to quantify a single sample prevented large experiments in which many studies were performed in parallel.

Syrian Hamsters

The identification of an inoculum that produced scrapie in the golden Syrian hamster (SHa) in about 70 days after intracerebral inoculation was an important advance (Marsh and Kimberlin 1975; Kimberlin and Walker 1977). In earlier studies, Syrian hamsters had been inoculated with prions, but serial passage with short incubation times was not reported (Zlotnik 1963). The relatively short incubation period in the hamster and the high prion titers in its brain made the hamster the preferred animal for biochemical research on the nature of the scrapie agent.

Incubation Time Bioassay

The development of an incubation time bioassay in hamsters reduced the time required to measure prions in samples with high titers by a factor of nearly 6; only 70 days were required instead of the 360 days previously needed. Equally important, 4 animals could be used in place of the 60 that were required for endpoint titrations, which made a large number of parallel experiments possible. However, there were disadvantages with the hamster compared to the mouse (Mo): (1) the lack of a large number of inbred hamster strains, (2) the higher purchase price and more expensive care of the hamster, and (3) the lack of procedures for transfer and ablation of genes in the hamster. No longer does the hamster possess the shortest incubation times; transgenic (Tg) mice have been developed that overexpress Mo or SHa PrP genes and have incubation times of about 40 days (Carlson et al. 1994).

Inbred and congenic strains of mice, in conjunction with molecular genetics, showed that the sequence of the PrP gene in mice can control the

length of the scrapie incubation time (Carlson et al. 1994; Moore et al. 1998). For many years, two types of mice were studied by the Edinburgh group: Most mice had short incubation times when inoculated with the Me7 strain of scrapie agent, but VM mice had long incubation times (Dickinson and Fraser 1977). Subsequently, it was shown that VM mice, like Iln/J mice from which they were derived, have different amino acids at positions 108 and 189 compared to mice with short incubation times such as C57BL mice (Westaway et al. 1987; Carlson et al. 1988).

Transgenic Mice

The expression of foreign and mutant PrP transgenes in mice has created a wealth of knowledge about prions that was previously unattainable. Such studies have elucidated the molecular mechanism of prion formation, begun to define the biochemical and genetic basis of the "species barrier," demonstrated an inverse relationship between the level of PrP^C expression and the incubation time, established the de novo synthesis of prion infectivity from mutant PrP, and revealed the molecular basis of prion strains. Additionally, in PrP-deficient mice neither prion disease nor replication has been found.

Cultured Cells

To date, no cell culture system has been devised for the bioassay of prions. No readily detectable, specific morphologic change in cultured cells infected with prions has been observed. Many reports of chronically infected cells with low levels of prions have been published (Clarke 1979); these low titers have prevented further studies using the cells as a source of prions. In contrast, mouse neuroblastoma cells (N2a) were found to propagate prions derived from sheep with scrapie and humans with Creutzfeldt-Jakob disease (CJD) if they were first passaged through mice (Kingsbury et al. 1984; Race et al. 1987; Butler et al. 1988). More recently, a mouse hypothalamic neuronal cell line (GT-1) immortalized by expression of TAg has been found to support scrapie infection in culture (Schätzl et al. 1997). Rat pheochromocytoma cells (PC12) have been reported to propagate mouse prions (Rubenstein et al. 1984, 1991, 1992), but the biology of this system remains unclear.

RODENT MODELS OF PRION DISEASE

Mice and hamsters are commonly used in experimental studies of prion disease. The shortest incubation times are achieved with intracerebral

inoculation of homologous prions; under these conditions all of the animals develop prion disease within a narrow interval for a particular dose (Fig. 1). The term homologous prions indicates that the PrP gene of the donor in which the prion was previously passaged has the same sequence as that of the recipient. When the PrP sequence of the donor differs from that of the recipient, the incubation time is prolonged, the length of the incubation becomes quite variable, and often, many of the inoculated animals do not develop disease (Carlson et al. 1989; Telling et al. 1994, 1995; Tateishi et al. 1996). This phenomenon is often called the prion "species barrier" (Pattison 1965).

Kinetics of Prion Replication and Incubation Times

When the titer of prions reaches a critical threshold level, the animals develop signs of neurologic dysfunction (Fig. 1). The time from inocula-

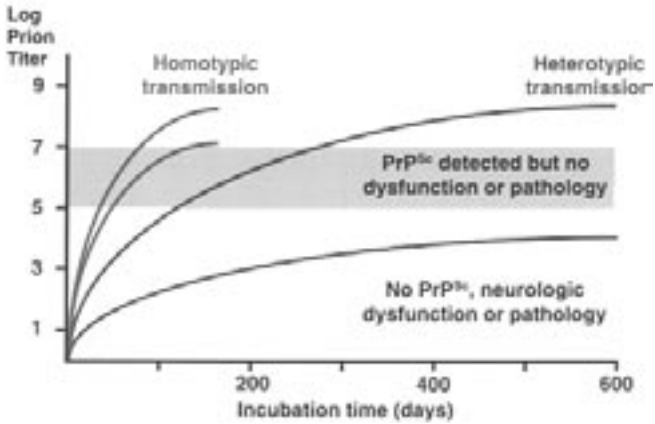

Figure 1 Kinetics of experimental prion infection in rodents. The infection can be divided into three phases: early, preclinical, and clinical. In the early phase, only prion infectivity is detectable, and the titer is generally $<10^5$ ID_{50} units/ml of 10% (w/v) homogenate. In the preclinical phase (*shaded rectangular area*), the titer is typically 10^5–10^7 ID_{50} units/ml, which is sufficiently high for PrP^{Sc} detection by immunoblotting, but few neuropathologic changes can be seen. In the clinical phase, the titer is $>10^7$ ID_{50} units/ml, PrP^{Sc} is readily detected, clinical signs of neurologic dysfunction are obvious, and frank neuropathology is seen. The left-hand set of curves depicts transmission of prions between homologous animals, and the right set of curves is for heterologous transmission, i.e., crossing the "species barrier," where there is much more variation in transmission of disease.

tion to reach this threshold level of prions is generally referred to as the incubation time or incubation period. The length of the incubation time can be modified by (1) the dose of prions, (2) the route of inoculation, (3) the level of PrP^{C} expression, (4) the species of the prion (i.e., the sequence of PrP^{Sc}), and (5) the strain of prion. The higher the dose of prions, the shorter is the time required to reach the threshold level for PrP^{Sc} at which signs of illness appear (Prusiner et al. 1982b). The intracerebral route of inoculation is substantially more efficient than other routes (Prusiner et al. 1985). The higher the level of PrP^{C}, the more rapid is the accumulation of PrP^{Sc} and, hence, the shorter the incubation time to reach the threshold level for PrP^{Sc} at which the animals exhibit clinical signs of CNS dysfunction (Prusiner et al. 1990). When the amino acid sequence of PrP^{Sc} in the inoculum is the same as that of the PrP^{C} expressed in the recipient, the incubation times are generally the most abbreviated (Scott et al. 1989, 1997a). At present, the mechanism by which strains modify the incubation time is not well understood, but interactions between PrP^{Sc} and PrP^{C} undoubtedly play a major role (DeArmond et al. 1997; Scott et al. 1997a).

Endpoint Titrations

An endpoint titration for rodent-adapted scrapie prions is performed by serially diluting a sample at 10-fold increments. Each dilution is typically inoculated intracerebrally into four to six animals, after which the waiting process ensues. Since the highest dilutions at which scrapie develops are the only observations of interest, 10–12 months must pass before the titration may be scored if mice are used. A titer or concentration of the infectious agent in the original sample can be calculated from the score at the highest dilutions at which there is a positive result. Not only are the resources great with respect to animals and time, but accurate measurements are problematic because experiments require pipetting 10 serial 10-fold dilutions.

Mouse-passaged Prions

In a typical endpoint titration for murine-adapted scrapie prions, female Swiss Webster mice are used. Serial 10-fold dilutions of samples are prepared with phosphate-buffered saline. For each dilution, six mice are inoculated intracerebrally with 30 μl of diluted suspension. The inoculations are all made in the left parietal region of the cranium with a 26-

gauge needle inserted to a depth of about 2 mm. After 3 months, the animals are examined three times per week during the next 9 months for clinical signs of scrapie. These signs include bradykinesia, plasticity of the tail, waddling gait, and a coarse, ruffled appearance of the coat. Histologic examination of the brain and spinal cord should be done occasionally to confirm the clinical diagnosis. Generally, the animals die within 4–6 weeks after the onset of scrapie. The disease is rather stereotypic and progressive. Titers are calculated according to the method of Spearman and Kärber (Dougherty 1964). The standard errors of our titrations generally vary between 0.2 and 0.4 log units; 2 S.E. $\approx \pm$ 0.4 to 0.8 log units (~95% confidence limits).

Ovine and Caprine Prions

Measuring scrapie prions in sheep or goat specimens by endpoint titrations in mice is considerably more difficult than for mouse-adapted prions. The adaptation of the scrapie agent from sheep or goats into mice greatly extends the incubation period. When homogenates of sheep or goat brain are diluted 10-fold and Swiss mice are inoculated, incubation periods of 10–12 months are observed. Eighteen to 24 months are needed to score endpoint titrations. Typically, 10–12 mice are used at each dilution, since intercurrent illnesses begin to produce significant mortality at about 18 months of age. Most confusing are chromophobe adenomas of the pituitary, which cause neurologic signs that can be misinterpreted as scrapie. Under these circumstances, histopathologic confirmation is necessary (W.J. Hadlow, pers. comm.). Since many mice die of illnesses other than scrapie during such prolonged experiments, expanding the number of mice inoculated is necessary in order to have at least 6 mice scored at each dilution for reliably determining the endpoint.

Hamster-passaged Prions

Because of the increased cost, limited numbers of endpoint titrations have been performed in hamsters. Five to 6 months are required before an endpoint titration may be scored in hamsters. The principles of the methodology are the same with the following exceptions: (1) 50 µl is inoculated intracerebrally, (2) four or six hamsters are generally used for each dilution, and (3) the clinical signs of scrapie are slightly different as discussed below.

INCUBATION TIME INTERVAL ASSAY

Faced with cumbersome, slow, and expensive endpoint titrations, we questioned whether a new approach might be devised that would decrease the number of animals and the time required for assaying each sample. Initially, we searched for a scrapie-specific alteration in the immune system of mice 20–30 days after inoculation, when the titer of the agent in the spleen reaches a maximum (Garfin et al. 1978a,b). Unable to find a scrapie-specific alteration of sufficient magnitude to correlate with scrapie agent titer, we turned to earlier observations that showed the incubation period or the time interval from inoculation to onset of illness increased as the dose of agent decreased (Eklund et al. 1963; Hunter et al. 1963; Dickinson et al. 1969). We asked whether it would be possible to use the length of the incubation period to measure the titer of the agent in a sample reliably.

Although the length of the incubation period was carefully recorded in early studies on scrapie in sheep and goats, its relationship to the size of the injected dose was unclear (Table 1) (Pattison and Millson 1961a). In contrast, a clear relationship between the length of the incubation period and the dilution of the inoculum was evident in studies by Eklund and coworkers that used mice infected with scrapie (Eklund et al. 1963). The regular increases in incubation periods and in intervals from inoculation to death with increasing dilution prompted these investigators to suggest that the relationship might be used as an assay.

Encouraged by reports on conventional viruses that had been assayed by measuring the time intervals from inoculation to onset of symptoms or to death (Bryan 1957; Luria and Darnell 1967), we determined how precise and predictable this relationship was for the scrapie agent. Our studies clearly demonstrated that measurements of time intervals could be used to predict, with precision equal to or greater than that obtainable by endpoint titration, the titer of the scrapie agent in a sample (Prusiner et al. 1980b, 1982b).

It is of interest that prior to the advent of cell culture assays for viruses, incubation period assays were used successfully to measure encephalomyelitis viruses (Gard 1940), rabbit papillomavirus (Bryan and Beard 1939), avian erythromyeloblastic leukosis (Eckert et al. 1954), Rous sarcoma virus (Bryan 1956), and several viruses of the psittacosis-lymphogranuloma venereum group (Golob 1948; Gogolak 1953; Crocker 1954).

The incubation time interval assay reduced the number of test animals, the time required for bioassay, and potential pipetting errors compared with the endpoint titration (Prusiner et al. 1980b, 1982b). With hamsters, studies on the structure of the scrapie agent were dramatically

accelerated by the development of a bioassay based on measurements of incubation times. This bioassay made it possible to quantify the concentration of prions in a sample with four animals. If the titer of the scrapie agent in the sample was greater than 10^8 ID_{50} units/ml, only about 70 days was required for a positive response.

Nomenclature

In discussions of slow infectious diseases, the incubation period is the time from inoculation to the onset of illness. The term incubation time interval assay was adopted to describe the bioassay procedure. The assay uses two measurements: (1) the time from inoculation to the onset of illness (y) and (2) the time from inoculation to death (z). The difference between z and y is the duration of clinical illness.

The dilution (D) of the sample to be assayed refers to the fractional concentration of prions. Conventional serial 10-fold dilutions were made and the dilution is expressed as $1/10^d$; thus, the $\log_{10}$ D equals –d. We recognize that the dilution process itself has a positive exponent.

It is noteworthy that the median infective dose (ID_{50}) and median lethal dose (LD_{50}) values for a given sample are identical since scrapie is uniformly fatal. The ID_{50} is expressed in units.

Preparation of Inocula

Rodent brains are generally homogenized in 320 mM sucrose, and the homogenate is clarified by centrifugation at 1000*g* for 10 minutes at 4ºC. The supernatant fraction must be diluted at least 3-fold or the inoculum is toxic; generally, we dilute such supernatant fractions 10-fold. For intracerebral inoculations described below, we typically inject 50 µl into female weanling hamsters and 30 µl into weanling mice. For LVG/Lak Syrian hamsters from Charles River Laboratories inoculated with Sc237 prions, the titer of the inoculum used in many of our studies is a median infective dose (ID_{50}) of $10^{9.5}$ units/g of brain tissue, as determined by endpoint titration using the method of Spearman and Kärber (Dougherty 1964). For CD-1 Swiss mice from Charles River Laboratories inoculated with RML prions, the titer is $10^{8.5}$ ID_{50} units/g of brain tissue.

Inoculation of Rodents

The shortest incubation periods are observed after intracerebral inoculation. Peripheral routes of inoculation, such as intraperitoneal, subcuta-

neous, and intravenous, result in longer incubation periods for a given dose (Prusiner et al. 1985; Kimberlin and Walker 1986). Studies on the intracerebral route of inoculation with bacteriophage or India ink have shown that 90% of the inoculum is absorbed systemically (Schlesinger 1949; Cairns 1950). Less than 10% of the inoculum remained in the brain. Presumably, the extreme vascularity of the brain and the pressure during inoculation are responsible for dispersal of most of the inoculum.

Weanling female hamsters or mice of either sex are inoculated intracerebrally with 50 or 30 μl, respectively, of a given sample at a specified dilution using a 26-gauge needle. The inoculations are all made in the left parietal region of the cranium with the needle inserted to a depth of approximately 2 or 3 mm for mice or hamsters, respectively. The diluent used is phosphate-buffered saline. We have used additives, including 0.5 units/ml penicillin, 0.5 μg/ml streptomycin, 2.5 μg/ml amphotericin, and 5% w/v recrystallized fraction V bovine serum albumin (Pentex-Miles Laboratories, Elkhart, Indiana), but they have not been found to affect the outcome of our studies.

Immunohistochemical studies show that prion amyloid filaments accumulate along the needle tract where the inoculum was injected (Bendheim et al. 1984). These amyloid deposits of prion proteins are synthesized de novo since immunoreactive prion polymers were not detected until 60 days after inoculation (DeArmond et al. 1985).

In attempts to increase the deposition of inoculated prions in hamster brains, subarachnoid injections were compared to intraparenchymal parietal lobe injections. No difference in incubation periods was observed for the two different intracerebral routes of inoculation (Table 2). Additional studies using agarose to hold the inoculum in the brain were performed, but with agarose plugs, neither the subarachnoid nor intraparenchymal injections showed any change in the incubation period.

Oral Inoculation

Studies on the oral transmission of prions have gained importance with the bovine spongiform encephalopathy (BSE) epidemic and the possible transmission of bovine prions to humans. Investigations of oral transmission of prions using Syrian hamsters were readily performed since these animals are avid cannibals; they devour their offspring at the least provocation. These natural cannibalistic activities of hamsters were exploited to examine the transmission of scrapie prions among laboratory animals (Prusiner et al. 1985). Weanling hamsters were inoculated intracerebrally with 10^7 ID_{50} units of scrapie prions, and 30–80 days later they were used

Table 2 Comparison of intracranial and subarachnoid inoculations

Route	Inoculum vol. (μl)	(*n*)	Onset of illness (mean days)	Death (mean days)	Agarose[a] (type/%conc.)	Temperature (ºC)
i.c.	50	12	64	81	—	4
s.a.	50	12	63	84	—	4
s.a.	100	12	70	86	—	4
i.c.	50	12	69	89	—	45
s.a.	50	8	73	94	—	45
s.a.	100	8	75	89	—	45
i.c.	50	6	71	87	HGT 0.5%	45
s.a.	50	5	71	90	HGT 0.5%	45
i.c.	50	4	70	88	LGT 0.5%	45
s.a.	50	6	78	92	LGT 0.5%	45
i.c.	50	5	70	90	LGT 0.75%	45
s.a.	50	3	74	89	LGT 0.75%	45

Intracerebral (i.c.) or subarachnoid (s.a.) inoculations were performed with 27-gauge needles.
[a]Agarose solidifying at high temperature (HGT) or low temperature (LGT).

in the experiments described here. In an initial set of experiments designed to reproduce what we had inadvertently observed earlier, scrapie-inoculated and uninoculated hamsters were allowed to cohabit. Those animals inoculated with prions developed a progressive neurological disorder characterized by ataxia, difficulty righting from a supine position, generalized tremor, and head bobbing. The terminal animals were eventually killed by their uninoculated cage mates. In all cases, the heads of the dead hamsters were consumed by their healthy cage mates. Frequently, the genital and abdominal organs were also eaten.

When healthy cannibal hamsters were placed in cages with scrapie-infected hamsters, all hamsters developed scrapie 110–135 days after eating their cage mates. Histopathologic examination of the brains of cannibal hamsters that developed scrapie was performed. Sections of the cerebral hemispheres demonstrated mild but widespread vacuolation in the cerebral cortex and adjacent hippocampus. Vacuoles appeared to be localized to the neuropil and were occasionally seen within neuronal perikarya. The most conspicuous vacuolation was seen in the hippocampus adjacent to the layer of pyramidal cells and in the subiculum. This distribution of vacuolar changes is similar to that seen after intracerebral injection of scrapie agent in the hamster brain. Adjacent to sections taken for histopathology, brain tissue samples were taken from three different cannibals for assay of the scrapie agent. The titers of prions in the three brains were 9.7 ± 0.10, 9.5 ± 0.60, and 9.6 ± 0.13 ID_{50} units/gram of tissue ± S.E. We assume that these titers are representative of the entire

brain, since other studies have shown no regional differences in the titers of scrapie agent in brain after unilateral intracerebral inoculation (Baringer et al. 1983).

To determine whether a dose-dependent relationship for oral transmission of scrapie could be found, scrapie-infected hamsters were sacrificed by cervical dislocation and then placed in the cages with healthy animals. As shown in Table 3, no dose-dependent relationship could be demonstrated when the number of experimental animals in each group was increased. There were no significant differences among the incubation times of the experimental groups, which ranged in their cannibal-to-victim ratios from 1 to 16. Furthermore, there were no differences between male and female cannibals with respect to times of onset of illness and death. For three experimental groups where the ratios of cannibals to victims were 1, 2, and 16, more than 80% of the cannibals developed clinical signs of scrapie at 120–140 days. Likewise, more than 80% of the cannibals died after 130–150 days. In similar experiments with uninoculated, healthy victims as well as victims previously inoculated with normal brain extracts, all the cannibals failed to develop scrapie.

Repeated consumption of scrapie-infected animals was also examined in an attempt to shorten the incubation and death times. In control experiments, healthy animals repeatedly cannibalized dead normal ani-

Table 3 Transmission of prions to hamsters after ingestion of infected hamsters—scrapie-infected hamsters were sacrificed prior to ingestion

				Incubation time intervals	
	Sex	Ratio (cannibals/victims)	(*n*)	onset of illness (days ± S.E.)	death (days ± S.E.)
Scrapie	F	16[a]	80	128 ± 4.2	145 ± 6.6
	F	8	36	127 ± 6.4	147 ± 7.0
	F	4	20	126 ± 6.7	146 ± 6.7
	F	2	80	121 ± 6.6	139 ± 3.9
	F	1	45	124 ± 10.2	144 ± 10.2
	M	16	58	132 ± 6.7	153 ± 5.7
	M	2	85	126 ± 6.7	152 ± 5.8
Normal	F	2[b]	42	>300	
	F	2[c]	41	>300	
	F	—	35	>300	
	M	2[b]	28	>300	

[a]Victims were inoculated with 10^7 ID_{50} units of scrapie prions 70 days prior to sacrifice. At this time, they displayed clinical signs of scrapie.

[b]Victims were uninoculated normal animals sacrificed by cervical dislocation prior to cannibalism.

[c]Victims were inoculated with normal brain extract (0.05 ml) 70 days prior to sacrifice.

mals prior to eating scrapie-infected hamsters once. There was a slight but probably insignificant reduction in the incubation times for animals repeatedly fed scrapie-infected victims compared with those consuming scrapie-infected animals only once. Repeated eating of uninfected controls prior to consuming scrapie-infected animals once gave incubation times comparable to those found with multiple feedings. More than 80% of the cannibals developed clinical signs of scrapie at 105–125 days, and death occurred at 130–150 days. When healthy, uninoculated animals were used as the only victims, the cannibals never developed scrapie.

To investigate the possibility that abrasion of the oral mucosa caused by consumption of tissues such as the calvarium might have influenced oral transmission, healthy hamsters were given pieces of infected brain. This protocol slightly accelerated the onset of disease and death compared with cannibalism of infected animals (Table 4). Again, no dose-dependent relationship was apparent, but the 6-fold range over which this was tested was probably too small to be measurable. More than 80% of the animals that developed clinical signs of scrapie at 110–120 days died at 130–150 days. When brains removed from healthy, uninoculated animals were used, the cannibal hamsters never developed scrapie.

Studies on the oral transmission of scrapie prions to mice showed a much higher rate of transmission after abrasion of the oral mucosa compared to unscarred controls (Carp 1982). In contrast to hamsters, most of the control mice did not develop signs of scrapie after oral exposure.

The comparative efficiencies of oral and intraperitoneal routes of infection compared to intracerebral inoculation are shown in Table 5. Cannibalism or oral ingestion routes are 10^9 times less efficient than intracerebral inoculation. Intraperitoneal injections are 10^5 times less effi-

Table 4 Transmission of prions by oral consumption of scrapie-infected brain tissue

	Brain tissue consumed (grams)	(n)	Incubation time intervals: onset of illness (days ± S.E.)	Incubation time intervals: death (days ± S.E.)
Scrapie	1/6[a]	40	119 ± 17.5	140 ± 8.8
		36	114 ± 3.3	138 ± 6.0
	1[a]	38	113 ± 0.5	133 ± 4.8
		40	118 ± 4.5	138 ± 6.5
Normal	1/6[b]	18/40	>370	
	1[c]	13/40	>370	

[a]Brain tissue was from scrapie-infected hamsters that were sacrificed 70 days after intracerebral inoculation with 10^7 ID_{50} units. At this time, the animals had clinical signs of scrapie.

[b]Brain tissue was from healthy hamsters that were sacrificed.

Table 5 Comparative efficiencies of scrapie prion inoculation routes in Syrian hamsters

Peripheral route of inoculation	Inocula dose (log ID_{50} units)	Incubation time intervals: onset of illness (mean days)	Incubation time intervals: death (mean days)	Equivalent intracerebral dose (log ID_{50} units)	Comparative efficiency of inoculation route
Cannibalism	9.5	120	147	0.4	$10^{-9.1}$
Intraperitoneal	8.3	92	112	2.9	$10^{-5.4}$

cient than inoculation into the brain. These findings, coupled with differences in the human PrP^{Sc} amino acid sequence in the inoculum (Goldfarb et al. 1990, 1994) and the PrP^{C} sequence of the recipient apes and monkeys (Schätzl et al. 1995), may explain the inability of investigators to transmit kuru prions orally to these animals initially (Gibbs et al. 1980).

Care of Rodents

Long-term holding of rodents is mandatory for most studies of experimental scrapie and CJD. First passage of scrapie prions from sheep to mice (W.J. Hadlow, unpubl.) or CJD prions from human to mice (Tateishi et al. 1983) may take 2 years or more. Typically, 10–12 mice were inoculated for scrapie sheep and goat studies at each dilution in order to be able to score endpoint titrations at the end of 1.5–2 years (Hadlow et al. 1974, 1982). About 50% of the mice would die of illness other than scrapie prior to developing signs of neurologic dysfunction.

In our experience with hamsters, holding them is easily accomplished for 100–200 days. Sc237 scrapie prions inoculated intracerebrally require less than 150 days for bioassay. In contrast, long-term experiments extending beyond 400 days present some difficulties. By 1 year, about 40% of the hamsters will have died. The attrition is slow and begins to be significant at about 6 months (Fig. 2). The animals generally die of traumatic injuries, because hamsters readily fight even when housed together since weaning. In all our studies, males and females were held in separate cages. No difference in survival between males and females was noted, although the males seemed more aggressive. The hamsters are housed in polyurethane cages 20 cm high, 20 cm wide, and 42 cm deep and covered with a tight-fitting wire top that holds the food. Four animals are housed per cage. Water is supplied by water bottles, and Purina rat chow is provided ad libitum. A generous supply of pine wood shavings is placed on the bottom of each cage and is changed two times per week. All person-

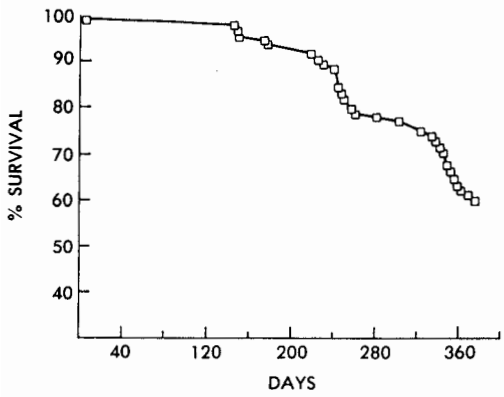

Figure 2 Survival of uninoculated Syrian hamsters. (Reprinted, with permission, from Prusiner 1987.)

nel entering the room are required to wear masks, gowns, rubber boots, gloves, head covers, and eye protection.

Clinical Signs of Experimental Prion Disease

The most reliable clinical signs of prion disease in rodents consist of (1) limb and truncal ataxia, (2) rigidity of the tail, (3) forelimb flexion instead of extension when suspended by the tail, and (4) difficulty righting from a supine position. The diagnosis of probable prion disease requires progression of at least two of these signs over a period of a few days, although slow progression over 10–20 days is more reliable. In some Tg mice the clinical phase of disease is very rapid and lasts for only one day or less; in such cases, histopathologic examination is necessary in a portion of the animals. In mice, we routinely look for limb and truncal ataxia, rigidity of the tail, and forelimb flexion instead of extension when suspended by the tail. In hamsters, we routinely look for limb and truncal ataxia, forelimb flexion instead of extension when suspended by the tail, and difficulty righting from a supine position. Spongiform degeneration and reactive astrocytic gliosis are required to diagnose prion disease. Nonobligatory neuropathologic features include PrP amyloid plaques and protease-resistant PrP^{Sc} on histoblotting.

Besides limb and truncal ataxia, rigidity of the tail, forelimb flexion instead of extension when suspended by the tail, and difficulty righting from a supine position, generalized tremors and head bobbing are frequently seen. The bobbing movements of the head are progressive and

may result from visual difficulties due to degenerative changes in the retina (Hogan et al. 1981). Between 1% and 10% of rodents show generalized convulsions at this early stage of the illness. With further deterioration, the ataxia becomes so pronounced that balance is maintained with considerable difficulty. Kyphotic posture, bradykinesia, and weight loss appear 7–15 days after the onset of illness. Over the next week the rodents become unable to maintain an erect posture; they lie quietly on their sides and exhibit frantic movements of the extremities when disturbed. Death follows in 3–5 days.

Calibration Curves

The development of an incubation time bioassay for prions, in which incubation periods are used to predict scrapie titers, requires calibration of the system. For each strain of prions and host, a calibration curve relating prion dose to the incubation time must be constructed.

The construction of reliable calibration curves probably requires samples with a wide range of titers. The titers are determined by endpoint titrations, and the time intervals from inoculation to onset of illness and to death are measured for each dilution. In developing calibration curves for Syrian hamsters, four animals were inoculated intracerebrally with a given sample at a specified dilution. After 55 days, the animals were examined two times weekly for clinical signs of scrapie. From the number of animals positive at each dilution, the titer was calculated using the method of Spearman and Kärber (Dougherty 1964). The injected dose is then calculated by multiplying the titer times the dilution.

Curves relating the injected dose to the time intervals from inoculation to onset of clinical illness, as well as from inoculation to time of death, are shown in Figures 3 and 4. The titers of the samples used to construct these curves varied over a range from 10^3 to $10^{8.5}$ ID_{50} units/ml, as determined by endpoint titration. As illustrated in Figure 3, the interval from inoculation to onset of illness minus a time factor of 40 is a linear function of the inoculated dose. The time factor was determined by maximizing the linear relationship between time interval and dose. With a factor of 40, the regression coefficient for the line is 0.87.

Although the onset of illness requires clinical judgment with respect to the diagnosis of scrapie, the time of death is a completely objective measurement. As shown in Figure 4, the time interval from inoculation to death minus a time factor of 61 is a linear function of the injected dose. As with the analysis of data for onset of illness, the time factor was determined by maximizing the linear relationship between this time

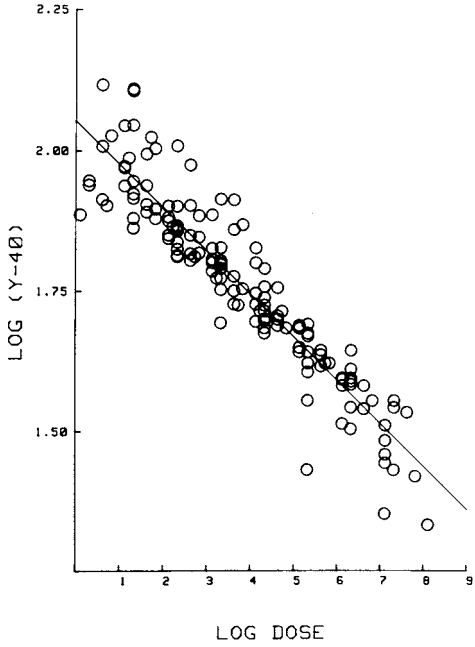

Figure 3 Incubation times from inoculation to onset of illness in Syrian hamsters as a function of the dose of Sc237 scrapie prions. (Reprinted, with permission, from Prusiner et al. 1982b [Lippincott Williams & Wilkins http://lww.com].)

interval and the dose. With a factor of 61, the regression coefficient for the line is 0.86.

Dose-response Relationships: Derivation of Equations

From the linear relationships described, equations relating titer, dilution, and time intervals can be written as follows:

$$\text{Log } T_y = 26.66 - (12.99)\ \log(\overline{y}\text{-}40) - \log \text{D}$$
$$\text{Log } T_z = 25.33 - (12.47)\ \log(\overline{z}\text{-}61) - \log \text{D}$$

where T is the titer, expressed in ID_{50} units/ml; D is the dilution, defined as the fractional concentration of the diluted sample; y is the mean interval from inoculation to onset of clinical illness in days; and z is the mean interval from inoculation to death in days. The most precise estimate of titer is obtained by calculating a weighted average of T_y and T_z.

Similar linear relationships were obtained when the reciprocals of the time intervals were plotted as a function of the logarithm of the dose.

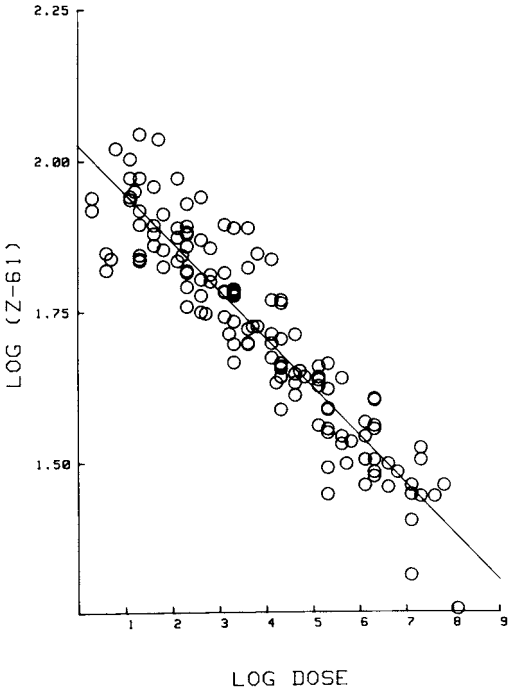

Figure 4 Incubation times from inoculation to death in Syrian hamsters as a function of the dose of Sc237 scrapie prions. (Reprinted, with permission, from Prusiner et al. 1982b [Lippincott Williams & Wilkins http://lww.com].)

Regression coefficients of 0.87 and 0.88 were obtained for lines relating $1/y$ and $1/z$, respectively, to the logarithms of the dose. Equations describing these functions gave similar results to those obtained with the two equations above.

About a decade after developing the incubation time bioassay for prions in Syrian hamsters, we reevaluated the system with a series of endpoint titrations where the animals were scored only for the onset of neurologic dysfunction. After the hamsters showed a clear progression of neurologic signs, the animals were euthanized. In these studies, we were unable to find any substantial difference between the newly obtained titration data and those gathered in the original investigation. We found that the simple relationship shown below, which was derived from a plot of the incubation time as a function of the dose, was highly reliable. We have used the following equations for bioassays in Syrian hamsters over the past decade.

For incubation times <104 days: Log T = 17 + [Log D] – (0.138Y)

For incubation times >104 days: Log T = 8.9 + [Log D] – (0.059Y)

where T is the titer, expressed in ID_{50} units/ml; D is the dilution, defined as the fractional concentration of the diluted sample; and Y is the mean interval from inoculation to onset of clinical illness in days.

After developing the bioassay for Syrian hamster prions, we asked whether the same approach could be used for mouse prions, despite earlier studies that concluded such an approach would not work with mice. From endpoint titrations, the reciprocal of the incubation time was plotted against the dose of prions (Fig. 5). From those data, the equation for CD-1 Swiss mice was derived:

$$\text{Log T} = 1.52 + [\text{Log D}] + ((185 - \text{Y})12.66)$$

where T is the titer, expressed in ID_{50} units/ml; D is the dilution, defined as the fractional concentration of the diluted sample; and Y is the mean interval from inoculation to onset of clinical illness in days.

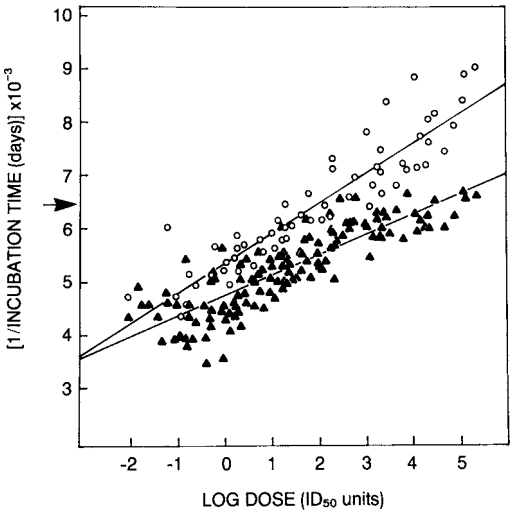

Figure 5 Calibration curve for the Chandler isolate of mouse scrapie prions in Swiss mice plotted as the reciprocal of the incubation time versus the average dose given each animal. The curve was obtained from endpoint titration of 29 independent preparations (18 spleen samples, 10 sucrose gradient fractions, and 1 brain homogenate) (Butler et al. 1988). The experiments were terminated 226 days after inoculation; the LD_{50} units were calculated by dividing the dose as given above by the volume of the inoculum given to each mouse (30 μl). Circles represent incubation time, triangles the time of death. (Reprinted, with permission, from Butler et al. 1988.)

Validation

The validity of the time interval measurements is supported by many lines of evidence. The titers of the hamster scrapie prion obtained by the time interval method agree in general with those found by endpoint titration within ±0.5 log ID_{50} units/ml. An estimate of the precision of the incubation time interval assays was made by calculating the 95% confidence intervals for each of the titer measurements at 10^{-1} dilution in Table 6. These confidence intervals were computed for two different degrees of freedom ($n - 1$, where n is the number of observations). It can be argued that the number of observations for 4 hamsters is 8, since the times of both onset of illness and death were determined. Conversely, it can be argued that the onset of illness and death are closely linked and that each animal was inoculated only once; thus, only four observations are independent and can be used to compute the degrees of freedom. If 7 degrees of freedom are chosen, then the 95% confidence interval ranges from $10^{0.5}$ to $10^{1.6}$ ID_{50} units/ml with an average interval of $10^{0.9}$. If 3 degrees of freedom are chosen, then the 95% confidence interval ranges from $10^{0.6}$ to $10^{2.1}$ ID_{50} units/ml with an average interval of $10^{1.2}$. The analysis agrees well with our experience, which dictates that samples must differ by more than 10^2 ID_{50} units/ml for the difference to be significant. On occasion we have identified differences of approximately 10^1 ID_{50} units/ml that appeared to be significant since these proved to be reproducible at least five times.

Scrapie agent titers were measured by the incubation time interval assay and endpoint titration for a series of samples subjected to heat inactivation. Table 6 compares these results. No significant difference between the two sets of data can be discerned. However, the endpoint

Table 6 Comparison of titers determined by endpoint titration and incubation time interval assays

Endpoint titration	Incubation time interval assay			
	10^{-1} dilution		10^{-3} dilution	
($\log_{10}$ titer)	($\log_{10}$ titer)	(ρ value)	($\log_{10}$ titer)	(ρ value)
8.3 ± 0.35	8.3 ± 0.10	1.00	8.7 ± 0.16	0.27
8.3 ± 0.35	7.7 ±.012	0.08	8.2 ± 0.47	0.84
8.1 ± 0.25	8.2 ± 0.25	0.76	8.0 ± 0.28	0.76
8.3 ± 0.35	8.1 ± 0.33	0.69	7.6 ± 0.13	0.04
8.1 ± 0.25	8.2 ± 0.17	0.48	7.8 ± 0.11	0.23
8.0 ± 0.44	8.2 ± 0.16	0.62	8.5 ± 0.18	0.24

Titer is expressed as ID_{50} units/ml ± S.E. Probability (ρ) values represent the proportion of sample means farther from the endpoint titration mean titer than the incubation time interval mean titer actually obtained.

titration assay required 40 hamsters for each of the eight points, or 320 animals, while the time interval assay gave the same data using 4 hamsters for each point, or a total of 32 animals. For nearly 200 days after inoculation, no information about the titers of the agent was available using the endpoint titration method. In contrast, at approximately 75 days after inoculation, the stability of the agent up to 90ºC was apparent using the incubation time interval assay. From this comparison, the advantages of the incubation time interval assay are obvious.

Measurements of the time intervals from inoculation to onset of illness are so reproducible that calibration curves developed 1.5 and 10 years apart were virtually superimposable upon one another as noted above (Prusiner et al. 1980c, 1982b). No differences between the molecular properties of the agents from hamster and murine sources have been detected, using primarily the time interval method with the former and endpoint titration with the latter (Prusiner et al. 1980c). Sedimentation profiles, detergent stability studies, and gel electrophoresis experiments yield the same data whether assays are performed by the incubation period method or by endpoint titration (Prusiner et al. 1978a,b, 1980a,b,c).

Some investigators have questioned the validity of the incubation time assay (Lax et al. 1983; Somerville and Carp 1983). Criticisms of the incubation time assay have involved situations where prions are being disaggregated by detergent treatment. Such difficulties with obtaining accurate measurements are not due to the assay but emanate from the amphipathic character of scrapie prions.

Advantages

The advantages of the incubation time interval assay over the endpoint titration assay are numerous. First, the number of animals required for determination of a particular sample is decreased by a factor of 10–15. Second, the imprecision that arises during pipetting the serial dilutions of samples is obviated by the incubation time method. Third, determination of the titer in samples with high titers is considerably faster using the incubation time method. As illustrated here, an inverse relationship exists between the length of the incubation period and the titer of the inoculum. To establish the endpoint of a titration for the scrapie agent, it is necessary to wait until all the animals become sick at the highest positive dilution. Fourth, the incubation time interval assay will always give a quantitative estimate of the concentration of scrapie prions in a given sample, unlike the endpoint titration assay. Attempts to economize with endpoint titrations will, at times, result in a series of dilutions that do not span the

endpoint of the sample. In such cases, considerable time will have already elapsed, and it would be necessary to begin a second titration.

An integral part of the incubation time interval assay for the scrapie agent is a computerized database. Biweekly updating of titers has greatly facilitated our research efforts. By 70 days after inoculation, samples with high titers are already causing disease in animals. During the next 2–4 weeks, samples with substantially lower titers are just beginning to cause disease. Frequent revisions of the database involving calculations of titers and plotting of experimental determinations would not be practical without the use of computers.

The development of an incubation time interval assay for measuring scrapie prions has had a profound effect on the progress of studies on the molecular biology of these infectious pathogens. Clearly, purification of scrapie prions could never have advanced far enough to allow identification of the prion protein, PrP 27-30, without the incubation time interval assay (Prusiner et al. 1982a). The discovery of PrP 27-30 is the cornerstone upon which considerable progress in understanding the molecular structure of prions is based.

A comparison of the endpoint titration and incubation time interval assays is shown in Table 6. The economics of both time and resources afforded by the incubation time interval assay are highly significant. We estimate that our research was accelerated more than 100-fold by the use of the incubation time interval assay. It is doubtful that the purification and characterization methods described below could have been developed if the endpoint titration method had been used to assay samples.

TRANSGENIC MICE

Tg mice overexpressing PrP^C from various species may prove to be of considerable value in the development of more rapid incubation time assays. For instance, Tg(SHaPrP$^{+/+}$)7/*Prnp*$^{0/0}$ mice overexpressing $SHaPrP^C$ approximately 8-fold compared to Syrian hamsters display incubation times of about 40 days when inoculated with ~10^7 ID_{50} units of Sc237 prions previously passaged in Syrian hamsters (Scott et al. 1997a). The Tg(SHaPrP$^{+/+}$)/7*Prnp*$^{0/0}$ mice are homozygous for the SHaPrP transgene array and were crossed onto a mouse PrP-deficient background (Büeler et al. 1992). Similarly, Tg(MoPrP-A) mice overexpressing $MoPrP^C$-A exhibit incubation times of about 45 days upon inoculation with about 10^6 ID_{50} units of RML prions previously passaged in CD-1 Swiss mice (Carlson et al. 1994; Telling et al. 1997).

With such abbreviated incubation times, Tg(SHaPrP$^{+/+}$)7/*Prnp*$^{0/0}$ mice overexpressing $SHaPrP^C$ were used for a series of endpoint titrations. The

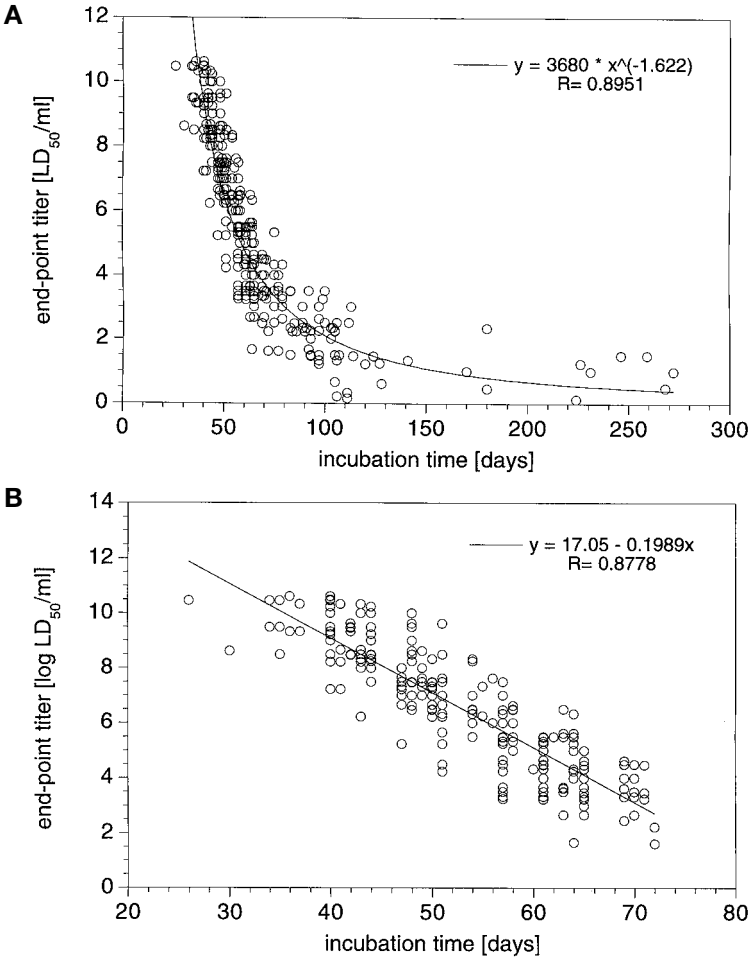

Figure 6 Calibration curve for the Sc237 isolate of hamster scrapie prions in Tg(SHaPrP$^{+/+}$)7/*Prnp*$^{0/0}$ mice. (*A*) The correlation between incubation time and injected prion dose was obtained from 12 independent endpoint titration experiments (2 hamster brain homogenates and 2 sucrose gradient fractions in triplicates) on 532 mice. The experiments were terminated 300 days after the inoculation and the endpoint titer was calculated by the method of Spearman and Kärber (Dougherty 1964). The curve is the best fit of the data by nonlinear least-squares regression analysis. Circles represent incubation time of each transgenic mouse. (*B*) Detailed linear range of the calibration curve from *A* for Sc237 isolate of SHa prions inoculated into Tg(SHaPrP$^{+/+}$)7/*Prnp*$^{0/0}$ mice. The incubation time cutoff at 75 days was established from curve deflection point and from sensitivity limit of endpoint prion assay (2 log LD_{50}/ml). Circles represent incubation time of each transgenic mouse; curve is the best fit of the data by linear least-squares regression analysis.

incubation times were plotted as a function of the dose of prions (Fig. 6A). The data from the linear portion of the curve were replotted in Figure 6B. At low doses of prions, the incubation times became greatly prolonged, which makes the proof of sterility using bioassays difficult. Such results emphasize the need for mice with highly abbreviated incubation times.

Although the Tg(MoPrP-A) mice offer a substantial advantage over non-Tg mice, the advantage of Tg(SHaPrP$^{+/+}$)*Prnp*$^{0/0}$ mice compared to Syrian hamsters is less clear. Because Tg mice are extremely expensive to produce, the cost of maintaining a supply of such mice available for bioassays on demand is considerable. Thus, the availability of Syrian hamsters still makes them quite useful for many bioassays. When the incubation times for bioassays in Tg mice become less than 20 days, the advantage will be so great that it seems very likely that such animals will become the system of choice for most basic studies.

Production of Transgenic Animals

Tg mice expressing a foreign or mutant PrP gene are generally produced using eggs from donor animals in which the MoPrP gene was ablated (*Prnp*$^{0/0}$) (Büeler et al. 1992). These *Prnp*$^{0/0}$ mice were produced from an embryonic stem cell line derived from a 129Sv line and backcrossed to C57BL/6 before intercrossing to obtain the homozygous *Prnp*$^{0/0}$ mice. In our experience, this genetic background (129Sv and C56BL/6) was suboptimal for the production of Tg mice; therefore, we crossed the *Prnp*$^{0/0}$ mice onto the FVB background. FVB mice produce large eggs that can be readily microinjected (Hogan et al. 1994). Typically, 20 superovulated females are placed with males and sacrificed early the next morning for embryo collection of 0.5-day embryos. From these, we obtain 10–15 fertilized females, which generally provide 150–300 zygotes, of which 80–90% are amenable to microinjection. Embryos are cultured using standard M2/M16 media (Hogan et al. 1994). Microinjection is performed using an inverted Leica stereomicroscope and Nomarski illumination. Successfully microinjected embryos are selected after a few hours of culture and implanted back into recipient CD-1 pseudopregnant females (30 embryos/female).

Preparation of Transgenes and Vectors

The vector commonly used is cosmid-derived and contains 40 kbp from the Syrian hamster PrP locus (CosSHa.tet) (Scott et al. 1992). The PrP open reading frame was replaced by a convenient tetracycline cassette

flanked by unique *Sal*I sites, which facilitates the insertion of transgenes in this otherwise cumbersome vector. This vector offers the advantage of reliable copy-dependent transgene expression levels (Scott et al. 1989; Prusiner et al. 1990).

Transgenes are excised from plasmid sequences, separated by agarose gel electrophoresis, purified using β-agarase, extracted using phenol/chloroform and ether, precipitated, dissolved in TE (10 mM Tris, 0.1 mM EDTA, pH 7.5), and dialyzed for 30 minutes against TE using a Millipore membrane filter (0.025 μm). The DNA is diluted to 1.5–2.5 ng/μl using TE and freed from residual particulate contaminants using a spin column (Spin-X, Costar).

With this protocol, we created 457 founder Tg mice over a 2-year period. Using transgenes derived from the CosSHa.tet vector, the live birth rate was 10.7% and the percentage of transgenics (over the total number of eggs microinjected and transplanted) was 1.9%. Using plasmid-derived transgenes, the live birth rate was 12.9% and the transgenic rate was not significantly different at 2.3%.

Breeding of Tg Mice

All information pertaining to the genealogy, inoculation, and health status of animals is maintained on a custom-made animal database (based on Paradox), and animal health status entry is facilitated by the use of bar codes (LabelRight, Qbar) and handheld bar code readers (Telzon). All the experimental information is uploaded on a central database and updated daily to peripheral terminals for availability to all investigators.

Since we currently maintain 150–250 strains of Tg mice, production of these animals for experimental studies can easily become a logistical problem. To facilitate the breeding of animal strains, experimental requests are entered on the central animal database, which generates a list of Tg strains requested. Tg mice are produced and bred with $Prnp^{0/0}$ animals that have been backcrossed for 10 generations with FVB mice ($Prnp^{0/0}$ × FVB)N10F1. This offers a homogeneous background that is suitable to both the production of new transgenic animals by microinjection and the maintenance of transgenic mouse lines by breeding.

Screening of Tg Mice

Some Tg lines are kept in the homozygous state and do not require constant screening for the transgene. Unfortunately, problems such as infertility, circling behavior, or embryonic death due to the transgene are not

uncommon in homozygous lines. Therefore, most lines are maintained as heterozygotes and the Tg status of every newborn mouse must be determined. Potential Tg mice are weaned at 3 weeks of age, at which time they receive an individual identification number which is tattooed directly on their tail at 4 weeks. A small piece of tail is then cut off and frozen for screening purposes. To handle large numbers of Tg mice, part of the screening procedures such as DNA extraction and slot blotting were automated. A robotic workstation is used to decrease the labor costs, to eliminate human errors, and to strengthen the consistency of the results. The system that we have adapted for our procedures is an automated pipetting station, Biomek 2000 (Beckman Instruments). Potential contaminations are avoided by the use of disposable materials. This workstation is easy to customize and we have added a slot blot apparatus to facilitate our screening. On a weekly basis, more than 1000 samples can be typed by two trained operators.

Genomic DNA is automatically extracted from the tail tissue with a phenol/chloroform-free method (Laird et al. 1991). The tails are lysed and digested with proteinase K; the DNA is directly precipitated in isopropanol, washed with 70% ethanol, dried, and dissolved in Tris-EDTA buffer. Determination of the Tg status is performed by polymerase chain reaction (PCR) with multiple primer pairs and/or slot blots of the genomic DNA followed by hybridization with ^{32}P-labeled probes. Although a variety of screening probes are routinely used, most Tg lines have been established using the CosSHa.tet cosmid vector (Scott et al. 1992) and are screened using a probe that specifically recognizes the 3′-untranslated region of the SHaPrP open reading frame. Use of a vector-specific probe rather than a probe specific to the open reading frame to be expressed facilitates the large-scale typing of the Tg mice. However, it also poses a potential disadvantage because mixing of animals between different Tg lines may remain undetected.

Besides the hybridization procedure and PCR reactions, most of the screening is performed with a robotic workstation. Although human intervention is still needed for the loading of the work surface and the mixing and spinning steps, the system has been found to be reasonably flexible and can be adapted to the constantly changing screening requirements that are mandated by new scientific questions.

TRANSMISSION OF HUMAN AND BOVINE PRIONS TO Tg MICE

For many years, non-Tg mice were used in studies of human prions derived from the brains of patients with Creutzfeldt-Jakob disease (CJD).

In such studies, the incubation times usually exceeded 500 days, and a minority of the animals developed prion disease (Tateishi et al. 1996). This problem was overcome with the introduction of Tg mice expressing either a chimeric Hu/Mo PrP gene or a HuPrP gene in the absence of MoPrP (Telling et al. 1994, 1995). Typically, these Tg mice exhibit signs of neurologic dysfunction in 200 days or less. Such abbreviated incubation times greatly facilitate experimental studies and drastically reduce the possibility of artifacts.

Two recent studies on the transmission of bovine prions to mice illustrate the value of Tg(BoPrP)*Prnp*$^{0/0}$ mice and demonstrate how ignoring several fundamental concepts in prion biology can lead to erroneous conclusions. BSE prions were inoculated into non-Tg mice, and between 300 and 700 days later, the mice developed an illness characterized by "hind limb paralysis, tremors, hypersensitivity to stimulation, apathy, and a hunched posture" (Lasmézas et al. 1997). All of the mice were said to have prion disease based on this constellation of clinical signs, yet only about 50% showed the neuropathologic changes of prion disease, i.e., spongiform degeneration and reactive astrocytic gliosis. In those mice with spongiform degeneration and reactive astrocytic gliosis, PrP^{Sc} was found by immunoblotting, whereas the mice lacking these neuropathologic changes did not have detectable PrP^{Sc}. Brain extracts prepared from mice with spongiform degeneration, reactive astrocytic gliosis, and PrP^{Sc} transmitted disease to recipient mice in about 150 days, whereas those without these features required >250 days for transmission to recipient mice. Such results are consistent with the interpretation that the mice lacking spongiform degeneration, reactive astrocytic gliosis, and PrP^{Sc} carried low titers of prions (Fig. 1). In the mice with low titers of prions, clinical illness was not due to prion disease, a contention supported by the lack of spongiform degeneration and reactive astrocytic gliosis. The apparent absence of PrP^{Sc} was expected since the prion titers were low; PrP^{Sc} could not be detected by the relatively insensitive immunoassay that was used (Lasmézas et al. 1996).

Why these investigators chose to equate seemingly apoptotic neurons in mice lacking spongiform degeneration and gliosis with prion disease is unclear. By doing so, they were faced with the mice that have a new form of "prion disease" in which they could not find PrP^{Sc} (Lasmézas et al. 1997). As noted above, the absence of detectable PrP^{Sc} would be expected since the mice did not have bona fide prion disease and the titers of prions were low based on the prolonged incubation times for transmission to recipient animals. In fact, the neuropathologic changes they depict can be attributed to artifacts from immersion fixation in formalin, including

the hyperchromatic Purkinje cells (so called "spiky" artifact) and the pseudo-apoptotic appearance of the Purkinje cell nucleus by electron microscopy.

A much different set of data was obtained when Tg(BoPrP)*Prnp*$^{0/0}$ mice were inoculated with extracts from BSE brains (Scott et al. 1997b). We found transmission of BSE prions to one Tg(BoPrP)*Prnp*$^{0/0}$ line that exhibited incubation times of about 230 days. Like most cattle with BSE, vacuolation and astrocytic gliosis were confined in the brain stems of these Tg mice. Unexpectedly, mice expressing a chimeric Bo/Mo PrP transgene were resistant to BSE prions, whereas mice expressing Hu or Hu/Mo PrP transgenes were susceptible to Hu prions as noted above (Telling et al. 1994, 1995). A comparison of differences in Mo, Bo, and Hu residues within the carboxyl terminus of PrP defines an epitope that modulates conversion of PrP^{C} into PrP^{Sc} and, as such, controls prion transmission across species. Development of susceptible Tg(BoPrP) mice provides a means of measuring bovine prions that may prove crucial in minimizing future human exposure.

All of the Tg(BoPrP)*Prnp*$^{0/0}$ mice inoculated with BSE prions that developed clinical signs of neurologic dysfunction were found to have protease-resistant $BoPrP^{Sc}$. This contrasts with the results of the study described above in which non-Tg mice inoculated with BSE prions were thought to have developed neurologic deficits, yet the brains lacked neuropathologic evidence of prion disease and were devoid of detectable protease-resistant PrP^{Sc} (Lasmézas et al. 1997). Although a credible explanation for these results is provided above, it is noteworthy that the lack of protease-resistant PrP has been reported in ill Tg mice expressing PrP with the P102L mutation of Gerstmann-Sträussler-Scheinker disease (GSS) at high levels (Hsiao et al. 1990). These findings parallel the absence of protease-resistant PrP in the brain of GSS patients. When these GSS mice develop progressive neurologic dysfunction, they lack protease-resistant PrP but do exhibit spongiform degeneration, astrocytic gliosis, and PrP amyloid plaques. This constellation of findings leaves no doubt about the diagnosis. Although prion disease was transmitted to Tg mice expressing the mutant transgene at low levels by inoculation of extracts from the foregoing Tg mice or from patients with GSS, these ill, recipient Tg mice likewise do not exhibit protease-resistant PrP (Hsiao et al. 1990, 1994; Telling et al. 1995, 1996). The recipient Tg mice did not develop prion disease spontaneously but did show spongiform degeneration, astrocytic gliosis, and PrP amyloid plaques after inoculation.

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Transmission and Replication of Prions

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One of the most remarkable features of slow infections is the clockwork precision with which the replication of prions occurs. Inoculation of numerous animals with the same dose of prions results in illness at the same time months later. The molecular mechanisms controlling this extraordinarily precise process are unknown.

The term infection implies that a pathogen replicates during this process. When the infectious pathogen has achieved a high titer, a disease in the host often appears. Prior to the recognition of the existence of prions, all infectious pathogens contained a nucleic acid genome that encoded their progeny. Copying of this genome by a polynucleotide polymerase provided a means of replicating the pathogen; in the case of prions, another mechanism functions.

GENERAL FEATURES OF PRION TRANSMISSION AND REPLICATION

As we learn more about prions, some general features and rules of prion replication are beginning to emerge. Although many of the results described here can be found in other chapters, we felt that it was important to collect information on prion transmission and replication in one place within this monograph.

Prion Replication and Incubation Times

When the titer of prions reaches a critical threshold level, the animals develop signs of neurologic dysfunction. The length of the interval from

inoculation to reach the threshold prion concentration, at which CNS dysfunction becomes evident, is referred to as the incubation time or incubation period.

The length of the incubation time can be modified by (1) the dose of prions, (2) the route of inoculation, (3) the level of PrP^{C} expression, (4) the species of the prion (i.e., the sequence of PrP^{Sc}), and (5) the strain of prion. The higher the dose of prions, the shorter is the time required to reach the threshold level for PrP^{Sc} at which signs of illness appear (Prusiner et al. 1982b; Chapter 3). The intracerebral route of inoculation is substantially more efficient than other routes (Prusiner et al. 1985). The higher the level of PrP^{C}, the more rapid is the accumulation of PrP^{Sc} and, hence, the shorter the incubation time to reach the threshold level for PrP^{Sc} at which the animals exhibit clinical signs of CNS dysfunction (Prusiner et al. 1990). When the amino acid sequence of PrP^{Sc} in the inoculum is the same as that of the PrP^{C} expressed in the recipient, the incubation times are generally the most abbreviated (Scott et al. 1989, 1997). At present, the mechanism by which strains modify the incubation time is not well understood, but interactions between PrP^{Sc} and PrP^{C} undoubtedly play a major role (DeArmond et al. 1997; Prusiner 1997; Scott et al. 1997).

TRANSMISSION OF PRIONS AMONG MAMMALS

More than 170,000 cattle, primarily dairy cows, have died of bovine spongiform encephalopathy (BSE) over the past decade (Anderson et al. 1996; Chapter 11). BSE is a massive common source epidemic caused by prion-contaminated meat and bone meal (MBM) fed primarily to dairy cows (Wilesmith et al. 1991). The MBM was prepared from the offal of sheep, cattle, pigs, and chickens as a high-protein nutritional supplement. In the late 1970s, the hydrocarbon-solvent extraction method used in the rendering of offal began to be abandoned, resulting in MBM with a much higher fat content (Wilesmith et al. 1991). It is now thought that this change in the rendering process allowed scrapie prions from sheep to survive rendering and to be passed into cattle. The mean incubation time for BSE is about 5 years. Most cattle therefore did not manifest disease because they were slaughtered between 2 and 3 years of age (Stekel et al. 1996).

The natural route by which scrapie prions are transmitted among sheep and goats is unknown. Some investigators have suggested that horizontal spread occurs orally (Palsson 1979). The suggested source of scrapie prions is placentae from infected sheep and goats (Pattison et al.

1972). For many years, studies of experimental scrapie were performed exclusively with sheep and goats. The disease was first transmitted by intraocular inoculation (Cuillé and 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 and Stamp 1969; Hadlow et al. 1980, 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).

Routes of Transmission

Oral transmission has also been suggested as the means by which kuru was spread among New Guinea natives (Gajdusek 1977; Alpers 1979). Ritualistic cannibalism of dead relatives has been implicated in the epidemic spread of kuru. However, the role of cannibalism in the etiology of kuru has been questioned because oral transmission of kuru to nonhuman primates has been demonstrated in only a few instances (Gibbs et al. 1980). These experimental hosts regularly contracted a kuru-like illness many months or years after intracerebral or peripheral inoculation (Gajdusek 1977). The oral transmission results have prompted hypotheses suggesting that the kuru agent might have entered only through abrasions of mucous membranes within the oropharynx or conjunctivae as well as through open wounds on the hands (Gajdusek 1977).

Hamsters have been used to study natural modes of transmission of scrapie prions as described above (Prusiner et al. 1985). In the course of studies on the molecular biology of scrapie prions, we noticed that scrapie was regularly transmitted by cannibalism in hamsters. Because of the putative role of ritualistic cannibalism in the spread of kuru, we investigated the oral spread of scrapie among hamsters.

The natural mode of transmission of prions among animals remains very poorly understood in the cases of sheep with scrapie as well as in the cases of mule deer and elk with chronic wasting disease (Spraker et al. 1997). It seems unlikely that Creutzfeldt-Jakob disease (CJD) is spread among humans by infection except in the cases of accidental inoculations; indeed, most CJD cases are sporadic and are likely to be the result of a somatic mutation or the spontaneous conversion of PrP^{C} into PrP^{Sc}. Many attempts to identify an infectious source of prions to explain sporadic CJD have been unsuccessful (Brown et al. 1987; Harries-Jones et al. 1988; Cousens et al. 1990).

KINETICS OF PRION REPLICATION

Studies of scrapie prion replication in mice showed that after subcutaneous inoculation the titers in systemic organs increased slowly (Table 1) (Eklund et al. 1967). The most rapid increase in prion titer occurred in the spleen, where it reached a maximum 1 month after inoculation and remained constant. The titer in brain rose more slowly, but after 3 months it began to exceed that of the spleen. By 4 months, the titer in brain was at a maximum. It is the central nervous system (CNS) where physiologic dysfunction and pathologic changes occur. The kinetics of scrapie prion replication in murine spleen and brain after intracerebral inoculation are similar to those described above for subcutaneous inoculation.

In Figure 1, the kinetics of scrapie prion replication in hamster brain is shown. Hamsters were inoculated intracerebrally with about 10 ID_{50} units; the animals were sacrificed at 1- to 2-week intervals and the prion titers were measured by incubation time interval assays or endpoint titrations. As shown, the prion titer in brain increases 10^4-fold over 50 days and then plateaus. Histopathologic changes were first seen in brain about 50 days after inoculation. By 70 days, widespread vacuolation of the neuropil and extensive astrocytic proliferation were observed. Between 75 and 85 days, most of the animals died.

In Figure 2A, survival curves for 200 hamsters inoculated intracerebrally with 10^7 ID_{50} units are plotted. The remarkable synchrony of the disease is well illustrated. All the animals were inoculated on day zero. Eighty percent of the hamsters developed clinical signs of scrapie between 65 and 69

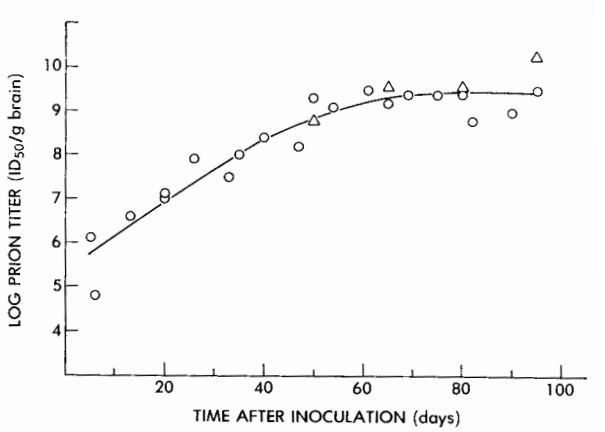

Figure 1 Kinetics of prion replication. Circles indicate titers determined by inoculation time assays; triangles are titers by endpoint titrations. (Reprinted, with permission, from Oesch et al. 1985 [copyright Cell Press].)

Table 1 Kinetics of prion replication in mice

Weeks after inoculation	1	4	8	12	16	20	24	28	29[a]	32	36	42
Percent of surviving mice sick with scrapie							6.8	26	40	60	63	25
Percent of total mice dead of scrapie								4.5	6.9	25	61	73
Tissues examined[b]												
Spleen	4.5[c]	3.5	5.6	5.6	6.2	6.2	5.5	5.5	5.7	5.6	5.2	5.5
Peripheral lymph nodes		3.4	5.6	4.7	4.7	5.2	4.5	4.8	5.4	5.5	5.6	4.6
Thymus			4.2	4.8	5.5	5.0	5.4	4.5	0.8[d]	5.2	5.6	4.5
Submaxillary salivary gland		5.8	5.5	6.5	5.2	6.2	6.0	6.4	5.6	3.4	2.5	
Lung				3.5	3.4	3.2	3.2	2.4	2.5		2.2	3.8
Intestine				2.2	2.2	3.4	5.3	5.5	4.6	5.4	5.2	4.5
Spinal cord				1.4	5.6	4.5	6.6	6.5	7.4	7.4	6.7	6.6
Brain					4.4	3.2	5.7	6.3	6.7	6.5	7.2	7.4
Bone marrow (femur)							1.7	2.8	4.8	3.5	5.0	3.6
Uterus						+[e]			+		+	+
Liver		———	-not examined	———		+	+	+	+	+		
Kidney		———	-not examined	———					+	+		

Data from Eklund et al. (1967).

[a]From the 29th week on, only sick mice were examined.

[b]Blood clot, serum, and tests were also examined, but virus was never detected in them.

[c]Negative $\log_{10}$ of dilution of tissue suspension that contained 1 LD_{50} per 0.03 ml when inoculated intracerebrally into mice. Blank spaces indicate virus was not detected in any dilution.

[d]Questionable whether thymus was removed.

[e]Virus was detected in 10^{-1} dilution only and not all mice were affected.

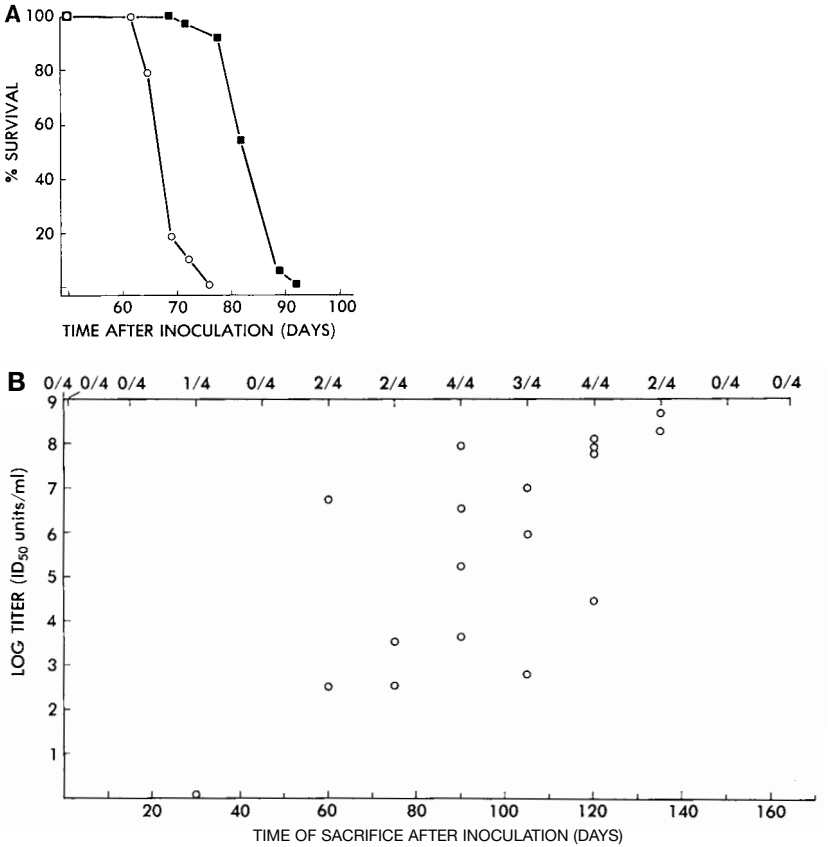

Figure 2 Survival curves for Syrian hamsters and kinetics of scrapie prion replication. (*A*) Inoculated with ~10^7 ID_{50} units of prions. Open circles indicate onset of clinical signs of scrapie; filled squares indicate death of hamsters. (*B*) Inoculated with ~10 ID_{50} units of prions. Four hamsters were sacrificed at times specified on the lower *x*-axis, and titers of prions in brains were determined by bioassays. Fraction of hamsters with detectable levels of brain prions denoted on the *x*-axis at the top of the graph. (Reprinted, with permission, from Prusiner 1987.)

days after inoculation; more than 70% of the animals died between 78 and 86 days after inoculation. This remarkable synchrony of disease onset and death after intracerebral inoculation with a given dose of prions is the basis of the incubation time interval bioassay (Prusiner et al. 1980b, 1982b.).

When the intracerebral inoculum was reduced to <10 ID_{50} units, the incubation period was doubled (120–130 days). The kinetics of prion replication in hamsters inoculated with <10 ID_{50} units is shown in Figure 2B. The titer rises more slowly and the scatter of data points is much

greater when compared with Figure 1. However, as shown, the titer rises 10^8-fold over 130 days. These studies clearly establish that prions can increase their number by a factor of about 100 million.

PRION TRANSMISSION BETWEEN SPECIES

A prolongation of the incubation time upon passage of prions from one species to another has often been referred to as the "species barrier" (Pattison 1965). Once the first passage of prions in a new species occurred, the reduction in incubation time on subsequent passage in the same species was frequently called "adaptation" (Kimberlin and Walker 1977; Gibbs et al. 1979; Manuelidis and Manuelidis 1979b; Tateishi et al. 1979; Kingsbury et al. 1982). On second passage in the same host, an additional, albeit small, decrement in the incubation time was sometimes reported.

The incubation period for first passage of scrapie prions from sheep to Swiss mice is typically 10–12 months for a 10-fold dilution of 10% (w/v) homogenates (W.J. Hadlow, unpubl.). When brain homogenates prepared from goats with drowsy-type scrapie were inoculated into Swiss mice, 6–7 months were required before the mice showed signs of experimental scrapie (Chandler and Fisher 1963). On subsequent passage from Swiss mouse to Swiss mouse, the incubation period was reduced to 4–5 months. In two subsequent Swiss mouse passages, the incubation period remained unchanged. Passage from mouse to white rats prolonged the incubation period to more than 16 months; however, subsequent passage to rats reduced the incubation time to 7 months (Pattison and Jones 1968). Passage from rats back to mice required an incubation period of 7 months. Subsequent passages of the mouse inoculum derived from rats caused scrapie in mice in 4–5 months (Table 1).

Table 2 Human prion transmission to nontransgenic mice

	Incubation times for transmission to mice					
	first passage		second passage		third passage	
Case number	(*n*)	(days ± S.D.)	(*n*)	(days ± S.D.)	(*n*)	(days ± S.D.)
1	6	318 ± 92	20	128 ± 14		
2	8	711 ± 115				
3	11	665 ± 122	18	111 ± 10	13	125 ± 43
4	7	447 ± 191	18	122 ± 9	3	120 ± 5
5	4	705 ± 53	2	215		
6	13	769 ± 71				

Data from Tateishi et al. (1996).

In studies on the transmission of CJD from humans to rodents, the species barrier is quite apparent (Tateishi et al. 1996). Primary transmissions from humans to mice required between 300 and 800 days (Table 2). On the second passage from mouse to mouse the incubation period was reduced to about 120 days; the same incubation period was seen on the third passage from mouse to mouse. Purification protocols and studies on the properties of CJD prions have been performed with mice. As with studies on scrapie prions, investigations on CJD prions have been dramatically accelerated by the use of incubation time assays (Walker et al. 1983; Bendheim et al. 1985).

Hadlow observed that the scrapie agent when passaged in mink retained its ability to infect goats but lost its ability to infect mice (W.J. Hadlow, unpubl.). Interestingly, the infectious agent causing mink encephalopathy has a similar host range (Marsh and Kimberlin 1975). The transmissible mink agent can be passaged in goats but not in mice. The molecular changes that distinguish the scrapie agent propagated in mink from that found in goats and mice are undoubtedly due to the conformation adopted by PrP^{Sc} as it is passaged from one host to another. This scenario is similar to that described for the Me7 scrapie mouse prion passaged directly into Syrian hamsters (Kimberlin et al. 1987) or through Tg(MH2M) mice and subsequently into hamsters (Scott et al. 1997).

Transgenetics and Species Barriers

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 a constant length observed for all subsequent passages, and transmission becomes a nonstochastic process. The species barrier is of practical importance in assessing the risk for humans of acquiring CJD after consumption of scrapie-infected lamb or BSE-infected beef.

To test the hypothesis that differences in PrP gene sequences might be responsible for the species barrier, Tg mice expressing SHaPrP were constructed (Scott et al. 1989; Prusiner et al. 1990). The PrP genes of Syrian hamsters and mice encode proteins differing at 16 residues. Incubation times in four lines of transgenic mice inoculated with mouse scrapie prions were prolonged compared to those observed for nontransgenic, control mice (Fig. 3A). Transgenic mice inoculated with SHa prions showed abbreviated incubation times in a nonstochastic process (Fig. 3B) (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 the transgenic mice (Fig. 3B,C) (Scott et al. 1989; Prusiner et al. 1990). $SHaPrP^{Sc}$ concentrations in the brains of clinically ill mice were similar in all four transgenic lines inoculated with hamster prions (Fig. 3D). Bioassays of brain extracts from clinically ill transgenic mice inoculated with mouse prions revealed that only mouse prions but no hamster prions were produced (Fig. 3E). Conversely, inoculation of transgenic mice with hamster prions led to the synthesis of only hamster prions (Fig. 3F). Thus, the de novo synthesis of prions in transgenic mice is species specific and reflects the genetic origin of the inoculated prions. Similarly, the neuropathology of transgenic mice is determined by the genetic origin of the prion inoculum. Mouse prions injected into transgenic mice produced neuropathologic changes characteristic of mice with scrapie. A moderate degree of vacuolation in both the gray and white matter was found, but amyloid plaques were rarely detected (Fig. 3G). Inoculation of transgenic mice with SHa prions produced intense vacuolation of the gray matter, sparing of the white matter, and numerous SHaPrP amyloid plaques, characteristic of Syrian hamsters with scrapie (Fig. 3H).

These studies with Tg mice established that the PrP gene influences virtually all aspects of scrapie including the species barrier, replication of prions, incubation times, synthesis of PrP^{Sc}, and neuropathologic changes.

VARIATIONS IN PATTERNS OF DISEASE

The lengths of the incubation times have been used to distinguish prion strains inoculated into sheep, goats, mice, and hamsters. Dickinson and his colleagues developed a system for "strain typing" by which mice with genetically determined short and long incubation times were used in combination with the F_1 cross (Dickinson et al. 1968, 1984; Dickinson and Meikle 1971). For example, C57BL mice exhibited short incubation times of about 150 days when inoculated with either the Me7 or Chandler isolates; VM mice inoculated with these same isolates had prolonged incubation times of about 300 days. The mouse gene controlling incubation times was labeled *Sinc* and because of prolonged incubation times in F_1 mice, long incubation times were said to be a dominant trait. Prion strains were categorized into two groups based on their incubation times: (1) those causing disease more rapidly in "short" incubation time C57BL mice and (2) those causing disease more rapidly in "long" incubation time VM mice. Noteworthy are the 22a and 87V prion strains, which can be passaged in VM mice while maintaining their distinct characteristics.

PrP Gene Dosage

More than a decade of studies was required to unravel the mechanism responsible for the "dominance" of long incubation times; not unexpect-

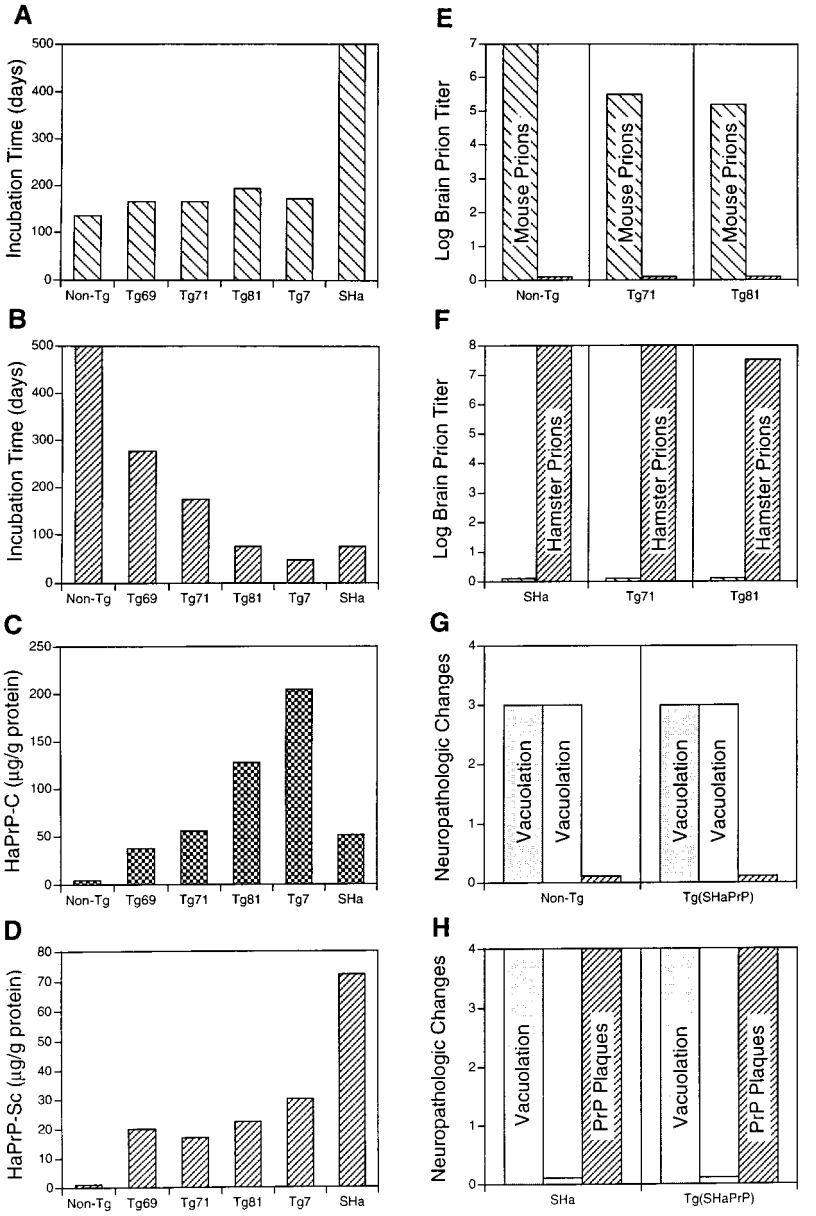

Figure 3. (See facing page for legend.)

edly, long incubation times were found not to be dominant traits. Instead, the apparent dominance of long incubation times is due to a gene dosage effect (Carlson et al. 1994).

The identification of a widely available mouse strain with long incubation times was a crucial step in elucidating the mechanism of incubation time control in mice. ILn/J mice inoculated with RML prions were found to have incubation times exceeding 200 days (Kingsbury et al. 1983), a finding that was later confirmed by other investigators (Carp et al. 1987). Once molecular clones of the PrP gene were available, a restriction fragment length polymorphism (RFLP) of the PrP gene was used to follow the segregation of MoPrP genes (*Prnp*) in the F_1 backcross and F_2 progeny of short incubation period NZW or C57BL mice crossed with long incubation period ILn/J mice. This approach demonstrated genetic linkage between *Prnp* and a gene modulating incubation times (*Prn-i*) (Carlson et al. 1986). Other investigators confirmed the genetic linkage,

Figure 3 Transgenic mice expressing Syrian hamster (SHa) PrP exhibit species-specific scrapie incubation times, infectious prion synthesis, and neuropathology (Prusiner et al. 1990). (*A*) Scrapie incubation times in nontransgenic mice (non-Tg), four lines of transgenic mice expressing SHaPrP, and Syrian hamsters inoculated intracerebrally with ~10^6 ID_{50} units of Chandler mouse prions serially passaged in Swiss mice. The four lines of transgenic mice have different numbers of transgene copies: Tg69 and 71 mice have 2–4 copies of the SHaPrP transgene whereas Tg81 mice have 30–50 and Tg7 mice have >60. The incubation time is number of days from inoculation to onset of neurologic dysfunction. *(B)* Scrapie incubation times in mice and hamsters inoculated with ~10^7 ID_{50} units of Sc237 prions serially passaged in Syrian hamsters as described in *A*. (*C*) Brain $SHaPrP^C$ in transgenic mice and hamsters. $SHaPrP^C$ levels were quantified by an enzyme-linked immunoassay. (*D*) Brain $SHaPrP^{Sc}$ in transgenic mice and hamsters. Animals were sacrificed after exhibiting clinical signs of scrapie. $SHaPrP^{Sc}$ levels were determined by immunoassay. (*E*) Prion titers in brains of clinically ill animals after inoculation with mouse prions. Brain extracts from non-Tg, Tg71, and Tg81 mice were bioassayed for prions in mice (*left*) and hamsters (*right*). (*F*) Prion titers 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 gray (*left*) and white matter (*center*); PrP amyloid plaques (*right*). Vacuolation score: 0 = none, 1 = rare, 2 = modest, 3 = moderate, 4 = intense. PrP amyloid plaque frequency: 0 = none, 1 = rare, 2 = few, 3 = many, 4 = numerous. (*H*) Neuropathology in Syrian hamsters and transgenic mice inoculated with hamster prions. Degree of vacuolation and frequency of PrP amyloid plaques as in *G*. (Reprinted, with permission, from Prusiner 1991 [copyright AAAS].)

and one group showed that the incubation time gene *Sinc* is also linked to PrP (Hunter et al. 1987; Race et al. 1990). It now seems likely that the genes for PrP, *Prn-i* and *Sinc*, are congruent (Moore et al. 1998); the term *Sinc* is no longer used (Ziegler 1993). The PrP gene sequences of NZW and I/Ln with short and long scrapie incubation times, respectively, differ at codons 108 (L→F) and 189 (T→V) (Westaway et al. 1987).

Although the amino acid substitutions in PrP that distinguish $Prnp^a$ from $Prnp^b$ mice argued for the congruency of *Prnp* and *Prn-i*, experiments with $Prnp^a$ mice expressing $Prnp^b$ transgenes demonstrated a "paradoxical" shortening of incubation times (Westaway et al. 1991). We had predicted that these Tg mice would exhibit a prolongation of the incubation time after inoculation with RML prions based on ($Prnp^a \times Prnp^b$) mice, which exhibit long incubation times. We described those findings as paradoxical shortening because we and others had believed for many years that long incubation times are dominant traits (Dickinson et al. 1968; Carlson et al. 1986). From studies of congenic and transgenic mice expressing different numbers of the *a* and *b* alleles of *Prnp* (Table 3), we now realize that these findings were not paradoxical; indeed, they resulted from increased PrP gene dosage (Carlson et al. 1994). When the RML isolate was inoculated into congenic and transgenic mice, increasing the number of copies of the *a* allele was found to be the major determinant in reducing the incubation time; however, increasing the number of copies of the *b* allele also reduced the incubation time, but not to the same extent as that seen with the *a* allele (Table 3).

The discovery that incubation times are controlled by the relative dosage of $Prnp^a$ and $Prnp^b$ alleles was foreshadowed by studies of Tg(SHaPrP) mice in which the length of the incubation time after inoculation with SHa prions was inversely proportional to the transgene product, $SHaPrP^C$ (Prusiner et al. 1990). The PrP gene dose determines not only the length of the incubation time but also the passage history of the inoculum, particularly in $Prnp^b$ mice. The PrP^{Sc} allotype in the inoculum produced the shortest incubation times when it was the same as that of PrP^C in the host (Carlson et al. 1989). The term "allotype" is used to describe allelic variants of PrP. To address the issue of whether gene products other than PrP might be responsible for these findings, we inoculated B6 and B6.I-4 mice carrying $Prnp^{a/a}$ as well as I/Ln and B6.I-2 mice (Carlson et al. 1993, 1994) with RML prions passaged in mice homozygous for either the *a* or *b* allele of *Prnp*. CD-1 and NZW/LacJ mice produced prions containing PrP^{Sc}-A encoded by $Prnp^a$, whereas I/LnJ mice produced PrP^{Sc}-B prions. The incubation times in the congenic mice reflected the PrP allotype of the previous host rather than other factors

Table 3 MoPrP-A expression is a major determinant and MoPrP-B is a minor determinant of incubation times in mice inoculated with the RML scrapie prions

Mice	*Prnp* genotype	*Prnp* transgenes (copies)	Alleles *a* (copies)	Alleles *b* (copies)	Incubation time (days ± S.E.M.)	*n*
Prnp$^{0/0}$	0/0		0	0	>600	4
Prnp$^{+/0}$	a/0		1	0	426 ± 18	9[a]
B6.I-1	b/b		0	2	360 ± 16	7
B6.I-2	b/b		0	2	379 ± 8	10[b]
B6.I-3	b/b		0	2	404 ± 10	20
(B6 x B6.I-1)F1	a/b		1	1	268 ± 4	7
B6.I-1 x Tg(MoPrP-B$^{0/0}$)15	a/b		1	1	255 ± 7	11[c]
B6.I-1 x Tg(MoPrP-B$^{0/0}$)15	a/b		1	1	274 ± 3	9[d]
B6.I-1 x Tg(MoPrP-B$^{+/0}$)15	a/b	bbb/0	1	4	166 ± 2	11[c]
B6.I-1 x Tg(MoPrP-B$^{+/0}$)15	a/b	bbb/0	1	4	162 ± 3	8[d]
C57BL/6J (B6)	a/a		2	0	143 ± 4	8
B6.I-4	a/a		2	0	144 ± 5	8
non-Tg(MoPrP-B$^{0/0}$)15	a/a		2	0	130 ± 3	10
Tg(MoPrP-B$^{+/0}$)15	a/a	bbb/0	2	3	115 ± 2	18
Tg(MoPrP-B$^{+/+}$)15	a/a	bbb/bbb	2	6	111 ± 5	5
Tg(MoPrP-B$^{+/0}$)94	a/a	>30b	2	>30	75 ± 2	15[e]
Tg(MoPrP-A$^{+/0}$)B4053	a/a	>30a	>30	0	50 ± 2	16

[a]Data from Prusiner et al. (1993).

[b]Data from Carlson et al. (1993).

[c]The homozygous Tg(MoPrP-B$^{+/+}$)15 mice were maintained as a distinct subline selected for transgene homozygosity two generations removed from the (B6 x LT/Sv) F_2 founder. Hemizygous Tg(MoPrP-B$^{+/0}$)15 mice were produced by crossing the Tg(MoPrP-B$^{+/+}$)15 line with B6 mice.

[d]Tg(MoPrP-B$^{+/0}$)15 mice were maintained by repeated backcrossing to B6 mice.

[e]Data from Westaway et al. (1991).

acquired during prion passage. The effect of the allotype barrier was small when measured in *Prnp*$^{a/a}$ mice but was clearly demonstrable in *Prnp*$^{b/b}$ mice. B6.I-2 congenic mice inoculated with prions from I/Ln mice had an incubation time of 237 ± 8 days compared to times of 360 ± 16 days and 404 ± 4 days for mice inoculated with prions passaged in CD-1 and NZW mice, respectively. Thus, previous passage of prions in *Prnp*^{b} mice shortened the incubation time by about 40% when assayed in *Prnp*^{b} mice, compared to those inoculated with prions passaged in *Prnp*^{a} mice (Carlson et al. 1989).

Overdominance

The phenomenon of "overdominance," in which incubation times in F_1 hybrids are longer than those of either parent (Dickinson and Meikle

1969), contributed to the confusion surrounding control of scrapie incubation times. When the 22A scrapie isolate was inoculated into B6, B6.I-1, and (B6 × B6.I-1)F_1, overdominance was observed: the scrapie incubation time in B6 mice was 405 ± 2 days, in B6.I mice 194 ± 10 days, and in (B6 × B6.I-1) F_1 mice 508 ±14 days (Table 4). Shorter incubation times were observed in Tg(MoPrP-B)15 mice that were either homozygous or hemizygous for the *Prnp*^{b} transgene. Hemizygous Tg(MoPrP-B$^{+/0}$)15 mice exhibited a scrapie incubation time of 395 ± 12 days, whereas the homozygous mice had an incubation time of 286 ± 15 days.

As with the results with the RML isolate (Table 3), the findings with the 22A isolate can be explained on the basis of gene dosage; however, the relative effects of the *a* and *b* alleles differ in two respects. First, the *b* allele is the major determinant of the scrapie incubation time with the 22A isolate, not the *a* allele. Second, increasing the number of copies of the *a* allele does not diminish the incubation time but prolongs it: The *a* allele is inhibitory with the 22A isolate (Table 4). With the 87V prion isolate, the inhibitory effect of the *Prnp*^{a} allele is even more pronounced, since only a few *Prnp*^{a} and (*Prnp*^{a} × *Prnp*^{b}) F_1 mice develop scrapie after >600 days post-inoculation (Carlson et al. 1994).

The most interesting feature of the incubation time profile for 22A is the overdominance of the *a* allele of *Prnp* in prolonging the incubation period. On the basis of overdominance, Dickinson and Outram put forth the replication site hypothesis postulating that dimers of the *Sinc* gene product feature in the replication of the scrapie agent (Dickinson and Outram 1979). The results in Table 4 are compatible with the interpreta-

Table 4 Influence of MoPrP-B transgene expression on incubation times in mice inoculated with 22A scrapie prions

Mice	*Prnp* genotype	*Prnp* transgenes (copies)	Alleles *a* (copies)	Alleles *b* (copies)	Incubation time (days ± S.E.M.)	*n*
B6.I-1	b/b		0	2	194 ± 10	7
(B6 × B6.I-1)F1	a/b		1	1	508 ± 14	7
C57BL/6J (B6)	a/a		2	0	405 ± 2	8
non-Tg(MoPrP-B$^{0/0}$)15	a/a		2	0	378 ± 8	3[a]
Tg(MoPrP-B$^{+/0}$)15	a/a	bbb/0	2	3	318 ± 14	15[a]
Tg(MoPrP-B$^{+/0}$)15	a/a	bbb/0	2	3	395 ± 12	6[b]
Tg(MoPrP-B$^{+/+}$)15	a/a	bbb/bbb	2	6	266 ± 1	6[a]
Tg(MoPrP-B$^{+/+}$)15	a/a	bbb/bbb	2	6	286 ± 15	5[b]

[a]The homozygous Tg(MoPrP-B$^{+/+}$)15 mice were maintained as a distinct subline selected for transgene homozygosity two generations removed from the (B6 × LT/Sv) F_2 founder. Hemizygous Tg(MoPrP-B$^{+/0}$)15 mice were produced by crossing the Tg(MoPrP-B$^{+/+}$)15 line with B6 mice.

[b]Tg(MoPrP-B$^{+/0}$)15 mice were maintained by repeated backcrossing to B6 mice.

tion that the target for PrP^{Sc} may be a PrP^{C} dimer or multimer. The assumptions under this model are that PrP^{C}-B dimers are more readily converted to PrP^{Sc} than are PrP^{C}-A dimers and that PrP^{C}-A:PrP^{C}-B heterodimers are even more resistant to conversion to PrP^{Sc} than PrP^{C}-A dimers. Increasing the ratio of PrP-B to PrP-A would lead to shorter incubation times by favoring the formation of PrP^{C}-B homodimers (Table 4). A similar mechanism may account for the relative paucity of individuals heterozygous for the Met/Val polymorphism at codon 129 of the human PrP gene in spontaneous and iatrogenic CJD (Palmer et al. 1991). Alternatively, the PrP^{C}–PrP^{Sc} interaction can be broken down into two distinct aspects: binding affinity and efficacy of conversion to PrP^{Sc}. If PrP-A has a higher affinity for 22A PrP^{Sc} than does PrP^{C}-B but is inefficiently converted to PrP^{Sc}, the exceptionally long incubation time of *Prnp*$^{a/b}$ heterozygotes might reflect a reduction in the supply of 22A prions available for interaction with the PrP^{C}-B product of the single *Prnp*^{b} allele. Additionally, PrP^{C}-A may inhibit the interaction of 22A PrP^{Sc} with PrP^{C}-B, leading to prolongation of the incubation time. This interpretation is supported by prolonged incubation times in Tg(SHaPrP) mice inoculated with mouse prions in which $SHaPrP^{C}$ is thought to inhibit the binding of $MoPrP^{Sc}$ to the substrate $MoPrP^{C}$ (Prusiner et al. 1990).

STRAINS OF PRIONS

Experimental transmission of prion diseases to laboratory animals has been extensively studied over the past three decades (Ridley and Baker 1996). The diversity of scrapie prions was first appreciated in goats inoculated with "hyper" and "drowsy" isolates (Pattison and Millson 1961b). Scrapie isolates or "strains" from goats with a drowsy syndrome transmitted a similar syndrome to inoculated recipients, whereas those from goats with a hyper or ataxic syndrome transmitted an ataxic form of scrapie to recipient goats. Studies in mice also demonstrated the existence of prion strains where extracts producing a particular pattern of disease could be repeatedly passaged (Dickinson and Fraser 1979; Bruce and Dickinson 1987; Kimberlin et al. 1987; Dickinson and Outram 1988). Although the clinical signs of scrapie for different prion strains in mice tended to be similar, the isolates could be distinguished by the incubation times, the distribution of CNS vacuolation that they produced, and amyloid plaque formation.

The existence of prion strains raises the question of how heritable biological information can be enciphered in any molecule other than nucleic acid (Dickinson et al. 1968; Kimberlin 1982, 1990; Dickinson

and Outram 1988; Bruce et al. 1991; Weissmann 1991; Ridley and Baker 1996). These strains or isolates bred true during propagation through multiple passages in mice and, thus, suggested that the scrapie pathogen has a nucleic acid genome that encodes progeny prions (Dickinson et al. 1968; Bruce and Dickinson 1987). However, no evidence for a scrapie-specific nucleic acid encoding information that specifies the incubation time and the distribution of neuropathologic lesions has emerged from considerable efforts using a variety of experimental approaches.

The typing of prion strains in C57BL, VM, and F_1 (C57BL x VM) inbred mice began with isolates from sheep with scrapie. The prototypic strains called Me7 and 22A gave incubation times of about 150 and about 400 days in C57BL mice, respectively (Dickinson et al. 1968; Dickinson and Meikle 1969; Bruce and Dickinson 1987). The PrP genes of C57BL and I/Ln (and later VM) mice encode proteins differing at two residues and control scrapie incubation times (Carlson et al. 1986, 1988, 1994; Hunter et al. 1987; Westaway et al. 1987; Moore et al. 1998).

Strains or varieties of prions were initially defined by incubation times and the distribution of neuronal vacuolation (Dickinson et al. 1968; Fraser and Dickinson 1973). Subsequently, the patterns of PrP^{Sc} deposition were found to correlate with vacuolation profiles, and these patterns were also used to characterize strains of prions (Bruce et al. 1989; Hecker et al. 1992; DeArmond et al. 1993). Moreover, mice expressing PrP transgenes demonstrated that the level of PrP expression is inversely related to the incubation time (Prusiner et al. 1990). Furthermore, the distribution of CNS vacuolation and attendant gliosis are a consequence of the pattern of PrP^{Sc} deposition, which can be altered by both PrP genes and non-PrP genes (Prusiner et al. 1990). These observations taken together began to build an argument for PrP^{Sc} as the information molecule in which prion "strain"-specific information is encrypted (Prusiner 1991; Cohen et al. 1994).

In retrospect, another important clue to the mechanism of prion strains lay in studies on the passage of prions to a host of a different species. New strains of prions were isolated when inocula from mice were passaged into hamsters (Kimberlin and Walker 1978). With the isolation of PrP 27-30 and subsequently PrP^{Sc}, it became clear that each species encodes a different PrP. Tg mouse studies showed that the "species barrier" for transmission between mice and hamsters could be abrogated by expression of the hamster PrP gene (Scott et al. 1989). Recently, passage of mouse prions into mice expressing chimeric SHa/Mo PrP transgenes has resulted in the isolation of new strains with novel features that become evident when passaged in hamsters (Scott et al. 1993, 1997). For example, Me7 prions that had been passaged and cloned by limiting dilu-

tion in C57BL mice gave rise to a strain designated Me7H with an incubation time of about 260 days when passaged directly into Syrian hamsters (Kimberlin et al. 1987). When Me7 prions from C57BL mice were passaged into Tg mice expressing chimeric MHu2M PrP and then into Syrian hamsters, a new strain with an incubation time of about 80 days was isolated. By changing the PrP gene during the initial passage from non-Tg mice to a new host, different strains of prions were isolated, which argues that prion diversity resides in PrP (Scott et al. 1997).

Until recently, support for the hypothesis that the tertiary structure of PrP^{Sc} enciphers strain-specific information (Prusiner 1991) was minimal except for the DY strain isolated from mink with transmissible encephalopathy (Marsh et al. 1991; Bessen and Marsh 1992, 1994). PrP^{Sc} in DY prions showed diminished resistance to proteinase K digestion, as well as a peculiar site of cleavage. The DY strain presented a puzzling anomaly since other prion strains exhibiting similar incubation times did not show this altered susceptibility to proteinase K digestion of PrP^{Sc} (Scott et al. 1997). For example, the 139H and Me7H strains of prions passaged in hamsters also exhibit prolonged incubation times like DY prions but the protease resistance of PrP^{Sc} is similar to that of the Sc237 strain (McKinley et al. 1983; Hecker et al. 1992; Scott et al. 1997). Indeed, such comparisons argue for an auxiliary molecule such as a scrapie-specific nucleic acid (Dickinson and Outram 1979, 1988; Kimberlin 1990; Weissmann 1991). Although the binding of radiolabeled PrP^{C} to PrP^{Sc} isolated from DY-infected hamster brain provides additional evidence for the specificity of binding, conversion of PrP^{C} into PrP^{Sc} was not demonstrable (Bessen et al. 1995). Although the binding of PrP^{C} in register with DY PrP^{Sc} is of interest, the inability to isolate the bound PrP^{C} and to measure its physical and biological properties severely limits the conclusions that can be legitimately drawn. Also notable are studies on the variations in PrP^{Sc} structure when one strain of hamster prions and two strains of mouse prions were compared (Kascsak et al. 1985). However, the results were confusing because hamster and mouse PrP have different sequences and thus, the interpretation was inconclusive (Merz et al. 1984a,b; Carp et al. 1985, 1994, 1997; Özel and Diringer 1994).

PrP^{Sc} Conformation Enciphers Diversity

Persuasive evidence that strain-specific information is enciphered in the tertiary structure of PrP^{Sc} comes from transmission of two different inherited human prion diseases to mice expressing a chimeric MHu2M PrP transgene (Telling et al. 1996). In fatal familial insomnia (FFI), the protease-resistant

Table 5 Distinct prion strains generated in humans with inherited prion diseases and transmitted to transgenic mice

Inoculum	Host species	Host PrP genotype	Incubation time (days ± S.E.M.)	(n/n_o)	PrP^{Sc} (kD)
None	human	FFI(D178N,M129)			19
FFI	mouse	Tg(MHu2M)	206 ± 7	(7/7)	19
FFI→Tg(MHu2M)	mouse	Tg(MHu2M)	136 ± 1	(6/6)	19
None	human	fCJD(E200K)			21
fCJD	mouse	Tg(MHu2M)	170 ± 2	(10/10)	21
fCJD→Tg(MHu2M)	mouse	Tg(MHu2M)	167 ± 3	(15/15)	21

Data from Telling et al. (1996 and in prep.).

fragment of PrP^{Sc} after deglycosylation has an M_r of 19 kD, whereas in fCJD(E200K) and most sporadic prion diseases, it is 21 kD (Table 5) (Monari et al. 1994; Parchi et al. 1996). This difference in molecular size was shown to be due to different sites of proteolytic cleavage at the amino termini of the two human PrP^{Sc} molecules, which reflects different tertiary structures (Monari et al. 1994). These distinct conformations were understandable since the amino acid sequences of the PrPs differ.

Extracts from the brains of FFI patients transmitted disease to mice expressing a chimeric MHu2M PrP gene about 200 days after inoculation and induced formation of the 19-kD PrP^{Sc}, whereas fCJD(E200K) and sCJD produced the 21-kD PrP^{Sc} in mice expressing the same transgene (Telling et al. 1996). On second passage, Tg(MHu2M) mice inoculated with FFI prions showed an incubation time of about 130 days and a 19-kD PrP^{Sc}, whereas those inoculated with fCJD(E200K) prions exhibited an incubation time of about 170 days and a 21-kD PrP^{Sc} (Prusiner 1997). The experimental data demonstrate that MHu2M PrP^{Sc} can exist in two different conformations based on the sizes of the protease-resistant fragments; yet, the amino acid sequence of MHu2M PrP^{Sc} is invariant.

The results of our studies argue that PrP^{Sc} acts as a template for the conversion of PrP^{C} into nascent PrP^{Sc}. Imparting the size of the protease-resistant fragment of PrP^{Sc} through conformational templating provides a mechanism for both the generation and propagation of prion strains.

Interestingly, the protease-resistant fragment of PrP^{Sc} after deglycosylation with an M_r of 19 kD has been found in a patient who developed a sporadic case of prion disease similar to FFI but with no family history. Since both PrP alleles encoded the wild-type sequence and a methionine at position 129, we labeled this case fatal sporadic insomnia (FSI). At autopsy, the spongiform degeneration, reactive astrogliosis, and PrP^{Sc}

deposition were confined to the thalamus (Mastrianni et al. 1997). These findings argue that the clinicopathologic phenotype is determined by the conformation of PrP^{Sc}, in accord with the results of the transmission of human prions from patients with FFI to Tg mice (Telling et al. 1996).

Selective Neuronal Targeting

Besides incubation times, profiles of spongiform change (Fig. 4) have been used to characterize prion strains (Fraser and Dickinson 1968), but recent studies argue that such profiles are not an intrinsic feature of strains (Carp et al. 1997; DeArmond et al. 1997). The mechanism by which prion strains modify the pattern of spongiform degeneration was perplexing since earlier investigations had shown that PrP^{Sc} deposition precedes neuronal vacuolation and reactive gliosis (Jendroska et al. 1991; Hecker et al. 1992). When FFI prions were inoculated into Tg(MHu2M) mice, PrP^{Sc} was confined largely to the thalamus (Fig. 5A), as is the case for FFI in humans (Medori et al. 1992; Telling et al. 1996). In contrast, fCJD(E200K) prions inoculated into Tg(MHu2M) mice produced widespread deposition of PrP^{Sc} throughout the cortical mantle and many of the deep structures of the CNS (Fig. 5B), as is seen in fCJD(E200K) of humans. To examine whether the diverse patterns of PrP^{Sc} deposition are influenced by asparagine-linked glycosylation of PrP^{C}, we constructed Tg mice expressing PrPs mutated at one or both of the asparagine-linked glycosylation consensus sites (DeArmond et al. 1997). These mutations resulted in aberrant neuroanatomic topologies of PrP^{C} within the CNS, whereas pathologic point mutations adjacent to the consensus sites did not alter the distribution of PrP^{C}. Tg mice with mutation of the second PrP glycosylation site exhibited prion incubation times of >500 days and unusual patterns of PrP^{Sc} deposition. These findings raise the possibility that glycosylation can modify the conformation of PrP and affect either the turnover of PrP^{C} or the clearance of PrP^{Sc}. Regional differences in the rate of deposition or clearance would result in specific patterns of PrP^{Sc} accumulation.

SPREAD OF PRIONS AMONG CELLS AND ORGANS

It is not known how prions spread from one cell to another. It seems most likely that PrP^{C} on the surface of cells acts as a receptor for PrP^{Sc}. Recent studies suggest that PrP^{C} is converted into a metastable state (PrP*) that is then capable of interacting with PrP^{Sc}. PrP* has been generated in vitro

by exposure of PrP^C to 3 M guanidine hydrochloride (GdnHCl) and subsequent dilution to 0.3 M GdnHCl (Kocisko et al. 1994, 1995; Kaneko et al. 1995, 1997a). It appears that PrP* formation can be prevented in cultured cells by compounds that stabilize protein conformation such as

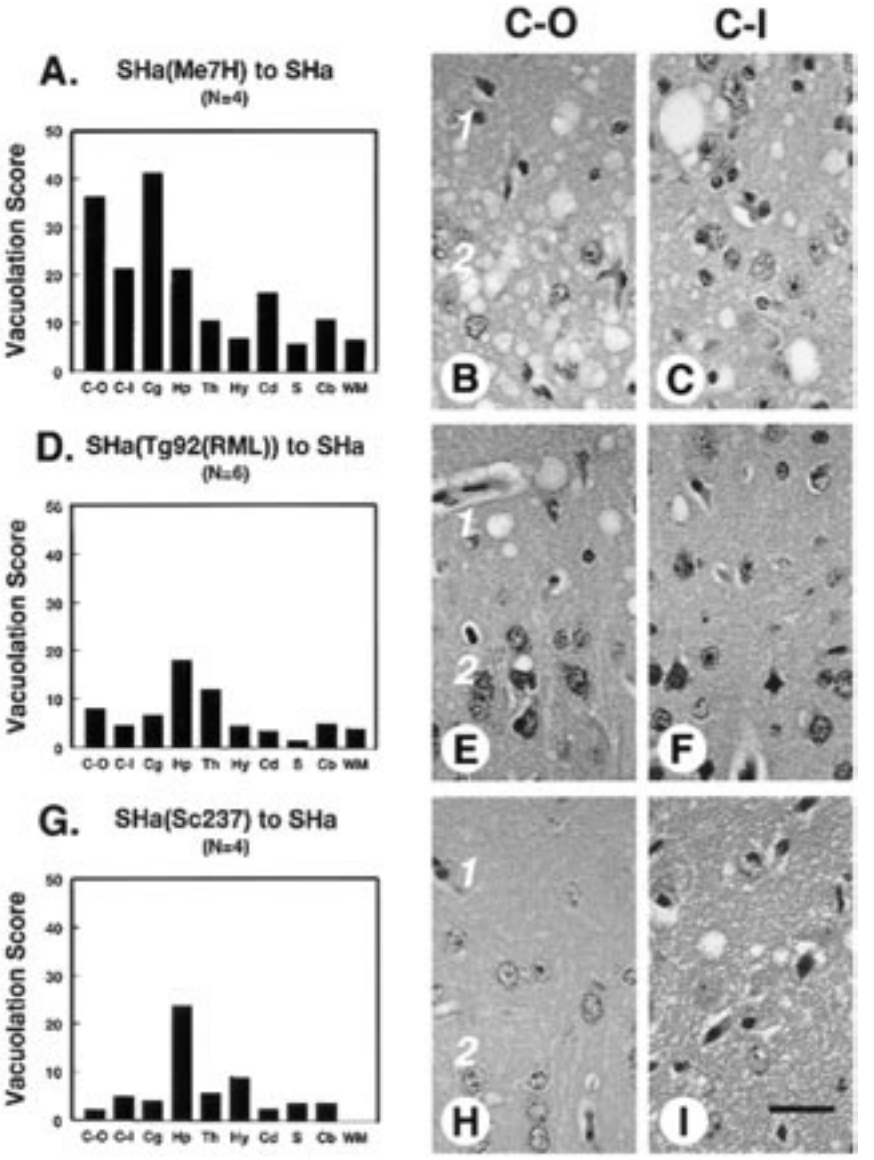

Figure 4 (See facing page for legend.)

glycerol and dimethylsulfoxide (DMSO) (Tatzelt et al. 1996a). The formation of PrP* in vivo probably occurs as a consequence of the binding of PrP^{C} to protein X; the PrP*/protein X complex is then capable of binding PrP^{Sc} (Telling et al. 1995; Kaneko et al. 1997b). Protein X is likely to be a chaperone-like macromolecule that facilitates PrP^{Sc} formation.

Prions have been found in blood in both scrapie and CJD. Studies with the buffy coat from rodents have shown the presence of CJD prions within white blood cells (Manuelidis et al. 1978; Tateishi 1985; Chapter 12). Low levels of scrapie prions have been found in the blood of hamsters through the course of infection (Diringer 1984; Casaccia et al. 1989). Both scrapie and CJD prions have also been detected in cerebrospinal fluid (Pattison et al. 1959, 1964; Gajdusek et al. 1977).

Peripheral inoculation studies suggest that prions may also spread along neural tracts within both the central and peripheral nervous systems

Figure 4 Neuroanatomic distribution of spongiform degeneration in the gray and white matter is specified by prion strains. Three scrapie prion strains were passaged in Syrian hamsters (SHa) and were inoculated intrathalamically with 30 µl of brain extracts prepared from animals infected with different strains of prions designated Me7H (*A–C*), the RML(SHa) (*D–F*), and Sc237 (*G–I*). The Me7H strain was generated by passage of mouse Me7 prions into SHa (Kimberlin et al. 1989). The RML(SHa) prion strain was generated by passage of RML mouse prions through $Tg(MH2M)92/Prnp^{0/0}$ mice and then into SHa (Scott et al. 1997). Semiquantitative estimates of the intensity of vacuolation as a function of brain region are displayed as histograms (*A,D,G*) (Fraser and Dickinson 1968). Vacuolation score is an estimate of the area of a hematoxylin and eosin (H&E) stained brain section occupied by vacuoles (*N* is the number of animals examined) (Carlson et al. 1994). H&E stained histological sections of layers 1 and 2 of the outer cerebral cortex (C-O) and layer 5 of the inner cerebral cortex (C-I) are presented for each of the three strains. *(A)* With Me7H, there is a characteristic intense vacuolation of all layers of the cerebral cortex as shown in *B*, layers 1 and 2 of the outer cerebral cortex (labeled *1* and *2* in white), and in *C*, layer 5 of the inner cortex. The vacuolation score for RML(SHa) is similar to that for Sc237 (compare *D* and *G*); however, there are notable differences. For example, there is significant vacuolation of layer 1 of the C-O layers that is characteristic of RML(SHa) and none with Sc237 (compare *E* and *H*). Mild but definitive vacuolation occurred in layer 5 of the C-I with both RML(SHa) and Sc237 (compare *F* and *I*). Bar in *I* is 25 µm and applies to all H&E stained sections. (C-O) Outer half of cerebral cortex; (C-I) inner half of cerebral cortex; (Cg) cingulate gyrus; (Hp) hippocampus; (Th) thalamus; (Hy) hypothalamus; (Cd) caudate nucleus; (S) septal nuclei; (Cb) cerebellum; and (WM) white matter. (Photomicrographs and bar graphs prepared by Stephen J. DeArmond.)

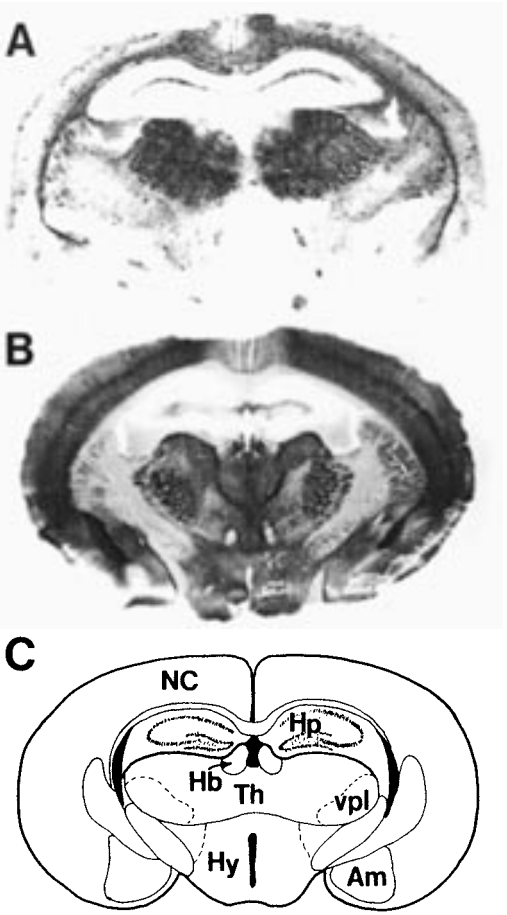

Figure 5 Regional distribution of PrP^{Sc} deposition in Tg(MHu2M)*Prnp*$^{0/0}$ mice inoculated with prions from humans who died of inherited prion diseases. Histoblot of PrP^{Sc} deposition in a coronal section of a Tg(MHu2M)*Prnp*$^{0/0}$ mouse through the hippocampus and thalamus (Telling et al. 1996). (*A*) The Tg mouse was inoculated with brain extract prepared from a patient who died of FFI. (*B*) The Tg mouse was inoculated with extract from a patient with fCJD(E200K). Cryostat sections were mounted on nitrocellulose and treated with proteinase K to eliminate PrP^{C} (Taraboulos et al. 1992). To enhance the antigenicity of PrP^{Sc}, the histoblots were exposed to 3 M guanidinium isothiocyanate before immunostaining using α-PrP 3F4 monoclonal antibody (Kascsak et al. 1987). (*C*) Labeled diagram of a coronal section of the hippocampus/thalamus region. (NC) Neocortex; (Hp) hippocampus; (Hb) habenula; (Th) thalamus; (vpl) ventral posterior lateral thalamic nucleus; (Hy) hypothalamus; (Am) amygdala. (Reprinted, with permission, from Prusiner et al. 1998 [copyright Cell Press].)

(Fraser 1982; Kimberlin et al. 1983; Fraser and Dickinson 1985; Beekes et al. 1996). Presumably, prions are transported by axonal retrograde transport systems (Borchelt et al. 1994). The retrograde transport of rabies virus through the nervous system is well documented.

Studies with immunodeficient SCID mice have shown that incubation times are prolonged after intraperitoneal but not intracerebral inoculation with scrapie prions compared to control immunocompetent mice (O'Rourke et al. 1994; K.L. Brown et al. 1996b; Fraser et al. 1996; Lasmézas et al. 1996). In studies with immunocompromised mice in which genes required for T- or B-cell function were ablated, B-lymphocytes were found to play an important role in neuroinvasiveness (Klein et al. 1997). In other studies, follicular dendritic cells in the spleens of mice were found to accumulate PrP^{Sc} early after intraperitoneal inoculation with CJD prions (Kitamoto et al. 1991).

In cell culture studies, prions have been found in the media. The prions in the media have been shown to be capable of infecting cells in culture that were previously not infected. Both N2a and GT1 cells have been used in such studies (Schätzl et al. 1997).

ROLE OF PrP IN PRION INFECTION

An absolute requirement for PrP^{C} in prion replication was surmised from the large body of evidence showing that PrP^{Sc} is a major component of the infectious prion particle (Prusiner 1991, 1992). That view was elegantly confirmed by experiments with mice in which the PrP gene had been genetically ablated ($Prnp^{0/0}$) (Büeler et al. 1992). $Prnp^{0/0}$ mice were found to be resistant to prion disease and not to replicate prions. In two initial studies, no evidence of prion disease could be found many months after inoculation of $Prnp^{0/0}$ mice with RML prions (Büeler et al. 1993; Prusiner et al. 1993; Chapter 7).

The brain of the Syrian hamster contains the highest levels of PrP poly$(A)^{+}$ RNA, whereas other organs have lower levels (Oesch et al. 1985). In the CNS, PrP mRNA is found primarily in neuronal cells (Kretzschmar et al. 1986). During scrapie infection, the highest final titers of prions in both hamsters and mice are found in the CNS. All other organs have lower prion titers (Table 1) (Eklund et al. 1967; Kimberlin and Walker 1977). These observations suggested that the accumulation of prions might be regulated by the level of PrP mRNA in cells; subsequently, studies of Tg(SHaPrP) mice showed that the length of the incubation time was inversely proportional to the level of PrP^{C} expression (Prusiner et al. 1990). Moreover, the level of PrP^{Sc} at the time of clinical

illness was independent of the incubation time. These findings argued that the rate of PrP^{Sc} formation is proportional to the level of PrP^{C} expression and that signs of neurologic dysfunction appear when PrP^{Sc} levels reach a threshold.

Since the extracellular accumulation of PrP^{Sc} in the form of amyloid plaques was shown to be a nonobligatory feature of prion disease in humans and mice, it was postulated that intracellular PrP^{Sc} must be required for neuronal vacuolation to develop (Prusiner et al. 1990). This hypothesis is supported by the findings with prion-infected grafts of CNS tissue in $Prnp^{0/0}$ mice that develop vacuolar changes while the surrounding PrP-deficient neurons remain healthy (Brandner et al. 1996). Interestingly, primary cultures of neurons from $Prnp^{0/0}$ mice do not exhibit neurotoxicity when exposed to PrP peptides, whereas those from normal mice do, under these conditions (D.R. Brown et al. 1994, 1996). While the neurons undergo degeneration during scrapie infection, the surrounding astrocytes become hypertrophic and exhibit high levels of GFAP mRNA and protein (Mackenzie 1983). Genetic ablation of the GFAP gene in mice did not alter the incubation time, which indicates that GFAP does not play a primary role in the neuronal degeneration found in prion disease (Gomi et al. 1995; Tatzelt et al. 1996b).

Consistent with the foregoing studies are results with newborn hamsters that do not have any detectable brain PrP mRNA as measured by Northern analysis and by immunoprecipitation of cell-free translation products. Detectable levels of PrP poly$(A)^{+}$ RNA appear about 1 day after birth and stay very low until 8 days after birth; PrP mRNA reaches a maximum level by 10 days of age (McKinley et al. 1987). After 10 days of age the level of PrP mRNA in brain remains constant. These observations are of interest with respect to scrapie infection in newborn mice. Inoculation of newborn mice resulted in a delay of prion replication for almost 1 year (Hotchin and Buckley 1977).

NATURAL AND EXPERIMENTAL SCRAPIE

Although scrapie was recognized as a distinct disorder of sheep as early as 1738, the disease remained enigmatic even with respect to its pathology for more than two centuries (Parry 1983). Some veterinarians thought that scrapie was a disease of muscle caused by parasites, while others thought that it was a dystrophic process (M'Gowan 1914).

Scrapie of sheep and goats appears to be unique among the prion diseases in that it is communicable within flocks. Although the transmissibility of scrapie seems to be well established, the mechanism of the nat-

ural spread of scrapie among sheep is so puzzling that it bears close scrutiny. The placenta has been implicated as one source of prions, which accounts for the horizontal spread of scrapie within flocks (Pattison and Millson 1961a; Pattison 1964; Pattison et al. 1972; Onodera et al. 1993). Whether or not this view is correct remains to be established. In Iceland, scrapied flocks of sheep were destroyed and the pastures left vacant for several years; however, reintroduction of sheep from flocks known to be free of scrapie for many years eventually resulted in scrapie (Palsson 1979). The source of prions that infected the sheep from flocks without a history of scrapie is unknown.

Parry argued that host genes were responsible for the development of scrapie in sheep. He was convinced that natural scrapie is a genetic disease that could be eradicated by proper breeding protocols (Parry 1962, 1983). 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). The incubation time gene for experimental scrapie in Cheviot sheep, called *Sip*, is said to be linked to a PrP gene RFLP (Hunter et al. 1989); however, the null hypothesis of non-linkage has yet to be tested and this is important, especially in view of earlier studies that argue that susceptibility of sheep to scrapie is governed by a recessive gene (Parry 1962, 1983).

Polymorphisms that produce amino acid substitutions at codons 136 and 171 of the PrP gene in sheep have been studied with respect to the occurrence of scrapie (Clousard et al. 1995). In Romanov and Ile-de-France breeds of sheep, a polymorphism in the PrP open reading frame (ORF) was found at codon 136 (A→V), which seems to correlate with scrapie (Laplanche et al. 1993b). Sheep homozygous or heterozygous for valine at codon 136 were susceptible to scrapie, whereas those that were homozygous for alanine were resistant. Unexpectedly, only one of 74 scrapied autochthonous sheep had a valine at codon 136; these sheep were from three breeds denoted Lacaune, Manech, and Presalpes (Laplanche et al. 1993a).

In Suffolk sheep, a polymorphism in the PrP ORF was found at codon 171 (Q→R) (Goldmann et al. 1990a,b). Studies of natural scrapie in the United States have shown that about 85% of the afflicted sheep are of the Suffolk breed. Scrapie was limited to Suffolk sheep homozygous for glutamine (Q) at codon 171, although healthy controls with QQ, QR, and RR genotypes were found (Westaway et al. 1994). These results argue that susceptibility in Suffolk sheep is governed by the PrP codon-171 poly-

morphism. Other studies of Suffolk sheep and some other breeds support this finding (Hunter et al. 1993, 1997a,b; Goldmann et al. 1994; Belt et al. 1995; Clousard et al. 1995; Ikeda et al. 1995; Bossers et al. 1996; O'Rourke et al. 1997). Recent studies mapping the site on PrP^C that interacts with protein X have shown that an R at sheep PrP residue 171 acts as a dominant negative by increasing the affinity of PrP^C for protein X (Kaneko et al. 1997b). The dominant negative explanation is consistent with the data showing that QR heterozygous sheep rarely develop scrapie. An attempt to explain the resistance of QR heterozygotes to scrapie by decreased binding of PrP^C with an R at position 171 to PrP^{Sc} appears fallacious (Bossers et al. 1997), since the PrP^C(Q171) would be expected to be readily converted to PrP^{Sc} in heterozygotes and to cause disease.

HUMAN PRION DISEASES

Although transmission of prions to humans features in a small minority of cases, such instances continue to receive considerable attention. Less than 1% of human prion diseases seem to have an infectious etiology whereas about 85% are sporadic and the remainder are dominantly inherited through germ-line mutations of the PrP gene.

Kuru and Cannibalism

Cannibalism is one of the most fascinating and macabre of all human activities. Cannibalism was a common but by no means universal practice among the groups of people inhabiting Papua New Guinea in the recent past (Alpers 1979). Among the Fore and their neighbors in the Eastern Highlands, the practice was that of endocannibalism, in which members of the group were eaten after death by their relatives.

The transmissibility of kuru and its disappearance in those born since the cessation of cannibalism strongly implicate cannibalism in the spread of this disorder (Alpers 1979). Cannibalism also explains the sex and age distribution of kuru in the past, since women and young children of both sexes, who ate the internal organs of their relatives, were the ones primarily afflicted (Table 6). Only 2% of the cases were found in adult males. Cannibalism also explains the clustering of cases in space and time that has been noted in multiple epidemiological studies of kuru (Alpers 1987).

Numerous attempts to transmit kuru by feeding nonhuman primates kuru-infected tissues have been unsuccessful, with a few exceptions (Gajdusek 1979; Gibbs et al. 1980). This contrasts with the rather uniform

Table 6 Infectious prion diseases of humans

Diseases	No. of cases
Kuru (1957–1982)	
Adult females	1739
Adult males	248
Children and adolescents	597
Total	2584
Iatrogenic Creutzfeldt-Jakob disease	
Depth electrodes	2
Corneal transplants	1
Human pituitary growth hormone	90
Human pituitary gonadotropin	5
Dura mater grafts	66
Neurosurgical procedures	4
Total	169

References cited in text.

susceptibility of these animals to kuru after intracerebral or peripheral inoculation (Gajdusek 1977). Our observations in hamsters may explain these puzzling results with apes and monkeys since intracerebral inoculation is 10^9 times more efficient than oral ingestion in hamsters (Prusiner et al. 1985).

Incubation Periods Exceeding Three Decades

No individual born in the South Fore after 1959, when cannibalism ceased, has developed kuru (Alpers 1987, 1979; Klitzman et al. 1984). Kuru has progressively disappeared, first among children and thereafter among adolescents. The number of deaths in adult females has decreased steadily and adult male deaths have remained almost invariant. Each year the youngest new patients are older than those of the previous year.

Of several hundred kuru orphans born since 1957 to mothers who later died of kuru, none has yet developed the disease. Thus, the many children with kuru seen in the 1950s were not infected prenatally, perinatally, or neonatally by their mothers, despite evidence for prions in the placenta and colostrum of a pregnant woman who died of CJD (Tamai et al. 1992). Attempts to demonstrate consistent transmission of prion disease from mother to offspring in experimental animals have been unsuccessful (Morris et al. 1965; Pattison et al. 1972; Manuelidis and Manuelidis 1979a; Amyx et al. 1981; Taguchi et al. 1993).

Whereas patients currently afflicted with kuru exhibit greatly prolonged incubation periods, children with kuru who were observed 30 years ago provide some information on the minimum incubation period. The youngest patient with kuru was 4 years old at the onset of the disease and died at age 5, but it is not known at what age young children were infected. CJD accidentally transmitted to humans has required only 18 months after intracerebral or intraoptic inoculation (Bernouilli et al. 1977; Duffy et al. 1974) to manifest. An incubation period of 18 months has also been found in chimpanzees inoculated intracerebrally with kuru prions.

The regular disappearance of kuru is inconsistent with the existence of any natural reservoirs for kuru besides humans. Indeed, there is no evidence for animal or insect reservoirs. Thus, patients dying of kuru over the past decade seem to have incubation periods exceeding 2 or even 3 decades (Prusiner et al. 1982a; Klitzman et al. 1984; Alpers 1987).

Iatrogenic Creutzfeldt-Jakob Disease

Accidental transmission of CJD to humans appears to have occurred by corneal transplantation (Duffy et al. 1974), contaminated electroencephalogram (EEG) electrode implantation (Bernouilli et al. 1977), and surgical operations using contaminated instruments or apparatus (Table 6) (Masters and Richardson 1978; Kondo and Kuroina 1981; Will and Matthews 1982; Davanipour et al. 1984). A cornea unknowingly removed from a donor with CJD was transplanted to an apparently healthy recipient who developed CJD after a prolonged incubation period. Corneas of animals have significant levels of prions (Buyukmihci et al. 1980), making this scenario seem quite probable. The same improperly decontaminated EEG electrodes that caused CJD in two young patients with intractable epilepsy were found to cause CJD in a chimpanzee 18 months after being experimentally implanted (Bernouilli et al. 1979; Gibbs et al. 1994).

Surgical procedures may have resulted in accidental inoculation of patients with prions during their operations (Gajdusek 1977; Will and Matthews 1982; Brown et al. 1992), presumably because some instrument or apparatus in the operating theater became contaminated when a CJD patient underwent surgery. Although the epidemiology of these studies is highly suggestive, no proof of transmission exists.

Dura Mater Grafts

Since 1988, more than 60 cases of CJD occurring after implantation of dura mater grafts have been recorded (Otto 1987; Thadani et al. 1988;

Masullo et al. 1989; Nisbet et al. 1989; Miyashita et al. 1991; Willison et al. 1991; Brown et al. 1992; Martínez-Lage et al. 1993; CDC 1997). All of the grafts were thought to have been acquired from a single manufacturer whose preparative procedures were inadequate to inactivate human prions (Brown et al. 1992). One case of CJD occurred after repair of an eardrum perforation with a pericardium graft (Tange et al. 1989).

Thirty cases of CJD in physicians and health care workers have been reported (Berger and David 1993); however, no occupational link has been established (Ridley and Baker 1993). Whether any of these cases represent infectious prion diseases contracted during care of patients with CJD or processing specimens from these patients remains uncertain.

Human Growth Hormone Therapy

The likelihood of transmission of CJD from contaminated human growth hormone (HGH) preparations derived from human pituitaries has been raised by the occurrence of fatal cerebellar disorders with dementia in more than 90 patients ranging in age from 10 to 41 years (Table 6) (Brown 1985; Buchanan et al. 1991; Fradkin et al. 1991; Brown et al. 1992; Billette de Villemeur et al. 1996; Public Health Service Interagency Coordinating Committee 1997). One case of spontaneous CJD in a 20-year-old woman has been reported (Gibbs et al. 1985; Brown 1985; Packer et al. 1980), CJD in patients under 40 years of age is very rare. These patients received injections of HGH every 2–4 days for 4–12 years (Gibbs et al. 1985; Koch et al. 1985; Powell-Jackson et al. 1985; Titner et al. 1986; Croxson et al. 1988; Marzewski et al. 1988; New et al. 1988; Anderson et al. 1990; Billette de Villemeur et al. 1991; Macario et al. 1991; Ellis et al. 1992). Interestingly, most of the patients presented with cerebellar syndromes that progressed over periods varying from 6 to 18 months (Brown et al. 1992). Some patients became demented during the terminal phase of illness. In some respects, this clinical course resembles kuru more than ataxic CJD (Prusiner et al. 1982a). Assuming these patients developed CJD from injections of prion-contaminated HGH preparations, the possible incubation periods range from 4 to 30 years (Brown et al. 1992). The longest incubation periods are similar to those (20–30 years) associated with recent cases of kuru (Gajdusek et al. 1977; Prusiner et al. 1982a; Klitzman et al. 1984). Many patients received several common lots of HGH at various times during their prolonged therapies, but no single lot was administered to all the American patients. An aliquot of one lot of HGH has been reported to transmit CNS disease to a squirrel monkey after a prolonged incubation period

(Gibbs et al. 1993). How many lots of HGH might have been contaminated with prions is unknown.

Although CJD is a rare disease with an annual incidence of approximately one per million population (Masters and Richardson 1978), it is reasonable to assume that CJD is present with a proportional frequency among dead people. About 1% of the population dies each year and most CJD patients die within 1 year of developing symptoms. Thus, we estimate that one per 10^4 dead people have CJD. Since 10,000 human pituitaries were typically processed in a single HGH preparation, the possibility of hormone preparations contaminated with CJD prions is not remote (P. Brown et al. 1985, 1994; Brown 1988).

The concentration of CJD prions within infected human pituitaries is unknown; it is interesting that widespread degenerative changes have been observed in both the hypothalamus and pituitary of sheep with scrapie (Beck et al. 1964). Forebrains from scrapie-infected mice have been added to human pituitary suspensions to determine whether prions and HGH copurify (Lumley Jones et al. 1979). Bioassays in mice suggest that prions and HGH do not copurify with currently used protocols (Taylor et al. 1985). Although these results seem reassuring, especially for patients treated with HGH over much of the last decade, the relatively low titers of the murine scrapie prions used in these studies may not have provided an adequate test (Brown 1985). The extremely small size and charge heterogeneity exhibited by scrapie (Alper et al. 1966; Prusiner et al. 1978, 1980a, 1983; Bolton et al. 1985) and presumably CJD prions (Bendheim et al. 1985; Bockman et al. 1985) may complicate procedures designed to separate pituitary hormones from these slow infectious pathogens. Although additional investigations argue for the efficacy of inactivating prions in HGH fractions prepared from human pituitaries using 6 M urea (Pocchiari et al. 1991), it seems doubtful that such protocols will be used for purifying HGH since recombinant HGH is available.

Molecular genetic studies have shown that most patients developing iatrogenic CJD after receiving pituitary-derived HGH are homozygous for either methionine or valine at codon 129 of the PrP gene (Collinge et al. 1991; P. Brown et al. 1994; Deslys et al. 1994). Homozygosity at the codon 129 polymorphism has also been shown to predispose individuals to sporadic CJD (Palmer et al. 1991). Interestingly, valine homozygosity seems to be overrepresented in these HGH cases compared to the general population.

Five cases of CJD have occurred in women receiving human pituitary gonadotropin (Cochius et al. 1990, 1992; Healy and Evans 1993).

CONCLUDING REMARKS

Many principles of prion replication are so unprecedented that some investigators have difficulty embracing these mechanisms. Although prion replication resembles viral replication superficially, the underlying principles are quite different. For example, in prion replication, the substrate is a host-encoded protein, PrP^{C}, which undergoes modification to form PrP^{Sc}, the only known component of the infectious prion particle. In contrast, viruses carry a DNA or RNA genome that is copied and directs the synthesis of most, if not all, of the viral proteins. The mature virus consists of a nucleic acid genome surrounded by a protein coat, whereas a prion appears to be composed of a dimer of PrP^{Sc}.

When viruses pass from one species to another, they often replicate without any structural modification, whereas prions undergo a profound change. The prion adopts a new PrP sequence that is encoded by the PrP gene of the current host. Differences in the amino acid sequences can result in a restriction of transmission for some species, while making the new prion permissive for others. In viruses, the different properties exhibited by distinct strains are encoded in the viral genome, but in prions, strain-specific properties seem to be enciphered in the conformation of PrP^{Sc}.

As the body of data on prions continues to grow, changes in our understanding of how prions replicate and cause disease will undoubtedly emerge. However, we hasten to add that many of the basic principles of prion biology are becoming well understood.

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5
Structural Studies of Prion Proteins

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Although many aspects of prion disease biology are unorthodox, perhaps the most fundamental paradox is posed by the coexistence of inherited, sporadic, and infectious forms of these diseases. Sensible molecular mechanisms for prion propagation must explain all three forms of prion diseases in a manner that is compatible with the formidable array of experimental data derived from histopathologic, biochemical, biophysical, human genetic, and transgenetic studies. In this chapter, we explore the phenomenologic constraints on models of prion replication with a specific emphasis on biophysical studies of prion protein structures. We examine how an inherited disease can also present as a sporadic or infectious illness in the context of the structural data on PrPs that are currently available.

THEORY OF PRION DISEASES

The inherited prion diseases include Gerstmann-Sträussler-Scheinker disease (GSS), familial Creutzfeldt-Jakob disease (fCJD), and fatal familial insomnia (FFI). These patients present with characteristic clinical and neuropathologic findings as early as their third or fourth decade of life, and their family histories are compatible with an autosomal dominant pattern of inheritance (Chapter 13). Molecular genetic studies argue that these diseases are caused by mutations in the prion protein (PrP) gene based on high LOD scores for 5 of the 20 known mutations (Hsiao et al. 1989; Dlouhy et al. 1992; Petersen et al. 1992; Poulter et al. 1992; Gabizon et al. 1993). As with many inherited disorders, the pathogenesis of the inherited prion disease is due to the aberrant behavior of the protein encoded by the mutant PrP gene. The altered physical properties of mutant PrP probably result from a change in the conformation of the

mutant protein akin to an allosteric effect; the magnitude of this conformational change can be quite variable. For example, the conformational change in sickle cell hemoglobin or in transthyretin mutants associated with familial amyloid neuropathy (FAP) is largely at the level of quaternary structure, whereas there is evidence that the conformational reorganization of the Alzheimer's βAPP fragment occurs at both the tertiary and quaternary structure levels (Lee et al. 1995; Colon et al. 1996; Kelly 1997). Unfortunately, these multimers are long-lived, exhibit pathologic properties, and have a histopathologic record of their existence. Considered in this context, it is not surprising that the conformation of the normal cellular isoform of the wild-type (wt) prion protein (PrP^{C}) is distinct from the disease-causing isoform of the mutant prion protein ($PrP^{Sc/fCJD}$, $PrP^{Sc/FFI}$, $PrP^{Sc/GSS}$) in both conformation and oligomerization states. However, the magnitude of the conformational rearrangement owing to a point mutation is unexpected.

To explain inherited prion diseases, one need only postulate that the protein can exist in two distinct conformations, one that prefers a monomeric state and a second that multimerizes where the wild type exhibits a dramatic preference for the monomeric state and the mutant preferentially adopts the multimeric state. The origin of this distinction could be kinetic or thermodynamic. Either the differential stability of the wild-type and mutant proteins in the monomeric and multimeric states is large, or a kinetic barrier that essentially precludes the conversion of the wild-type monomer is abrogated by the disease-causing mutations. These two scenarios are contrasted in Figure 1.

Results from a variety of site-directed mutagenesis studies of protein stability suggest that the impact of a single point mutation on the free energy of folding is unlikely to exceed 2–3 kcal (Matthews 1996). If the simple thermodynamic model were operative and the conversion of multimeric PrP^{C} into monomeric were controlled by the differences in the free energies of the ground state, one would expect that wild-type PrP^{Sc} production would be approximately 1% as efficient as mutant PrP^{Sc} formation. Given the resistance of the core of wild-type PrP^{Sc} to proteolytic digestion, PrP^{Sc} would accumulate in the wild-type setting due to the difficulties associated with the metabolic clearance of this molecule. This scenario is at odds with neuropathologic and epidemiologic data on the incidence of sporadic CJD (sCJD). Ionizing irradiation experiments have suggested that the minimally infectious PrP^{Sc} particle is a dimer (Bellinger-Kawahara et al. 1988). If the energetics of dimer formation is simply the sum of the monomeric terms, then wild-type PrP^{Sc} formation would be 0.01% as likely as mutant PrP^{Sc} production. This level of infec-

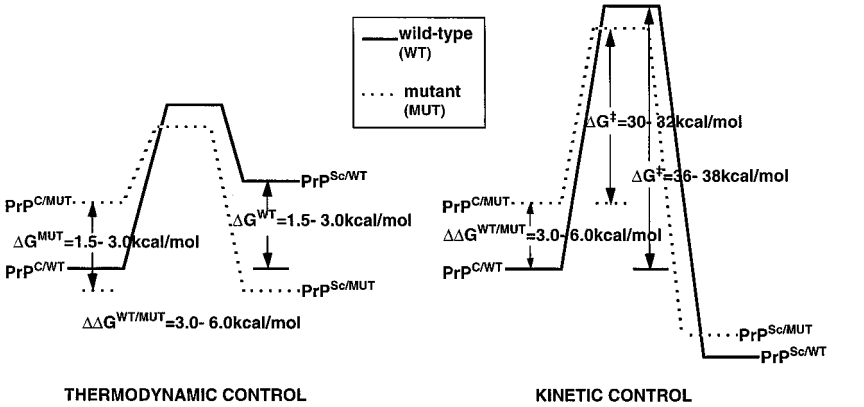

Figure 1 An illustration of the distinction between thermodynamic and kinetic models for the energetics of the conversion of PrP^{C} carrying the wild type (WT) and mutant (MUT) sequences into PrP^{Sc}. $\Delta G^{\ddagger}$ is the free-energy difference between the PrP^{C} and PrP^{Sc} states and ΔG is the activation energy barrier separating these two states. $\Delta\Delta G^{WT/MUT}$ is the difference between ΔG^{WT} and ΔG^{MUT}. The free-energy diagrams for the wild-type sequences are shown in solid lines and the mutant sequences in broken lines.

tivity would be detectable using a variety of immunoassays and is in contrast to experimental results. Sensibly, the cooperativity component of dimer formation is unlikely to be substantially different for the wild-type and mutant forms, because disease-causing mutations exist in several distinct regions of the sequence and are distributed throughout the core of the structure (Huang et al. 1994; Riek et al. 1996; James et al. 1997). Thus, the cooperativity component of the free-energy difference between the cellular and scrapie isoforms of the wild-type and mutant proteins ($\Delta\Delta G^{WT/MUT}$) is unlikely to exceed 2.0–3.0 kcal/mole. Under these extreme assumptions, wild-type PrP^{Sc} in normal cohorts should be 10^{-6} as common as mutant PrP^{Sc} in carriers from affected families. This level of wild-type PrP^{Sc} would be detected in bioassays of infectivity, a prediction that is at odds with a number of inoculation studies of PrP derived from natural and recombinant sources (Mehlhorn et al. 1996; Kaneko et al. 1997a). In contrast, kinetic control over the conversion of PrP^{C} to PrP^{Sc} provides a simple explanation for the observed clinical and experimental results. PrP^{Sc} would need to be only marginally more stable than PrP^{C} (ΔG = –2.0 to –3.0 kcal/mole). To explain the normal absence of PrP^{Sc}, the energetic barriers separating the two states would need to be quite large ($\Delta G^{\ddagger}$= 36 to 38 kcal/mole assuming a transition-state argument). In this

setting, a small change in the activation barrier (e.g., $\Delta\Delta G^{\ddagger}_{WT/MUT} = 2.0$ to 3.0 kcal) would result in approximately 100-fold slowing of the rate of conversion. Thus, the 30- to 40-year prodromal period for inherited prion disease in the mutant setting would become 3000–4000 years with the wild-type protein if this were the rate-limiting step in disease progression. Cooperative effects could further amplify the distinction between the normal cellular and disease-associated isoforms. For example, if the disease-associated isoform were a dimer, then interactions between the monomeric components could provide additional stability from the thermodynamic perspective. The disease-associated isoform could also impact the kinetic aspects of the conversion process by acting as a template that lowers the activation barrier ($\Delta G^{\ddagger}$) for the conformational change in a manner reminiscent of the way an enzyme's active site orchestrates the positioning of substrates to speed the rate of a reaction. In this setting, the disease-associated isoform would also be disease-causing because its presence would dramatically enhance the likelihood of conversion of the normal cellular isoform.

In this context, the sporadic occurrence of CJD could arise for two reasons. First, a somatic cell mutation could give rise to a mutant PrP that would prefer the conformation of the disease-causing isoform (Prusiner 1989). Initially, propagation of the mutant PrP^{Sc}-like conformer would be limited to the cell in which the somatic mutation had occurred. Either within that cell or within surrounding cells, this mutant PrP^{Sc}-like conformer would have to be capable of triggering the conversion of wild-type PrP^{C} into PrP^{Sc}. If the particular somatic mutation produces a PrP^{Sc}-like conformer that cannot interact with wild-type PrP^{C}, then prion propagation will not occur and disease will not develop. This scenario of sporadic disease caused by a somatic mutation differs from the inherited prion diseases where mutant PrPs with the characteristics of PrP^{Sc} accumulate. Because extracts from the brains of patients carrying the D178N, E200K, or V210I point mutation have transmitted to mice expressing the wt chimeric MHu2M transgene (Telling et al. 1996b; J.A. Mastrianni and S.B. Prusiner, in prep.), it seems likely that any of these point mutations could initiate sporadic prion disease. In contrast, human prions carrying the P102L mutation could not be transmitted to Tg(MHu2M) mice, which argues that this mutation could not initiate sporadic prion disease. Although expression of the P102L mutation in either humans or mice causes neurodegeneration, detection of these prions was greatly facilitated when mice expressing PrPs carrying the same mutation were used as recipients of inocula from ill humans or Tg mice (Hsiao et al. 1994; Telling et al. 1995, 1996a). A second explanation for sporadic disease is

a corollary of the kinetic ideas advanced in the discussion of inherited disease. The kinetic barrier separating the cellular and disease-causing conformers of PrP provides only a stochastic barrier that will be crossed given a sufficient time. Although this event will be vanishingly rare in any individual human lifetime, following the logic of the ergodic theorem, the likelihood of a rare event will increase as the size of the population enlarges. In the case of sporadic CJD with an incidence of one per million people, a barrier height of 36 to 38 kcal/mole would be required following a transition-state argument. Once this misfolded conformer is formed, it would be available to act as a template to direct the rapid replication of the disease-causing conformer. If the infectious efficiency of an individual PrP^{Sc} oligomer were small, then this would act as a prefactor in the rate equation that would lower the expected $\Delta G^{\ddagger}$ for a single PrP^{Sc} formation event.

From the perspective of inherited and sporadic neurodegenerative diseases, Alzheimer's and the prion diseases could share similar pathogenic mechanisms. However, a fundamental point of departure arises from the transmissibility of the prion diseases, which has not been demonstrated for Alzheimer's disease (Goudsmit et al. 1980; Godec et al. 1994). Inoculation of tissues from animals suffering from prion disease causes disease in the recipient host. The infectious pathogen can be purified and treated with reagents that modify or hydrolyze polynucleotides without loss of infectivity. Although prions are remarkably resistant to proteolytic degradation, they can be inactivated by prolonged digestion with proteases or by exposure to high concentrations of salts known to denature proteins, such as guanidinium thiocyanate (Prusiner et al. 1981a,b, 1993b). Although these initial biochemical results were viewed with great skepticism, it has become increasingly clear how this protein could replicate. The mechanism of infectious prion disease follows from the second explanation for sporadic disease with the minor modification that the initiation of the process is not truly stochastic at a molecular level but relates to facilitated industrial or ritualistic cannibalism (Gajdusek 1977; Wilesmith et al. 1991). The efficiency of the infection relates to the titer of the inoculum, the mode of entry, and the frequency of exposure; intracerebral inoculation of a large dose of prions most efficiently initiates prion replication (Prusiner et al. 1985). In contrast, a single ingestion of foodstuffs containing a small dose of prions is likely to be exceedingly inefficient. The efficiency of this process is also governed by the strength of the interaction of PrP^{C} with PrP^{Sc}. When both isoforms contain the same sequence (homotypic interaction), experimental data confirm that conversion is most likely (Prusiner et al. 1990). When the sequences are

different, especially in certain regions of the structure, conversion is less likely (Scott et al. 1993; Kocisko et al. 1995). The inefficiency of heterotypic conversion is commonly referred to as the "species barrier" and has been used to explain why humans have not contracted scrapie from sheep and why nontransgenic mice are largely resistant to human $PrP^{Sc/CJD}$ inocula (Telling et al. 1995; Prusiner 1997).

A REPLICATION CYCLE FOR PrP^{Sc}

A simple replication cycle for PrP^{Sc} can be constructed. PrP^{C} exists in equilibrium with a second state, PrP^{*}, which is best viewed as a transient intermediate that participates in PrP^{Sc} formation either through an encounter with PrP^{Sc} or with another PrP^{*} molecule. Under normal circumstances, PrP^{C} dominates the conformational equilibrium. With infectious diseases, PrP^{Sc} specified here minimally as a PrP^{Sc}/PrP^{Sc} dimer is supplied exogenously. It can bind PrP^{*} to create a heteromultimer that can be converted into a homomultimer of PrP^{Sc} (see Fig. 2A). Genetic evidence points to the existence of an auxiliary factor (protein X) in this conversion (Telling et al. 1995; Kaneko et al. 1997b). Protein X preferentially binds PrP^{C} and is liberated upon the conversion of PrP^{*} to PrP^{Sc}. Protein X can then be recycled and join another heteromultimeric complex (see Fig. 2B). The homomultimer can dissociate to form two replication-competent templates creating exponential growth of the PrP^{Sc} concentration. In inherited disease, the concentration of PrP^{*} rises due to either the destabi-

Figure 2 Initiation and replication of PrP^{Sc} synthesis. (*A*) Exogenous PrP^{Sc} initiates PrP^{Sc} synthesis by binding to a $PrP^{C/*}/X$ complex. Facilitated by protein X and directed by the PrP^{Sc} template, PrP^{*} changes conformation and forms PrP^{Sc}. PrP^{Sc} no longer binds protein X and so the heteromultimeric complex dissociates, yielding recycled protein X and endogenous PrP^{Sc}. (*B*) A replication cycle for PrP^{Sc} synthesis following the creation of endogenous PrP^{Sc}. Again PrP^{Sc} binds to the $PrP^{C/*}/X$ complex to form the activated template for conversion of PrP^{*} to PrP^{Sc}. When PrP^{Sc} forms, protein X dissociates and is recycled. The newly generated PrP^{Sc} can then facilitate two replication cycles leading to an exponential rise in PrP^{Sc} formation. (*C*) In inherited disease and perhaps with spontaneous disease in the presence of a somatic cell mutation, mutant PrP^{C} can bind protein X and form the $PrP^{*}/X/PrP^{*}/X$ encounter complex, which can then form PrP^{Sc}/Mut in the absence of a PrP^{Sc} template. Once this event occurs, replication follows the pattern in *B*. (*D*) In spontaneous disease, the rare formation of the $PrP^{*}/X/PrP^{*}/X$ complex could lead to de novo PrP^{Sc} formation. Once this rare event occurs, replication would follow the outline in *B*.

lizing effect of the mutation on PrP^{C} or the increased stability of a PrP^{C} or PrP^{*} dimer (multimer). This increases the likelihood of the presence of a PrP^{*}/PrP^{*} complex that can form PrP^{Sc}/PrP^{Sc} (see Fig. 2C) and initiate the replication cycle. Sporadic disease requires merely a rare molecular event, formation of the PrP^{*}/PrP^{*} complex (see Fig. 2D), or a somatic cell mutation that follows the mechanism for the initiation of inherited disease. Once formed, the replication cycle is primed for subsequent conversion.

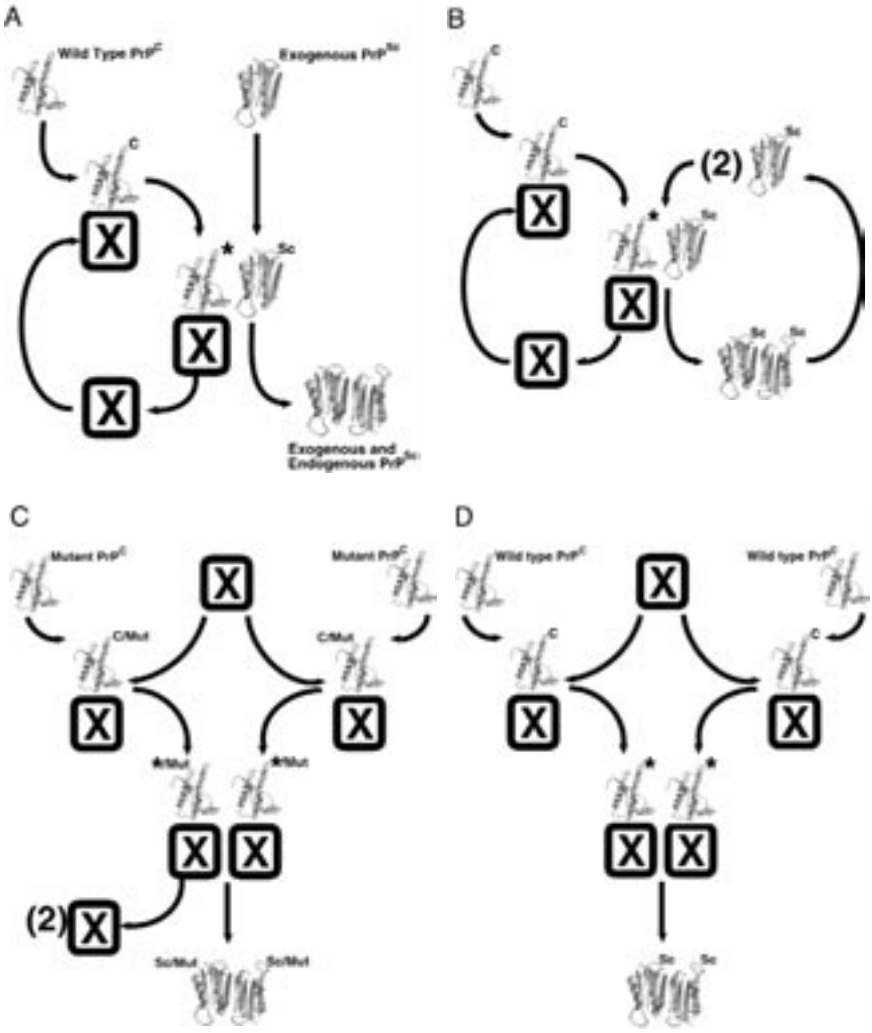

Figure 2 (See facing page for legend.)

From an analysis of the thermodynamics and kinetics of prion replication and the replication cycle for the inherited, sporadic, and infectious scenarios, several inferences can be made about the biophysical properties of the normal cellular and disease-carrying PrP isoforms.

1. PrP^{Sc} replication requires the presence of the PrP gene in the host cell to direct PrP^{C} synthesis (Büeler et al. 1993; Prusiner et al. 1993b; Manson et al. 1994; Sakaguchi et al. 1995) . However, although PrP^{Sc} replication requires a PrP gene in the host cell, it does not need to be carried by the infectious pathogen (Oesch et al. 1985).
2. PrP^{Sc} must be more stable than PrP^{C}, and a sensible origin for this distinction is an extensive network of intermolecular interactions between PrP monomers in a PrP^{Sc} multimer (Bellinger-Kawahara et al. 1988). Protease resistance could be a corollary of this increased stability and not necessarily the origin of the increased metabolic stability of PrP^{Sc} (Prusiner et al. 1982; McKinley et al. 1983).
3. A large conformational distinction between PrP^{C} and PrP^{Sc} would create a substantial kinetic barrier rendering prion disease extremely uncommon in the wild-type setting. As a corollary, some region or regions of PrP must exhibit extreme conformational plasticity (Nguyen et al. 1995; Zhang et al. 1995, 1997).
4. For PrP^{Sc} to provide a useful and efficient template to facilitate PrP^{C} conversion, the molecular interaction between PrP^{Sc} and PrP^{C} must be quite specific (Prusiner et al. 1990; Scott et al. 1993; Kocisko et al. 1995). Thus, differences in the sequences of PrP^{C} and PrP^{Sc} should disrupt or attenuate conversion.
5. When the PrP gene carries amino acid substitutions that destabilize the protein in the PrP^{C} isoform or stabilize PrP^{*}, the incidence of inherited disease should rise (Chapman et al. 1994; Spudich et al. 1995). Other amino acid substitutions that do not alter the stability of PrP^{C} or PrP^{*} significantly will provide sites for polymorphisms between or within species (for review, see Prusiner and Scott 1997).
6. If conformational conversion provides the rate-limiting step for prion replication, disease progression should depend on the concentration of PrP (Prusiner et al. 1993b; Büeler et al. 1994; Carlson et al. 1994; Manson et al. 1994). As this is most logically a first order process, halving the concentration of PrP should double the time to disease and doubling the concentration of PrP should halve the time to disease. The standard deviation of measurements of time to disease (t) should be $t^{1/2}$.
7. Small molecules that stabilize the PrP^{C} isoform could act as thera-

peutic agents by decreasing the concentration of PrP^{*}, thereby slowing PrP^{Sc} replication (Cohen et al. 1994). Similarly, molecules that abrogate the PrP/protein X interaction or stabilize it sufficiently to prevent protein X recycling could have therapeutic potential (Kaneko et al. 1997b; Prusiner 1997).

We return to these inferences in the context of the experimental results that follow.

EXPERIMENTAL AND COMPUTATIONAL STUDIES OF PrP^{C} AND PrP^{Sc}

The failure to detect a polynucleotide associated with the infectious prion particle created a "replication" conundrum (Prusiner 1982, 1984, 1991). Purification of PrP 27-30 (Prusiner et al. 1982, 1983), the 27- to 30-kD protease-resistant core of PrP^{Sc}, and subsequent microsequencing provided sufficient partial sequence information (Prusiner et al. 1984) to discover the endogenous PrP gene (Chesebro et al. 1985; Oesch et al. 1985). This gene resides on chromosome 20 in man and on the syntenic chromosome 2 of mouse (Sparkes et al. 1986). This is consistent with inference 1 on the requirements of prion replication. Genomic DNA sequencing revealed that the entire PrP coding region was contained in a single open reading frame (Basler et al. 1986). The sequence revealed many well-known features, including a signal sequence and two sites for N-linked glycosylation. In addition, several unusual features were noted, including an eight-residue repeating sequence (octarepeat) P-H-G-G-G-W-G-Q that is unlike any known protein structural motif and an alanine-rich region A-G-A-A-A-A-G-A for residues 113 to 120. Until recently, no extended regions of the PrP sequence were recognizably analogous to non-PrP gene products (Bamborough et al. 1996).

Although PrP^{C} and PrP^{Sc} share a common sequence and pattern of posttranslational modification as judged by a variety of biochemical and mass spectroscopic studies, substantial differences at a structural level have been demonstrated between these two conformers. These are summarized in Table 1. Although the magnitude of these conformational distinctions was unexpected by most, it is clear that this feature is an essential aspect of prion biology.

Biochemical Characterization

PrP 27-30 was purified as the protease-resistant core of the major, and probably the only, component of the infectious prion particle (Prusiner et al. 1982, 1983). PrP 27-30 aggregates into rod-shaped polymers that are

Table 1 Structural differences between PrP^{C} and PrP^{Sc}

Property	PrP^{C}	PrP^{Sc}
Protease resistance	no	stable core containing residues 90–231
Disulfide bridge	yes	yes
Molecular mass after deglycosylation	16 kD [rPrP(90-231)]	16 kD (PrP 27-30)
Glycosylation	2 N-linked sugars	2 N-linked sugars
Glycoforms	multiple	multiple
Secondary structure	dominated by α-helices	rich in β-structure
Sedimentation rate	consistent with monomeric species	multimeric aggregated species
Accessible epitopes[a]	109–112 138–165 225–231	225–231
Free energy of stabilization	$\Delta G = {}^{-}6$–$^{-}8$ kcal/mole	
Predicted secondary structure	α: 109–122; 129–141; 179–191; 200–217	
Experimental data	at pH 4.5[b] α: 144–154; 179–193; 200–217 β: 128–131; 161–164 at pH 5.2[c] α: 144–157; 172–193; 200–227 β: 129–131; 161–163	

[a]Peretz et al. (1997).
[b]Riek et al. (1996),
[c]James et al. (1997).

insoluble in aqueous and organic solvents as well as nonionic detergents. These purified prion rods exhibit the tinctorial and ultrastructural properties of amyloid (Prusiner et al. 1983). PrP 27-30 is stable at high temperatures for extended periods of time, consistent with its unusual stability of prion infectivity by comparison to globular proteins from mesophiles. Protein denaturants (e.g., 3 M GdnSCN) that modify the structure of PrP 27-30 also inactivate prion infectivity (Prusiner et al. 1993a). In contrast, prion infectivity resists inactivation by reagents that disrupt nucleic acid polymers including nucleases, psoralens, and UV irradiation (Alper et al. 1967; Latarjet et al. 1970; Diener et al. 1982; Prusiner 1982; Bellinger-Kawahara et al. 1987a,b). Microsequencing of the amino terminus of PrP 27-30 (Prusiner et al. 1984) led to the cloning and sequencing of the PrP gene (Chesebro et al. 1985; Oesch et al. 1985). The amino terminus of PrP 27-30 corresponds to approximately codon 90 in the full-length coding sequence (Basler et al. 1986; Locht et al. 1986).

Under physiologic conditions, cells synthesize PrP in the endoplasmic reticulum (ER) with the cleavage of a hydrophobic leader sequence (residues 1–22). Asparagine-linked carbohydrates that were attached to residues 181 and 197 in the ER are remodeled in the Golgi as PrP transits to the cell surface (Endo et al. 1989). A glycosyl phosphatidylinositol (GPI) moiety is attached to residue 231 as 23 carboxy-terminal residues are removed in the ER (Stahl et al. 1990). The biogenesis of PrP is complicated by the presence of a stop transfer signal contained within residues 95–110 (Yost et al. 1990). Whereas the majority of PrP biosynthesis leads to a GPI-anchored form, the channel for PrP export into the ER may disassemble during biogenesis, resulting in a transmembrane form of PrP (De Fea et al. 1994; Hegde et al. 1998).

PrP secreted to the cell surface under normal conditions is known as PrP^{C}, a soluble, monomeric protein containing a single disulfide bridge between residues 179 and 214. This protein was initially purified from hamster brain in its glycosylated form (Pan et al. 1992, 1993; Turk et al. 1988) and subsequently produced by recombinant (r) sources as either the truncated 142-residue molecule SHa rPrP (90-231) corresponding to PrP 27-30 or, more recently, the nearly full-length molecule SHa rPrP (29-231) (Mehlhorn et al. 1996; Donne et al. 1997; Riek et al. 1997). The recombinant protein can be oxidized and solubilized in GdnHCl between pH 5 and pH 8. Dilution of the denaturant and incubation at room temperature for 2–12 hours yields a folded material that shares the spectral and immunologic properties of PrP^{C} purified from SHa brain (Mehlhorn et al. 1996; Peretz et al. 1997; Zhang et al. 1997). Thermal and GdnHCl denaturation of rPrP (90-231) indicates a three-state process with a well-defined intermediate (Zhang et al. 1997). The free-energy change between the folded and intermediate states was calculated to be 1.9 ± 0.4 kcal/mole. The second transition to the unfolded state was associated with a free-energy change of 6.5 ± 1.2 kcal/mole. PrP^{C} unfolds with a T_m of 50–55ºC, substantially lower than PrP^{Sc}. Analytical ultracentrifugation studies reveal that rPrP (90-231) is monomeric with a monomer-dimer equilibrium association constant of $5.4 \times 10^{-5} M^{-1}$. rPrP (90-231) refolded under acidic conditions yields a molecule with less helicity as judged by circular dichroism spectroscopy. This form has a greater tendency to aggregate (Zhang et al. 1997).

In contrast, PrP^{Sc}, the full-length infectious conformer of PrP^{C}, has a tendency to form aggregates but not amyloid fibrils (McKinley et al. 1991). Attempts to identify a covalent distinction between PrP^{C} and PrP^{Sc} have been unsuccessful (Stahl et al. 1993). In both PrP^{C} and PrP^{Sc}, the two cysteines form a disulfide bridge that is needed for PrP^{Sc} formation (Turk

et al. 1988; Muramoto et al. 1996). PrP^{Sc} can be denatured with GdnSCN, but refolding conditions that permit recovery of prion infectivity have not been identified (Prusiner et al. 1993a). In the absence of conditions for reversible unfolding of PrP^{Sc}, it has not been possible to establish the relative stability of PrP^{Sc}, PrP^{C}, and the unfolded state. However, the difference in T_m values between the molecules suggests that PrP^{Sc} is more stable than PrP^{C}. This is consistent with inference 2. rPrP (90-231), when refolded at high concentration, forms a β-rich structure that melts at higher temperatures than rPrP (90-231) in the α-helical state. However, the melting curves for β rPrP (90-231) are not reversible, and so realistic free-energy estimates cannot be derived for this transition (Zhang et al. 1997). Analytic ultracentrifugation reveals a multimer containing 6–7 β-rPrP molecules. Although these recombinant molecules folded into a β-rich form share many spectroscopic and biochemical features of PrP^{Sc}, they are not infectious. This limits the conclusions that one can reach from a study of these molecules.

CD and FTIR Spectroscopy

Studies of purified PrP 27-30 showed that it assembled into polymers with the properties of amyloid (Prusiner et al. 1983), whereas earlier investigations had shown that other amyloids have a high β-sheet content (Glenner et al. 1972; Glenner 1980). In the prion diseases, the amyloid deposits were subsequently shown to contain large amounts of the PrP gene product (Bendheim et al. 1984; DeArmond et al. 1985; Kitamoto et al. 1986; Roberts et al. 1988). Subsequently, Fourier transform infrared (FTIR) spectroscopy was used to measure the high β-sheet content of PrP 27-30 (Caughey et al. 1991; Pan et al. 1993). FTIR and circular dichroism (CD) studies showed that PrP^{Sc} contains about 30% α-helical structure and about 40% β-sheet (Pan et al. 1993; Safar et al. 1993). These data are in marked contrast to the structural studies of PrP^{C} purified from normal brain (Pan et al. 1993; Pergami et al. 1996) and of rPrP(90-231) (Mehlhorn et al. 1996), which are soluble molecules with substantial α-helical structure (40%) and little β-structure (~3%) by CD and FTIR spectroscopy. Taken together, these studies confirm inference 3 concerning a large conformational distinction between PrP^{C} and PrP^{Sc}.

Computational Studies

With experimental evidence of two distinct conformational isoforms in hand, efforts were made to predict the secondary and tertiary structures of

PrP^{C} and PrP^{Sc} (see Fig. 3) (Huang et al. 1994, 1996). A series of secondary structure prediction algorithms were applied to a collection of PrP sequences ranging from species as divergent as chicken and human. No regular secondary structure was identified for the octarepeat region from residues 23 to 90. Four or five putative structural regions were identified, but there was a relative lack of consistency in the designation of these regions as α-helices or β-strands (Huang et al. 1994). In an effort to fit the spectroscopic data, four of the regions were identified as structured. We presumed that all of these regions adopted α-helical conformation in PrP^{C} and two of these regions formed the component strands of a β-sheet in the PrP^{Sc} isoform (Huang et al. 1996). Combinatorial packing algorithms were applied to obtain plausible models of the tertiary structures of PrP^{C} and PrP^{Sc}. Each alternative structure was examined for inconsistencies with experimental data from peptide studies, with mutation information derived from patients with inherited prion diseases, and with sequence polymorphism data derived from an extensive effort to sequence PrP genes from a variety of species. The result was low-resolution models of the structures of PrP^{C} and PrP^{Sc} that facilitated the design of a variety of peptides and transgenic constructs.

Antibody Studies

Early studies of distinctions in the antigenic surface of PrP^{C} and PrP^{Sc} were hampered by the limited antigenicity of PrP^{Sc} (Prusiner et al. 1993b; Williamson et al. 1996). Operationally, only two monoclonal antibodies

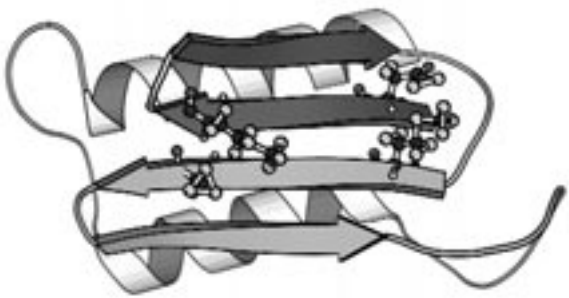

Figure 3 Ribbon diagram of a predicted structure for PrP^{Sc}. The two carboxy-terminal helices correspond to helices B and C in PrP^{C}. The *red* and *green* strands correspond to the region between residues 90 and 145 that are predicted to adopt a β structure in PrP^{Sc}. Side chains of some residues implicated in the species barrier are also included and noted to cluster on one face of the putative β-sheet.

have been developed that recognize PrP: 13A5 and 3F4 (Barry and Prusiner 1986; Kascsak et al. 1987). Although both bind to PrP^{C} and denatured PrP^{Sc}, neither binds to folded PrP^{Sc} effectively (see Fig. 4). Recently, we have surveyed a diverse set of Fab fragments of recombinant origin (Peretz et al. 1997). Seven Fabs were identified that bind to distinct linear PrP epitopes: D4, R10, D13, R72, R1, R2, and D2. The last three of these can reliably recognize active PrP^{Sc} as well as PrP^{C}. Epitope-mapping studies demonstrate that 3F4, D4, R10, and D13 bind to an epitope bounded by residues 94–112 (I) whereas 13A5 and R72 recognize a distinct epitope formed by residues 138–165 (II). The extreme carboxyl terminus of the molecule, residues 225–231, contains a binding site for R1, R2, and D2 (III). The large conformational rearrangement between PrP^{C} and PrP^{Sc} demonstrated spectroscopically is supported by a clearly differentiable antibody-binding pattern. From epitope-mapping studies, we can now localize the conformationally flexible region to include residues 90–112. This region could extend to residue 138. In contrast, the region between residues 225 and 231 is likely to adopt a similar structure in PrP^{C} and PrP^{Sc} based on the relatively uniform binding of rFabs R1, R2, and D2 to both isoforms. Whether this conformationally rigid zone extends to include residues 166–224 remains to be determined. This evaluation will

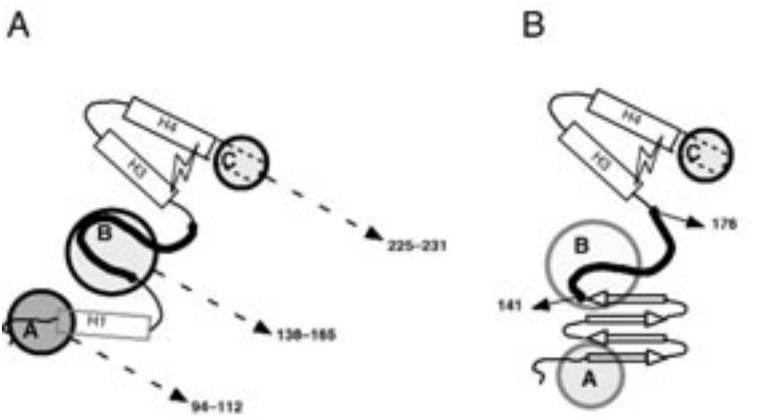

Figure 4 Schematic diagram of the secondary structure of PrP^{C} (residues 90-231) and a model of PrP^{Sc}. The distinct epitopes A, B, and C have been identified and the boundaries of these linear epitopes mapped. Antibodies 3F4, D4, R10, and D13 recognize epitope A in PrP^{C} but this epitope is cryptic in PrP^{Sc}. Antibodies 13A5 and R72 recognize epitope B in PrP^{C} and to a lesser extent, epitope B in PrP^{Sc}. Finally, antibodies R1, R2, and D2 recognize epitope C in PrP^{C} and PrP^{Sc}.

require the identification of monoclonal antibodies or Fab fragments that recognize this region. Recent work by Oesch and colleagues has identified an IgM that appears to recognize PrP^{Sc} specifically (Korth et al. 1997). These results are consistent with the conformational dimorphism of PrP^{C} and PrP^{Sc} and support a model for the structure of PrP^{Sc} similar to that proposed by Huang et al. (1996).

PrP^{C} BINDS PrP^{Sc} DURING SCRAPIE PRION FORMATION

A cardinal feature of prion transmission studies is the existence of a "species barrier" (Pattison 1965). That is, prions derived from a particular host species are more effective in transmitting disease upon inoculation into an animal of the same species than into one of a more evolutionarily distant species. This concept can be explicitly tested by creating mice expressing a Syrian hamster (SHa) PrP transgene (Scott et al. 1989). When inoculated with mouse PrP^{Sc} ($MoPrP^{Sc}$), this transgenic animal produces $MoPrP^{Sc}$. In contrast, when transgenic (Tg) mice expressing SHaPrP were inoculated with SHa prions containing $SHaPrP^{Sc}$, the Tg animals produced $SHaPrP^{Sc}$ (Prusiner et al. 1990). Thus, the transgene product $SHaPrP^{C}$ must recognize and bind $SHaPrP^{Sc}$ to template a sequence-specific conversion. A similar situation is obtained with the $MoPrP^{C}/MoPrP^{Sc}$ interaction. Thus, homotypic interactions between identical PrP sequences in the PrP^{C} and PrP^{Sc} isoforms are more favorable than the heterotypic alternatives (Prusiner 1991). This principle of prion replication was extended using chimeric SHa/Mo PrP transgenes in which only the central domain (residues 96–167) rendered the mice susceptible to SHa prions (Scott et al. 1992, 1993). Subsequent biochemical studies demonstrated that PrP^{C} binds PrP^{Sc} respecting sequence-specific preferences (Kocisko et al. 1994, 1995; Kaneko et al. 1995). These studies provide evidence in support of inference 4 concerning the specificity of the PrP^{C}/PrP^{Sc} heterodimer interface.

PROTEIN X IN PrP^{Sc} FORMATION

On the basis of the results with chimeric SHa/MoPrP transgenes, mice expressing chimeric Hu/MoPrP transgenes (MHu2M) were created. These Tg mice that coexpressed MHu2M and $MoPrP^{C}$ were susceptible to Hu prions from the brains of patients who had died of inherited, sporadic, and infectious prion diseases, whereas mice coexpressing Hu and $MoPrP^{C}$ were resistant to Hu prions (Telling et al. 1995). Only when Tg(HuPrP) mice were crossed onto the $Prnp^{0/0}$ background did they

become susceptible to Hu prions. To achieve comparable incubation times, the expression of HuPrP needed to be at least 10-fold higher than that in Tg(MHu2M)Prnp$^{0/0}$ mice. These findings were interpreted in terms of an auxiliary macromolecule that facilitates the conversion of PrP^{Sc} formation. Since this molecule is most likely a protein, it was provisionally designated "protein X." To explain the resistance of Tg mice expressing both MoPrP and HuPrP to Hu prions, MoPrP^{C} must bind to protein X with a higher avidity than does HuPrP^{C}. Because the conversion of MHu2M PrP^{C} into PrP^{Sc} was only weakly inhibited by MoPrP^{C}, it was surmised that either the amino- or carboxy-terminal domain of PrP, but not the central domain, binds to protein X (Telling et al. 1995). Since the amino terminus of PrP is not required for either transmission or propagation of prions (Rogers et al. 1993; Fischer et al. 1996; Muramoto et al. 1996), it was then postulated that the binding site for protein X lies at the carboxyl terminus (Telling et al. 1995). To test this hypothesis, a chimeric Hu/Mo gene was constructed in which the amino-terminal and central domains were composed of MoPrP and the carboxy-terminal region of HuPrP. This construct (M3Hu PrP) did not support PrP^{Sc} formation in scrapie-infected mouse neuroblastoma (ScN2a) cells, which suggests that its carboxyl terminus carrying the HuPrP sequence did not bind to Mo protein X. Subsequent studies of the PrP^{C}/protein X interaction in ScN2a cells have shown that MoPrP residues 167, 171, 214, and 218 are essential for protein X binding (Kaneko et al. 1997b). Biochemical efforts to purify protein X are under way.

NMR SPECTROSCOPY

Solution phase nuclear magnetic resonance (NMR) spectroscopic studies have been completed on three nonglycosylated fragments of PrP derived from synthetic or recombinant sources and refolded in an effort to replicate the conformation of the native glycosylated membrane-anchored PrP^{C}. These fragments are (1) SHaPrP(90-145), a synthetic peptide that begins with the residues at the amino terminus of PrP 27-30 (Prusiner et al. 1984) and ends at a position corresponding to a truncation mutation observed in a Japanese patient diagnosed with a prion disease (Kitamoto et al. 1993; Zhang et al. 1995); (2) MoPrP(121-231), a recombinant molecule found to form a stable folding domain (Hornemann and Glockshuber 1996) and corresponding to most of PrP^{C}-II, a normal degradation product of PrP^{C} (Haraguchi et al. 1989; Pan et al. 1992; Chen et al. 1995; Taraboulos et al. 1995); and (3) SHaPrP(90-231), a recombinant protein corresponding to the sequence of PrP 27-30 (Mehlhorn et al. 1996).

In an effort to investigate the structural basis of the conversion of α-helical structure in PrP^{C} into β-sheet structure in PrP^{Sc}, chemically synthesized peptide fragments corresponding to the SHaPrP(90-145) and the subfragment 109-141 were studied. These peptides include regions of the PrP sequence that, from an evolutionary perspective, are most conserved (Bamborough et al. 1996) as well as regions previously shown to be conformationally plastic (Nguyen et al. 1995). A shorter PrP peptide composed of residues 109–121 corresponding to the first putative α-helix was unexpectedly found to adopt a β-sheet conformation (Gasset et al. 1992), the structure of which has been examined by solid-state NMR (Heller et al. 1996). A slightly larger peptide composed of residues 106–126 has been studied with respect to both its structure and neurotoxicity using primary cultures of hippocampal neurons (Forloni et al. 1993; De Gioia et al. 1994; Brown et al. 1996). The longer SHaPrP(90-145) and SHaPrP(109-141) peptides could be induced to form α-helical structures in some organic solvents (e.g., trifluoroethanol) and detergents (e.g., sodium dodecyl sulfate) (Zhang et al. 1995). In contrast, acetonitrile or physiologic concentrations of NaCl facilitated the formation of a β-sheet-rich structure. Ultrastructurally, the β-sheet-rich form assembled into rod-shaped polymers. Stabilized by the hydrophobic support of detergent micelles, the peptide forms one stable and one more transient helix as judged by NMR spectroscopy. Chemical shift data on PrP 90-145 in 20 mM sodium acetate and 100 mM SDS collected at pH 3.7 and 25ºC suggested a stable α-helix in residues 113–122 and a more transient helical structure from 128 to 139. Although it was possible to assign all the proton resonances, only short-range nuclear Overhauser effects (NOEs) were identified, suggesting that this PrP fragment has only transient long-range organization, at best. At pH 5.0, and to a lesser extent at pH 7.2, PrP(90-145) will form a β-sheet-rich structure as measured by FTIR.

In efforts to identify a conformationally stable PrP fragment for NMR characterization, some investigators chose a MoPrP fragment of 111 residues (Hornemann and Glockshuber 1996) that is approximately 8 residues smaller than PrP^{C}-II (Chen et al. 1995). Using this *Escherichia coli*-derived MoPrP(121-231) fragment, a three-dimensional structure was obtained using standard heteronuclear spectroscopic methods (Riek et al. 1996). At pH 4.5 and 20ºC in the absence of any buffer, three α-helical and two β-strand regions were identified. As anticipated by the computational and biochemical studies, a disulfide bridge joined the two carboxy-terminal helices. Several regions of the chain were judged to be too flexible to locate specifically in the three-dimensional structure of this fragment, including residues 121–128, 167–176, and 220–231.

The structure of SHaPrP(90-231), a recombinant protein derived from *E. coli* with a sequence corresponding to PrP 27-30 isolated from scrapie-infected Syrian hamsters (Mehlhorn et al. 1996), has been determined (James et al. 1997). Biochemical and immunologic studies of SHaPrP(90-231), also designated rPrP or rPrP(90-231) (Mehlhorn et al. 1996), suggest a pH-labile structure below pH 5.2. As rPrP retains much if not all of its secondary structure at low pH, it is likely that rPrP adopts an acid-induced "A-state" reminiscent of that observed in apomyoglobin. Fortunately, the James et al. studies were carried out at pH 5.2 and 30ºC in 20 mM sodium acetate.

From the NOE cross-peaks, 2401 experimental distance restraints were used to generate low-resolution structures via standard constrained refinement calculations (Güntert et al. 1991). A best-fit superposition of backbone atoms for residues 113–228 of rPrP is shown in Figure 5A (James et al. 1997). The helices and β-sheet are fairly well defined, and loop regions can also be defined, although with less precision. Helix A spans residues 144–157 with the last turn quite distorted, corresponding to helix 144–154 found for MoPrP 121-231. Helix B includes residues 172–193, with the first turn irregular at the present stage of structure refinement. This is about two turns longer than the 179–193 helix found for MoPrP(121-231) (Riek et al. 1996), which agrees well with predicted helix H3 (179-191) (Huang et al. 1994). Helix C extends from residue 200 to 227 with the 225–227 turn irregular. This is substantially longer than the helix corresponding to residues 200–217 in MoPrP(121-231) (Riek et al. 1996). It is notable that predicted helix H4 (residues 202–218) (Huang et al. 1994) corresponds well with that found in MoPrP(121-231). Two 4-residue β-strands (128–131 and 161–164) were identified in the MoPrP(121-231) structure (Huang et al. 1994). In rPrP(90-231), a similar antiparallel β-sheet, with S2 spanning residues 161–163 and S1 spanning 129–131 possessing β-sheet characteristics, was found, but the two strands do not manifest standard β-sheet geometry (James et al. 1997). In fact, a β-bridge occurs only between Leu-130 and Tyr-162, and there are extensive cross-strand connectivities of residues in segment 129–134 with proximate residues on the antiparallel segment 159–165.

The loop between S2 and helix B (i.e., residues 165–171) yields resonances clearly exhibiting long-range as well as medium-range restraints in rPrP (James et al. 1997); this contrasts with the absence of resonances for the backbone atoms of residues 167–176 in the shorter MoPrP(121-231) (Riek et al. 1996). The results of studies on rPrP(90-231) indicate that the loop is reasonably ordered (James et al. 1997), whereas it was concluded that this region is disordered in MoPrP(121-231) (Riek et al. 1996).

The NMR results for rPrP(90-231) (James et al. 1997), compared with the structure reported for MoPrP(121-231)(Riek et al. 1996), support the notion that the core of the PrP^{C} structure is formed by parts of helices B and C, which correspond largely to the predicted H3 and H4

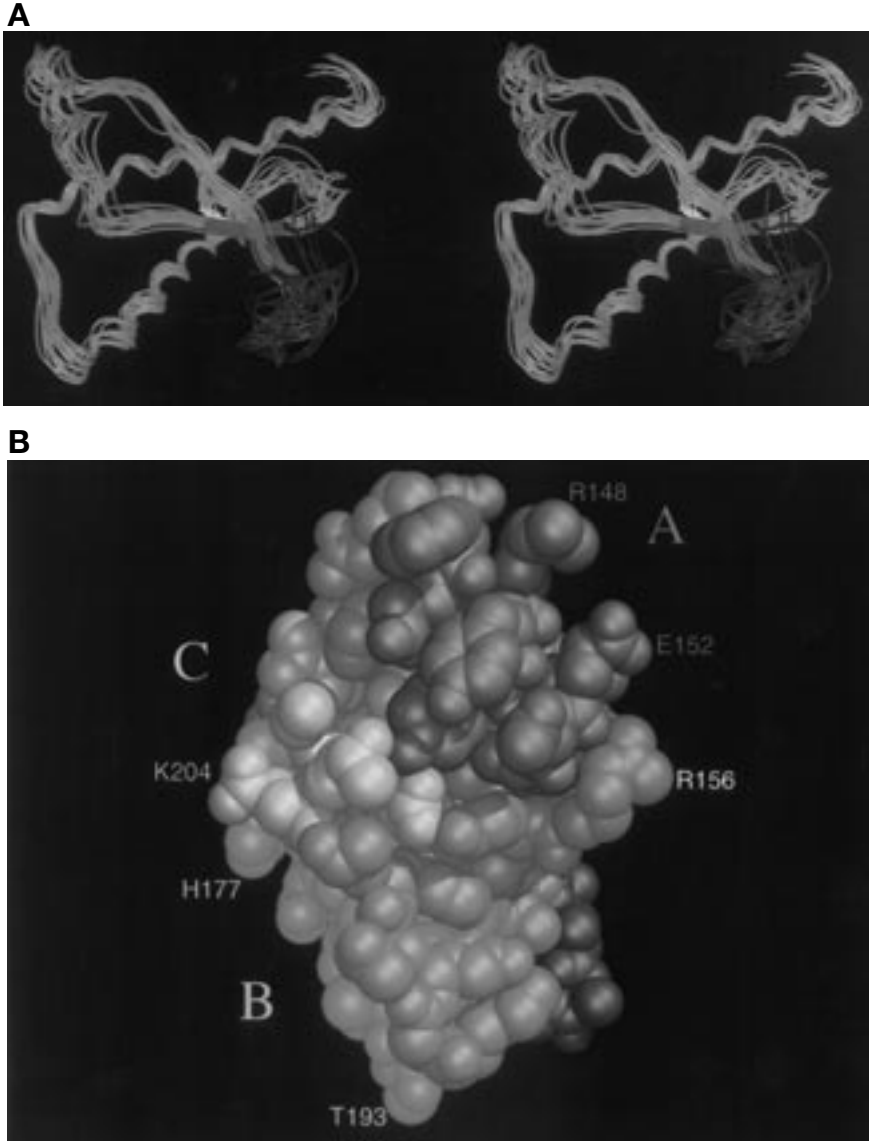

Figure 5 (See following pages for rest of figure and legend.)

regions (Huang et al. 1994), and is stabilized by the disulfide, which is essential for α-helical folding (Mehlhorn et al. 1996; Muramoto et al. 1996). As seen in Figure 5, helices B and C essentially form one side of the protein structure (James et al. 1997). This core is further stabilized by

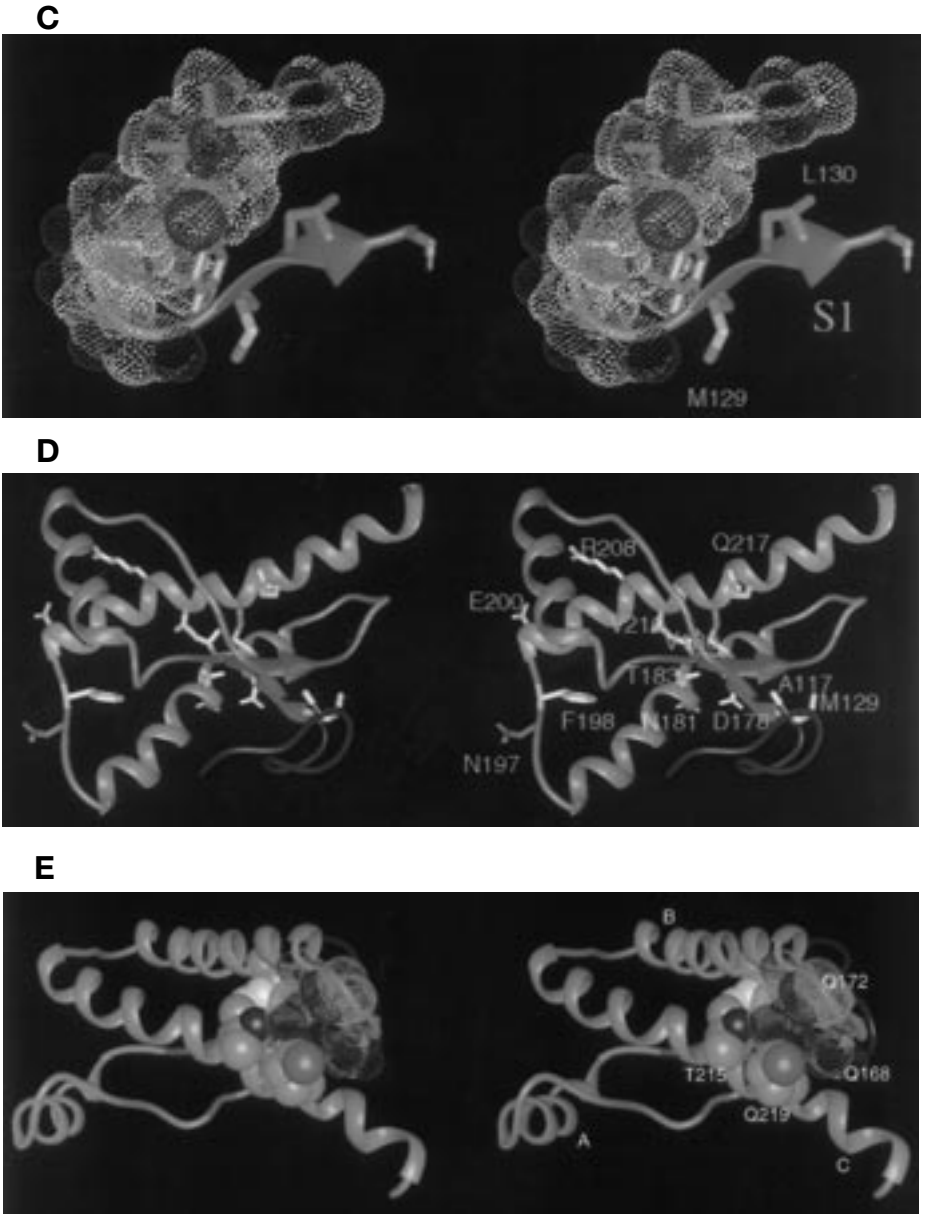

Figure 5 (Continued; see facing page for legend.)

helix A, which lies across helix C with side chains between the two helices interacting (Fig. 5B). Strand S2 also lies on this side of the protein and interacts predominantly with helices B and C as well as S1. With or without S2 and S1, we presume this relatively stable folding core is associated with the second unfolding transition.

Under the conditions used for NMR studies, it appears that rPrP(90-231) forms dimers a measurable fraction of the time (James et al. 1997). Recent unfolding experiments on rPrP(90-231) as a function of concentration suggest that unfolding follows a two-state regime at low protein concentrations; thus, it is likely that the initial unfolding transition at high protein concentration represents the disruption of a dimer interface (S. Marqusee, pers. comm.). Since MoPrP(121-231) exhibits biphasic unfolding at high protein concentration (Hornemann and Glockshuber

Figure 5 NMR structure of SHa rPrP(90-231). (*A*) Comparison of the 15 best-scoring structures of rPrP shown with a best-fit superposition of backbone atoms for residues 113–227 (stereo view). In all figures except *B*, the color scheme is: disulfide between Cys-179 and Cys-214, *yellow*; sites of glycosidation in PrP^C, i.e., Asn-181 and Asn-197, *gold*; hydrophobic cluster composed of residues 113–126, *red*; helices, *pink*; loops, *gray*; residues 129–134, *green*, encompassing strand S1 and residues 159–165, *blue*, encompassing strand S2; the arrows span residues 129–131 and 161–163, as these show a closer resemblance to β-sheet. The structures were generated with the program DIANA (Güntert et al. 1991), followed by energy minimization with AMBER 4.1 (Pearlman et al. 1995). Structure generation parameters are as follows: 2401 distance restraints (intraresidue, 858; sequential ($i \rightarrow i+1$), 753; ($i \rightarrow i+2$), 195; ($i \rightarrow i+3$), 233; ($i \rightarrow i+4$), 109; and ($i \rightarrow i + \geq 5$), 253 for amino acid *i*); hydrogen bond restraints, 44; distance restraint violations >0.5 Å per structure, 30; AMBER energy, -1443 ± 111 kcal/mole. Precision of structures: atomic RMSD for all backbone heavy atoms of residues 128–227, <1.9 Å. The distance restraint violations and precision in some molecular moieties reflect the conformational heterogeneity of rPrP. (*B*) van der Waals surface of rPrP turned approximately 180º from *A*, illustrating the interaction of helix A with helix C. Helices A, B, and C are respectively colored *magenta, cyan*, and *gold*. (*C*) Residues 113–132 illustrating (stereo view) in one representative structure the interaction of the hydrophobic cluster, with van der Waals rendering of atoms in residues 113–127, with the first β strand. (*D*) Stereo view, highlighting in *white* the residues corresponding to point mutations that lead to human prion diseases. (*E*) Stereo view, using ribbonjr, illustrating the proximity of helix C to the 165–171 loop and the end of helix B, where residues Gln-168 and Gln-172 are depicted with a low-density van der Waals rendering and helix C residues Thr-215 and Gln-219 are depicted with a high-density van der Waals rendering. Illustrations were generated with *Midasplus*.

1996), we believe that an essential component of the PrP^{*}:PrP^{*} dimer interface will include residues 90–120. This segment is sequentially and spatially distinct from the protein-X-binding site and includes some of the species polymorphisms that are likely contributors to the PrP^{*}:PrP^{*} interface and may be part of the molecular basis of the species barrier (Schätzl et al. 1995; Bamborough et al. 1996). Figure 6 highlights the side chains that are polymorphic in the 90–120 segment. In addition, other polymorphic residues that are spatially adjacent to this subgroup are presented. We hypothesize that this interface is the PrP^{*} portion of the dimerization interface that is essential for PrP^{Sc} replication and that small molecules that bind to this region will disrupt PrP^{Sc} replication. In contrast, polypeptides including antibodies that mimic this interface could act as templates to facilitate PrP^{Sc} replication.

Presence of the additional 31 amino-terminal residues of rPrP(90-231), relative to MoPrP(121-231), induces substantial changes in the structure of PrP including alterations in the carboxyl terminus (James et al. 1997). Helix C is extended by at least 9 residues, helix B is up to 7

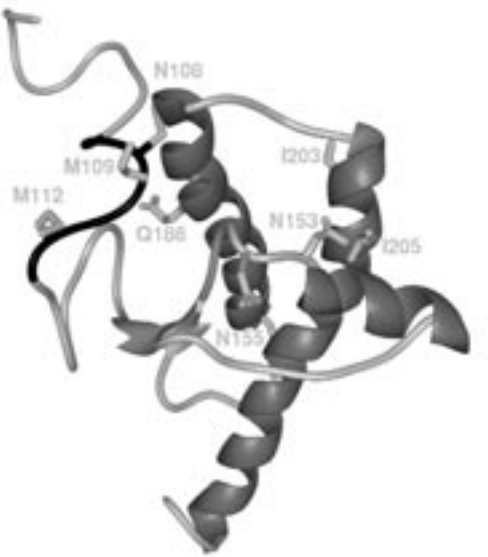

Figure 6 The NMR structure of SHaPrP (90-231) with the conformation of residues 107–112 constrained to follow the structure of this peptide as seen in an X-ray structure of the peptide bond to a Fab fragment of the 3F4 antibody (Z. Kanyo, pers. comm.). Residues with substantial polymorphisms across known sequences (Schätzl et al. 1995) that are not part of the protein X binding interface are shown. These residues form a likely site for the PrP^{C}:PrP^{Sc} interaction.

residues longer, and the loop comprising residues 165–171 is sufficiently ordered that many long-range restraints can be observed. A hydrophobic region without regular secondary structure (residues 113–125) predominantly interacts with S1 in the β-sheet (Fig. 5C). This may serve to stabilize the observed extension of helix B from 179 in MoPrP 121-231 to 172 in rPrP(90-231). Stability may also be conferred by hydrophobic interactions of Tyr-128 with Tyr-163 in the β-sheet, which, in turn, interacts with Val-176. The relative stability of the 165–171 loop and the three additional helical turns in helix C are presumably connected to stabilization of the other structural elements.

Within the irregular hydrophobic cluster (James et al. 1997) is a palindromic sequence, A^{113}GAAAAGA, that, together with surrounding residues, is conserved in all species examined to date (Bamborough et al. 1996). In humans, the A117V mutation causes GSS (Doh-ura et al. 1989; Hsiao et al. 1991; Mastrianni et al. 1995), whereas an artificial set of mutations consisting of A113V, A115V, and A118V in Tg mice causes spontaneous neurodegeneration and promotes β-sheet formation in recombinant PrP (Scott et al. 1997b; Hegde et al. 1998). Residues where point mutations lead to human diseases (for review, see Prusiner and Scott 1997) are highlighted in Figure 5D. A point mutation in the PrP gene that changes Asp-178 to Asn-178 causes FFI if residue 129 is methionine (Medori et al. 1992); the double mutation with Met-129 changed to valine results in a subtype of CJD instead (Goldfarb et al. 1992). Residue 178, which is in the extension of helix B seen in rPrP(90-231) (James et al. 1997) but not in MoPrP(121-231) (Riek et al. 1996), and residue 129 are located opposite one another with strand S2 partially intervening (Fig. 5E). If the mutation D178N destabilizes the structure, part of helix B could unravel. This part of helix B is near Arg-164 in S2, which in turn is adjacent to Met-129. Depending on the identity of residue 129, the structure of the codon 178 mutants may vary in a fashion anticipated by Gambetti and co-workers (Petersen et al. 1996).

Studies on the transmission of Hu prions to Tg mice suggest that a species-specific non-PrP protein designated protein X as noted above acts to facilitate PrP^{Sc} formation (Telling et al. 1995). Information gained from mutagenesis and NMR studies performed in concert suggests the side chains of MoPrP residues Gln-167, Gln-171, Thr-214, and Gln-218, which correspond to SHaPrP residues Gln-168, Gln-172, Thr-215, and Gln-219, respectively, form the site at which protein X binds to PrP^{C} (James et al. 1997; Kaneko et al. 1997b). Although sequentially distant, these residues cluster on one face of the molecule (James et al. 1997). The glycosylation sites, Asn-181 and Asn-197, are evidently not very near this

putative binding site (Fig. 5E). A comparison of the structure of rPrP(90-231) with MoPrP(121-231) (Riek et al. 1996) suggests that omission of residues 90–120 destabilizes helix C, resulting in its truncation and consequent disordering of residues 167–176. Perhaps this explains why MoPrP(121-231) is ineligible for conversion into PrP^{Sc} and does not appear to be able to bind to protein X (Muramoto et al. 1996; L. Zulianello et al., in prep.). As seen in Figure 5D, Thr-215 and Gln-219 lie in register one turn apart on helix C and interact with the loop containing residues 167 and 171. This information also allows us to understand the structural basis of genetic resistance to scrapie. Residue 171 is a glutamine in most species. Studies of Suffolk sheep in the US revealed codon 171 to be polymorphic, encoding either glutamine or arginine. All Suffolk sheep with scrapie were found to be Gln/Gln, which indicates that arginine conferred resistance (Westaway et al. 1994). These data suggest that the basic side chain of arginine acts to increase the affinity of PrP^{C}(R171) for protein X such that PrP^{C} is not readily released from the complex. Such a scenario where R171 acts through a negative dominant mechanism seems likely, since heterozygous Arg/Gln sheep are also resistant to scrapie. Susceptibility to scrapie in other breeds of sheep is also determined largely by the nature of residue 171 (Hunter et al. 1993, 1997a,b; Goldmann et al. 1994; Belt et al. 1995; Clousard et al. 1995; Ikeda et al. 1995; O'Rourke et al. 1997). Equally important is the observation that about 12% of the Japanese population encode lysine instead of glutamic acid at position 219 (Kitamoto and Tateishi 1994). No cases of CJD have been found in people with Lys-219 which, like arginine, is basic (Shibuya et al. 1998).

Recently, the NMR structures of MoPrP(23-231) and SHaPrP(29-231) have been characterized (Donne et al. 1997; Riek et al. 1997). This work suggests that the amino-terminal residues from 23 or 29 to 124 are flexible, under the experimental conditions chosen as judged by the negative NOEs and patterns of chemical shift data. Despite this flexibility in SHaPrP(29-231), the octarepeat region makes transient interactions with the carboxy-terminal end of helix B as judged by changes in the C_{α} chemical shifts for these residues in SHaPrP(29-231) compared with rPrP(90-231). However, it is unlikely that the octarepeat region is truly flexible and disordered under biologically relevant conditions given that full-length and not truncated PrP^{C} is normally purified from animal brain preparations and that the addition of a sixth octarepeat gives rise to an inherited prion disease in humans.

It now seems clear that PrP^{C} is a metalloprotein and that the highly flexible octarepeat region seen in the NMR experiments may be much

more structured in the presence of copper. Each SHaPrP(29-231) molecule has been shown to bind copper preferentially over other divalent cations following a 4:1 stoichiometry with spectroscopic evidence of tryptophan burial upon metal binding (Stöckel et al. 1998). This work extends earlier work on the binding of copper to synthetic peptides containing the octarepeat sequence (Hornshaw et al. 1995a,b; Miura et al. 1996). The biological relevance of these findings is supported by the recent findings of alterations of copper levels in Cu/Zn superoxide dismutase activity in PrP-deficient ($Prnp^{0/0}$) mice (Brown et al. 1997). The paramagnetic properties of Cu(II) will delay NMR characterization of the complete structure of this metalloprotein.

PRION STRAINS

The existence of prion strains has been considered difficult to reconcile with the protein-only model and the evidence that prion diseases involved protein folding and distinct folded conformations (Bruce and Dickinson 1987; Dickinson and Outram 1988; Prusiner 1991; Ridley and Baker 1996). However, recent results on the creation of new prion strains and their subsequent biochemical characterization have shown how the strain phenomenon is entirely compatible with the protein-only conformational conversion model (Telling et al. 1996b). Prion strains were initially isolated on the basis of different clinical syndromes in goats with scrapie (Pattison and Millson 1961); subsequently, strains were isolated in rodents on the basis of different incubation times and neuropathologic profiles (Fraser and Dickinson 1973; Dickinson and Fraser 1977). New strains have been produced upon passage from one species to another (Kimberlin et al. 1987) or passage from non-Tg mice to mice expressing a foreign or artificial PrP transgene (Scott et al. 1997a).

Studies of the drowsy (DY) and hyper (HY) prion strains isolated from mink by passage in Syrian hamsters showed that two strains produced PrP^{Sc} molecules with protease-resistant cores (PrP 27-30) of different molecular sizes as judged by gel electrophoresis (Bessen and Marsh 1994). Since the smaller size of the protease-resistant fragment of the DY strain did not correlate with a particular phenotype, the significance of these findings was unclear (Scott et al. 1997a). In seemingly unrelated studies, brain extracts from patients who died of FFI exhibited a deglycosylated, protease-resistant core of $PrP^{Sc/FFI}$ of 19 kD, whereas that found in extracts from patients with fCJD(E200K) and sCJD was 21 kD (Monari et al. 1994). The smaller size of the $PrP^{Sc/FFI}$ fragment was attributed to the distinct sequence of the $PrP^{Sc/FFI}$ protein. Studies on the

transmission of prions from patients with FFI, fCJD(E200K), or sCJD to Tg(MHu2M)Prnp$^{0/0}$ mice have shown that different sequences were not required to maintain the structural distinctions as judged by the molecular sizes of the deglycosylated, protease-resistant core of PrP^{Sc} molecules (Telling et al. 1996b). The Tg(MHu2M)Prnp$^{0/0}$ mice inoculated with extracts from FFI patients developed signs of CNS dysfunction about 200 days later and exhibited a deglycosylated, protease-resistant core of PrP^{Sc} that was 19 kD in size, whereas mice inoculated with extracts from fCJD(E200K) patients developed signs at about 170 days and exhibited a PrP^{Sc} of 21 kD. On second passage in Tg(MHu2M)Prnp$^{0/0}$ mice, the animals receiving the mouse FFI extract developed neurologic disease after about 130 days and displayed a 19-kD PrP^{Sc} molecule, and those injected with the mouse fCJD extract exhibited disease after about 170 days and showed a 21-kD PrP^{Sc} molecule. The second passage of these distinct prion strains with identical PrP amino acid sequences demonstrates that the size of the protease-resistant core is a stable feature of the strain. Thus, the two prion strains represent distinct PrP^{Sc} structures that can be enciphered on a proteinaceous template that is stable in passaging experiments.

What is the structural basis of these alternative PrP^{Sc} conformers? Work on diphtheria toxin identified distinct crystal forms that displayed different tertiary and quaternary structures for a single polypeptide sequence (Bennett et al. 1995). To describe this observation, the notion of domain swapping was introduced, where a region of one monomer displaced the corresponding region in another monomer to create an interlocking molecular handshake (Bennett et al. 1995). This phenomenon has now been observed in a variety of other protein structures with the swapped elements as small as an isolated α-helix or β-strand and as large as an entire folded domain. We suspect that a similar phenomenon is responsible for prion strains.

Imagine that the protease core of PrP^{Sc} has two subdomains joined by a linking region and that PrP^{Sc} exists as a dimer. Figure 7 shows a schematic of the fCJD-derived strain that shields a cleavage site near residue 105 but retains the cleavage site near residue 90. In the FFI-derived strain, the smaller subdomain of one monomer would swap with its partner in the dimer in a manner reminiscent of alternative ribonuclease A structures. In this way, the heterosubdomain interface (90-105:110-231) and the homosubdomain interface (90-105: 90-105) are maintained, but the proteolytic remnant is quite different. Moreover, the templates provided by each protease-resistant core would present a distinct interface for the conversion of PrP^{C} to PrP^{Sc} during prion replication. Many lines

of evidence suggest that the residues between 90 and 120 play a major role in the creation of the PrP^{C}/PrP^{Sc} interface.

In addition to a structure for PrP^{C} that is distinct from PrP^{Sc}, these results on prion strains suggest that there are multiple approximately isoenergetic PrP^{Sc} conformers. This is an obvious point of departure from earlier work demonstrating that for most proteins, there was a single folded structure that was uniquely encoded in the sequence (Anfinsen 1973). In an effort to probe the limits of this conformational pluralism for PrP, we have explored this phenomenon on a thermodynamically well understood model of protein structure, the H-P model on a square and cubic lattice (Chan and Dill 1996). In this model, two amino acid types are allowed, hydrophobic (H) and hydrophilic (P) residues, and the conformations of the chain are limited to those that can be exactly embedded upon the lattice. The energetics of this system are equally discrete, H-H interactions are worth one favorable energy unit, and all other interactions are negligible. Although this model lacks many features of real polypep-

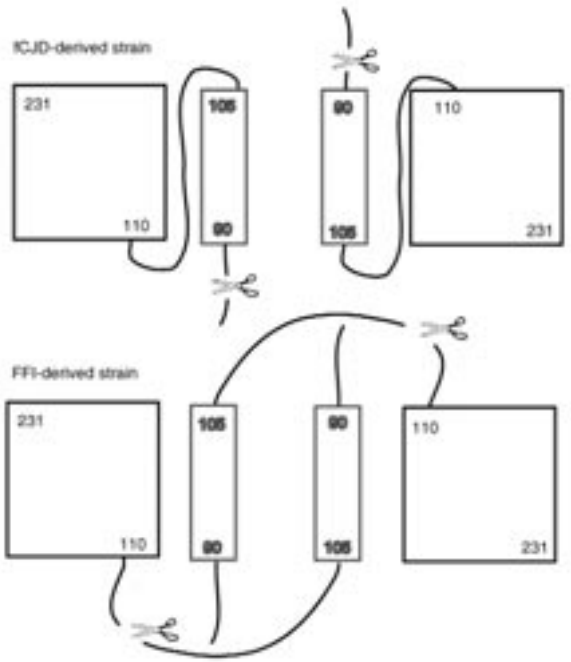

Figure 7 A cartoon of two strains of PrP^{Sc} formed by domain swapping. The two schematic structures are approximately isoenergetic but are expected to have distinct proteolytic cleavage patterns.

tide chains, for relatively short chains it is possible to enumerate all chain conformations and to identify all sequences with a unique lowest energy monomeric structure.

For H-P sequences of length 27 on a cubic lattice, one could ask how many sequences that have a unique monomeric structure would prefer a different conformation upon forming a symmetric dimer. With this simplified system, we found that about 5% of all sequences are prion-like in that the lowest energy structure for the monomer is unlike the most stable dimeric structures (Harrison et al. 1999). Taking this analogy one step further, it is possible to identify domain swaps between the amino- or carboxy-terminal regions of each monomeric member of the dimer that are isoenergetic with the new interleaved dimeric arrangement. This is reminiscent of the experimental work on strains. In this lattice limit, it is easy to see how the strains could act as templates to direct the formation of distinct "scrapie" molecules. It would also follow that proteolysis experiments could reveal different resistant cores. One could also ask how often a mutation to the 27-mer sequence would differentially affect the stability of the dimeric structures. Our preliminary calculations document that this is a relatively common phenomenon holding true for 20–30% of the residues, especially when residues are mutated from Ps to Hs.

CONCLUSIONS

A wide array of biochemical and biophysical experimental results indicate that a dramatic change in protein conformation is a central feature of the prion diseases. Although the transmissible aspect of prion biology has led to a variety of quite disparate hypotheses about the molecular basis of prion diseases, these mechanistic issues are more easily understood when one begins with the tenet that these diseases are fundamentally autosomal dominant inherited diseases. From a historical perspective, the inherited prion diseases are likely to have been the first to arise, because the stochastic nature of sCJD would seem to require a substantial population of individuals over the age of 60, and cannibalism could lead only to infectious CJD if the victim had preexisting familial or sporadic CJD.

Many autosomal dominant inherited diseases act by distorting the structure of a protein. The experimental results on the prion diseases are entirely compatible with this notion. In general, the change in protein structure could be under kinetic or thermodynamic control. For the prion diseases, the evidence points toward kinetic control. A special feature of the prion diseases is that the disease-causing isoform, PrP^{Sc}, appears to be capable of acting as a template to lower the kinetic barriers that normally

separate PrP^{C} from PrP^{Sc}. This provides a simple explanation for the inherited prion diseases that can be easily adapted to explain sporadic and infectious prion diseases.

To date, the majority of structural studies have focused on the soluble cellular isoform PrP^{C}. If we are truly to understand this disease process at a molecular level, we must endeavor to find routes to solubilize the PrP^{Sc} isoform or to identify polypeptides with PrP^{Sc}-like structures (Muramoto et al. 1996).

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6

Prions of Fungi: [URE3], [PSI], and [Het-s] Discovered as Heritable Traits

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In 1994, it was proposed that two non-Mendelian genetic elements of *Saccharomyces cerevisiae*, called [URE3] and [PSI], were in fact prion (infectious protein) forms of the chromosomally encoded proteins, Ure2p and Sup35p, respectively (Wickner 1994). Recently, it has been suggested that [Het-s], a non-Mendelian genetic element of the filamentous fungus *Podospora anserina*, is a prion of the protein encoded by *het*-s (Coustou et al. 1997). Here we describe the properties of these phenomena, the evidence that they are prions, and their general implications for prion biology.

The concept of an infectious protein was first proposed to explain the unusual properties of the agent producing the transmissible spongiform encephalopathies (Griffith 1967). The term "prion" was coined to mean the scrapie agent, including the possibility that it may have no essential nucleic acid component, but not restricted to this case (Prusiner 1982). We shall use the term prion here to mean infectious protein (the protein-only model), regardless of the organism or protein, and making no assumptions about the mechanism involved. In general, a prion is a protein that has undergone a change such that it no longer carries out its normal function but has acquired the ability to convert the normal form of the protein into the same form as itself, the prion form. By this definition, the prion change need not be one of conformation. A protein methylase might methylate another molecule of itself by mistake and, having done

so, the methylated methylase may no longer be able to modify its normal target proteins but may have become quite efficient at acting on normal molecules of itself. This chain reaction would be self-propagating if the protein had a means to spread to other cells or individuals. We recognize that this usage of the term prion differs from its original definition (Prusiner 1982), and we hope that this does not cause unnecessary confusion.

The three candidate prions (infectious proteins) discussed here were discovered by genetic analysis of the yeast, *S. cerevisiae,* and the filamentous fungus, *P. anserina*. New evidence of another prion, also in *S. cerevisiae*, has recently appeared. In this case as well, the discovery was made by genetic means.

Even though infectious bacteriophage or viruses often integrate into the host genome or enter a plasmid state and are then inherited, it is customary to separate the concepts of infection and inheritance. The virus is being infectious when it passes from cell to cell as a virus particle and is being inherited when it is integrated into the host genome or becomes a plasmid. However, the study of yeast dsRNA viruses has blurred this distinction (for review, see Wickner 1996). These viruses are functionally and structurally similar to the cores of Reoviridae but have no extracellular route of infection. The same may be said of fungal viruses, all of which spread horizontally by mating or mitotic fusion of hyphae (cell processes). Thus, one would expect a prion of yeast or filamentous fungi to likewise spread by this means and to appear as a non-Mendelian genetic element. In the 1950s to 1970s, non-Mendelian genetic elements were considered of great interest because, in this pre-cloning era, they were thought to be potential sources of small nucleic acids that could be studied. This was particularly critical in yeast where no classic lytic viruses had (or have even now) been found. Among the non-Mendelian genetic elements described in this period were [PSI] (Cox 1965), [URE3] (Lacroute 1971), and [Het-s] (Rizet 1952).

CHARACTERISTICS OF NON-MENDELIAN GENETIC ELEMENTS

A non-Mendelian genetic element is typically first recognized by its failure to segregate properly in meiosis. Mating a strain carrying the element with one lacking the element usually produces meiotic offspring, all of which have the element. This is called 4+ : 0 segregation in yeast, since there are four haploid meiotic progeny from a single diploid cell. In contrast, a single chromosomal gene difference between the parents leads to 2+ : 2– segregation.

A second characteristic of non-Mendelian genetic elements is their efficient transfer from cell to cell by cytoplasmic mixing (cytoduction or heterokaryon formation). In yeast, one uses a mutant that fails to undergo nuclear fusion after mating, the *kar1* mutant (Conde and Fink 1976). Cells with the element and the *kar1* mutation are mated for a few hours with cells of opposite mating type and lacking the element. The cells form mating pairs, but because of the *kar1* mutation, nuclei do not fuse in the heterokaryon formed. The nuclei separate into the daughter cells at the next cell division, but the cytoplasm of the two parent cells has mixed, so a cytoplasmic genetic element initially present in only one parent will be found in both daughter cells. Usually the donor parental strain has mitochondrial DNA (ρ^+) and so can grow on glycerol as a carbon source, but the recipient lacks mtDNA (ρ^o) and is glycerol$^-$. Cells from the mating mixture that have the recipient nuclear genotype but have acquired mtDNA must have received cytoplasm from the donor. They will generally also have received other nonchromosomal genetic elements initially present in the donor. The same method is available in filamentous fungi, which naturally form heterokaryons when two strains grow together. This is discussed in some detail below for *Podospora*.

A third trait of non-Mendelian genetic elements is their curability by relatively nonmutagenic agents. These are specific to each element. For example, levels of ethidium bromide that are only mildly mutagenic to nuclear genes result in rapid and total elimination of the mitochondrial genome from all cells in the population (Goldring et al. 1970).

GENETIC PROPERTIES EXPECTED OF A YEAST PRION

As discussed above, an infectious protein should, like other infectious elements of yeast, appear as a non-Mendelian genetic element. Certain special properties of a non-Mendelian genetic element make it a candidate to be a prion (Fig. 1) (Wickner 1994). Each of these properties provides evidence against the non-Mendelian trait's being based on a nucleic acid replicon, such as a virus or plasmid.

Reversible Curability

If a prion can be cured from a strain, and the cured strain purified by multiple single colony isolation, the cured strain should nonetheless be capable of giving rise to subclones which again carry the prion *without its introduction from another cell*. Whatever the change of the normal protein that constitutes the prion state, it can again occur in the cured cell

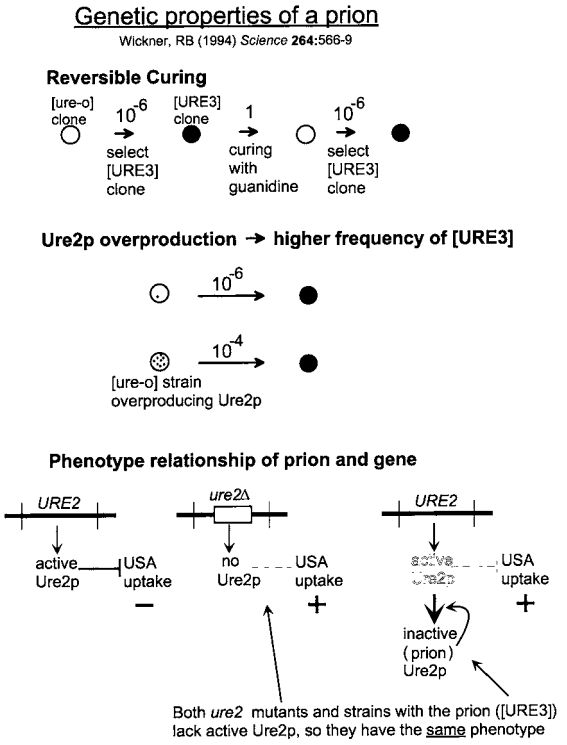

Figure 1 Genetic properties of yeast and fungal prions. (Reprinted, with permission, from Wickner 1994, 1997b.)

(at some low frequency) and give rise to a clone of prion-containing cells. Curing of nucleic acid-based non-Mendelian genetic elements, such as the yeast viruses L-A and M or the mtDNA, is irreversible. Once gone, they will not return unless reintroduced from outside the cell.

Overproduction of the Normal Form Increases the Frequency with Which the Prion Arises

If the cell has more of the normal form (a chromosomally encoded protein), then the stochastic process that gives rise to the prion form is expected to lead to the prion's arising with higher frequency. In contrast, overproducing one of the chromosomally encoded proteins on which a virus or plasmid depends would not give rise to the virus or plasmid in that cell.

Phenotypic Relation of the Prion and the Chromosomal Gene Encoding the Protein

Deletion of the chromosomal gene encoding the protein capable of undergoing the prion change will result in loss of the function carried out by the normal form of the protein. The presence of the prion should likewise give rise to the *same* or a similar result, since the prion form of the protein is unlikely to be able to carry out the normal function. This phenotypic relation is opposite that customarily found between mutation of a chromosomal gene needed to propagate a non-Mendelian genetic element and the presence of the element (Table 1). In that case, the absence of the chromosomal gene results in loss of the nucleic acid and loss of whatever function it encodes. This is the *opposite* of the phenotype of the presence of the nucleic acid replicon. For example, chromosomal mutants that lose mtDNA cannot grow on glycerol, but the presence of the mtDNA results in the ability to grow on glycerol.

The chromosomal gene encoding the protein capable of undergoing the prion change will be found to be necessary for prion propagation. The prion propagates by converting the normal form into the prion form. Without the normal form, the prion form will be diluted out as the cells grow and will be lost. Thus, in looking for new prions, one should pay attention to chromosomal genes necessary for the propagation of the non-Mendelian genetic element that is a candidate prion (Wickner 1994).

Defective interfering mutants of viruses or plasmids can arise which complicate this picture (Table 1). For example, a deletion mutant of the mtDNA may interfere with the replication of the wild-type mitochondrial genome. In this case, the defective genome would be dominant.

Table 1 Relation of phenotypes of a prion and mutants in the gene for the protein

Non-Mendelian element	Presence of non-Mendelian element	Chromosomal mutant	Relation	Does replacing the chromosome mutant gene restore the phenotype?
	Phenotypes			
M dsRNA	killer +	killer –	opposite	no
mtDNA	glycerol +	glycerol –	opposite	no
mtDNA-DI	glycerol –	glycerol –	same	no
Theoretical prion	defective	defective	same	yes
[URE3]	USA uptake +	USA uptake +	same	yes
[PSI]	suppressor +	suppressor +	same	yes

Reprinted, with permission, from Wickner (1997b). See text for details.

Mutation of a chromosomal gene needed to replicate the mutant mtDNA would result in loss of either the wild-type mitochondrial genome or the mutant. Thus, the glycerol minus phenotype of the chromosomal mutant would be the same as that due to the presence of the defective interfering mtDNA mutant (Table 1) because each would result in loss of the normal mtDNA, and the phenotype relationship would mimic that of a prion. However, the distinction could still be made. If the chromosomal mutation was corrected by introducing the normal gene, the phenotype would *remain defective* because the mitDNA would not be replaced (Table 1). This contrasts with the prion case, in which a deletion of the chromosomal gene is complemented by introduction of the normal gene, with return of the normal phenotype, because there is no normal plasmid or virus to replace.

[URE3], A PRION OF URE2P, A NITROGEN REGULATORY PROTEIN

In 1971 François Lacroute, studying uracil biosynthesis, described mutants of *S. cerevisiae*, called *ure* mutants, which could take up *ure*idosuccinate to allow growth of a mutant deficient in aspartate transcarbamylase (the first step in the uracil pathway, whose product is ureidosuccinate) (Lacroute 1971). This work uncovered the chromosomal *URE2* gene, a key player in regulation of nitrogen metabolism (Drillien et al. 1973), and the non-Mendelian genetic element, [URE3]. It was the early observation of Aigle and Lacroute (1975) that propagation of [URE3] requires *URE2* which led eventually to the suggestion that [URE3] is a prion (Wickner 1994).

Wild-type yeast can take up ureidosuccinate from the medium if it is growing on a poor nitrogen source, such as proline, but the uptake system is repressed if there is a rich nitrogen source, such as asparagine, glutamine, glutamate, or ammonia. The control of uptake of ureidosuccinate, an intermediate in uracil biosynthesis, by the nitrogen source in the medium is an accident of the structural resemblance of ureidosuccinate and allantoate (Fig. 2). Allantoate is a poor nitrogen source for yeast, but it can be used (Cooper 1982). However, in the presence of a good nitrogen source, enzymes needed for utilization of allantoate (or other poor nitrogen sources) are repressed. This repression occurs through the action of Ure2p, Gln3p, and other controlling proteins (Fig. 2) (for review, see Cooper 1982; Magasanik 1992). Gln3p is a positive transcription regulator that recognizes the sequence GATAA upstream of many genes whose products are involved in catabolism of nitrogen sources. Ure2p blocks the action of Gln3p and other positive transcription factors related to nitrogen metabolism, apparently by binding to Gln3p at its site of action on the DNA

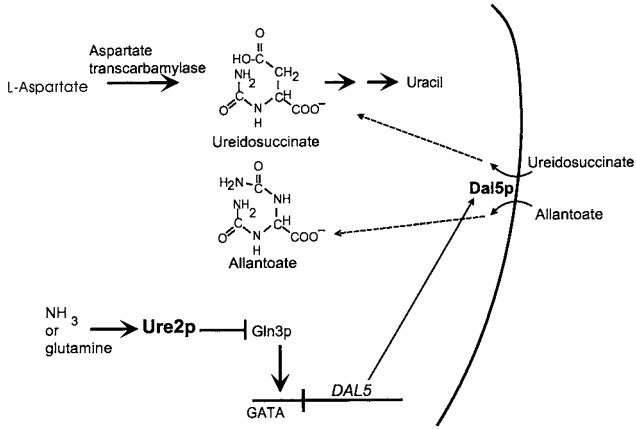

Figure 2 Ureidosuccinate, allantoate, Ure2p, and nitrogen regulation. Allantoate is a poor, but usable, nitrogen source for yeast. In the presence of a good nitrogen source, such as ammonia, glutamine, or asparagine, the proteins needed for allantoate utilization are transcriptionally turned off. These proteins include the allantoate transporter, Dal5p (Rai et al. 1987; Turoscy and Cooper 1987). Because allantoate chemically resembles ureidosuccinate, an intermediate in uracil biosynthesis, ureidosuccinate uptake (by the Dal5p transporter) is under control of the nitrogen catabolite repression system. Ure2p plays a key role in this system, sensing the nitrogen source (by an as yet unknown means) and blocking positive transcription activation by Gln3p and Put4p (Courchesne and Magasanik 1988; Xu et al. 1995).

(Blinder et al. 1996). However, Ure2p also has homology with glutathione-*S*-transferases, suggesting that it might act by posttranslational modification of Gln3p (Coschigano and Magasanik 1991). It is suggested that Ure2p senses intracellular glutamine (Magasanik 1992), but there is as yet no direct evidence for interaction of Ure2p and any small molecule.

Genetic Properties of [URE3] Suggest It Is a Prion

Lacroute found mutations in two chromosomal genes (*ure1* and *ure2*) that made cells able to take up ureidosuccinate in the presence of ammonia and glutamate (Schoun and Lacroute 1969; Lacroute 1971), and one non-Mendelian "mutant," called [URE3-1] (Lacroute 1971). [URE3] was shown to segregate 4+:0 in meiosis (Lacroute 1971), to be transferred by cytoduction (Aigle and Lacroute 1975), and to be cured by growth on low concentrations of guanidine (M. Aigle, cited in Cox et al. 1988), all prop-

erties of a nonchromosomal genetic element. [URE3] was shown to be unrelated to the mitochondrial genome (Lacroute 1971), the only well-documented nonchromosomal nucleic acid replicon in yeast at the time. Later studies showed it was not related to the yeast dsRNA viruses, the 2-micron DNA plasmid or [PSI] (Leibowitz and Wickner 1978).

Although *ure2* and [URE3] strains have the same phenotypes (Drillien et al. 1973), it was impossible to obtain *ure2* [URE3] strains (Aigle and Lacroute 1975). This key finding led eventually to the prion explanation for [URE3] (Wickner 1994).

[URE3] Is Reversibly Cured

[URE3] is efficiently cured by growth on rich medium containing 5 mM guanidine HCl (M. Aigle, cited in Cox et al. 1988; Wickner 1994). This in itself is certainly not evidence that [URE3] is a prion, since the mechanism of curing is completely unknown and it is not believable that 5 mM would be a high enough concentration to denature Ure2p and not other essential proteins in the cell. Moreover, similar low concentrations of guanidine have been shown to block replication of poliovirus by acting on the viral RNA-dependent RNA polymerase (Tamm and Eggers 1963). From a cured [URE3] strain, purified by single colony isolation, [URE3] derivatives can again be isolated (Wickner 1994). These arise at a frequency of about 10^{-6} or 10^{-7}.

"Spontaneous" Creutzfeldt-Jakob disease (CJD) could be due to somatic mutation of the PrP gene, with the mutated cell killed early in the pathogenic process. This is nearly impossible to rule out in mammals. However, the reversible curability of [URE3] (Wickner 1994) (and [PSI]; see below) shows that the analog of somatic mutation is not the basis for its arising. Spontaneous [URE3] clones arise from a wild-type strain with a frequency of about 10^{-6}. After curing [URE3] from such a clone, [URE3] again arises in the cured strain with about the same frequency, not a higher frequency, showing that the original [URE3] did not arise from a particularly susceptible cell.

Overexpression of Ure2p Increases the Frequency with Which [URE3] Arises

Introducing *URE2* on a high-copy plasmid into a [ure-o] strain increased by 100-fold the frequency with which [URE3] arose (Wickner 1994). When *URE2* was introduced under control of the galactose-inducible *GAL1* promoter, the increased frequency of [URE3] was seen only when cells had been grown on galactose. Thus, it was overexpression of the

gene, not simply the high copy number of the gene, that induced [URE3] (Wickner 1994).

To show that it was Ure2p, and not the mRNA, whose overexpression induced [URE3], two frameshift mutations were introduced into the overexpressed *URE2* gene. By removing one base in codon 44 and inserting a base in codon 80, most of the prion domain (see below) was completely changed in sequence. This resulted in only a minimal change in the mRNA sequence, no decrease in the mRNA amount, and an altered Ure2p sequence, which carried out nitrogen regulation normally but was reduced 10,000-fold (to undetectable levels) in its ability to induce [URE3] (Masison et al. 1997). This strongly indicates that it is the Ure2 protein and not the mRNA that is responsible for [URE3] induction.

Relation of Phenotypes of ure2 and [URE3] Strains

The *ure2* mutants cannot propagate [URE3] (Aigle and Lacroute 1975; Wickner 1994), but these strains have the *same* phenotypes (Drillien et al. 1973). This is the relation expected for a prion and the gene encoding it (Wickner 1994).

Spontaneous Generation of [URE3]

The original [URE3] strains were isolated as apparent mutants in a laboratory strain that did not have [URE3]. Could [URE3] be a mutant derivative of a nucleic acid replicon present in the parent that happens to depend on *URE2* for its propagation and, like suppressive petites (deletion derivatives of mtDNA that prevent the normal functional mtDNA from replicating), has a phenotype dominant to the normal genome? In this case, a *ure2*Δ strain should lack this normal replicon (analogous to a ρ^o strain) and so should be unable to give rise to [URE3] derivatives. A plasmid expressing Ure2p from a *GAL1* promoter was introduced into a *ure2*Δ strain. The resulting transformants did not grow on ureidosuccinate if the *GAL1* promoter was turned on by galactose, indicating that no normal Ure2p-dependent replicon is responsible for nitrogen regulation. Spontaneous USA+ derivatives were isolated and several were shown to carry [URE3] (Masison et al. 1997). Thus, it is Ure2p itself, and not some replicon dependent on Ure2p, which is responsible for nitrogen regulation and [URE3] appearance.

Protease Resistance of Ure2p in [URE3] Strains

The prion model for [URE3] predicts that Ure2p will be altered in [URE3] strains compared to [ure-o] strains. The proteinase K resistance

of PrP facilitated its discovery (Bolton et al. 1982). Although the alteration need not be one of conformation, sensitivity to proteases is a simple method to detect alterations when one has neither purified protein nor advance knowledge of the nature of the change.

Ure2p was more resistant to proteinase K digestion in extracts of [URE3] strains than in extracts of wild-type cells (Masison and Wickner 1995). The 40-kD Ure2p was converted in less than 1 minute to fragments of 30 kD or 32 kD that persisted for 15–20 minutes. The normal protein was digested in less than 1 minute to fragments too small to detect on the gel. The protease-resistant part of Ure2p in [URE3] strains included the amino-terminal part of Ure2p. Curing the [URE3] strain resulted in a return of the protease sensitivity to the wild-type pattern, and independent isolates of [URE3] showed similar patterns of protease resistance (Masison and Wickner 1995).

One alternate explanation of [URE3] is that it is a heritable state of a regulatory system, such as has been described for the *lac* operon, bacteriophage λ, and *Drosophila* sex determination (see, e.g., Novick and Weiner 1957; for review, see Riggs and Porter 1996). This hypothesis is particularly important to consider because Ure2p is known to be a transcriptional regulator. In this model, the protease resistance of Ure2p would be explained as a change that happens as part of derepression of nitrogen metabolism, a normal alteration of the protein. However, it was found that growing normal cells on a poor nitrogen source such as proline did not make Ure2p detectably protease resistant, so this hypothesis is unlikely (Masison et al. 1997).

Prion Domain Versus N-regulation Domain and Their Interactions

Overexpressed fragments of Ure2p were examined for their ability to complement a chromosomal *ure2*Δ mutation and for their ability to induce the appearance of the [URE3] non-Mendelian genetic element (Fig. 3) (Masison and Wickner 1995). The amino-terminal 65-residue fragment was sufficient to induce [URE3] at high frequency, about 100 times more efficiently than overexpression of the intact Ure2p. Deletion of the same amino-terminal 65 residues left a carboxy-terminal fragment that could completely, though inefficiently, complement the *ure2*Δ mutation but had no activity in inducing the appearance of [URE3] (Masison and Wickner 1995). Even a small deletion of the carboxyl terminus of Ure2p resulted in loss of complementing activity and increase of [URE3]-inducing activity, suggesting that in some way the active protein stabilizes

the protein in the normal form. The amino-terminal 65 residues was thus denoted the *prion domain*, since its overexpression was both necessary and sufficient for prion induction. The carboxy-terminal part of the molecule was named the *nitrogen regulation domain*.

The roles of these domains have been further clarified by experiments modeled after the 1906 Chicago White Sox famous double-play combination, Tinker-to-Evers-to-Chance (Esteban and Wickner 1987; Masison et al. 1997). [URE3] was introduced from Tinker by cytoduction into a strain (Evers) deleted for its chromosomal copy of *URE2* but expressing the prion domain, the nitrogen regulation domain, or both (as separate molecules) from plasmids. The phenotypes of Evers cytoductants were noted, and then cytoplasm was transferred to Chance, a normal strain, to determine whether [URE3] was propagated in Evers.

It was found that the amino-terminal prion-inducing domain is sufficient to propagate [URE3], even in the complete absence of the carboxy-terminal nitrogen regulation domain. The carboxy-terminal domain, lack-

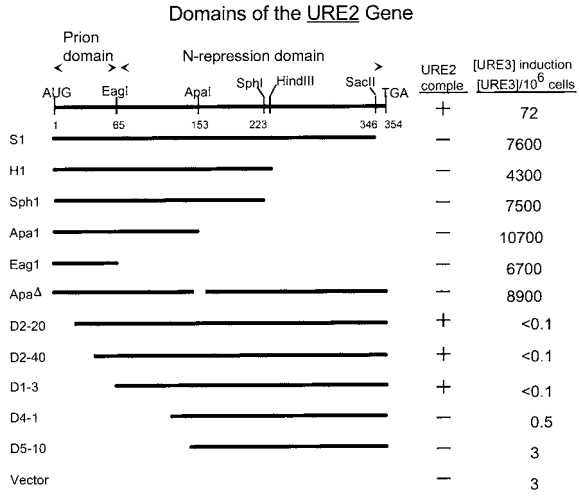

Figure 3 Domains of Ure2p. Parts of Ure2p were overexpressed from a GAL1 promoter and tested for induction of [URE3] appearance (in a wild-type host) or for complementation of a chromosomal deletion of *URE2*. The amino-terminal 65 amino acid residues were sufficient to induce [URE3], but could not complement *ure2*Δ, and so this part of Ure2p is called the prion domain. The carboxy-terminal domain lacking these first 65 residues could complement *ure2*Δ but could not induce [URE3] and is called the nitrogen regulatory domain. (Reprinted, with permission, from Masison and Wickner 1995.)

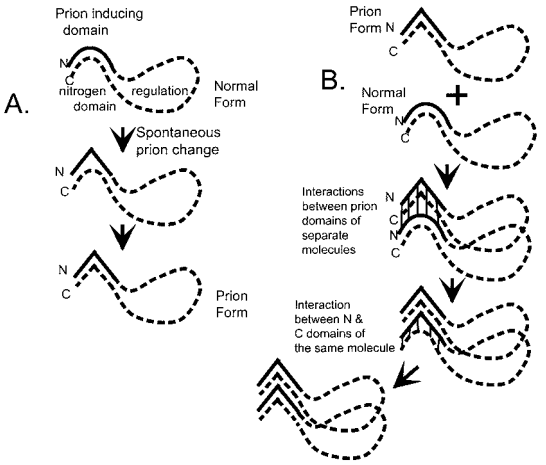

Figure 4 Model of [URE3] prion propagation. (*A*) The prion change of Ure2p arises in the amino-terminal domain and this change then inactivates the carboxy-terminal nitrogen regulation domain. (*B*) The [URE3] change is communicated from one molecule to another by an interaction of their respective amino-terminal prion domains. The altered amino-terminal domain of the second molecule then inactivates its own carboxy-terminal domain. A similar model may apply to [PSI]. (Modified from Masison et al. 1997.)

ing a covalently attached amino-terminal domain, could not respond to [URE3] when it was introduced. Thus, a strain in which the two domains are separately expressed shows repressed nitrogen metabolism (carboxy-terminal domain active). Nonetheless, [URE3] is propagated in this strain and is detected in strain Chance (Masison et al. 1997).

These results are summarized in a model (Fig. 4) in which only prion domain/prion domain and prion domain/regulatory domain interactions occur in the prion propagation process (Wickner et al. 1995; Masison et al. 1997).

The glutathione-*S*-transferase homology of Ure2p (Coschigano and Magasanik 1991) is restricted to the carboxy-terminal domain. The prion domain of Ure2p is 40% asparagine and 20% threonine + serine, an unusual composition (Fig. 5). The relation of this composition to prion induction is not yet clear. The association of expanded polyglutamine domains in several dominant inherited diseases of humans suggested to Perutz that polyglutamine might mediate aggregation of the affected proteins (Perutz et al. 1994). This notion was supported by the finding that

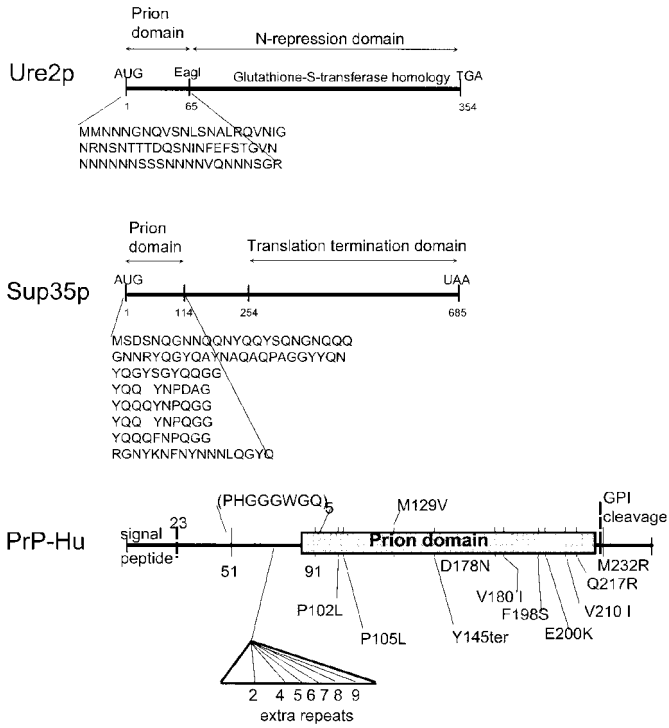

Figure 5 Comparison of Ure2p, Sup35p, and PrP.

insertion of an artificial polyglutamine domain into a soluble monomeric protein promoted its self-association (Stott et al. 1995). It is possible that the high asparagine and glutamine contents of the prion domains of Ure2p and Sup35p (see below and Fig. 5) may lead to self-association by a similar mechanism.

[PSI], A PRION OF Sup35P, A TRANSLATION TERMINATION FACTOR

History and Phenomenology of [PSI]

Discovery of [PSI] by Brian Cox

Nonsense codons in yeast, as well as in other organisms, can be read through (suppressed) by mutant tRNAs. In 1965, B. Cox found that a tRNA mutation may cause more efficient nonsense suppression in some strains (called [PSI+]) and less efficient nonsense suppression in other

strains (called [psi–]) (Cox 1965). [PSI+] strains exhibit a low-level nonsense suppression even in the absence of any known tRNA mutations (Liebman and Sherman 1979). Although tRNA suppressor mutants are usually codon-specific, [PSI+] can suppress all three types of nonsense codons (Ono et al. 1986; Cox et al. 1988; Tikhodeev et al. 1990), a so-called "omnipotent" suppressor.

Non-Mendelian Behavior of [PSI]

Genetic crosses between [PSI+] and [psi–] haploid strains resulted in [PSI+] diploids, which produced exclusively [PSI+] meiotic progeny (Cox 1965; Cox et al. 1988). [PSI+] can also be transmitted from cell to cell by cytoduction (Cox et al. 1988), wherein cytoplasmic fusion occurs without nuclear fusion. The efficiency of cytoduction-mediated transfer of [PSI] points to the cytoplasm as the most probable location of this genetic element. Despite many years of extensive search, [PSI] was not linked to any extrachromosomal DNA or RNA element in the yeast cell (see Cox et al. 1988; for review, see Cox 1994). It was argued (Dai et al. 1986) that [PSI] might reside on the so-called 3-micron DNA, extrachromosomal self-replicating copies of the ribosomal RNA genes (Meyerink et al. 1979; Clark-Walker and Azad 1980; Larionov et al. 1980). However, later experiments showed that [PSI+] strains remained [PSI+] and [psi–] strains remained [psi–] after all endogenous rRNA genes were deleted (Y. Chernoff and S. Liebman, unpubl.) and substituted with a multicopy plasmid expressing a whole rRNA cluster under a normal promoter (Chernoff et al. 1994), or all Pol-I-transcribed rRNA genes were inactivated by Pol-I mutation and substituted by exogenous rRNA expressed from a Pol-II promoter (Nierras and Cox 1994).

Reversible Curing of [PSI]

Another important feature of [PSI+] cells is that they can be efficiently changed into [psi–] cells ("cured") by treatments, such as osmotic stress (Singh et al. 1979), which are not known to efficiently induce gene mutations (for review, see Cox et al. 1988). Cured [psi–] cells are usually able to spontaneously "revert" back to the [PSI+] state (Lund and Cox 1981). Growth in the presence of 1–5 mM guanidine HCl (GdnHCl) is the most efficient cure for [PSI] (Tuite et al. 1981). Some [PSI+] strains were 100% cured after 7–8 generations in 5 mM GdnHCl. It was believed that [psi–] strains generated by 5 mM GdnHCl treatment do not revert back to the [PSI+] state (Tuite et al. 1981; Cox et al. 1988), but cases of such reversions in 5 mM GdnHCl-generated [psi–] strains have been described

previously (Tikhodeev et al. 1990; Chernoff et al. 1993) and [PSI] appearance in these strains can be induced by overproduction of the prion-forming domain of Sup35p (see below).

SUP35 as a [PSI] Maintenance Gene

Sup35 is Release Factor eRF3

Nonsense suppression by [PSI] points to a possible translational termination defect in [PSI+] strains. Translational termination in eukaryotic cells is performed by a complex of two release factors, eRF-1 (Frolova et al. 1994) and eRF-3 (Zhouravleva et al. 1995). The latter contains GTP-binding domains and is probably responsible for GTP hydrolysis, which is required for termination. The yeast counterpart of eRF-3 is a 79-kD (685 amino acids) protein called Sup35p (Stansfield et al. 1995; Zhouravleva et al. 1995). *Sup35* mutations were known for years to cause omnipotent suppression (Inge-Vechtomov 1964; Hawthorne and Mortimer 1968; Inge-Vechtomov and Andrianova 1970; Surguchov et al. 1984; Hinnebusch and Liebman 1991) and to increase the efficiency of nonsense suppression by mutant tRNAs (Surguchov et al. 1984; Ono et al. 1986). Thus, *sup35* mutants exhibit phenotypes similar to those caused by [PSI].

[PSI] Induction by SUP35 Overproduction

Unexpectedly, a multicopy plasmid bearing a wild-type *SUP35* gene was also shown to cause nonsense-suppression (Chernoff et al. 1988, 1992a) as had previously been observed for [PSI+]. The mechanism of this phenomenon is not entirely clear. Sup35 overproduction is not accompanied by a proportional increase of the ribosome-associated Sup35p (Didichenko et al. 1991), indicating that most of the overproduced Sup35p is misplaced due to a limited number of terminating ribosomes. Misplaced Sup35p may bind functional Sup35p and/or other components required for normal termination, thus resulting in decreased efficiency of termination, and nonsense suppression (Fig. 6).

Most interestingly, a significant subpopulation (in some strains, up to 20%) of cells bearing a multicopy *SUP35* plasmid retained suppression after loss of the plasmid (Chernoff et al. 1993). These cells acquired a new nonsense suppressor, which, like [PSI], was inherited cytoplasmically and cured by GdnHCl. Experiments with *SUP35* under the control of a galactose-inducible (GAL) promoter (Derkatch et al. 1996) show that it is overproduction of Sup35p and not the excess of *SUP35* DNA or mRNA that causes appearance of [PSI]. Induction of [PSI] appearance due to

Sup35p overproduction, which resembles induction of [URE3] appearance due to Ure2p overproduction, served as crucial evidence for the prion model of [PSI] (Wickner 1994).

SUP35 Requirement for [PSI] Maintenance

Nuclear *PNM* ("PSI no more") mutations result in cells being unable to propagate [PSI], even if it is reintroduced (Cox et al. 1988). One of the *PNM* mutations, *PNM2*, is a mutant allele of *SUP35* that contains a glycine-to-aspartic acid substitution at amino acid position 58 (Doel et al. 1994). Although *SUP35* is essential for cell viability (Kikuchi et al. 1988; Wilson and Culbertson 1988; Kushnirov et al. 1990b; Ter-Avanesyan et al. 1993), the amino-terminal 253 amino acids of the *SUP35* ORF can be deleted without any apparent effect on the growth (Ter-Avanesyan et al. 1994). However, this *sup35N* deletion manifests itself as a recessive *pnm* mutation, as does deletion of residues 21 to 69 (Ter-Avanesyan et al. 1994). Thus, the amino-proximal portion of Sup35p is required for [PSI] maintenance (see below, Fig. 7). Although it has initially been hypothesized that Sup35p and [PSI] are interacting in *trans* (Ter-Avanesyan et al.

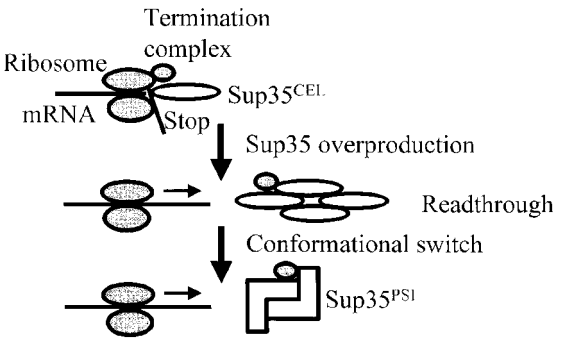

Figure 6 Induction of suppression and [PSI] appearance by Sup35 overproduction. ($Sup35^{CEL}$) Normal cellular form of the Sup35 protein; ($Sup35^{PSI}$) prion form of the Sup35 protein; (Stop) termination codon; (●) components of the translational termination complex (other than Sup35). $Sup35^{CEL}$ overproduction facilitates aggregation of the Sup35 protein and increases a possibility of the conformational switch. Sup35 aggregates titrate out other components of the termination complex, thus blocking termination and leading to the readthrough.

1994), such a result would also be expected (Wickner 1994) if [PSI+] is a prion-like derivative of Sup35p.

Evidence for Sup35p Aggregation in [PSI+] Strains

As expected from the phenotypes, stop codons of heterologous templates are read through in cell-free lysates of [PSI+] strains but not in those of

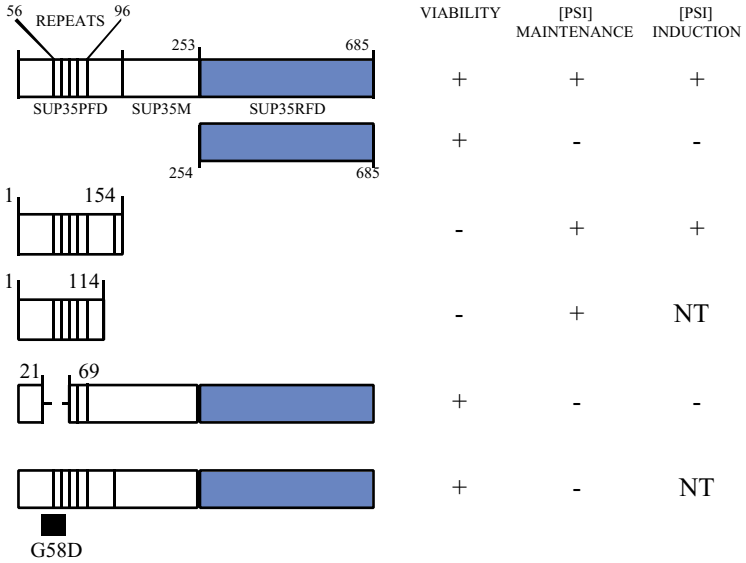

Figure 7 Structural and functional organization of the Sup35 protein. Sup35p domains (Sup35PFD: prion-forming domain; Sup35M: middle domain; Sup35RFD: release factor domain) and the region of oligopeptide repeats are shown. Amino acid positions are indicated by numbers. Position of the *PNM2* mutation (58Gly-to-Asp; Doel et al. 1994) is indicated. Construction of the in-frame *SUP35* deletions is described by Kushnirov et al. (1990a) and Ter-Avanesyan et al. (1993). For viability (Ter-Avanesyan et al. 1993) and [PSI] maintenance (Doel et al. 1994; Ter-Avanesyan et al. 1994) experiments, the corresponding *SUP35* fragments were either transplaced into the chromosome, thus substituting the normal chromosomal *SUP35* copy, or combined in *trans* with previously constructed *sup35* deletions which are not able to confer viability or to maintain [PSI] by themselves. For suppression (Chernoff et al. 1992a,b; Chernoff and Ono 1992; Ter-Avanesyan et al. 1993; Derkatch et al. 1996) and [PSI] induction (Chernoff et al. 1993; Derkatch et al. 1996) experiments, the corresponding *SUP35* fragments were multiplied or overexpressed in the presence of the intact chromosomal *SUP35* copy. (NT) Not tested.

[psi–] strains (Tuite et al. 1983). The earliest evidence that Sup35p was altered in [PSI+] strains was that a factor from [psi–] extracts that corrected this defect was ribosome-associated (Tuite et al. 1987), like Sup35p (Eustice et al. 1986; Didichenko et al. 1991). That readthrough of stop codons (i.e., [PSI+]) was dominant in vivo, but recessive in vitro, is an argument for the prion model of [PSI] (Wickner et al. 1995). Apparently, the prion conversion that is responsible for [PSI] dominance in vivo does not occur in the conditions of this in vitro system. Thus, the Sup35p in the [psi–] lysate remains functional and acts efficiently in vitro.

Recent experiments (Patino et al. 1996; Paushkin et al. 1996) clearly demonstrate that although Sup35p is soluble in [psi–] cells, it is assembled into insoluble aggregates in [PSI+] strains. $Sup35^{PSI}$ aggregates are so huge that they are precipitated by centrifugation at 12,000*g* (Patino et al. 1996) and even at 7,000*g* (M. Patino and S. Lindquist, pers. comm.; G. Newnam and Y. Chernoff, in prep.). By itself, Sup35 aggregation in [PSI+] strains does not prove a prion model of [PSI]. An alternative model would have aggregation of the release factor secondary to the effects of a product of a nucleic acid replicon. However, in light of the genetic data discussed above, such an explanation is unlikely. $Sup35^{PSI}$ aggregation provides an explanation of the termination defect caused by [PSI+]. Aggregated $Sup35^{PSI}$ is probably inactive in translational termination, leading to translational readthrough.

Sup35p in [PSI+] strains also shows increased proteinase resistance (Patino et al. 1996; Paushkin et al. 1996; Y. Chernoff et al., unpubl., [quoted in Patino et al. 1996]), a characteristic of mammalian PrP in scrapie-infected animals (Prusiner 1984, 1991), and yeast Ure2p in [URE3+] strains (Masison and Wickner 1995). However, the proteinase resistance of $Sup35^{PSI}$ is significantly lower than that of PrP^{Sc} and $Ure2^{URE3}$.

Eukaryotic release factor includes two subunits, eRF3 (Sup35) and eRF1 (Sup45) (Frolova et al. 1994; Zhouravleva et al. 1995), which physically interact with each other (Stansfield et al. 1995). Patino et al. (1996) reported that Sup45 protein remains soluble in the [PSI+] cells, but Paushkin et al. (1997b) have observed Sup45 aggregates, the presence or absence of which correlates with presence or absence of [PSI]. Strain differences might have contributed to these discrepancies. Additionally, Paushkin et al. used a high-speed (200,000*g*) centrifugation through sucrose pads, an approach that enabled them to recognize smaller oligomers, compared to those identified by Patino et al., who employed 12,000*g* centrifugation in most of the experiments. Perhaps Sup45 is associated with the small $Sup35^{PSI}$ oligomers or is self-aggregating in the

absence of available soluble Sup35p, but is not in the huge $Sup35^{PSI}$ aggregates. Unlike Sup35p, Sup45p does not become proteinase resistant in [PSI+] strains (Paushkin et al. 1997b), as would be expected if Sup45p were in the huge aggregates.

Structural and Functional Analysis of the Sup35 Prion and Release Factor Domains

S. cerevisiae Sup35p consists of two distinct regions: the nonessential amino-terminal 253 amino acids (called Sup35N) necessary for [PSI] propagation, and the carboxy-terminal 432 amino acids (Sup35C) necessary for translation termination and growth and homologous to elongation factor EF-1α (see Figs. 5, 7) (Kushnirov et al. 1988, 1990a; Wilson and Culbertson 1988; Ter-Avanesyan et al. 1993, 1994; Doel et al. 1994). Overexpression of the *SUP35N* portion alone causes a nonsense-suppressor effect in the same way (and even more efficiently) as does overexpression of the whole *SUP35* gene (Kushnirov et al. 1990a; Ter-Avanesyan et al. 1993). In contrast, overexpression of the *SUP35C* portion alone decreases translational readthrough (Ter-Avanesyan et al. 1993), as expected for a release factor.

Overproduction of Sup35N was essential for [PSI] induction (Derkatch et al. 1996). The shortest piece of Sup35p that was still able to induce [PSI] contained just the first 154 amino acids. Deletion of residues 21 to 69 eliminated both [PSI] induction (Derkatch et al. 1996) and ability to propagate [PSI] (Ter-Avanesyan et al. 1994; see above). The *PNM2* glycine-to-aspartic acid substitution, which cures [PSI], is also located within this region (Doel et al. 1994).

Overproduction of the Sup35p lacking just the 21-69 region does not increase [PSI] appearance, but it still causes nonsense suppression (Kushnirov et al. 1990a; Ter-Avanesyan et al. 1993). Apparently, nonsense suppression caused by Sup35p overproduction cannot be interpreted as a simple consequence of [PSI] appearance. Rather, Sup35p (or Sup35N) overproduction results in two distinct phenomena: (1) a translational termination defect, which disappears after the Sup35p-overproducing plasmid is lost; (2) formation of $Sup35^{PSI}$, which also causes a translational termination defect but remains in the cell after loss of the plasmid (see above and Fig. 6). The 21-69 region is required for [PSI] formation, but not for the translational termination defect. Perhaps the 21-69 region is essential for the Sup35/Sup35 interactions that lead to the formation of prion "seeds."

Nonsense suppression and induction of [PSI] by overproduction of the Sup35N region were detected only in the strains that contain an intact chromosomal copy of the *SUP35* gene and not in those in which *sup35N* was deleted. However, expression of the *SUP35N* region in *trans* rescued [PSI] in the haploid strain that contained only the *SUP35C* region in the chromosome (Ter-Avanesyan et al. 1994). Although nonsense suppression by [PSI] could not be detected in the strain in which Sup35N and Sup35C domains are physically separated, [PSI] rescue could be verified by the subsequent cytoduction into the strain bearing an intact chromosomal copy of the SUP35. The minimal fragment that was still able to rescue [PSI] contained the first 114 amino acids of the Sup35N. Thus, Sup35p consists of at least two structurally and functionally distinct parts: the amino-proximal prion-forming domain (PFD) and carboxy-proximal release factor domain (RFD). These two domains are linked to each other by the portion Sup35M ("middle"), which appears dispensable for both prion and release factor functions.

Existence of the prion and functional domains within Sup35p resembles the situation of another yeast prion, Ure2 (Masison and Wickner 1995; see above). It has been shown that Sup35N (PFD-containing) region fused to the green fluorescent protein (GFP) causes formation of the fluorescent aggregates in Psi+ but not in Psi– strains (Patino et al. 1996). Patterns of these aggregates resemble patterns expected for the [PSI] factor, indicating that Sup35 PFD fused to another protein (not to the Sup35 RFD) may be able to convert this protein into a prion. However, the ability of the Sup35N-GFP aggregates to reproduce themselves generation after generation in prion-like fashion is yet to be proved.

The amino-terminal 123 amino acid residues of Sup35, which roughly correspond to the Sup35PFD, are high in glycine, glutamine, asparagine, and tyrosine residues (Kikuchi et al. 1988; Kushnirov et al. 1988, 1990; Wilson and Culbertson 1988). The high concentration of glycine residues, which usually mark the positions of polypeptide chain turns, may point to a high potential for structural flexibility. Moreover, Sup35 PFD contains glycine-rich oligopeptide repeats, which are similar to those found at approximately the same position of the mammalian PrP (Cox 1994; Kushnirov et al. 1995). The region of repeats includes the Sup35 amino acid residues 56–90 and overlaps a piece that is required for [PSI] maintenance and induction (amino acid residues 21–69). Although the corresponding region of repeats in PrP does not seem to be essential for prion infection in mammals (Fischer et al. 1996), mutations within repeats and/or duplica-

tions of the repeats were shown to be associated with an inherited form of prion disease (Goldfarb et al. 1994). The Sup35M region (amino acid residues 124–253) contains a high percentage of glutamic acid and lysine residues (Kikuchi et al. 1988; Kushnirov et al. 1988, 1990a; Wilson and Culbertson 1988).

Evolution of Sup35p from Yeast to Humans

Sup35 (eRF3) is a well-conserved eukaryotic protein. Southern hybridization at low stringency, using a piece of *SUP35C* as probe, uncovered *SUP35* homologs from yeast to humans (Chernoff et al. 1992b), and those from *Pichia methanolica* (Kushnirov et al. 1990b), *Homo sapiens* (Hoshino et al. 1989; Jean-Jean et al. 1996), and *Xenopus laevis* (Zhouravleva et al. 1995; Jean-Jean et al. 1996) have been cloned and sequenced.

Distinct Sup35p domains exhibit different rates of evolution (Fig. 8). The Sup35C (RFD) region was conserved from *Saccharomyces* to *Pichia* (76%) to humans (57%), and the *Pichia SUP35* could complement a dele-

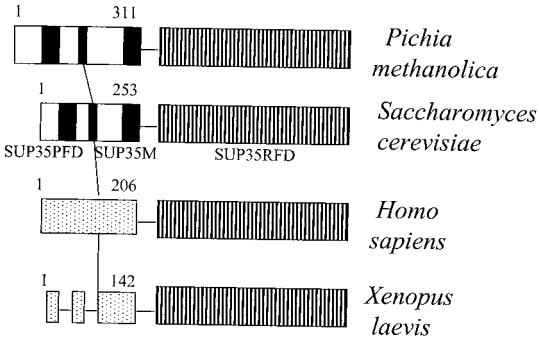

Figure 8 Evolution of the Sup35 protein from yeast to humans. Regions of homology are filled in the same way. Blank boxes correspond to the divergent regions. Amino acid positions are indicated. Data are from Kushnirov et al. (1988) and Wilson and Culbertson (1988) for *S. cerevisiae*; Kushnirov et al. (1990a) and Chernoff et al. (1992b) for *P. methanolica*; Hoshino et al. (1989) for *H. sapiens*; and Zhouravleva et al. (1995) and Jean-Jean et al. (1996) for *X. laevis*. Additional 138 amino acids are added to the *H. sapiens* Sup35 sequence according to the resequencing data (Jean-Jean et al. 1996) and computer analysis results (M. Borodovsky and Y. Chernoff, unpubl.).

tion of the *S. cerevisiae sup35* (Kushnirov et al. 1990b). Both human (Hoshino et al. 1989) and *Xenopus* (Zhouravleva et al. 1995) *SUP35* cDNA clones conferred temperature resistance to a temperature-sensitive *S. cerevisiae sup35* mutant, although attempts to rescue a *sup35* disruption with the human *SUP35* gene failed (Y. Chernoff et al., unpubl.).

In contrast, the Sup35N region is highly variable, even between two yeast genera (Kushnirov et al. 1990b; Chernoff et al. 1992b). The *Pichia* Sup35N of 311 residues contains only three stretches (55–88, 124–160, and 231–253) homologous to the *Saccharomyces* Sup35N (Kushnirov et al. 1990b). The first piece is a region of oligopeptide repeats similar to those in *Saccharomyces* involved in [PSI] maintenance (see above). The *Pichia* Sup35 PFD-corresponding region is also followed by a portion of the protein which, like the *S. cerevisiae* Sup35M, is rich in glutamic acid and lysine residues.

The human homolog of *SUP35* (first called *GST1*-Hs; Hoshino et al. 1989), reported at first to have only a short amino-terminal domain of 68 residues, was found by computer analysis to be likely to have a longer amino terminus (M. Borodovsky and Y. Chernoff, unpubl.), and resequencing (Jean-Jean et al. 1996) revealed a frameshift error in the original sequence. Thus, human Sup35N in fact has 206 amino acids. Although hSup35N has no significant homology with the corresponding *Saccharomyces* or *Pichia* domains, the first (PFD-corresponding) half of the human Sup35N contains a high percentage of proline (12%) and glycine (26%) residues, which underlies a high probability of turns. Interestingly, the highest similarity was found in the portion corresponding to the *S. cerevisiae* Sup35N oligopeptide repeats. However, instead of the regular oligopeptide repeats found in the yeast Sup35N regions, the human Sup35N contains long poly-glycine stretches, which are similar to those found in so-called "homopeptide" proteins (Karlin and Burge 1996). The second half of the human Sup35N region is enriched in glutamic acid residues (26%), like the *Saccharomyces* and *Pichia* Sup35M. The human and *Xenopus* Sup35N sequences have long stretches of homology, but the *Xenopus* PFD-corresponding region is 64 amino acids shorter (Jean-Jean et al. 1996).

Thus, Sup35N protein domains of different origins possess unusual amino acid composition and high potential for structural flexibility. Overproduction of the *Pichia* Sup35p has been shown to cause nonsense suppression (Kushnirov et al. 1990b) and to induce [PSI] appearance (Y. Chernoff and G. Newnam, unpubl.) in *S. cerevisiae*. Thus, *S. cerevisiae* Sup35p can be turned into a prion by overproduction of Sup35p of different evolutionary origin. It is possible that [PSI]-like phenomena can

also be found in eukaryotes other than yeast and may either have a regulatory function or cause a disease.

Physiological and Environmental Factors That Influence [PSI] Propagation

Yeast as a Model to Study Chaperone Effects on Prion Propagation

Although it has been hypothesized that other proteins, in addition to the prion protein, are required for the propagation of mammalian prions, the first evidence that directly connects a chaperone to a prion came from the yeast systems (Chernoff et al. 1995b). Chaperone involvement may explain why some prion conformational conversions are so difficult to reproduce in vitro, that is, in the absence of chaperones. Since chaperones are involved in protein folding by definition, the requirement for Hsp104 in [PSI] propagation suggests that the prion change of Sup35p is a conformational change. Chaperones may also provide an important mechanism for environmental and physiological factors to influence prion appearance and propagation. If confirmed for other prions, these data may lead to new anti-prion treatments that use variations of chaperone levels or activity.

Hsp104 Role in [PSI] Maintenance

Among 6,400 colonies screened from a yeast centromeric (single-copy) genomic library, one colony was found in which nonsense suppression by [PSI] was inhibited by the library clone (Chernoff and Ono 1992). This clone encoded the chaperone Hsp104 (Chernoff et al. 1995b; K. Kounakov et al., unpubl.), previously cloned and sequenced by Y. Sanchez and S. Lindquist (1990). The presence of a functional extra copy of *HSP104* was proved to be responsible for [PSI] inhibition, while transient overproduction of the Hsp104 protein completely cured [PSI] (Chernoff et al. 1995b). Interestingly, [PSI] was also cured by an *hsp104* disruption (Chernoff et al. 1995b). Neither genetic cross nor *SUP35* overexpression was able to reintroduce [PSI] into an *hsp104* deletion strain. Mutations that inactivated both Hsp104 ATP-binding domains also cured [PSI] (Chernoff et al. 1995b), possibly due to a dominant negative effect, since Hsp104 is a hexamer (Lindquist et al. 1995). Interestingly, simultaneous overexpression of *HSP104* and *SUP35* in the Psi+ strain partially protected [PSI] against Hsp104-mediated curing (Y. Chernoff and G. Newnam, unpubl.). Clearly, there is a balance between the Hsp104 and Sup35 proteins that is responsible for the stable maintenance of [PSI]. The

Hsp104 effect on [PSI] appears to be highly specific. Despite an extensive search, no other genes were identified in the yeast overexpression libraries that affect [PSI] in the same way as does Hsp104 (Y. Chernoff and G. Newnam, in prep.).

Hsp104 is nonessential for growth but protects yeast against heat and other stresses and is responsible for induced stress tolerance (Sanchez and Lindquist 1990; Sanchez et al. 1992). Usually, this protein is not expressed during most of the exponential phase, although it is activated in late exponential and in stationary phase, as well as during sporulation (Sanchez et al. 1992). Hsp104 disaggregates protein complexes induced by stress, implying an effect on protein folding (Parsell et al. 1994).

It is not clear yet whether Hsp104 acts on newly synthesized Sup35p, or on the preexisting Sup35p, or on both. Perhaps an optimal level of Hsp104 chaperone is required for maintaining the balance between normal Sup35p ($Sup35^{CEL}$) and an unstable derivative (Sup35*), which serves as an intermediate for prion conversion (Fig. 9) (Chernoff et al. 1995b). The prion form ($Sup35^{PSI}$) converts the intermediate into $Sup35^{PSI}$, rather than into the normal cellular Sup35 ($Sup35^{CEL}$). The prion form is more resistant to chaperone action, so that Sup35*-$Sup35^{PSI}$ conversion is irreversible under normal conditions. However, the $Sup35^{PSI}$ can be converted into the Sup35* if excess Hsp104 is present.

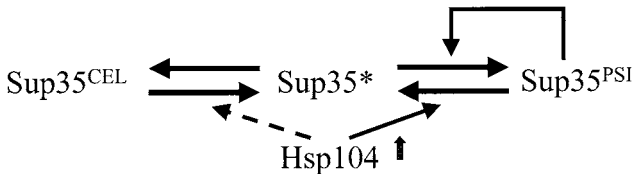

Figure 9 Model for Hsp104 role in [PSI] propagation. ($Sup35^{CEL}$) Normal cellular Sup35 protein; ($Sup35^{PSI}$) prion form of Sup35 protein; (Sup35*) unstable intermediate; (Hsp104 ↑) Hsp104 overproduction. An interaction not required by the model of Paushkin et al. (1996) is indicated by the dashed line. According to the template assistance concept (Cohen et al. 1994), Sup35* is a partially unfolded protein that is susceptible to the prion action (Chernoff et al. 1995b). According to the nucleated polymerization concept (Lansbury and Caughey 1995), Sup35* is an unstable oligomer that can seed formation of the stable prion polymers (Patino et al. 1996; Paushkin et al. 1996). Note that the model of Chernoff et al. (1995b), which was initially proposed within the framework of the template assistance concept, can be interpreted in terms of the nucleated polymerization concept as well. Neither model suggests a role for Hsp104 in the Sup35*→$Sup35^{PSI}$ conversion step.

It is also possible that an increase in the Hsp104/Sup35 ratio makes it improbable for one Hsp104 hexamer to bind more than one Sup35 molecule at a time, so that physical interactions between Sup35 molecules that are required for prion formation become difficult to achieve (Patino et al. 1996). As a result, $Sup35^{PSI}$ cannot be reproduced and is subsequently lost as cells grow. Another possibility is that the role of Hsp104 is to produce enough fragments of the $Sup35^{PSI}$ aggregate to ensure efficient segregation of [PSI] into the daughter cells (Paushkin et al. 1996). Such a model would not require Hsp104 action on the normal $Sup35^{CEL}$. Neither model suggests a direct role for Hsp104 in a Sup35*-$Sup35^{PSI}$ conversion step.

Growth in the presence of elevated levels of Hsp104 indeed results in both curing cells of [PSI] (Chernoff et al. 1995b) and solubilization of the Sup35 aggregates (Patino et al. 1996; Paushkin et al. 1996). It is not clear yet whether overproduced Hsp104 actually disaggregates the $Sup35^{PSI}$ complexes, leading to loss of [PSI], or prevents newly synthesized $Sup35^{CEL}$ protein from conversion and aggregation so that [PSI] is diluted and lost in a generation-dependent manner. Even a transient (30–60 min) increase in Hsp104 caused 50–95% loss of [PSI] in some strains, suggesting the direct solubilization model (Chernoff et al. 1995a), but Hsp104 is a very stable protein (S. Lindquist, pers. comm.), so that excess protein could still be present even after overexpression has been turned off. In vitro, excess Hsp104 failed to solubilize preexisting $Sup35^{PSI}$ aggregates (G. Newnam and Y. Chernoff, unpubl.).

Low levels of excess Hsp104 activity convert [PSI] into a "cryptic" state such that cells are phenotypically Psi^{-}, but return to the Psi+ state when the plasmid is lost (Chernoff et al. 1995b). This intermediate level of Hsp104 activity is sometimes accompanied by conversion of most of Sup35p into the soluble form but causes only a slight curing of [PSI] (Patino et al. 1996; Paushkin et al. 1996). It has been suggested that the remaining $Sup35^{PSI}$ molecules or oligomers serve as templates or seeds for the prion conversion after excess Hsp104 activity is turned off. Those seeds are eliminated if Hsp104 activity is further increased. Interestingly, in some "strong" [PSI+] strains (see below), Sup35 protein remains mostly insoluble in the cryptic [PSI] state, despite the apparent disappearance of translational termination defect (G. Newnam and Y. Chernoff, in prep.). It is possible that excess Hsp104 "releases" other components of the translational machinery that are titrated out by $Sup35^{PSI}$ aggregates. Recent data (Paushkin et al. 1997b) suggest that Sup45 protein becomes soluble in the cryptic [PSI] strains.

These models predict that Hsp104 and Sup35 proteins physically interact. Indeed, some characteristics of Hsp104 were specifically

changed in the presence of Sup35p in vitro (Schirmer and Lindquist 1997). However, conventional biochemical assays, such as affinity chromatography or co-immunoprecipitation, failed to provide evidence for Hsp104/Sup35p interaction (Schirmer and Lindquist 1997), and Hsp104 has not been found in $Sup35^{PSI}$ aggregates (Patino et al. 1996). Thus, if Hsp104/Sup35p interaction occurs in the cell, this interaction is likely to be transient. Alternatively, the Hsp104 effect on Sup35p may be mediated by other as-yet-unidentified proteins. Preliminary data suggest that the absence of Hsp104 affects in vivo interactions between Sup35p and some other proteins (P. Boilleul and Y. Chernoff, unpubl.).

Differential Effects of Stress-related Proteins on Prions

Hsp104 is induced by most of the [PSI]-curing treatments, such as osmotic stress (Sanchez et al. 1992), ethanol stress (Sanchez et al. 1992), or GdnHCl (Lindquist et al. 1995). Moreover, Hsp104 is induced by UV and methyl methanesulfonate (MMS) (T. Magee and K. McEntee [unpubl.] quoted in Chernoff et al. 1995b), which are also able to convert [PSI+] cells into [psi–] (Cox et al. 1988). Since DNA damage has been implicated as a trigger that "switches on" at least some UV- and MMS-inducible genes (McClanahan and McEntee 1988; Treger et al. 1988; Muramatsu et al. 1993), one could expect that repair of the DNA damage (e.g., by photoreactivation) would decrease the level of Hsp104 induction and correspondingly decrease the frequency of [psi–] "mutations" induced by UV or MMS. Such a hypothesis (Chernoff et al. 1995b) removes a major obstacle to the prion model for [PSI] (see Cox 1994; Wickner et al. 1995) by explaining why the [PSI+]-to-[psi–] mutagenesis caused by DNA-damaging agents depends on DNA repair, as has been reported by M. Tuite and B. Cox (1980).

Can all [PSI]-curing phenomena be explained by Hsp104 induction? One objection (Wickner 1995) is that Hsp104 levels are increased dramatically in cells growing at 37ºC or entering stationary phase (Sanchez and Lindquist 1990; Sanchez et al. 1992), but neither of these conditions cures [PSI] (Singh et al. 1979; Tuite et al. 1981; Cox et al. 1988; Chernoff et al. 1995a). Temperature-induced or stationary-phase-induced proteins (other than Hsp104) may protect [PSI] from Hsp104 effect (Chernoff et al. 1995a). Indeed, changes of Hsp70 levels did affect [PSI]-mediated suppression in a strain-dependent fashion, although they did not cure [PSI] (Chernoff et al. 1995b; Lindquist et al. 1995). Simultaneous overproduction of Hsp104p and heat-induced protein of the Hsp70 family significantly decreased the efficiency of [PSI] curing by Hsp104 (G. Newman et al., in prep.). Hsp70p may antagonize the Hsp104p effect by stabilizing prion intermediates in the "conversion-prone" state.

Hsp104 affects [PSI] and [URE3] differently (Y. Chernoff and S. Liebman, unpubl.). Disruption of *HSP104* in a yeast strain containing both [PSI] and [URE3] cured [PSI] but did not cure [URE3], showing that [URE3] propagation does not require Hsp104 function. Moreover, [URE3] remained GdnHCl-curable in the *hsp104* deletion strain, showing that prion curing by GdnHCl cannot be explained exclusively by Hsp104 induction. Hsp104 overproduction did inhibit [URE3] expression; however, [URE3] was recovered in most of the cells after the HSP104 plasmid was lost (Y. Chernoff and S. Liebman, unpubl.). Although the prion form of the Ure2 protein exhibits an increase in protease resistance (Masison and Wickner 1995), no evidence of Ure2 protein aggregation in [URE3+] cells has been reported thus far (Wickner 1994). Possibly, [PSI] and [URE3] fall into two distinct groups of prions, which are folded via different pathways and use different chaperone "helpers." In that case, one might speculate that the [PSI]-specific pathway is more similar to that of mammalian prions, since amino-proximal regions of the Sup35 and PrP proteins exhibit some similarities in sequence organization and amino acid composition (see above), whereas much less similarity was found between any of those proteins and Ure2 protein (Cox 1994; Kushnirov et al. 1995).

[PSI] Biology

In Vivo Translational Suppression by [PSI]

Although tRNA suppressor mutations were initially used in combination with [PSI] to clearly distinguish Psi+ from Psi– strains (Cox 1965; Cox et al. 1988), nonsense alleles that are efficiently suppressed by [PSI] alone were described later (Liebman and Sherman 1979; Ono et al. 1986; Tikhodeev et al. 1990). It is unknown whether [PSI] affects termination at natural stop codons that are supposed to be protected by context from high efficiency of misreading.

Some readthrough proteins may give cells a selective advantage under certain conditions. Some yeast genes containing in-frame nonsense codons have been described. At least in one case, it has been reported that such a nonsense codon is efficiently read through, although whether the strain was [psi–] or [PSI+] was not determined (Gozalbo and Hohmann 1989).

Is [PSI] a Disease?

[PSI+] strains grow as fast as [psi–] (Cox et al. 1988; Y. Chernoff et al., unpubl.), and both have been identified in yeast genetic collections. However, some *sup35* and *sup45* mutants (Tikhodeev et al. 1990), as well

as some strong tRNA suppressors (Cox 1971), are inviable if [PSI+]. Severe growth defects were detected if Sup35p was overproduced in the presence of [PSI]. As a result, moderate copy number *SUP35*-containing plasmids exhibit extreme instability and undergo unusually frequent integration in the [PSI+] strains (Chernoff et al. 1988; Chernoff et al. 1992a; Dagkesamanskaya and Ter-Avanesyan 1991). At 100 copies per cell, the *SUP35* gene also becomes toxic to [psi–] strains (Derkatch et al. 1996).

Deletional analysis showed that the same region of Sup35p is required for both prion formation and growth inhibition effects in both [PSI+] and [psi–] strains (Ter-Avanesyan et al. 1993; Derkatch et al. 1996). Apparently, Sup35 hyperproduction in Psi– strains causes frequent appearance of [PSI], which results in growth inhibition due to incompatibility between [PSI] and the Sup35-overproducing plasmid. Interestingly, growth inhibition cannot be explained simply as a direct consequence of the high-level translational readthrough caused by [PSI]. Overproduction of Sup35 fragments lacking the 21-69 region but retaining the rest of the PFD caused suppression but did not lead to growth inhibition (Ter-Avaresyan et al. 1993; Derkatch et al. 1996). Perhaps prion aggregates either physically damage the cell or bind and titrate out the proteins that are essential for growth.

Preliminary data also show that at least in some genotypic backgrounds, [PSI+] cells are significantly less viable in the deep stationary phase (that is, in the expired medium) than isogenic [psi–] cells (Y. Chernoff et al., in prep.). Hsp104 protein, which is required for [PSI] propagation (Chernoff et al. 1995b), also plays a crucial role in the deep stationary phase survival (Sanchez et al. 1992). No significant loss of [PSI] was detected in the deep stationary cells, even under conditions in which [psi–] derivatives have a selective advantage. Moreover, some [psi–] strains accumulated [PSI+] cells upon incubation in the expired medium (Y. Chernoff et al., unpubl.). It has also been observed (M. Aigle, pers. comm.) and confirmed (Chernoff et al. 1995b) that spontaneous appearance of [URE3] is increased at about 10- to 100-fold in the yeast cultures, kept refrigerated on the expired synthetic medium for 2–3 weeks. It appears that deep stationary phase conditions provoke or induce appearance of the yeast prions, although presence of the prion may decrease cell survival rates.

A report that a [PSI+] strain contained more Sup35p than the isogenic [psi–] strain, particularly in stationary phase (Paushkin et al. 1996), has not been reproduced when other [PSI+] and [psi–] strains were studied (Patino et al. 1996; Y. Chernoff and G. Newnam, unpubl.). However, proteinase resistance of Sup35p in both [PSI+] and [psi–] strains is increased

proportionally in stationary phase (Y. Chernoff and G. Newnam, unpubl.), and a larger proportion of Sup35p is in insoluble aggregates in the stationary [PSI+] cells (Patino et al. 1996). Formation of aggregates may trigger cell death when nutrients are in short supply. In starving [psi–] cells, the appearance of Sup35p oligomers may result from a decrease in the number of actively translating ribosomes, so that release factor molecules lacking their normal target begin to aggregate. In this model, Sup35 overproduction reproduces the same effect in exponentially growing cells by shifting the balance from ribosome-associated to ribosome-non-associated release factor. Oligomers form the aggregation seeds from which the huge aggregates are formed. These aggregates may eventually kill the cells, but they may also lead to the formation of the self-reproducible $Sup35^{PSI}$ form in the cells that remain viable. The whole process is accelerated in the [PSI+] cells, where $Sup35^{PSI}$ seeds are present from the very beginning.

"Strains" of [PSI]

Mammalian prions exist in different variants (strains), distinguished by their incubation periods (Dickinson and Meikle 1971) and protease digestion patterns (Kascsak et al. 1986; Bessen and Marsh 1994). These strain differences are stably propagated in animals. The yeast non-Mendelian factor [ETA] (Liebman and All-Robyn 1984) is apparently the first example of a [PSI] strain variant. [ETA] causes lethality in a haploid strain containing a sup35 mutation. Like [PSI], [ETA] was cured by GdnHCl, but [ETA] did not cause detectable nonsense suppression, although it was able to increase efficiency of other suppressors. In contrast to [PSI], [ETA] was not transmitted to all the meiotic progeny. Genetic crosses between [PSI+] and [ETA+] strains seemed to indicate an independent transmission of [PSI] and [ETA] to the meiotic progeny. However, interpretation of these results was complicated by the differences in genetic background that might influence expression of [PSI] and [ETA] features.

Suppression efficiency of [PSI] varies in different strains. Although differences in genetic background contribute to such variations (Cox et al. 1988), it has been shown that [PSI]-like factors of different properties may be identified in the isogenic derivatives of one and the same yeast strain. [PSI] factors of different origins transferred into the same strain using cytoduction produced different suppression efficiencies (Tikhodeev et al. 1990).

[PSI] factors induced de novo in a single yeast strain by overproduction of wild-type Sup35p differ from each other in both suppressor effi-

ciency and mitotic stability (Derkatch et al. 1996). These differences were stably inherited in mitotic divisions. Interestingly, [PSI] mitotic stability and efficiency of [PSI]-mediated suppression were positively correlated. "Weak" [PSI+] strains showed 0.1–1% loss during growth in complete medium, whereas "strong" [PSI+] factors were absolutely stable. In addition, the weak [PSI+] strains were cured more efficiently with GdnHCl or by Hsp104 overproduction. Cured [psi–] derivatives gave rise to the same spectrum of newly induced [PSI+] factors upon Sup35 overproduction. Thus, [PSI] strain differences were not determined by changes in the "host" cell genotype (Fig. 10).

Strains of [PSI] appear to be formally analogous to the mysterious strains of mammalian prions. The current model for the mammalian prion strains (Bessen et al. 1995) states that there is either more than one prion conformation of PrP^{Sc} or more than one shape of the prion aggregate ("crystal"), so that different conformations (or different shapes) correspond to the different incubation periods and are stably reproduced in prion-directed conformational conversion events. A similar interpretation has been suggested to explain differences between [PSI] strains (Derkatch et al. 1996).

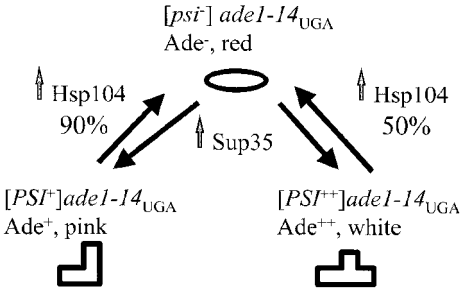

Figure 10 "Strains" of [PSI]. In one and the same [psi–] strain, Sup35 overproduction (↑Sup35) may result in formation of the different $Sup35^{PSI}$ isoforms, which are characterized by the different efficiencies of translational readthrough leading to the detectable variations of color and growth on the specific media. $Sup35^{PSI}$ isoforms are stably inherited in cell generations and are presumably determined by the different shapes of the $Sup35^{PSI}$ molecules (or aggregates). $Sup35^{PSI}$ isoforms also differ from each other by their sensitivities to the [PSI] curing agents, such as GdnHCl or overproduced Hsp104. Cured [psi–] strains exhibit the same spectrum of the newly induced $Sup35^{PSI}$ isoforms as the original [psi–] strain. (Data are from Derkatch et al. 1996.)

Pin+ Versus Pin–

Surprisingly, GdnHCl-induced [psi–] strains, [psi–] strains induced by inactivation of Hsp104, and [psi–] strains induced by excess Hsp104 are not necessarily identical to each other (Derkatch et al. 1997). All excess Hsp104-cured [psi–] strains tested can be efficiently reinduced back to the [PSI+] state by overproduction of the complete Sup35p. Such "*P*si re*IN*ducible" strains were called Pin+. Among the GdnHCl-cured [psi–] strains of the same genotypic background, both Pin+ and Pin– strains were found. Overproduction of the complete Sup35 in Pin– strains did not result in [PSI] appearance. Strains cured of [PSI] by inactivation of Hsp104 were also Pin–, even if a wild-type *HSP104* gene was reintroduced. Moreover, [psi–] Pin+ strains could be converted to [psi–] Pin– strains by growth in the presence of GdnHCl. This observation may indicate that GdnHCl-mediated curing cannot be explained only by Hsp104 induction and may include an additional component. It has been proposed that GdnHCl, which is a protein-denaturing agent, may act on a prion protein directly (Tuite 1994). Such a possibility seems unlikely due to the low (1–5 mM) concentrations of the GdnHCl in the [PSI]-curing medium. However, it cannot be completely ruled out, since we have no information about the intracellular levels of GdnHCl under such circumstances. An alternative explanation could be that GdnHCl induces or affects some other proteins, in addition to Hsp104, which are involved in prion propagation.

Quite remarkably, multiple copies of the *SUP35* fragment coding for only the Sup35 PFD induced [PSI] appearance in both Pin+ and Pin– strains (Derkatch et al. 1997). This confirms that GdnHCl-induced curing of [PSI] remains reversible in principle. One possibility is that GdnHCl action, direct or indirect, results in a conformational change of Sup35p. This change is reproduced as cells grow and prevents the spontaneous conversion of Sup35p into a prion in the absence of preexisting prion protein. The Sup35p region responsible for such a change is apparently located outside the Sup35 PFD. This is why overproduction of the Sup35 PFD alone results in [PSI] induction. Formally speaking, a determinant of the Pin+ phenotype manifests itself as a self-reproducing non-Mendelian element, suggesting that regions of the Sup35p located outside its PFD may influence Sup35p prion-forming abilities in a prion-like fashion.

Cell-free Propagation of [PSI]

Cell-free propagation of the $Sup35^{PSI}$ aggregates has been described by Paushkin et al. (1997a). They incubated a mixture of cell-free extracts

from [psi–] and [PSI+] strains. In addition to the chromosomal copy of the *SUP35* gene, the [psi–] strain contained a plasmid bearing the *SUP35N* (that is, *SUP35PFD* or *SUP35PFD+SUP35M*) region only. Thus, it was possible to distinguish between the shorter Sup35N protein supplied by the [psi–] strain only and the full-size Sup35 protein coming from both [PSI+] and [psi–] strains. After as little as 20 minutes of incubation, a significant portion of the Sup35N protein was converted into the insoluble and protease-resistant $Sup35^{PSI}$ form. In 2 hours, conversion of the Sup35N protein was essentially complete. This process did not occur if the [PSI+] extract was not added. $Sup35^{PSI}$ aggregates were purified and added to the newly prepared [psi–] cell-free extracts to initiate the subsequent cycles of conversion. Again, the differences in molecular weights of the Sup35 derivatives were used to monitor the conversion products. They proved that it was the addition of the polymerized $Sup35^{PSI}$ protein that triggered the conversion process.

Another approach to in vitro formation of [PSI] is based on the ability of the whole Sup35 protein (Glover et al. 1997) and its PFD-corresponding region (King et al. 1997), expressed in and purified from *Escherichia coli*, to form highly ordered β-sheet-enriched fibers in vitro. Possibly, the normally stable $Sup35^{CEL}$ conformation cannot be formed in bacteria, so that purified Sup35 protein deprived of its normal functional counterparts tends to aggregate in amyloid-like fashion. The resulting fibers were remarkably similar to the amyloids formed by the mammalian prions (Glover et al. 1997; King et al. 1997). Preexisting fibers greatly accelerated formation of the new fibers by the newly added soluble protein, thus reproducing themselves in a prion-like fashion. Such an acceleration has also been achieved when the cell-free extracts of [PSI+] strains were used instead of in vitro generated fibers (Glover et al. 1997). The soluble Sup35 protein used in these experiments has been denatured by high concentrations of urea in order to provide the $Sup35^{PSI}$ prion with uniform substrate. Quite remarkably, the same Sup35 fragments, overproduction of which induced [PSI] appearance in vivo (Chernoff et al. 1993; Derkatch et al. 1996), were able to form amyloid fibers in vitro (Glover et al. 1997). The whole process appears to reproduce [PSI] induction upon Sup35 overproduction (Chernoff et al. 1993; Derkatch et al. 1996) in the test tube. The ability of free Sup35p to form self-seeded aggregates in vitro in the absence of other proteins (Glover et al. 1997) seems to contradict the chaperone requirement for [PSI] propagation in vivo (Chernoff et al. 1995b). This could be explained by suggesting that self-aggregating and prion-forming potential of Sup35p is suppressed by its interaction with other proteins in vivo. These interactions are modified

by Hsp104, and possibly by other chaperones, leading to generation of "conversion-prone" intermediates. In vitro, other proteins are not present, or protein complexes are destroyed in the process of cell breakage, so that Sup35p is able to exhibit its prion-forming potential.

In general, cell-free systems for $Sup35^{PSI}$ reproduction are easier to handle and more efficient than the similar systems for in vitro reproduction of the mammalian prions (Kocisko et al. 1994; Bessen et al. 1995). In vitro reproduction of the prion-like protein in the several consecutive cycles (Paushkin et al. 1997a) has not been achieved for the mammalian PrP^{Sc} thus far. Once in vivo "infectivity" of in-vitro-generated $Sup35^{PSI}$ protein is proved, cell-free systems for [PSI] formation could be considered to be established.

PODOSPORA HETEROKARYON INCOMPATIBILITY AND A NEW PRION

Heterokaryon Incompatibility

Recently, another long-known non-Mendelian genetic element has been identified as a likely prion. Filamentous fungi have two forms of mating. One mode is sexual, occurs only between strains differing in sex, and is determined in *Podospora* (as in *Saccharomyces*) by alleles of a single locus. Strains with the + allele will mate only with those with the – allele. This sexual mating leads to meiosis. A second form of mating is called hyphal anastomosis or heterokaryon formation (for review, see Begueret et al. 1994). A colony of a filamentous fungus is composed of cells connected physically by the cell walls and communicating by cytoplasmic bridges between cells. Nuclei can migrate from one "cell" to another relatively freely, so that the entire mass is a syncytium, rather than a cell. The fungal cellular processes are called hyphae, and anastomosis means fusion. Heterokaryons are cells having two or more unfused nuclei of different genotype in the same cell. When two fungal colonies of identical genotype grow toward each other, the approaching hyphae will fuse to form a continuous communicating multicellular individual. This is called hyphal anastomosis and produces cells (heterokaryons) with two types of nuclei, one from each parent. Viruses are found in many fungi (for review, see Wickner 1996), and hyphal anastomosis results in infection of both hyphal mats by any viruses initially present in either one of the parental colonies. Probably to limit the spread of such viruses, hyphal anastomosis (heterokaryon formation) occurs only between strains that are nearly identical, and so probably already have the same viruses. Cells judge each other to be close enough for heterokaryon formation by whether they have

the same alleles at a set of loci, distinct from the mating type locus, called the *het* loci.

One *het* locus, called *het-s* (Rizet 1952), has alleles *het-s* and *het-S*, differing by 14 amino acid residues in a 289-residue protein (Turcq et al. 1990). When *het-S* and *het-s* strains meet, hyphae of the two strains fuse, but the fused cells die (Beisson-Schecroun 1962), forming a barrier that prevents the two colonies from growing together and fusing further. This is called heterokaryon incompatibility.

A Non-Mendelian Genetic Element Affects *Podospora het-s*

Early studies of the *het-s/het-S* incompatibility system showed that cells having the *het-s* genotype could assume either of two phenotypes, now called [Het-s] and [Het-s*] (Rizet 1952). Clones with the [Het-s] phenotype are compatible with other *het-s* strains, but not with *het-S* strains, as above. Clones with the [Het-s*] phenotype are "neutral" in that they can form heterokaryons with either *het-s* or *het-S* strains (Fig. 11). Mating female [Het-s] and male [Het-s*] clones produced meiotic progeny, all of which were [Het-s]. This indicates the presence of something in the [Het-s] strains that is absent in [Het-s*] strains. [Het-s] and [Het-s*] strains are also compatible for heterokaryon fusion, and it is found that following such fusions, the [Het-s] trait spreads throughout what was the [Het-s*] colony, including into regions that do not have the nucleus of the [Het-s] parent. Thus, this trait is controlled by a non-Mendelian genetic element (Rizet 1952). That the [Het-s] non-Mendelian genetic element is restricted to the cytoplasm is suggested by the fact that male [Het-s] strains, when crossed with female [Het-s*] strains, give almost all [Het-s*] progeny (Rizet 1952).

[Het-s] Represents a Prion Form of the *het-s* Protein

Rizet found that the meiotic progeny of mating *het-s* [Het-s] strains with *het-S* strains included *het-S* segregants and *het-s* [Het-s*] progeny, but none that was *het-s*[Het-s] (Rizet 1952). In effect, this meiotic cross efficiently cured the [Het-s] non-Mendelian genetic element. However, among cells that had lost the [Het-s] trait via meiosis, the trait could arise again at low frequency, and when it arose in a colony, it could be shown to spread within the colony, presumably because the colony consists of interconnected cells in which cytoplasmic genetic elements can spread from cell to cell (Rizet 1952). This represents reversible curing of [Het-s].

Overproduction of the *het-s* protein has recently been shown to induce conversion of [Het-s*] strains to [Het-s] (Coustou et al. 1997).

This is reminiscent of overproduction of Ure2p and Sup35p inducing generation of [URE3] and [PSI], respectively (see above).

Deletion mutants of *het-s* (*het-s*^{o}) are healthy and can mate and carry out hyphal anastomosis, but they show no heterokaryon incompatibility. They have the neutral phenotype, like the *absence* of [Het-s]. Moreover, *het-s*^{o} strains are unable to propagate the [Het-s] trait (Coustou et al. 1997), confirming the intimate relation of the *het-s* protein and the [Het-s] non-Mendelian genetic element. Note that unlike [URE3] and [PSI], which confer phenotypes like those of *ure2* and *sup35* mutants, respectively, the [Het-s] element phenotype is the opposite of that of *het-s*^{o} mutants.

These genetic arguments for [Het-s] being a prion form of the *het-s* protein are supported by the finding that this protein is relatively protease resistant in [Het-s] strains compared to [Het-s*] strains (Coustou et al. 1997).

Questions about the [Het-s] System

Several phenomena of the [Het-s] system pose interesting questions in view of the prion interpretation. Crossing *het-s* [Het-s] strains (carrying

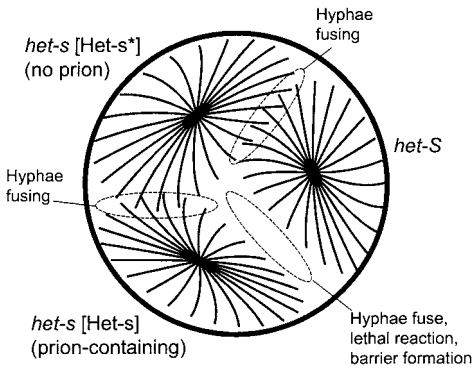

Figure 11 Diagram of vegetative incompatibility in *Podospora.* Heterokaryon incompatibility is controlled by a non-Mendelian genetic element whose properties suggest it is a prion (Coustou et al. 1997). Three strains were inoculated on a plate of growth medium. Chromosomal alleles *het-s* and *het-S* and the presence ([Het-s]) or absence ([Het-s*]) of the prion form of the *het-s* protein are shown. After several days, the *het-s* [Het-s] strain and *het-S* strain show the incompatibility reaction, marked by death of fused hyphae and lack of pigmentation near the barrier. (Reprinted, with permission, from Wickner 1997a.)

the prion form) with a *het-S* strain results in all *het-s* segregants being [Het-s*] (lacking the prion). How does this occur? One could speculate that the *het-S* protein is sufficiently similar to be incorporated into the prion "crystal," but that it is sufficiently different to poison the crystal. If this is true, why, in the vegetative incompatibility reaction, does the interaction of the *het-S* protein with the prion form of the *het-s* protein result in death of the fused hyphae? Could the cell recognize and react to the "poisoned crystal?"

The *het-s* protein has no similarity to other proteins in the database, and it is not yet known what part of the *het-s* protein mediates the [Het-s] prion phenomenon.

Comparison of the *Podospora* [Het-s] Prion with Other Prion Systems

The proposal of genetic criteria for prions (Wickner 1994) has led to the identification of three strong candidates: [URE3], [PSI], and now [Het-s] (Coustou et al. 1997). It has been suggested that fungi are particularly good candidates to develop prions because their mating and hyphal anastomosis involves fusion of all the cell contents of the two parents (Wickner and Masison 1996). In contrast, mating in bacteria results in the passage of only DNA from cell to cell, so prions would not as easily arise.

Among the striking features of the *Podospora* [Het-s] system is that a putative prion helps the cells carry out a normal function (Coustou et al. 1997). Heterokaryon incompatibility is viewed as a normal function of many fungi (Begueret et al. 1994) whose purpose may be to limit the spread of fungal viruses. Another possibility is that the spread of the prion form of the *het-s* protein into the *het-S* strain triggers some sort of fungal apoptosis and that prions will be found to be involved in more than one of the vegetative incompatibility systems.

[Het-s] shows a sort of meiotic curing (Rizet 1952): When *het-s* [Het-s] cells (male or female) are mated with *het-S* strains, all *het-s* progeny are [Het-s*]. It is not the process of meiosis that produces this effect because crosses of the type *het-s* [Het-s] × *het-s* [Het-s*] yield only *het-s* [Het-s] offspring if [Het-s] was in the female parent. Presumably, the presence of the *het-S* protein makes the difference. [URE3] is also often cured by meiosis. Diploid strains that are stably [URE3] may produce meiotic progeny that all lack [URE3] and have returned to normal (R. Wickner, unpubl.; F. Lacroute, pers. comm.). The mechanism of this effect is not yet clear.

The prion domains of Ure2p and Sup35p are similar in their high content of asparagine and glutamine, whereas Sup35p and PrP both have

octapeptide repeats. However, the *het-s* protein lacks both of these features and has no detectable similarity to any of the other putative prion proteins.

Applications of Yeast and Fungal Prions

The availability of microbial systems showing the whole spectrum of phenomena known in scrapie and its various forms has already been useful in providing some evidence for the existence of a prion, some of a type impossible to obtain in a mammalian system.

What would prove the existence of a prion? Probably no one experiment would be sufficient. However, one type of experiment not yet successful in any of the systems is to propagate the altered form in vitro under conditions incompatible with nucleic acid replication and show that infectivity is increased. Infectivity assays have not yet been described for any of the yeast or fungal systems.

Microbial systems are also suited for the development of screening methods to detect compounds that can induce or cure prions. Both the [URE3] and [PSI] systems can be used for either positive or negative selection systems. To test for [URE3] induction, for example, one could spot a compound on a lawn of a *ura2* strain on medium lacking uracil but containing ureidosuccinate and look for colonies surrounding the spot. The disappearance of the red color due to buildup of phosphoribosylamino-imidazole in *ade2* mutants graphically signals the generation of [PSI]. [URE3] cells should be more sensitive than normal to some amino acid analogs whose uptake is sensitive to nitrogen catabolite repression, so rare cells that have lost [URE3] should be selectable. These would signal curing by a tested drug. Likewise, curing of [PSI] makes the red color of an *ade2* strain reappear.

The facility of the yeast system has already paid off in the discovery of the involvement of Hsp104, a chaperone, in propagation of [PSI] (Chernoff et al. 1995b). It is likely that other cellular factors will be found to influence prion formation or propagation.

Microbial systems will also be useful in the search for other new prions. The composite nature of Ure2p and Sup35p suggests that other protein segments may be substituted for their prion domains and the library of hybrid proteins screened for prion-generating clones.

The existence of heritable prions in yeast and fungi suggests that information encoded in protein structure, and not in the primary sequence, can be transmitted to the next generation if preexisting structure serves as a model or template during formation of the new structure. Cytoskeletal networks, formation of which involves a nucleated polymer-

ization mechanism, could serve as likely candidates for such systems of structural coding. Indeed, Sonneborn has shown that a surgically altered patch of the cortical structure of *Paramecium* is propagated with the same morphologically altered structure in subsequent generations of cells (Beisson and Sonneborn 1965). Evidently, the altered structure serves as a template in the formation of the new structure, a phenomenon called cytotaxis or cortical memory (for review, see Hyver and LeGuyander 1995). Similar mechanisms may underlie cytoskeleton formation or other organelle biogenesis. In development, as in de novo prion formation, changes initiated in stem cells are stably propagated even after the original stimulus is removed. It is possible that in some cases a prion-like mechanism underlies this phenomenon.

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7

Knockouts, Transgenics, and Transplants in Prion Research

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Compelling linkage between the infectious scrapie agent, or prion, and PrP was established by biochemical and genetic data (Prusiner 1982, 1989; Gabizon et al. 1988; Hsiao et al. 1989; Scott et al. 1989; Weissmann 1989), leading to the prediction that animals devoid of PrP should be resistant to experimental scrapie and fail to propagate infectivity. This prediction was indeed borne out, adding support to the protein-only hypothesis. In addition, the availability of PrP knockout mice provided an approach to new lines of investigation.

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GENERATION AND PROPERTIES OF MICE DEVOID OF PrP

Generation of PrP Knockout Mice

Three lines of mice devoid of PrP^C have been generated by homologous recombination, using different strategies. Büeler et al. (1992) replaced PrP codons 4–187 (of a total of 254 codons), about 80% of the sequence encoding the mature protein (residues 22–230), by a *neo* cassette. Mice homozygous for the disrupted gene on a mixed C57BL-129/Sv genetic background (designated in this chapter as $Prnp^{0/0}$[Zürich]) expressed truncated PrP mRNA but no detectable PrP fragment and developed normally. Manson et al. (1994b) prepared a line of PrP knockout mice ($Prnp^{-/-}$[Edinburgh]) in which the PrP gene was disrupted by the insertion of a *neo* cassette into a unique *Kpn*I site (following codon 93) of the PrP open reading frame. Mice homozygous for the knockout allele, on a pure 129/Ola background, also developed normally, and no PrP mRNA or PrP-related protein was detected in the brain.

A third line of PrP knockout mice, $Prnp^{-/-}$[Nagasaki], in which 200–300 bp of the second intron, all of the coding sequence, and 452 bp of the 3′ noncoding sequence of the PrP gene were deleted and replaced by a *neo* cassette was generated by Sakaguchi et al. (1995). In this case, mice homozygous for the deleted allele, on a mixed 129/Sv-C57BL background, also developed normally but suffered from ataxia in later life, as described below.

Phenotype of PrP Knockout Mice

The normal function of PrP is not known. PrP or the PrP gene has been found in all vertebrates examined, including chicken (Gabriel et al. 1992) and salmon (Gibbs and Bolis 1997). In mouse embryonic development, the PrP gene is expressed by mid-gestation in the developing central and peripheral nervous tissue. It continues to be expressed throughout development, and mRNA can be detected in a number of nonneuronal tissues both within the embryo and in the extraembryonic tissue (Manson et al. 1992). The PrP gene is expressed at high levels in neurons of the adult brain and has also been detected in astrocytes and oligodendrocytes (Moser et al. 1995). Lower levels of PrP mRNA can be detected in other tissues such as heart, lung, and spleen (Oesch et al. 1985). PrP itself is present predominantly in brain tissue (Bolton et al. 1982) but also at quite high levels in heart and skeletal muscle, whereas it is barely detectable in liver (Bendheim et al. 1992).

The hope that the generation of PrP null mice would cast light on the normal function of PrP has not been fully realized. The offspring of mat-

ing pairs of mice heterozygous for the disrupted PrP allele yielded about one quarter of homozygous offspring, showing that embryonic development of these mice was not impaired. PrP null mice developed and reproduced normally (Büeler et al. 1992; Manson et al. 1994b). Behavioral studies of the Zürich PrP null mice revealed no significant differences from wild-type mice in the Morris water maze, the Y maze discrimination test, and the two-way avoidance test (Büeler et al. 1992).

No difference was found between muscles from wild-type and PrP null animals regarding acetylcholine receptor expression (Brenner et al. 1992). Electrophysiological studies on *Prnp*$^{0/0}$[Zürich] mice by Collinge and his colleagues led to a report that in the hippocampus GABA-A receptor-mediated fast inhibition was weakened, long-term potentiation (LTP) was impaired (Collinge et al. 1994; Whittington et al. 1995), and Ca^{++}-activated K^{+} currents were disrupted (Colling et al. 1996). The inbred Edinburgh PrP null mice consistently showed an absence of LTP in the CA1 region of the hippocampus and, in its place, a short-term potentiation that decayed to control levels within one hour (Manson et al. 1995). Since the two lines of mice were produced by different targeting strategies and have different genetic backgrounds, these results indicate that the impairment of synaptic plasticity is a result of the loss of PrP and not an artifact of gene targeting and subsequent mouse production. Reintroduction of high copy numbers of the human PrP gene into PrP null mice restored the LTP response to that seen in the wild-type controls (Whittington et al. 1995). In contrast, no electrophysiological abnormalities were found by other investigators in the hippocampus (Lledo et al. 1996) or in the cerebellum (Herms et al. 1995) of Zürich null mice.

Mice devoid of PrP were reported to have alterations in both circadian activity rhythms and sleep (Tobler et al. 1996). PrP null mice exhibit a longer circadian period than the wild-type mice. During prolonged recording in constant darkness, the circadian period of the null mice remained stable, in contrast to the wild-type mice, in which it became more variable and progressively shorter. The PrP mice also showed a more fragmented sleep than the wild-type mice and a greater response to sleep deprivation; the increase in electroencephalographic slow wave activity during recovery sleep was almost twice as large in PrP null as in wild-type mice. The effects on circadian activity rhythms and sleep in the PrP null mice can be attributed to an absence of PrP because two different lines of PrP null mice showed similar phenotypes and because the effect was abolished by PrP transgenes (Tobler et al. 1996). However, it is not clear whether the differences between knockout and wild-type mice are due to the absence of a specific function of PrP or to compensatory

effects of the organism elicited by the absence of PrP. Transgenic mice with controllable expression of PrP might provide insight into this question.

In contrast to the findings with the Zürich and Edinburgh knockout mice, the *Prnp*$^{-/-}$[Nagasaki] mice showed progressive symptoms of ataxia and an extensive loss of Purkinje cells (Sakaguchi et al. 1996) at about 70 weeks of age. Because such cerebellar symptoms were not observed in the Zürich and Edinburgh knockout lines, they may be due not to the deletion of the PrP coding region, but rather to the partial deletion of the 3′ noncoding region or of the second intron, which is believed to contain a Purkinje-cell-specific enhancer(s) and which might be active on genes other than PrP (Fischer et al. 1996). In any event, it will be of interest to determine whether the cerebellar deficit of the *Prnp*$^{-/-}$[Nagasaki] mice can be reversed by introducing a PrP expression sequence devoid of the large intron or by the *Prnp*$^{0/0}$ allele of the Zürich knockout mice.

In summary, ablation of PrP has no discernible effects on the development of the mouse nor on its learning ability or ability to perform difficult tasks, but there seem to be discrete effects on the circadian rhythm and sleep pattern; the generality and significance of electrophysiological changes and Purkinje cell degeneration in aging *Prnp*$^{-/-}$[Nagasaki] mice remain to be established. The mode of action of PrP at the molecular level continues to be elusive. It is unknown whether PrP is functionally redundant, but if it is, the compensating protein(s) is unlikely to show extensive structural homology inasmuch as all data bank searches have failed to reveal candidate molecules.

PrP Null Mice Are Resistant to Scrapie

Prnp$^{0/0}$[Zürich] mice, wild-type littermates (both with a genetic background derived from 129/Sv and C57BL/6J animals), as well as Swiss CD-1 control mice were inoculated intracerebrally with about 10^7 LD_{50} units of the Chandler isolate of mouse-adapted prions (Chandler 1961). CD-1 mice showed typical neurologic symptoms at 140 ± 6 days (Fig. 1) and died at 153 ± 7 days. All of 31 *Prnp*$^{+/+}$ mice with the C57BL–129/Sv background showed symptoms at 158 ± 11 days (Fig.1) and died at 171 ± 11 days. In stark contrast, 23 of 25 *Prnp*$^{0/0}$ mice were alive and free of symptoms for at least 2 years (Fig. 1), while 2 died of intercurrent disease other than scrapie. They showed no scrapie-specific pathology as late as 57 weeks after inoculation with prion-containing brain homogenates and were indistinguishable from those 56 weeks after inoculation with normal brain homogenate, whereas *Prnp*$^{+/+}$ mice, 23–25 weeks after inoculation, showed pronounced

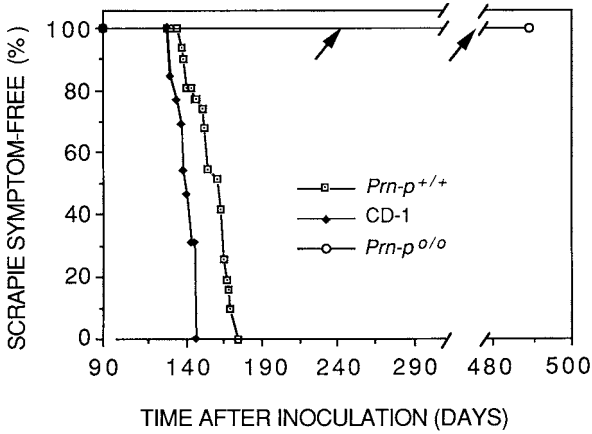

Figure 1 Scrapie resistance of mice with disrupted PrP genes. $Prnp^{0/0}$ mice remain symptom-free after inoculation with mouse scrapie prions. $Prnp^{+/+}$ littermates or wild-type CD-1 mice show scrapie symptoms at the times indicated. *Arrows:* Five mice were killed at various times; none had scrapie symptoms. (Modified from Büeler et al. 1993.)

astrogliosis and vacuolation mainly in the cortex, thalamus, and hippocampus, and in some cases, neuronal loss in the hippocampus and thalamus. Similar results were reported for the $Prnp^{0/0}$[Zürich] mice bred in San Francisco inoculated with the Chandler scrapie agent (Prusiner et al. 1993), for the $Prnp^{-/-}$[Edinburgh] mice (Manson et al. 1994a) inoculated with the ME7 strain of scrapie agent, and for the $Prnp^{-/-}$[Nagasaki] mice inoculated with the CJD-derived Fukuoka-1 strain (Sakaguchi et al. 1995).

The next question concerned the propagation of infectious agent. In the experiments with the Zürich PrP null mice, intracerebral inoculation was performed with non-heated Chandler-agent-infected brain extract or with brain extract heated at 80ºC for 20 minutes to inactivate any adventitious infectious agent. Scrapie infectivity of pooled homogenates of four brains or four spleens was determined by endpoint titration at different times after inoculation. No infectivity was detected in the brains of wild-type mice at 4 and 14 days after inoculation, but infectivity reached about 5.4 and 8 log LD_{50} units/ml at 8 weeks and at terminal disease around 23–25 weeks, respectively. Similar results were obtained with heated and non-heated inocula, within the limits of error. In the case of $Prnp^{0/0}$[Zürich] mice inoculated with heated inocula, no infectivity was detected in brain except for traces at 4 days after inoculation, likely due to residual inoculum. In the experiment with the non-heated inoculum,

the pooled brain sample taken at 20 weeks gave a titer of 3.2 log LD_{50} units/ml, whereas all other samples, taken at 8, 12, 25, 33, and 48 weeks, were negative (Büeler et al. 1993). The experiment was repeated; analyses for infectivity were performed after 16, 18, 20, and 22 weeks, on 4 mice for each time point. Only the 20-week sample gave rise to scrapie disease in one of 8 indicator mice; all other samples in 56 indicator mice were negative (Sailer et al. 1994). Prusiner et al. (1993) performed a similar experiment and found no infectivity in the brains of 8 PrP null mice tested between 120 and 315 days after inoculation, but borderline infectivity was detected in 4 out of 5 mice examined after 5 to 60 days p.i.

Inoculation experiments performed by Sakaguchi et al. (1995) on the Nagasaki null mice showed that low levels of infectivity were found even as late as 29 weeks in all but one of seven samples (each sample was a pool of five mouse brains from different time points) and that this infectivity was diminished or abolished if the samples were first heat-treated. The findings with the Edinburgh mice are not yet available.

From these reports we conclude that there is no evidence for net generation of scrapie prions in PrP null mice and surmise that the occasional low-level infectivity detected in the brains of such mice after i.c. inoculation is due to residual inoculum or, less likely, to contamination. However, it has been speculated that in the absence of PrP, a temperature-sensitive form or otherwise labile form of scrapie agent might be generated (Chesebro and Caughey 1993; Sakaguchi et al. 1995).

Properties of Mice Hemizygous for *Prnp*^{0}

Not surprisingly, mice carrying a single *Prnp* allele (*Prnp*$^{0/+}$ mice) showed no abnormal phenotype as regards behavior and development (Büeler et al. 1992; Manson et al. 1994b). However, they showed enhanced resistance to prion disease, as manifested by a significant delay in onset and progression of clinical disease (Büeler et al. 1994). Whereas lines of wild-type mice (CD-1 or littermates of the knockout mice) showed incubation times of 130–158 days, the "Zürich" *Prnp*$^{0/+}$ mice took 290 days to show disease, the "Nagasaki" hemizygotes 259 days, and mice hemizygous for the "Zürich" ablated PrP allele but bred in San Francisco (Prusiner et al. 1993) took 400–465 days. The reason for the difference between the results with the San Francisco and the Zürich mice, both of which were inoculated with the RML strain of mouse prions, is not clear; the criteria for diagnosing clinical disease may have been different, and/or the genetic background of the mice may play a role. The

Prnp$^{+/-}$[Edinburgh] mice were challenged with three different strains of scrapie agent, ME7, 301V, and 301C; in all cases disease symptoms appeared with a delay, from 80 to 150 days, as compared with wild-type mice (Manson et al. 1996). There appeared, however, to be no difference in the severity of the clinical signs and the pathology in the brain at the terminal stages of disease. PrP gene dosage therefore appears to affect the timing of disease but not the final pathology (Manson et al.1994a).

In summary, whereas in wild-type animals and *Prnp*$^{-/+}$[Edinburgh] mice inoculated with the ME7 strain the increase in PrP^{Sc} levels is followed within weeks by scrapie symptoms and death, *Prnp*$^{0/+}$[Zürich] mice remained free of symptoms for many months despite similar levels of scrapie infectivity and PrP^{Sc} (Fig. 2). These findings suggest that clinical symptoms are not necessarily correlated with the overall accumulation of PrP^{Sc}; there are, in fact, several reports in which prion disease leads to death without substantial accumulation or even detectable levels of PrP^{Sc} (Hsiao et al. 1994; Collinge et al. 1995; Telling et al. 1996; Lasmézas et al. 1997; Manuelidis et al. 1997). Moreover, it is of practical interest that clinically healthy mice can harbor high levels of infectivity for long periods of time because it suggests that cattle or humans in apparent good health may also contain infectious agent in their central nervous system and perhaps fail to show clinical symptoms during their natural lifetimes. This is a point to consider when animal tissues are used for preparing pharmaceuticals or for grafting procedures.

Restoration of Susceptibility to Prion Disease and Prion Propagation by PrP Transgenes

When a certain phenotype is generated by gene ablation—in this case resistance to scrapie—it is important to show that this is indeed the consequence of the targeted genetic intervention and not of some unintended or subsidiary event, such as obliteration of an enhancer governing another gene or disruption of an unidentified reading frame. The most effective way to link a phenotype to the ablation of a specific protein is to introduce into the knockout animal a cDNA encoding the protein in question. Although this may be a problem when a gene gives rise to various protein species as a consequence of transcriptional or splicing variants, this is not an issue in the case of PrP because it is encoded in a single exon. It had been shown earlier that wild-type mice transgenic for a murine cosmid containing the PrP gene gave rise to overexpression of PrP (Westaway et al. 1994). Because the gene contains two introns, one of which is about

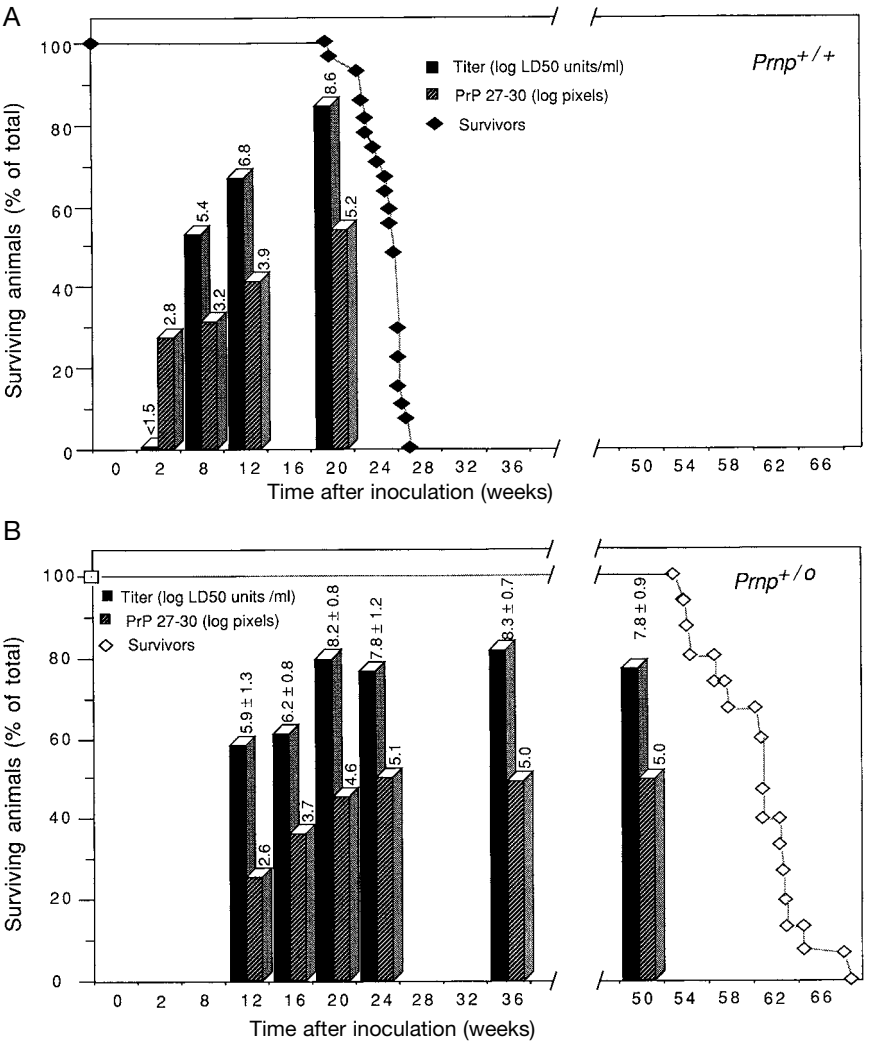

Figure 2 Survival, prion titers, and PrP^{Sc} in brains of scrapie-inoculated mice. (*A*) Wild-type and (*B*) $Prnp^{0/+}$ mice at various times after inoculation with mouse prions. (Reprinted, with permission, from Büeler et al. 1994 [copyright *Molecular Medicine*].)

10 kb in length, we compared the efficacy of constructs from which the large intron (half-genomic *Prnp*) or both introns had been removed with that of the complete murine *Prnp* cosmid mentioned above. Not too surprisingly, in view of findings reported for other transgenes (Brinster et al. 1988), no significant PrP expression was found in mice transgenic for the

intronless PrP construct, even when several gene copies were integrated, whereas efficient overexpression was achieved with both the cosmid and the half-genomic *Prnp* construct. At first blush the expression pattern of both kinds of transgenic mice appeared to be the same, but a more detailed examination later revealed that the Purkinje cells expressed neither PrP nor PrP mRNA at detectable levels. Thus, the half-genomic construct lacks a Purkinje-cell-specific enhancer, which may be located in the large intron or in the downstream 1.6 kb of the 3′ noncoding region, both of which are absent in the half-genomic construct (Fischer 1995).

Although overexpression of the cosmid transgene leads to a profound necrotizing myopathy involving skeletal muscle, a demyelinating polyneuropathy, and focal vacuolation of the central nervous system as the mice age (Westaway et al. 1994), no impairments were ever observed with mice transgenic for the half-genomic construct, even though PrP expression was at equal levels (up to seven times the wild-type PrP level). It is possible that different expression patterns elicited by the two constructs are responsible for the contrasting phenotypes.

Two lines of mice transgenic for the half-genomic *Prnp* construct, expressing PrP at about 3–4 and 6–7 times the level of wild-type mice, were challenged with mouse prions. As shown in Figure 3A, they succumbed to scrapie even more rapidly than wild-type CD-1 mice, confirming that the incubation times are inversely related to PrP expression levels (Scott et al. 1989). Because of its short incubation time, the mouse line *tga20* was rendered homozygous and used for a more rapid scrapie infectivity assay (Brandner et al. 1996b; Fischer et al. 1996). In summary, these experiments confirm that the scrapie-resistant phenotype of the Zürich PrP knockout mice is indeed due to ablation of PrP.

REVERSE GENETICS: STRUCTURE-FUNCTION STUDIES ON PRP

Reconstitution of PrP Knockout Mice with Modified PrP Transgenes

The ability to reconstitute susceptibility to scrapie in mice devoid of PrP paved the way to structure–function analysis of this protein. It had been shown earlier that treatment of scrapie prion preparations with protease cleaves off about 60 amino-terminal residues of PrP^{Sc}, up to residue 88 of the mature protein (Hope et al. 1988), but does not abrogate infectivity of the sample (McKinley et al. 1983). Within the framework of the protein-only hypothesis, and under the assumption that the infectious form of PrP is PrP^{Sc} (Weissmann et al. 1996), this means that the truncated PrP^{Sc} was able to elicit conversion of normal PrP^{C} into PrP^{Sc}. To ascertain whether

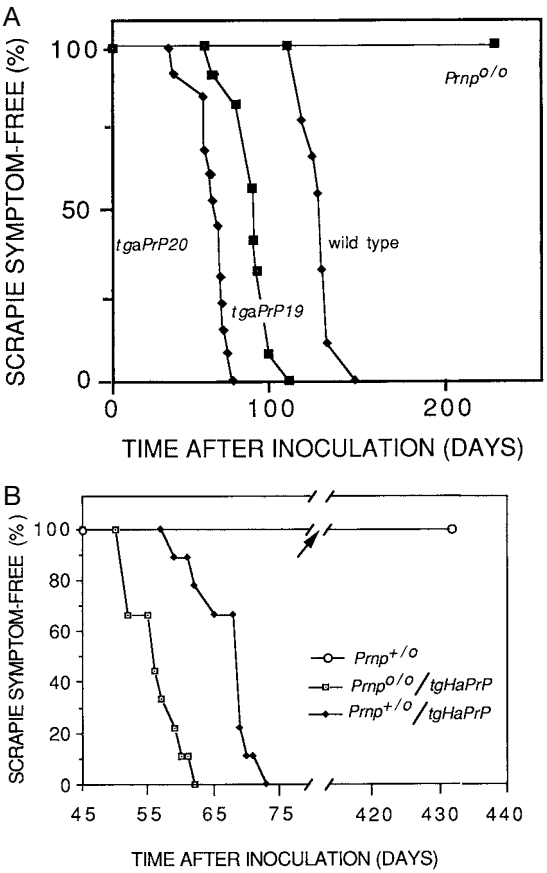

Figure 3 Susceptibility to scrapie of PrP knockout mice carrying various PrP transgenes. (*A*) *Prnp*$^{0/0}$ mice were rendered transgenic for Prnp genes. *tga19/+* mice had 3–4 times the normal PrP^{C} level; *tga20/+* mice had 6–7 times the normal level. (Reprinted, with permission, from Fischer et al. 1996.) (*B*) *Prnp*$^{0/0}$ and *Prnp*$^{0/+}$ mice with hamster PrP transgenes were inoculated with the Sc237 isolate of hamster prions. *Arrow:* One animal died spontaneously without scrapie symptoms and one was killed because of a tumor. Mice with a mouse PrP allele are completely resistant to hamster prions, whereas the presence of hamster PrP in a PrP knockout mouse renders it very susceptible. The additional presence of a mouse PrP allele reduces susceptibility to hamster prions, as evidenced by increased incubation times. (Modified from Büeler et al. 1993.)

amino-terminally truncated PrP^{C} could serve as substrate for the conversion and sustain susceptibility to scrapie in the mouse, we generated a series of transgene-encoding PrP molecules that retained the signal

sequence but had a deletion between residues 32 and 80, 93, 106, 121, and 134 of the mature sequence. PrP knockout mice overexpressing PrP with deletions to positions 80 (Fischer 1995) and 93 (Shmerling et al. 1998) yielded normal mice, which after intracerebral inoculation with scrapie prions developed disease, propagated prions, and exhibited protease-resistant truncated PrP, albeit with longer incubation times and with a lower level of infectivity and PrP^{Sc} in the case of the longer deletion (D. Shmerling, unpubl.). Thus, at least 60 residues from the amino-proximal region of PrP are expendable, which include not only the segment that is cleaved off PrP^{Sc} by proteinase K, but also the entire octarepeat region. This is remarkable because the octarepeat region has been implicated in pathogenesis of familial Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker disease (GSS) cases where the number of repeats is increased from 5 to 9, 10, 11, 12, or 13 (Goldfarb et al. 1992). It has been shown by nuclear magnetic resonance (NMR) that the first 98 residues of mature murine PrP^{C} form a flexible random coil (Donne et al. 1997; Riek et al. 1997), and it was proposed that this segment would be converted to β-sheets in PrP^{Sc} (Riek et al. 1997). If so, our results show that at most only 38 of these residues, starting at position 93, would be essential for the conversion. Indeed, a peptide comprising residues 106–126, which are contained in the flexible region, can form β-sheet structures (Tagliavini et al. 1993; Nguyen et al. 1995) and is toxic to primary neuronal cultures (Forloni et al. 1993).

Pathological Phenotypes Elicited by Truncated PrP Transgenes

Interestingly, transgenic mice overexpressing PrP with deletions extending beyond position 106 to positions 121 and 134 developed ataxia spontaneously at 3–8 weeks of age. In one line of mice with the 32-134 deletion (F11), the granular layer of the cerebellum, which seemed normal at 3 weeks of age, virtually disappeared by 6 weeks (Fig. 4), while the adjoining Purkinje cells and the cerebrum in general showed normal appearance. Crossing a single wild-type allele into mice overexpressing the deleterious transgenes completely abolished the pathologic features, showing that the pathology was not due to an unspecific toxic effect. Offspring devoid of the normal allele, obtained by crossing these heterozygotes with *Prnp*$^{o/o}$ mice again presented the pathologic phenotype. This effect is thus specific for truncations extending to positions 121 and 134 and perhaps beyond because mice overexpressing wild-type PrP or PrP truncated to positions 80 and 93 to the same level or higher remain in

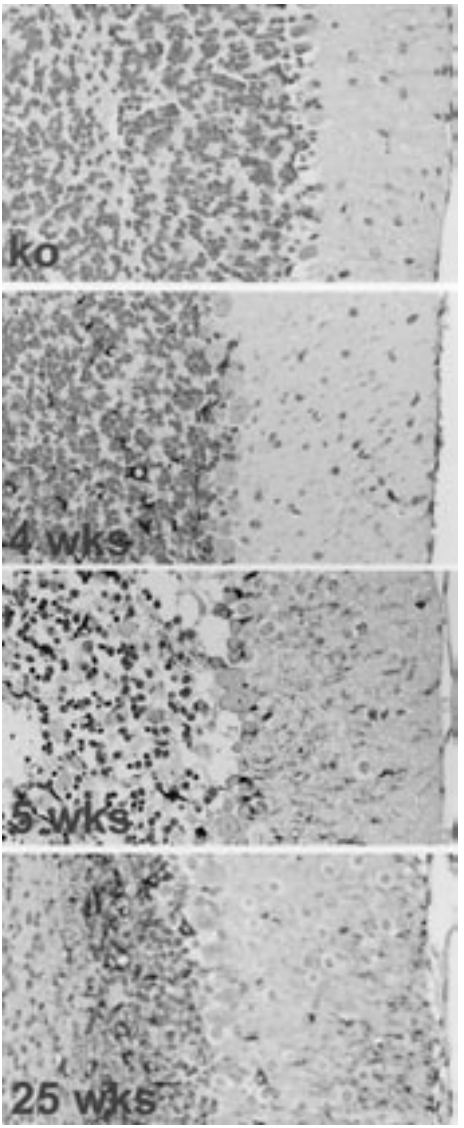

Figure 4 Cerebellar sections from mice expressing Δ32-134 truncated PrP at different ages, showing progressive degeneration of the granule cell layer. Development of the cerebellum proceeds normally until early postnatal life, and leads to formation of molecular layer, Purkinje cell layer, and granule cell layer (right to left in the figure) of normal thickness. However, at 4 weeks, some degree of pathological astrogliosis can already be discerned. At 5 weeks, massive degeneration of granule cells by apoptosis is ongoing. Note strong gliosis affecting also the molecular layer. At the end stage of disease, mice suffer from a profound cerebellar syndrome and the thickness of the granule cell layer is considerably reduced (Shmerling et al. 1998). In some areas of the cerebellar cortex, granule cells disappear completely (not shown).

good health up to at least 2 years and, in the case of the Δ32-106 deletion, up to at least 8 months (Shmerling et al. 1998). Prusiner and his colleagues have reported on a variety of internal PrP deletion mutants other than those described by us and have noted that mice with deletions of either of the two carboxy-terminal α-helices of PrP showed cytoplasmic inclusions of PrP-derived deposits and spontaneously developed fatal

CNS illnesses similar to neuronal storage diseases at ages ranging mostly from 90 to 227 days (Muramoto et al. 1997). This phenotype was observed on a wild-type *Prnp* background and is clearly caused by a different mechanism than the one described above.

How could such effects elicited by transgenes come about? Strong overexpression of wild-type and mutant PrP might result in accumulation of the product, interfere with synthesis and metabolism of other proteins, or interfere or compete with their function; such an effect may well be specific for certain cells or tissues. It is striking that the pathologic phenotype caused by the overexpression of the truncated PrP's Δ32-121 and Δ32-134 is abolished by coexpression of a single wild-type PrP allele. One explanation could be that the truncated PrP competes with both PrP and another, as yet unknown, functional homolog of PrP for a common ligand or receptor without, however, eliciting the appropriate physiological response. This would explain why absence of PrP does not give rise to a phenotype, but expression of certain truncated PrPs does (a transdominant inhibitory effect) and why wild-type PrP, inasmuch as it has a higher affinity for the receptor, can reverse the phenotype (Shmerling et al. 1998).

PrP Transgenes and the Species Barrier

Introduction of PrP transgenes from other species into wild-type mice has been shown to reduce or abolish the so-called species barrier to prions (Scott et al. 1989; Prusiner et al. 1990; Büeler et al. 1993). This effect is even more pronounced if the recipient mouse is devoid of its endogenous PrP genes, as shown by a comparison of the incubation times of $Prnp^{0/+}$ and $Prnp^{0/0}$ mice carrying the same cluster of hamster PrP genes (Fig. 3B) (Büeler et al. 1993). More recently, it has been shown that PrP knockout mice but not wild-type mice containing human PrP transgenes are susceptible to human prions (Telling et al. 1995). As discussed elsewhere in this volume, the sequence of the PrP gene is not the only determinant involved in the species barrier; rather, a component designated protein X is believed to be implicated in this phenomenon (Telling et al. 1994, 1995).

INTRODUCTION OF TARGETED POINT MUTATIONS INTO THE ENDOGENOUS MURINE PRP GENE

Double Recombination Gene Targeting

Variant forms of PrP have been shown to be associated with human transmissible spongiform encephalopathies (TSEs) (for a review, see Collinge and Palmer 1994), and amino acid differences have been shown to be asso-

ciated with different incubation periods in mice (Westaway et al. 1987), hamsters (Lowenstein et al. 1990), and sheep (Goldmann et al. 1991). Recent advances in gene targeting technology now allow the role of specific polymorphisms in the PrP gene to be analyzed by introducing point mutations into the endogenous murine PrP gene (Moore et al. 1995).

A double-replacement gene-targeting strategy was used to introduce point mutations into the PrP locus of the hypoxanthine phosphoribosyltransferase (HPRT)-deficient embryonic stem cell line HM1. In the first step, the entire PrP coding region, located in exon 3, was deleted and replaced with an HPRT minigene. This was achieved by homologous recombination with a targeting vector comprising the 8.5-kb *Bam*HI fragment of the PrP gene in which the coding region of PrP was replaced with an HPRT minigene and to which an HSV-TK gene was appended to allow selection against random integration. Clones with this targeting event were isolated by selection in hypoxanthine, aminopterin, and thymidine (HAT) and gancyclovir.

The second step targeted the inactive PrP allele, replacing the HPRT minigene with a PrP coding sequence into which a specific point mutation had been introduced. This second round of homologous recombination rendered the cells resistant to 6-thioguanine and gave rise to a PrP allele that was indistinguishable from the wild-type allele, except for the desired mutation.

PrP Mutations and Spontaneous Neurodegenerative Disease

A mutation at codon 102, resulting in the replacement of a proline residue for a leucine, has been shown to be linked to GSS in man (Hsiao et al. 1989). Standard transgenic approaches have been used to produce mice (containing wild-type PrP genes) that express high levels of a chimeric hamster-murine PrP gene with the mutation in the equivalent amino acid (101) of the murine gene. These transgenic mice have been shown to spontaneously develop neurodegeneration, spongiform changes in the brain, and astrogliosis late in life (Hsiao et al. 1990). Subsequent work showed that the same mutant PrP gene cluster placed in a PrP knockout mouse caused early disease and more severe neuropathology. Brain extracts from these spontaneously ill mice transmitted disease to transgenic mice expressing the same 101 mutant PrP gene at a lower level (which did not cause spontaneous disease by itself), but not to wild-type mice (Telling et al. 1996).

To study the role of murine PrP with the 101P→L replacement in development and disease, a double-replacement gene-targeting strategy

was used to introduce the mutation into the appropriate position of the endogenous mouse PrP gene in embryonic stem cells. Mice both heterozygous and homozygous for the mutant gene were produced (Moore et al. 1995), allowing the 101P→L mutation to be studied without the complications of disease resulting from overexpression of the PrP gene (Westaway et al. 1994). No symptoms of spontaneous neurodegenerative disease were detected in these mice in up to 650 days, and no abnormal pathology was detected in the brains of the mutant mice. The presence of the mutated PrP at physiological levels is therefore not sufficient to lead to spontaneous neurodegeneration in mice.

Why should PrP with the (101)102P→L mutation give rise to disease in man but not in mouse when expressed at physiological levels? It is assumed that this mutation may facilitate the occasional spontaneous conversion of PrP^{C} to PrP^{Sc} in man. This event may be so rare that it occurs with high probability within the lifetime of a human but not of a mouse, whose brain is more than a thousand times smaller, unless there is very considerable overproduction of the protein. In addition, the interaction of the mutated PrP with other components of the human brain, not present or different in the mouse, may be critical.

PrP Mutations Linked to Incubation Period of Scrapie in Mice

The *Sinc* gene has been shown to be the major gene controlling survival time of mice exposed to scrapie (Dickinson et al. 1968; Carlson et al. 1986). Animals homozygous for the *Sinc* s7 allele have short incubation times when infected with Chandler isolate of scrapie agent and those with the *Sinc* p7 allele have long incubation times. With a different agent, the mouse-adapted bovine spongiform encephalopathy (BSE) strain 301V, incubation times are long for the *Sinc* s7 allele and short for the *Sinc* p7 allele (Bruce et al. 1994). The *Sinc* s7 allele is linked to the *Prnp*a allele characterized by the residues Leu-108 and Thr-189, whereas the *Sinc* p7 allele is linked to the *Prnp*b with Phe-108 and Val-189 (Westaway et al. 1987). The close linkage of the *Sinc* and *Prnp* genes suggested that they might be congruent (Hunter et al. 1987, 1992; Westaway et al. 1987); however, definitive proof remained elusive (Carlson et al. 1986).

Using the double-replacement gene-targeting technique, PrP codons 108 and 189 in murine embryonic stem cells from a *Sinc* s7 (*Prnp*a allele) mouse were altered to Phe-108 and Val-189, which are characteristic for the *Prnp*b allele. Embryonic stem (ES) clones with the mutant PrP gene were used to produce chimeras, and these were bred with 129/Ola mice to

generate an inbred line of mice carrying the PrP gene with Phe-108 and Val-189. Mice homozygous for the *Prnp*a allele and the mutated, *Prnp*b-like allele were inoculated with the 301V agent. The incubation time in the mutant mice carrying the *Prn*b-like allele was 133 days, about 110 days shorter than that of their wild-type littermates carrying the *Prn*a allele. Thus, the point mutations in the PrP gene in mice with otherwise identical genetic background caused a dramatic change in incubation time, establishing that the *Sinc* and *Prnp* genes are indeed congruent (Moore et al. 1998). The results of inoculations with other strains of scrapie agent are, unfortunately, not yet available.

ECTOPIC EXPRESSION OF PrP

In most mouse scrapie models, infectivity appears in the spleen within days after intracerebral inoculation and rises slowly or remains constant throughout the lifetime of the mouse, whereas infectivity in the brain starts rising a few weeks after inoculation and reaches titers two orders of magnitude higher than in the spleen (Eklund et al. 1967; Kimberlin 1979; Büeler et al. 1993). The kinetics of these events depends on the strain of prion as well as on the genotype of the mouse. PrP is expressed in neurons and in astrocytes (Moser et al. 1995), but it is not evident in which cell type infectious agent is generated. In the spleen, PrP is present on the surface of T and B cells, albeit at very low levels (Cashman et al. 1990); after infection, PrP^{Sc} was found mainly in follicular dendritic cells (Kitamoto et al. 1991). The question remained as to whether the infectious agent found in spleen is generated in that organ or scavenged either from the inoculum or from the brain. In any event, if scavenging from the inoculum and storage in the spleen were involved, the process would be dependent on PrP because in PrP knockout animals no infectivity is detected in the spleen 2 weeks after inoculation, while wild-type spleen has a titer of 6.2 log LD_{50} units/ml (Büeler et al. 1993).

An important question in this regard is whether the presence of PrP in or on a cell suffices to allow propagation of prions. To approach this problem, the PrP gene was placed under the control of a variety of tissue-specific promoters. Not surprisingly, expression of hamster PrP under the control of the neuron-specific enolase (NSE) promoter, which is considered to be neuron specific, rendered transgenic mice susceptible to hamster prions (Race et al. 1995). In conjunction with the fact that a neuron-derived cell line, N2a, can support prion replication (Race et al. 1987; Butler et al. 1988; Borchelt et al. 1990), one may conclude that neurons propagate prions. Expression of hamster PrP under direction of the astro-

cyte-specific glial fibrillary acid protein (GFAP) promoter in PrP knockout mice rendered the animals susceptible to hamster prions and led to clinical disease and prion propagation. Because no expression of PrP could be detected in neurons (within the sensitivity of the immunohistochemical analysis), it would seem that astrocytes are competent for prion replication (Fig. 5). Interestingly, the neuropathology exhibited by these transgenic mice is quite similar to that found in scrapie-infected wild-type mice (Raeber et al. 1997).

Transgenic mice expressing PrP under the control of a Eμ enhancer /IRF-1 promoter (Yamada et al. 1991) overexpressed PrP in the spleen, in both T and B cells. There were also low levels of PrP expression in brain, lung, intestine, heart, liver, and kidney, due at least in part to the presence of lymphoid cells. After inoculation with mouse prions, infectivity appeared in the spleen after 2 weeks at a level similar to that in wild-type mice and was not detectable in the brain at 6 months p.i. Fractionation of spleen cells, using B- and T-cell-specific antibodies, showed that most infectivity was associated with B cells and some with T cells, but curiously no infectivity was detected in circulating lymphocytes (Raeber et al. 1996). No statement could be made about follicular dendritic cells that were not purified. Transgenic mice overexpressing PrP under the control of the T-cell-specific lck promoter (Chaffin et al. 1990) showed high levels of PrP in T cells, both in thymus and spleen. High levels of PrP mRNA were found in kidney and low levels in brain and lung. No PrP protein was detected in brain. Inoculation led to no symptoms, and no infectivity was detected in spleen, thymus, or brain up to one year after inoculation. Because in these mice the T cells expressed at least ten times as much PrP as those in the mice expressing PrP under the direction of the Em enhancer/IRF-1 promoter, it would seem that T cells alone are unable to generate infectivity, but are able to pick it up from other sources.

From these experiments it can be concluded that the presence of PrP on the cell surface does not suffice to support prion replication; perhaps location within a particular plasma membrane region is required (Taraboulos et al. 1995) and/or other components are necessary, such as the postulated protein X (Telling et al. 1995) or a receptor.

NEUROGRAFTING AS A TOOL IN PRION RESEARCH

Among the many unresolved questions of prion diseases are those concerning the molecular mechanisms of pathogenesis in the CNS triggered by prion replication and the mechanism of prion spread in the infected organism.

Damage to the CNS, which is observed in animal models of prion diseases and in human spongiform encephalopathies, ranges from widespread destruction of CNS tissue (e.g., in the panencephalopathic form of CJD) to minimal damage circumscribed to specific areas of the thalamus (e.g., fatal familial insomnia [FFI]) to prominent deposition of amyloid plaques (e.g., scrapie, kuru, and vCJD).

How can all these different manifestations be reconciled? Is the damage related to the actual replication of the prion? Or is encephalopathy the result of accumulation of toxic metabolites, be it within or around neurons? One prime candidate for a toxic metabolite is certainly PrP^{Sc}, a possibility that is supported by the results of several in vitro experiments (Brown et al. 1994, 1996). If this is the case, can PrP^{Sc} damage nerve cells when acting from outside, or is it toxic only when generated within cells?

This hypothesis cannot be tested by inoculating wild-type mice intracerebrally with PrP^{Sc} because the mice would of course develop scrapie, and a one-time inoculation of PrP-knockout mice would not be meaningful because infectivity (and therefore presumably PrP^{Sc}) is cleared very rapidly from the brain (Eklund et al. 1967; Büeler et al. 1993). The question would be best approached by installing a constant source of PrP^{Sc} in the brain of PrP-knockout animals and determining its effect on neighboring tissue.

Figure 5 Expression of PrP mRNA (*black*) and GFAP protein (*red*) in brains of PrP knockout mice with a PrP transgene under an astrocyte-specific promoter compared to wild-type and PrP knockout mouse brains. PrP mRNA (*black*) was visualized by in situ hybridization and GFAP protein (*red*) by immunohistochemistry. (*Left* column) $Prnp^{0/0}$ mice carrying hamster PrP genes under the control of the astrocyte-specific GFAP promoter (Tg(GFAP-HaPrP)3/$Prnp^{0/0}$); (*middle* column) $Prnp^{0/0}$ mice; (*right* column) wild-type mice. To enhance transcription from the GFAP promoter, mice were subjected to "cryolesioning" of the cortex (duration, 60 sec), which induces intense gliosis. At low-power magnification (*A,B,C*), the necrotic zone and the penumbra of the cryolesion appear demarcated by reactive astrocytes. Labeled rectangles correspond to a detailed view of the respective area. In Tg(GFAP-HaPrP)3/$Prnp^{0/0}$ mice, coexpression of *Prnp* mRNA (*black*) and GFAP (*red*) is visible after strong activation of the GFAP promoter in the penumbra zone of the cryolesion (*G*), whereas *Prnp* mRNA is transcribed below detection threshold in resting astrocytes (*D*). $Prnp^{0/0}$ mice show activated astrocytes around cryolesioned areas (*H*) and resting astrocytes in the hippocampus (*E*) but no detectable PrP mRNA. Wild-type mice show PrP mRNA in hippocampal (*F*) and cortical neurons (*I*) but not in activated or resting astrocytes. (Reprinted, with permission, from Raeber et al. 1997.)

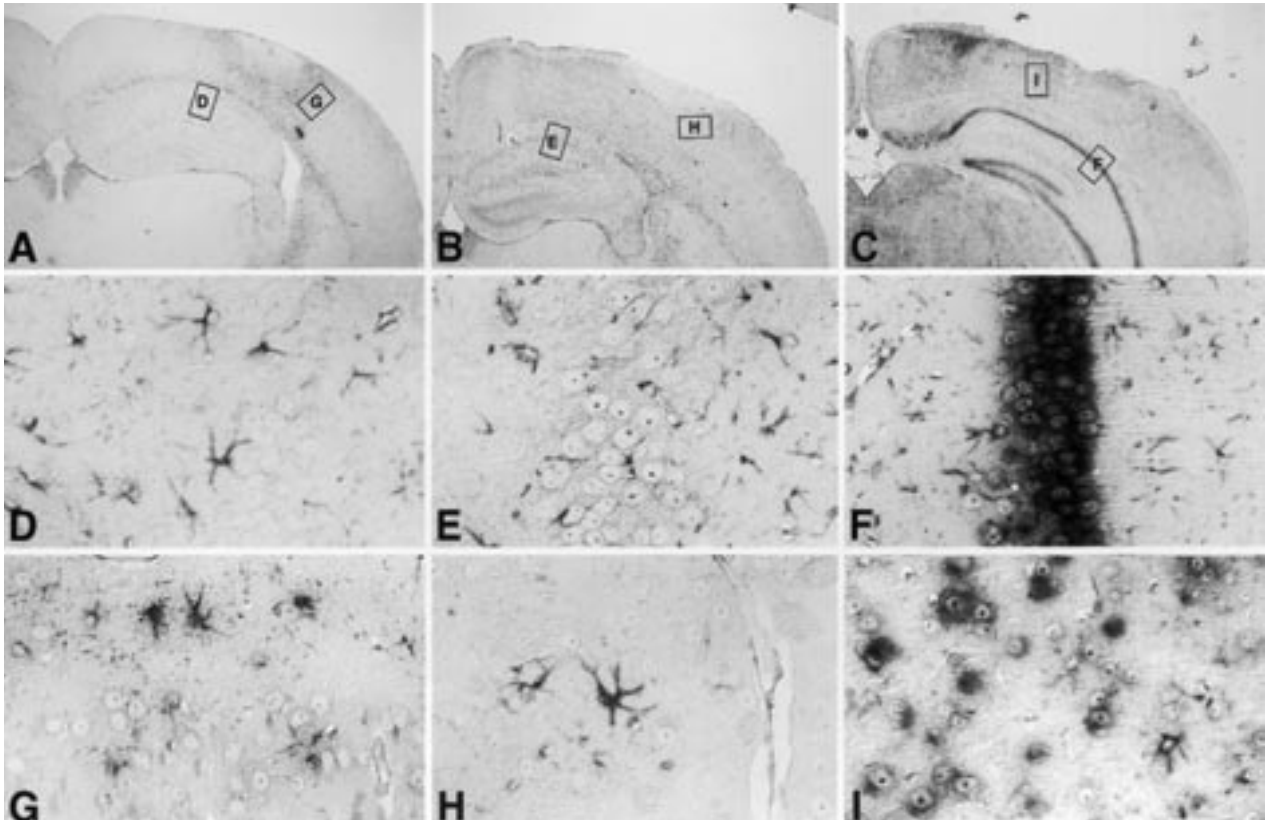

Figure 5 (*See facing page for legend.*)

As far as studying the mechanism of prion spread, it was desirable to have a PrP knockout mouse with a scrapie-susceptible target in the CNS in order to determine whether prions could spread through tissue devoid of PrP, and if not, to determine which PrP-expressing tissues were required for this process. As described below, brain grafts of PrP-expressing neuroectodermal tissue introduced into *Prnp*$^{o/o}$ mice could be used both as a target to monitor transport of prions to the brain and, if prion-infected, as a constant source of PrP^{Sc}. Tissue-specific expression of PrP could be achieved by ectopically expressing *Prnp* transgenes in PrP null mice, as outlined above, or by transplanting into PrP-deficient mice various types of PrP-expressing tissues.

The Neurografting System: Technical and Biological Aspects

Typically, neural grafting is used to address questions related to developmental neurobiology (Fisher and Gage 1993) or to investigate the establishment of neuronal organization and interactions with the host CNS. In the field of neurodegenerative disorders, grafting studies have been mainly aimed at reconstituting certain pathways or particular functions after surgical or toxic lesions to selected functional systems (Dunnett 1990; Lindvall 1994).

We have developed a conceptually different approach, based on the use of neurografts for studying growth or degeneration of genetically modified tissue in the context of a primarily nonaffected host. In a first series of studies, we engrafted retrovirally transduced cells into the rodent CNS to address the tumorigenic potential of various oncogenes. Later, we studied the differentiation and maintenance of neural tissue from mutant mice with premature lethal genotypes. The grafting procedure (Aguzzi et al. 1991; Isenmann et al. 1996a) is quite straightforward; neuroectoderm is derived from embryos at defined stages of gestation (ideally E12.5–13.5), optionally radiolabeled for later identification by autoradiography, and injected into the caudoputamen or lateral ventricles of recipient mice using a stereotactic frame. If histocompatible strains of mice are used, signs of graft rejection, such as lymphocytic infiltration and tissue necrosis, are seldom seen. Embryonic neuroepithelial tissue grafted into an adult host brain follows a program of maturation and differentiation similar to the in vivo time course (Isenmann et al. 1996b).

The blood-brain barrier (BBB) maintains the homeostatic environment in the brain by preventing blood-borne compounds from entering the CNS parenchyma. From the perspective of the scrapie agent entering the host from peripheral sites, the BBB may represent a hurdle. The

impact of an intact or leaky BBB on the neuroinvasion of prions must therefore be considered. We studied the kinetics of reconstitution of the BBB after grafting-induced disruption by using postmortem techniques, such as immunohistochemistry for serum components (which are normally not found in cerebrospinal fluid and brain parenchyma) and magnetic resonance imaging (MRI) in vivo, after administration of gadolinium as a marker of BBB leakage. The results consistently indicated that the BBB is reconstituted in two-thirds of all grafts after 3 weeks, and in 90% of the grafts 7 weeks after grafting (Isenmann et al. 1996c).

Neurografts in Prion Research

A neurograft from a donor mouse that dies young from a lethal disease can be kept alive in a healthy recipient. Therefore, we undertook application of this technique to the study of mouse scrapie. As discussed above, *Prnp*$^{0/0}$ mice, which are devoid of PrP^{C}, are resistant to scrapie and do not propagate prions (Büeler et al. 1993; Sailer et al. 1994). Because these mice show normal development and behavior, it has been argued that scrapie pathology may be induced by neurotoxic PrP^{Sc} depositions (Forloni et al. 1993), rather than by depletion of cellular PrP^{C}. However, acute depletion of PrP^{C} may be much more deleterious than chronic deficiency throughout development, since the organism may then not have the time to enable compensatory mechanisms.

To address the question of neurotoxicity, we exposed brain tissue of *Prnp*$^{0/0}$ mice to a continuous source of PrP^{Sc}. This was achieved by transplantation of embryonic telencephalic tissue from transgenic mice overexpressing PrP (*Tga20*; Fischer et al. 1996) into the forebrain of *Prnp*$^{0/0}$ mice and inoculating the pseudochimeric brains with scrapie prions. Because protease-resistant PrP cannot be equated with infectivity, we determined the amount of infectivity in the graft and in regions of the brain at various distances from the graft by bioassay titration (Brandner et al. 1996b; Fischer et al. 1996). All grafted and scrapie-inoculated mice remained free of scrapie symptoms for at least 70 weeks; this exceeded at least sevenfold the survival time of the scrapie-infected donor mice. Therefore, the presence of a continuous source of PrP^{Sc} and of scrapie prions does not seem to exert any clinically detectable adverse effects on the physiological functions of a mouse devoid of PrP^{C}.

On the other hand, histologic analysis revealed that grafts developed characteristic histopathologic features of scrapie after inoculation (Fig. 6). The course of the disease in the graft was very similar to that observed in the brain of scrapie-inoculated, non-grafted mice. Since uninfected or

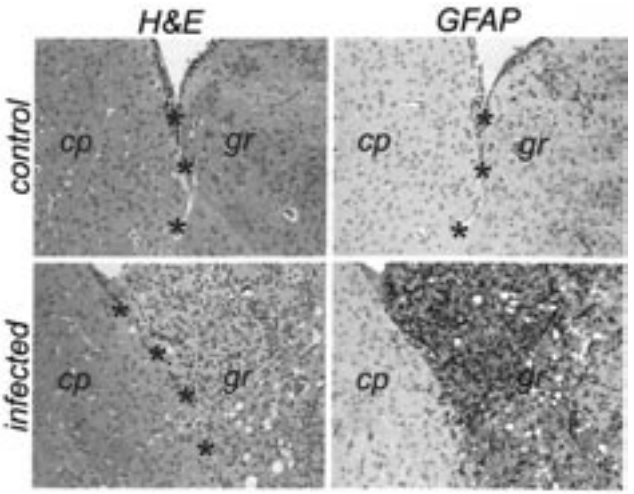

Figure 6 Typical histological appearance of a PrP^{C}-expressing graft (*left* side of each panel) at the interface to the PrP knockout host brain (*right* side). Knockout mice were grafted with PrP-expressing neuroectodermal tissue and inoculated intracerebrally with mouse scrapie prions where indicated. Animals were sacrificed 232 days after inoculation. Note the spongiform microcystic changes (*left* panel: hematoxylin-eosin) and the brisk astrocytic reaction evidenced by the immunocytochemical stain for glial fibrillary acidic protein (*right* panel: GFAP). For further details, see Brandner et al. (1996b).

mock-infected grafts never showed scrapie pathology, high expression of PrP by itself does not induce neurodegeneration in grafts. Intriguingly, the grafts underwent progressive disruption of the BBB during the course of the disease (S. Brandner et al., unpubl.).

Importantly, grafts had extensive contact with the recipient brain, and prions could navigate between the two compartments, as shown by the fact that inoculation of wild-type animals engrafted with PrP-expressing neuroectodermal tissue resulted in scrapie pathology in both graft and host tissue.

When engrafted *Prnp*$^{0/0}$ recipients were inoculated, histopathology never extended into host tissue, even at the latest stages (>450 days); however, PrP^{Sc} was detected in both grafts and recipient brain, and immunohistochemistry revealed PrP deposits in the hippocampus, and occasionally in the parietal cortex, of all animals (Brandner et al. 1996b). It is unlikely that such deposits were produced locally by *tga20* cells emigrating from the graft, since (1) the graft borders were always sharply demarcated, (2) PCR analysis of host brain regions containing PrP deposits

failed to reveal PrP-encoding DNA, and (3) graft-derived cells were never detected distant from the graft by in situ hybridization or autoradiography of brain engrafted with [^{3}H]thymidine-labeled tissue. In addition, infectivity was observed in the same regions in which PrP deposits were present. Thus, infectious prions moved from the grafts to some regions of the PrP-deficient host brain without causing pathologic changes or clinical disease. The distribution of PrP^{Sc} in the white matter tracts of the host brain suggests diffusion within the extracellular space (Jeffrey et al. 1994) rather than axonal transport (S. Brandner and A. Aguzzi, unpubl.).

The results described above led us to the conclusion that the expression of PrP^{C} by a cell, rather than the extracellular deposition of PrP^{Sc}, is the critical determinant in the development of scrapie pathology. Perhaps PrP^{Sc} is inherently nontoxic and PrP^{Sc} plaques found in spongiform encephalopathies are an epiphenomenon rather than a cause of neuronal damage. One may therefore propose that availability of PrP^{C} for some intracellular process elicited by the infectious agent is directly linked to spongiosis, gliosis, and neuronal death. This would be in agreement with the fact that in several instances, and especially in FFI, spongiform pathology is detectable although very little PrP^{Sc} is present (Collinge et al. 1995; Aguzzi and Weissmann 1996).

Spread of Prions in the CNS

Intracerebral (i.c.) inoculation of tissue homogenate into suitable recipients is the most effective method for transmission of spongiform encephalopathies and may even facilitate circumvention of the species barrier. However, prion diseases can also be initiated from the eye by conjunctival instillation (Scott et al. 1993), corneal grafts (Duffy et al. 1974), and intraocular injection (Fraser 1982). The latter method has proved particularly useful for studying neural spread of the agent, since the retina is a part of the CNS, and intraocular injection does not produce direct physical trauma to the brain, which may disrupt the BBB and impair other aspects of brain physiology. The assumption that spread of prions occurs axonally rests mainly on the demonstration of diachronic spongiform changes along the retinal pathway following intraocular infection (Fraser 1982).

In the preceding sections of this chapter, we discussed how knockout mice were useful in establishing that expression of PrP^{C} is required for prion replication and also for neurodegenerative changes to occur. It therefore seemed reasonable to ask whether spread of prions within the CNS is also dependent on PrP^{C} expression on neural pathways. Again, we

used the neurografting approach—in this instance, however, intracerebrally placed grafts served as an indicator for infectivity in an otherwise scrapie-resistant host. After inoculation of prions into the eyes of engrafted $Prnp^{0/0}$ mice, no signs of spongiform encephalopathy or deposition of PrP^{Sc} were detected in the grafts. One graft of an intraocularly inoculated mouse was bioassayed and found to be devoid of infectivity. In control experiments, unilateral intraocular inoculation led to progressive appearance of scrapie pathology along the optic nerve and optic tract in *tga20* mice, which then extended into the rest of the brain as described previously by other workers (Fraser 1982; Kimberlin and Walker 1986). We conclude that infectivity administered to the eye of PrP-deficient hosts cannot induce scrapie in a PrP-expressing brain graft.

Since $Prnp^{0/0}$ mice, by definition, have not been exposed to PrP during maturation of their immune system, engraftment of $Prnp^{0/0}$ mice with PrP^{C}-producing tissue might lead to an immune response to PrP and possibly to neutralization of infectivity. Indeed, analysis of sera from grafted mice revealed significant anti-PrP antibody titers. Because even mock-inoculated and uninoculated grafted $Prnp^{0/0}$ mice showed an immune response to PrP, while intracerebral inoculation of nongrafted $Prnp^{0/0}$ mice did not (Büeler et al. 1993), PrP^{C} presented by the intracerebral graft (rather than the inoculum or graft-borne PrP^{Sc}) was clearly the offending antigen. To test whether grafts would develop scrapie if infectivity were administered before they mounted a potentially neutralizing immune response, we inoculated mice 24 hours after grafting. Again, no disease was detected in the graft of two mice inoculated intraocularly.

To definitively rule out the possibility that prion transport was disabled by a neutralizing immune response, we repeated the experiments in mice tolerant to PrP. We used $Prnp^{0/0}$ mice transgenic for multiple copies of a hybrid gene consisting of a PrP coding sequence under the control of the *lck* promoter. As described above, these mice, designated *tg33*, overexpress PrP on T lymphocytes but are resistant to scrapie and do not contain scrapie infectivity in brain and spleen after inoculation with scrapie prions (Brandner et al. 1996a). *tg33* mice engrafted with PrP-overexpressing *tga20* neuroectoderm did not develop antibodies to PrP after intracerebral or intraocular inoculation ($n = 9$) even 31 weeks after grafting, presumably due to clonal deletion of PrP-immunoreactive lymphocytes. As before, intraocular inoculation with prions did not provoke scrapie in the graft, supporting the conclusion that lack of PrP^{C}, rather than immune response to PrP, prevented spread.

The prion itself is therefore surprisingly sessile—we speculate that it will not move freely within an intact nervous system *unless* supported by

PrP-expressing cells. Spread might proceed along a PrP^C-paved chain of cells (Brandner et al. 1996a). Perhaps prions require PrP^C for propagation across synapses: PrP^C is present in the synaptic region (Fournier et al. 1995) and, as discussed above, certain synaptic properties are altered in *Prnp*$^{0/0}$ mice (Collinge et al. 1994; Whittington et al. 1995). Perhaps transport of prions within (or on the surface of) neuronal processes is PrP^C-dependent. Within the framework of the protein-only hypothesis, these findings may be accommodated by a "domino-stone" model in which spreading of scrapie prions in the CNS occurs *per continuitatem* through conversion of PrP^C by adjacent PrP^{Sc} (Aguzzi 1997).

Spread of Prions from Extracerebral Sites to the CNS

As discussed above, PrP^C seems indispensable for prion spread within the CNS; but prions are not normally delivered *directly* to the CNS. More commonly, spongiform encephalopathies have been transmitted by feeding (Wells et al. 1987; Kimberlin and Wilesmith 1994; Anderson et al. 1996) as well as by intravenous, intraperitoneal (Kimberlin and Walker 1978), and intramuscular injection (Buchanan et al. 1991). Which are the pathways exploited by prions to spread across the body and reach the CNS? Do "breeding sites" exist in the body, in which prions multiply silently during the incubation phase of the disease? Answering these questions may be of eminent practical significance and may help devise ways to interfere with the march of prions from peripheral sites to the CNS.

A wealth of early studies (for review, see Kimberlin and Walker 1988) points to the importance of prion replication in lymphoid organs, and probably also in man (Hill et al. 1997). Replication of the infectious agent in the spleen typically precedes intracerebral replication, even if infectivity is administered intracerebrally. Infectivity can accumulate in all components of the lymphoreticular system (LRS), including lymph nodes and intestinal Peyer's patches, where it replicates almost immediately following oral administration of prions (Kimberlin and Walker 1989). Intraperitoneal (i.p.) infection of mice does not lead to replication of prions in the spleen nor to cerebral scrapie in SCID (severe combined immune deficiency) mice, and transfer of wild-type spleen cells to SCID mice restores susceptibility of the CNS to i.p. inoculation (Lasmézas et al. 1996). The results mentioned above suggest that components of the immune system are required for efficient transfer of prions from the site of peripheral infection to the CNS. Do immune cells suffice to transport the agent all the way from LRS to CNS? This is unlikely, since lymphocytes do not normally cross the BBB (unless they are instructed to do so,

e.g., by cytokines in inflammatory conditions). Moreover, disease and prion replication occur first in the CNS segments to which the sites of peripheral inoculation project (Kimberlin and Walker 1980; Beekes et al. 1996), implying that the agent spreads through the peripheral nervous system, analogously to rabies and herpesviruses. Perhaps prions injected i.p. are first brought to lymphatic organs (specifically, to germinal centers) by mobile immune cells. Then, invasion of peripheral nerve endings may occur in a lymphocyte-dependent fashion. Eventually, the CNS is reached, and further spread occurs transsynaptically and along fiber tracts.

Is it possible to interfere with this chain of events without resorting to ablation of a functional immune system, as in the case of SCID mice? Again, PrP^C may offer an intriguing handle. We reasoned that, since PrP^C is crucial for prion spread within the CNS (Brandner et al. 1996a), it may be required also for spread of prions from peripheral sites to CNS. Indeed, PrP-expressing neurografts in $Prnp^{0/0}$ mice did not develop scrapie histopathology after intraperitoneal (i.p.) or intravenous (i.v.) inoculation with scrapie prions. We then reconstituted the hematopoietic system of $Prnp^{0/0}$ mice with cells expressing PrP. Prion titers were undetectable in spleens of inoculated $Prnp^{0/0}$ mice, but were restored to wild-type levels upon reconstitution of the host lymphohematopoietic system with PrP-expressing cells. Surprisingly, however, i.p. or i.v. inoculation failed to produce scrapie pathology in the neurografts of 27 out of 28 animals, in contrast to intracerebral inoculation. We concluded that transfer of infectivity from spleen to CNS is crucially dependent on the expression of PrP in a tissue compartment that cannot be reconstituted by bone marrow transfer (Blättler et al. 1997). For reasons outlined above, this is likely the peripheral nervous system (Kimberlin and Walker 1980; Beekes et al. 1996). Reconstitution of $Prnp^{0/0}$ mice with either a PrP-expressing lymphoreticular system , a PrP-producing peripheral nervous system, or both together should allow this question to be resolved.

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Transgenetic Investigations of the Species Barrier and Prion Strains

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The history of prion strains started in 1936, with the first reported claim by Cuillé and Chelle (1939) of the successful transmission of scrapie by intraocular injection of healthy sheep with spinal cord from an afflicted sheep. This milestone event provided the first evidence that the disease was transmissible and led inevitably to a search for the infectious agent responsible. At first, the logical conclusion was that it must be a virus. Because of the extended incubation times, these diseases were often referred to as either slow virus diseases or unconventional viral diseases (Sigurdsson 1954; Gajdusek 1977, 1985). As detailed in other chapters in this book, considerable effort was expended searching for the "scrapie virus"; yet, nothing was found either with respect to a virus-like particle or a genome composed of RNA or DNA. Over the ensuing period of more than 60 years, it has become clear that scrapie is only one of a growing number of diseases known to be caused by prions and that prions comprise a unique class of pathogens that multiply by an entirely novel mechanism (Prusiner 1982a). In this study, we focus on the mechanisms that determine susceptibility to prions, that support prion diversity, and that govern the emergence of distinct prion strains during serial transmission.

EVIDENCE AGAINST A CONVENTIONAL GENOME

Aficionados of the virus hypothesis were challenged by a large body of evidence showing that the agent had properties unlike any known virus.

The infectious agent showed a remarkable resistance to treatments, such as UV irradiation or treatment with nucleases (Alper et al. 1966; Hunter 1972; Prusiner 1982b), that would normally inactivate the nucleic acid genome of a virus composed of either RNA or DNA. Following the discovery of the prion protein, PrP (Bolton et al. 1982; Prusiner et al. 1982a), came the formulation of the prion hypothesis (Prusiner 1982a). The original definition of prions was "small *pro*teinaceous *in*fectious particles that resist inactivation by procedures which modify nucleic acids." A more contemporary working definition of a prion might be "a proteinaceous infectious particle that lacks an essential nucleic acid." However, it might be better not to think about prions as infectious particles at all, since transmission of the disease is accomplished not through the action of a foreign agent, but rather through the action of an abnormal conformation of a protein which is itself a normal component of the host. For this reason, some of us prefer to refer to prions as transmissible, rather than infectious.

THE PRION STRAIN PROBLEM

It was understood that the etiological agent of the transmissible encephalopathies was unusual: There was no detectable immune response, fever, or other sign of infection, and the resistance to treatments that inactivate viruses was also well established. In recognition of the unusual nature of the agent responsible, a number of hypotheses were advanced, including the "unconventional virus" alluded to earlier (Gajdusek 1977) and the slightly more radical "virino" (Kimberlin 1982; Dickinson and Outram 1988), a particle composed partly of proteins encoded by the host. However, even the virino hypothesis stipulated that a nucleic acid, albeit small, must be involved. Later, it was conceded that the "genome" might not actually comprise a nucleic acid, but that a separate "informational component" distinct from PrP must still be present in the infectious particle (Bruce and Dickinson 1987; Bruce et al. 1992; Bruce 1993).

Why was it so difficult to lay to rest the notion of a nucleic acid genome, or at least an "independent self-determining informational component?" One would think that the biophysical evidence against an essential nucleic acid would have been sufficiently compelling. The concept of a "protein-only" nature for the agent was not hard to grasp and had even been entertained previously (Griffith 1967). Indeed, in the era preceding the elucidation of the structure of DNA, it was widely believed that genes must be made of protein, since only proteins seemed capable of forming

structures of sufficient complexity (Watson 1968). If there is a single obstacle to the idea that prions contain no genome, let alone one composed of nucleic acid, it has to be the existence of distinct strains of the agent. Since the sequence of the PrP gene is contributed by the host, it is easy to visualize how changes in prion properties could occur during serial passage from one host to another, in response to changes in the sequence of PrP, and some aspects of strain behavior can be attributed to this mechanism (Westaway et al. 1987). However, the existence of prion strains with properties that remain distinct in hosts that share a PrP gene of identical sequence has been championed as evidence for an independently replicating informational molecule or genome (Bruce and Dickinson 1987). To accommodate multiple strains in the context of the protein-only model, PrP must be able to sustain separate information states within the same amino acid sequence faithfully. In the absence of another molecule, this can be accomplished only by a covalent modification or through the adoption of multiple conformations (Prusiner 1991). Opponents of the prion hypothesis have used the argument that the protein-only model could never support the high degree of variation observed. However, as we shall argue, this level of diversity may be largely illusory, as the number of truly distinct prion strains seems entirely consistent with experimental data showing that prion strain characteristics are enciphered in multiple conformations of PrP^{Sc} (Bessen and Marsh 1994; Telling et al. 1996; Safar et al. 1998).

PRION STRAIN CHARACTERISTICS

Prion strains with distinct biological properties have long been recognized since the identification of two strains of scrapie in goats, described originally as "scratching" and "drowsy" (Pattison and Millson 1961b). In this case, the difference in clinical presentation of the disease is apparent from the terminology. However, behavioral differences are only one of several criteria that can be adopted to classify strains. Others include differences in the histopathology observed in animals inoculated with different strains (Fraser and Dickinson 1973) and variations in the pattern of deposition of PrP^{Sc} (Hecker et al. 1992; DeArmond et al. 1993). Perhaps the most reliable and important characteristic for classifying prion strains has been that of incubation period (Dickinson et al. 1968).

The incubation period can be defined as the time interval between inoculation and appearance of clinical signs or death. There are several remarkable properties of incubation period that should be noted. First, the

length of the incubation period is related inversely to the titer of the inoculum (Pattison and Smith 1963; Prusiner et al. 1981). Second, the relationship between incubation period and titer can differ between distinct prion strains (Hecker et al. 1992), and the incubation period observed using a standardized inoculum (usually 1% brain homogenate) is a reliable marker for distinguishing prion strains. Third, the incubation period in animals of one species was always found to be shorter if the donor animal was of the same species as the host animal, a phenomenon termed the "species barrier" (Pattison 1965). Typically, in the first passage from animals of one species to another, the longer incubation period was accompanied by atypical clinical signs and unusual histopathology. Once the initial passage had been accomplished, the incubation period shortened and became fixed, as did other characteristics, such as histopathology (Pattison 1965). The concept of the species barrier is of great importance in understanding both the mechanisms that underlie prion propagation and the etiology of prion diseases and is discussed at great length below.

STRAINS OF PRIONS DERIVED FROM NATURAL SCRAPIE

Parry argued that host genes were responsible for the development of scrapie in sheep. He was convinced that natural scrapie is a genetic disease that could be eradicated by proper breeding protocols (Parry 1962, 1983). He considered its transmission by inoculation of importance primarily for laboratory studies and considered 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).

In sheep, polymorphisms that produce amino acid substitutions at codons 136, 154, and 171 of the PrP gene in sheep have been studied with respect to the occurrence of scrapie in sheep (Goldmann et al. 1990a,b; Laplanche et al. 1993; Clousard et al. 1995). Studies of natural scrapie in the US have shown that about 85% of the afflicted sheep are of the Suffolk breed. Only those Suffolk sheep homozygous for glutamine (Q) at codon 171 were found with scrapie, although healthy controls with QQ, QR, and RR genotypes were also found (Westaway et al. 1994; Hunter et al. 1997a,b). These results argue that susceptibility in Suffolk sheep is governed by the PrP codon-171 polymorphism. In Cheviot sheep, as in Suffolks, the PrP codon-171 polymorphism has a profound influence on susceptibility to scrapie, and codon 136 seems to play a less pronounced role (Goldmann et al. 1991a; Hunter et al. 1991).

DEPENDENCE OF SCRAPIE PRION STRAIN PROPERTIES ON PASSAGE HISTORY

Before we review the published history of many established scrapie strains, we first attempt to clarify the interpretation of these results, in light of our current understanding of the mechanism of prion propagation. As previously stated, much of the work on scrapie strains reported in the literature was performed with the apparent intent being to prove that the agent possessed an independent genome. Accordingly, the nomenclature used, as well as the sheer enormity of the number of passages, can become bewildering. For example, the convention has been to give new isolates alphanumeric names, such as 22A or 139A, which in large part reflect an identity that the investigators believed best explains the relationships between strains. We would argue that this can be misleading. For example, we should not assume that 22A and 22C are any more closely related in structure than 22A is to 139A. In some cases, the opposite is true; for example, the following strains, 87A, 31A, 51C, 125A, and 138A, are proposed to represent "re-isolation of the same strain" (Bruce and Dickinson 1979). Other examples abound and have been extensively reviewed previously (Ridley and Baker 1996).

Since an extensive review of the history of mouse prion isolates has already been presented (Ridley and Baker 1996), we focus on specific examples which seem to be particularly significant. Strikingly, many of the well-characterized prion strains appear to be derived from the same original pool of brains of scrapied sheep, termed SSBP/1. This was a pooled brain homogenate derived from one Cheviot and two Cheviot × Border Leicester crossbred sheep (Dickinson 1976). Passaging of SSBP/1 in sheep and then to mice resulted in the so-called "22 family" of strains (Dickinson and Fraser 1979). Transmission of SSBP/1 to goats at Compton led to isolation of the scratching and drowsy strains. The drowsy goat line is of particular importance. Passaging of the goat drowsy source to mice led to isolation of the "Chandler" mouse scrapie isolate (Chandler 1961), from which the strain we term RML was obtained and, through a series of passages performed over a number of years, to isolation of several other well-characterized Mo prion strains, including 139A, which seems to be similar to RML. In addition, passaging of the drowsy goat isolate to $Prnp^{a/a}$ mice led to the 79A strain, and passage of the drowsy isolate to $Prnp^{b/b}$ animals resulted in the 87V strain (Bruce et al. 1976; Bruce and Dickinson 1985). In contrast to the experience with the drowsy inoculum, passaging of the goat scratching isolate to mice led to re-isolation of members of the 22 family. To explain this, Dickinson and colleagues proposed that in reality the drowsy goat source had become

contaminated with a case of natural scrapie in one of the original goats, since he apparently believed that the passage in mice was simply isolating strains that previously existed in the original SSBP/1 inoculum (Dickinson 1976). An alternative explanation is possible, and seems more likely to us on the basis of our current knowledge. The action of passaging prions from one species to another causes the generation of new strains because of differences in the sequences of the host and donor PrP (Scott et al. 1997a). If a general picture from all of this emerges, it is that prion strains are frequently created during passage, and that in many instances where distinct prion strains have been identified, the two strains being compared differ with respect to their passage history. In other instances, the concept of agent "mutation" was used to explain the frequent emergence of new strains (Bruce and Dickinson 1987; Kimberlin et al. 1987). Once again, we believe these results can be better explained by changes enforced upon the prion by a change in the sequence of PrP encoded by the host. It is intriguing to note that mutation alone does not seem sufficient to explain all of the variation observed, prompting speculation that some of the data could best be explained if a host-encoded protein formed a functional part of the infectious agent (Kimberlin et al. 1987).

Since all of the above-mentioned strains may be considered to originate from a single source, strains that can be traced to a completely different natural source are of particular biological interest. Passage of the spleen of a scrapied Suffolk sheep into Moredun ($Prnp^{a/a}$) mice and subsequent cloning by limiting dilution led eventually to isolation of the Me7 strain (Zlotnik and Rennie 1965; Dickinson and Meikle 1969; Dickinson et al. 1969). As we noted earlier, this same strain was reportedly obtained from several independent isolations and appears identical to 7D and 58A (Ridley and Baker 1996). It is important to emphasize that 87A, which was derived from the Cheviot source, was found to produce a strain indistinguishable from Me7 even following passage at high dilution ("cloning") (Ridley and Baker 1996). This introduces a point we shall return to later: that identical strains can be obtained from entirely different primary sources, consistent with the view that prion diversity is fundamentally limited in scope.

More recently, the epidemic of bovine spongiform encephalopathy (BSE) among cattle in Britain has provided a new source of mouse prion strains; transmission of BSE to mice yielded two new isolates, 301V and 301C (Bruce et al. 1994). As we discuss below, 301V and 301C are probably identical, differing only with respect to the sequence of PrP supplied by the host.

STRAINS OF PRIONS IN RODENTS: THE Sinc GENE

Studies of scrapie in inbred mice demonstrated the existence of a genetic locus, *Sinc* (for *s*crapie *inc*ubation), which profoundly influenced the scrapie incubation period of mouse prion strains (Dickinson et al. 1968; Dickinson and Meikle 1969). Two alleles of the Sinc gene, termed s7 and p7, were found to produce short and prolonged incubation periods, respectively, upon inoculation with the Chandler scrapie isolate. In a later study using different strains of mice, a major determinant of scrapie incubation period, termed *Prn-i*, was found to be either congruent with or closely linked to the structural gene for PrP, designated *Prnp* (Carlson et al. 1986; Westaway et al. 1987). Subsequent molecular genetic studies showed that the *Sinc* locus was probably synonymous with the scrapie incubation period determinant *Prn-i* (Carlson et al. 1986; Hunter et al. 1987). Mice with different alleles of the *Sinc/Prn-i* gene encoded PrPs that differed at two amino acid residues; these *Prnp* alleles were designated $Prnp^a$ and $Prnp^b$ (Westaway et al. 1987). The $Prnp^a$ allele corresponds to the s7 allele of the *Sinc* locus and encodes leucine at codon 108 and threonine at codon 189 of the Mo PrP open reading frame (ORF), whereas the $Prnp^b$ allele corresponds to p7 and encodes phenylalanine and valine at these locations in Mo PrP. It is now widely accepted that the scrapie incubation time genes (*Sinc* and *Prn-i*) are congruent with the PrP gene; hence the use of the *Sinc/Prn-i/Prnp* terminology is becoming largely redundant. Indeed, it is often more convenient to refer to the short and long allelic products simply as PrP-A and PrP-B, respectively.

PARADOXICAL RESULTS FROM TRANSGENIC STUDIES

Although the amino acid substitutions in PrP that distinguish $Prnp^a$ from $Prnp^b$ mice argued for the congruency of *Prnp* and *Prn-i*, experiments with $Prnp^a$ mice expressing $Prnp^b$ transgenes demonstrated a paradoxical shortening of incubation times (Westaway et al. 1991). We had predicted that these Tg mice would exhibit a prolongation of the incubation time after inoculation with RML prions on the basis of our previous studies with ($Prnp^a \times Prnp^b$) F_1 mice, which do exhibit long incubation times. We described those findings as paradoxical shortening because we and other investigators had believed for many years that long incubation times are dominant traits (Dickinson et al. 1968; Carlson et al. 1986). From studies of congenic and transgenic mice expressing different numbers of the *a* and *b* alleles of *Prnp*, we learned that these findings were not paradoxi-

cal; indeed, they result from increased PrP gene dosage (Carlson et al. 1994). As we discuss in more detail below, increased gene dosage was found to be inversely related to incubation period in studies using transgenic mice expressing hamster PrP (Prusiner et al. 1990). When the RML strain was inoculated into congenic and transgenic mice, increasing the number of copies of the *a* allele was found to be the major determinant in reducing the incubation time; however, increasing the number of copies of the *b* allele also reduced the incubation time, but not to the same extent as that seen with the *a* allele.

PRION STRAIN VARIATION AND INCUBATION PERIOD

The relationship between prion titer and incubation period is of great significance in tracing prion strain properties during passage, particularly when comparing inocula prepared by different methods. For example, inefficient routes of inoculation, such as oral dosing, can prolong incubation period, and titers of prions in tissues other than brain may be much lower, leading to prolongation of incubation period. It is therefore crucial to always be aware that a variation in incubation period could be caused either by a change in titer or a change in the strain of prion, or both.

To clarify the relationship between incubation period and titer, two distinct Syrian hamster prion strains, Sc237 and 139H, were compared (Hecker et al. 1992). Sc237 and 139H exhibit incubation periods of about 75 and 165 days, respectively, in hamsters. Since previous studies had disclosed that a single LD_{50} of the Sc237 strain causes disease at about 140 days after inoculation (Marsh and Kimberlin 1975; Kimberlin and Walker 1977; Prusiner et al. 1982b), the possibility that the relatively long incubation period of 139H could arise due to a defect in 139H that prevented accumulation of high titers of infectious prions was investigated by endpoint titration (Fig. 1) (Hecker et al. 1992). Significantly, no difference was found in the titer of 10% (w/v) brain homogenates prepared from hamsters showing clinical signs of scrapie after inoculation with either strain. The relationship between incubation period and titer was different, however; a comparison of any two doses of 139H gave a larger change in incubation period than between corresponding doses of Sc237 (Fig. 1) (Hecker et al. 1992). Thus, it seems that distinct incubation periods can arise because of differences in the rate of accumulation of prion strains. It is important to stress that PrP gene dosage also affects the kinetics of prion propagation, as discussed below.

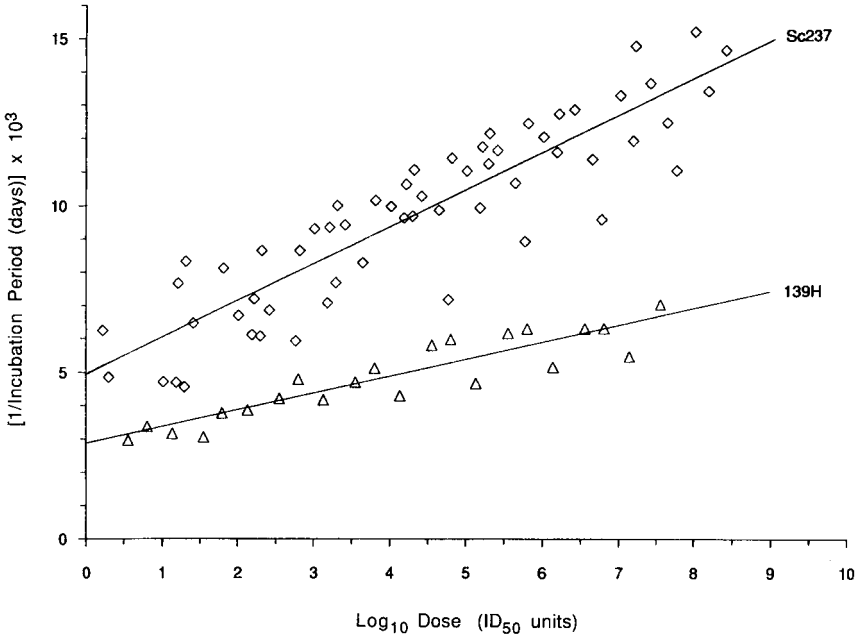

Figure 1 Scrapie incubation times plotted as a function of the dose of inoculum for two distinct prion isolates. The $\log_{10}$ dose, which is calculated from the titer x dilution, is on the *x* axis; the reciprocal of incubation time is on the *y* axis. The curves were fitted by linear regression analysis. Sc237 regression coefficient = 0.88, slope = 1.11, and *y* intercept = 4.88; 139H regression coefficient = 0.91, slope = 0.51, and *y* intercept = 2.82. (Reprinted, with permission, from Hecker et al. 1992.)

PRION PROTEIN INTERACTIONS AND THE SPECIES BARRIER

The molecular mechanisms that underlie the species barrier are of great importance with respect to both the mechanism of prion propagation and our understanding of the etiology of prion diseases. The latter is of particular significance in assessing the risk to the human population from the ongoing epidemic of BSE in Britain and is discussed in much depth below. Much has been learned about the mechanisms that underlie the species barrier and prion susceptibility through the use of transgenic mice expressing foreign PrP genes. When transgenic mice were constructed expressing Syrian hamster (SHa) PrP, they were found to be highly susceptible to prions that had been passaged in hamsters, in contrast to control nontransgenic mice (Scott et al. 1989; Prusiner et al. 1990). The incu-

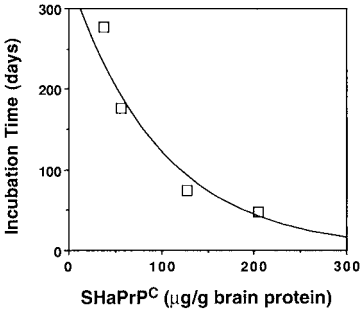

Figure 2 Plot of scrapie incubation times as a function of the HaPrP^{C} steady-state levels in the four Tg (HaPrP) Mo lines. An exponential curve was fitted to the data points. (Reprinted, with permission, from Prusiner et al. 1990 [copyright Cell Press].)

bation period was found to be inversely proportional to the level of expression of SHaPrP (Fig. 2) (Prusiner et al. 1990). Significantly, there was no difference in incubation period whether the prions were derived from hamsters or by prior transmission in Tg(SHaPrP) mice, establishing that no detectable change in the prion could be attributed to other host factors. Prions passaged through Tg(SHaPrP) mice also appeared unaltered when transmitted back to hamsters.

From these studies, we can conclude that the PrP gene governs the species barrier, and expression of a PrP gene from one species can be both necessary and sufficient to confer susceptibility to prions derived from the species of animal from which the PrP transgene was derived (Scott et al. 1989; Prusiner et al. 1990). Exceptions to this general rule exist, however. On the basis of observations with Tg mice discussed in the next section, it was proposed that PrP^{C} may need to bind specifically to a host-encoded cellular factor termed protein X as a prerequisite for conversion to PrP^{Sc} (Telling et al. 1995). If PrP and protein X are derived from distantly related species, the affinity of interaction is reduced (Telling et al. 1995). It appears that variations in the sequence of PrP that affect the binding site to protein X can prevent PrP from being efficiently converted (Kaneko et al. 1997). These observations have importance to the design of efficient transgenic mouse models for bioassays of prions from humans and ruminant sources and underscore an unfortunate weakness in the terminology coined by Pattison. Classically, the species barrier refers to the specific situation in which prions undergo a change during passage from one species to another. It is clear from the aforementioned trans-

genic mouse studies, as well as from extensive DNA sequence analysis of the PrP genes of numerous species (Gabriel et al. 1992; Schätzl et al. 1995), that this change is attributable to a corresponding difference in the sequence of PrP of the donor and host animals. In other cases, however, the host species may be simply incapable of supporting prion propagation because it expresses a PrP molecule that acts in a dominant negative fashion, preventing prion propagation by sequestering protein X (Kaneko et al. 1997). Certain breeds of sheep, generated through selective breeding to be resistant to scrapie, appear to derive this resistance due to a polymorphism (Q171R) affecting the binding site to protein X (Kaneko et al. 1997). A similar mechanism may explain the absence of Creutzfeldt-Jakob disease (CJD) in Japanese individuals who carry a similar polymorphism (Kaneko et al. 1997). It is important to stress that this type of resistance to foreign prions does not arise through the same mechanism as that observed during most interspecies transmissions. However, it may be of great practical significance to the production of farm animals resistant to prions, as well as to the development of novel therapies for patients suffering from prion disease or bearing a prion mutation causing familial prion disease.

It has become popular to equate the use of the term species barrier with the relative lack of susceptibility of some host species to prions derived from another species. A more complex picture is emerging, however. As we discuss below, the species barrier originally described by Pattison may sometimes reflect a modulation from one strain into another, enforced by a change in the primary structure of PrP (Scott et al. 1997a). In other cases, it may arise due to differences in the sequence of PrP that prevent interaction of PrP^{C} encoded by the host and PrP^{Sc} in the inoculum (Prusiner et al. 1990; Scott et al. 1993).

CHIMERIC PrP TRANSGENES AND PROTEIN X

Attempts to abrogate the prion species barrier between humans and mice by using an approach similar to that described for the abrogation of the species barrier between Syrian hamsters and mice were initially unsuccessful. Mice expressing HuPrP transgenes did not develop signs of CNS dysfunction more rapidly or frequently than non-Tg controls (Telling et al. 1994). Like the Tg(SHaPrP) mice that preceded them, these mice were created prior to the availability of mouse strains that lacked endogenous MoPrP (Büeler et al. 1992). However, in studies with Tg(SHaPrP) mice, although $SHaPrP^{C}$ appeared to compete with $MoPrP^{C}$ for binding to $SHaPrP^{Sc}$, the effect of this competition was a slight prolongation of

incubation period (Prusiner et al. 1990). Viewed in this context, the complete resistance of Tg(HuPrP) mice to Hu prions was initially puzzling.

The successful breaking of the species barrier between humans and mice has its origins in a set of studies with Tg mice expressing chimeric PrP genes derived from SHa and Mo PrP genes (Scott et al. 1992). One SHa/MoPrP gene, designated MH2M PrP, contains five amino acid substitutions encoded by SHaPrP, whereas another construct designated MHM2 PrP has two substitutions. Tg(MH2M PrP) mice were susceptible to both SHa and Mo prions, whereas three lines expressing MHM2 PrP were resistant to SHa prions (Table 1) (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 favoring propagation in mice that express the corresponding chimeric PrP but were also transmissible, albeit at reduced efficiency, to non-Tg mice and hamsters. These findings provided additional genetic evidence for homophilic interactions between PrP^{Sc} in the inoculum and PrP^{C} synthesized by the host (Scott et al. 1993).

With the recognition that Tg(HuPrP) mice were not suitable recipients for the transmission of Hu prions, we constructed Tg(MHu2M) mice analogous to the Tg(MH2M) mice described above. Hu PrP differs from Mo PrP at 28 of 254 positions (Kretzschmar et al. 1986), whereas chimeric MHu2MPrP differs at 9 residues. The mice expressing the MHu2M trans-

Table 1 Incubation times for chimeric prions passaged in Tg(MH2M PrP)92 mice or Syrian hamsters

		Scrapie incubation times			
		illness		death	
Inoculum	Host	(n/n_0)	(days ± S.E.)	(n/n_0)[a]	(days ± S.E)
SHa(Sc237)	Tg(MH2M PrP)92	34/34	134 ± 3.6	26/26	142 ± 4.4
SHa(Sc237)→MH2M	Tg(MH2M PrP)92	22/22	73 ± 0.7	12/12	85 ± 1.2
SHa(Sc237)→MH2M→MH2M	Tg(MH2M PrP)92	10/10	64 ± 1.9	8/8	76 ± 2.9
SHa(Sc237)	Syrian hamsters	48/48	77 ± 1.1[b]	48/48	89 ± 1.7
SHa(Sc237)→MH2M	Syrian hamsters	23/23	116 ± 1.9	10/10	136 ± 3.5
SHa(Sc237)→MH2M→MH2M	Syrian hamsters	6/6	161 ± 3.8	4/4	187 ± 2.4
SHa(Sc237)→MH2M→SHa	Syrian hamsters	8/8	77 ± 0.6	4/4	85 ± 1.2

(Reprinted, with permission, from Scott et al. 1993 [copyright Cell Press].)

[a]The reduced number of animals in the death column reflects sacrifice of some animals for immunoblotting and neuropathology.

[b]Scott et al. (1989).

gene are susceptible to human prions and exhibit abbreviated incubation times of about 200 days (Telling et al. 1994). In these initial studies, the chimeric MHu2M transgene encoded a methionine at codon 129, and all of the patients were homozygous for methionine at this residue.

From Tg(SHaPrP) mouse studies, prion propagation is thought to involve the formation of a complex between PrP^{Sc} and the homotypic substrate PrP^{C} (Prusiner et al. 1990). Propagation of prions may require the participation of other proteins, such as chaperones, which might be involved in catalyzing the conformational changes that feature in the formation of PrP^{Sc} (Pan et al. 1993). Notably, efficient transmission of HuCJD prions to Tg(HuPrP)/$Prnp^{0/0}$ mice was obtained when the endogenous MoPrP gene was inactivated, suggesting that $MoPrP^{C}$ competes with $HuPrP^{C}$ for binding to a cellular component (Telling et al. 1995). In contrast, the sensitivity of Tg(MHu2M) mice to HuCJD prions was not affected by the expression of $MoPrP^{C}$. One explanation for the difference in susceptibility of Tg(MHu2M) and Tg(HuPrP) mice to Hu prions in mice may be that mouse chaperones catalyzing the refolding of PrP^{C} into PrP^{Sc} can readily interact with the $MHu2MPrP^{C}/HuPrP^{CJD}$ complex but not with $HuPrP^{C}/HuPrP^{CJD}$. The identification of protein X is an important avenue of research, since isolation of this protein or complex of proteins would presumably facilitate studies of PrP^{Sc} formation. To date, attempts to isolate specific proteins that bind to PrP have been disappointing (Oesch et al. 1990). Whether or not identification of protein X will require isolation of a ternary complex composed of PrP^{C}, PrP^{Sc}, and protein X remains to be determined.

To accomplish the conversion of PrP^{C} into PrP^{Sc}, participation of one or more molecular chaperones may be required, as discussed above for protein X. Scrapie-infected cells in culture display marked differences in the induction of heat-shock proteins (Tatzelt et al. 1995, 1996), and Hsp70 mRNA has been reported to increase in scrapie of mice (Kenward et al. 1994). By two-hybrid analysis in yeast, PrP has been shown to interact with Bcl-2 and Hsp60 (Edenhofer et al. 1996; Kurschner and Morgan 1996). Weiss and coworkers have used a similar approach to show that PrP binds the laminin receptor protein (Weiss 1997). Although these studies are suggestive, no direct identification of a molecular chaperone involved in prion formation in mammalian cells has been accomplished.

SELECTIVE NEURONAL TARGETING OF PRION STRAINS?

In addition to incubation times, neuropathologic profiles of spongiform change have been used to characterize prion strains (Fraser and Dickinson

1968). Although this is still an essential parameter in categorizing prion strains, the development of a new procedure for in situ detection of PrP^{Sc}, designated histoblotting (Taraboulos et al. 1992), made it possible to localize and quantify PrP^{Sc} as well. This technique makes it possible to determine whether or not distinct strains produce different, reproducible patterns of PrP^{Sc} accumulation; the patterns of PrP^{Sc} accumulation were found to be different for each prion strain if the genotype of the host was held constant (Hecker et al. 1992; DeArmond et al. 1993). This finding was in accord with earlier studies showing that the distribution of spongiform degeneration is strain specific (Fraser and Dickinson 1973), since PrP^{Sc} accumulation precedes vacuolation and reactive gliosis (DeArmond et al. 1987; Jendroska et al. 1991; Hecker et al. 1992).

Although studies with both mice and Syrian hamsters established that each strain has a specific signature as defined by a specific pattern of PrP^{Sc} accumulation in the brain (Hecker et al. 1992; DeArmond et al. 1993; Carlson et al. 1994), any such comparison must be done on an isogenic background (Scott et al. 1993; Hsiao et al. 1994). When a single strain is inoculated into mice expressing different PrP genes, variations in the patterns of PrP^{Sc} accumulation were found to be at least equal to those seen between two strains (DeArmond et al. 1997). On the basis of the initial studies, which were performed in animals of a single genotype, we suggested that PrP^{Sc} synthesis occurs in specific populations of cells for a given distinct prion isolate (Prusiner 1989; Hecker et al. 1992). More recent data using transgenic mice supplied another striking example of the localized accumulation of prion strains. When fatal familial insomnia (FFI) prions were inoculated into Tg(MHu2M) mice, PrP^{Sc} was confined largely to the thalamus, as is the case for FFI in humans (Medori et al. 1992b; Telling et al. 1996). In contrast, fCJD(E200K) prions inoculated into Tg(MHu2M) mice produced widespread deposition of PrP^{Sc} throughout the cortical mantle and many of the deep structures of the CNS, as is seen in fCJD(E200K) of humans (Telling et al. 1996). Thus, the distinct pattern of PrP^{Sc} accumulation exhibited by each strain can be faithfully reproduced in another species.

Although these data appear to support the hypothesis of targeted propagation of distinct prion isolates, other studies with PrP transgenes argue that such profiles do not depend entirely on the strain (Carp et al. 1997; DeArmond et al. 1997). To examine whether the diverse patterns of PrP^{Sc} deposition are influenced by asparagine-linked glycosylation of PrP^{C}, we constructed Tg mice expressing PrPs mutated at one or both of the asparagine-linked glycosylation consensus sites (DeArmond et al.

1997). These mutations resulted in aberrant neuroanatomic topologies of PrP^C within the CNS, whereas pathologic point mutations adjacent to the consensus sites did not alter the distribution of PrP^C. Tg mice with mutation of the second PrP glycosylation site exhibited prion incubation times of more than 500 days and unusual patterns of PrP^{Sc} deposition. These findings raise the possibility that glycosylation can modify the conformation of PrP and affect either the turnover of PrP^C or the clearance of PrP^{Sc}. Regional differences in the rate of deposition or clearance would result in specific patterns of PrP^{Sc} accumulation. Because a single prion strain produced many different patterns when inoculated into mice expressing various PrP transgenes, we concluded that the pattern of PrP^{Sc} deposition is a manifestation of the particular strain but not an essential feature for successful propagation (DeArmond et al. 1997). The results of studies of three prion strains prepared from three brain regions and spleens of inbred mice support this contention (Carp et al. 1997).

ISOLATION OF NEW HAMSTER PRION STRAINS USING MICE AND TRANSGENIC MICE

The successful passaging of scrapie to Syrian hamsters led to the discovery and purification of the prion protein (Bolton et al. 1982), and hamsters are unrivaled as a source for purified prions. In addition to Syrian hamsters, prions have been passaged to two other species of hamsters, Armenian and Chinese hamsters. These three distinct species carry different PrP alleles and exhibit distinctive characteristic incubation periods when inoculated with prions passaged in either of the other two species (Lowenstein et al. 1990). Initial transmission of rat-adapted 139A strain to Syrian hamsters reportedly yielded two distinct hamster strains, termed 302K and 431K (Kimberlin and Walker 1978), leading the authors to suggest that the original inoculum must have been a mixture of the two strains. Following serial transmission in hamsters for several generations, however, a new strain, termed 263K, emerged that was no longer transmissible to mice (Kimberlin and Walker 1978). The 263K strain was derived from the same source as our standard hamster strain Sc237 (Marsh and Kimberlin 1975; Kimberlin and Walker 1977, 1978; Kimberlin et al. 1987). Passaging of the 139A mouse strain (indistinguishable from RML) to hamsters led to isolation of 139H, and serial transmission of Me7 to Syrian hamsters led to the stable isolate Me7-H (Kimberlin et al. 1987).

Although a few mouse-passaged scrapie prion strains had been successfully transmitted directly to hamsters, as described above, a new approach was developed for increasing the number of distinct prion isolates available in the Syrian hamster genetic background. Transgenic mice expressing a chimeric SHa/Mo PrP (MH2M) gene are susceptible to Mo prion strains (Scott et al. 1993). Following a single passage in these Tg(MH2M) mice, the chimeric prions are able to efficiently infect hamsters (Scott et al. 1993). In the course of these studies, evidence was obtained showing that strain variation could be created by altering the sequence of PrP encoded by the host animal, even using prion strains that had been previously cloned by limiting dilution in mice (Dickinson et al. 1969). When the "cloned" Me7 strain was transmitted to hamsters, a stable strain with an extremely long incubation period, termed Me7-H, was obtained (Kimberlin et al. 1987). It is important to note that Me7-H could also be transmitted back to mice, and that the resultant mouse isolate was restored to a state indistinguishable from Me7 (Kimberlin et al. 1989). However, a single passage of Me7 through Tg(MH2M) mice followed by transmission to hamsters led to formation of a new strain of hamster-passaged Me7, which we termed SHa(Me7). In contrast to Me7-H, SHa(Me7) produced short incubation periods and a noticeably distinct distribution of pathology (Scott et al. 1997a). In each passage, 100% of the inoculated animals exhibited clinical signs with minimal spread in the time of first onset of symptoms, making it extremely unlikely that selection for a rare variant could occur. Thus, it seems that the change in the strain was caused entirely by a change in the primary structure of PrP. In the same study, strains once thought to be distinct, which had originally been isolated from different breeds of scrapied sheep, were shown to have indistinguishable properties. Thus, prion strain properties depend on passage history with respect to the sequence of PrP, not on a separate, distinct informational component (Scott et al. 1997a), and both the generation and propagation of prion strains seem to depend on the structure of PrP^{Sc} and PrP^{C}.

HAMSTER PRION STRAINS DERIVED FROM TRANSMISSIBLE MINK ENCEPHALOPATHY EXHIBIT BIOCHEMICAL DIFFERENCES

A remarkable series of discoveries followed the successful transmission of transmissible mink encephalopathy (TME) to hamsters. An epidemic of TME in farmed mink in North America occurred simultaneously at five farms sharing the same feed source, strongly suggesting a food-borne

route of infection (Marsh 1992). Interestingly, although BSE has been successfully transmitted to mink, the resulting disease does not resemble TME (Robinson et al. 1994). Attempts to transmit TME to mice were unsuccessful (Ridley and Baker 1996); the disease could be transmitted to Chinese hamsters (Kimberlin et al. 1986) and subsequently to Syrian hamsters (Bessen and Marsh 1992b). Two distinct strains were obtained, termed "HYPER" (HY) and "DROWSY" (DY), creating a remarkable parallel to the initial passage of sheep scrapie to goats years earlier, which had produced two distinct strains, termed "scratching" and "drowsy" (Pattison and Millson 1961a). The drowsy syndrome bears a striking resemblance to that of its namesake in goats (Bessen and Marsh 1992a,b).

The isolation of the DY strain turned out to be a milestone in the study of prion strains. Unlike the HY isolate, the DY strain was found to differ significantly from other known hamster prion strains in its biochemical and physical properties. Marked differences were identified by sedimentation analysis, protease sensitivity, and the migration pattern of PrP^{Sc} proteolytic fragments on SDS gels (Bessen and Marsh 1992b). PrP^{Sc} produced by the DY prions showed diminished resistance to proteinase K digestion and truncation of the amino terminus compared to HY and many other strains (Fig. 3) (Bessen and Marsh 1994). Since both HY and DY were prepared by passaging in outbred Syrian hamsters, the markedly different properties of the two isolates could not be attributed to differences in the sequence of PrP. For the first time, there was evidence that different strains might represent different conformers of PrP^{Sc} (Prusiner 1991; Bessen and Marsh 1992b). An alternative explanation, that the distinct properties of HY and DY were caused by differences in posttranslational modification, was also considered (Bessen and Marsh 1992a). As far as we are aware, the remarkable properties of DY remain unique among hamster prion strains; when several distinct hamster prion isolates were studied in an attempt to identify a similar change in the size of the partially protease-resistant fragment, all except DY produced a fragment of the same size as the HY strain (Scott et al. 1997a).

The unusual properties of DY were also valuable in attempts to replicate prions in cell-free systems. The altered sensitivity to protease displayed by the DY strain in vivo was mimicked in vitro when partially denatured, radiolabeled PrP^{C} was mixed with an excess of PrP^{Sc} (Bessen et al. 1995). Although it has not yet been possible to demonstrate the propagation of infectious prions using this system, the faithful reproduction of this evidence of a conformational change is suggestive of a highly specific interaction.

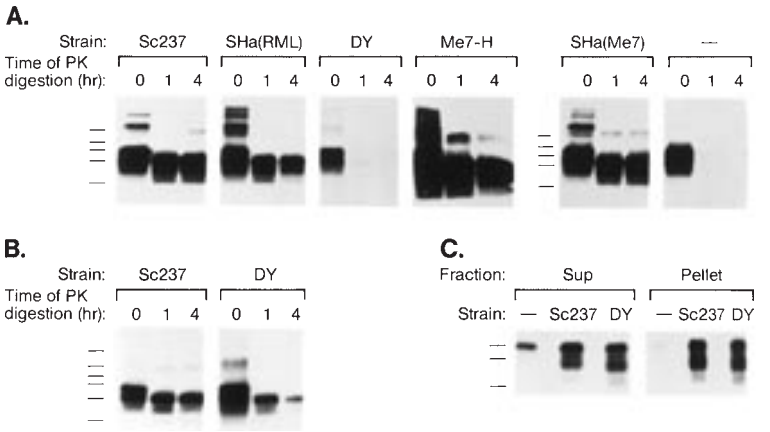

Figure 3 Comparison of protease sensitivity and 27-30 products of different prion strains. (*A*) Brain homogenates (1 mg ml^{-1} total protein) of SHa infected with several SHa prion strains were treated with 100 μg ml^{-1} proteinase K for 0, 1, or 4 hr, and analyzed by Western blotting, using α-PrP 3F4 MAb, which recognizes SHaPrP (Kascsak et al. 1987). The inocula used in this experiment were Sc237, SHa(RML), DY, Me7-H, SHa(Me7), and uninoculated control. (*B*) Brain microsomal membrane fractions (1 mg ml^{-1} total protein) of SHa infected with Sc237 or DY were treated with 100 μg ml^{-1} proteinase K for 0, 1, or 4 hr, and analyzed by Western blotting, using α-PrP 3F4 MAb. (*C*) Brains from normal or scrapie-ill hamsters infected either with Sc237 or the DY inoculum were solubilized in cold buffer A (1% Triton X-100 and 1% sodium deoxycholate in phosphate-buffered saline) incubated on ice and then centrifuged at 15,000*g* for 20 min at 4ºC. The supernatant was removed, and then an equal percentage of the supernatant (Sup) and the pellet (Pellet) fractions were analyzed by Western blotting using 3F4 anti-PrP antibody. Molecular-size markers are indicated on the left of the panels and in descending order represent 84, 53, 35, 29, and 21 kD (panels *A,B*) or 35, 29, and 21 kD (panel *C*). (Reprinted, with permission, from Scott et al. 1997a [copyright American Society for Microbiology].)

DE NOVO GENERATION OF HUMAN PRION STRAINS FROM MUTANT PrP GENES

In humans, a mutation of the PrP gene at codon 178 that results in the substitution of asparagine for aspartic acid causes a prion disease called fatal familial insomnia (FFI), provided the polymorphic codon 129 encodes methionine (Goldfarb et al. 1992; Medori et al. 1992a). In this disease, adults generally over age 50 present with a progressive sleep disorder and die within about a year of onset (Lugaresi et al. 1986). In their brains, deposition of PrP^{Sc} is confined largely within the anteroventral and the

dorsal medial nuclei of the thalamus. In contrast, the same D178N mutation with a valine encoded at position 129 produces fCJD in which the patients present with dementia, and widespread deposition of PrP^{Sc} is found postmortem (Goldfarb et al. 1991). The first family to be recognized with CJD was recently found to carry the D178N mutation (Meggendorfer 1930; Stender 1930; Kretzschmar et al. 1995).

When the PrP^{Sc} molecules produced in the two prion diseases with the D178N mutations were examined by Western immunoblotting after limited digestion with proteinase K and deglycosylation with PNGase F, the PrP^{Sc}(D178N, M129) of FFI exhibited an M_r of 19 kD and that of fCJD(D178N, V129) an M_r of 21 kD (Monari et al. 1994). This difference in molecular size was shown to be due to different sites of proteolytic cleavage at the amino termini of the two PrP^{Sc} molecules and interpreted as reflecting different tertiary structures (Monari et al. 1994). These distinct conformations were not entirely unexpected, since the amino acid sequences of the PrPs differ.

Persuasive evidence that strain-specific information is enciphered in the tertiary structure of PrP^{Sc} comes from transmission of two different inherited human prion diseases to mice expressing a chimeric MHu2M PrP transgene (Telling et al. 1996). Extracts from the brains of FFI patients transmitted disease to mice expressing a chimeric MHu2M PrP gene about 200 days after inoculation and induced formation of the 19-kD PrP^{Sc}; fCJD (E200K) and sCJD produced the 21-kD PrP^{Sc} in these mice (Table 2) (Telling et al. 1996). The experimental data demonstrate that MHu2MPrP^{Sc} can exist in two different conformations based on the sizes of the protease-resistant fragments; yet, the amino acid sequence of MHu2MPrP^{Sc} is invariant. These findings, together with those of the DY

Table 2 Distinct prion strains generated in humans with inherited prion diseases and transmitted to Tg mice

Inoculum	Host species	Host PrP genotype	Incubation time [days ± S.E.M.]	Incubation time (n/n_o)	PrP^{Sc} (kD)
None	human	FFI(D178N,M129)			19
FFI	mouse	Tg(MHu2M)	206 ± 7	(7/7)	19
FFI→Tg(MHu2M)	mouse	Tg(MHu2M)	136 ± 1	(6/6)	19
None	human	fCJD(E200K)			21
fCJD	mouse	Tg(MHu2M)	170 ± 2	(10/10)	21
fCJD→Tg(MHu2M)	mouse	Tg(MHu2M)	167 ± 3	(15/15)	21

Data from Telling et al. (1996). (Reprinted, with permission, from Prusiner 1997 [copyright AAAS].)

strain discussed earlier, suggest that propagation of prion strains results in the faithful copying of specific conformations of PrP from a "template" molecule of PrP^{Sc} into a "substrate" molecule that we have termed PrP*, which may itself derive from PrP^{C} following interaction with protein X (Prusiner 1998; Prusiner et al. 1998).

Interestingly, the 19-kD protease-resistant fragment of PrP^{Sc} formed after deglycosylation has also been found in a patient who died after developing a clinical disease similar to FFI. Since both PrP alleles encoded the wild-type sequence and a methionine at position 129, we labeled this case fatal sporadic insomnia (FSI). At autopsy, the spongiform degeneration, reactive astrogliosis, and PrP^{Sc} deposition were confined to the thalamus (Mastrianni et al. 1997). These findings argue that the clinicopathologic phenotype is determined by the conformation of PrP^{Sc} in accord with the results of the transmission of human prions from patients with FFI to Tg mice (Telling et al. 1996).

CONSERVATION OF BSE "PHENOTYPE" DURING SERIAL PASSAGE

Beginning in 1986, an epidemic of a previously unknown prion disease named bovine spongiform encephalopathy (BSE) or "mad cow" disease appeared in cattle in Great Britain (Wells et al. 1987) in which protease-resistant PrP was found in brains of ill cattle (Hope et al. 1988; Prusiner et al. 1993). It has been proposed that BSE represents a massive common-source epidemic that was caused by meat and bone meal (MBM) fed primarily to dairy cows (Wilesmith et al. 1991). The MBM was prepared from the offal of sheep, cattle, pigs, and chickens as a high-protein nutritional supplement. In the late 1970s, the solvent extraction method used in the rendering of offal began to be abandoned, resulting in an MBM with a much higher fat content (Wilesmith et al. 1992). It is now thought that this change in the rendering process allowed scrapie prions from sheep to survive rendering and to pass into cattle. Alternatively, bovine prions that had caused clinical CNS dysfunction at such a low level as not to be recognized survived the rendering process and were passed back to cattle through the MBM. Recent statistics argue that the epidemic is now disappearing as a result of the 1988 food ban.

Brain extracts from BSE cattle have transmitted disease to mice, cattle, sheep, pigs, and mink after intracerebral inoculation (Fraser et al. 1988; Robinson et al. 1994). Primary transmissions to mice generally produce an incubation period of about 400 days (Fraser et al. 1992). On subsequent passages in mice, the incubation period shortens considerably

and eventually stabilizes at a value that depends on the *Sinc/Prnp* genotype of the host mice (Bruce et al. 1994). Although the initial report concluded that two distinct strains, termed 301V and 301C, could be discriminated in several separate transmissions from BSE-afflicted cows, it now appears more likely that 301V and 301C are fundamentally the same strain, differing only with respect to the *Sinc/Prnp* identity of PrP encoded by the donor and recipient mice used in these transmissions (Bruce et al. 1994; Ridley and Baker 1996).

It is interesting to note that, in contrast to sheep, different breeds of cattle have no specific PrP polymorphisms other than a variation in the number of octarepeats: Most cattle, like humans, have five octarepeats, but some have six (Goldmann et al. 1991b; Prusiner et al. 1993). Humans with seven octarepeats develop fCJD (Goldfarb et al. 1993), but the presence of six octarepeats does not seem to be overrepresented in BSE (Goldmann et al. 1991b; Prusiner et al. 1993; Hunter et al. 1994). The number of octarepeats does not appear to affect the specificity of transmission of prions (Fischer et al. 1996), in contrast to polymorphic changes within the 90-231 residue core of the protein, which we have elaborated upon above. Since the majority of evidence for prion strain diversity can be traced to differences in the sequence of PrP in successive hosts for transmission, it is possible that the lack of PrP polymorphism in cattle may to some degree account for the remarkable consistency of BSE strain properties observed.

ARE BSE AND vCJD RELATED?

The emergence of vCJD in teenagers and young adults in the UK and France has raised the possibility that transmission of BSE to humans may occur (Chazot et al. 1996; Will et al. 1996). The average age of these individuals was 27 years, much younger than for any other group of people who have died of CJD except for those who received pituitary HGH. Why such cases should be confined to young people is unclear. Whether the young CNS is more vulnerable to invasion by bovine prions or the dietary habits of these young individuals exposed them to a greater dose of bovine prions is unknown. Not only does age set these teenagers and young adults apart from other individuals who died of prion disease, but so does the neuropathology. The deposition of PrP in the brains of these patients is extreme, and numerous multinucleated PrP amyloid plaques surrounded by intense spongiform degeneration have been observed. These neuropathologic changes seem to be unlike any observed in other forms of prion disease.

To investigate this possibility, transmissions of BSE to primates have been performed. Significantly, BSE could be transmitted to the marmoset after intracerebral inoculation and, more recently, to the macaque (Baker et al. 1993; Lasmézas et al. 1996). In addition, a young adult monkey born and reared in Britain has died of a neurodegenerative disorder thought to be a prion disease in a Montpelier zoo (Bons et al. 1996). Whether this monkey's illness is due to bovine prions in contaminated foodstuffs or is a case of sporadic prion disease remains to be established.

Further evidence that vCJD and BSE could be related has arisen through the use of Tg(HuPrP)$Prnp^{0/0}$ mice (Collinge et al. 1995). These mice are homozygous for valine at codon 129 and routinely develop illness approximately 200–250 days after inoculation with Hu fCJD, sCJD, and iCJD prions (Collinge et al. 1995; Telling et al. 1995). A similar incubation period was reported upon inoculation with six separate vCJD isolates (Collinge et al. 1995); however, it is hard to assess how efficient the transmission was, since only 25 out of a total of 56 animals inoculated were affected. This contrasts with other human prion transmissions in Tg(HuPrP)$Prnp^{0/0}$ mice, in which the transmission efficiency is usually close to 100% (Collinge et al. 1995; Telling et al. 1995). Unlike fCJD and BSE, vCJD was also found to transmit to FVB mice after a long incubation period (>350 days), and about 40% of Tg(HuPrP)$Prnp^{0/0}$ mice inoculated directly with BSE developed neurological disease, albeit with extremely protracted incubation periods (>600 days) (Collinge et al. 1995). The patterns of deposition of PrP when BSE and vCJD were inoculated into FVB or Tg(HuPrP)$Prnp^{0/0}$ were compared. Although the two strains of mice differed in the pattern of deposition, as expected from other studies (DeArmond et al. 1997), vCJD and BSE were found to produce similar patterns when compared in both strains of mice. In addition, the relative distribution of various glycoforms of PrP^{Sc} revealed by Western blot was found to be similar when BSE and vCJD-inoculated animals were compared (Collinge et al. 1997). A separate study using three inbred mouse strains (RIII, C57BL, and VM) reached a similar conclusion (Bruce et al. 1997).

Although a link between vCJD and BSE has certainly been forged by the preceding study, it is still impossible to evaluate the potential risk to humans exposed to BSE-tainted beef. The number of reported cases of vCJD does not seem to be increasing. It is also important to note that epidemiological studies over nearly three decades have failed to establish convincing evidence for transmission of sheep prions to humans (Cousens et al. 1990; Malmgren et al. 1979). Of interest is the high incidence of CJD among Libyan Jews that was initially attributed to the consumption of lightly cooked sheep brain (Kahana et al. 1974); however,

subsequent studies showed that this geographical cluster of CJD is due to the E200K mutation (Goldfarb et al. 1990; Hsiao et al. 1991).

TRANSGENIC MOUSE MODELS OF BSE

Mice inoculated intracerebrally with BSE prions require more than a year to develop disease (Fraser et al. 1992; Taylor 1991; Lasmézas et al. 1997). Other attempts at assaying BSE prions have used animals from various species. Brain extracts from BSE cattle cause disease in cattle, sheep, mice, pigs, and mink after intracerebral inoculation (Fraser et al. 1988; Dawson et al. 1990a,b; Bruce et al. 1993). All of these alternative bioassay systems suffer from severe limitations to their usefulness. Apart from the cost involved, the long incubation periods and low efficiency of transmission of prions, heightened in most cases by a species barrier, caused by lack of PrP sequence identity, have conspired to severely impede progress in performing routine measurements of titers of BSE prions. Since no alternative bioassay has offered any significant advantage over the use of normal mice, these have become the species of choice for the majority of BSE bioassays, until recently. Of available mouse strains, RIII mice give the shortest incubation periods, with mean incubation periods reportedly ranging from 302 to 335 days for transmission of frozen brain samples (Bruce et al. 1994). Even RIII mice suffer from limitations, however, which restrict their usefulness in bioassays of bovine prions. BSE prions that have been serially passaged in mice exhibit dramatically shortened incubation periods, in some cases less than 140 days (Bruce et al. 1994). Although some investigators appear to disagree that this is problematic (Bruce et al. 1994), it seems clear to us from these data that BSE prions passaged in mice and those passaged in cattle are not equivalent; instead there is clear evidence of a species barrier (Pattison 1965) to the transmission of BSE from cattle to mice.

The evidence for a significant species barrier to the transmission of BSE prions to mice has important ramifications that make mice inappropriate for use in bioassays aimed at detecting low doses of BSE prions. As we have argued elsewhere (Scott et al. 1997a) and previously in this chapter, a species barrier can be indicative of a change in strain of prion enforced by differences in the sequence of PrP of the donor and recipient host animals. Some investigators argue that this is not the case with BSE; the characteristic BSE strain "signature" is reportedly unchanged by serial transmission from numerous species to mice (Bruce et al. 1994). Even if we assume this to be true, only one other explanation for the enormous

difference in incubation period seems possible: Since incubation period and the titer of inoculum are inversely related (Pattison and Smith 1963; Prusiner et al. 1981), the extended incubation period during primary transmission must represent an effective reduction in titer, caused presumably by the inefficient interaction of Bo PrP^{Sc} in the inoculating prions with Mo PrP^{C} of the host (Prusiner et al. 1990). In support of this reasoning, endpoint titrations of BSE prions in cattle suggest much higher titers of prions in bovine brain than those determined by mouse bioassays (J. Wilesmith, pers. comm.). To overcome this limitation, we created transgenic mice expressing Bo PrP transgenes (Scott et al. 1997b).

Three lines of Tg(BoPrP)$Prnp^{0/0}$ mice expressing BoPrP were inoculated with a 10% homogenate derived from the medulla of a Hereford bull (case PG31/90) clinically ill with BSE (Scott et al. 1997b). One line, Tg(BoPrP)E4125/$Prnp^{0/0}$ mice, which showed the highest level of BoPrP expression, was found to be highly susceptible to BSE prions, with 100% of animals exhibiting clinical signs within 250 days after inoculation. Another line, Tg(BoPrP)E4092/$Prnp^{0/0}$, had an intermediate level of BoPrP expression and exhibited a longer incubation period with the same inoculum; they had a mean incubation period of about 320 days (Table 3). Similar incubation periods were obtained when the lines were inoculated with other BSE isolates. Like most cattle with BSE, vacuolation and astrocytic gliosis were confined in the brain stems of these Tg mice (Fig. 4) (Scott et al. 1997b).

Many previous studies with transgenic mice over the last decade have clearly established that expression of a foreign PrP transgene abrogates the species barrier (Scott et al. 1989, 1993, 1997b; Prusiner et al. 1990; Hsiao et al. 1994; Telling et al. 1994, 1995); hence we can reasonably expect that these Tg(BoPrP) mice will make possible, for the first time, an accurate determination of BSE prion titers in brain and other tissues. Determining the titers of BSE prions in muscle, pancreas, liver, and intestine, which are commonly consumed by humans, will be of utmost importance. If the current cases of vCJD are due to bovine prions, then it is likely the exposure occurred prior to the specified bovine offals ban of November 1989 that prohibited human consumption of CNS and lymphoid tissues from cattle older than 6 months of age. This legislation was based on studies in sheep showing that the highest titers of scrapie prions are found in these tissues (Hadlow et al. 1982). In those scrapie studies, sheep tissues were inoculated into non-Tg Swiss mice, which are slightly more susceptible to sheep prions than bovine prions. Because the bioassay for bovine prions in ordinary mice is so insensitive (Taylor 1991), the

Table 3 Susceptibility and resistance of transgenic mice to BSE prions

Inoculum	Recipient	Transgene expression	Incubation time (days ± S.E.)	n/n_o
	A. Mice deficient for PrP (Prnp$^{0/0}$)			
BSE(PG31/90)	Tg(BoPrP)4125	8-16x	234 ± 8	10/10
BSE(PG31/90)	Tg(BoPrP)4092	4–8x	319 ± 15	8/8
BSE(GJ248/85)	Tg(BoPrP)4125	8–16x	281 ± 19	10/10
BSE(GJ248/85)	Tg(BoPrP)4092	4–8x	343 ± 18	8/8
BSE(GJ248/85)	Tg(MBo2M)14586	8–16x	>600	0/15
BSE(PG31/90)	Tg(MBo2M)14586	8–16x	>600	0/13
BSE(574C)	Tg(MBo2M)14586	8–16x	>600	0/13
	B. Mice expressing MoPrP-A			
BSE(GJ248/85)	FVB	0	628 ± 47	2/3
BSE(PG31/90)	FVB	0	448 ± 29	2/2
BSE(574C)	FVB	0	525 ± 34	4/4
BSE(PG31/90)	Tg(MoPrP-A)4053	8–16x	>310	4/10
BSE(GJ248/85)	Tg(MoPrP-A)4053	8–16x	322 ± 6	8/9
BSE(PG31/90)	Tg(BoPrP)333	0[a]	426 ± 11	8/8
BSE(PG31/90)	Tg(BoPrP)833	0[a]	395 ± 22	9/9
BSE→Tg(BoPrP)333	CD-1	0	163 ± 5	8/8
BSE→Tg(BoPrP)333	CD-1	0	148 ± 0	8/8

(Reprinted, with permission, from Scott et al. 1997b [copyright National Academy of Sciences].)
[a]No BoPrP was detected by Western immunoblotting.

levels of prions in bovine muscle remain unknown. If the distribution of bovine prions proves to be different from that presumed for sheep, then assumptions about the efficacy of the offal ban will need to be reassessed.

VARIATIONS IN GLYCOSYLATION OF PrP^{Sc} ASSOCIATED WITH PARTICULAR STRAINS

Recent studies of PrP^{Sc} from brains of patients who died of vCJD show a pattern of PrP^{Sc} glycoforms different from those found for sCJD or iCJD (Collinge et al. 1996, 1997). It closely resembled that found in the brains of mice and cats infected with BSE (Collinge et al. 1996, 1997). We have found a similar pattern of glycoforms in cattle with BSE, and this pattern appears to be reproduced upon transmission to Tg(BoPrP) mice, providing further support for the notion that these mice represent a faithful biological model for the bovine prion disease (Scott et al. 1997b). The sig-

nificance of this pattern is questionable, however, in trying to relate BSE to vCJD (Collinge et al. 1997; Parchi et al. 1997; Somerville et al. 1997). From our own studies, prion strains are often unstable when transmissions are effected between animals with distinct PrP sequences (Scott et al. 1997a). Although the glycoform ratios appear similar when BSE or vCJD passaged into normal mice are compared with extracts of Hu vCJD patients, the larger glycoform from extracts derived from the murine background appears to migrate more rapidly than that from human extracts (Collinge et al. 1997), suggesting that BSE and vCJD PrP^{Sc} preparations from mice are underglycosylated compared to their corresponding protease-resistant fragments in Hu vCJD brain. In addition, since PrP^{Sc} is formed after the protein is glycosylated (Borchelt et al. 1990; Caughey and Raymond 1991), and enzymatic deglycosylation of PrP^{Sc} requires denaturation (Endo et al. 1989; Haraguchi et al. 1989), either some form of selective conversion of PrP^{C} into PrP^{Sc} or variations in the rate of clearance of different PrP^{Sc} glycoforms (Prusiner et al. 1998) would have to account for the glycoform ratios observed.

If different PrP^{Sc} glycoform ratios do arise, as we suspect, due to variations in either the rate of synthesis or the rate of clearance of the various PrP^{Sc} glycoforms, then it seems reasonable to suggest this is itself dependent on the strain of prion and hence the conformation of PrP^{Sc}.

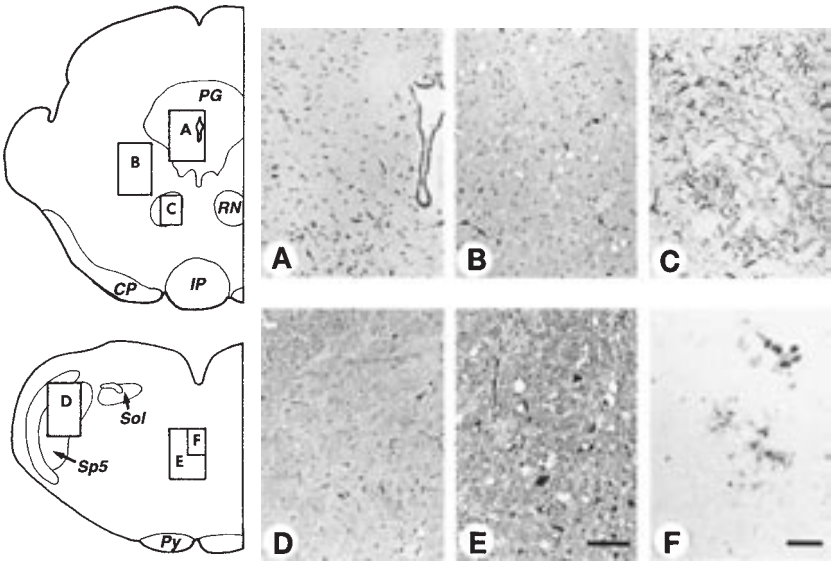

Figure 4 (See facing page for legend.)

Apparently, the BSE "strain" will preferentially form fully glycosylated PrP^{Sc}, producing the "type 4" pattern (Collinge et al. 1996). By optimizing the sequence of PrP to facilitate formation of the higher-molecular-mass glycoforms, we may be able to accelerate the rate of conversion and consequently reduce the incubation period dramatically. PrP mutations that selectively increase the conversion of diglycosylated PrP^{C} may assist in the creation of Tg mice with more rapid incubation times when inoculated with BSE and/or vCJD prions.

PrP STRUCTURAL CONSTRAINTS MODULATING PRION TRANSMISSION

Because of our success in transmitting Hu CJD prions to Tg mice expressing a chimeric Hu/Mo PrP gene termed MHu2M (Telling et al. 1994, 1995), we also created mice expressing an analogous Bo/Mo PrP transgene, which we termed MBo2M (Scott et al. 1997b). Unexpectedly, when Tg(MBo2M) mice expressing high levels of chimeric PrP were inoculated with BSE prions, they did not develop signs of disease, even more than 600 days following exposure to BSE (Table 3) (Scott et al. 1997b). This was surprising, since the MBo2M ORF comprised only Bo PrP and Mo PrP-A sequences, and both mice and cattle have been shown to be susceptible to BSE prions. A comparison of the Mo PrP-A, MBo2M PrP, and

Figure 4 Neuropathology of $Tg(BoPrP)Prnp^{0/0}$ mice inoculated with BSE prions. (*A*) No pathologic changes were found in the periaqueductal gray of the midbrain. (*B*) Mild to moderate vacuolar degeneration was found in the reticular formation of the midbrain tegmentum. (*C*) Reactive astrocytic gliosis colocalized with sites of vacuolar degeneration: Astrogliosis in the red nucleus is shown here. (*D*) Little or no vacuolar degeneration was found in the tract or the nucleus of the spinal tract of the trigeminal nerve in the medulla. (*E*) Moderate to severe vacuolar degeneration occurred in the medial tegmentum of the medullary reticular formation. (*F*) Small PrP-immunopositive primitive plaque-like deposits colocalized with sites of the most severe vacuolar degeneration. Diagrams show locations of photomicrographs. Hematoxylin and eosin stain used in *A,B,D,E*. Glial fibrillary acidic protein immunohistochemistry used in *C*. PrP immunohistochemistry used in *F*. Bar in *E* = 100 μm; also applies to *A, B*, and *D.* Bar in *F* = 50 μm; also applies to *C*. Italicized letters identify selected brain stem structures: (CP) cerebral peduncle; (IP) interpeduncular nucleus; (PG) periaqueductal gray; (Py) pyramidal tract; (RN) red nucleus; (Sol) nucleus and tractus solitarius; (Sp5) nucleus of the spinal tract of the trigeminal nerve. (Reprinted, with permission, from Scott et al. 1997b [copyright National Academy of Sciences].)

MHu2M translated sequences showed that Hu residue substitutions in MHu2M extended from 97 to 168, whereas Bo substitutions in MBo2M included additional differences between 168 and 186. This finding raised the possibility that residues 184 and 186, which are not homologous in Mo and Bo PrP and lie at the end of the chimeric region, might account for this difference in susceptibility. Alternatively, residue 203, which is valine in Mo and Hu PrP and isoleucine in BoPrP, might be responsible for this difference in susceptibility. Since residue 203 is valine in MBo2M, and isoleucine in Bo PrP, this might prevent efficient formation of MBo2M PrP^{Sc} (Scott et al. 1997b).

When viewed in a spatial context, the potential impact of these differences is more easily appreciated (Fig. 5) (Scott et al. 1997b). Residues 184, 186, 203, and 205 were identified within the three-dimensional structure of SHa PrP(90-231) derived by solution NMR (Fig. 5) (James et al. 1997). These residues were seen to cluster on one side of the PrP^{C} structure and are spatially distinct from the discontinuous epitope comprising residues 168, 172, 215, and 219 that binds protein X (Fig. 5) (Kaneko et al. 1997). We next compared these residues to others known to be polymorphic from sequence comparisons of the PrP ORFs of many other species (Bamborough et al. 1996) which are thus candidates for contributing to the species barrier. As can be seen from Figure 5, these residues appear to cluster and enlarge the epitope formed by residues 184,186, 203, and 205. It is also interesting to note that seven point mutations (178, 180, 183, 198, 200, 208, 210) known to cause inherited prion diseases, including those which have been shown to create a transmissible encephalopathy, map to this region of structure as well (Fig. 5) (Scott et al. 1997b).

Figure 5 PrP residues governing the transmission of prions. (*A*) NMR structure of recombinant SHaPrP region121-231 (James et al. 1997) shown with the putative epitope formed by residues 184, 186, 203, and 205 highlighted in red. Residue numbers correspond to SHaPrP. Additional residues (138, 139, 143, 145, 148, and 155) that might participate in controlling the transmission of prions across species are depicted in green. Residues 168, 172, 215, and 219 that form the epitope for the binding of protein X are shown in blue. (*A*, Reprinted, with permission, from Scott et al. 1997b [copyright Natinoal Academy of Sciences.]) (*B*) Within and adjacent to the epitope formed by residues 184, 186, 203, and 205 shown in red are known pathologic mutations at residues 178, 180, 183, 198, 200, 208, and 210 colored in magenta. The three helices (A, B, and C) are highlighted in brown. Illustration was generated with Biosym/Insight II.

The identification of a species-specific epitope that modulates the conversion of PrP^{C} into PrP^{Sc} has important implications for the future design of PrP transgenes. Whether optimal Bo/Mo chimeric transgenes should contain Mo or Bo residues at positions 184, 186, 203, and 205 remains to be established. Similarly, it is unknown whether improved

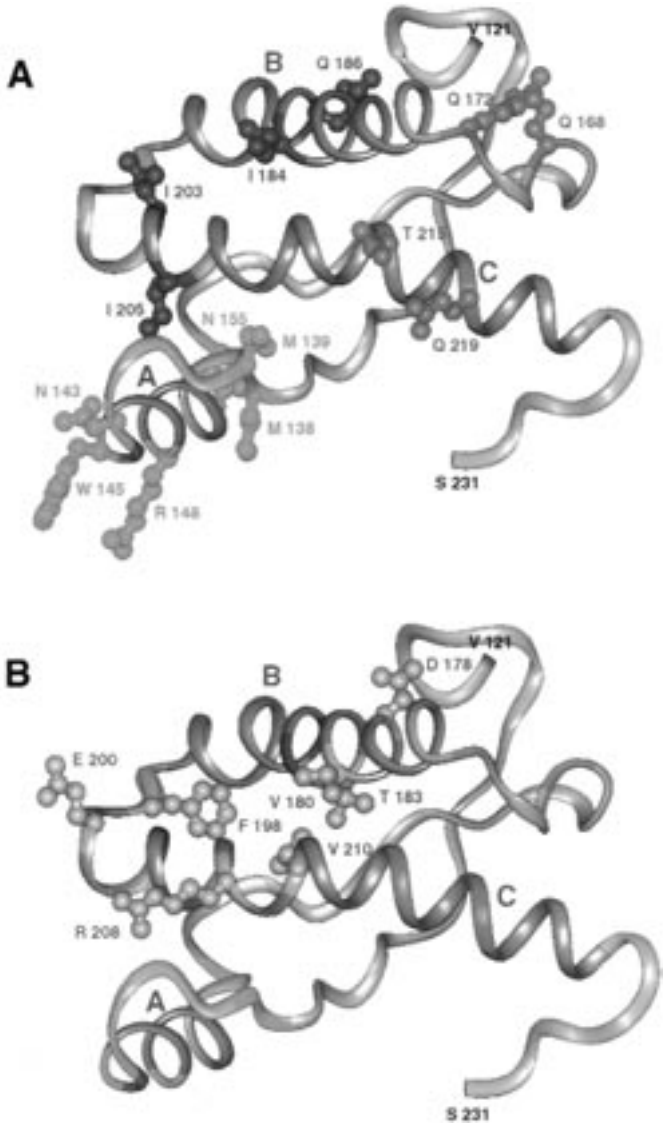

Figure 5 (See facing page for legend.)

Hu/Mo chimeric transgenes can be constructed by simultaneously mutating Hu residues at these same positions to Mo residues (Fig. 5). Mutagenesis at any or all of these positions may overcome the paradoxically long incubation times found in Tg(MHu2M)$Prnp^{0/0}$ mice expressing high levels of the transgene product (G. Telling and S.B. Prusiner, in prep.) as well as the resistance of Tg(MBo2M) mice to Bo prions.

ESTIMATING THE LIMITS OF PRION DIVERSITY

As we stated at the outset, the only significant obstacle to understanding prion strains within the context of a protein-only model for prion replication has been that of the existence of prion diversity. It now seems clear that prion strains encipher their properties in PrP conformers (Telling et al. 1996; Safar et al. 1998). In a recent study, eight distinct hamster prion isolates were analyzed using a conformational dependent immunoassay (CDI) (Safar et al. 1998). Using the CDI, the ratio of denatured/native PrP as a function of Prp^{Sc} concentration before and after limited digestion with proteinase K (Safar et al. 1998) could be used to discriminate each of the eight prion isolates. Notably, only the DY strain could be distinguished from the other seven isolates by Western blotting after limited proteolysis (Scott et al. 1997a). Intriguing evidence that each individual prion strain itself comprises multiple distinct conformers of PrP^{Sc} was obtained when several strains were compared using CDI following limited GdnHCl denaturation and protease digestion (Safar et al. 1998). It will be of great interest to investigate whether these distinct conformers exist in a stoichiometric arrangement unique to each strain.

In this review, and in others (Ridley and Baker 1996), we have argued that, in reality, the number of possible strains may be much lower than has been claimed. If prion strains within a single PrP genetic background are found to appear coincident in their properties, despite having been derived from completely different primary sources, geographically isolated from one another, prion diversity must be restricted. The frequent "reisolation" of Me7 illustrates this point (Ridley and Baker 1996). In other studies, the apparent identity of Sc237 and SHa(Me7) when passaged between identical hosts (Scott et al. 1997a) is made all the more striking in view of the fact that Me7 appears to be derived from an entirely different primary source, a Suffolk sheep spleen, whereas the ancestry of both Sc237 and RML can be traced to the Compton "drowsy goat" source, which in turn was derived by transmission from scrapied Cheviot sheep (Pattison et al. 1959). In contrast, we were able to obtain two completely different SHa strains from the same, "cloned" primary source,

Me7, by including one additional passage in Tg(MH2M) mice (Scott et al. 1997a). Thus, the passage history, rather than the original source, determines strain characteristics in any particular host.

We have also described how differences in prion strain characteristics can be directly traced back to changes in PrP sequence during serial transmission, suggesting that strain characteristics are maintained and propagated through protein-protein interactions between PrP isoforms. This is entirely consistent with a model in which prion strain diversity is contained entirely within PrP. The spectrum of possible PrP conformations must be constrained by the sequence of PrP, and it seems likely that only a limited number of distinct conformations for a PrP^{Sc} of any particular sequence are possible. In turn, each prion strain appears to be composed of several of these individual conformers of PrP^{Sc} (Safar et al. 1998). Admittedly, more detailed analysis of the molecular architecture of distinct prion strains will be required before the exact mechanism of strain diversity is completely resolved. Despite this, the picture that is rapdily emerging from these studies is one of a mechanism which seems easily capable of supporting a large variety of distinct prion strains encoded by a single PrP polypeptide sequence.

THE SPECIES AND STRAIN OF PRIONS REFLECT THE RELATIONSHIP BETWEEN SEQUENCE AND CONFORMATION

The recent advances described above in our understanding of the role of the primary and tertiary structures of PrP in the transmission of disease have given new insights into the pathogenesis of the prion diseases. It appears that the amino acid sequence of PrP encodes the species of the prion (Scott et al. 1989; Telling et al. 1995) and the prion derives its PrP^{Sc} sequence from the last mammal in which it was passaged (Bockman et al. 1987). Whereas the primary structure of PrP is likely to be the most important, or even the sole, determinant of the tertiary structure of PrP^{C}, existing PrP^{Sc} seems to function as a template in determining the tertiary structure of nascent PrP^{Sc} molecules as they are formed from PrP^{C} (Prusiner 1991; Cohen et al. 1994). In turn, prion diversity is enciphered in the conformation of PrP^{Sc}, and prion strains may represent different conformers of PrP^{Sc} (Bessen and Marsh 1992a; Telling et al. 1996). Furthermore, we have argued that the species barrier observed during transmission of prions between hosts with distinct PrP genes may, at least in some instances, reflect a change in the strain of prion (Scott et al. 1997a). This can easily explain how multiple different isolates are identified when prions cross species barriers—they are generated, not selected

from a preexisting mixture. Presumably, new strains are created because the original strain conformer is energetically unfavorable when folded from the new PrP sequence.

Although the foregoing scenario seems to be unprecedented in biology, considerable experimental data now support these concepts. Two prion-like elements, [URE3] and [PSI], have been described in yeast, and another in another fungus denoted [Het-s*] (Wickner 1994; Chernoff et al. 1995; Coustou et al. 1997; Glover et al. 1997; King et al. 1997; Paushkin et al. 1997). Conversion to the prion-like [PSI] state in yeast requires the participation of the molecular chaperone Hsp104 (Chernoff et al. 1995; Patino et al. 1996). It may prove to be highly significant that different strains of yeast prions have already been identified (Derkatch et al. 1996). Other parallels exist in higher organisms. Refolding studies of hen and human lysozyme have clearly shown that the folding pathways of homologous proteins can differ markedly as a result of amino acid replacements (Hooke et al. 1994).

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Cell Biology of Prions

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The cell biology of prions has emerged as an important area of investigation. Studies of the biosynthesis of PrP^{C} and its trafficking through the cell as it is either degraded or transformed into PrP^{Sc} have yielded a series of unexpected findings.

BIOGENESIS OF PrP^{C}

The biosynthesis of PrP^{C} is similar to that of many secreted glycoproteins. PrP^{C} is translated from the mRNA in the endoplasmic reticulum (ER) (Caughey et al. 1989; Borchelt et al. 1990). As the Syrian hamster (SHa) PrP polypeptide chains are being elongated, presumably the signal peptide of 22 amino acids at the amino terminus is removed proteolytically, as for other secretory proteins (Basler et al. 1986; Hope et al. 1986; Turk et al. 1988). After assembly of the polypeptide chain is complete, a signal sequence of 23 amino acids is removed from the carboxyl terminus as a glycosyl phosphatidylinositol (GPI) anchor is added to serine at position 231 (Stahl et al. 1987, 1990). In the ER, the remaining 209 residues of the SHaPrP polypeptide contain a disulfide bond and two asparagine-linked CHOs, as well as the GPI anchor attached to the carboxyl terminus through an ethanolamine (Fig. 1).

As PrP^{C} is transported through the Golgi apparatus, the sugar chains of the asparagine-linked CHOs and GPI anchor are modified. The high mannose cores of the two asparagine-linked sugar chains are remodeled

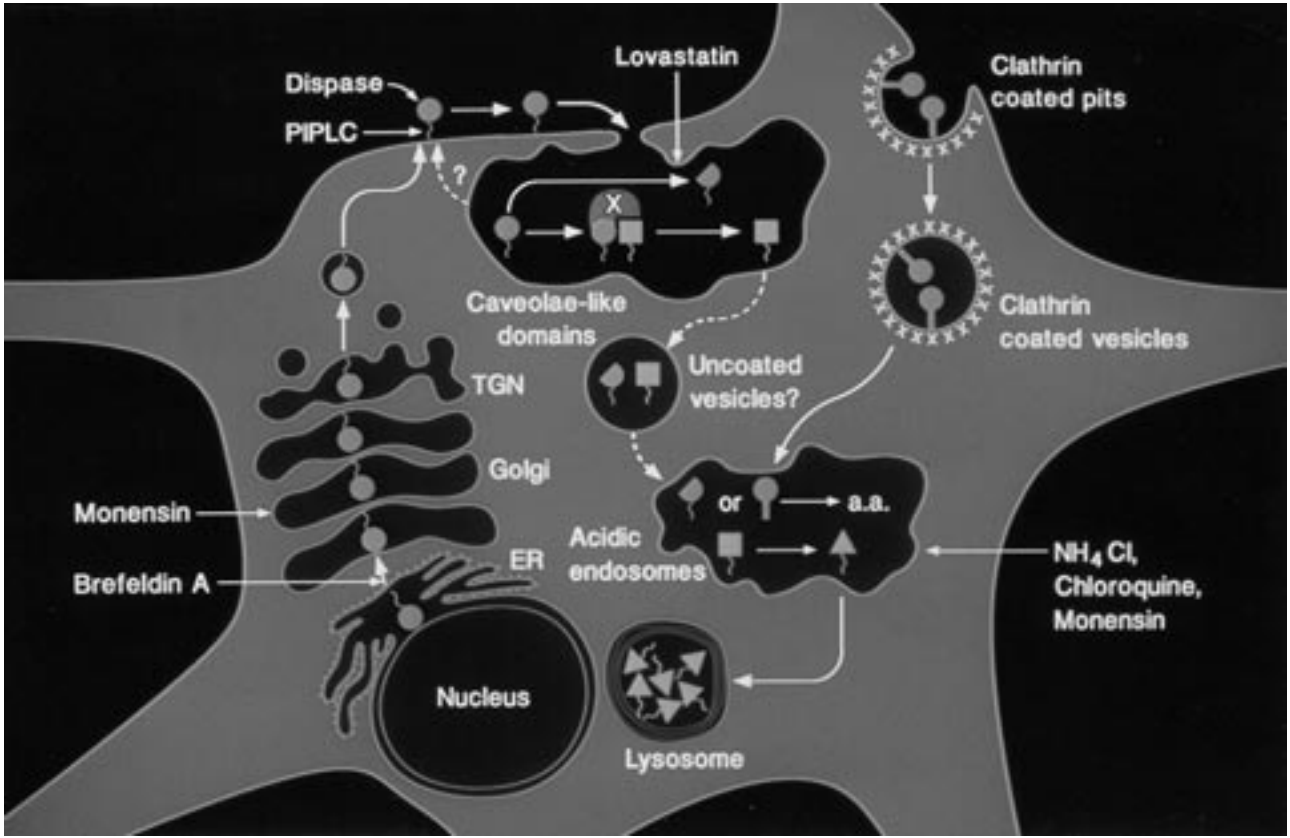

Figure 1 (*See facing page for legend.*)

and subsequently sialylated, and the GPI anchor also undergoes sialylation. The sequences of the asparagine-linked sugar chains were determined on fractions of PrP 27-30 purified from SHa brain (Endo et al. 1989; Haraguchi et al. 1989). The diversity of complex type oligosaccharides was impressive: more than 400 different glycoforms could be created from the diverse array of sugar chains. Bi-, tri-, and tetra-antennary structures were found, some of which exhibited branched fucosylation as well as varying degrees of sialylation.

From the Golgi, mature PrP^C molecules continue their journey to the cell surface in secretory vesicles. As PrP^C emerges on the cell surface, it is attached to the external surface of the plasma membrane by the GPI anchor. PrP^C can be released from the cell surface by hydrolyzing an ester bond within the GPI anchor that links the diacylglycerol to the phosphoinositol (Stahl et al. 1987, 1990). The GPI anchor targets PrP^C to caveolae-like domains (CLDs) where PrP^C is either initially degraded by proteolytic cleavage to form PrP^C-II or converted into PrP^{Sc} (Taraboulos et al. 1995). PrP^C-II is derived from cleavage of full-length PrP^C apparently at residue 113 by a specific but unknown protease (Haraguchi et al. 1989; Pan et al. 1992; Chen et al. 1995). When PrP^C molecules carrying a transmembrane (TM) carboxyl terminus were expressed, they trafficked to clathrin-coated pits and failed to be converted into PrP^{Sc} (Kaneko et al. 1997a).

The asparagine-linked oligosaccharides of PrP^C are thought to modify the structure of the protein, in turn altering the rate at which PrP^C is converted into PrP^{Sc} (DeArmond et al. 1997). Differences in the rates of

Figure 1 Pathways of prion protein synthesis and degradation in cultured cells. PrP^{Sc} is denoted by green squares; olive circles designate PcP^C. The squiggly tail attached to PrP^C and PrP^{Sc} denotes the GPI anchor. Prior to becoming protease resistant, the PrP^{Sc} precursor transits through the plasma membrane and is sensitive to dispase or PIPLC added to the medium. PrP^{Sc} formation seems to occur in rafts or caveolae-like domains (CLDs); rafts and CLDs are regions of the plasma membrane that are enriched for cholesterol and glycosphingolipids, and to which GPI-anchored proteins are targeted. The synthesis of nascent PrP^{Sc} seems to require the interaction of PrP^C with protein X denoted by the purple polygon; the PrP^C/protein X complex binds existing PrP^{Sc}. In cultured cells, but not brain, the amino terminus of PrP^{Sc} is trimmed to form PrP 27-30 denoted by the green triangles; PrP^{Sc} then accumulates primarily in secondary lysosomes. Redirecting PrP^C to clathrin-coated pits was accomplished by substitution of a carboxy-terminal transmembrane sequence for the GPI anchor addition sequence. This mutant PrP^C is denoted by an olive rectangular stem attached to a circle.

conversion of PrP^{C} into PrP^{Sc} may be responsible for the strain-specific patterns of PrP^{Sc} accumulation (DeArmond et al. 1987, 1993; Bruce et al. 1989; Hecker et al. 1992).

The pathway by which PrP^{C}-II and the amino-terminal PrP fragment generated during PrP^{C}-II formation are internalized appears to involve the fusion of CLDs to endosomes (P.J. Peters et al., in prep.). Whether some PrP^{C}-II fragments enter the cell through clathrin-coated pits is unknown, but there are reports showing that a portion of the PrP^{C} molecules do enter cells through clathrin-coated pits and that a fraction of these molecules recycle to the cell surface (Harris et al. 1993a). Notable are studies on the overexpression of chicken PrP (ChkPrP) in N2a cells (Shyng et al. 1993, 1994, 1995). Immunogold labeling of N2a cells showed that the concentration of ChkPrP within coated pits was 3–5 times higher than over other areas of plasma membrane, and gold particles were also seen within coated vesicles and deeply invaginated coated pits. These studies also provided evidence for the recycling of ChkPrP from endosomes to the cell surface. Furthermore, internalization of ChkPrP was reduced about 70% after N2a cells were incubated in hypertonic medium, a treatment that inhibits endocytosis by disrupting clathrin lattices. However, the effect of this treatment on non-clathrin endocytotic pathways is unknown. Localization of recombinant ChkPrP to clathrin-coated pits may have resulted from the high-level expression of the ChkPrP in mouse N2a cells as well as the divergent sequence of this protein. ChkPrP is only about 30% homologous with mammalian PrP (Harris et al. 1989); most mammalian species have PrPs that are >90% homologous with that of other species (Gabriel et al. 1992; Schätzl et al. 1995; Bamborough et al. 1996). Biochemical analyses demonstrated that ChkPrP is highly concentrated in CLDs and not in clathrin-coated pits (Gorodinsky and Harris 1995).

Overexpression of $SHaPrP^{C}$

Syrian hamster (SHa) PrP^{C} and a truncated SHaPrP lacking the carboxy-terminal signal sequence for GPI anchor addition were expressed in Chinese hamster ovary (CHO) cells using a glutamine synthetase selection and amplification system to establish a mammalian cell system capable of generating PrP^{C} at high levels of expression (Bebbington and Hentschel 1987; Bebbington et al. 1992). The CHO cell clones expressing the PrP lacking the GPI addition sequence secreted the majority of the PrP into the media, whereas most of the PrP produced by clones expressing the full-length protein with the GPI anchor was located on the cell surface, as

demonstrated by its release upon treatment with phosphatidylinositol-specific phospholipase C (PIPLC) (Blochberger et al. 1997).

To obtain PrP that was structurally similar to native PrP^C but expressed at higher levels than in brain, clones expressing full-length PrP were subcloned. The full-length recombinant PrP was hyperglycosylated and migrated as a diffuse band between 19 kD and 40 kD (Blochberger et al. 1997). *N*-Glycosidase F (PNGase F) treatment of the CHO cell lysate resulted in three bands: 19 kD, 17 kD, and 15 kD. The 19-kD species corresponded to the correct size of unglycosylated, full-length PrP and had also been reported for PNGase F-treated PrP in ScN2a cells (Taraboulos et al. 1990a). The 17-kD and 15-kD species may represent degradation products caused by proteases possibly present in the PNGase F or possibly deglycosylated PrP(II) species that are generated in vivo during PrP^C degradation (Tarentino et al. 1985; Taraboulos et al. 1990b, 1992). Lower molecular weight products were also seen with the PNGase F-treated ScN2a cells. Quantification of the 19-kD band corresponding to deglycosylated full-length PrP revealed that expression was at a level about 14-fold higher than that of PrP^C found in Syrian hamster brain.

Characterization of the GPI anchor of recombinant PrP revealed some interesting features. PIPLC treatment of the PrP-expressing CHO cells resulted in the release of the majority of PrP. These results are similar to those of Lin et al. (1990), who reported that PIPLC treatment of CHO cells expressing recombinant T-cell-antigen receptor released about 90% of the receptor from the cell surface. The residual cell-associated PrP may be located inside the cell and thus inaccessible to the PIPLC. An alternative explanation may be that a subset of PrP molecules exist that are resistant to PIPLC release due to acylation of inositol hydroxyl groups (Mayor et al. 1990).

Since a conformational change in the structure of PrP seems to be the fundamental event underlying the prion diseases (Pan et al. 1993), structural studies of PrP are of utmost importance in deciphering the process whereby PrP^C is transformed into PrP^{Sc}. Although the level of PrP^C in rodent brain is high compared to that in other tissues, it still represents less than 0.1% of the total CNS protein (Turk et al. 1988; Pan et al. 1992, 1993). To overcome this difficulty, many investigators have tried to express recombinant PrP^C at high levels in cultured cells (Table 1). The need for such systems has been emphasized by recent reports on the nuclear magnetic resonance (NMR) structure of fragments of PrP synthesized and labeled in bacteria (Riek et al. 1996; James et al. 1997). In one case, a PrP fragment of 111 amino acids, which is slightly shorter than the PrP^C degradation product PrP^C-II (Pan et al. 1992; Chen et al. 1995), was

Table 1 Summary of systems employed for recombinant PrP expression

Cell type	Protein expressed	Reference
Escherichia coli	SHaPrP*	Weiss et al. (1995); Mehlhorn et al. (1996)
	MoPrP†	Hornemann and Glockshuber (1996)
Spodoptera frugiperda	SHaPrP*	Scott et al. (1988); Weiss et al. (1995)
Drosophila melanogaster	$SHaPrP^{c}$	Raeber et al. (1995)
Xenopus oocytes	$SHaPrP^{c}$	Hay et al. (1987)
Mouse C127	$MoPrP^{c}$	Caughey et al. (1988)
Mouse N2a	$SHaPrP^{c}$	Scott et al. (1988); Chesebro et al. (1993)
	$ChkPrP^{c}$	Harris et al. (1993a); Gorodinsky and Harris (1995)
	$Mo/ChkPrP^{c}$	Shyng et al. (1995)
	$Mo/SHaPrP^{c}$	Chesebro et al. (1993)
Mouse ScN2a	$Mo/SHaPrP^{c}$	Scott et al. (1992); Rogers et al. (1993)
Mouse NIH-3T3	$SHaPrP^{c}$	Scott et al. (1988); Kocisko et al. (1994)
Mouse Sc^{+}-MNB	$SHaPrP^{c}$	Priola et al. (1994)
	$SHa/MoPrP^{c}$	
	$MoPrP^{c}$	
Mouse MNB	$MoPrP^{c}$	Kocisko et al. (1995)
	$Mo/SHaPrP^{c}$	
Hamster HJC	$SHaPrP^{c}$	Scott et al. (1988)
Hamster CHO	$SHaPrP^{c}$	Borchelt et al. (1993)
	$MoPrP^{c}$	Lehmann and Harris (1996a)
	$Mo/SHaPrP^{c}$	
Rat RAT-1	$SHaPrP^{c}$	Scott et al. (1988)
Monkey COS-7	$SHaPrP^{c}$	Scott et al. (1988)
Monkey CV-1	$SHaPrP^{c}$	Rogers et al. (1991)
	$SHa/MoPrP^{c}$	
	$MoPrP^{c}$	

*Fusion protein formed from PrP and glutathione-*S*-transferase.
†Carboxy-terminal fragment corresponding to mouse residues 111–231.

prepared from *Escherichia coli* for structural studies (Hornemann and Glockshuber 1996). In another case, a PrP fragment of 142 amino acids that corresponds in sequence to PrP 27-30, the protease-resistant core of PrP^{Sc} (Prusiner et al. 1984), was prepared from *E. coli* for structural studies (Mehlhorn et al. 1996). Subsequently, full-length PrP molecules corresponding to the PrP sequences in SHa and mouse were expressed (Donne et al. 1997; Riek et al. 1997). Such studies with *E. coli*-derived proteins suffer from the lack of posttranslational modifications, which may substantially alter the structure of PrP.

IMMUNOLOCALIZATION OF SHaPrP^C TO CAVEOLAE

As it has become increasingly important to establish the site(s) of conversion of PrP^C into PrP^{Sc}, a better understanding of the subcellular trafficking routes of PrP^C has become critical. To date, the qualitative and quantitative localization of PrP^C at the ultrastructural level has not been documented. To establish subcellular localization of PrP^C, the sensitive technique of cryo-immunogold electron microscopy was employed.

Cryo-immunogold Electron Microscopy

Cells on plastic tissue-culture dishes were fixed at 37ºC with either paraformaldehyde (PFA), 2% for 24 hours; glutaraldehyde (GA), 1% for 1 hour; or PFA/GA 2%/0.2% for 2 hours (Peters 1999). Post-fixation, the cells were scraped and collected in 0.2% PFA. The cell pellet was then embedded in gelatin, infiltrated in sucrose, frozen in liquid nitrogen, and sectioned with a Leica ultra-cryomicrotome and Diatome diamond knife. Ultrathin cryosections of 45–60 nm were cut at –120ºC and collected with a mixture of sucrose and methyl cellulose. Cryosections were labeled with primary antibodies followed by protein A conjugated to gold particles. Sections were contrasted and embedded in a mixture of methyl cellulose and uranyl acetate. Sections were viewed with a JEOL 1010 transmission electron microscope at 80 kV.

Fourteen different α-PrP polyclonal and monoclonal antibodies as well as recombinant (r) Fabs (3F4, W5512, N10, N12, N20, W5511, W5512, W5514, W5517, 13A5, R1, R2, D2, and R10) were tested on three cell lines that were fixed in either PFA, GA, or PFA/GA. The cell lines that were used consisted of CHO fibroblasts stably expressing SHaPrP (30-C3) or the neuronal cell lines N2a and GT-1 (Butler et al. 1988; Blochberger et al. 1997; Schätzl et al. 1997). No specific labeling with any of the conventional monoclonal and polyclonal antibodies was found in any cell type. However, very specific labeling for PrP was seen with three of the rFabs (R1, R2, and D2) (Williamson et al. 1996; Peretz et al. 1997), in all three of the cell lines. To our knowledge, this is the first report of such rFabs being successfully used for cryo-immunogold electron microscopy. Remarkably, the fixation step required the use of GA.

Figure 2A shows the distribution of PrP in the 30-C3 cells. PrP was found to be present in small omega-shaped indentations of the plasma membrane that morphologically resemble caveolae. Multiple omega-shaped indentations were also seen on deep membrane invaginations and were morphologically reminiscent of alveolar micro-sacs. In addition,

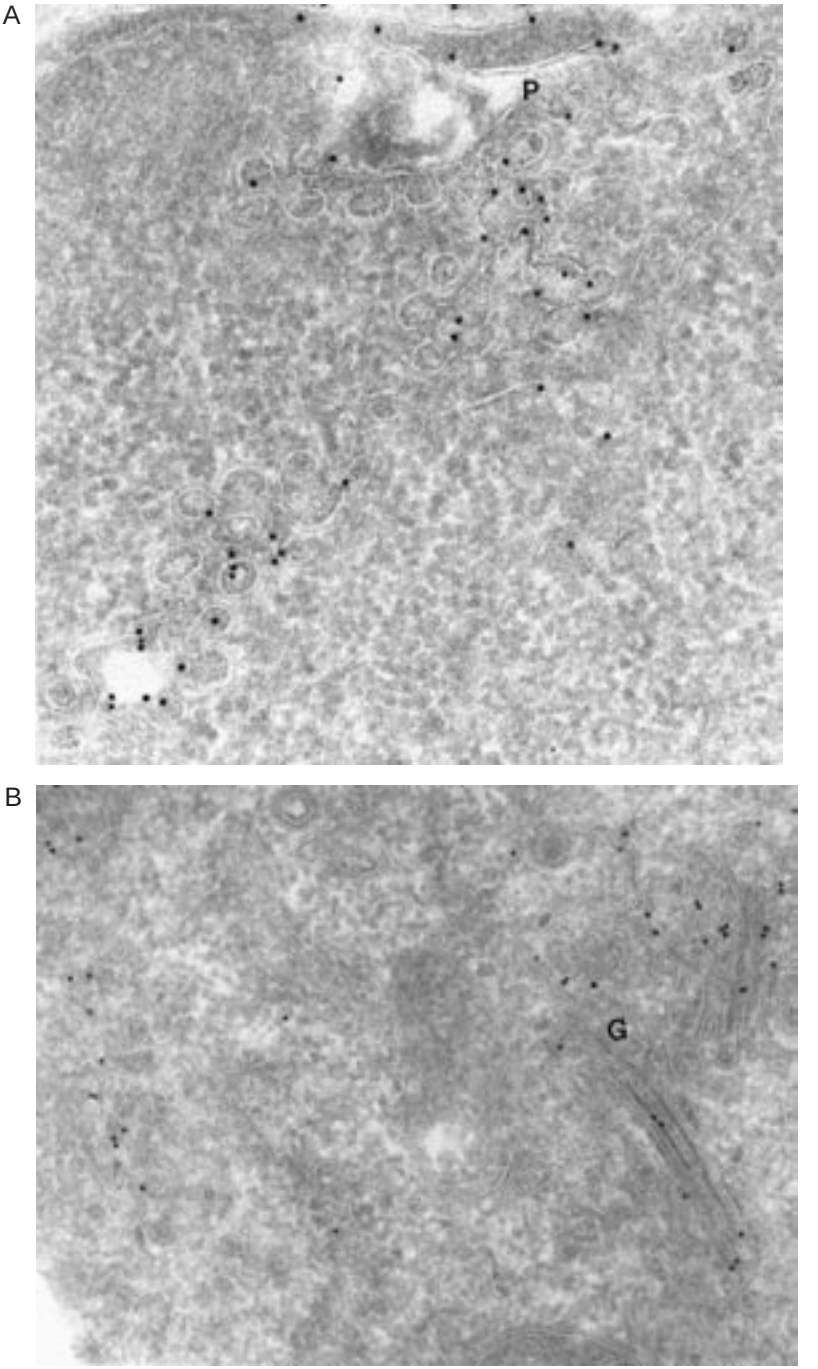

Figure 2 (*Continued on facing page.)*

labeling for PrP was seen on microvilli-like domains of the plasma membrane, although these were essentially devoid of caveolae.

Similar caveolae structures that labeled for PrP were also seen in the *trans*-Golgi network (TGN) and on early endosomes. In addition, PrP was also seen in the ER and normal Golgi cisternae (Fig. 2B), although these were without caveolae. Remarkably, in the neuronal cell lines, N2a and GT-1, which do not exhibit caveolae structures, a diffuse low intensity of labeling for PrP was detected along the plasma membrane.

To confirm that the membrane indentations observed in CHO cells were indeed caveolae, cells were labeled with antibodies against caveolin-

C

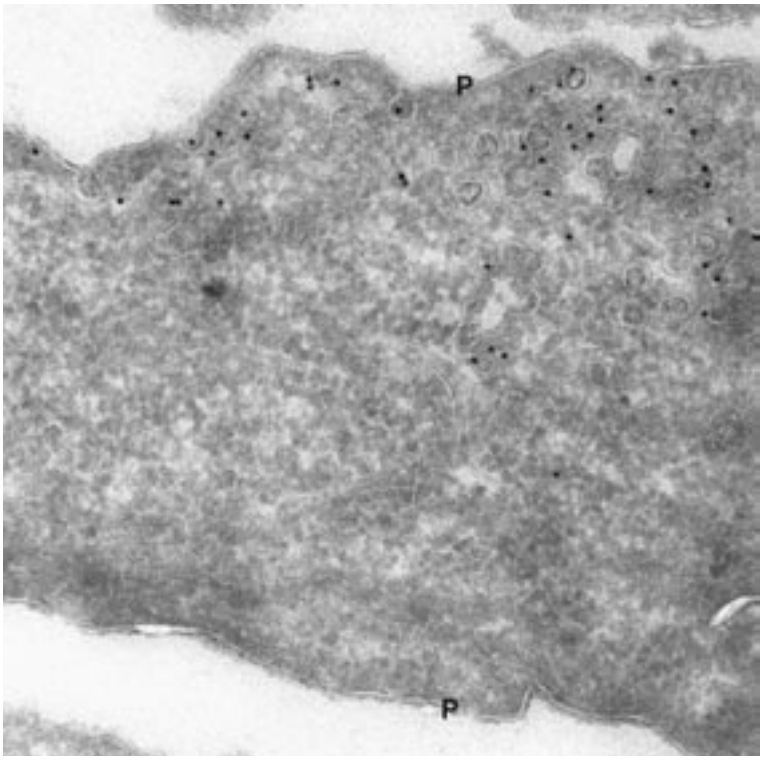

Figure 2 (*continued*) Expression of PrP^C in CHO cells. PrP was immunolabeled on ultrathin cryosections using a recombinant antibody (Fab) against PrP (R1) or a polyclonal antibody against caveolin 1. (*A*) Labeling for PrP^C was observed on microvilli and the plasma membrane (P) and was enriched in caveolae-like structures. (*B*) Labeling for PrP^C was also abundant in the Golgi (G) and caveolae-like structures in the pericentriolar region. (*C*) Caveolae-like structures in PrP-expressing CHO cells labeled positively for caveolin 1.

1, a resident caveolae protein. Most omega-shaped invaginations on the plasma membrane, early endosomes, and at the TGN were labeled for caveolin-1 (Fig. 2C). Unfortunately, the sensitivity of the α-caveolin-1 antibodies and α-PrP rFabs to glutaraldehyde and paraformadehyde fixation, respectively, precluded double-labeling procedures with both these probes. Nevertheless, we believe that our data unambiguously show that PrP resides in caveolae present at the plasma membrane, TGN, and early endosomes. These studies are in agreement with the results of subcellular fractionation studies (Gorodinsky and Harris 1995; Taraboulos et al. 1995; Vey et al. 1996; Naslavsky et al. 1997) and provide new approaches to investigate the role of caveolae in the intracellular trafficking of PrP.

FORMATION OF PrP^{Sc} IS A POSTTRANSLATIONAL PROCESS

Metabolic labeling studies of scrapie-infected cultured cells have shown that PrP^{C} is synthesized and degraded rapidly, whereas PrP^{Sc} is synthesized slowly by a posttranslational process (Fig. 3) (Borchelt et al. 1990, 1992; Caughey and 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 make it likely that PrP^{Sc} is formed during a posttranslational event (Basler et al. 1986).

Both PrP isoforms appear to transit through the Golgi apparatus where their asparagine-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 GPI moiety (Stahl et al. 1987, 1992; Safar et al. 1990). In contrast, PrP^{Sc} accumulates primarily within cells where it is deposited in cytoplasmic vesicles, many of which appear to be lysosomes (Taraboulos et al. 1990a, 1992; Caughey et al. 1991; McKinley et al. 1991b; 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 argue that PrP molecules destined to become PrP^{Sc} exit to the cell surface as does PrP^{C} (Stahl et al. 1987) prior to their conversion into PrP^{Sc} (Caughey and Raymond 1991; Borchelt et al. 1992; Taraboulos et al. 1992). Interestingly, the GPI anchors of both PrP^{C} and PrP^{Sc}, which presumably feature in directing the subcellular trafficking of

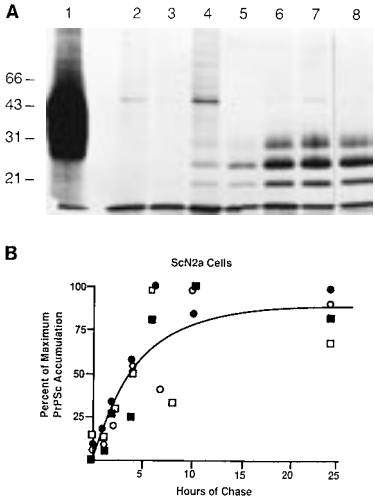

Figure 3 Kinetics of PrP^{Sc} formation in cultured ScN2a cells. The cells were metabolically radiolabeled for 1 hr. Excess [^{35}S]methionine was removed by washing the cells in PBS before a chase in unlabeled medium. Radiolabeled PrP molecules were identified by immunopurification with antiserum R073. To identify radiolabeled PrP^{Sc} molecules, cell extracts were digested with proteinase K and denatured in 3 M GdnSCN before dispersion in DLPC and immunopurification with PrP antiserum R073. This antiserum recognizes the same radiolabeled molecules as other PrP antisera, and preimmune serum R073 does not recognize any protease-resistant molecules. (*A*) Lane *1* is a control in which cells were labeled, then PrP was extracted without protease digestion. Lanes *2–8* contain protease-resistant PrP^{Sc} molecules recovered from one confluent 25-cm^2 flask of cells after a 1-hr labeling and increasing chase times. (Lane *2*) No chase; (lane *3*) 1 hr chase; (lane *4*) 2 hr chase; (lane *5*) 4 hr chase; (lane *6*) 6 hr chase, (lane *7*) 10 hr chase; (lane *8*) 24 hr chase. (*B*) The amount of radioactive PrP in each lane was quantified with a Molecular Dynamics PhosphoImager (*filled squares*). The optical density of autoradiographic bands was also measured in three separate experiments (*filled* and *open circles*, *open squares*). The values for the three PrP^{Sc}-specific bands (27-30, 25, and 19 kD) were summed, then plotted as a percentage of the maximum attainable value for a given experiment. The values for the four experiments were combined and fit to a single curve with a previously described computer program that calculates a best fit to a first-order reaction. The calculated $t_{1/2}$ for the acquisition of protease resistance by PrP^{Sc} was 3 hr. (Reprinted, with permission, from Borchelt et al. 1992.)

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

Brefeldin A Inhibits PrP^{Sc} Synthesis

Studies with brefeldin A indicate that PrP^{Sc} synthesis does not occur in the ER-Golgi and that transport down the secretory pathway is required for this synthesis (Taraboulos et al. 1992). Experiments with monensin demonstrate that PrP^{Sc} precursors traverse the *mid*-Golgi in the same time frame as PrP^{C}. These PrP molecules continue along the secretory pathway to the cell surface where they are bound by a GPI anchor (Caughey and Raymond 1991; Borchelt et al. 1992). A minority of these PrP molecules are then converted to PrP^{Sc}, presumably either in the endocytic pathway or on the plasma membrane. Brefeldin A is the first compound found to inhibit the synthesis of PrP^{Sc}.

Digestions with PIPLC and Dispase Inhibit PrP^{Sc} Synthesis

Those PrP molecules that are destined to become PrP^{Sc} appear transiently on the cell surface and can be released with PIPLC (Caughey and Raymond 1991; Borchelt et al. 1992) or hydrolyzed by dispase (Borchelt et al. 1992). The synthesis of PrP^{Sc} was dramatically reduced when nascent PrP^{C} was digested with PIPLC at 18ºC, but digestion of nascent PrP^{Sc} with PIPLC at 37ºC did not reproducibly diminish the synthesis of PrP^{Sc} by more than a factor of 2. Digestion of nascent PrP^{C} with dispase significantly reduced the synthesis of PrP^{Sc}, and this phenomenon could be partially prevented by delaying the exposure of cells to dispase. Although trypsin digestion (2 mg/ml for 20 minutes at 37ºC) of nascent PrP^{C} did on occasion cause a large decrease in PrP^{Sc} synthesis as reported by other workers (Caughey and Raymond 1991), these conditions were so harsh that we could not determine whether the reduction in PrP^{Sc} synthesis was due to digestion of cell-surface protein or to nonspecific effects of the treatment, such as the dislodging and reattachment of the cells (Borchelt et al. 1992). Dispase proved to be a more gentle protease that did not dislodge cells from culture vessels.

Is PrP^{Sc} Derived from a Specific Precursor Pool?

Whether PrP^{Sc} is synthesized from a subset of PrP molecules or all PrP^{C} molecules are eligible for conversion remains to be established. Less than 10% of the radiolabeled nascent PrP molecules that have been synthesized by the end of a 1-hour metabolic radiolabeling pulse are converted into PrP^{Sc} (Borchelt et al. 1992). At present, we and other investigators

have been unable to identify any differences between those PrP molecules that are destined to become PrP^{Sc} and the pool of PrP^{C} molecules (Borchelt et al. 1990, 1992; Caughey and Raymond 1991). Both PrP^{Sc} precursor molecules and PrP^{C} can be released from the surface of cells by PIPLC digestion or hydrolyzed by dispase. Furthermore, both PrP^{C} and those PrP molecules destined to become PrP^{Sc} appear to be susceptible to cellular degradation.

Synthesis of PrP^{Sc} from Unglycosylated PrP

Although most of the difference in the mass of PrP 27-30 predicted from the amino acid sequence and that observed after posttranslational modification is due to complex-type oligosaccharides, these sugar chains are not required for PrP^{Sc} synthesis in scrapie-infected cultured cells based on experiments with the asparagine-linked glycosylation inhibitor tunicamycin and site-directed mutagenesis studies (Taraboulos et al. 1990b). ScN2a cells produce three species of PrP^{Sc} that differ in their degree of N-linked glycosylation (Taraboulos et al. 1990b). Since the formation of any particular PrP^{Sc} glycoform could not be uncoupled from another, all three species of PrP^{Sc} seem to traverse the same biosynthetic pathway.

Studies with cultured cells showed that unglycosylated PrP produced in the presence of tunicamycin was converted to unglycosylated PrP^{Sc} (Taraboulos et al. 1990b), but unglycosylated PrP^{C} is not readily detected at the cell surface (Caughey et al. 1989). Recombinant PrP molecules lacking consensus sites for N-linked glycosylation were not detected on the cell surface (Rogers et al. 1990) but could be converted to unglycosylated PrP^{Sc} in ScN2a cells (Taraboulos et al. 1990b). Although these results suggest that unglycosylated PrP need not transit to the cell surface before conversion into PrP^{Sc}, we could not eliminate the possibility that a small fraction of PrP was transported to the cell surface in these experiments and was converted to PrP^{Sc} upon re-entry into the cell. Alternatively, in the presence of tunicamycin, PrP may be transported directly from the ER or Golgi to the endocytic pathway, where it may be converted to PrP^{Sc} prior to entering the lysosomes (Taraboulos et al. 1990b; Stoorvogel et al. 1991).

SUBCELLULAR SITE OF PrP^{Sc} FORMATION

Since infectious prions seem to be composed entirely of PrP^{Sc} molecules (Prusiner 1991), it was important to identify the site of PrP^{Sc} synthesis and to define the molecular events involved in this process. Our earlier

observations argued that PrP^{Sc} synthesis occurred within the endocytic pathway (Borchelt et al. 1992), but more recent studies have refined those data and contend that PrP^{Sc} formation takes place in CLDs.

How exogenous prions initiate infection is unknown, but the presence of PrP^{C} on the cell surface suggests a mechanism whereby PrP^{Sc} binds either to PrP^{C} or to PrP*, which is already bound to protein X. PrP* is thought to be a conformationally distinct metastable variant of PrP^{C} that is competent for conversion into PrP^{Sc}. Whether PrP^{Sc} can bind to PrP^{C} on the cell surface or PrP^{C} must first be transformed into PrP* remains uncertain. Once PrP^{Sc} is formed in CLDs, it enters the endocytic pathway and eventually is deposited in lysosomes.

Evidence for PrP^{Sc} Formation in Caveolae-like Domains

Studies on the localization of PrP^{C} in mouse neuroblastoma (N2a) cells indicate that it is bound to the external surface of the plasma membrane by a GPI anchor (Stahl et al. 1987) and it is clustered in caveolae or CLDs (Ying et al. 1992); in accord with these results, PrP^{C} was found to be insoluble in cold Triton X-100 (Gorodinsky and Harris 1995; Taraboulos et al. 1995). Caveolae appear as raft-like membranous domains or as coated invaginations of plasma membranes; in the presence of Triton X-100, they form detergent-insoluble complexes that are enriched for GPI-anchored proteins, cholesterol, and glycosphingolipids (Brown and Rose 1992). The presence of these domains does not seem to be dependent on the marker protein caveolin, because cells lacking caveolin, such as N2a cells, also contain detergent-insoluble CLDs in which GPI-anchored proteins are clustered (Gorodinsky and Harris 1995; Taraboulos et al. 1995). Caveolae are involved in diverse cellular functions such as transcellular movement (Simionescu 1983), folate uptake (Anderson et al. 1992), signal transduction (Anderson 1993; Sargiacomo et al. 1993; Smart et al. 1995), and down-regulation of blood-clotting activity (Sevinsky et al. 1996).

The localization of PrP^{C} to CLDs and the finding that PrP^{Sc} formation was inhibited by lovastatin, which diminishes cellular cholesterol levels, suggested that glycosphingolipid- and cholesterol-rich CLDs might be the site where prions are propagated (Taraboulos et al. 1995). Replacing the GPI anchor addition signal sequence of PrP with the CD4 transmembrane carboxy-terminal 62 residues targeted the chimeric PrP molecule to clathrin-coated pits and prevented PrP^{Sc} formation. Furthermore, carboxy-terminal truncation of PrP, which deleted the sig-

nal sequence for GPI anchor addition, substantially reduced PrP^{Sc} formation (Rogers et al. 1993). Studies described below have extended those observations by showing that squalestatin, a more specific inhibitor of cholesterol biosynthesis, and three other transmembrane carboxy-terminal segments inhibit PrP^{Sc} formation (Kaneko et al. 1997a). Earlier investigations had shown that PrP^{Sc} interacts with PrP^{C} during the formation of nascent PrP^{Sc} (Prusiner et al. 1990; Telling et al. 1995) and that this process occurs after PrP^{C} transits to the plasma membrane (Caughey and Raymond 1991; Borchelt et al. 1992).

CLDs seem to be the site where PrP^{C} is converted into PrP^{Sc}, but lysosomes appear to be at least one of the subcellular compartments in which PrP^{Sc} accumulates (McKinley et al. 1991b; Arnold et al. 1995). After lysis of ScN2a cells in cold Triton X-100, both PrP isoforms and an amino-terminally truncated PrP^{C}-II were found concentrated in detergent-insoluble complexes resembling CLDs that were isolated by flotation in sucrose gradients (Fig. 4) (Vey et al. 1996). Similar results were obtained when CLDs were purified from plasma membranes by sonication and gradient centrifugation; with this procedure no detergents are used, which minimizes artifacts that might arise from redistribution of proteins among subcellular fractions (Fig. 5). The caveolar markers ganglioside GM1 and H-ras were found concentrated in the CLD fractions.

Although substantial amounts of PrP^{Sc} accumulate in lysosomes (Taraboulos et al. 1990a; McKinley et al. 1991b; Arnold et al. 1995), PrP^{Sc} can be labeled by addition of the membrane-impermeable reagent sulfo-NHS-biotin added to the outside of ScN2a cells, which indicates that some of the PrP^{Sc} is bound to the external surface of plasma membrane (Fig. 6). The biotinylated PrP^{Sc} was subsequently recovered in CLDs isolated by the detergent-free method; such results contend that PrP^{Sc} on the plasma membrane is concentrated in CLDs. The foregoing findings, taken together with results from other studies that showed PrP^{Sc} formation was abolished by replacement of the GPI anchor of PrP^{C} with a transmembrane segment that targets PrP to clathrin-coated pits (Taraboulos et al. 1995; Kaneko et al. 1997a), build a substantial case for the formation of PrP^{Sc} within CLDs.

PrPs Targeted to Clathrin-coated Pits

To test the hypothesis that efficient formation of PrP^{Sc} requires targeting PrP^{C} by GPI anchors to CLDs, a series of chimeric PrP molecules that redirect PrP^{C} to clathrin-coated pits were created. Each of four chimeric

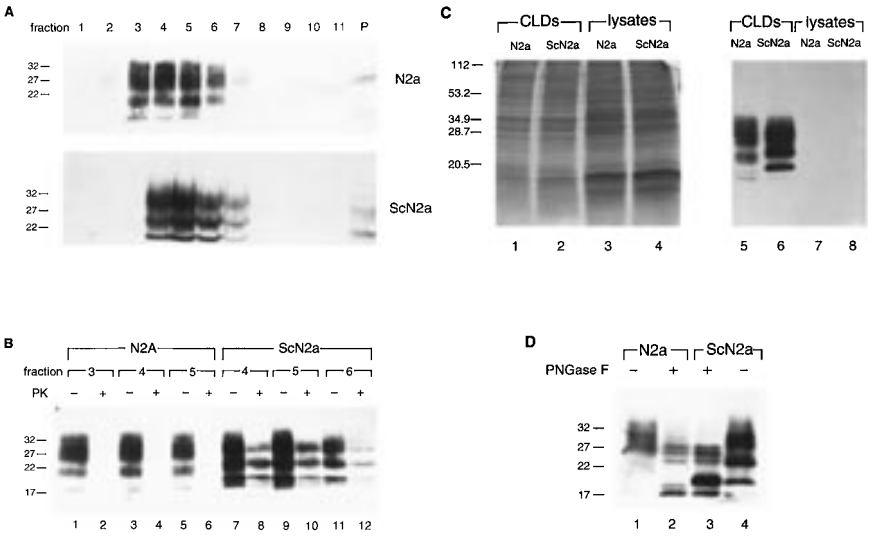

Figure 4 PrP^{c} and PrP^{Sc} are concentrated in CLDs isolated from neuroblastoma cells using the cold Triton X-100 detergent method. (*A*) Distribution of PrP after lysis of N2a and ScN2a cells in cold Triton X-100 and separation of CLDs by flotation into sucrose gradients. Aliquots of gradient fractions were immunoblotted with the α-PrP polyclonal R073 rabbit antiserum (Serban et al. 1990). (Lanes *1–7*) Fractions of the sucrose gradients; (lanes *8–11*) lysate fractions; (lanes *P*) pellets. (*B*) Detection of PrP^{Sc} in gradient fractions. Immunoblot of gradient fractions from *A* before (–) and after (+) treatment with proteinase K (PK). (*C*) Concentration of PrP and other proteins in CLDs. Immunoblot (*right* panel) and silver-stained gel (*left* panel) of CLD proteins and cell lysate proteins from N2a and ScN2a cells. (*D*) Detection of PrP^{c} degradation products in CLDs from N2a and ScN2a cells. Immunoblot of CLD proteins before (–) and after (+) treatment with PNGase F (Tarentino et al. 1985). (Reprinted, with permission, from Vey et al. 1996 [copyright National Academy of Sciences].)

PrPs with different carboxy-terminal transmembrane (TM) domains prevented the formation of PrP^{Sc} (Fig. 7). To determine if these carboxy-terminal TM segments prevented PrP^{C} from refolding into PrP^{Sc} by altering the structure of the polypeptide, we fused the 28-amino-acid carboxyl termini from the Qa protein. Two carboxy-terminal Qa segments differing by a single residue direct the transmembrane protein to clathrin-coated pits or the GPI form to CLDs; PrP^{Sc} was formed from GPI-anchored PrP^{C} but not from transmembrane PrP^{C}.

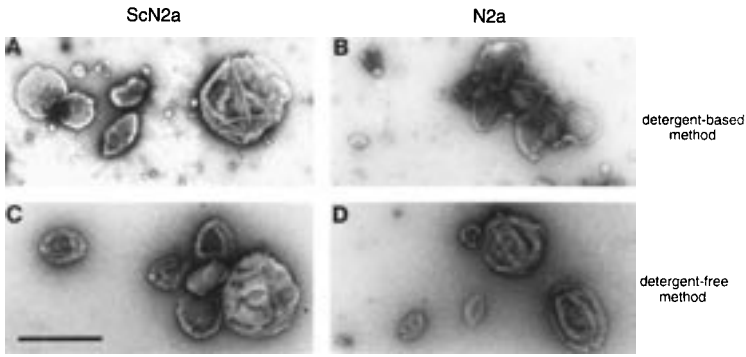

Figure 5 Ultrastructure of isolated CLDs. (*A,B) C*LDs were isolated by the cold Triton X-100 detergent method. *(C,D)* CLDs isolated by the detergent-free procedure. Samples were prepared from ScN2a *(A,C)* and N2a cells (*B,D).* Bar, 0.5 μm. (Reprinted, with permission, from Vey et al. 1996 [copyright National Academy of Sciences].)

The carboxy-terminal signal sequence that is cleaved when a GPI anchor is added has been shown to contain at least three elements. At the first and third positions of this sequence, the amino acid residues must have small side chains. Between these residues and a hydrophobic domain

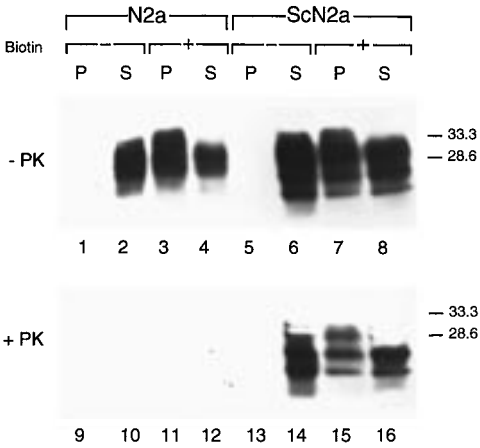

Figure 6 PrP^{c} and PrP^{Sc} are concentrated in CLDs on the surface of ScN2a cells. Detection of streptavidin-agarose-precipitated PrP from CLDs derived from biotinylated plasma membranes of N2a and ScN2a cells isolated with the detergent-free method. Immunoblots of CLD proteins precipitated with streptavidin-agarose (lanes *P*) and unbound proteins of the respective supernatants (lanes *S*) using α-PrP R073 antiserum without (–PK) or with (+PK) limited digestion by proteinase K. (Reprinted, with permission, from Vey et al. 1996 [copyright National Academy of Sciences].)

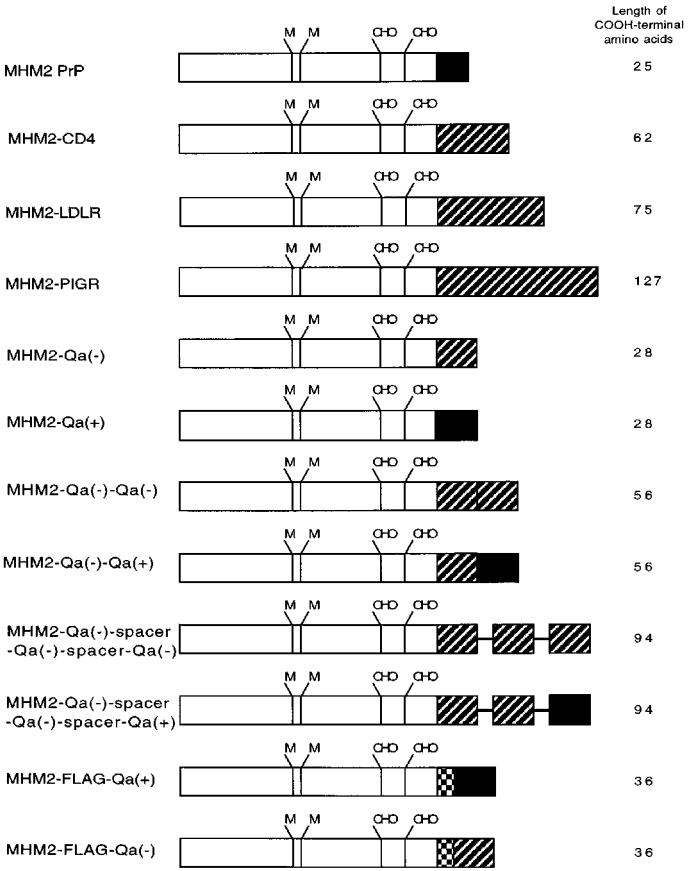

Figure 7 Map of recombinant DNA constructs expressed in ScN2a cells using the pSPOX vector. (*Filled bars*) GPI attachment sequences from wild-type MoPrP or Mo Qa(+). (*Hatched bars*) Transmembrane and cytoplasmic sequences from either Mo CD4, rabbit LDLR, human PIGR, or Mo Qa(–). (*Checkered bar*) FLAG sequence of Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys. Bold horizontal lines represent spacer sequence of Ser-Ala-Gly-Ser-Ala. (CHO) N-glycosylation sites. (M) Met at codon 109 and 112 of SHaPrP.

of at least 11 amino acids at the carboxyl terminus is a spacer domain that has no specific requirements (Coyne et al. 1993; Kodukula et al. 1993). Although no consensus sequence has been recognized on the amino-terminal side of the cleavage site where the GPI anchors are added (Ferguson and Williams 1988), our data demonstrate that insertion of the

Qa(–) transmembrane segment did prevent GPI-anchor addition. When the Qa(–) transmembrane segment was placed between PrP on the amino-terminal side and the Qa(+) segment on the carboxyl-terminal side so that the two Qa sequences were expressed in tandem, no release of the recombinant PrP^C by PIPLC digestion was found. It is of interest that the mRNAs encoding N-CAM, which can exist as either a transmembrane or GPI-anchor protein, undergo splicing so that both the transmembrane and GPI-anchor-addition sequences do not exist in the same mature protein (He et al. 1987). Even though each of the four constructs containing multiple Qa sequences was translated (Fig. 8), none of the resulting proteins was GPI-anchored, as evidenced by a lack of release from the surface of cells by PIPLC digestion.

Our inability to create a GPI-anchored PrP molecule containing the Qa(–) transmembrane segment demonstrates that cells regulate the biogenesis of proteins in a manner that prevents the formation of chimeras with the structures that we sought. In view of these results, we substituted the hydrophilic sequence FLAG for Qa(–) and produced the chimeric construct MHM2-FLAG-Qa(+). Unlike the constructs containing the Qa(–) segment, the MHM2-FLAG-Qa(+) protein acquired a GPI anchor and was converted into PrP^{Sc}. The results with this construct support our contention that steric hindrance by foreign carboxy-terminal sequences is not responsible for the inhibition of PrP^{Sc} formation.

It is noteworthy that the carboxy-terminal α-helix of SHaPrP(90-231) as determined by solution NMR extends from residue 200 to 227. This contrasts with the structure reported for MoPrP(121-231), in which the carboxy-terminal α-helix was stated to extend from position 200 to 217 and that no assignments could be made for the residues from 218 to 231 (Riek et al. 1996). On the basis of that report, we argued that these carboxy-terminal 14 residues beyond the helical segment provide a flexible tether to which TM segments were attached in the chimeric PrPs described above. As such, we contended that it is unlikely that the TM segments in these chimeric PrPs would interfere with refolding into PrP^{Sc}. We now know that the structural data for MoPrP(121-231) are incorrect and thus cannot be used in such an argument. However, fusion of the hydrophilic FLAG sequence followed by the Qa(+) sequence at the carboxyl terminus of PrP^C did not prevent conversion into PrP^{Sc}, which lends important support for our contention. Although this chimeric PrP was GPI-anchored, a similar chimeric PrP differing by one amino acid with the hydrophilic FLAG sequence at the carboxyl terminus followed by TM Qa(–) sequence was not converted into PrP^{Sc} (Fig. 8).

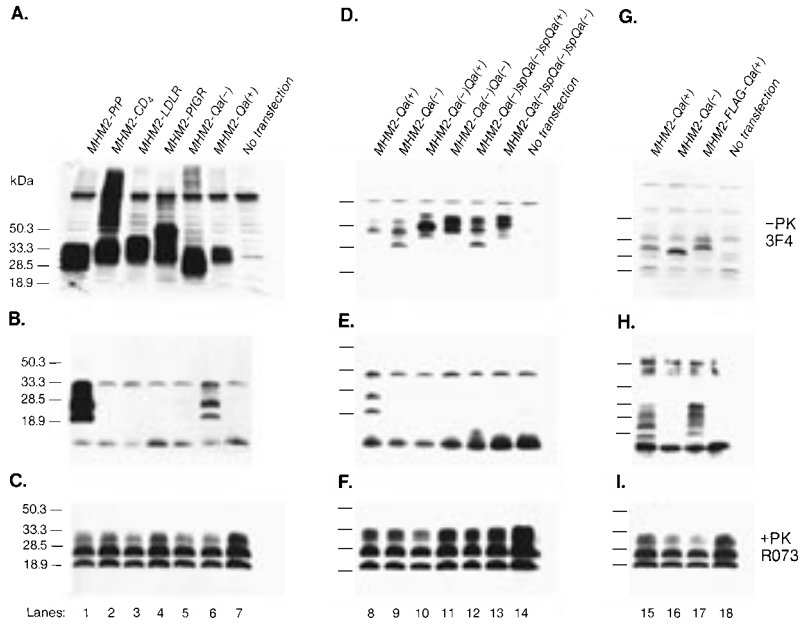

Figure 8 GPI-anchored MHM2 PrP is converted into PrP^{Sc} but the transmembrane form is not. Western blotting of each MHM2-chimeric construct expressed in ScN2a cells is shown. *A*, *B* and *C* (lane *1*) MHM2 PrP; (lane *2*) MHM2-CD4; (lane *3*) MHM2-LDLR; (lane *4*) MHM2-PIGR; (lane *5*) MHM2-Qa(–); (lane *6*) MHM2-Qa(+); and (lane *7*) untransfected control ScN2a cells. *D*, *E*, and *F* demonstrate that the Qa(–) transmembrane signal predominates in the chimeric constructs with Qa(+) GPI-anchor addition signal. (Lane *8*) MHM2-Qa(+); (lane *9*) MHM2-Qa(–); (lane *10*) MHM2-Qa(–)-Qa(+); (lane *11*) MHM2-Qa(–)-Qa(-); (lane *12*) MHM2-Qa(–)-spacer-Qa(–)-spacer-Qa(+); (lane *13*) MHM2-Qa(–)-spacer-Qa(–)-spacer-Qa(–); and (lane *14*) untransfected control ScN2a cells. *G, H*, and *I* (lane *15*) MHM2-Qa(+); (lane *16*) MHM2-Qa(-); (lane *17*) MHM2-FLAG-Qa(+); (lane *18*) untransfected control ScN2a cells. Panels *A, D*, and *G* demonstrate the expression of each chimeric MHM2 PrP construct: 40 µl of undigested cell lysates was applied to each lane and MHM2 PrP was detected by staining with α-PrP 3F4 MAb. Panels *B*, *E*, and *H* demonstrate the conversion of MHM2 PrP^{c} into MHM2 PrP^{Sc} and were stained with α-PrP 3F4 MAb. Panels *C*, *F*, and *I* show endogenous $MoPrP^{Sc}$ detected with RO73 antiserum. In panels *B–C*, *E–F*, and *H–I*, 500 µl of cell lysate was digested with proteinase K (20 µg/ml) at 37ºC for 1 hr followed by centrifugation at 100,000*g* for 1 hr at 4ºC and the resuspended pellet was loaded onto the gels.

Endocytic Pathway

In earlier studies, multiple lines of evidence were gathered that argued that PrP acquires protease resistance after it is transported to the cell sur-

face through the secretory pathway (Borchelt et al. 1990, 1992; Caughey and Raymond 1991). When ScN2a cells were exposed to dispase immediately after the labeling pulse, no PrP^{Sc} was formed during the chase at 37ºC (Borchelt et al. 1992). In contrast, when ScN2a cells were chased for 2 hours at 37ºC prior to dispase exposure, some PrP became inaccessible to the dispase in the media and subsequently acquired resistance to proteinase K. When ScN2a cells were chased at 18ºC but not at 37ºC, PrP^{C} was released by PIPLC digestion, and PrP^{Sc} synthesis was abolished in one set of studies (Borchelt et al. 1992). However, PrP^{C} was reported by others to be released at 37ºC in another set of investigations (Caughey and Raymond 1991). Since PIPLC catalyzed the release of PrP^{C} from the cell surface relatively slowly, prolonged digestions with PIPLC at 18ºC were required to remove those molecules destined to become PrP^{Sc} (Borchelt et al. 1992). At 37ºC, radiolabeled PrP molecules probably transit to the surface and are endocytosed too rapidly for PIPLC digestion to be completely effective. The formation of PrP^{Sc} in ScN2a cells was inhibited when the chase was performed at 18ºC, and PrP remained largely accessible to PIPLC throughout a long 18ºC chase. As predicted from the work of other investigators, endocytosis and vesicular compartmentalization of membrane glycoproteins were retarded at 18ºC in ScN2a cells. If ScN2a cells were held at 37ºC for 1–2 hours of chase prior to shifting to 18ºC, then some PrP^{Sc} was formed at 18ºC (Borchelt et al. 1992). Presumably, some PrP was endocytosed at 37ºC and acquired protease resistance via a process that is less temperature-dependent.

Inhibition of PrP^{Sc} Synthesis at 18ºC

Although many investigators have observed that the endosomal transport of membrane glycoproteins to lysosomes is inhibited at 18ºC (Dunn et al. 1980; Weigel and Oka 1981; Marsh et al. 1983; Hare and Huston 1984; Wolkoff et al. 1984; Griffiths et al. 1988; Hare 1988; Salzman and Maxfield 1989), it is unlikely that 18ºC is highly specific. Although it seems likely that exposure to 18ºC inhibited endosome function in ScN2a cells based on studies of the endocytosis of FITC-WGA and the lack of formation of well-defined cytoplasmic vesicles (Borchelt et al. 1992), it is possible that exposure to 18ºC also inhibited one or more processes in CLDs, including PrP^{Sc} formation.

Numerous studies have shown that most membrane glycoproteins are transported to lysosomes via endosomes, which act as receptacles for plasma membrane proteins that are destined for degradation or recycling (Dunn et al. 1980; Marsh and Helenius 1980; Willingham and Pastan

1980; Stoorvogel et al. 1991). After endosomes have received membrane glycoproteins from endocytic vesicles, they appear to mature as they change from "early" to "late" endosomes before delivery of their contents to lysosomes (Stoorvogel et al. 1991).

Many membrane proteins are degraded after endocytosis and transit through endosomes to lysosomes (Dunn et al. 1980; Steinman et al. 1983; Hare and Huston 1984; Wolkoff et al. 1984; Hare 1988). Although PrP^{Sc} accumulates in cytoplasmic vesicles, many of which are lysosomes (McKinley et al. 1991a), recent studies described above argue that PrP^{Sc} is formed in CLDs before they fuse with endosomes and later lysosomes. First, although lysosomotropic amines block the digestion of the amino-terminal 90 amino acids of PrP^{Sc}, they do not interfere with the formation of PrP^{Sc} (Caughey et al. 1991; Taraboulos et al. 1992). Second, lysosomotropic amines do not alter the degradation of PrP^{C} substantially, which suggests that it might be degraded before reaching lysosomes. Third, kinetic studies indicate that PrP^{Sc} acquires protease resistance approximately 1 hour before exposure to lysosomal proteases and digestion of the amino terminus (Taraboulos et al. 1992).

Restricted Subcellular Localization of PrP^{Sc} Formation

The foregoing studies are all consistent with the hypothesis that PrP^{Sc} formation is confined to CLDs and although this is a pathologic process, it occurs within a specific subcellular domain. The apparent restriction of PrP^{Sc} formation to CLDs would seem to argue that such a process is likely to involve auxiliary macromolecules found within this compartment. Such an auxiliary factor has been implicated in the conversion of PrP^{C} into PrP^{Sc} based on the results of transgenetic studies where a chimeric Hu/Mo PrP molecule but not Hu PrP rendered mice susceptible to Hu prions from patients who died of CJD (Telling et al. 1995). Additionally, the recognition site on PrP^{C} to which protein X binds has been mapped using a series of chimeric PrP constructs expressed in ScN2a cells as described previously (Kaneko et al. 1997c).

PRION REPLICATION

In an uninfected cell, PrP^{C} with the wild-type sequence exists in equilibrium in its monomeric α-helical, protease-sensitive state or bound to protein X (Fig. 9). We denote the conformation of PrP^{C} that is bound to protein X as PrP* (Cohen et al. 1994); this conformation is likely to be different from that determined under aqueous conditions for monomeric recombinant PrP.

The PrP*/protein X complex will bind PrP^{Sc}, creating a replication-competent assembly. Order of addition experiments demonstrate that for PrP^{C}, protein X binding precedes productive PrP^{Sc} interactions (Kaneko et al. 1997c). A conformational change takes place wherein PrP, in a shape competent for binding to protein X and PrP^{Sc} (denoted $PrP^{*/Sc}$), represents the initial phase in the formation of infectious PrP^{Sc}. It is noteworthy that PrP* has also been used to denote a subgroup of PrP^{Sc} molecules that are infectious (Weissmann 1991); however, we have no evidence for such a subset.

Several lines of evidence argue that the smallest infectious prion particle is an oligomer of PrP^{Sc}, perhaps as small as a dimer (Prusiner et al. 1978; Bellinger-Kawahara et al. 1988). Upon purification, PrP^{Sc} tends to aggregate into insoluble multimers that can be dispersed into liposomes (Gabizon et al. 1988; McKinley et al. 1991a).

In attempts to form PrP^{Sc} in vitro, PrP^{C} has been exposed to 3 M guanidine hydrochloride (GdnHCl) and then diluted 10-fold prior to binding to PrP^{Sc} (Kocisko et al. 1994; Kaneko et al. 1997b). On the basis of these results, we presume that exposure of PrP^{C} to GdnHCl converts it into a PrP*-like molecule. Whether this PrP*-like protein is converted into PrP^{Sc} is unclear. Although the PrP*-like protein bound to PrP^{Sc} is protease-resistant and insoluble, this PrP*-like protein has not been reisolated in order to assess whether or not it was converted into PrP^{Sc}. It is noteworthy that recombinant PrP can be refolded into either α-helical or

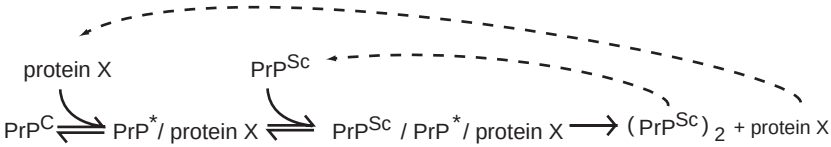

Figure 9 Schematic diagram showing template-assisted PrP^{Sc} formation. In the initial step, PrP^{C} binds to protein X to form the PrP*/protein X complex. Next PrP^{Sc} binds to PrP* that has already formed a complex with protein X. When PrP* is transformed into a nascent molecule of PrP^{Sc}, protein X is released and a dimer of PrP^{Sc} remains. The inactivation target size of an infectious prion suggests that it is composed of a dimer of PrP^{Sc} (Bellinger-Kawahara et al. 1988). In the model depicted here, a fraction of infectious PrP^{Sc} dimers dissociate into uninfectious monomers as the replication cycle proceeds, while most of the dimers accumulate in accord with the increase in prion titer that occurs during the incubation period. The precise stoichiometry of the replication process remains uncertain. (Reprinted, with permission, from Prusiner et al. 1998 [copyright Cell Press].)

β-sheet forms, but none have been found to possess prion infectivity as judged by bioassay (Zhang et al. 1997).

Inherited and Sporadic Prion Diseases

For inherited and sporadic prion diseases, the major question is how the first PrP^{Sc} molecules are formed. Once these are formed, replication presumably follows the mechanism outlined for infectious disease. Several lines of evidence suggest that PrP^{Sc} is more stable than PrP^{C} and a kinetic barrier precludes the formation of PrP^{Sc} under normal conditions. In the case of the initiation of inherited prion diseases, the barrier to PrP^{Sc} formation must be lower for the mutant (ΔPrP^{C}) than the wild type, and thus ΔPrP* can spontaneously rearrange to form ΔPrP^{Sc}. Although the known mutations would appear to be destabilizing to the structure of PrP^{C}, we lack useful information about the structure of the transition state for either the mutant or wild-type sequences. Studies of PrP in the brains of patients who were heterozygous for the E200K mutation revealed ΔPrP^{Sc}(E200K) molecules that were both detergent-insoluble and resistant to limited proteolysis, whereas most wild-type PrP was detergent-insoluble but protease-sensitive (Gabizon et al. 1996). These results suggest that in fCJD(E200K), insoluble wild-type PrP might represent a form of PrP* (Gabizon et al. 1996). In studies with CHO cells, expression of ΔPrP(E200K) was found to be accompanied by the posttranslational acquisition of resistance to limited proteolysis (Lehmann and Harris 1995, 1996a,b), but such results could not be obtained with other cell lines expressing ΔPrP(E200K), including cultured fibroblasts obtained from a patient who was homozygous for the E200K mutation (Meiner et al. 1992 and in prep.). It is noteworthy that levels of proteinase K used in those studies, where ΔPrP(E200K) was expressed in CHO cells, were lower by a factor of 10 to 100 compared to digestions of PrP^{Sc} derived from brain or ScN2a cells. Whether these alterations in the properties of ΔPrP(E200K) in CHO cells provide evidence for ΔPrP* or such changes lie outside the pathway of ΔPrP^{Sc}(E200K) formation remains to be determined.

Initiation of sporadic disease may follow from a somatic mutation and thus follow a path similar to that for germ-line mutations in inherited disease. In this situation, the mutant PrP^{Sc} must be capable of co-opting wild-type PrP^{C}, a process known to be possible for some mutations (e.g., E200K, D178N) but less likely for others (e.g., P102L) (Hsiao et al. 1994; Telling et al. 1995, 1996). Alternatively, the activation barrier separating

wild-type PrP^{C} from PrP^{Sc} could be crossed on rare occasions when viewed in the context of a population. Most individuals would be spared, and presentations in the elderly with an incidence of about 1 per million would be seen.

PrP^{Sc} ACCUMULATION IN PRION DISEASES

Pathologic changes in prion diseases seem to be confined to the nervous system. Such changes include vacuolation of neurons and hypertrophy of astrocytes, as well as the extracellular accumulation of fragments of PrP^{Sc} that polymerize to form amyloid fibrils. The vacuolation of neurons and hypertrophy of reactive astrocytes are generally obligatory features of prion disease, whereas PrP amyloid deposits are a variable feature (Prusiner et al. 1990). When PrP deposits in the form of amyloid plaques are present, the diagnosis of prion disease cannot be in question.

The cell biology of PrP^{Sc} accumulation that leads to neuronal vacuolation remains unclear. A variety of studies argue that it is the intracellular accumulation of PrP^{Sc} that induces vacuolation of neurons (DeArmond et al. 1987). As vacuolated neurons die, these vacuoles coalesce to form the spongy change that is always seen in prion disease, with rare exception. However, it is important to note that spongiform degeneration is frequently confined to very limited regions of the brain as in fatal familial insomnia of humans, scrapie of sheep, and bovine spongiform encephalopathy of cattle (Zlotnik and Stamp 1961; Lugaresi et al. 1986; Wells et al. 1989). The reactive astrocytic gliosis that accompanies neuronal degeneration is most easily assessed by glial fibrillary acid protein (GFAP) immunostaining. In the prion diseases, increased GFAP mRNA levels are found in addition to elevated levels of the protein and intermediate filaments composed of GFAP. It is notable that GFAP-deficient ($GFAP^{0/0}$) mice were as susceptible to inoculated prions as their nonablated controls (Gomi et al. 1995; Tatzelt et al. 1996). A much more extensive description of the processes underlying the pathology of the prion diseases can be found in Chapter 14.

When cultured neurons isolated from mouse brains were exposed to a PrP peptide corresponding to residues 100–126, the cells died (Forloni et al. 1993). When cultured neurons isolated from brains of PrP-deficient ($Prnp^{0/0}$) mice were exposed to the same PrP peptide, the cells were unharmed (Brown et al. 1994). Consistent with these results are studies with brain grafts infected with prions where the graft was transplanted into the brains of $Prnp^{0/0}$ mice (Brandner et al. 1996). Although the cells

of the graft produced large amounts of PrP^{Sc}, the surrounding cells remained healthy. These studies argue that PrP^{Sc} is cytotoxic only for cells that express PrP^{C} and that extracellular PrP^{Sc} does not cause cellular dysfunction (Prusiner et al. 1990).

Subcellular Localization of PrP^{Sc}

Recent studies suggest that more than 50% of PrP^{Sc} in ScN2a cells may remain attached to the plasma membrane (S.J. DeArmond, unpubl.). Whether a similar localization will be found in the brains of mammals with prion diseases remains to be determined. It is unknown whether the accumulation of PrP^{Sc} exerts its effect on neuronal function when it is newly formed in CLDs or after it is transported into the interior of the cell. How prions spread through the CNS is also unknown. Several studies argue that prions transit along axons by both retrograde and anterograde transport (Kimberlin and Walker 1979; Fraser 1982; DeArmond et al. 1987; DeArmond and Prusiner 1997).

PrP TOPOGENESIS

Cell-free translation studies have demonstrated two forms of PrP: a transmembrane (TM) form that spans the bilayer at least once and a secretory form (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 topologic conundrum. Using cell-free translation systems, three forms of PrP could be identified: (1) ^{sec}PrP, which is a secretory form that is completely protected from proteolysis in the absence of detergent, (2) ^{Ntm}PrP, which is a TM form in which the amino terminus protrudes into the lumen and is protected from proteolysis in the absence of detergent, and (3) ^{Ctm}PrP, which is another TM form in which the carboxyl terminus protrudes into the lumen and is also protected from proteolysis (Fig. 10A) (Hegde et al. 1998). The proteolysis protection assays used in these studies are distinct from those used for detection of PrP 27-30 generated from PrP^{Sc} in the presence of high concentrations of proteinase K and detergent.

Evidence That Transmembrane PrP Is Pathogenic

The role of the different topological forms of PrP was explored using Tg mice carrying PrP mutations that alter the relative ratios of these forms.

Expression of ^{Ctm}PrP produced neurodegenerative changes in mice similar to some inherited human prion diseases (Hegde et al. 1998). Brains from these mice contained ^{Ctm}PrP, but not PrP^{Sc}, as defined by the lack of protease resistance and transmissibility. Furthermore, in one heritable prion disease of humans denoted GSS(A117V), brain tissue contained ^{Ctm}PrP but not PrP^{Sc}. Thus, considerable evidence argues that aberrant regulation of PrP biogenesis and topology is involved in the pathogenesis of at least some prion diseases (Hegde et al. 1998).

Synthesis of two different transmembrane forms of PrP is dependent on discrete sequences within the PrP coding region (Hay et al. 1987; Yost et al. 1990; De Fea et al. 1994). Two adjacent domains within PrP (see Fig. 10B), the hydrophobic, potentially membrane-spanning stretch from amino acids Ala-113 to Ser-135 (termed TM1) and the preceding hydrophilic domain (termed STE, and presently narrowed to residues Lys-104 to Met-112), appear to act in concert to generate both transmembrane forms of PrP. Mutations, deletions, or insertions within these domains can alter the relative amounts of each topological form of PrP that is synthesized at the ER (Lopez et al. 1990; Yost et al. 1990). Given these complex and unusual features of PrP biogenesis, it seemed plausible to think that it may have dramatic consequences for the physiology of an organism.

To explore the hypothesis that misregulation of PrP biogenesis might cause neurodegeneration, four mutations within the STE–TM1 region were identified (Fig. 10C) that greatly alter the ratio of the topological forms when assayed by cell-free translation. Two of these mutations (KH→II and ΔSTE) were engineered into SHaPrP, while the other two (AV3 and G123P) were put into MH2MPrP, a mouse/hamster chimera in which residues 94–188 are from SHaPrP (Scott et al. 1993). We found that the species variation between SHaPrP and MH2MPrP (differing at 8 residues) had little effect on topology. However, a comparison of mutant SHaPrP(KH→II) with wild-type SHaPrP showed a dramatic increase (from ~10% to ~50%) in the relative amount of ^{Ctm}PrP synthesized and a concomitant decrease in ^{sec}PrP (Fig. 10D). The amount of ^{Ntm}PrP remained essentially unchanged. Similar results were obtained when MH2MPrP(AV3) was compared to MH2MPrP. In contrast, both SHaPrP(ΔSTE) and MH2MPrP(G123P) were synthesized exclusively in the ^{sec}PrP form. These results, quantified and summarized (Fig. 10D), provided the basis for examination of the effects of aberrant ^{Ctm}PrP synthesis in vivo. To do this, PrP transgenes encoding each of these mutations were expressed in mice that lacked the PrP gene (FVB/Prnp$^{0/0}$). These

mice were then observed for clinical signs and symptoms and examined for histopathology, and the PrP molecules in their brains were analyzed biochemically for transmembrane topology.

^{Ctm}PrP and the Development of Neurodegeneration

Mice expressing the SHaPrP(KH→II) transgene (designated TgSHaPrP(KH→II)$_H$, line 1198) developed signs of neurodegeneration. All 29 Tg mice spanning three generations have developed clinical signs of prion disease including ataxia and paresis (Fig. 11A). In the F_2 generation of mice harboring the transgene (n = 24), the average age of onset was 58 days ± 11 days, with the earliest development of symptoms being 41 days (Fig. 11B). In contrast, none of the nontransgenic littermates exhibited any signs of illness. Neither the FVB/Prnp$^{0/0}$ mice nor FVB/Prnp$^{0/0}$ mice expressing the wild-type SHaPrP transgene (designated Tg(SHaPrP), line 3922) developed any signs of neurologic dysfunction. Quantification of PrP expression levels demonstrated that TgSHaPrP(KH→II)$_H$ expressed PrP at approximately half the level seen in Tg(SHaPrP)/3922/Prnp$^{0/0}$ mice, indicating that disease was not a trivial consequence of massive overexpression (which has been shown to cause a neuromyopathy at ages of 1 year or more [Westaway et al. 1994]).

Transgenic mice expressing MH2MPrP(AV3) at high levels also showed neurological signs of illness and death within 2 months (Scott et

Figure 10 Analysis of the topology of mutant PrP molecules at the ER membrane using cell-free translation. (*A*) Schematic of the standard proteolysis assay for PrP topology determination. The three topologic forms of PrP are shown before (*left*) and after (*right*) digestion with cytosol-disposed proteinase K (PK). The approximate sizes of the fragments generated from each form are indicated above the diagram. The positions of the epitopes recognized by the 3F4 and 13A5 antibodies (Rogers et al. 1991; Scott et al. 1992) are indicated by the white and gray boxes, respectively. (*B*) Schematic of topogenic domains in PrP. Topogenic sequences shown are the amino-terminal signal sequence (signal), the stop transfer effector sequence (STE), the potential membrane spanning domain (TM1), and glycosylation sites (CHO). Amino acid positions are shown below the diagram. (*C*) Mutations used in this study. The amino acid number of hamster PrP is indicated above the figure. Changes are indicated by shading. (*D*) Quantitative representation of the relative amounts of ^{sec}PrP (*black bars*), ^{Ctm}PrP (*shaded bars*), and ^{Ntm}PrP (*white bars*) for each PrP construct analyzed in panels *C–E*. (Reprinted, with permission, from Hegde et al. 1998 [copyright AAAS].)

al. 1997). Biochemical analyses of mutant PrP in selected ill founder animals revealed the expression level to be equal to or lower than in the brains of Tg(SHaPrP) mice, again militating against the explanation of simple overexpression. Thus, similar to the KH→II mutation, expression of the AV3 mutation caused neurodegeneration in mice.

Transgenic mice expressing SHaPrP(ΔSTE) and MH2MPrP(G123P) were also constructed, and a high-expressing line of each—designated

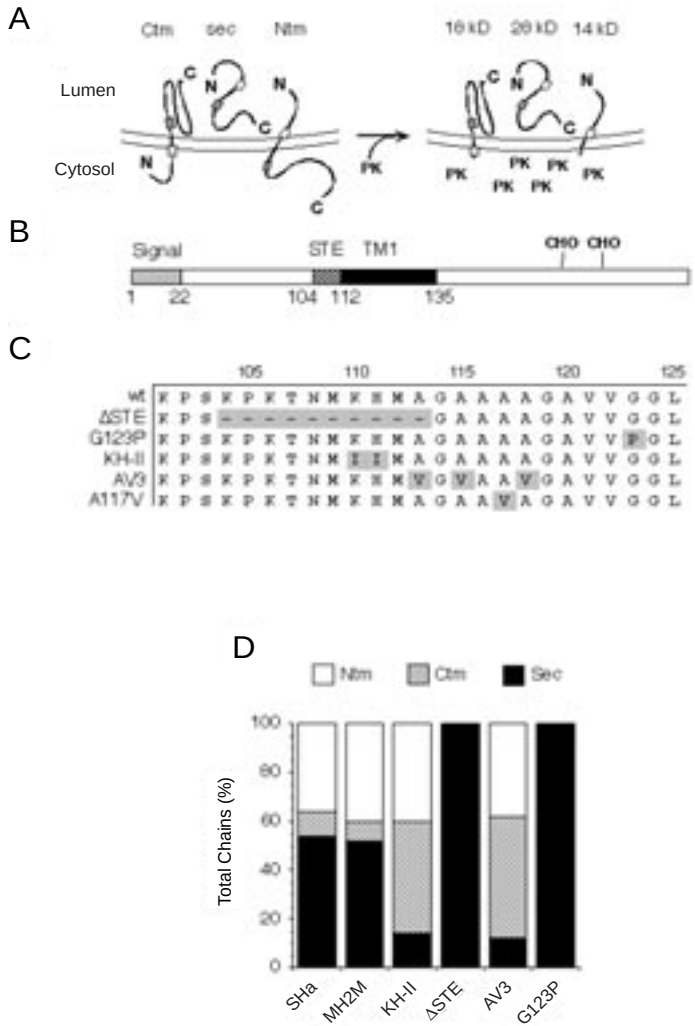

Figure 10 (See facing page for legend.)

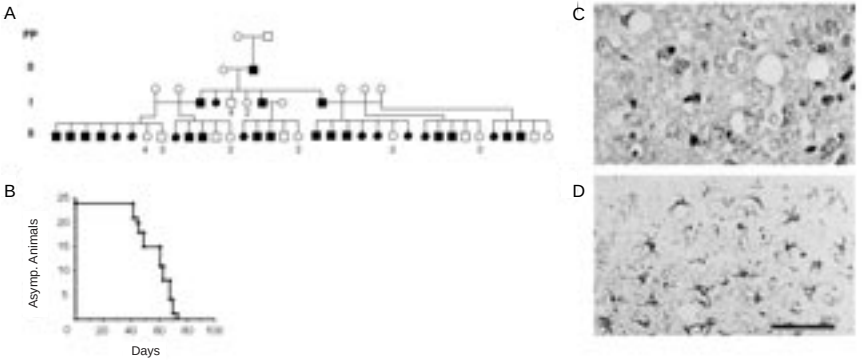

Figure 11 Production and histologic characterization of a transgenic mouse line expressing SHaPrP[KH→II)]. (*A*) Genealogy of Tg[SHaPrP(KH→II)H] transgenic line (Scott et al. 1992). Individuals carrying the transgene are indicated by symbols that are blackened (each of which succumbed to neurodegenerative disease). Open symbols indicate non-transgenic littermates. Males are indicated with a square and females with a circle. Numerals below some symbols indicate multiple individuals represented by that symbol. FP indicates the founding parents; 0, I, and II indicate the founder, F_1, and F_2 generations, respectively. (*B*) The F_2 transgenic animals ($n = 24$) were observed for signs of neurodegenerative disease, including ataxia and paresis. Plotted on the ordinate axis are the number of asymptomatic animals in the group at any given age (in days). (*C*) Hematoxylin and eosin stain of immersion fixed brain sections (Hsiao et al. 1990) from Tg[SHaPrP(KH→II)$_H$] mice showing mild to moderate vacuolation. (*D*) Analysis of Tg[SHaPrP(KH→II)$_H$] mice by immunohistochemical staining of immersion fixed brain section with antibodies to glial fibrillary acidic protein (Hsiao et al. 1990), demonstrating reactive astrocytic gliosis. (Reprinted, with permission, from Hegde et al. 1998 [copyright AAAS].)

Tg[SHaPrP(ΔSTE)], line 1788, and Tg[MH2MPrP(G123P)], line 13638—was selected for further study (Table 2). In contrast to mice carrying the KH→II or AV3 mutation in PrP, neither Tg[SHaPrP(ΔSTE)] nor Tg[MH2MPrP(G123P)] mice showed any signs of illness. Furthermore, even at ages significantly beyond the life span of the Tg[SHaPrP(KH→II)$_H$] mice, histological analysis of Tg[SHaPrP(ΔSTE)] and Tg[MH2MPrP(G123P)] mice revealed no abnormal neuropathologic changes. Taken together with the above findings, these results are suggestive of the notion that favored synthesis in the ^{Ctm}PrP form, as judged by the cell-free translocation assay, is indicative of the pathogenicity of the PrP mutation in vivo.

The topology of PrP in the brains of these Tg mice was examined using microsomal membranes prepared from brain. These intact vesicles

Table 2 Mutational analysis of PrP in vitro and in vivo

	^{Ctm}PrP cell-free (rel to wt)	Level of expression (rel to SHa)	Detection of ^{Ctm}PrP in brain	Clinical symptoms	Neuropathology	PrP^{Sc} detection in brain
Wild type	=	4x	–	–	–	–
ΔSTE	<	2.5–3x	–	–	–	–
G123P	<	2x	–	–	–	–
$KH(II)_H$	>	2x	+	+	+	–
$KH(II)_L$	>	0.25–0.5x	–	–	–	–
AV3[a]	>	2–4x	+	+	+	–
A117V[b]	>	n/a	+	+	+	–

[a]The AV3 mutation is composed of three valine substitutions for alanine at residues 113, 115, and 118.

[b]The A117V mutation is in human PrP, and the data for this mutant derive from analysis in vitro or from human brain tissue. The data for the other mutants derive from analysis of Tg mice.

were then subjected to protease digestion, and the accessibility of PrP to protease was assessed by immunoblotting. Generation of a proteolytic fragment encompassing the carboxyl terminus of PrP would indicate that those molecules were in the ^{Ctm}PrP orientation, whereas full protection from protease would indicate that the PrP was in the ^{sec}PrP orientation. Analysis of the proteolytic fragments was simplified by the removal, just prior to SDS-PAGE, of the highly heterogeneous carbohydrate trees with the enzyme PNGase F. This allowed a search for the 18-kD carboxy-terminal fragment characteristic of the ^{Ctm}PrP form without the complications of differential electrophoretic migration of variably glycosylated PrP molecules. To ensure that vesicle integrity was maintained during the proteolytic reaction, the accessibility to protease of GRP94, an ER luminal protein (Welch et al. 1983), was also evaluated.

Only the Tg[$SHaPrP(KH{\rightarrow}II)_H$] and Tg[MH2MPrP(AV3)] samples contained PrP molecules spanning the membrane, which, after protease digestion in the absence of detergent, produced an 18-kD fragment (Hegde et al. 1998). This fragment produced by protease digestion was determined to be a carboxy-terminal fragment of PrP, based on its detection by the 13A5 monoclonal antibody and its observed slower migration on SDS-PAGE as a heterogeneous set of bands if polysaccharides were not removed from PrP prior to analysis. Thus, brains from Tg[$SHaPrP(KH{\rightarrow}II)_H$] and Tg[MH2MPrP(AV3)] mice contained ^{Ctm}PrP (comprising ~20–30% of the PrP in the microsomes). Similar results were obtained from multiple Tg[$SHaPrP(KHII)_H$] and Tg[MH2MPrP(A3)] animals, but in no instance

was any ^{Ctm}PrP detected in Tg(SHaPrP), Tg[SHaPrP(ΔSTE)], or Tg[MH2MPrP(G123P)] animals. Taken together, these data indicate that the presence of the ^{Ctm}PrP form of PrP in the brains of Tg mice correlates well with observations made using the cell-free translocation system, although the absolute amount of ^{Ctm}PrP in brain was consistently less than that observed in cell-free assays.

Level of ^{Ctm}PrP Expression Modulates Disease

The observation that the percentage of PrP molecules found in the ^{Ctm}PrP topology in vivo was consistently lower than that found in vitro raises the possibility that cells normally have mechanisms to prevent the accumulation of this potentially pathogenic form. Thus, the basis for the modest ^{Ctm}PrP accumulation in brain in the KH→II or AV3 mutant may be a combination of overexpression and the severe skew toward ^{Ctm}PrP synthesis, which together exceed the capacity of the cell to eliminate ^{Ctm}PrP (for example, by rapid degradation at the ER). As a result, ^{Ctm}PrP would accumulate, exit the ER, and trigger disease. If this were the case, one would predict that lower levels of expression of a ^{Ctm}PrP-favoring mutant should fall below such a threshold and, thus, produce only ^{sec}PrP. Such mice would be predicted not to get sick despite the mutation in the PrP gene, due to the absence of ^{Ctm}PrP.

This idea was explored by first identifying a Tg line of mice (designated Tg[SHaPrP(KH→II)$_L$], line 12485) expressing the KH→II mutation at a low level. The line contained approximately one-fourth to one-half the level of PrP found in normal Syrian hamsters, corresponding to levels about fivefold lower than the Tg[SHaPrP(KH→II)$_H$] mice. Upon biochemical examination of the brain, ^{Ctm}PrP was not detected in Tg[SHaPrP(KH→II)$_L$] mice, with all of the PrP being in the ^{sec}PrP form. Thus, by decreasing the level of transgene expression by a factor of about 5, the percentage of PrP in the ^{Ctm}PrP form was reduced from about 30% to undetectable levels, even upon overexposure of the blots, under conditions where ^{sec}PrP was readily detectable. Corresponding to this lack of ^{Ctm}PrP generation, observation of the animals from the Tg[SHaPrP(KH→II)$_L$] line have thus far revealed no signs of illness at ages greater than 300 days. This is in sharp contrast to the Tg[SHaPrP(KH→II)$_H$] line, which showed both ^{Ctm}PrP and signs of disease at about 60 days of age (Fig. 11B). These data support the hypothesis that generation of ^{Ctm}PrP leads to neurodegeneration in mice, with the role of the mutation being limited to one of favored synthesis of ^{Ctm}PrP.

Spontaneous Disease without PrP^{Sc} Accumulation

Whether an increase in ^{Ctm}PrP production is the basis of disease pathogenesis for the KH→II and AV3 mutants remains to be determined. Whether the spontaneous disease caused by the KH→II and AV3 mutations is transmissible remains to be determined. The brains of these ill Tg mice were analyzed for the presence of protease-resistant PrP^{Sc}, but no protease-resistant PrP was detected in either the Tg[SHaPrP(KH→II)$_H$] or Tg[MH2MPrP(AV3)] mice, even after overloading the gels. Immunohistochemistry of brain sections from Tg[SHaPrP(KH→II)$_H$] and Tg[MH2MPrP(AV3)] mice following hydrolytic autoclaving failed to detect PrP^{Sc}. The lack of evidence for PrP^{Sc} lends support to the notion that ^{Ctm}PrP accumulation is responsible for the neurodegeneration observed in these Tg mice.

^{Ctm}PrP Accumulation in GSS(A117V)

Given that the distribution of newly synthesized PrP between transmembrane and secretory topologic forms is readily manipulated by mutations, we asked if an inherited human prion disease might be caused by elevated levels of a TM form of PrP. A Gerstmann-Sträussler-Scheinker disease (GSS)-causing mutation in PrP (Doh-ura et al. 1989; Hsiao et al. 1991; Mastrianni et al. 1995), an alanine to valine substitution at position 117, was a likely candidate for several reasons. First, this mutation lies in the hydrophobic domain (TM1) that has been shown to be crucial to the biogenesis of the transmembrane forms of PrP (Yost et al. 1990). Second, the pathologic findings in these cases of GSS(A117V) (Doh-ura et al. 1989; Hsiao et al. 1991; Mastrianni et al. 1995) appear to share some features with mice that become ill due to ^{Ctm}PrP overexpression. Third, the biochemical examination of brain tissue from these cases of GSS has revealed little or no protease-resistant PrP (Tateishi et al. 1990; Hegde et al. 1998). These observations raised the possibility that a mechanism other than PrP^{Sc} accumulation was involved in the pathogenesis of these cases of GSS(A117V).

To explore the mechanism by which the A117V mutation causes disease, the biogenesis in a cell-free system of this mutant PrP was compared to its wild-type counterpart (both of which contained a valine at the polymorphic position 129). Translocation reactions demonstrated that the A117V mutation significantly favored the synthesis of both ^{Ctm}PrP and ^{Ntm}PrP, with a concordant decrease of the ^{sec}PrP form (Hegde et al. 1998).

Assay of PrP topology in human brain was problematic, since fresh tissue suitable for subcellular fractionation is not readily available. To exploit differences in the native conformations that would be expected of the different topologic forms of PrP, limited proteolysis was employed. Although harsh digestion at elevated levels of proteinase K (at >500 μg/ml) or elevated temperature (37ºC) digested all topologic forms of PrP (but not PrP^{Sc}), a subset of PrP was only partially digested under the milder conditions of 250 μg/ml proteinase K and 0ºC. The fragment generated under these conditions comigrated on SDS-PAGE with the protected carboxy-terminal domain generated in the cell-free translation-topology assay.

Digestion of each of the topological mutants of PrP under the same conditions revealed that the generation of the protease-resistant fragment correlated well with the amount of ^{Ctm}PrP form. Mutations that increased the relative amount of PrP in this form (A117V, KH→II, and AV3) resulted in increased generation of the proteinase K-resistant fragment, whereas mutations that abolished synthesis of this form (ΔSTE and G123P) did not yield a proteinase K-resistant fragment. As expected, all of the topological forms were completely digested by harsher treatment under which PrP^{Sc} is amino-terminally truncated to form PrP 27-30.

To confirm that the procedure described above could distinguish ^{Ctm}PrP from both ^{sec}PrP and PrP^{Sc} in frozen brain tissue samples, assays were performed on samples from Tg mice in which the distribution of PrP among the topologic forms had previously been established. Brain tissue from Tg(SHaPrP), Tg[SHaPrP(KH→II)$_L$], and Tg[SHaPrP(KH→II)$_H$] mice was analyzed by the conformational assay, and a protease-protected fragment was detected only in the Tg[SHaPrP(KH→II)$_H$] sample. Thus, the presence of this carboxy-terminal fragment, generated under mild but not harsh digestion conditions, appeared to be diagnostic for the presence of ^{Ctm}PrP.

With the ability to distinguish ^{Ctm}PrP from PrP^{C}, ^{sec}PrP, and PrP^{Sc} in frozen brain tissue, samples from GSS(A117V) brains were analyzed for ^{Ctm}PrP. Although similar levels of PrP were found in both normal and GSS human brain, the GSS brain contained increased levels of ^{Ctm}PrP. Neither control nor GSS(A117V) brain contained detectable protease-resistant PrP^{Sc} under conditions where it was readily found in brain tissue from a sporadic CJD patient. These results were confirmed by analysis of multiple samples of tissue from the same patient, and also with brain tissue from a second patient carrying the A117V mutation. The lack of accumulation of protease-resistant PrP^{Sc} was also confirmed for the second patient. Thus, consistent with observations in vitro, the A117V mutation

resulted in increased generation of the ^{Ctm}PrP form in vivo, suggesting that this is the basis of at least some of the neuropathological changes seen in these cases of GSS(A117V).

Expanding the Spectrum of Prion Diseases

The finding of elevated levels of ^{Ctm}PrP in both GSS(A117V) and Tg[SHaPrP(KH→II)$_H$] mice argues that this topologic form of PrP may be pathogenic. Although the mechanism by which ^{Ctm}PrP causes neurodegeneration is unknown, this finding expands the spectrum of prion diseases.

How many different forms of prion disease will be found is of considerable interest. At present, three different forms of heritable prion disease can be defined by the nature of the PrP molecule that accumulates. First, mutant PrP^{Sc} accumulates in fCJD(E200K) and fatal familial insomnia. Second, ^{Ctm}PrP is found at elevated levels in both GSS(A117V) and Tg[SHaPrP(KH→II)$_H$] mice. Third, protease-sensitive MoPrP^{Sc}(P101L) is found in Tg mice and sometimes in GSS(P102L).

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Scrapie, Chronic Wasting Disease, and Transmissible Mink Encephalopathy

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Transmissible spongiform encephalopathies (TSE) such as scrapie in sheep and goats, transmissible mink encephalopathy (TME), and chronic wasting disease (CWD) of cervids belong to a group of diseases that affect both humans and animals, known as prion diseases. They are associated with alterations in conformation of a neural cell-surface glycoprotein, the prion protein (PrP^{C}) that aggregates and accumulates in the brain of affected animals or humans. This abnormal form of PrP (PrP^{Sc}, also called PrP-res) is thought to be an essential component of the infectious particle causing TSEs, named prion (Prusiner 1982). In mice, it has been shown that TSE development is dependent on the agent strain, the infectious dose, and the host genotype (Bruce et al. 1991). Recent data now indicate that development of scrapie in sheep, CWD, and TME follows the same rules.

SCRAPIE

Epidemiology

Sheep, goats, and moufflon (*Ovis musimon*) (Wood et al. 1992) are susceptible to natural scrapie, and the disease occurs primarily in sheep of breeding age. Scrapie is an enzootic fatal neurodegenerative disorder of unknown etiology. The disease is also called *tremblante* (trembling) in France, *traberkrankheit* in Germany (trotting disease), or *rida* in Iceland (ataxia or tremor). Scrapie was the first spongiform encephalopathy whose transmissibility was demonstrated (Cuille and Chelle 1936). Epidemiological studies have been conducted on the potential risk of transmission of the scrapie agent to humans but have never supported a causal relationship (Chatelain et al. 1981).

Scrapie was initially reported in Europe in 1732 in England and in 1759 in Germany. The etiology of scrapie was already a matter of debate at the beginning of the nineteenth century. Some authors considered that the disease appeared spontaneously and became hereditarily transmissible while others supposed it was caused by an animalcule (Girard 1830).

In the following decades, scrapie endemically affected flocks in several countries following the importation of affected or incubating sheep, but its exact prevalence and incidence remain obscure. In the Netherlands, the prevalence of flocks with scrapie cases was recently established between 3.8% and 8.4%, with an incidence in the sheep population of 0.1 case/100 ewes/year (Schreuder et al. 1993). The disease appears to be much more prevalent in the northern hemisphere than in the southern hemisphere. Whether this could reflect the influence of climate on scrapie development or is an expression of the difference between intensive or extensive breeding strategies is unknown. New Zealand and Australia are fortunate because they are among the few sheep-raising countries recognized by most as free from scrapie. This status results from extensive protective measures designed to prevent the introduction of scrapie from imported sheep (MacDiarmid 1996). The importation strategy is based on embryo transfer, bioassay of the material from the lymph nodes of donor sheep in young goats, and a 3-year quarantine period for the embryo-derived offspring. Other countries such as Denmark, Finland, Sweden, Israel, and Zimbabwe have a low scrapie risk. Scrapie was also eradicated from the Republic of South Africa before it was widely disseminated through the national flock (MacDiarmid 1996).

There is little information on scrapie in goats and moufflon. Most reported occurrences of natural scrapie in goats have been associated with the presence of scrapie in sheep (Brotherston et al. 1968). However, Hourrigan and colleagues (1979) observed that scrapie could be spread

from goat to goat without sheep contact. In the UK, six cases of scrapie have been confirmed in two different flocks of moufflon, and it has not been possible to determine the origin of the disease in either case. No relationship has been made with sheep scrapie.

Despite progress made on many aspects of prion diseases, important points concerning scrapie are still unanswered or controversial. Among them are the precise characteristics of the infectious agent, the number of naturally existing strains, the measurement of the infective dose in the field, the mode of transmission, the route of contamination, and efficient strategies for disease prevention. In the last decade, geneticists have made major contributions to the understanding of this old disease. In this strange "host-pathogen" relationship where little is known about the pathogen, studies have focused on the host in attempts to characterize the genetic factors that determine its susceptibility or resistance to the disease. The results thus obtained should still be viewed with caution; however, management of sheep scrapie through genetic selection may be anticipated in the near future.

Clinical Presentation of Scrapie

Typical clinical signs of scrapie in sheep (Dickinson 1976) begin with mildly impaired social behavior such as unusual restlessness and signs of nervousness, which are often noticeable only to an experienced shepherd. Overt illness can last from 2 weeks to 6 months. In later stages of the clinical course, the general condition of the animal begins to deteriorate and is sometimes accompanied by a change in fleece color. Pruritis (itching with resulting skin damage) can result from the animal attempting to relieve what seems to be an intense irritation by scratching against fence posts or by biting the affected area. Despite ataxia (loss of motor coordination), affected sheep will walk considerable distances to indulge in bouts of scratching (Parry 1984). Specific parts of the skin are continually rubbed and suffer wool loss; for example, around the base of the tail and, occasionally, an entire side of the body. In the final stages of scrapie, sheep waste away, cannot walk very far, and become very agitated by any (even mild) stress. The appetite may appear normal but the animals lose the ability to feed themselves. Scrapie does not alter reproductive ability until muscle wasting interferes with motor activity. Lambs can be born successfully to mothers in the clinical phase of the disease and rams remain fertile and active even when showing scrapie symptoms (Parry 1984; J.D. Foster and C. MacKenzie, unpubl.).

Reported clinical descriptions often vary, however. In a group of scrapie-affected sheep from Shetland between 1985 and 1991 (Clark and

Moar 1992), most animals showed pruritis and emaciation; others had pruritis, emaciation, and hyperaesthesia (oversensitivity), and others showed all of these signs plus ataxia. These authors also described sheep apparently with scrapie that exhibited such a short clinical course that they were simply "found dead." In a report of clinical signs in some Japanese sheep (Onodera and Hayashi 1994), some animals (Suffolks and Corriedales) showed signs of pruritis, but others (Corriedales) died for no obvious reason; scrapie was diagnosed after histopathologic examination. These differences both in symptoms and clinical course may simply be due to breed characteristics, or they may indicate the presence in the field of different strains of scrapie.

Affected goats are less likely to rub against fixed objects but scratch vigorously with hind feet and horns (Detwiler 1992). In moufflon, clinical signs were indistinguishable from sheep scrapie (Wood et al. 1992).

It is very difficult to detect those animals with scrapie that are not visibly affected by the disease by histopathology alone. At terminal stages, however, common neurologic lesions in the brain include neuronal degeneration with the formation of intracytoplasmic vacuoles, proliferation of astroglia but no demyelination, or other overt inflammatory responses. There appears to be some involvement of cytokines in early responses to infection, at least in mouse models (Williams et al. 1996), but this may be a nonspecific reaction to tissue damage. Vacuolation is not present in all areas of the brain; for example, one study of scrapie-affected sheep in Britain described seven patterns of vacuolation (Wood et al. 1997) in ten brain areas: medulla, pons, cerebellum, substantia nigra, mesencephalon, hypothalamus, thalamus, septal area, corpus striatum, and neocortex. Some patterns of vacuolar damage were very similar to each other; however, others were markedly different. In another study of both naturally affected and experimentally challenged sheep, vacuolation occurred in areas such as the dorsal vagus nucleus, cerebellum, and thalamic nuclei (Foster et al. 1996a). Vacuolation was described as "seldom apparent" without detection of PrP^{Sc} in the vicinity; however, PrP^{Sc} was sometimes present in areas with no vacuolation. The presence of the disease-associated PrP^{Sc} can be detected in the preclinical phase and is therefore of greater potential interest for diagnosis than vacuolation.

Infectivity in Scrapie-affected and Preclinical Sheep Tissues

In sheep with natural scrapie and in rodent models of the disease, the concentration of PrP^{Sc}, which is a major component of scrapie-associated fibrils (SAF) or scrapie prion, correlates closely with scrapie infectivity titer

(Diringer et al. 1983; McKinley et al. 1983; Somerville et al. 1986). Many scrapie studies using rodent models have resulted in a serviceable understanding of scrapie. However, distribution of scrapie infectivity or PrP^{Sc} in tissues from natural hosts, sheep, and goats during the incubation period is less well studied because of the difficulty in diagnosing preclinical scrapie. Studies in natural hosts have inherent problems that are not encountered in the rodent models. Investigations with sheep born and raised in scrapie-infected flocks come with the risk that some sheep are not infected (Hadlow et al. 1977, 1982). In experimentally infected sheep killed at various intervals after infection, the timing of PrP^{Sc} appearance varies according to the different levels of scrapie susceptibility of individual sheep (Ikegami et al. 1991). Currently, estimation of the sheep's susceptibility to scrapie by genotyping the *PrP* gene can overcome some of these problems (Laplanche et al. 1993b).

Distribution of Scrapie Infectivity in Sheep Tissues

Cuille and Chelle (1936) first demonstrated scrapie infectivity by transmitting it to sheep using scrapie-affected sheep brain. Distribution of infectivity titer in sheep and goat tissues has been examined by bioassay using mice (Hadlow et al. 1977, 1980, 1982; Groschup et al. 1996). Based on the data of Hadlow and colleagues (1980, 1982), WHO (1991) tissues were separated into four categories based on relative levels of infectivity: I, highly infective; II, moderately infective; III, minimally infective; IV, no detectable infectivity. Category I tissues include brain and spinal cord; category II tissues are the spleen, tonsils, lymph nodes, ileum, and proximal colon; category III includes the sciatic nerve, pituitary gland, adrenal glands, distal colon, nasal mucosa, cerebrospinal fluid, thymus, bone marrow, liver, lung, and pancreas; and category IV tissues are skeletal muscles, heart, mammary glands, colostrum, milk, blood clots, serum, feces, kidneys, thyroid gland, salivary glands, saliva, ovaries, uterus, testes, and seminal vesicles. Skin is classified as category IV (Stamp et al. 1959). Sometimes, the ovaries, uterus, placenta, and amniotic fluid are positive for scrapie infectivity (Hourrigan 1990; Onodera et al. 1993). Groschup et al. (1996) have recently shown that substantial amounts of infectivity could also be detectable in several peripheral nerves except nervus saphenicus.

Infectivity of sheep tissues is usually lower when assayed in mice than in sheep, apparently due to the species barrier. Failure to detect infectivity in certain tissues in mice, therefore, may not always mean that the tissue is free from scrapie prions. Fetal membranes, in which infectivity is

demonstrable by sheep bioassay (Pattison et al. 1972, 1974), the placenta, and amniotic fluid from infected ewes are all thought to be important sources of natural infection.

Hadlow and colleagues (1977, 1982) have examined temporal distribution of scrapie infectivity in Suffolk sheep from scrapie-infected flocks. They describe no infectivity before 8 months of age. From 10 to 11 months of age, infectivity is detected at a low level in various lymph nodes, spleen and tonsil, and intestinal tissue. Subsequently, the infectivity in these tissues increases and reaches a moderate level. The appearance of the infectivity in the tissues of the central nervous system (CNS) including the spinal cord occurs later than that in the lymphoid tissues but before the onset of clinical disease; i.e., a low level of infectivity can be detected in the diencephalon and medulla oblongata in a 25-month-old clinically normal sheep. By the time animals show clinical disease, the infectivity in the CNS is at its highest level. Among the parts of the CNS with the highest level of infectivity are the diencephalon, midbrain, medulla oblongata, and cerebellar cortex. Infectivity appears first in the tonsils, retropharyngeal lymph nodes, and intestines, possibly reflecting an oral route of infection. Most frequently, sheep become infected with scrapie at or soon after birth. Thus, the age when infectivity was detected is roughly the same as the time after infection.

Biochemical Diagnosis of Sheep Scrapie: Detection of SAF and PrP^{Sc}

SAF have been detected in detergent-treated extracts of brain and spleen from natural and experimentally affected sheep using electron microscopy of negatively stained specimens (Gibson et al. 1987; Rubenstein et al. 1987; Stack et al. 1991). Sensitivity of SAF detection seems to be not very high. Detection of PrP^{Sc}, which is a major component of SAF, is more sensitive and practical for determining the presence of scrapie prion (Cooley et al. 1998).

PrP^{Sc} is detected in the tissues of sheep that are clinically infected and in preclinically infected sheep tissues by immunochemical methods and immunohistochemical staining using antibodies to prion or synthetic PrP-based peptides. Although these methods are less sensitive than the bioassay for scrapie prion infectivity detection, they have significant advantages over bioassay in time and expense. Relative concentrations of PrP^{Sc} in tissues may be assayed by Western blots and enzyme-linked immunosorbent assay (ELISA), and the distribution and localization of PrP^{Sc} in tissues is revealed by conventional immunohistochemistry and

histoblots in which cryostat sections are blotted onto membranes (Taraboulos et al. 1992).

Sheep PrP^{Sc} was first detected in brain extracts by Western blot (Takahashi et al. 1986; Rubenstein et al. 1987), and detection in CNS is one of the methods for scrapie diagnosis (Farquhar et al. 1989; Ikegami et al. 1991; Race et al. 1992; Mohri et al. 1992; Skarphedinsson et al. 1994). Although data describing concentrations of PrP^{Sc} in different parts of sheep brain are not available, PrP^{Sc} seems to distribute unevenly, with the brain stem being the best tissue for reliable Western-blot detection of PrP^{Sc}. Immunohistochemistry of PrP^{Sc} in CNS revealed that it is detected preferentially in the brain stem, especially at the obex, even when PrP^{Sc} detection in other parts of the brain is difficult. PrP^{Sc} deposits in neuronal cells, gray matter neuropile, sometimes around blood vessels, and outside of neuronal cells as plaques (McBride et al. 1988; Miller et al. 1993).

van Keulen and colleagues (1996) immunohistochemically examined the distribution of PrP^{Sc} in lymphoid tissues from scrapie-affected sheep and found that PrP^{Sc} is consistently detected in the spleen, retropharyngeal lymph node, mesenteric lymph node, and the palatine tonsil. PrP^{Sc} is deposited in a reticular pattern in the center of lymphoid follicles. As in the mouse model (Kitamoto et al. 1991; McBride et al. 1992), follicular dendritic cells appear to be the target of the deposition. Macrophages associated with lymphoid follicles contain PrP^{Sc} in their cytoplasm.

In a preclinical-stage analysis of naturally infected sheep using Western blots, PrP^{Sc} was detected in the spleen and visceral and surface lymph nodes, but not in the CNS from euthanized apparently healthy 5-month-old (Fig. 1, sheep A), 8-month-old, and 40-month-old Suffolk sheep (Ikegami et al. 1991). Lymphoid tissues can be collected by biopsy without killing sheep. Muramatsu and colleagues (1993) and Schreuder and colleagues (1996) examined the lymphoid tissues from sheep that were born and raised on farms experiencing scrapie outbreaks. Muramatsu's group detected PrP^{Sc} by Western blotting in a subiliac lymph node from a 12-month-old Suffolk-Corriedale mixed-breed sheep that developed scrapie 2 months after the biopsy (Fig. 1, sheep B), and Schreuder's group used immunohistochemistry to detect PrP^{Sc} in the palatine tonsils from six sheep genetically determined to be susceptible (see below) that were approximately 10 months of age, which was less than half the length of the expected incubation period. PrP^{Sc} was also detected in the lymph node from a Suffolk sheep experimentally infected intravenously at 14 months after infection (Ikegami et al. 1991).

Distribution of scrapie infectivity and PrP^{Sc} in affected sheep tissues is summarized in Table 1. Generally, less is known about the temporal dis-

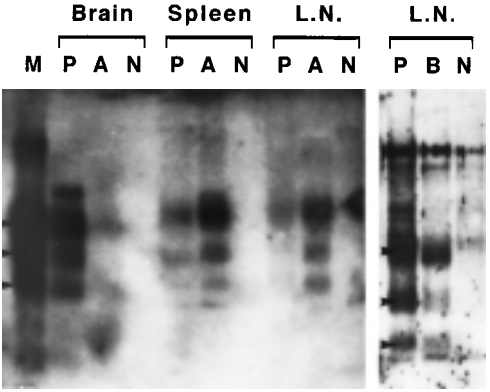

Figure 1 Detection of PrP^{Sc} in sheep spleen and lymph nodes at preclinical stage of scrapie. Apparently healthy sheep tissues were obtained by autopsy after euthanasia (sheep A, 5 months old) or by biopsy (sheep B, 12 months old). Sheep B showed scrapie signs 2 months after the biopsy. Brain, spleen, and mesenteric lymph nodes (L.N.) from sheep A and a subiliac lymph node from sheep B were examined for the presence of PrP^{Sc} by Western blot. (Lane *A*) Apparently healthy sheep A; (lane *B*) apparently healthy sheep B; (lane *P*) scrapie-affected sheep, (lane *N*) healthy sheep. Lane *M* is a partially purified prion protein fraction prepared from scrapie sheep brain, as positive control. PrP^{Sc} was detected as three broad bands between 19 kD and 30 kD, indicated by arrowheads.

tribution of PrP^{Sc} in sheep tissues than about scrapie infectivity. The appearance of PrP^{Sc} in lymphoid tissues precedes its appearance in the CNS, and the appearance of PrP^{Sc} seems to coincide with infectivity; scrapie infectivity titer correlates closely with the PrP^{Sc} concentration. Improvement of the biochemical methods for detecting PrP^{Sc}, especially in developing new antibodies (Somerville et al. 1997) and methods of tissue processing for Western blot and ELISA (Grathwohl et al. 1996, 1997), permit increased sensitivity of PrP^{Sc} detection. For some purposes, such as early diagnosis of scrapie through examination of peripheral lymphoid tissues and determination of the kinetics of prion accumulation, the infectivity bioassay using mice may be replaced by assaying PrP^{Sc}.

Host Control of Experimental Sheep and Goat Scrapie

Early experimental studies of scrapie in sheep left the scrapie world with a selection of contradictory findings. In a study published in 1966

(Gordon 1966), more than 1000 sheep of 24 different breeds were injected intracerebrally with a source of scrapie known as SSBP/1 (Dickinson 1976). The animals were observed for 2 years, during which time some of them developed scrapie with incubation periods ranging from 3.5 months to 23 months and some remained completely healthy. It was thought at the time that some breeds were more resistant than others and the results apparently confirmed this idea: Herdwicks, Scottish Blackface, Suffolks, and Dorset Horn breeds had disease incidences of 78%, 18%, 12%, and zero, respectively. Different results were obtained when others repeated the work with separate groups of sheep; for example, Herdwicks were later found to be only 30% susceptible (Pattison 1966). There were also problems with the original Dorset Downs, which were supposed to be resistant and were kept alive after the experimental observation period ended at 24 months: Some of these Dorset Downs developed SSBP/1 scrapie at later dates (Dickinson 1976). There was a clear need to develop lines of sheep with more predictable responses to scrapie if any progress was to be made, and three such flocks were developed in Britain—Cheviots, Herdwicks, and Swaledales.

The Cheviot sheep flock (Neuropathogenesis Unit [NPU] Cheviots) was founded by Alan Dickinson in 1960. Two lines of sheep were selected, a positive line and a negative line, depending on the incubation period in the animals following injection with SSBP/1 (Dickinson 1976). The gene that controls experimental scrapie incubation period in these sheep was called *Sip* (for *s*crapie *i*ncubation *p*eriod) and had two alleles, sA and pA (Dickinson and Outram 1988). Negative line Cheviots were described as Sip^{pApA} and these survived subcutaneous (s.c.) injection of SSBP/1. Positive line sheep were Sip^{sAsA} or Sip^{sApA} (Sip^{sA} being partially dominant; Foster and Hunter 1991) and these developed scrapie from SSBP/1 s.c. inoculation in 150–400 days. Different sources of scrapie inocula have different transmission characteristics; for example, although SSBP/1 (termed an A group isolate) has shorter incubation periods in Sip^{sA} carriers (positive line) than in Sip^{pApA} (negative line) sheep, CH1641 (a C group scrapie isolate) and bovine spongiform encephalopathy (BSE) have shorter incubation periods in some negative line sheep than in positive line animals (Foster and Dickinson 1988; Foster et al. 1993). More detailed elucidation of the action of the scrapie susceptibility/resistance gene, however, has come from the study of the genetics of the *PrP* gene.

Recent evidence has strongly implied that the *Sip* gene, as defined by classic genetics, is identical to the *PrP* gene, which has been extensively characterized by molecular genetics first in laboratory mouse models (Westaway et al. 1987; Hunter et al. 1992) and later in humans (Hsiao et

Table 1 Distribution of scrapie prion in affected-sheep tissues

Tissues	Infectivity in mice	PrP^{Sc}	
		WB	IH
Brain	+++	+	+
Spinal cord	+++	+	+
Nervus axillaris	++	ND	ND
Nervus ulnaris	++[a]	ND	ND
Nervus medianus	++[a]	ND	ND
Nervus ischiadicus	+~++	ND	ND
Nervus tibialis	++[a]	ND	ND
Nervus fibularis	++[a]	ND	ND
Nervus saphenus	–~+[a]	ND	ND
Spleen	++	+	+
Lymph nodes	++	+	+
Tonsil	++	ND	+
Ileum	++	ND	ND
Proximal colon	++	ND	ND
Cerebrospinal fluid	+	ND	ND
Pituitary gland	+	ND	ND
Thymus	+	ND	ND
Bone marrow	+	ND	ND
Nasal mucosa	+	ND	ND
Adrenal glands	+	ND	ND
Distal colon	+	ND	ND
Liver	+	ND	ND
Lung	+	ND	ND
Pancreas	+	ND	ND
Ovaries	+	ND	ND
Uterus	+	ND	ND
Skeletal muscle	–	ND	ND
Heart muscle	–	ND	ND
Mammary gland	–	ND	ND
Colostrum	–	ND	ND
Milk	–	ND	ND
Thyroid gland	–	ND	ND
Salivary gland	–	ND	ND
Saliva	–	ND	ND
Kidney	–	ND	ND
Testis	–	ND	ND
Blood clot	–	ND	ND
Serum	–	ND	ND
Feces	–	ND	ND
Skin	–	ND	ND
Placenta	+[b]	–	ND
Amniotic fluid	+[b]	ND	ND

Infectivity titers (log 10 LD_{50}/g or ml of tissues) shown by marks are as follows: +++= >5, ++= >2, += <2, – = no detectable infectivity. Infectivity was tested after intracerebral inoculation or [a] after intraperitoneal inoculation. [b] Infectivity titers not determined. In WB and IH rows, + or – indicates that PrP^{Sc} was detectable or undetectable by Western-blot (WB) and/or immunohistochemistry (IH). (ND) Not done. The data are from the following references: Scrapie infectivity assayed in mice by intracerebral inoculation (Hadlow et al. 1982; Hourrigan 1990; Onodera et al. 1993; Groschup et al. 1996), Western blot detection (Ikegami et al. 1991; Onodera et al. 1993; Race et al. 1992), and immunohistochemical detection (McBride et al. 1988; van Keulen et al. 1996).

al. 1989; Collinge et al. 1991). Studies of the NPU Cheviot sheep *PrP* gene revealed three polymorphisms at codons 136 (A/V), 154 (R/H), and 171 (Q/R) (Goldmann et al. 1991).

In NPU Cheviot sheep, one *PrP* allele (encoding valine at codon 136) is linked to Sip^{sA}, but there are so many Sip^{pA}-related *PrP* alleles (all encoding alanine at codon 136 but differing at other codons) that it has become more precise to use the *PrP* genotype rather than *Sip* terminology. Therefore, in NPU Cheviot sheep, valine at codon 136 (V^{136}) on both PrP alleles (VV^{136}, the old Sip^{sAsA}) is linked to short incubation following s.c. inoculation with SSBP/1, whereas those with longer incubation periods are heterozygotes with one V^{136} allele and one encoding alanine (AV^{136}, the old Sip^{sApA}) (Goldmann et al. 1994). Alanine encoded on both alleles (AA^{136}, the old Sip^{pApA}) is linked with resistance to s.c. challenge with SSBP/1. This linkage of SSBP/1-induced experimental disease with the V^{136} allele was confirmed in USA Cheviots (Maciulis et al. 1992), and so is not specific to the NPU flock.

Incidence of scrapie in NPU Cheviots following challenge with CH1641 or BSE, however, does not associate primarily with codon 136 variation, nor indeed, precisely with the *Sip* gene. Instead, animals encoding glutamine at codon 171(Q^{171}) on both *PrP* alleles (QQ^{171}) succumb to intracerebral (i.c.) inoculation, whereas those having one allele with arginine (R^{171}) have much longer incubation periods (Goldmann et al. 1994). The QQ^{171} animals that develop disease following such challenges could be $A^{(136)}Q^{(171)}/A^{(136)}Q^{(171)}$ and Sip^{pApA} or could be AQ/VQ and Sip^{sApA}, or could be VQ/VQ and Sip^{sAsA}, which emphasizes the need to move away from using "*Sip*" and instead to concentrate on the *PrP* genotypes that are more informative.

Although SSBP/1 targets animals carrying V^{136}, an influence of codon 171 is seen, since AV^{136} animals that are also QR^{171} have a longer incubation than those that are QQ^{171}. Similarly with CH1641 and BSE, although the major effect on incubation period depends on the codon 171 genotype (QQ^{171} being the most susceptible genotype), animals encoding V^{136} (VV^{136} or AV^{136}) have longer incubation periods than AA^{136} sheep (Table 2) (Goldmann et al. 1994).

Characterization of SSBP/1 and CH1641 scrapie has not been possible, as neither transmits well to mice (I. McConnell, pers. comm.), unlike BSE which transmits very easily to several mouse strains (Bruce et al. 1994). SSBP/1 originated from a pool of brains from about eight scrapie-affected positive line NPU Cheviots (therefore all encoding V^{136} but differing at other codons). CH1641 originated from a natural scrapie case in an NPU Cheviot positive line sheep but has subsequently been passaged in negative line (AA^{136}) sheep to provide the inoculum in current use.

Table 2 Sheep *PrP* genotypes of NPU Cheviots and incidence of experimental TSE

Infection source	Route	*Sip* genotype	*PrP* genotype	*n*	Incubation time (days ± S.D.)
SSBP/1	s.c.	sAsA	VQ/VQ	9	170 ± 16
		sApA	VQ/AQ	10	260 ± 15
		sApA	VQ/AR	15	364 ± 17
		pApA	AQ/AQ	>20	survive
		pApA	AQ/AR	>20	survive
		pApA	AR/AR	>20	survive
BSE	i.c.	sAsA	VQ/VQ	ND	–
		sApA	VQ/AQ	2	424, 880
		sApA	VQ/AR	2	1874, 1874
		pApA	AQ/AQ	2	440, 487
		pApA	AQ/AR	3	1886, 1923, 2353
		pApA	AR/AR	ND	–

Data simplified and taken from Goldmann et al. (1994) and W. Goldmann (pers. comm.). *PrP* genotypes are given at codon 136 (A = alanine, V = valine) and 171 (Q = glutamine, R = arginine). (s.c.) Subcutaneous; (i.c.) intracerebral; (ND) not done.

These sheep-passaged sources of scrapie therefore tend to cause disease more readily in animals of similar PrP genotype to that of the sheep that were used to produce the infectious inoculum (W. Goldmann and N. Hunter, unpubl.).

The other two selected flocks of sheep have not produced as much information as the Cheviots but have been able to confirm the results from the larger set of studies. The Herdwick lines, also positive and negative responders to SSBP/1, have similar *PrP* genetics to the Cheviots in that V^{136} is associated with susceptibility (N. Hunter, unpubl.). The Swaledale flock has only a negative line, selected for scrapie resistance using two pools of Swaledale natural scrapie (SW73 and SW75) as inocula (Davies and Kimberlin 1985). SW73 and SW75 were clearly selecting sheep with the same *PrP* genotypes as does SSBP/1, as the prevailing genotypes in the resistant animals are AA^{136}.

In Suffolk sheep, codon 136 has limited polymorphism and the *PrP* V^{136} allele is rare (Ikeda et al. 1995; O'Rourke et al. 1996; Hunter et al. 1997a). Suffolk sheep do, however, succumb to scrapie, and one study in Suffolk sheep in the US demonstrated that orally administered infectious material would cause disease in a proportion of animals (O'Rourke et al. 1997). The inoculum was pooled brain and spleen from scrapie-affected Suffolk sheep. Inoculated animals succumbed to disease in 622 days ±

Table 3 Association of goat *PrP* codon 142 genotype with experimental TSE

Infection source	Route	*PrP* genotype	*n*	Incubation time (days ± S.D.)
BSE	i.c.	II	7	551 ± 29
		IM	2	984, 985
CH1641	i.c.	II	4	396 ± 64
		IM	2	675, 894
spME7	i.c.	II	9	378 ± 45
		IM	2	640, 895

Adapted from Goldmann et al. (1996). (i.c.) Intracerebral, (spME7) sheep passaged ME7 scrapie strain. (I) Isoleucine; (M) methionine.

240 and were all AQ/AQ genotype. Not all AQ/AQ sheep succumbed, but the oral route is known to be less efficient than other routes. No AQ/AR or AR/AR animals developed scrapie.

Concerning goat scrapie, analysis of the caprine *PrP* gene revealed several different alleles. Four PrP variants were found due to variations at codons 142 (I/M), 143 (H/R), and 240 (S/P) (Obermaier et al. 1995; Goldmann et al. 1996). One PrP variant was identical to the most common PrP^{ARQ} sheep variant. The dimorphism at codon 142 appeared to be associated with differing disease incubation periods in goats experimentally infected with isolates of BSE, sheep scrapie CH 1641, or sheep-passaged ME7 scrapie (Table 3). All experiments showed that the change from isoleucine to methionine at codon 142 is associated with increased disease incubation periods. Preliminary analysis indicated that none of four natural scrapie goats carried the M^{142} allele (Goldmann et al. 1996).

Host Control of Natural Sheep Scrapie

Molecular genetic studies on natural scrapie started when it appeared that mutations in the *PrP* gene coding sequence influenced the incubation time of scrapie in mice (Westaway et al. 1987) and caused familial human prion diseases (Hsiao et al. 1989). At the same time, restriction fragment length polymorphism studies on the *PrP* gene from the NPU Cheviot flock showed a strong association between the *PrP* gene and susceptibility to experimental scrapie (Hunter et al. 1989). The cloning of the sheep *PrP* gene (Goldmann et al. 1990) marked the beginning of case-control studies in many countries to look for PrP disease-associated polymorphisms in natural scrapie. Although highly contributive, these case-control studies were sometimes limited by the low number of affected or con-

trol sheep included, often having different geographical origins and/or environmental conditions. These two last points could not take into account the different levels of infectivity pressure. The extension of the *PrP* genotyping to all sheep of the same flock, affected and unaffected, gave the opportunity to estimate and to compare the penetrance of the disease in different contaminated environments and breeding strategies, according to sheep genotypes.

The *PrP* gene coding sequence appeared highly polymorphic, and at least nine different sheep *PrP* allelic variants were described in the last 5 years, including those initially found in the NPU Cheviots, at codons numbers 112 (M/T), 136 (A/V), 137 (M/T), 141 (L/F), 154 (R/H), 171 (Q/R/H), and 211 (R/Q) (Fig. 2) (Laplanche et al. 1992; Belt et al. 1995; Clouscard et al. 1995; Bossers et al. 1996; Hunter et al. 1996). All polymorphisms seemed to be mutually exclusive. Some of them were rare and detected in only a few animals, making it difficult to conclude whether they influenced the development of the disease or not. The three main predicted amino acid substitutions that were demonstrated to play a significant role in both experimental and natural scrapie onset were at codons 136, 154, and 171. They defined five alleles: $A^{(136)}R^{(154)}Q^{(171)}$, VRQ, ARH, AHQ, and ARR. The frequency and distribution of these allelic variants can differ widely from breed to breed and also between flocks of the same breed, depending on the breeding strategy of each country. For

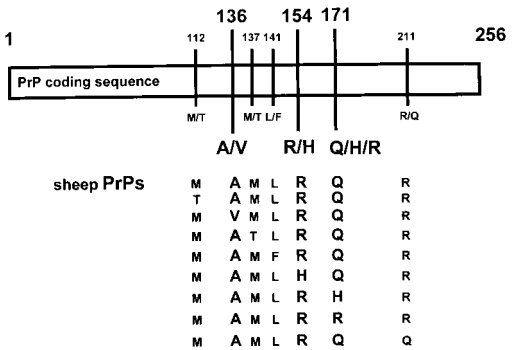

Figure 2 Schematic map of the known polymorphisms in the sheep *PrP* gene coding sequence and predicted sheep PrPs resulting from their combination. Polymorphisms at codons 136, 154, and 171 influence survival times of sheep with experimental or natural scrapie. (A) Alanine; (H) histidine; (F) phenylalanine; (L) leucine; (M) methionine; (A) arginine; (T) threonine; (Q) glutamine; (V) valine.

example, in Suffolk sheep in the US (Westaway et al. 1994; O'Rourke et al. 1996) or Lacaune in France (Clouscard et al. 1995) the VRQ allele is rarely found; the ARR allele has not yet been observed in Icelandic sheep (A. Palsdottir, pers. comm.)

Among the first breeds endemically affected with natural scrapie that were examined for *PrP* gene mutations were French Romanov and Ile-de-France (Laplanche et al. 1992, 1993b). In these two breeds, susceptibility to scrapie appeared highly associated with the V136 allele, which was also evidenced as shortening the survival time of Cheviot sheep subcutaneously inoculated with SSBP/1 (Goldmann et al. 1991). No homozygous Romanov or Ile-de-France VV^{136} sheep were found among their apparently healthy flockmates that were beyond the mean age at onset, nor in an Ile-de-France scrapie-free flock (Table 4), suggesting a complete disease penetrance of scrapie in VRQ/VRQ animals.

The strong association between VRQ and susceptibility to natural scrapie was then found in many other breeds in the UK (Bleu du Maine, Cheviot, Herdwick, Merino x Shetland, Scottish halfbred, Shetland, Swaledale) and the Netherlands (Flemish, Texel Swifter) (Hunter et al. 1993, 1994, 1997a; Belt et al. 1995; Ikeda et al. 1995; Bossers et al. 1996). In all these case-control studies, scrapie-affected groups had a proportion of VRQ/VRQ or ARQ/VRQ sheep higher than the control groups.

This defined the VRQ allele as a susceptibility factor for natural scrapie, as it was also described in experimental scrapie following SSBP/1 inoculation (Goldmann et al. 1991). VRQ/VRQ and ARQ/VRQ genotypes also influenced the survival time of affected animals with a gene dose effect: 77% of the 35 natural scrapie cases in the NPU Cheviot flock between 1986 and 1995 were VRQ/VRQ and died at the mean age of 907 days (range 497–1631), whereas 23% were ARQ/VRQ and died later, at 1462 days (range 1107–2250) (Hunter et al. 1996). A similar effect was also observed by Bossers et al. (1996) in a Flemish and Swifter sheep flock.

The recessive effect of the VRQ allele in V^{136}-coding breeds was reminiscent of the hypothesis made by Parry (1960), who believed that scrapie resulted from a recessive mutation. The 100% penetrance of scrapie observed in sheep homozygous for valine at codon 136 and the development of the disease in VRQ/VRQ and ARQ/VRQ sheep within defined times of birth have led to the question of whether scrapie could be a genetic disease caused by the V^{136} variant. However, Hunter et al. (1997b) have recently described healthy Cheviot sheep carrying the VRQ allele in Australia, a scrapie-free country. Two Cheviot were VRQ/VRQ

Table 4 *PrP* genotype frequencies (%) and natural scrapie

	Breeds with polymorphic *PrP* codon 136				Breeds with limited polymorphism at *PrP* codon 136			
	Ile-de-France		Swaledales		Suffolk		Lacaune	
PrP Genotype	S (20)	H[a] (33)	S (24)	H[b] (48)	S (31)	H[c] (57)	S (58)	H[d] (105)
VRQ/VRQ	35	0	37.5	4	0	0	0	0
ARQ/VRQ	50	3	62.5	15	0	0	14	2
ARQ/ARQ	0	0	0	35.5	100	56	76	9
VRQ/ARR	15	39.5	0	15	0	0	0	0
ARQ/ARH	0	0	0	0	0	0	10	1
ARQ/AHQ	0	0	0	8	0	0	0	2
VRQ/AHQ	0	0	0	4	0	0	0	0
ARQ/ARR	0	12	0	10.5	0	37	0	44.5
AHQ/ARR	0	0	0	2	0	0	0	2
AHQ/ARH	0	0	0	0	0	0	0	1
ARH/ARR	0	0	0	0	0	0	0	4
ARR/ARR	0	45.5	0	6	0	7	0	24.5
AHQ/AHQ	0	0	0	0	0	0	0	0

PrP genotypes are given at codon 136 (A=alanine, V=valine), 154 (R=arginine, H=histidine), and 171 (Q=glutamine, R=arginine, H=histidine). (S) Scrapie-affected sheep; (H) healthy sheep.

Data adapted from Laplanche et al. (1993b and unpubl.), Westaway et al. (1994), Clouscard et al. (1995), and Hunter et al. (1997a).

[a]From a single scrapie-free flock.

[b]Healthy flockmates.

[c]From three scrapie-free flocks.

[d]All the rams used for artificial insemination

and apparently healthy at age 36 and 96 months when sampled, indicating that scrapie is not a spontaneous genetic disease but likely results from an exogenous pathogenic agent. One present view is that there could exist at least one natural strain of the scrapie agent that, when contaminating sheep, would target individuals carrying PrP^{VRQ} with high affinity and mimic the effect of SSBP/1 (that probably contains a PrP^{VRQ}-associated scrapie agent).

The susceptibility of sheep with the ARQ/ARQ genotypes belonging to V^{136}-encoding breeds is an interesting point of debate in view of the characterization of the natural scrapie agent strain properties that affect these breeds. Previous studies focusing on codon 136 in single-flock investigations did not find, or found only at a very low frequency, AA^{136} scrapie-affected sheep (Laplanche et al. 1992; Hunter et al. 1993, 1994). Similarly, ARQ/ARQ NPU Cheviot sheep did not also spontaneously develop scrapie (Hunter et al. 1996). In a study taking into account the

three main *PrP* polymorphisms, this genotype was not found among 83 scrapie-affected sheep belonging to six British different flocks and breeds (Hunter et al. 1997a) although it was reported in diseased Dutch Texel sheep in a percentage (9%, $n = 34$) not different from that observed in the healthy control group (11%, $n = 91$; Belt et al. 1995). However, situations exist in which ARQ/ARQ sheep were severely affected with scrapie. For example, an unexpected scrapie outbreak following an experimental oral challenge with nematode parasites appeared in a French Romanov subflock previously free from scrapie (Clouscard et al. 1995; Laplanche et al. 1996). Not only all VRQ/VRQ ($n = 8$) and 80% of ARQ/VRQ ($n = 15$), but also 77% of ARQ/ARQ ($n = 13$) sheep developed scrapie at ages ranging from 704 to 1232 days (Table 5) (Elsen et al. 1998). The nearly complete penetrance of the disease in these three genotypes, including ARQ/ARQ, could reflect the high level of contamination by the scrapie agent, following the lesions made by the parasite in the gastrointestinal tract. A few months later, the disease spread to the non-parasited flock (NP) that was living in close contact with the other (Laplanche et al. 1996). In this flock, 891 sheep, including 244 scrapie-affected sheep born between 1986 and 1995 that lived at least 1 year in the flock after the outbreak, were genotyped. Sheep carrying VRQ/VRQ genotype appeared the most susceptible, followed by those with ARQ/VRQ and ARQ/ARQ (Table 5) (Elsen et al. 1998). These observations suggest that sheep carrying the ARQ/ARQ genotype would develop scrapie only when exposed to high levels of scrapie infectivity. This might be due to a lower affinity of the scrapie agent for PrP^{ARQ} than for PrP^{VRQ}. Therefore, a high number of diseased ARQ/ARQ sheep observed in a VRQ-encoding breed could reveal a high pressure of scrapie infectivity in the flock.

The extension to new breeds of the molecular screening of the *PrP* gene coding sequence complicated the situation when it appeared that codon 136 was not polymorphic in all breeds (Hunter et al. 1993; Laplanche et al. 1993a), indicating that VRQ was not the sole determinant of scrapie susceptibility. The frequency of the VRQ allele, when present, was particularly low in Lacaune, Manech, and Préalpes (Laplanche et al. 1993a; Clouscard et al. 1995), Suffolk (Westaway et al. 1994; Ikeda et al. 1995; O'Rourke et al. 1996), and Soay (Hunter et al. 1997a). Scrapie-affected sheep from these breeds mainly carried the ARQ/ARQ genotype, which defines it as the major susceptibility factor to scrapie in the breeds with limited polymorphism at codon 136 (Table 4). It is difficult to determine how susceptible to scrapie are the few heterozygous or homozygous VRQ-encoding sheep that could exist. However, there were reports of scrapie in these rare sheep (Clouscard et

al. 1995; Ikeda et al. 1995; Hunter et al. 1997a), indicating that they are also susceptible. Unfortunately, it was not mentioned if their survival time was longer than that of their ARQ/ARQ flockmates, as it was observed when CH1641 (probably a $PrP^{ARQ/ARQ}$-associated agent) or BSE isolates were used for challenging NPU Cheviots (Goldmann et al. 1994; Hunter et al. 1997a).

Of equal, if not more, importance as scrapie susceptibility factors are genetic factors associated with "reduced susceptibility" to the disease. This term seems better than "resistance" as it has not yet been unequivocally demonstrated that apparently healthy animals are not asymptomatic carriers of the agent. Preliminary observations had shown that among AV^{136} Ile-de-France or Romanov sheep living in three contaminated flocks, some animals had a long lifetime, well extended beyond the maximal age of clinical onset (Romanov >3.5 years; Ile-de-France >7 years). It was suggested that this longer survival time was related to their heterozygosity at codon 154 or 171 (Laplanche et al. 1992, 1993b). This observation was then confirmed and extended to many other breeds. Sheep with the AHQ or ARR alleles, in combination with all other possible alleles, were found with a low frequency, or not at all, in different samples of scrapie-affected sheep while they were significantly represented in the corresponding healthy population (Table 4) (Westaway et al. 1994; Belt et al. 1995; Clouscard et al. 1995; Ikeda et al. 1995; Hunter et al. 1996, 1997a; Laplanche et al. 1996).

Analysis of twin pairs from distinct breeds, Lacaune (Clouscard et al. 1995) and Cheviot (Hunter et al. 1996), living in their respective flocks, also indicated that healthy animals were genetically distinct from scrapie cases at the *PrP* gene level, and all had at least one ARR allele.

In Europe and the US, no scrapie case was reported in ARR/ARR sheep and this genotype would apparently determine their resistance to scrapie. This fit very well with the prolonged survival time observed in ARR-encoding sheep, experimentally contaminated with SSBP/1, CH1641, BSE isolates (Goldmann et al. 1991, 1994) or Suffolk-passaged scrapie agent (O'Rourke et al. 1997). Moreover, it should be noted that none of the five Romanov sheep carrying at least one ARR allele developed scrapie after the sudden outbreak following the experimental parasitic infestation described above (Table 5). This point was important as it indicated that ARR-encoding sheep maintained a low susceptibility to scrapie even in the case of an increased exposure to infection. Also, in the main NP Romanov flock, no case has been detected so far in the 217 sheep heterozygous for the ARR allele (Table 5) (Elsen et al. 1998). This confirmed the dominant protective effect of the

Table 5 Sheep *PrP* genotypes of two Romanov flocks and incidence of scrapie

	P flock		NP flock	
PrP genotype[a]	exposed	affected (%)	exposed	affected (%)
VRQ/VRQ	8	8 (100)	66	48 (73)
ARQ/VRQ	15	12 (80)	252	128 (51)
ARQ/ARQ	13	10 (77)	171	67 (39)
ARQ/AHQ	2	0 (0)	89	1 (1)
VRQ/AHQ	5	1 (20)	49	0 (0)
ARQ/ARR	3	0 (0)	93	0 (0)
VRQ/ARR	2	0 (0)	76	0 (0)
AHQ/ARR	0	0 (0)	37	0 (0)
ARR/ARR	0	0 (0)	47	0 (0)
AHQ/AHQ	0	0 (0)	11	0 (0)

(P) Experimentally parasited flock; (NP) non-parasited flock (see text for details). The youngest age at scrapie onset was 352 days. For this reason, only sheep that lived at least 1 year in the flock were considered. (Modified from Elsen et al. [1998] and J.M. Elsen [pers. comm.].)

[a] *PrP* genotype at codons 136, 154, 171.

ARR allele on the scale of a large flock living in a likely heavily contaminated environment.

There is only one report in Japan of a single scrapie-affected ARR/ARR sheep (Ikeda et al. 1995). This sheep was the second scrapie case examined in this country (1982) and died at 4 years and 3 months. PrP^{Sc} was detected in its brain and the disease was successfully transmitted to mice. This observation could reflect the existence in Japan of scrapie strains different from those in Western Europe or the US, but the sheep might also be different genetically. Hunter et al. (1996) have noted that in the Japanese Suffolk breed, R^{171} was linked to a particular series of restriction fragment length polymorphisms not found in British sheep (Muramatsu et al. 1992).

The AHQ allele often had a low frequency in most of the studied breeds, making it difficult to firmly establish whether it could also reduce scrapie sheep susceptibility, as is often suggested. However, in the NP Romanov-affected flock, only 2 ARQ/AHQ out of 186 carriers of the AHQ allele and 1 out of the 5 VRQ/AHQ sheep belonging to the parasited flock have developed scrapie so far (Table 5) (Elsen et al. 1998). These observations showed that, at least in this breed, the AHQ allele was also, like ARR, a reduced susceptibility allele and could overcome the effects of the VRQ and ARQ susceptibility alleles. However, it should be noted that there is no information about the possible carrier state of the ARQ- or AHQ-harboring sheep.

Epidemiological molecular data from different breeds living in different geographic and environmental conditions are now available. A general trend can be drawn from these data. It seems that sheep breeds can be divided into two groups according to the polymorphism of the codon 136 of their *PrP* gene. Breeds encoding V^{136} appear susceptible to strain(s) that target with higher affinity PrP^{VRQ} than PrP^{ARQ}, whereas breeds with limited polymorphism at codon 136 would be infected by strain(s) that target both PrP^{ARQ} and PrP^{VRQ} sheep with a nearly identical affinity. In both groups, it is interesting to note that the same genetic determinant of reduced susceptibility could play a role in disease prevention, i.e., in PrP^{ARR} individuals, even in a heavily contaminated environment.

Finally, recent experiments by Bossers et al. (1997) on the cell-free conversion of different ovine PrPs into PrP-res have elegantly reproduced in vitro what could happen in vivo. It was shown that PrP^{ScVQ} or PrP^{Sc} enriched from a ARQ/VRQ sheep brain (presumably containing mostly PrP^{ScVRQ}) converted three times more efficiently $PrP^{C\text{-}VRQ}$ than $PrP^{C\text{-}ARQ}$. PrP^{ScARQ} alone also converted both proteins but with equal efficiency. Consistently with the observed protective effect of the ARR allele in vivo, $PrP^{C\text{-}ARR}$ was poorly converted by the three previous PrP^{Sc}.

Other polymorphisms in the *PrP* gene or other undefined genes could perhaps modulate sheep susceptibility to scrapie and explained why some animals with susceptible genotypes (except VRQ/VRQ which all died in contaminated environment) escape from the disease. Data remain very limited: sheep major histocompatibility (OLA) complex has been linked to one locus of susceptibility/resistance to the disease in a single Ile-de-France flock (Millot et al. 1988), but the results were disputed by Cullen (1989) and could result from a founder effect. Moreover, the analysis for variation in the Class I ovine lymphocyte antigens in the naturally scrapie-affected NPU Cheviot flock has brought no information, resulting from inbreeding as the flock has been closed since 1962 (Hunter et al. 1996).

Natural Transmission of Scrapie

It is likely that scrapie is caused by an infectious agent whose spread is encouraged by close contact between animals (Brotherston et al. 1968). There are at least four potential routes for natural infection: ingestion of infected material, entry through skin abrasions, maternal transmission from ewe to lamb, and contact with an intermediate host or vector. Because of the apparent early involvement of the alimentary tract in one form of Suffolk sheep scrapie, an oral route of transmission was postulated (Hadlow et al. 1982). This early study has more recent support from

the finding of PrP^{Sc}, a marker for infectivity, in sheep tonsil at early preclinical stages (Schreuder et al. 1996). Pattison (Pattison et al. 1972; Pattison 1974) demonstrated that it was possible to produce scrapie in Herdwick sheep by feeding them placental material from scrapie-affected Swaledale ewes, and thus the idea of oral transmission of scrapie via ingested infected placental tissue was put forward. There is more recent evidence that scrapie can be transmitted from placental tissue samples (Onodera et al. 1993), but there have also been transmission failures. Contamination of pasture as a source of infection is also supported by Icelandic scrapie studies (Palsson 1979) and by the work of Greig (Greig 1940a,b, 1950) and could be quite long-lived because of infamous resistance of scrapie to inactivation. Such a source of infectivity could enter the body of a sheep through abrasions or cuts in the skin. The scarification route has not been explored experimentally in sheep, although the unpublished work of D.R. Wilson (Dickinson 1976) in the 1950s suggested that it was possible to inoculate sheep successfully with scrapie using scarification. Skin scratches have also been shown to be effective routes by which to experimentally infect mice with scrapie, and with much the same efficiency as inoculation by intraperitoneal, intravenous, or perivenous routes (Taylor et al. 1996).

It is part of the dogma of scrapie that the disease in sheep is maternally transmitted with the placenta as the prime suspect that brings scrapie infectivity in utero to the developing lamb. Telling the difference between genuine maternal transmission and lateral transmission by close contact during the perinatal period is difficult, and it is not always possible to know when an animal has acquired its infection. However, in a natural scrapie outbreak in the NPU Cheviot flock (Hunter et al. 1996 and unpubl.), it seems likely that most animals were infected at about the same age. The ages at death from scrapie were constant, with VRQ/VRQ animals dying at 700–900 days of age and heterozygotes ARQ/VRQ dying at 1100–1200 days of age. This difference between homozygotes and heterozygotes, expected from the experimental data, is not always seen in other flocks (Hunter et al. 1994). However, the survival of three VRQ/VRQ animals to more than 1900 days in this flock after stringently clean procedures were used around the time of their birth suggests that the perinatal period is a dangerous time for a susceptible lamb. This period is now being further investigated by comparing caesarian-derived and bottle-fed lambs with naturally birthed animals.

Embryo transfer (ET) procedures have also been investigated in the hope of bypassing any infection cycle. Although work with the NPU Cheviot flock was compromised by natural scrapie in the flock (Foster et

al. 1992, 1996b), it is clear that ET procedures coupled with "over the top" cleanliness at birth do not prevent scrapie from occurring in all lambs. These experiments generated the three surviving VRQ/VRQ animals discussed above but also generated 10 VRQ/VRQ sheep that did develop scrapie (9 at mean age 826 ± 24 days of age and one at 1267 days of age). This group of 10 animals was not protected by ET procedures even following International Embryo Transfer Society (IETS) protocols (Stringfellow and Seidel 1990). A similar experiment set up in the US (Foote et al. 1993) is difficult to interpret because of the lack of *PrP* genotype information. However, one of the groups involved Cheviot sheep inoculated with SSBP/1 scrapie, and it has already been shown that these animals give similar incubation periods to NPU Cheviots and SSBP/1 (Maciulis et al. 1992).

The incubation periods in scrapie-inoculated donors and recipients ranging from 6.1 months (183 days) to 14.9 months (447 days) plus survivors to 25 months (750 days) suggest that a mixture of *PrP* genotypes is present in the group. Of the positive control lambs (gestated and born naturally), 2 out of 9 developed scrapie at 31 months (930 days) and 49 months (1470 days) of age, whereas none of the 22 ET-derived Cheviot lambs succumbed to scrapie with ages at death ranging from 74.5 months (2235 days) to 96 months (2880 days). It is of course possible that there were no susceptible lambs among the ET group, but there is at least a suggestion that ET procedures have protected lambs from scrapie in this case, and this again implicates the possibility of maternal transmission as a route for scrapie transmission. Further experiments are being carried out to clarify the situation.

Finally, a number of intriguing reports suggest that scrapie infection may be spread by an intermediate host or vector. Nematodes taken at early stages from experimentally scrapie-infected animals were ground up and injected into various animals, resulting in no disease transmission (Fitzsimmons and Pattison 1968). However, an inadvertent experiment seemed to successfully transmit scrapie after live nematode infestation, quite a different and more natural situation (Clouscard et al. 1995 and above). In this latter study, sheep of QQ^{171} genotype succumbed to scrapie with survival times (after nematode challenge) that looked remarkably like those found in experimental challenges.

Hay mites have also been considered as scrapie vectors, this time by the oral route with sheep picking up infection by eating the mites along with hay (Wisniewski et al. 1996). Proteinase K-resistant PrP was apparently found in mites taken from scrapie-affected Icelandic farms, and injection of the mites into mice produced a few positive transmissions.

However, perhaps the most likely vector for scrapie is sheep themselves. The possibility that resistant animals can harbor subclinical infection, which can be passed on to more susceptible animals, is at the moment under intense investigation.

CHRONIC WASTING DISEASE AND TRANSMISSIBLE MINK ENCEPHALOPATHY

Chronic Wasting Disease

Epidemiology of CWD

CWD is a prion disease of captive and free-ranging mule deer (*Odocoileus hemionus*), white-tailed deer (*Odocoileus virginianus*), and Rocky Mountain elk (*Cervus elaphus nelsoni*) (Fig. 3) (Williams and Young 1992; Spraker et al. 1997). The clinical syndrome of wasting and eventual death was first recognized in the late 1960s by biologists working with captive mule deer in Colorado who observed the complex of clinical signs associated with the syndrome they designated chronic wasting disease. It was initially thought to be associated with stresses of captivity, nutritional deficiencies, or intoxications. CWD in mule deer was first recognized as a spongiform encephalopathy in 1977 (Williams and Young 1980). This was followed in the early 1980s by recognition of CWD in captive and free-ranging elk (Williams and Young 1982, 1992). By the mid-1980s, CWD was found in free-ranging mule deer in Colorado and Wyoming, and in 1990 the host range of CWD was expanded to include white-tailed deer. These species are the most common wild cervids (members of the deer family) in North America.

CWD is endemic only in north central Colorado and southeastern Wyoming (Fig. 4). Knowledge of the distribution of CWD in Wyoming

Figure 3 Wild cervids. (*A*) Mule deer (*Odocoileus hemionus*); (*B*) white-tailed deer (*Odocoileus virginianus*); (*C*) elk (*Cervus elaphus nelsoni*).

Figure 4 Map of US showing areas where CWD has been diagnosed.

and Colorado is based on decades of active monitoring of captive and free-ranging wildlife, including cervids, for naturally occurring diseases (Thorne et al. 1982). No cases of CWD in free-ranging deer or elk have been identified outside the known endemic area. CWD has been diagnosed in cervids in three zoological gardens that received animals directly from wildlife facilities in Wyoming or Colorado. After affected animals died, no additional cases of CWD were recognized on these premises.

A single case of CWD was diagnosed in a game farm elk from Saskatchewan, Canada, and the herd was depopulated. The affected animal had been imported from a captive herd in South Dakota; there was no documented tie to Colorado or Wyoming and the origin of the disease in that animal remains obscure. Recently, CWD occurred on two commercial elk farms in South Dakota; the epidemiologic investigations are not yet completed.

The mode of transmission of CWD is not known. Lateral transmission appears to occur in captivity, and population modeling suggests the prevalence of CWD could not be maintained at detected levels unless lateral transmission were occurring (M. Miller and C. McCarty, pers. comm.).

Since 1983, and with much greater intensity since 1992, surveillance for CWD in hunter-harvested deer and elk has been conducted in the endemic area to develop prevalence estimates. Brains were examined by routine microscopy and, beginning in 1995, by immunohistochemistry; selected cases were also examined by negative-stain electron microscopy

for scrapie-associated fibrils and by Western blot analysis. Estimated prevalences from harvest surveys were <1.1% in elk (1992–1996; n = 337) and about 2.5% in deer (1983–1996; n = 687; range 0–5.9% annually) in the endemic area (M. Miller, pers. comm.; E. Williams, unpubl.); none of >300 samples from cervids harvested outside endemic areas in Colorado and Wyoming was positive for CWD. Hunter-harvest surveillance is being expanded in the endemic area to include larger numbers of harvested animals and improve prevalence estimates. In addition, the surveillance area is being expanded within Wyoming and Colorado and other states in the US and provinces in Canada to better define the distribution of CWD. Initial surveillance activities during 1996 by examination of brains from hunter-harvested deer and elk from other western states were negative for CWD.

Clinical Presentation of CWD

Clinical signs of CWD in captive cervids have been described previously (Williams and Young 1992). The majority of CWD-affected animals are 3–5 years of age. Natural incubation period is not precisely known, but the youngest animal diagnosed with the disease was 17 months of age. Maximum incubation period has not been determined but the oldest animal with CWD was >15 years of age. As suggested by its name, the most prominent clinical sign of the disease is loss of body condition that pro-

Figure 5 Female elk with CWD. Notice the lowered head and ears, depressed demeanor, and poor body condition.

gresses to a state of emaciation if the disease is allowed to run its course (Fig. 5). Characteristic behavioral changes include alteration in behavior toward humans, changes in interaction with other members of the herd, stupor, walking repetitive patterns, and rarely hyperexcitability. Polydipsia and consequent polyuria are frequently present. Hypersalivation and difficulty swallowing may occur. A smaller percentage of affected animals, especially elk, display ataxia, incoordination, and/or fine head tremors. Pruritus has never been observed. Duration of clinical disease is usually protracted, but a few white-tailed deer have died acutely or after only a few days of recognizable illness. Pneumonia, often aspiration pneumonia, may be the actual cause of death in affected animals that live out the complete clinical course.

Pathological and Biochemical Diagnosis of CWD

Morphologic pathology of CWD has been described previously (Guiroy et al. 1993, 1994; Williams and Young 1993). Spongiform lesions are most striking in the medulla oblongata, especially the parasympathetic vagal nucleus; thalamus and hypothalamus; and olfactory cortex. Prominent lesions in the medulla oblongata at the obex are found in all cases of CWD, and examination of this region is used for surveillance and diagnostic purposes. PrP plaques surrounded by spongiform change are prominent in some cases of CWD (Williams and Young 1993), particularly in white-tailed deer or white-tailed × mule deer hybrids (Fig. 6) (E. Williams, unpubl.). These plaques morphologically resemble "florid plaques," which are a prominent feature of nvCJD and BSE in macaques (*Macaca mulatta*) (Lasmézas et al. 1996; Will et al. 1996), but the distribution of the plaques in CWD-affected cervid brain is considerably different, generally sparing the cerebellum, which is extensively affected in primates. SAFs are readily identified in brains and spleen of clinically affected deer and elk (Spraker et al. 1997; E. Williams, unpubl.). Techniques and antisera developed for scrapie research and diagnostics (Rubenstein et al. 1986; Miller et al. 1993) have been adapted for use in diagnosis of CWD. Immunohistochemistry (Guiroy et al. 1991a,b) and Western blotting for PrP-res in brains of affected and some presumably preclinical deer and elk are used for diagnostic, surveillance, and research purposes.

The relationship of the CWD prion and those of other transmissible spongiform encephalopathies is not yet clear. Strain typing by incubation period and lesion profiles in genotypically characterized mice has shown

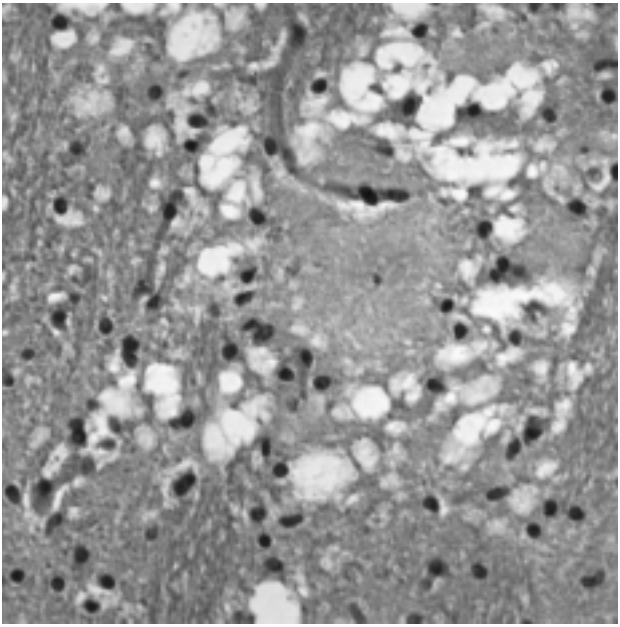

Figure 6 Amyloid plaque from the medulla oblongata of a mule deer with CWD. Note the marked spongiform change at the periphery of the plaque.

the CWD agent differs from the BSE agent (Bruce et al. 1997). The CWD agent also differs from all strains of scrapie tested in this system thus far (M. Bruce, pers. comm.).

Genetics

The genetics of CWD susceptibility is being studied, and only preliminary information is currently available. Sequencing of the *PrP* genes shows marked homology between mule deer, white-tailed deer, and elk (Cervenakova et al. 1997; R. Rohwer, pers. comm.) with an amino acid polymorphism recognized at codon 138 (serine or asparagine) of mule deer (Cervenakova et al. 1997; K. O'Rourke, pers. comm.). Sequences of the *PrP* gene of three captive elk from Germany (Schätzl et al. 1997) identified leucine at codon 129; Cervenakova et al. (1997) reported methionine at this location. In a larger sample of elk from Colorado, codon 129 was found to be polymorphic (leucine or methionine) (K. O'Rourke, pers. comm.). The relationship of these polymorphisms to CWD susceptibility has yet to be determined.

Transmissible Mink Encephalopathy

Epidemiology of TME

TME is a rare sporadic disease of ranched mink. It was first recognized in 1947 in Wisconsin and Minnesota; outbreaks occurring in the 1960s in Wisconsin were epidemiologically associated with consumption of feed of bovine or ovine origin (Hartsough and Burger 1965). Additional outbreaks have been identified in Idaho, Canada, Finland, East Germany, and Russia (Hadlow and Karstad 1968; Marsh 1976). The most recent occurrence of TME was in 1985 in Stetsonville, Wisconsin (Marsh et al. 1991). The origin of TME was hypothesized to be from ingestion of scrapie agent. Epidemiologic investigation failed to find evidence of exposure of the mink to sheep products. The Stetsonville incident was specially interesting because the rancher was a dead stock feeder who used mostly dairy cows collected around his ranch. This led to the suggestion that TME may arise by exposure to an unrecognized scrapie-like disease of cattle (Marsh et al. 1991). No such disease has been recognized in North American cattle.

Clinical Presentation and Pathology of TME

The clinical signs and features of TME were summarized by Marsh (1976). Only adult minks are affected by TME, and the minimum incubation period is 7 months with a maximum incubation period of 12 months observed in two outbreaks (Marsh 1976). Mortality may be nearly 100% of adult animals. Clinical signs of TME are characterized by behavioral alterations that include confusion, loss of cleanliness, and aimless circling. Affected animals lose weight, may develop matted fur, experience hind-quarter ataxia, and carry the tail arched over the back. Somnolence and progressive debilitation occur until death after a clinical course of 6–8 weeks.

Lesions of TME in mink are typical of other spongiform encephalopathies, although the distribution is somewhat different than in ruminants (Eckroade et al. 1979; Hadlow 1996). Spongiform degeneration involves the cerebral cortex, telencephalon, diencephalon, and mesencephalon with decreasing intensity in the caudal portions of the brain. No plaques are visible.

Transmission Studies

Mink inoculated intramuscularly, or fed brain tissue from animals affected with the Wisconsin source of TME, developed TME-like disease in 5 and 7 months, respectively. TME has also been successfully transmitted to other species (ferret, hamster, nonhuman primates, sheep, goat, and cattle).

TME was not reproduced in mink via oral exposure to various strains of sheep scrapie, although mink were susceptible by intracerebral route to certain sheep scrapie sources (Hanson et al. 1971; Marsh and Hanson 1979), showing that different sources of the agent can vary in their pathogenicity for mink. Interestingly, scrapie agent from naturally infected Suffolk sheep that was passaged three times in mink became nonpathogenic for mice (Marsh and Hadlow 1992). Relatively rapid incubation (15–25 months) and development of severe spongiform encephalopathy were found in cattle inoculated intracerebrally with TME agent (Marsh et al. 1991; Robinson et al. 1995), suggesting lack of a significant species barrier. In addition, bovine-passaged TME was highly pathogenic for orally infected mink, with incubation of 7 months (Marsh et al. 1991).

The mink *PrP* gene was cloned in 1992 (Kretzschmar et al. 1992). At the amino acid level, the greatest interspecific similarities are observed between the mink, sheep (97, 7%) and bovine (96, 9%) *PrP* sequences (Bartz et al. 1994). No information is available about possible mink PrP polymorphisms and their role in TME susceptibility.

Molecular Characterization of Hamster-adapted TME Agent Strains

Comprehensive biological characterization of the TME agent has been conducted by Marsh and colleagues. Through series of experimental infections in hamsters, mink, and primates, multiple strains of TME agent were identified from single outbreaks (Marsh and Hanson 1979; Bessen and Marsh 1992). Two TME strains from the Stetsonville outbreak produced distinctly different clinical syndromes in Syrian hamsters and had distinct brain titers. PrP from hamsters infected with these strains, called "drowsy (DY)" and "hyper (HY)" strains, differed in sedimentation in *N*-laurosylsarcosine, sensitivity to and differences in protein sequencing after proteinase K digestion, migration on polyacrylamide gels, immunoreactivity, and targeting in the brain, which suggested that the PrP-res protein structure determines strain variation (Bessen and Marsh 1992, 1994). Bessen et al. (1995) showed that PrP-enriched preparations from HY- or DY-infected animals were sufficient for converting PrP^{C} to PrP^{HY} or PrP^{DY}, demonstrating strain-specific conversion of PrP^{C} to PrP-res in vitro.

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Bovine Spongiform Encephalopathy and Related Diseases

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In Great Britain, a previously unrecognized neurologic disease in cattle was first defined in 1986, on the basis of a constellation of clinical signs associated with characteristic pathologic changes in the brain (Wells et al. 1987). There was the insidious onset of altered behavior (either fear or sometimes aggressive responses), ataxia (incoordinated gait with falling), and dysesthia or reflex hyperesthesia (abnormal responses to touch and sound). The relentless progression of these symptoms made it impossible to handle animals, requiring slaughter within 1–6 months (Wilesmith et al. 1988). Histologically, the brain exhibited spongiform lesions and astrogliosis (Wells et al. 1987). It was immediately recognized (Wells et al. 1987) that the lesions were similar to those characteristic of transmissible spongiform encephalopathies (TSEs) or prion diseases in other species, specifically scrapie of sheep, the prototype of the TSEs. In retrospect, cases of BSE had been seen in England as early as 1985, but probably not before that year. This new disease was made statutorily notifiable (Anonymous 1996a; Wilesmith 1996a) and the numbers of cases continued to increase each month, indicating the onset of a major epidemic (Fig. 1).

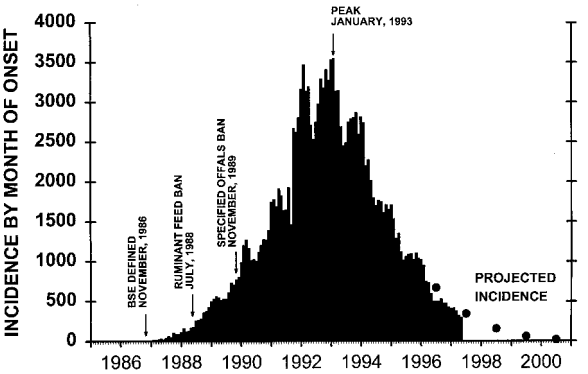

Figure 1 BSE by month of onset, UK, 1985 to April, 1995, and projections for 1996–2000. Reported cases from Anonymous (1996b, 1997a) and projections from Anderson et al. (1996).

EPIDEMIOLOGY OF BSE

The United Kingdom

The advent of a new prion disease in epidemic form led to a detailed investigation of its possible cause. An important clue was offered by mapping of the cases occurring within the first 18 months of the outbreak (Wilesmith 1991), which showed that they were widely distributed throughout much of England. The same pattern is reflected in Figure 2, which shows that the epidemic began almost simultaneously in each of 10 regions in Great Britain (Donnelly et al. 1997b). This is the classic pattern of a common source rather than a propagated epidemic and led to the search for a common source (Wilesmith et al. 1991). A series of case studies explored numerous possible sources (Table 1) and suggested that one common exposure was the use of a dietary protein supplement, meat and bone meal (MBM), that was regularly fed to dairy cattle beginning at weaning. A comparison of dairy with beef herds (Fig. 3) showed a strikingly higher incidence of disease in dairy herds, consistent with the possible role of MBM, which is fed in larger amounts to dairy than to beef cattle. Another clue was provided (Wilesmith et al. 1991) by the age at onset of animals in the first wave of BSE cases; most of these cases were 3–5 years old (Fig. 4). If it is assumed that exposure to a putative causal agent began shortly after birth, which is consistent with the initiation of MBM feeding, then it may be inferred that exposure began in the early 1980s. A computer simulation, based on the early incidence of the epidemic, estimated 1980–1981 as the date of first exposures (Wilesmith et

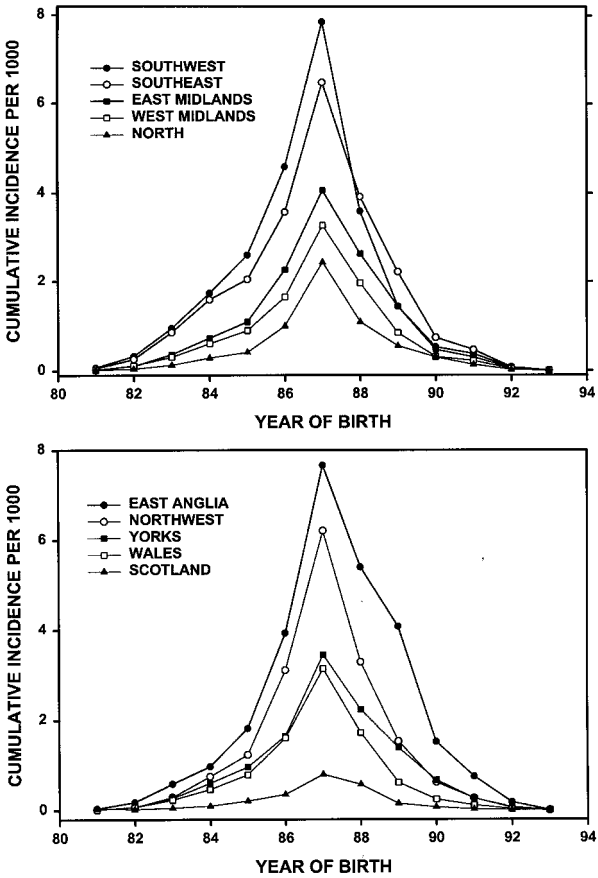

Figure 2 Cumulative BSE incidence by year of birth for 10 geographic regions of Great Britain. The data are shown in two panels for clarity. (Modified from Donnelly et al. [1997b] based on tabulations provided by C.A. Donnelly that were developed from data collected by the Central Veterinary Laboratory.)

al. 1991), and this is borne out when cases are tabulated by year of birth (Table 2).

What could explain the date of initial transmission of BSE, and how does this relate to the possible incrimination of MBM? MBM is manufactured in rendering plants, of which there were about 45 in Great Britain in the mid 1980s. These plants convert slaughterhouse refuse (offal) into two products: tallow (fat) and a defatted mixture of MBM. The process involves mixing, heating with steam, milling, and extraction with hydrocarbon organic compounds that act as fat solvents while precipitating the

Table 1 Risk factors among 169 early cases of BSE, UK, 1986

Risk factor	Number of cases
MBM dietary supplement	169
Dictocaulus viviparus vaccine	82
Leptospira hardjo vaccine	8
Salmonella dublin vaccine	5
Clostridia species vaccine	18
Viral respiratory vaccines	5
Bacterial antisera	9
Hormones	49
Pyrethroid insecticides	6
Organophosphorus insecticides	121

Data from Wilesmith et al. (1991).

protein. The organic solvent extraction process demanded a high energy input, and the increase in fuel prices during the 1970s made this process inefficient. This, together with a loss of the differential price between tallow and MBM, meant that extracting tallow from greaves (partially rendered offal), resulting in a fat content of about 1% in the MBM, was uneconomic. Other factors responsible for the decline in the use of the organic solvent extraction process were the increase in energy density provided by residual fat in MBM and the introduction of more stringent health and safety measures in industrial processes. The proportion of MBM processed with fat solvents fell from at least 70% in the mid 1970s to about 10% in the early 1980s, concurrent with the postulated first

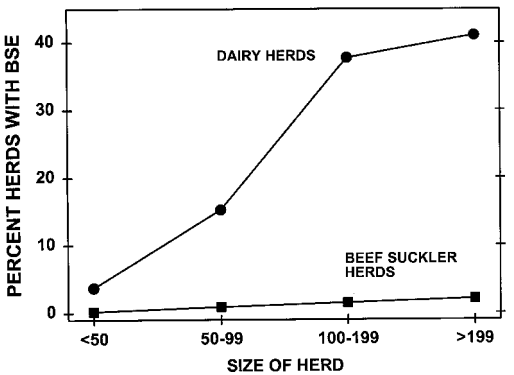

Figure 3 Frequency of BSE among cattle herds in the UK, by herd size, for dairy and beef cattle. Data taken from Wilesmith (1991).

Table 2 Confirmed cases of BSE by year of birth, UK, 1976–1993

Year	Percent of maximum year
1976	<0.1
1977	0.1
1978	0.1
1979	0.2
1980	0.7
1981	1.5
1982	3.6
1983	16.4
1984	30.8
1985	39.2
1986	68.1
1987	100.0
1988	88.2
1989	24.0
1990	9.3
1991	4.1
1992	0.6
1993	<0.1

Data were corrected for birth seasonality, and incidence is probably incomplete for cases with births 1989–1993, since some of these cases will have onsets through 1998. (Modeled after Donnelly et al. [1997b], based on tabulations provided by C.A. Donnelly that were derived from data provided by the Central Veterinary Laboratory.)

transmissions of BSE to cattle (Wilesmith et al. 1991). In the 1980s, there was also an increase in the use of MBM as a dietary supplement relative to other sources of protein, such as soya and fishmeal, because of the escalating costs of the latter (Taylor and Woodgate 1997).

At this point it is necessary to note the physical properties of prions, the agents of the TSEs, mainly based on studies of scrapie of sheep (Ernst and Race 1993; Taylor 1993; Taylor et al. 1994). Prions consist of a single protein, designated PrP (prion protein), which is attached to cellular lipid membranes by a glycosyl phosphatidylinositol (GPI) anchor. The infectivity of prions is notoriously resistant to heat (including steam under pressure) and treatment with some harsh denaturing agents, such as formaldehyde, but can be inactivated by lipid solvents (Ernst and Race 1993). It may be postulated that if petroleum distillates are used in preparing MBM, they would substantially reduce the infectivity of any prions in

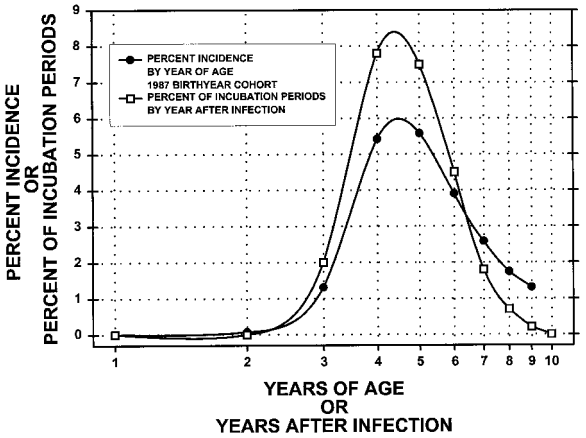

Figure 4 Age-specific incidence of BSE cases born in 1987 (with onsets in 1989–1996) and estimated distribution of incubation periods in years. The incubation period computation takes into account estimates of the age of transmission and corrects for truncation of the observations due to slaughter of a high proportion of cattle at ages 1.5–6 years. Incidence data from Wilesmith et al. (1988) and Anonymous (1997a) and incubation period data from Anderson et al. (1996), Donnelly et al. (1997b), and Ferguson et al. (1997a).

the raw tissues used by rendering plants, but that infectivity could survive heating in the absence of solvents (Safar et al. 1993; Taylor 1996).

Another critical fact is that there is a large sheep population in Great Britain (GB), so that ovine waste constitutes a substantial part of the offal treated by rendering plants. Furthermore, in GB, scrapie is enzootic in the ovine population at a relatively high prevalence roughly estimated at about 2 cases per 1000 sheep (Morgan et al. 1990). These considerations led to the hypothesis that the scrapie agent had always been present in slaughterhouse offal but was inactivated during the production of MBM. With the change in rendering practices in the late 1970s, it is postulated that inactivation became less effective, leading to the contamination of some batches of MBM beginning about 1980 (Wilesmith et al. 1988, 1991; Taylor 1989b, 1996).

Circumstantial evidence in support of this hypothesis is provided by studies of the geographic distribution of BSE in GB (Table 3). There was a marked gradient, with highest cumulative incidence in southern England decreasing toward the north, with the lowest rates in Scotland. MBM was produced in about 45 rendering plants in GB and most of the product was distributed locally. A survey revealed (Wilesmith et al. 1991)

Table 3 Geographic differences in BSE in GB, 1986–1989, and relationship to the production process used by rendering plants

Region	Dairy herds with BSE (%)	Use of solvents to process MBM	MBM from greaves (%)
Southern England	12.6	NO	0.2
Midlands	3.9	NO	8.6
Northern England	2.8	NO	25.5
Scotland	1.8	YES	39.0

Data from Wilesmith et al. (1991).

that in the late 1980s rendering practices differed in different regions, with the use of petroleum solvents and reprocessing of greaves inversely related to BSE incidence (Table 3).

Transmissible Spongiform Encephalopathy in Other Species

Throughout the BSE epidemic, there have been a total of 28 cases (through December 1997) of spongiform encephalopathy diagnosed in captive wild exotic animal species that had not been previously subject to

Table 4 Transmissible spongiform encephalopathy in zoo animals, 1986–1997

Species	Number of cases
Bovidae	
Ankole	2
Arabian oryx	1
Eland	6
Gemsbok	1
Kudu	6
Nyala	1
Scimitar-horned oryx	1
Bison	1
Felidae	
Cheetah	3
Ocelot	2
Puma	3
Tiger	1

Modeled after Kirkwood and Cunningham (1994), Taylor and Woodgate (1997), and G. Wells (unpubl.).

such diseases (Table 4) (Kirkwood and Cunningham 1994; Anonymous 1997a; Taylor and Woodgate 1997; G. Wells, unpubl.). Affected species are confined to two phylogenetic families: the bovidae and felidae. The bovid species in which cases have occurred were all fed MBM as a dietary supplement in a manner similar to domestic cattle. Captive wild felids were fed bovine carcass material, including spinal cord, on a regular basis (Kirkwood and Cunningham 1994).

In addition, there has been a total of 84 cases of spongiform encephalopathy diagnosed in domestic cats (through December 1997); the first with onset of clinical signs in 1989 (Wyatt et al. 1991; J. Wilesmith and G. Wells, unpubl.). Domestic cats are likely to have been fed pet foods containing bovine tissues and MBM (J. Wilesmith, unpubl.). In 1989, prior to the diagnosis of the first case of feline spongiform encephalopathy in 1990, the manufacturers of pet foods instituted a ban on the inclusion of certain bovine offals, including spleen, brain, and spinal cord. Through December 1997 all but one or two of the reported cases in domestic cats were in cats born prior to the time of the ban, suggesting that the epidemic has been almost completely controlled by the exclusion of specified bovine offals. TSE in domestic and wild feline species may have provided the first evidence of the contamination of beef carcasses and bovine tissue with infectious prions.

Incubation Period

Figure 4 shows the age-specific incidence of BSE cases for a cohort born in 1987, the birth cohort with the highest incidence (Table 2). Determination of the incubation period of BSE is complicated by several problems. First, it is difficult to determine the exact age of transmission of BSE for individual animals. The low rate of BSE even in herds with the highest incidence indicates that transmission was a relatively infrequent event, from which it may be inferred that transmission did not necessarily occur as soon as cattle were weaned and began to consume potentially contaminated MBM. Second, the high rate of slaughter of cattle, particularly between ages 1.5 and 6 years, means that no intact cohort could be followed throughout the incubation period. Although this is somewhat corrected by using age-specific incidence, it must be assumed that living cattle are representative of the total population. Finally, age-specific data in Figure 4 were limited to the cattle less than 10 years of age. Thus, the age-specific incidence is a poor surrogate for incubation period. A statistical analysis by Anderson and colleagues (Anderson et al. 1996) took these confounding variables into account and produced the distribution of

incubation times reproduced in Figure 4. The mean incubation period was estimated at about 5 years with a variance of about 1.6 years.

BSE in Europe

BSE has occurred in a number of European countries but the reported incidence has been much lower than in GB. It is likely that the higher incidence was due to the concatenation of several circumstances, some of which occurred only in GB. These include a high ratio of sheep to cattle, a high enzootic prevalence of scrapie in sheep, the intensive feeding of MBM to dairy cattle, and the changes in rendering practices. A comparison of BSE in GB and Switzerland illustrates these differences. The cumulative incidence of BSE in Switzerland is about 100-fold lower than in GB (Table 5). There are three rendering plants in Switzerland that supply MBM to the feedmill industry. A comparison was made of the relative risk of scrapie contamination of slaughterhouse waste produced in the two countries, based on the ratio of sheep to cattle and the relative prevalence of scrapie in sheep (Hörnlimann et al. 1996). If this relative ratio is normalized to 1 for GB, it is estimated at 0.002 for Switzerland, indicating that the likelihood of scrapie entering rendering plants was much lower in Switzerland (Table 5). The BSE outbreak in Switzerland occurred about 3 years later than the outbreak in GB (Fig. 5). Since the Swiss imported considerable amounts of MBM from other countries, it is likely that most

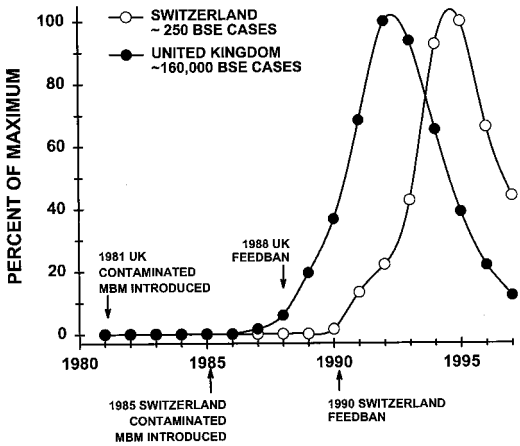

Figure 5 Comparison of BSE epidemic curves in the UK and Switzerland based on dates of onset. Data from Anonymous (1997a) and Swiss Federal Veterinary Office, with extrapolation for 1997.

Table 5 Comparison of UK and Switzerland to show differences in BSE incidence and differences in potential scrapie contamination of slaughterhouse offal

Parameter	United Kingdom	Switzerland
Cattle population	~12,000,000	~1,700,000
Cumulative BSE reported through May, 1996	~160,000	221
Cumulative incidence per 1,000,000[a]	~13,300	~125
Cumulative incidence: relative ratio	1	~0.01
Sheep population	~29,000,000	450,000
Ratio: sheep to cattle	2.4	0.26
Estimated prevalence of scrapie per 1,000,000 sheep	2,250	40
Estimated scrapie contamination of offal: relative ratio[b]	1	0.002

Based on an analysis of BSE risk factors by Hörnlimann et al. (1996). Cattle and sheep population in GB after FAO handbook (1994). Cattle and sheep population in Switzerland after Swiss Federal Veterinary Office (1996). BSE in GB after Wilesmith et al. (1996a,b). Scrapie in GB after Morgan et al. (1990).

[a]This estimate does not take account of the turnover of the population.

[b]Relative ratio calculated as the product of the ratio of sheep to cattle multiplied by the estimated prevalence of scrapie, normalized to 1 for GB.

of the BSE cases were due to imported rather than domestically manufactured protein supplement (Hörnlimann et al. 1994; Butler 1996). This inference is supported by studies of the transmission of BSE to mice which indicate that isolates from Swiss cows produced lesions identical to those produced by UK isolates of BSE (Taylor and Woodgate 1997).

Possible Horizontal and Vertical Transmission

The question of whether BSE could be transmitted horizontally from cow to cow or maternally from cow to calf has been considered from the beginning of the epidemic (Hoinville et al. 1995; Wilesmith 1996a). Because all animals in each age group in a herd would be exposed to the same dietary supplements, it could be difficult to distinguish horizontal from common source transmission. The low frequency of BSE in affected herds (overall <5% annual incidence) suggests that horizontal transmission was not occurring at a high frequency, although it does not preclude transmission at a low rate.

It has long been suggested that scrapie in sheep is transmitted from ewe to lamb (Hoinville 1996), perhaps via the placenta from which the scrapie agent has been isolated, although recent critical reviews (Ridley

and Baker 1995; Hoinville 1996) concluded that vertical transmission may be more myth than fact. In GB in 1994, a case-control study (Hoinville et al. 1995) was conducted of cases of BSE in animals born after the ban on feeding ruminant-derived protein that was introduced in July, 1988 (see below). This study indicated that maternal transmission—if it occurred—would be at a rate between 0 and 13% and would not be a major factor in the occurrence of cases of BSE in animals born after the feed ban.

Another study (Wilesmith et al. 1997) was initiated in 1989 to examine the question of maternal transmission or of a maternally associated risk factor, based on a long-term follow-up of selected cohorts of animals (Table 6). These results indicate a risk for offspring of dams that developed BSE of about 15% in calves born to BSE-infected dams compared to about 5% in calves whose dams did not develop BSE, a difference of about 10%. In addition, there was an as-yet-unexplained declining risk in successive birth cohorts and a major change in this statistic for animals born after the 1988 feed ban. In interpreting these data, it is difficult to distinguish between maternal transmission, genetic risk (Grobet and Vandevenne 1994; Hunter et al. 1994; Ferguson et al. 1998), differential

Table 6 Frequency of BSE in calves born of cows with and without BSE

Birth date	Mothers' status	Number of offspring studied	Number of offspring with BSE	Percent offspring with BSE	Percent difference
July–December 1987	with BSE	10	7	70	47
	without BSE	9	2	22	
January–June 1988	with BSE	12	5	42	35
	without BSE	14	1	7	
July–December 1988	with BSE	215	25	12	8
	without BSE	205	8	4	
January–December 1989	with BSE	64	5	8	5
	without BSE	73	2	3	
1987-1989	with BSE	301	42	14	10
	without BSE	301	13	4	
Both groups		602	55	9	

Calves born to mothers with BSE were compared to calves born to mothers without BSE in the same herds and birthyears. All study animals were followed until they had reached age 7 (unless BSE or another disease intervened), and BSE was confirmed by histologic examination. (Modeled after Wilesmith et al. 1997.)

exposure to contaminated MBM, or a combination of these possible factors. However, another study (Ferguson et al. 1997b) indicated that the later in the incubation period that the dam was at the time of birthing, the greater the risk to the calf. This is consistent with the hypothesis of maternal-offspring transmission, in contrast to a genetic effect that should be unaffected by incubation period.

It is also important to assess the potential impact of maternal transmission. For calves born after the ruminant feeding ban (1988), it appears that maternal BSE may elevate lifetime risk of BSE by no more than 5%. Thus, every 100 cases of MBM-transmitted BSE would give rise to no more than 5 cases of vertically transmitted BSE. This suggests that maternal transmission, if it occurs, would have a negligible impact on the waning epidemic (Hoinville et al. 1995).

Control of the Epidemic and Future Projection

The recognition that BSE was a common source epidemic, and the identification of contaminated MBM as the putative cause, led to a decision to ban ruminant offal as a raw material in the preparation of dietary supplements destined for feeding to cattle. The ruminant feed ban, introduced in July, 1988 (extended to all livestock species in 1996), represented a decisive administrative action to control the problem (Anonymous 1997a), as well as the ultimate test of the hypothesis that MBM was the source of the outbreak. In GB, the epidemic peaked in January, 1993 and has undergone a dramatic decline so that incidence in mid-1997 was about 1/10 of maximal levels (Fig. 1). The waning of the epidemic has followed a path that was roughly predicted at the time of the feed ban (Nathanson et al. 1993), providing strong support for the MBM causal hypothesis.

A recent elegant mathematical and statistical study of the BSE epidemic by Anderson and colleagues (Anderson et al. 1996) has used back calculation to reconstruct the dynamics of the outbreak, including the probable numbers of infected animals. This analysis indicates that infections did not cease in July, 1988, immediately following the feed ban, but continued in substantial but declining numbers in animals born through 1991 (estimated new infections: >300,000 in 1988 and ~30,000 in 1991). These results reflect observations on the occurrence of BSE cases in birth cohorts (Table 2), which show that cases occurred in animals born in 1989 through 1991, but in sharply decreasing numbers (Anonymous 1996b). In addition, these analyses were used to predict the course of the epidemic (Anderson et

al. 1996; Donnelly et al. 1997a), which projected a stepwise waning to minimal numbers by the year 2000 (projections shown in Fig. 1).

The occurrence of new infections after 1988 can be ascribed in part to contaminated feedstuffs, manufactured before July 1988, that were already in the feed supply chain. In addition, there is evidence after 1988 of accidental cross-contamination in feed mills of rations produced for consumption by monogastric animals (poultry and pigs) and ruminants (cattle and sheep), and of the possible feeding of cattle with rations produced for monogastric animals (Wilesmith 1996a,b; Hörnlimann et al. 1997; Taylor and Woodgate 1997). The risks of accidental contamination were greatest in the northern and eastern regions of England where pig and poultry populations are concentrated, and this has resulted in a change in the geographical variation in risk for animals born since the feed ban (Wilesmith 1996b).

Transmission Studies

The epidemiologic observations and reconstruction of the source of the BSE epidemic raise the question of whether BSE can be reproduced by the experimental transmission of sheep scrapie to cattle. A number of transmission experiments have been reported (Cutlip et al. 1996; Gibbs et al. 1996; Prusiner 1997) that have produced certain consistent results. Various strains of sheep and goat scrapie can be transmitted to cattle by intracerebral or multiple routes but not (as yet) by feeding. The resulting pathologic picture differs from BSE in cattle and resembles scrapie in sheep. Prions from the brains of cattle to which scrapie has been transmitted show different disease patterns when they are further transmitted into mice and hamsters (Gibbs et al. 1996), indicating that they represent different "strains."

Does the difference between the lesion profile of the spongiform encephalopathy produced in cattle by experimental transmission of sheep scrapie and the lesion profile of BSE (see below) undermine the postulated sheep scrapie origin of the BSE epidemic? Several points bear on this question. (1) Due to the cumbersome nature of the model, scrapie transmissions to cattle have not been extended to serial passages through cattle. (2) TSE agents often change their properties upon serial passage in a new host species. (3) BSE isolates represent prions that have had several serial passages in cattle and have become "fixed" in their properties. (4) Sheep scrapie occurs in many different strains, and not all of these have been transmitted experimentally to cattle. These considerations sug-

gest that the experimental data in no way exclude the possibility that one of the multiple strains of sheep scrapie could produce prions with the BSE phenotype upon serial passage in cattle.

NEUROPATHOLOGY OF BSE

From the first preliminary identification of BSE as a new disease in cattle, its distinctive pathologic presentation played a key role in establishing the syndrome as a nosological entity (Wells et al. 1987). Subsequent studies (Wells et al. 1991, 1994; Wells and Wilesmith 1995; Hawkins et al. 1996; Simmons et al. 1996a,b) established that BSE produced a consistent neuropathologic picture, in contrast to scrapie in sheep, where the lesion patterns are quite variable. The stereotypical pattern of the brain lesions was important since it became clear that a histopathologic examination could be used to confirm or refute clinical suspicion of BSE. A basis for confirming the disease independent of clinical presentation was useful because the signs can be confused with several other neurologic disorders of cattle, and about 20–25% of clinically suspect cases remain unconfirmed on neuropathologic examination. Furthermore, the consistency of the brain pathology made it possible to restrict the examination to a single brain section (medulla) that was invariably the site of lesions in affected cattle. In turn, this permitted the use of the pathologic examination as a routine confirmation method, despite the very large number (~200,000) of suspect cases, of which about 165,000 had been confirmed through July, 1997.

Qualitative and Quantitative Description of BSE Lesions

The pathologic changes in BSE are confined to the central nervous system. Qualitatively, the major brain lesions of BSE include spongiform changes, astrogliosis, and neuronal outfall. (1) Spongiform lesions are the most characteristic changes in BSE and can be seen with a routine hematoxylin and eosin (H and E) stain, which shows vacuolation of the neuropil that is mainly extracellular, although intraneuronal vacuoles are also present. (2) Using special stains, astrocytic hypertrophy is regularly seen, although usually it is not as marked as in sheep scrapie. (3) Neuronal degeneration is occasionally seen, including necrotic neurons, basophilic shrunken neurons, neurophagia, or dystrophic neurites. There is substantial neuronal loss in the vestibular nuclei, but this can be documented only by morphometric studies (Jeffrey et al. 1992; Jeffrey and Halliday 1994;

Wells and Wilesmith 1995). (4) Demonstrable amyloidosis, based on examination for birefringence of Congo Red-stained sections, is very infrequent (as it is in scrapie), in contrast to variant Creutzfeldt-Jakob disease, where it is very marked. However, immunohistochemical staining for PrP^{Sc} demonstrates diffuse staining, the intensity of which generally correlates with the distribution of vacuolar lesions.

It has already been remarked that one notable feature of BSE is the consistency of the brain lesions seen in different animals. This simplified a description of the distribution of brain lesions, since it justified aggregation of all the observations. A thorough study was conducted, based on 100 cases of BSE slaughtered from 1986 to 1989 that were examined for vacuolar lesions using H and E-stained sections (Wells et al. 1991, 1992, 1994; Wells and Wilesmith 1995; Simmons et al. 1996a). A scale of 0–4 was used to score 88 neuroanatomical regions to construct a lesion profile following the method originally introduced for mouse scrapie (Fraser and Dickinson 1968). The lesion profile is shown diagrammatically in Figure 6, in which the intensity of vacuolation in 7 different coronal sections of the brain and spinal cord is represented by the scores of 18 key gray matter areas (Simmons et al. 1996a). Areas with the most severe lesion scores were midbrain (central gray matter), pons and medulla (nucleus of the spinal tract of the trigeminal nerve), diencephalon (hypothalamic nuclei), and hippocampus. The spinal cord was evaluated for vacuolar lesions in 10 cases (Wells et al. 1994). Lesions were confined to the gray matter and were seen at cervical, thoracic, and lumbar regions; the most marked changes were always in the substantia gelatinosa of the dorsal gray horns. Over the whole central nervous system, vacuolation is most severe in the cord, medulla, pons, and midbrain, and is much milder in the rostral regions of the brain.

Ultrastructural studies (Liberski et al. 1992) have shown that BSE resembles other TSEs. There are membrane-bounded vacuoles within neuronal processes, hypertrophic astrocytes, and dystrophic cells contained accumulations of neurofilaments, mitochondria, lamellar bodies, and other electron-dense profiles. Consistent with the light microscopic picture, changes are mainly seen in gray matter, in contrast with some other TSEs, where the white matter shows more marked abnormalities.

Biological Inferences

The uniformity of the lesion profile in BSE is notable because it contrasts so strongly with the marked variation in lesion profiles seen in different

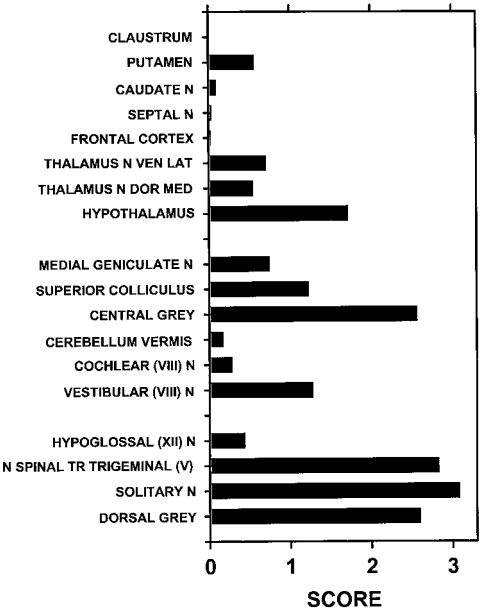

Figure 6 BSE lesions in the bovine brain. Cases of bovine BSE tend to exhibit a stereotypic lesion profile. The diagram shows the relative severity of vacuolar changes in different regions of the brain based on analysis of cases with onsets 1987–1994, showing the median for three groups of 100 cases. Lesion score: (1) a few vacuoles; (2) several vacuoles evenly distributed; (3) moderate or many vacuoles evenly distributed; (4) numerous vacuoles, some of which may be coalescent. (Modified from Wells and Wilesmith 1995.)

cases of scrapie in sheep. Studies in mice and sheep have shown that there are two major determinants of lesion profile, the host genotype and the prion strain (Fraser and Dickinson 1973). The marked uniformity of BSE lesions implies that there is little variation in host genotype, at least as regards susceptibility to BSE, and this is consistent with genetic studies that show little variation among different breeds of cattle in the sequence of their PrP genes (Prusiner et al. 1993; Hunter et al. 1994). In addition, the stereotypic lesions suggest that, regardless of origin, cattle have been infected with, or have selected, a single prion strain (Fraser et al. 1992; Bruce et al. 1997). Consistent with this interpretation, when different BSE isolates are transmitted to a given strain of mice, they all produce an identical picture as to incubation period and lesion profile (Bruce et al. 1994, 1997; Hill et al. 1997).

It should be emphasized that both the qualitative nature and the quantitative lesion profile of BSE in cattle are strikingly different from the pathologic picture of variant Creutzfeldt-Jakob disease and clearly different from the lesion profile seen in mice inoculated with BSE. These points are further discussed in the next section.

The pathogenesis of prion diseases following extraneural routes of infection is not well understood. However, a study of intragastric intraperitoneal infection of mice with scrapie led to the conclusion that the agent might invade the central nervous system along peripheral neural pathways, based on the sequential appearance of prions first in the thoracic spinal cord with subsequent spread in both caudal and rostral directions (Kimberlin and Walker 1988, 1989; Blattler et al. 1997). From this perspective, it could be significant that, in BSE, among the most severely affected neuronal centers are the nucleus of the spinal tract of the trigeminal nerve, the dorsal nucleus of the vagus nerve, and the nucleus solitarius (Fig. 6). In principle, if the site of prion entry following ingestion were either the buccal mucosa or the intestine, and if the agent invaded the central nervous system via peripheral neuronal pathways, it might spread along either the trigeminal or the vagus nerves that provide much of the innervation to the alimentary tract. In this regard, it is interesting that when BSE is injected intracerebrally into mice expressing a transgene for bovine PrP [Tg(BoPrP)], the subsequent PrP distribution in the CNS resembles that seen in BSE except for relatively minimal lesions in the nucleus of the spinal tract of the trigeminal nerve, the nucleus solitarius, and periaqueductal gray matter of the midbrain (Blattler et al. 1997); these differences may reflect a difference between invasion by the intracerebral route and invasion via the alimentary tract.

VARIANT CREUTZFELDT-JAKOB DISEASE AND ITS POSSIBLE ASSOCIATION WITH BSE

The potential transmission of BSE to humans by the consumption of beef or beef products prepared from cattle with the disease has been discussed from the outset of the epidemic (Taylor 1989a). There were several reasons to discount this possibility. Perhaps the most persuasive was a number of carefully conducted epidemiologic studies that failed to demonstrate an association between sporadic cases of CJD (or other human prion diseases) and consumption of lamb, mutton, or other sheep tissues, including brain (Brown et al. 1987; Taylor 1989a; Will 1993). It was reasonable to argue that if scrapie of sheep had never been transmitted to humans, it would be unlikely that a closely related spongiform

encephalopathy, arising by transmission of scrapie to another ruminant species, would pose a risk. Another relevant biological observation was the relatively low infectivity of scrapie when administered by feeding in contrast to intracerebral injection (Kimberlin 1979; Kimberlin and Wilesmith 1994); in one study using mice, 10^5 intracerebral LD_{50} were required to produce one intragastric LD_{50} (Kimberlin and Walker 1989). In particular, the failure to transmit kuru to chimpanzees by feeding of material shown to have high titer by intracerebral and other routes of injection (Gajdusek and Gibbs 1973; Gibbs et al. 1980; Brown and Gajdusek 1991) suggested that transmission of BSE to humans would be unlikely. Nevertheless, early in the BSE epidemic, some workers expressed concern (Dealler and Lacey 1990) about the potential transmission of BSE to humans.

Several actions were taken to minimize the theoretical risk of BSE transmission (Anonymous 1996a, 1997a; Wilesmith 1996a). First, BSE was made a reportable disease, with compensation to farmers for animals destroyed because of the disease. In GB, a diagnostic service was established to screen all suspect cases by neurohistologic examination of the brain; since 1987 about 200,000 suspect animals have been examined, of which about 80% have been confirmed as positive for BSE (Wilesmith 1996a). Second, to terminate the epidemic, a ruminant feed ban was introduced in July, 1988, very early in the epidemic, and this ban has succeeded in bringing the epidemic under control, as described above. Third, in 1989, a ban on specified bovine offals (SBO) was imposed, forbidding entry into the human food chain of certain bovine tissues (including brain and spleen) that might contain the BSE agent, and this ban was widened in 1992 (to prohibit the use of headmeat if the skull had been opened), in 1994 (to prohibit human consumption of thymus and intestines), and in 1995 (to include the whole bovine head as a specified banned offal) (Anonymous 1996a; Taylor and Woodgate 1997). Analysis of the epidemiology of TSE in domestic cats suggested that an SBO ban could effectively diminish or terminate the risk of consuming bovine tissues (J. Wilesmith, unpubl.).

Clinical and Epidemiologic Observations

In April, 1996, a new variant form of CJD (vCJD) was reported (Will et al. 1996) in 10 patients resident in GB with onsets of illness between February 1994 and October 1995. As of October 1, 1997 (Anonymous 1997b), there were 21 confirmed or probable cases with onsets in 1994 (7 cases), 1995 (9 cases), or 1996 (5 cases). There are three types of CJD,

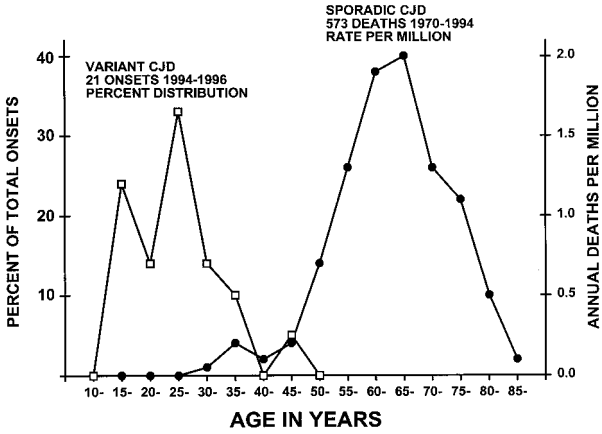

Figure 7 Annual age-specific death rates for sporadic Creutzfeldt-Jakob disease (573 cases) for the UK, 1970–1994, compared with the age distribution of the first 21 cases of variant Creutzfeldt-Jakob disease, for the UK, 1995–1996. Note that the two groups are plotted on different scales, which optimizes comparison of the age distributions but distorts the relative numbers of the two kinds of cases. Based on data from Will (1996), Will et al. (1996), Anonymous (1997b, 1997c).

sporadic (sCJD, ~85% of cases), familial (fCJD, ~15% of cases), and transmitted (tCJD, <1% of cases). Variant CJD was distinguished from sCJD by four major characteristics: (1) the cases were mainly under age 40 at onset, in contrast to CJD, where <1% of cases are this young (Fig. 7); (2) the clinical picture of vCJD, in which behavioral changes, dysesthesia, and ataxia were prominent early symptoms, contrasted with that of CJD in which dementia is usually seen early in the illness; (3) the absence of triphasic slow waves on the electroencephalogram in cases of vCJD differed from the pattern that is seen in most cases of sCJD; and (4) vCJD exhibited certain neuropathologic features (Ironside 1997), most notably amyloid plaques staining for PrP, which are rarely seen in CJD, although they are common in other spongiform encephalopathies of humans, such as kuru and Gerstmann-Sträussler-Scheinker syndrome. In addition, the cerebellum showed marked spongiform changes, neuronal dropout, and astrogliosis, which is unusual in sCJD. Notably, the lesion pattern of vCJD was very consistent among cases, in contrast to the variability seen in sCJD.

Two circumstantial aspects of vCJD, its appearance in 1994 and its localization in GB, raised the question whether vCJD could be related to BSE. Both of these points deserve careful consideration. First, is vCJD a truly new entity or only one that is newly recognized? To date (October

Table 7 Annual age-specific death rates per million for Creutzfeldt-Jakob disease in the United States, 1979–1994

	1979–82		1983–86		1987–90		1991-94	
Age group	deaths	rates	deaths	rates	deaths	rates	deaths	rates
0–44	22	0.04	18	0.02	21	0.03	27	0.04
45–59	160	1.17	170	1.26	148	1.06	174	1.13
60+	584	4.04	703	4.52	796	4.84	827	4.83

Modeled after Holman et al. (1996).

1997), there are no reports of cases similar to vCJD occurring prior to 1994. Second, is vCJD a worldwide phenomenon or is it mainly confined to GB? One case has been reported from France (Chazot et al. 1996), but at this time there are no additional reports of vCJD from other countries. Recent studies (Anonymous 1996c; Holman et al. 1996) in the US confirm that there are very few CJD cases with onsets under age 45, and there has been no evidence of an increase of younger cases in the last few years (Table 7), nor has intensive surveillance at a number of neurologic centers identified individual cases of vCJD (Anonymous 1996c).

The first 21 cases of vCJD have given no family history of prion diseases. More pertinent, there was no indication of exposure to growth hormone or other risk factors (brain surgery, tissue transplants) that have been associated with the transmission of CJD (Brown and Gajdusek 1991). Nor did these cases give a history of unusual consumption of beef or calf brains, or any occupational or casual exposure to cattle, slaughterhouses, or rendering plants. To date, this line of inquiry has failed to demonstrate any link to BSE.

A related question is whether infected cattle entered the human food chain, providing a potential source of exposure. During the period from 1985 to June 1997, a cumulative total of more than 165,000 cases of BSE were confirmed (Anonymous 1997a). These cases were distributed over about 32,000 affected herds (with one or more cases of BSE), or about 5 cases per herd (Anonymous 1995). Since the mean size (Fig. 3) of affected herds was about 100 cattle, in affected herds the incidence of diagnosed cases was approximately 5% of animals. This suggests that, in such affected herds, the 95% of cattle that were apparently normal included some animals that were incubating BSE. The Anderson study (Anderson et al. 1996) estimated, using back calculation methods, that more than 700,000 animals incubating BSE entered the human food chain during the period 1985–1995, in addition to the more than 150,000 diagnosed with BSE that did not enter the food chain. If more than 2.5 million cattle are slaughtered annually, the more than 700,000 animals represent less than

3% of the more than 25 million animals that entered the human food chain in GB during 1985–1995. Furthermore, the majority of these subclinically infected animals would have been slaughtered for prime beef at less than 30 months of age, at a time when they would have been less than halfway through the incubation period (~60 months); this appears to be significant, since infectivity has not been detected in the central nervous system 30 months after infection in an ongoing study of the pathogenesis of BSE following oral exposure (Wells et al. 1996). Nevertheless, more than 3% (>20,000) of the approximately 700,000 animals would have been slaughtered within 12 months of onset (Fig. 18 in Ferguson et al. 1997a) at a time when tissues would likely have a high level of infectivity.

Biological Studies

Several lines of inquiry shed light on the possible association of BSE and vCJD.

Genetic Studies

Among normal persons (Table 8), there is a polymorphism of the PrP gene at codon 129 (Owen et al. 1990). Cases of CJD usually are homozygous at codon 129, either for methionine or valine in the sporadic form of

Table 8 PrP genotype polymorphism at codon 129 and prion glycoforms in Creutzfeldt-Jakob disease: Comparison of transmitted and variant forms of the disease

Group	Codon 129 haplotype[a]			
	M/V	M/M	V/V	Glycoforms*
Healthy controls (N=519)	47%	41%	12%	None
Variant CJD (N=14)	0%	100%	0%	type 4
Transmitted CJD, central (N=15)	7%	87%	7%	type 2
Transmitted CJD, peripheral (N=41)	7%	51%	41%	type 3
				type 1

Haplotype data mainly after P. Gambetti et al. (unpubl.), based on Collinge et al. (1991, 1996), Palmer et al. (1991), Deslys et al. (1994), Brown et al. (1994b), Aguzzi and Weissmann (1996), Parchi et al. (1996), and Anonymous (1997b), and glycoform data mainly after Parchi et al. (1996) and Collinge et al. (1996).

[a]Codon 129 alleles: (M) Methionine; (V) valine. Glycoforms: glycosylation pattern of prions after proteinase K digestion and PAGE. Transmitted CJD: (central) transmitted by corneal or dura mater grafts or neurosurgical instruments; (peripheral) transmitted by injection of cadaveric pituitary-derived growth hormone or ritual endocannibalism. The peripherally transmitted cases of CJD showed the following glycoform patterns: haplotype VV or MV, type 3 glycoform; haplotype MM, type 1 glycoform (Collinge et al. 1996).

the disease (Palmer et al. 1991), but predominantly for valine in the cases transmitted by injection of cadaver-derived growth hormone (Collinge et al. 1991). All of the 14 cases of vCJD that have been tested were homozygous for methionine at codon-129 of the PrP gene (Collinge et al. 1996). This codon-129 methionine homozygosity bolsters the grouping of cases of vCJD as a distinct prion disease of humans. However, it does not help to distinguish between different hypotheses about its origin.

Transmission of BSE

Other relevant information is provided by experimental studies of BSE transmission to primates. It was reported (Lasmézas et al. 1996) that a single pool of bovine BSE, injected intracerebrally into three rhesus macaques, produced a disease (after a 3-year incubation period) that resembled vCJD, both clinically and pathologically. This stands in contrast to the transmission of sCJD to primates, which produces a syndrome similar to the same kind of CJD in humans (Masters et al. 1976; Gibbs et al. 1979; Brown et al. 1994a; Aguzzi 1996; de Silva 1996). It is noteworthy that cynomolgus macaques are homozygous for methionine at codon 129 of the PrP gene (Aguzzi 1996).

Recently, Bruce and coworkers (Bruce et al. 1997) published preliminary results of a study of transmission of BSE and vCJD to several strains of inbred mice. There was a striking similarity in the results of transmissions to one inbred mouse line, the RIII strain, with several BSE and vCJD inocula (Table 9), and the incubation period and particularly the neuropathologic lesion pattern were different from those seen following transmission with samples from patients dying with sCJD.

Table 9 Transmission of BSE and CJD to RIII mice

Prion source (number of cases)	Disease transmission (%)	Incubation period in days: Mean and range	Pathologic signature[a]
BSE (8)	100	317 302–335	"BSE"
vCJD (3)	100	~307 288–351	"BSE- like"
sCJD (6)	0[b]	>600	other

[a]Pathologic signature refers to the distribution of lesions in different brain regions; "BSE" is the pattern produced by BSE inocula and "other" is a different pattern. (Modeled after Bruce et al. 1997.)

[b]RIII mice failed to exhibit clinical evidence of disease to 600 days after injection, but 130/156 asymptomatic mice dying of intercurrent illnesses >500 days after injection showed neuropathologic stigmata of spongiform encephalopathy.

Transgenic mice have also been used to determine the potential for transmission of BSE to humans. Collinge and colleagues (Collinge et al. 1995; Hope 1995; Hill et al. 1997) have employed mice in which the native mouse PrP gene was "knocked out" and a transgene for human PrP was introduced. Table 10 summarizes the patterns of transmission that were observed. Sporadic and iatrogenically transmitted CJD were readily transmitted to transgenic mice, whereas wild-type mice were almost totally resistant. In contrast, BSE was transmitted to wild-type mice quite readily and somewhat less readily to transgenic mice. The transmission pattern for vCJD closely resembled that for BSE and was markedly different from that shown by other kinds of CJD. Furthermore, preliminary observations suggested that there were other similarities between mice injected with BSE and vCJD, such as "backwards walking" and brain distribution of PrP^{Sc}. The human transgene used in these mice carried valine at codon 129 (Hope 1995), and it remains to be determined whether the result would be different if the experiment were repeated with a mouse bearing a transgene carrying methionine at codon 129, in view of the occurrence of CJD exclusively in humans bearing M/M alleles.

Overall, it appears that BSE, vCJD, and each mouse strain injected with BSE show a very consistent pattern of brain lesions. However, each of these lesion patterns is distinctly different. The common denominator is the similarity between the lesions produced when inocula from BSE or

Table 10 Transmission of BSE and CJD to transgenic and wild-type mice

	FVB transgenic		FVB wild-type	
Prion source (number of inocula)	disease transmission	mean incubation period (days)	disease transmission	mean incubation period (days)
BSE (5)	38%	~600	87%	~470
	10/26		21/24	
vCJD (6)	45%	~230	77%	~370
	25/56		33/43	
sCJD and tCJD (16)	99%	~200	6%	~425
	114/115		6/98	

(sCJD and tCJD) Both sporadic and transmitted cases, representing the three possible genotypes (V/V, M/M, V/M) at codon 129. (Wild-type) FVB strain; (transgenic) FVB background $MoPrP^{0/0}$ $Tg(HuPrP^{+/+}$ codon 129 V/V). (Incubation periods). Rounded means are shown for animals that developed disease (asymptomatic mice excluded). Some transgenic animals inoculated with either BSE or vCJD showed an unusual backwalks walking behavior, both wild-type and transgenic animals inoculated with either BSE or vCJD showed similar distribution of PrP^{Sc} in their brains. (Modeled after Hill et al. 1997.)

vCJD are used to infect RIII mice, which is consistent with the hypothesis that a single prion strain produces both diseases.

Pathogenesis of BSE

The pathogenesis of BSE is currently the subject of studies that require prolonged experimentation. These studies should shed light on the mechanism of transmission, the sites and dynamics of sequential spread of prions from the site of entry, prion titers in extraneural tissues during the course of infection, and the route of invasion of the central nervous system. This information will help to understand the potential sources of infectivity of beef products, the plausibility of maternal transmission, whether there is any potential for horizontal spread of infection, and the routes by which BSE might be transmitted to humans.

Bovines experimentally inoculated with BSE by either oral or intracerebral routes will develop BSE with a minimal incubation period of about 3 years (Wells et al. 1996). When infected animals are sacrificed and tissues assayed for evidence of infectivity, transmissible prions have been detected only in the brain and spinal cord, and only at more than 30 months post-injection; extraneural tissues have as yet failed to exhibit infectivity (Middleton and Barlow 1993). However, mouse strains heretofore available provide a suboptimal assay system (Fraser et al. 1992; Prusiner 1997), since they may be as much as 1000-fold less sensitive than bovines (Middleton and Barlow 1993). Thus, Table 10 shows less than 100% transmission of BSE to FVB mice even with concentrated inocula. Transgenic mice carrying the bovine PrP sequence (Tg(BoPrP)$Prnp^{0/0}$) may provide a more sensitive assay system, since the species barrier should be markedly reduced or completely abrogated in these mice (Scott et al. 1997).

Biochemical Signature

Collinge and colleagues have provided further important evidence in studies (Aguzzi and Weissmann 1996; Collinge et al. 1996; Hill et al. 1997) that utilized the approach of Gambetti and coworkers (Parchi et al. 1996) to compare the patterns of pathologic prion proteins (PrP^{Sc} or PrP^{res}) in polyacrylamide gel electrophoresis (PAGE). PrP^{Sc} shows microheterogeneity due to different glycosylation patterns that produce three discrete bands on PAGE, and differences in the banding pattern of PrP^{Sc} from different prion diseases provide a biochemical "signature" (Telling et al. 1997). Strikingly, the signature of PrP^{Sc} from vCJD is similar to that of PrP^{Sc} extracted from cattle with BSE and from macaques or mice to whom

BSE has been transmitted (Aguzzi and Weissmann 1996; Collinge et al. 1996; Hill et al. 1997), and this pattern is distinct from that of CJD that has been transmitted from human to human (Table 8). Other investigators (Parchi et al. 1997) have pointed out that, after deglycosylation, PrP^{Sc} from human cases of sCJD shows only two (rather than four) patterns, dependent on the size of the PrP peptide (21 kD or 19 kD, respectively), and that vCJD corresponds to one of these two patterns; on this basis the significance of the four glycoform types has been questioned.

Comment

The question of the relationship between BSE and vCJD has been the subject of much debate (Aguzzi 1996; Almond et al. 1996; Smith and Cousens 1996; Cousens et al. 1997). In our view, the potential association has been strengthened by two recent biological observations: (1) the similar pathologic lesion profile in mice to which BSE and vCJD have been transmitted (Bruce et al. 1997) and (2) the similar distinctive glycoform signature (Collinge et al. 1996) of prions in cattle with BSE and humans with vCJD and in mice and monkeys injected with BSE.

However, some important questions remain unanswered.

1. Is there significant prion infectivity in beef products prepared from cows that are incubating BSE, and are the titers adequate to infect humans by the oral or any other route? The development of prion infectivity assays of increasing sensitivity will help to clarify this question.
2. How is BSE transmitted to humans? Did the cases of vCJD have subtle dietary or other exposures that would explain their selection, or is transmission a rare event governed only by the laws of chance? Unfortunately, the impossibility of obtaining precise and detailed dietary histories precludes a definitive answer to this question.
3. If cases of vCJD have no remarkable BSE exposure, are there other host risk factors, in addition to homozygosity at codon 129, that explain the occurrence of vCJD in these individuals?
4. Why are there so few cases of vCJD in persons over age 40?
5. If the only epidemiologic connection between BSE and vCJD is an association in place and time, will ongoing surveillance confirm that CJD is a new prion disease that first appeared in 1994 and continues to occur mainly in GB?
6. If the cases with onsets in 1994–1996 represent the beginning of an epidemic, why is there no evidence of a stepwise increase each year?

Will the future incidence of vCJD follow predictions (Cousens et al. 1997; Skegg 1997) based on the calculated curves of putative exposure to BSE and the postulated incubation period?

SUMMARY

Bovine spongiform encephalopathy (BSE) is a transmissible spongiform encephalopathy (TSE) or prion disease of cattle that was first recognized in GB in 1986, where it produced a common source epidemic that peaked in January, 1993, and has subsided markedly since that time. Through June 1997, about 167,000 cases have been confirmed, representing over 95% of the total projected incidence. The epidemic began simultaneously at many geographic locations and was traced to contamination of meat and bone meal (MBM), a dietary supplement prepared from rendering of slaughterhouse offal. It appears that the epidemic was initiated by the presence of the agent of scrapie (a longstanding TSE of sheep) that was first transmitted to cattle beginning in the early 1980s, when most rendering plants abandoned the use of organic solvents in the preparation of MBM. The epidemic was accelerated by the recycling of infected bovine tissues, probably prior to the recognition of BSE. To terminate the epidemic, a prohibition on the feeding of ruminant-derived protein to ruminants was introduced in GB in July, 1988 and accounts for the decline of the epidemic after an interval of about 5 years, approximately equivalent to the average incubation period of BSE. Relatively few cases of BSE have occurred in cattle born after 1993, and it is predicted that the epidemic will terminate about the year 2000, based on an extrapolation of the present declining curve. A comparison of information from GB and relatively low-incidence countries, such as Switzerland, indicates that this epidemic has been mainly confined to GB because of a unique concatenation of risk factors including (1) a high ratio of sheep to cattle, (2) a relatively high rate of endemic scrapie, (3) the heavy feeding of MBM to dairy cattle, and (4) changes in the rendering process used to prepare MBM (Wilesmith et al. 1991).

Recently, cases of a variant form of Creutzfeldt-Jakob disease (vCJD, a TSE of humans) have been reported in GB. These cases, over 20 of which have now been identified, with onsets in 1994–1996, are distinguished by their occurrence almost exclusively in subjects under age 40, by their clinical presentation, and by their neurohistopathologic picture. The recent appearance of this novel disease and its concentration in GB have strongly suggested that it represents the transmission of BSE to humans. The possibility of a causal association has been enhanced by

finding that transmission of BSE and of vCJD to mice produces a similar neuropathologic pattern of disease and that the biochemical pattern of prion glycoforms is similar in BSE, vCJD, and in animals to which the two diseases have been transmitted. However, there remain some observations about vCJD that are not readily explained and require further study.

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Infectious and Sporadic Prion Diseases

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Creutzfeldt-Jakob disease (CJD) was first identified in 1921 by Jakob (Jakob 1921), who referred to a previous case described by Creutzfeldt in 1920 (Creutzfeldt 1920). These original cases were clinically heterogeneous, and recent review of pathologic material provided confirmation of the diagnosis of CJD in only two of the original five cases (Masters and Gajdusek 1982). Over subsequent decades the nosology of CJD was confused and confusing. Wilson regarded CJD as a "dumping ground for several rare cases of presenile dementia" (Wilson 1940). Although an important monograph by Kirschbaum (1968) listed the clinical and pathologic features of all 150 cases identified before 1965, it included cases such as Creutzfeldt's original case, which would not now fulfil clinical or pathologic criteria for the diagnosis of CJD. In 1954 (Jones and Nevin 1954)

and 1960 (Nevin et al. 1960) Nevin and Jones described the typical clinical course, the "characteristic" electroencephalogram (EEG) and neuropathologic changes, including spongiform change, which in combination are now recognized as the paradigm features of sporadic CJD.

In the late 1950s and early 1960s with remarkable perseverance in difficult conditions, Gajdusek and colleagues investigated kuru, a fatal ataxic syndrome restricted to the Okapa area of the highlands of Papua New Guinea (Gajdusek and Zigas 1957). The similarity of the neuropathologic findings in scrapie and kuru was recognized in 1959 by Hadlow (Hadlow 1959) with the implication that kuru, like scrapie, might be transmissible in the laboratory. Successful laboratory transmission of kuru in 1966 (Gajdusek et al. 1966) was followed in 1968 (Gibbs et al. 1968) by the laboratory transmission of CJD following intracerebral inoculation of primates, leading to a transformation in the level of scientific interest in these diseases. CJD had been regarded as a nontransmissible degenerative condition, as there was no evidence of a host immune response either serologically or histologically. The confirmation that CJD was transmissible in the laboratory led to a range of important scientific questions, including whether and if so, to what extent, CJD might be naturally transmitted. Epidemiological research in a number of countries established baseline characteristics for CJD, such as incidence rates, but no environmental source of infection was identified (Brown et al. 1987). Paradoxically for a transmissible disease, about 5–15% of cases were found to be familial with a dominant pattern of inheritance (Brown et al. 1987). By the 1970s convincing evidence implicated iatrogenic transmission of the disease in a small number of cases through transfer of a corneal transplant (Duffy et al. 1974) or CNS tissue from patient to patient in the course of medical practice (Will and Matthews 1982).

The identification of prion protein (PrP) accumulation as a specific feature of CJD and other prion diseases (McKinley et al. 1983) has led to a revolution in the understanding of the pathogenesis of these conditions. The discovery that hereditary forms of human prion diseases were linked to mutations of the PrP gene (Hsiao et al. 1989) provided critical support for a central role of PrP in disease causation and expression.

Although these advances stimulated much scientific debate, a major increase in public interest in prion diseases, particularly in the UK, only began after confirmation of bovine spongiform encephalopathy (BSE) as an emerging epizootic disease in UK cattle in 1987 (Wells et al. 1987). The leading hypothesis for the origin of BSE included the transmission of scrapie to cattle by ingestion of contaminated meat and bone meal (Wilesmith et al. 1988). The emergence of BSE raised important ques-

tions regarding the possibility of a risk to public health. Many scientists regarded this possibility as remote, in part because of the presumed link between BSE and scrapie (Southwood Committee 1989). Epidemiological studies had not identified scrapie in sheep as a risk factor for the development of CJD (Brown et al. 1987). Apart from its economic importance, scrapie was largely of scientific interest because many of the advances in the understanding of prion diseases were based on laboratory models of scrapie transmission in rodents. In 1996, however, a new type of CJD, designated new variant CJD (nvCJD), was reported in the UK (Will et al. 1996b). The emergence of nvCJD raised concerns that its temporal and geographic association with the emergence of BSE might reflect a causal relationship and indicate that BSE had different properties from scrapie in its pathogenicity to humans. The accumulating reports of nvCJD and a series of scientific studies strengthening the link between nvCJD and BSE (Bruce et al. 1997; Hill et al.1997b) have led to an exponential increase in public interest and concern about CJD and other prion diseases.

Systematic studies of CJD in many countries have established that the crude average annual incidence of CJD is about one case per million population and that there are three main forms of disease: (1) sporadic cases with no known environmental source, (2) familial cases, and (3) cases transmitted from a known or presumed environmental source, such as iatrogenic cases (Brown et al. 1992). Sporadic CJD accounts for about 85% of all cases (Will 1998) and these cases are characterized by a relatively stereotyped and rapidly progressive clinical course, although rare variants with an extended duration of illness are well recognized (Brown et al. 1984). The cause of sporadic CJD is unknown. Cases appear to occur without predictable geographic or temporal clustering (Will et al. 1986). Case-control studies have failed to establish any common environmental risk factor for the development of disease (Wientjens et al. 1996b).

Molecular biological analyses have demonstrated an increased risk of disease in relation to the codon-129 polymorphism of the PrP gene (Palmer et al. 1991). Familial CJD, unlike sporadic CJD, is associated with mutations of the PrP gene (Pocchiari 1994). Since the original mutation in human prion disease was found in two kindreds with Gerstmann-Sträussler-Scheinker syndrome (GSS), 21 point or insertional mutations have now been discovered, the majority in CJD families. Genetic forms of CJD account for about 5–15% of cases (Will et al. 1998).

Transmitted CJD from a known environmental source such as iatrogenic CJD is rare (Brown et al. 1992). Transmission has involved cross-contamination through neurosurgical instruments, corneal grafts, human dura mater grafts, and human pituitary-derived hormones. Iatrogenic

forms of CJD associated with a CNS route of inoculation are clinically similar to sporadic CJD. In contrast, iatrogenic forms of CJD associated with a peripheral route of inoculation result in a predominantly ataxic syndrome. Kuru is clinically similar to these latter cases. Unlike them, however, kuru has been specifically related to direct human-to-human transmission through ingestion or peripheral inoculation of the kuru agent during ritual cannibalism practiced by the Fore people of Papua New Guinea (Gajdusek 1990; Alpers 1968). nvCJD is clinically similar to human growth hormone (HGH)-related CJD and kuru, consistent with a probable oral or peripheral route of exposure. Even if the causal link between nvCJD and BSE were established, currently available data are insufficient to estimate the future number of cases of nvCJD (Cousens et al. 1997).

There is no in vivo diagnostic marker for the presence of infectivity in prion diseases, including CJD, and a firm diagnosis rests on identifying the characteristic neuropathologic changes, usually postmortem. The accuracy of routine histologic diagnosis has been enhanced by recent techniques, including immunocytochemical staining for PrP and the identification of protease-resistant PrP from brain tissue by histoblot or Western blot analysis. The transmissibility of individual cases or subtypes of CJD can be confirmed by laboratory experiments, but these investigations are time-consuming and expensive. The development of transgenic rodents with copies of human PrP genes may allow more sensitive bioassay, but this remains to be established. Diagnostic criteria for CJD were first proposed in 1979 (Masters et al. 1979) and have been adapted in the light of scientific developments (Budka et al. 1995). These criteria are useful in epidemiologic studies for ensuring data consistency and comparability. In any clinically suspect case of CJD, a firm diagnosis still depends on neuropathologic examination.

SPORADIC PRION DISEASE

Clinical Features

Sporadic CJD is typified clinically by progressive multifocal neurologic dysfunction, myoclonic involuntary movements, a terminal state of severe cognitive impairment, and death within a few months (Roos et al. 1973).

Prodromal symptoms prior to the occurrence of frank neurologic dysfunction are common and include alteration of personality or behavior, insomnia, anorexia, and depression (Table 1). Because such symptoms may occur as part of other background illnesses, their presence may not always be related to CJD. In about 5% of patients with sporadic CJD, the

Table 1 Prodromal symptoms in sporadic CJD (n=93) and comparison with frequency of these symptoms in hospital controls (n=186): England and Wales 1980–1984

	CJD cases (%)	Controls (%)
Depression	37	34
Tiredness	47	50
Sleep disturbance	28	58
Abnormal sweating	13	16
Abnormal appetite	22	19
Weight loss	46	24
Diarrhea	6	8
Headache	12	9

onset of neurologic dysfunction is abrupt, mimicking stroke (McNaughton and Will 1997). For example, patients may present with the acute development of hemiparesis or brain stem dysfunction. In these patients, the diagnosis may become apparent when their condition fails to stabilize or improve and progressive neurologic dysfunction, particularly myoclonus, develops.

The presenting symptoms and signs of sporadic CJD are varied, presumably reflecting the disparate areas of cerebral cortex that may be initially involved in the pathologic process. The most common initial symptoms and signs are listed in Table 2, with cognitive impairment and ataxia predominating. Rare presentations include initial cortical blindness (the Heidenhain variant) (Heidenhain 1929), which is usually associated with

Table 2 Clinical characteristics of sporadic CJD: symptoms at presentation (percentages)

	England and Wales 1970–1979 (n=124)	France 1968–1977 (n=124)	US 1963–1993 (n=232)	UK 1990–1994 (n=144)
Dementia	21	29	48	19
Ataxia	19	29	33	39
Behavioral disturbance	18	30	29	15
Dizziness	11	8	13	N/A
Visual	9	17	19	10
Involuntary movements	5	1	4	2
Dysphasia	5	N/A	N/A	4
Sensory	4	2	6	N/A
Headache	3	10	11	N/A

visual hallucinations, and a pure cerebellar syndrome (the Brownell-Oppenheimer variant) (Brownell and Oppenheimer 1965). The most striking feature of sporadic CJD is the relentless and multifocal progression of neurologic deficits: Patients who are initially ataxic may develop cognitive impairment, dysphasia, and myoclonus whereas patients presenting initially with cognitive impairment may develop ataxia and cortical blindness. Although there is great variation in the symptoms in individual cases, the rapid evolution of deficits involving multiple cerebral areas is typical of CJD and relatively distinctive.

The signs in sporadic CJD reflect this variation in clinical symptomatology. Initial cognitive impairment or ataxia occurs most frequently (Table 3), but as the disease evolves, a range of neurologic signs are seen, most importantly myoclonus (Brown et al. 1986). In addition to the progressive cognitive impairment, which occurs in all cases of sporadic CJD, the great majority of cases develop ataxia and myoclonus, and a proportion develop dysphasia, cortical blindness, primitive reflexes, and paratonic rigidity of the limbs. Lower motor neuron signs are rare, although peripheral neuropathy has been described in some cases (Sadeh et al. 1990), and epilepsy occurs in only a small minority of cases despite the predominantly cortical neuropathology.

Historically, cases with a combination of dementia and motor neuron

Table 3 Clinical characteristics of sporadic CJD: signs during course of illness (percentage)

	England and Wales 1970–1979 (n=124)	France 1968–1977 (n=124)	US 1963–1993 (n=232)	UK[a] 1990–1994 (n=144)
Dementia	100	100	100	100
Myoclonus	82	84	78	85
Pyramidal	79[b]	44	62	62
Dysphasia	62	N/A	N/A	58
Cerebellar	42	56	71	85
Akinetic mutism	39	N/A	N/A	75
Primitive reflexes	30	N/A	N/A	58
Cortical blindness	13	N/A	N/A	52
Extrapyramidal	3	60	56	34
Lower motor neuron	3	12	12	17
Seizures	9	9	19	13

[a]In this series, cases of CJD were examined in life, which may explain the higher incidence of primitive reflexes and cortical blindness.

[b]Rigidity alone classified as pyramidal.

disease, the amyotrophic variant, were regarded as a form of CJD, but these cases are no longer classified as prion diseases because transmission studies have been almost uniformly unsuccessful (Salazar et al. 1983).

The differential diagnosis of CJD is wide (Table 4) and the clinical distinction from other forms of dementia rests on the occurrence of focal neurologic signs early in the course of the illness, the rapid clinical evolution, and, in particular, the total illness duration. In systematic surveys the mean duration from first symptom to death in CJD is about 5 months (Will and Matthews 1984), which is relatively distinct from other more common forms of dementia. Furthermore, in CJD many patients decline rapidly to a state of akinetic mutism and then survive for some weeks or months. In these cases, the rapidity of disease evolution is quite different from more common forms of dementia and raises the possibility of other diagnoses including stroke, encephalitis, or brain tumor.

About 90% of CJD cases are recognized clinically, but there are cases with a prolonged clinical illness lasting for months or years, and in some of these cases, the insidious progression may mimic other conditions such as Alzheimer's disease (Brown et al. 1984). The clinical distinction from Alzheimer's disease may be particularly difficult if there is associated myoclonus, which is often not recognized to be a feature of Alzheimer's disease. A definitive diagnosis of CJD requires neuropathologic confirmation. In cases of CJD with long clinical duration the diagnosis may only be recognized postmortem.

FAMILIAL CJD

The features of familial CJD vary according to the underlying mutation associated with the particular case. Overall, there is an earlier age of onset in familial cases and a more prolonged duration of illness. A description of the clinical features of familial CJD can be found in Chapter 10.

EPIDEMIOLOGY

In 1968 it was established that CJD was experimentally transmissible (Gibbs et al. 1968), and this stimulated epidemiologic research aimed at identifying the mechanism of natural transmission, should this indeed occur. Epidemiologic surveys of CJD have varied in their scope, ranging from localized study to systematic national studies, and also in their methodologies for case ascertainment and criteria for case definition. Nonetheless, the conclusions of these studies are remarkably consistent

Table 4 Differential diagnosis of CJD in systematic surveys

	England and Wales[a] 1970–1979 (n=42)	England and Wales[b] 1980–1984 (n=95)	UK[c] 1990–1994 (n=41)
Alzheimer's disease (ATD)	20 (3)[d]	28 (5)[d]	17
ATD + multi infarct dementia (MID)	—	—	5
MID	3 (1)[d]	4	4
Motor neuron disease	5 (1)[d]	2 (1)[d]	2
Corticostriatonigral degeneration	3 (3)[d]	—	—
Idiopathic encephalopathy	2 (2)[d]	45 (5)[d]	1
Hydrocephalus	—	4 (3)[d]	—
Parkinson's disease	3 (2)[d]	1	—
Pick's disease	1 (1)[d]	1 (1)[d]	1
Others: (n=1)	ATD and MS limbic encephalitis herpes simplex encephalitis familial spinocerebellar degeneration multiple cerebral abscess	stroke limbic encephalitis atypical demyelination carcinomatous meningitis glioblastoma post-anoxic encephalopathy cerebral metastases hepatic encephalopathy catatonic schizophrenia progressive supranuclear palsy	diffuse Lewy body diseases (DLBD) DLBD + ATD cerebellar degeneration multi-system atrophy cortico-basal degeneration viral encephalomyelitis metastatic carcinoma hypoxia epilepsy progressive supranuclear palsy spongiform myelinopathy

[a]Case certified as dying of CJD in which the diagnosis was reclassified after review of clinical data or pathology.

[b]Cases referred as suspect CJD in which the diagnosis was reclassified after review of clinical data or pathology.

[c]Cases referred as suspect CJD with neuropathologic confirmation of alternative diagnosis.

[d]Numbers of cases with neuropathologic data in parentheses.

and baseline epidemiologic parameters for CJD have been established (Brown et al. 1979; Galvez et al. 1980; Kondo 1985; Will et al. 1986), which have been important in assessing hypotheses generated from basic scientific research and in the identification of any change due to novel risk factors for human prion disease.

The majority of studies indicate that males and females are affected with equal frequency, although some studies have shown a relative female excess of cases (Will et al. 1986). In the US, 1979–1994 (Holman et al. 1996), 52.9% of the reported CJD deaths were in women, but the age-adjusted CJD death rate in men was slightly higher than in women. The higher absolute number of deaths from CJD in women primarily reflected the greater proportion of women in the older age groups that were at highest risk for CJD. The annual incidence of CJD is often estimated to be about one case per million population. Annual incidence and mortality rates are similar in CJD because the illness is invariably fatal and its mean duration is relatively short. Annual mortality rates in some early studies were lower than the quoted figures, ranging from 0.09 (Matthews 1975) case per million in one study including only neuropathologically verified cases to about 0.5 case per million in more systematic and comprehensive surveys (Brown et al. 1979; Cousens et al. 1990). More recent systematic surveys of CJD have identified incidence or mortality rates (Table 5) with figures approaching or exceeding one case per million in many countries. The efficiency of case ascertainment may have improved with increased awareness of CJD among the neurologic community and, more recently, by other health practitioners and indeed the community at large. In 1994, for example, the estimated annual incidence in terms of cases per million population was 0.91 in the UK and 1.01 in the Netherlands (Wientjens et al. 1996a). In Austria, annual incidence rates of CJD of up to 1.5 cases per million per annum have been reported (Hainfellner et al. 1996). Whether this estimate reflects the very high postmortem rate in Austria or a variation about a mean is not yet established. In the US, based on reviews of national death certificate data, the recent annual CJD death rate has been 1.0 (1994–1996) (L.B. Schonberger, pers. comm.).

CJD almost certainly occurs worldwide, and countries in which the condition has not been reported are mainly in central Africa where diagnostic facilities are limited and in which the majority of the population may not live to the age at which CJD is likely to develop (Fig. 1). Since the age-adjusted CJD mortality rate for African-Americans in the US, however, is only 40% of that for whites, the age-adjusted incidence of CJD in many African countries may, in fact, be unusually low.

Table 5 Systematic studies of CJD worldwide

Country	Period	Incidence: cases/ million	Country	Period	Incidence: cases/ million
Austria	1969–1985	0.18	Italy	1958–1971	0.05
	1986–1994	0.67		1993–1994	0.11
	1995	1.5		1993–1995	0.56
Australia	1970–1980	0.66	Japan	1975–1977	0.45
	1987–1996	1.07		1985–1996	0.58
Chile	1955–1972	0.10	Netherlands	1993–1995	0.81
	1973–1977	0.31			
	1978–1983	0.69	New Zealand	1980–1989	0.88
Czechoslovakia	1972–1986	0.66	Slovakia	1993–1995	0.62
France	1968–1977	0.34	Switzerland	1988–1997	1.14
	1978–1982	0.58			
	1993–1995	0.84	United Kingdom	1964–1973	0.09
				1970–1979	0.31
Germany	1979–1990	0.31		1980–1984	0.47
	1993a–1995	0.55		1985–1989	0.46
				1993–1995	0.77
Israel	1963–1972	0.75			
	1963–1987	0.91	US	1973–1977	0.26
				1986–1988	0.83
				1979–1990	0.90

[a]Extrapolated from part-year data.

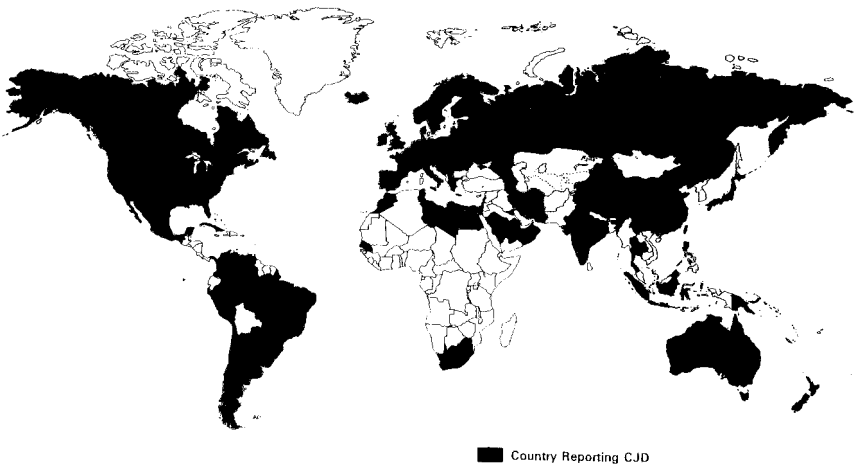

Figure 1 Countries in which CJD has been reported.

There are two populations, Libyan-born Israelis (Goldberg et al. 1979) and certain populations in restricted areas of Slovakia (Ferak et al. 1981), in which the incidence of CJD is 60–100 times greater than expected. The hypothesis that these clusters might be causally related to dietary exposure to the scrapie agent was not supported by a case-control study, and a high index of inbreeding was identified many years ago in the Slovak population (Ferak et al. 1981). The crucial study linking familial forms of prion disease to a specific mutation of the PrP gene (Hsiao et al. 1989) led to genetic investigation of the high-incidence populations in Israel and Slovakia. It is now clear that the increased incidence in these populations is linked to a high prevalence of codon-200 mutations of the PrP gene (Goldfarb et al. 1990; Hsiao et al. 1991).

Systematic national surveys of CJD suggest that cases are randomly distributed within individual countries (Fig. 2) (Cousens et al. 1990), and cluster analysis in many surveys indicates an apparently random distribution of cases in space and time. An increased incidence has been found in urban populations in some studies (Brown et al. 1979; Galvez et al. 1980), but this may be due to improved ascertainment as a result of more available medical services in urban areas. In the US, 1979–1990, the proportion of CJD deaths in metropolitan counties of residence (75.4%) was similar to the proportion of the US population in such areas in 1980 and 1990 (76.2% and 77.5%, respectively) (Holman et al. 1995). With the potentially long incubation period, analysis of case distribution by residence of death might be misleading. One study that analyzed residence throughout life showed no convincing evidence of space-time clustering (Cousens et al. 1990).

Sporadic CJD is largely a disease of late middle age with a peak incidence and mean age at death in the 65–69-year age group (Fig. 3). The rarity of cases of sporadic CJD in patients aged less than 30 years has been confirmed in all systematic surveys, although there have been occasional case reports of CJD in teenagers (Monreal et al. 1981; Brown et al. 1985). A sharp drop in incidence over the age of 75 years has been consistent in all surveys, although some doubt persists about the efficacy of case identification in older patients. Recent studies in Europe have shown higher incidence rates in the elderly than in previous surveys (Will et al. 1998), but the age-specific incidence rate in those over the age of 75 years remains significantly lower than in the 65–69-year age group. The identification of clinically unrecognized cases of CJD in postmortem studies in the elderly demented is exceptional. Current evidence suggests that the progressive fall in incidence in older patients is accurate and not simply an artifact of less efficient case ascertainment.

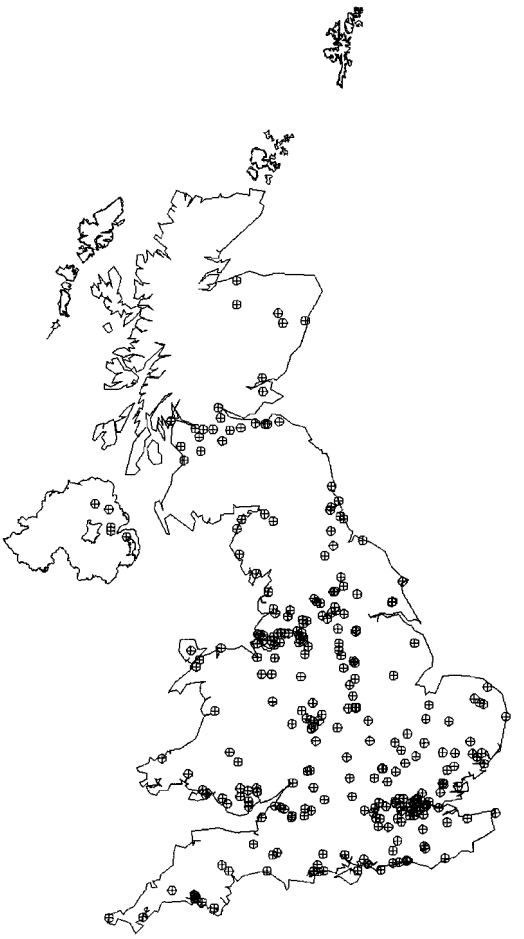

Figure 2 Map of the UK showing the distribution of cases of definite or probable sporadic CJD according to residence at death (1990–1998).

Case-control studies have been carried out in a number of populations in order to look for risk factors for the development of CJD (Bobowick et al. 1973; Kondo and Kuroiwa 1982; Davanipour et al. 1985; Harries-Jones et al. 1988; van Duijn et al. 1998). The methodologies of these studies have varied; all have necessarily included only small numbers of cases and controls and the findings have been contradictory (Table 6). A meta-analysis of case-control studies has found an increased risk of CJD in those individuals with a family history of the condition and also an increase in risk in those with a family history of other neurodegenerative

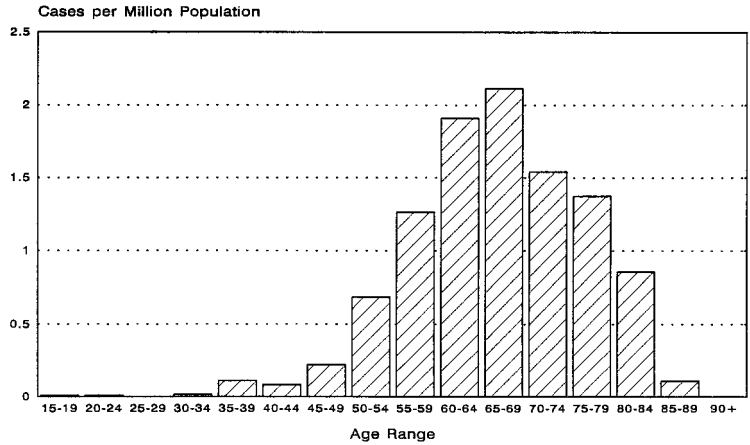

Figure 3 Age-specific mortality rates for sporadic CJD (UK 1970–1998).

diseases including Parkinson's disease (Wientjens et al. 1996b). The latter finding is unexpected and may, in some studies, be related to the exclusion of control subjects with dementia. Such an exclusion could artificially lower the prevalence of a family history of dementia in control subjects in comparison to the CJD cases. Although fewer than 700 CJD cases have been enrolled in published case-control studies to date and not all risk factors have been consistently examined, these studies are important. They provide no convincing evidence of an increased risk of CJD through dietary habits, occupation, animal contact, or a history of blood transfusion. Because of inherent statistical and other limitations of case-control studies, however, they cannot rule out possibly small increases in risk.

The epidemiologic studies have not resulted in an explanation for the cause of sporadic CJD. The apparently random geographic distribution of cases and the absence of any common risk factor for disease development has led some experts to hypothesize that most sporadic cases do not have an environmental source of infection and that a spontaneous alteration in the structure of PrP is the mechanism of disease initiation, possibly facilitated by an occasional somatic mutation (Prusiner 1994). This theory fits much, but not all, of the epidemiologic evidence. For example, the decrease in incidence of CJD over the age of 75 years remains puzzling. It is also important to note that exclusion of risk factors can be difficult in a disease with a prolonged incubation period, particularly when evidence of risk factors is derived from surrogate witnesses.

Table 6 Significant risk factors for CJD in controlled studies

Author	Method	Risk factors
Bobowick et al. (1973)	38 "selected" cases; healthy controls	none
Kondo and Kuroiwa (1982)	population study: 60 cases; healthy controls	trauma in males
Kondo (1985)	88 autopsied cases; autopsied controls	organ resection
Davanipour et al. (1985)	26 cases; 40 controls	trauma or surgery to head or neck other trauma surgery needing sutures tonometry
Davanipour et al. (1985)	as above	roast pork, ham, underdone meat, hot dogs
Davanipour et al. (1985)	as above	contact with fish, rabbits, squirrels
Harries-Jones et al. (1988)	92 cases; 184 controls	herpes zoster keeping cats contact with pets other than cats/dogs dementia in family
van Duijn et al. (1988)	405 cases; 405 controls	consumption of raw meat consumption of brain frequent exposure to leather products exposure to fertilizer consisting of hoof and horn

INFECTIOUS PRION DISEASES

Iatrogenic CJD

The very fact that CJD has been demonstrated to be transmissible in laboratory studies raises the possibility that transmission from person to person might occur in the course of medical treatment. Iatrogenic transmission of CJD was first suggested in 1974 by a case of CJD in a recipient of a corneal transplant derived from a patient who also died of CJD (Duffy et al. 1974), although in retrospect it is likely that three of Nevin and Jones's original cases were transmitted by contaminated neurosurgi-

Table 7 Total cases of iatrogenic CJD worldwide

Mode	Number of cases	Mean incubation period (years)	Clinical
Neurosurgery	4	1.6	visual/cerebellar/dementia
Depth electrodes	2	1.5	dementia
Corneal transplant	2	15.5[a]	dementia
Dura mater	69	6[b]	visual/cerebellar/dementia
Human growth hormone	>100	12[b]	cerebellar
Human gonadotrophin	4	13	cerebellar

[a]Range 1.5–30 years.
[b]Estimated on incomplete data.

cal instruments in the early 1950s (Jones and Nevin 1954; Nevin et al. 1960). Transmission of CJD by depth electrodes in two cases was suggested by an article in 1977 (Bernoulli et al. 1977), and the tragedy of CJD in human growth hormone recipients was first recognized in 1985 (Koch et al. 1985). More recently, the iatrogenic transmission of CJD by human dura mater grafts has been identified (Thadani et al. 1988). All these transmissions have involved cross-contamination with material in or adjacent to the brain where the expected titers of infectivity would be highest (Table 7). All have involved parenteral inoculation either by surgery or by intramuscular injection. Strong evidence exists that the risk, if any, of transmission of CJD by blood products is low; no such transmission has yet been documented. It is uncertain, however, whether this undetected risk reflects the absence of the agent of CJD in the blood of infected persons or the safety of a low-titer tissue.

Neurosurgical Transmission

The seminal publications by Nevin and Jones were crucial to the definition of clinical, investigative, and neuropathologic features of sporadic CJD. In retrospect, three of these cases may have been caused by iatrogenic transmission via contaminated neurosurgical instruments. Review of the cases in the original papers indicated that some of the cases were investigated in the same hospitals and that hospital admissions overlapped. A small number of patients with CJD underwent invasive procedures, including brain biopsy, and concurrent inpatients underwent neurosurgery for other conditions; e.g., removal of meningioma or cortical

undercut. The readmission of three of these cases 18 months to 2 years later to the same hospitals with CJD provides strong circumstantial evidence of transmission through contaminated neurosurgical instruments. A similar case has been described in France (Foncin et al. 1980). The clinical features are indistinguishable from sporadic CJD in these putative cases of neurosurgical transmission by an intracerebral inoculation.

Corneal Grafts and Depth Electrodes

The first description of potential iatrogenic transmission of CJD was in 1974 (Duffy et al. 1974). A 55-year-old woman developed CJD 18 months after transplantation of a cadaveric corneal graft, which had been obtained from a 55-year-old man who had died of pathologically confirmed CJD. A second case of CJD in association with corneal grafting was published in 1994 (Uchiyama et al. 1994), but in this case the diagnosis in the corneal donor was not established. A third case was described in 1997 in which both donor and recipient died from pathologically confirmed CJD, although the latency between corneal transplant and the development of CJD was 30 years (Heckmann et al. 1997). These cases provide strong circumstantial evidence of transmission of CJD through cadaveric corneal grafts. Although epidemiologic data indicate that the risks to any individual patient from corneal graft are very low, the importance of screening donors is underscored by the documentation that this route of transmission is possible.

In 1977 two patients, aged 25 years and 19 years, developed CJD 2 years after stereotactic EEG recordings (Bernoulli et al. 1977). The instruments had previously been used in a 69-year-old patient with rapidly progressive dementia and myoclonus, who was later confirmed as having died of CJD. The electrodes had been "sterilized" with ethanol and formaldehyde vapor. The suspicion that CJD had been transmitted through inadequately sterilized depth electrodes, raised by the young age of the two secondary cases, was subsequently supported by transmission of CJD to a chimpanzee 18 months after intradural implantation of the suspect electrodes (Brown et al. 1992).

Dura Mater Grafts

The potential for the transmission of CJD from patient to patient by cadaveric dura mater grafts was first recognized after a case report in the US in 1987. There have now been at least 66 cases worldwide, including

43 cases recently identified in Japan (CDC 1997). Almost all of these cases, including at least 41 of the 43 recently reported cases in Japan, involved the insertion of Lyodura brand grafts processed before May 1987. Other known or possible exceptions to the use of such Lyodura grafts include one case in the UK in which the source was unknown (Will and Matthews 1982) and one case in Italy in which locally produced dura was implanted (Masullo et al. 1989). In 1987, the US Centers for Disease Control (CDC) published a description of differences between the processing of Lyodura and other similar products and suggested that Lyodura may be associated with a higher risk for transmitting CJD than other dura mater products used in the US (CDC 1987). Also in June 1987, representatives of B. Braun Melsungen A.G. reported that as of May 1, 1987, their procedures for collecting the processing dura were revised to reduce the risk of CJD transmission (Janssen and Schonberger 1991). These revised procedures included conversion from batch to individual processing of dura mater and treatment of each dura mater graft with 1.0 N sodium hydroxide. The large number of Lyodura-associated cases of CJD has provided strong evidence for cross-contamination within the production process. The risk of transmission of CJD through dura mater grafts had been thought to be low because only a small number of recipients would receive contaminated material from any individual infected donor.

For the recently reported 43 dura mater-associated cases of CJD in Japan, the mean latency period from receipt of the graft to the onset of CJD was 7.4 years, range 1.3–16.1 years (CDC 1997). For the 23 earlier reported cases outside Japan, the interval from the implantation of the graft to the development of clinical disease ranged from 1 to 12 years with a mean of about 4 years.

Clinically, the majority of patients with dura mater-related CJD present with symptoms and signs consistent with sporadic CJD. In some cases the presentation is less typical, and in a few patients a cerebellar syndrome has been described. The anatomical site of the graft may influence clinical presentation, and two cases in the UK presented with a cerebellar syndrome 5–10 years after receiving posterior fossa grafts following excision of cerebellar tumors.

Estimating the risk of developing CJD following dura mater grafts is hampered by the absence of precise information on the number of persons who received grafts each year and their subsequent survival. The major risk, however, appears to be in those individuals who received grafts between 1981 and 1987 (Fig. 4). It was roughly estimated in Japan that between 1983 and 1987, up to 100,000 patients may have received a Lyodura graft. The 33 reported recipients of Lyodura, 1983–1987, who died of CJD with-

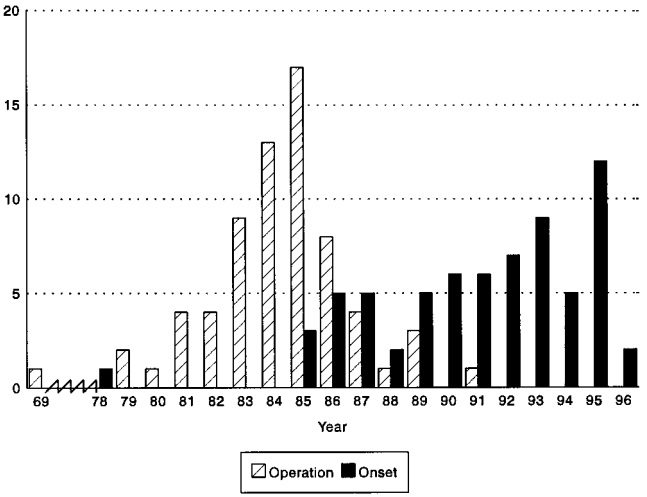

Figure 4 Dura mater cases worldwide shown by year of operation and year of onset of symptoms for CJD. Mean incubation period from operation to onset of symptoms: 6.8 years (range 1–16).

in 12 years after receipt of the grafts represented a minimum risk of approximately one case of CJD per 3,000 Lyodura graft recipients.

In many countries, human dura mater grafts have been withdrawn from use, but some countries continue to use this material. In the US, it has been recommended that human dura mater grafts may still be used provided there is stringent donor screening, including neuropathologic evaluation of donors, and the processing of each dura separately to avoid possible cross-contamination. In addition, treatment of dura with 1 N sodium hydroxide for one hour, or some other comparable inactivating procedure, has been recommended. Because even these steps may not completely eliminate the potential for an infectious graft, some surgeons may choose the alternative use of autologous fascia lata grafts, fascia temporalis, or synthetic substitutes. Whether autologous fascia lata grafts, which involve more extensive surgery, or other forms of dural implant that may have other disadvantages carry significantly lower risks to neurosurgical patients than human dura mater grafts remains controversial.

Human Pituitary Hormones

The treatment of short stature in children, frequently endocrinologically determined, with human pituitary-derived growth hormone was initiated

in the late 1950s, and the effectiveness of this therapy resulted in the subsequent treatment of large numbers of individuals. About 30,000 children had been treated with HGH worldwide by 1985. Small numbers of women were treated for infertility with human pituitary gonadotrophin over a similar period in a number of countries. In 1985, the occurrence of CJD in a HGH recipient (Koch et al. 1985) indicated the possibility of iatrogenic transmission because of the remarkably young age of the patient, and the occurrence of a further case in the US (Gibbs et al. 1985) and a case in the UK (Powell-Jackson et al. 1985) in the same year provided strong circumstantial evidence of transmission of CJD via HGH. Since then, CJD has developed in over 100 HGH recipients in a number of countries including the US, UK, France, New Zealand, and the Netherlands (Table 8). In 1996, the reported overall proportion of CJD cases in the total recipient population of these countries was about 1 in 150, consistent with laboratory decontamination studies (Pocchiari et al. 1991), but this proportion varies between countries for reasons that have not been fully established. In the US, the proportion of cases in relation to the total number of recipients was approximately 1 in 500; in the UK, 1 in 100; and in France, 1 in 50 (Brown 1996). In addition to differences in periods of follow-up, these differences in proportions may reflect differences in the characteristics of the donor and recipient populations and the methods of hormone preparation. Recent evidence suggests that, for unknown reasons, the proportion of cases to recipients in New Zealand may be higher, particularly among recipients of HGH produced in the US.

The methodology of growth hormone production has varied with time and between production centers, and it had been hoped that HGH produced using a column chromatography step might be of low risk on the basis of decontamination studies. However, cases of CJD have developed in recipients of HGH produced using at least one column chromatographic method (Billette de Villemeur et al. 1991).

Table 8 Numbers of deaths from HGH-related CJD by country

Country	Number of deaths
France	55
UK	26
US	16
New Zealand	4
Netherlands	1
Brazil	1

Contamination of the hormone preparation occurred when pituitary glands derived from patients who died from or were incubating CJD were included in the production process. The necessity to pool many thousands of glands in order to produce HGH, and cross-contamination of multiple lots during production, presumably aggravated the risk. The powerful circumstantial evidence suggesting a causal link between HGH and CJD has been supported by transmission studies in which a squirrel monkey developed a spongiform encephalopathy following inoculation with 1 of 76 potentially contaminated lots of HGH in the US (Gibbs et al. 1993). The long incubation period in this animal experiment and the inability to transmit CJD from any of the other potentially contaminated lots of HGH support the conclusion that the infectivity levels in affected lots were extremely low. In the US, the mean incubation period calculated from the midpoint of therapy to the onset of CJD has approached 20 years.

Cases of HGH-related CJD continue to occur, but the current numbers of cases indicate that the large majority of HGH recipients are unlikely to develop the disease. In Europe, in contrast to the US, the incubation period ranges from 4.5 to over 25 years with a mean of about 12 years. The reasons for the shorter incubation periods in Europe are unknown but suggest exposure to possibly higher levels of infectivity. In the absence of any test for the presence of infectivity, there is a population of many young individuals at risk of CJD. HGH was withdrawn in most countries in 1985 and human pituitary gonadotrophin has also been withdrawn in many countries following the occurrence of CJD in four recipients in Australia (Cochius et al. 1990, 1992).

The clinical features of human pituitary hormone-related CJD are distinct from sporadic CJD. In the great majority of cases, the initial presentation involves a progressive cerebellar syndrome, and other features including dementia develop late, if at all. It is possible that the route of inoculation of the infectious agent may be an important determinant to clinical expression of disease. In kuru, presumed to be due to a peripheral route of infection, cerebellar signs predominate in the early stages as in human pituitary hormone recipients, whereas in iatrogenic CJD due to central inoculation the clinical features are similar to classic CJD.

Possibility of Transmission through Other Routes, e.g., Blood

All cases of iatrogenic CJD have involved cross-contamination with potentially high titers of infectivity by a parenteral route. However, the possibility of transmission of CJD via other tissues must be considered.

Some laboratory transmission studies of prion diseases have demonstrated the presence of infectivity in blood, although the validity of the positive studies in patients with CJD has been questioned (Brown 1995). In rodent models, the infectivity of blood is present early in the incubation period well before development of clinical disease (Diringer 1984; Casaccia et al. 1989). These studies of infectivity usually maximize the chances of successful transmission by the inoculation of concentrated blood samples, such as buffy coat, by the most efficient, intracerebral route. Infectivity has been reported in blood derived from CJD patients in four separate laboratories involving rodent inoculations. In contrast, no infectivity of blood was reported with CJD or kuru in tests using primates as the assay animal. The primate assays are more sensitive to human prions than rodent assays. Thus, the negative results using the primate assays, and a number of "puzzling" findings in the four positive experiments using rodents, indicate that the presence of CJD infectivity in human blood is not clearly established. In contrast to the difficulties in clearly demonstrating the infectivity of blood in CJD patients, the infectivity of blood in rodent models of CJD is well established.

The studies of the infectivity of blood in prion diseases suggest a theoretical possibility that blood or blood products might pose a risk of iatrogenic transmission of CJD. The existing reports of infectivity in the blood of CJD patients, whether valid or not, however, do not establish the magnitude or even the existence of the possible risk of iatrogenic transmission through blood or blood products. To help assess this risk, epidemiologic data are used.

Up until mid 1998, no convincing instance of transmission of CJD by blood or blood products has been reported. A claim that there were four transfusion-related cases of CJD in Australia (Klein and Dumble 1993) has not been substantiated, and the report of a French case that potentially implicated transmission by albumin or liver transplant was most likely fortuitous, as suggested by the remarkably short potential incubation period (Creange et al. 1995). There have been no cases of CJD identified in hemophiliacs, and case-control studies do not suggest an increased risk of CJD through previous blood transfusion (Esmonde et al. 1993; Wientjens et al. 1996b; van Duijn et al. 1998). This evidence cannot preclude the possibility that CJD may be occasionally transmitted through blood or blood products, but the balance of evidence indicates that if such transmission occurs at all, it is very rare. As a precaution, in many countries patients at higher risk of CJD, including those with a family history of CJD or those with a past history of HGH treatment, are excluded as blood donors.

The relatively reassuring evidence in relation to classic CJD may not be applicable to nvCJD, as this condition is caused by a different infectious agent (Bruce et al. 1997). There is preliminary evidence that the level of PrP in peripheral tissues may be higher in nvCJD than in classic CJD (Hill et al. 1997b). The proportion of the population in the UK who are incubating nvCJD and may be acting as blood donors is unknown. Blood products derived from patients who subsequently develop classic CJD have been withdrawn in some countries, and similar withdrawals of blood products derived from nvCJD blood donors have been initiated in the UK. The degree to which such a preventive measure reduces the theoretical risk of transmission of CJD or nvCJD largely depends on the proportion of the potentially infectious blood or blood products that are withdrawn before they are used. Since for the endemic form of CJD this proportion is low, CJD-related withdrawals can have only a very limited effect on reducing the theoretical risk of transmission of CJD by blood or blood products.

Infectivity titers in blood are relatively low in experimental studies, and procedures that reduce infectivity, for example, filtration, centrifugation, or column chromatography, may significantly reduce risk. In the UK other measures such as leuco-depletion of all blood donations are being considered in response to the theoretical risks of nvCJD. Blood and blood products are an important part of medical treatment and may be lifesaving. Efforts to reduce or avoid the theoretical risk of transmission of CJD or nvCJD must be balanced against the public health consequences of such efforts, including the potential creation or aggravation of shortages of blood products for specific therapies.

Iatrogenic CJD: Precautions and Implications for Public Health

The occurrence of iatrogenic CJD is a medical tragedy. The first recommendations for precautions in tissue transfer in CJD were published in 1977 (Gajdusek et al. 1977), years before the identification of HGH- or dura mater-related CJD. The long latency between the initiation of specific therapies and the identification of a CJD-related risk indicates the need for caution and continued vigilance regarding the case-to-case transmission of human prion diseases.

Guidelines have been drawn up in some countries to minimize the risk of iatrogenic transmission of CJD and modern sterilization techniques, for example porous load autoclaving at 134ºC for 18 minutes, have been demonstrated to significantly reduce infectivity. In some countries the destruction of all neurosurgical or ophthalmological instruments

used on cases of CJD is obligatory. However, no cases of transmission of CJD via neurosurgical instruments have been documented since 1980.

HGH was withdrawn in many countries in 1985 and has been replaced by recombinant therapy. Dura mater grafts are no longer used in many countries, and donor screening will significantly reduce any risk in countries that continue to use this material. There has been no well-documented case of transmission of CJD through blood or blood products, and individuals at greater risk of developing CJD have been excluded as blood donors in many countries.

These measures significantly reduce the possibility of iatrogenic transmission of CJD but do not exclude all risk. Guidelines may not be followed and tissues may be derived from individuals incubating CJD with no clinical evidence of disease. It is important to continue to study CJD systematically in order to maintain confidence in the safety of blood products and to identify any novel and perhaps unexpected route of iatrogenic transmission.

KURU

Kuru is a fatal CNS disease that is geographically restricted to the Okapa area of the highlands of Papua New Guinea (Gajdusek and Zigas 1957). At its peak in the early 1960s, it was the cause of over half of all deaths of the affected population, and more than 3,000 cases have occurred in a total at-risk population of about 30,000 individuals (Alpers 1987). The identification of the mechanism of disease transmission followed years of meticulous research carried out in very difficult conditions by Dr. Carleton Gajdusek and colleagues (Glasse 1967), and the transmissibility of human prion disease was first confirmed in 1966 following the intracerebral inoculation of kuru brain tissue in primates (Gajdusek et al. 1966).

The epidemiologic pattern of kuru has been unusual (Alpers 1987). The disease predominantly affected women and their children of either sex in the early years of the epidemic. With time the incidence of the disease has declined and the proportion of affected adult males and females has become more similar (Fig. 5), and no children born after 1959 have been affected. The cause of kuru was unknown, and theories included a toxic, dietary exposure, infection with conventional viruses or bacteria, or dietary deficiency. All these possibilities were excluded by investigation including a detailed study of dietary factors and examination of tissues, including brain histology. Neuropathologically there was no evidence of an inflammatory response (Fowler and Robertson 1959), and the possibility of a genetic basis of kuru was raised by the unusual sex and age distribution of cases. The possibility of transmission through endo-cannibal-

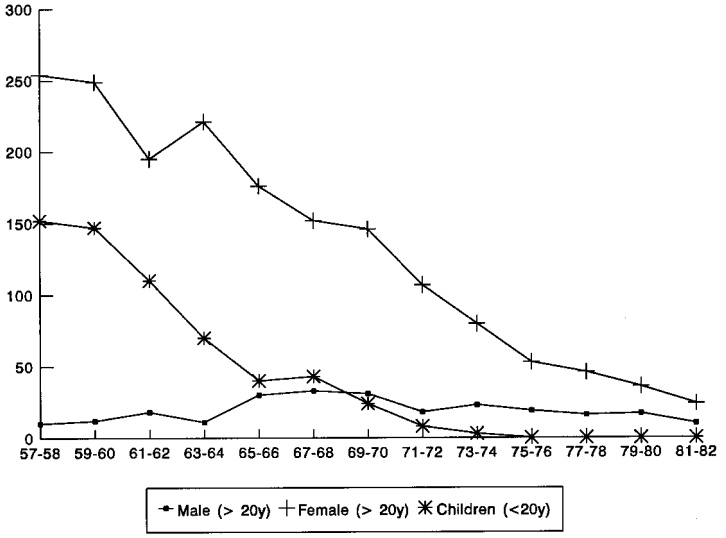

Figure 5 Number of deaths from kuru in 2-year periods between 1957 and 1982 in adults aged >20 years and children aged <20 years.

ism was raised by anthropological enquiry (Glasse and Lindenbaum 1992), and the decline in the epidemic following the cessation of cannibalism in the late 1950s is consistent with this proposed mechanism of transmission.

Women and children consumed diseased relatives as a mark of respect, leading to a familial aggregation of cases. Virtually all tissues were eaten, including brain, viscera, and even powdered bone. In addition to the possible oral transmission of kuru, there is also the possibility of transcutaneous transmission through rubbing infected material on the skin. Males, and in particular elderly males, were involved at times in the cannibalistic rituals, but the excess of cases in females and children is consistent with the available descriptions of the rituals, since they, and not the men, ate the internal organs, in particular the brain. In recent years the decline in incidence of kuru has led to the linking of cases with individual cannibalistic rites in which several members of a family, including those resident in different areas, have been affected by kuru (Klitzman et al. 1984). The incubation period ranges from 4 1/2 years to at least 40 years, and cases are still occurring, albeit at a very low rate. No precise figure for the mean incubation period is available, as affected individuals may have taken part in multiple funeral rituals and the critical exposure cannot be identified.

Clinically, cases of kuru presented with a pure cerebellar syndrome (Alpers 1987). A prodromal phase characterized by headache and limb pains was universal. Initial signs of the cerebellar syndrome included astasia, midline trunkal ataxia, trunkal tremors, and titubation, followed by gait ataxia and dysarthria. Hypotonia was common in the early stages, and occasional patients developed marked flaccidity of limb musculature. In contrast to CJD, other neurologic signs such as upper motor neuron signs and myoclonus did not occur. In the later stages communication was often difficult because of severe dysarthria, but in the majority of patients, dementia was conspicuous by its absence. Even in the terminal moribund, akinetic, and mute state, most patients could make eye contact and attempt to carry out simple commands. The total illness duration in adults ranged from 12 to 18 months.

In juveniles the clinical picture was similar to the adult form of disease. Brain stem signs were common and included titubation, nystagmus, strabismus, facial weakness, and sometimes ptosis. Other features included transient generalized hyper-reflexia and clonus. The clinical course in children was shorter than in adults, ranging from 3 to 12 months.

The scientific study of kuru has been crucial to current understanding of prion diseases. Following Hadlow's recognition of a similarity of the pathology in kuru and scrapie, transmission studies were initiated which proved that human prion diseases were caused by transmissible agents, despite any evidence of infection histologically or serologically. This remarkable discovery led to the successful laboratory transmission of CJD and initiated research into the epidemiology and pathogenesis of human prion disease and to further investigation of the nature of the infectious agent. From a clinical perspective, there is a remarkable consistency in the clinical picture in kuru, following exposure to an exogenous infectious agent, and the epidemiologic pattern of kuru has demonstrated that human diseases can be transmitted through a peripheral route, either orally or transdermally. It is also important to note that there was no evidence of case-to-case transmission of kuru through routes other than cannibalism, and in particular, there was no evidence of vertical transmission of infectivity in kuru, despite the breastfeeding of infants by many hundreds of clinically affected mothers (Gajdusek 1990; Alpers 1968).

NEW VARIANT CJD

Surveillance of CJD was instituted in the UK in 1990 in order to identify any change in this condition that might indicate transmission of BSE to the human population. In 1993, a collaborative system for the study of

CJD, sharing common methodologies and criteria for case definition, was established in Europe and included national surveillance programs in France, Germany, Italy, the Netherlands, Slovakia, and the UK (Wientjens et al. 1994). One aim of this collaborative study was to identify any change in CJD that might correlate with presumed prior exposure to the BSE agent.

In 1995 and early 1996, a number of cases of CJD were identified in the UK with a clinicopathologic phenotype distinct from previous experience. By March 1996, 10 cases of CJD had been identified with a young age at onset, mean 29 years in comparison to 66 years in sporadic CJD; a long duration of illness, mean 14 months in comparison to 4.5 months in sporadic CJD; and an unusual and remarkably uniform clinical presentation, relatively different from that previously seen in CJD (Will et al. 1996b). The cases all developed early psychiatric symptoms including depression and withdrawal for months prior to the development of neurologic signs. The terminal stages were similar to classic CJD, but the EEG did not show the characteristic changes so often seen in these cases. Crucially, the neuropathologic appearances, including extensive florid plaque formation, were thought to be distinct from the neuropathology observed in CJD in the past. There have now been 30 cases of this form of CJD, designated nvCJD, identified in the UK and 1 case in France. All these cases have been confirmed neuropathologically, with the exception of one case in the UK. The features that distinguish nvCJD from sporadic CJD are listed in Table 9.

The distinction between individual clinical features in nvCJD and classic CJD is not absolute, but as a group, the cases of nvCJD exhibit a different phenotype (Zeidler et al. 1997b,c). Occasionally cases of classic CJD may share many of the features of nvCJD, and the identification of nvCJD cases still rests on neuropathologic verification. The hypothesis that the neuropathologic features are distinctive is supported by the failure to identify cases with similar pathologic features in other countries, even after review of archival material.

Table 9 Differences between classic CJD and nvCJD

	Classic	New variant
Mean age (years)	66	29
Median illness duration (months)	4	14
EEG	typical	atypical
Pathology	kuru plaques (10%)	florid plaques (100%)

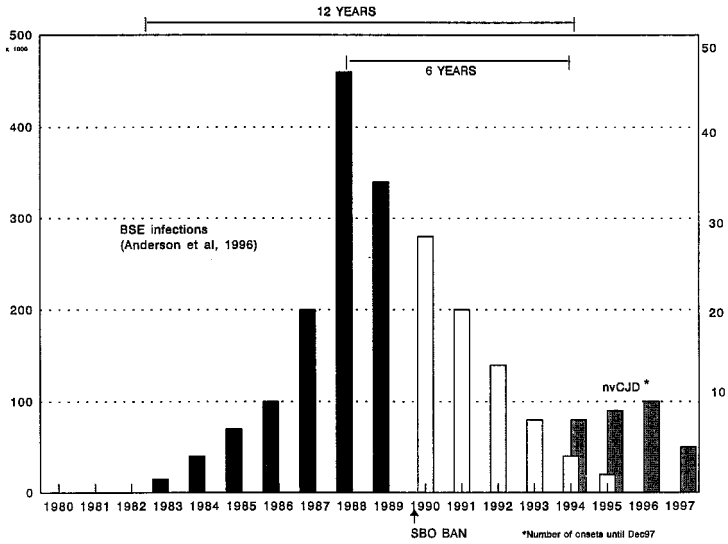

Figure 6 BSE "exposure" and the incidence of nvCJD by year of onset.

The identification of a novel form of CJD does not in itself necessarily imply a novel etiological factor. Variants of CJD have been described in the past; for example, the Heidenhain and Brownell-Oppenheimer variants. The hypothetical causal link with BSE depends from an epidemiologic perspective on the timing of the occurrence of nvCJD and the colocalization of nvCJD with BSE, as a potential novel risk factor for human prion disease (Will 1998). Evidence on incubation periods in CJD comes from iatrogenic cases and in particular HGH-related CJD and kuru, which both involved a peripheral route of infection. The minimum incubation period in these types of human prion diseases is 4.5 years, with a mean in HGH-related CJD of about 13 years. The transmission of BSE to the human population would necessarily involve crossing a species barrier, which usually results in a relative prolongation of incubation period compared to within-species transmission. Human exposure to the BSE agent probably started around 1983, probably peaked in 1989, and declined subsequently (Fig. 6). The first cases of nvCJD had disease onset in 1994, implying a minimum incubation period of CJD to the human population of approximately 6–12 years, consistent with data from HGH-related CJD and kuru. The slightly longer incubation period in nvCJD may reflect a species barrier effect.

Through the coordinated surveillance system for CJD in Europe, it has been possible to establish that nvCJD is occurring almost exclusively

Table 10 Numbers of cases of BSE per country and numbers of cases of nvCJD per country

Country	Number of BSE cases	Number of nvCJD cases
UK	175,451	32
Republic of Ireland	306	0
France	42	1
Belgium	5	0
Switzerland	275	0
Portugal	147	0
Canada	1	0
Germany	6	0
Oman	2	0
Denmark	1	0
Netherlands	3	0
Luxembourg	1	0
Italy	2	0
Falklands	1	0
US	0	0

in the UK (Table 10). The exposure of the human population to the BSE agent was potentially very much higher in the UK than in any other country, and the occurrence of a BSE-related human prion disease in the UK is consistent with a causal link (Will et al. 1998). A single case of nvCJD has been identified in France (Chazot et al. 1997), which was a major importer from the UK of meat products, cattle food in the form of meat and bone meal, and cattle that may have been incubating BSE.

Analysis of risk factors has been carried out in nvCJD. None of the cases has a history of neurosurgery, corneal graft, or human pituitary hormone therapy. One hypothesis was that these cases might be hereditary forms of CJD and, indeed, some of the cases do have a family history, often distant, of neurodegenerative disease. However, full sequencing of the PrP gene in these cases has excluded all known mutations of the PrP gene (Zeidler et al. 1997a), and preliminary analysis of the promoter region has also failed to identify any specific abnormality. On current evidence, these cases are not associated with mutations of the PrP gene. All cases have been methionine homozygotes at codon 129 of the PrP gene, but evidence from the French cohort of HGH recipients indicates that variation at this locus may influence incubation period rather than overall susceptibility to exogenous infection (Deslys et al. 1996). It is possible that cases of nvCJD expressing valine at codon 129 may occur in the future, and it is possible that the clinicopathologic phenotype may be different for the cases already identified.

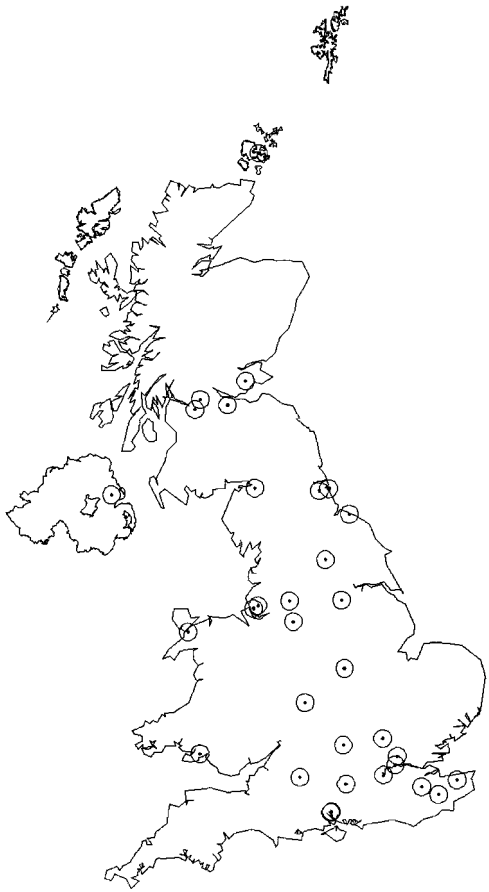

Figure 7 Map of the UK showing the distribution of cases of definite or probable nvCJD according to residence at disease onset.

nvCJD occurs throughout the UK (Fig. 7), and cluster analysis suggests that these cases are randomly distributed, with the implication that any risk factor must have been widely disseminated (National CJD Surveillance Unit 1997). A case-control study comparing risk factors in nvCJD cases with age- and sex-matched hospital controls has identified no increased risk for nvCJD in relation to past medical history, including operative procedures, occupation, or dietary factors, although all cases to date did eat beef or beef products in the 1980s. The negative findings of the dietary study do not exclude the possibility that nvCJD is caused by oral exposure to the BSE agent. Information on dietary history is neces-

sarily obtained from surrogate witnesses. In nvCJD any causal exposure is likely to have taken place many years ago, probably in the 1980s, and information on past dietary exposure is known to be unreliable even with direct interview and, furthermore, there is evidence of recall bias in the study of dietary factors in sporadic CJD (National CJD Surveillance Unit 1995). Most importantly, there may have been dietary exposure to the BSE agent in a range of products that may have been contaminated intermittently. The retrospective identification of specific dietary risk factors in nvCJD may be impossible.

The epidemiologic evidence implying a causal link between BSE and nvCJD has been supported by other research. The neuropathologic features in macaque monkeys inoculated with BSE are similar to nvCJD (Lasmézas et al. 1996), and the protein subtypes deposited in the brains of nvCJD cases are similar to BSE and distinct from other forms of CJD (Collinge et al. 1996). Transmission studies in mice have demonstrated that the incubation periods in inbred strains of mice are almost identical in BSE and nvCJD (Bruce et al. 1997) and that the anatomic distribution of neuropathology is also very similar in mice following transmission of either BSE or nvCJD. Incubation periods and anatomic lesion profile in nvCJD transmissions are distinct from sporadic CJD. These studies provide strong evidence supporting the proposed causal link (Bruce et al. 1997; Hill et al. 1997b).

The mechanism of transmission of BSE to the human population remains speculative, although transmission through the oral route is the only reasonable current hypothesis. Some information is available on the use of bovine material in the human food chain in the UK in the 1980s with particular reference to bovine CNS tissue, which on current data is the only tissue known to contain infectivity in BSE, apart from retina, dorsal root ganglia, and possibly bone marrow. Furthermore, the high titers of infectivity found in bovine CNS tissues in comparison to any other tissue suggest that consumption of CNS tissue is the most likely mechanism of cross-contamination to humans. Brain tissue is unlikely to have entered the human food chain in large quantities, but spinal cord tissue almost certainly did enter the human food chain in the UK through the production of mechanically recovered meat. This material was used in a range of foodstuffs including burgers, sausages, meat pies, and pate, and there may have been widespread human consumption of this material in the 1980s prior to the institution of the specified bovine offals ban in 1989/1990.

Current evidence suggests that nvCJD is a novel condition, clinically and pathologically. The epidemiologic evidence is consistent with BSE as

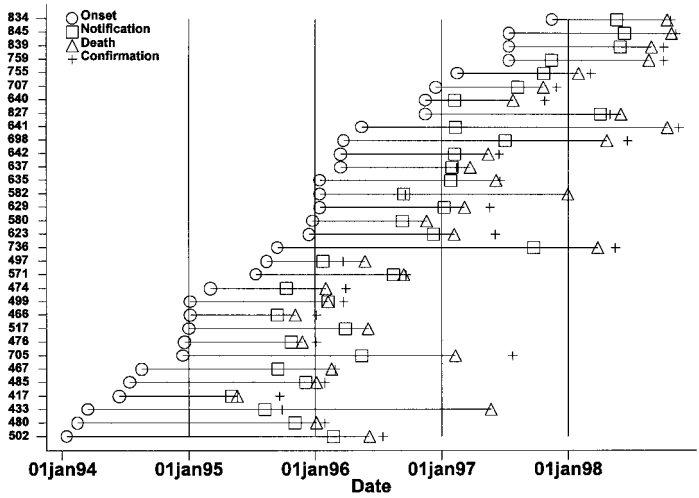

Figure 8 Cases of nvCJD in the UK.

the causal agent, and the recent laboratory evidence provides strong support to the hypothesis of a causal link between BSE and nvCJD. The number of future cases of nvCJD that may occur is uncertain. Mathematical modeling utilizing data from the first 14 cases indicated that the possible future number of cases could vary widely, with estimates ranging from a few hundred cases to over 80,000 cases (Cousens et al. 1997), with predictions crucially dependent on variables including the incubation period. Currently, cases of nvCJD are occurring at a steady rate (Fig. 8), with for-

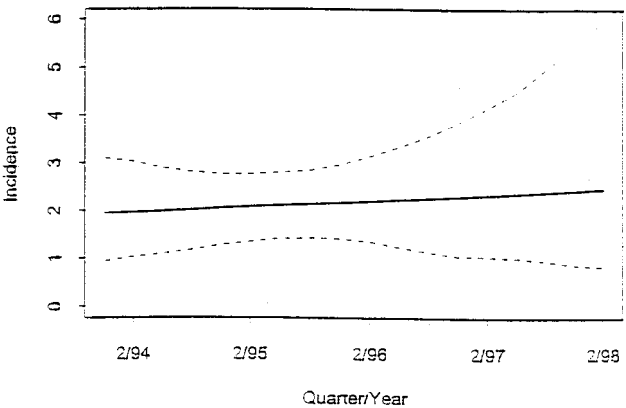

Figure 9 Mathematical estimate of the incidence of nvCJD onsets (exponential model with gamma delays and 90% limits).

mal analysis showing no overall change in trend to either more or fewer cases per quarter (Fig. 9). The fact that only a limited number of cases of nvCJD have been reported in the past 2 years is somewhat reassuring, but the possibility of a large number of future cases cannot yet be excluded.

DIAGNOSIS AND INVESTIGATION OF HUMAN PRION DISEASES

The diagnosis of CJD is usually suspected because of the relatively stereotyped and characteristic features, including multifocal neurologic dysfunction, involuntary myoclonic movements, and rapid progression. A small proportion of sporadic cases present atypically with relatively insidious onset and progression, and in familial cases the clinical features may depend on PrP gene analysis. Iatrogenic cases may be diagnosed on the basis of progressive neurologic dysfunction occurring in the context of a patient with a recognized risk factor for CJD. In nvCJD the age of the patient remains an important clue to diagnosis, and progressive and unexplained neurologic deficits are not common in neurologic practice in patients under the age of 50 years. In all forms of CJD the diagnosis may be supported by investigations; a major research objective is to identify early diagnostic tests.

INVESTIGATIONS

Routine hematological and biochemical indices are usually normal in all forms of CJD. Liver function tests are abnormal in about a third of cases of sporadic CJD, with either a raised bilirubin, raised liver enzymes, or both (Roos et al. 1971). These abnormalities may be an epiphenomenon reflecting side effects of medication; for example, chlorpromazine therapy or the poor general state of patients with CJD in the terminal stages of the illness.

EEG shows triphasic generalized periodic complexes (Fig. 10) in about two-thirds of all cases of sporadic CJD (Will and Matthews 1984), and the chances of finding this abnormality are enhanced if serial EEG recordings are obtained. The EEG appearances are not specific to CJD, occurring in a variety of conditions, including metabolic diseases such as hyponatremia or hepatic encephalopathy, and in toxic states; for example, lithium overdose or metrizamide encephalopathy (Will 1991). However, the clinical distinction between CJD and these other conditions is usually apparent, and in the appropriate clinical context, a "typical" EEG suggests that the diagnosis of CJD is highly likely. Preliminary criteria for the classification of the EEG changes in CJD have been proposed previously (Steinhoff et al. 1996).

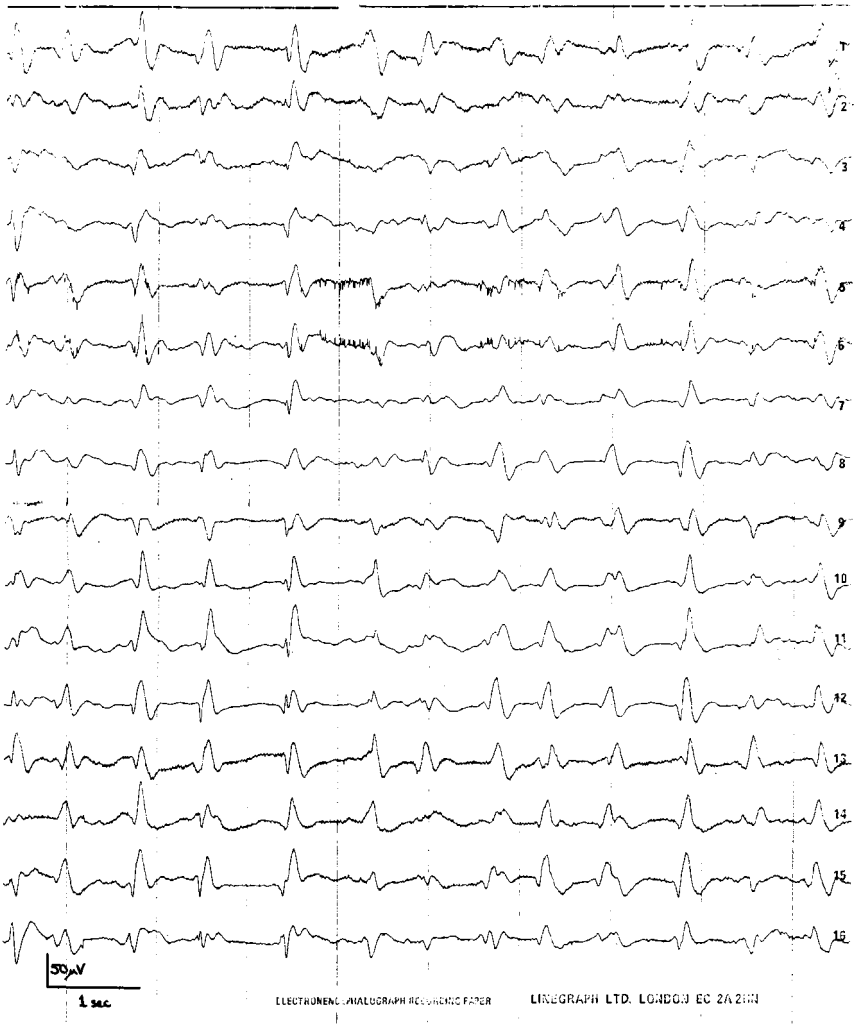

Figure 10 Typical electroencephalogram in sporadic CJD.

Cerebrospinal fluid (CSF) examination is usually normal. An elevated CSF protein is found in about one-third of cases (Will and Matthews 1984), and rarely, evidence of localized IGG synthesis within the CNS has been observed. The CSF is acellular, and a pleocytic CSF is very much against the diagnosis of any form of CJD. Immunoassay for the 14-3-3 protein has been developed from the two-dimensional gel electrophoresis abnormalities originally described by Harrington (Blisard et al. 1990). The original publication describing the 14-3-3 immunoassay implied a high diagnostic sensitivity and specificity of over 90% (Hsich et al. 1996),

which has been supported in classic CJD by other studies. In the appropriate clinical context, i.e., in patients with rapidly progressive and unexplained dementia, the 14-3-3 immunoassay may be diagnostically helpful, but the test does not distinguish between subtypes of CJD, and preliminary analysis of 14-3-3 immunoassay in nvCJD suggests a lower sensitivity and specificity than in classic CJD (Will et al. 1996a).

Cerebral imaging is important in suspect cases of CJD in order to exclude other conditions such as brain tumor. Computed tomography (CT) scanning does not provide specific diagnostic information in any form of CJD, but recent findings suggest that magnetic resonance imaging (MRI) brain scanning may be more useful. The Gottingen group has reported a high incidence of high signal abnormalities on T2 imaging in the putamen and caudate regions in classic CJD (Fig. 11) (Finkenstaedt et al. 1996). In nvCJD a high proportion of cases exhibit high signal in the pulvinar region of the posterior thalamus (Fig. 12), which correlates with

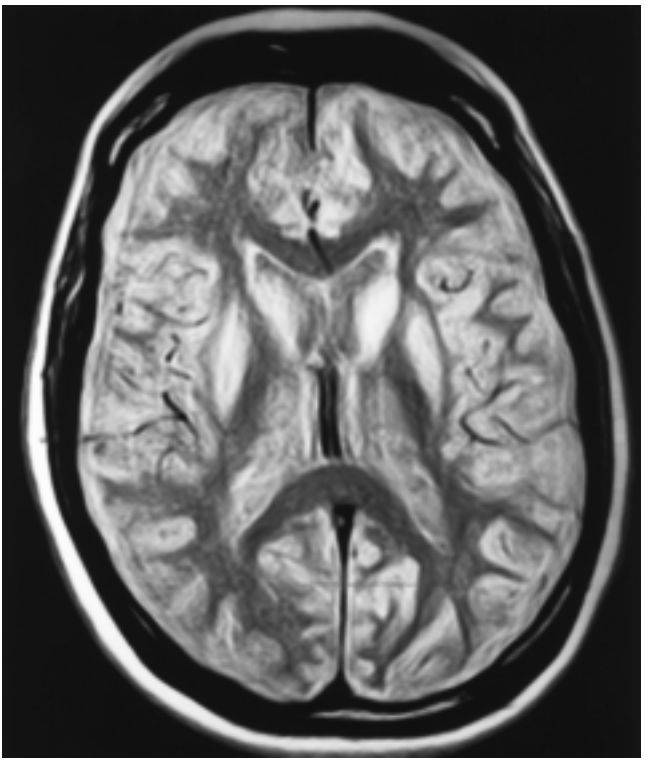

Figure 11 MRI scan in sporadic CJD showing bilateral increase in signal in the caudate and putamen.

the neuropathologic findings in that the most marked gliotic changes are found in this region of the thalamus (Sellar et al. 1997). Further "blinded" studies of MRI abnormalities in CJD and nvCJD are necessary to confirm the diagnostic value of MRI scanning.

Brain biopsy has been used to diagnose CJD in life and may have a high diagnostic accuracy. However, a negative biopsy result does not exclude the diagnosis of CJD because of the possibility of sampling error, and the procedure does have risks to the patient, for example, brain hemorrhage or abscess formation. There are also theoretical risks to surgical staff and risk of onward transmission of infection to other patients via surgical instruments. The main role of brain biopsy is the exclusion of other potentially treatable conditions such as cerebral vasculitis, and the procedure is mainly indicated in younger suspect cases.

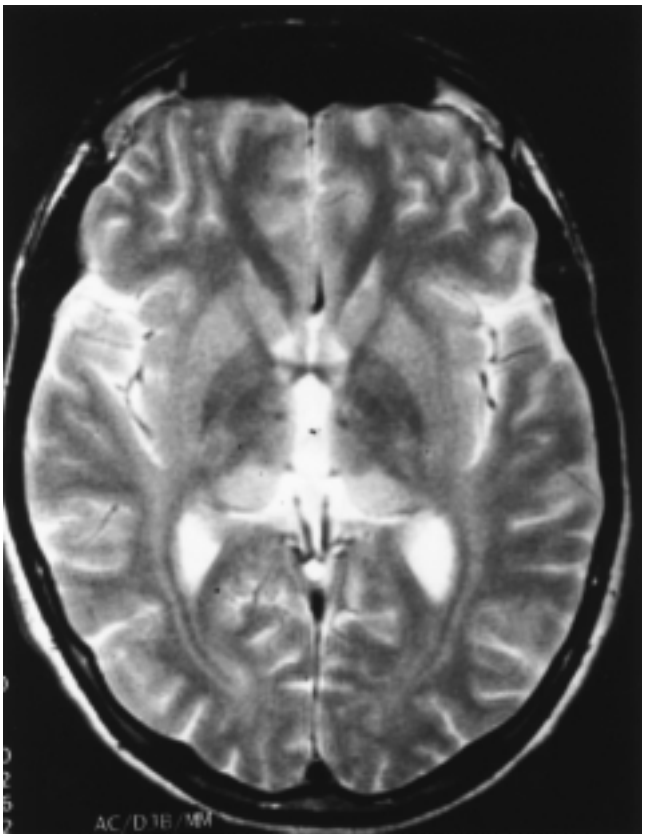

Figure 12 MRI scan in nvCJD showing increased signal in the posterior thalamic regions.

Tonsillar tissue obtained postmortem from a case of nvCJD has been shown to exhibit PrP immunostaining in germinal centers (Hill et al. 1997a), and limited information suggests that such staining is not found in classic CJD. Tonsillar biopsy may have to be performed using general anesthesia in suspect cases of nvCJD, and there are attendant risks of hemorrhage; biopsy instruments should be destroyed after use. Whether or not this procedure should be included as part of the diagnostic workup of suspect nvCJD cases remains controversial, as a positive biopsy is of no therapeutic value to the patient, although it may allow early diagnosis.

In systematic surveys of CJD, about 15% of cases are found to be familial. In these cases, about one-third have a positive family history of CJD, about one-third a family history of some other form of neurologic disease, and about one-third no significant family history of any form of neurologic disease (Laplanche et al. 1994). Utilizing a family history alone to identify hereditary forms of human prion disease is unreliable, and screening of the PrP gene in suspect cases is important in systematic surveys if hereditary forms of CJD are to be identified. Because of the implications for other family members, in most centers informed consent is necessary prior to PrP gene analysis.

A firm diagnosis of CJD or nvCJD depends on neuropathologic examination postmortem, and the accurate identification of cases of CJD in population surveys is dependent on a high postmortem rate. The clinical diagnosis of sporadic CJD is relatively accurate, particularly if there is an atypical EEG, and hereditary forms of CJD may be confirmed by PrP gene analysis. Recognition of a relevant risk factor is essential to the diagnosis of iatrogenic CJD. The limited experience in nvCJD and the absence of any specific diagnostic investigation makes accurate clinical diagnosis problematic. The evolution of the illness and perhaps the presence of posterior thalamic abnormalities on MRI may be important in diagnosis of nvCJD.

SUMMARY AND CONCLUSIONS

Infectious and sporadic forms of human prion disease are rare and, although symptomatic treatment may be helpful in individual cases, these conditions are incurable and invariably fatal. There has been a remarkable increase in awareness and interest in these diseases, partly because the conditions are caused by unique causal agents, prions, and partly because of concerns about the potential public health implications of the probable transmission of an animal prion disease to the human population. Basic scientific research, particularly using transgenic models of prion disease,

has led to insights into mechanisms of disease causation that may be relevant to other currently unexplained neurologic diseases. A major objective is to identify a marker for the presence of infectivity prior to the onset of clinical illness, and this may in turn lead to novel therapeutic approaches. The occurrence of nvCJD is a stimulus to further research in view of the uncertainty about the future course of this disease and the possibility of large numbers of affected individuals. CJD in any form is a tragedy for the individuals and their relatives, and it is hoped that further scientific developments may lead to progress in therapeutic strategies.

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13
Inherited Prion Diseases

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HISTORICAL OUTLINE

The first report of an inherited prion disease can be traced to an affected member of the "H" family carrying a neurologic disorder through multiple generations. It was presented at a meeting of the Viennese Neurological and Psychiatric Association in 1912 (Dimitz 1913). Two decades passed before Gerstmann in 1928 and Gerstmann, Sträussler, and Scheinker in 1936 reported clinical presentations and neuropathologic findings for several affected members of the "H" family. These reports establish the disease that is currently referred to as Gerstmann-Sträussler-Scheinker disease or GSS (Gerstmann 1928; Gerstmann et al. 1936).

The subject reported by Creutzfeldt in 1920 and 1921, which had a positive family history (Creutzfeldt 1920, 1921), is unlikely to have been affected by the condition now called Creutzfeldt-Jakob disease (CJD). The first authentic familial case of CJD was recorded in 1924 by Kirschbaum (1924). However, it was Meggendorfer in 1930 who showed that the subject described by Kirschbaum was a member of a large kindred that became known as the "Backer" family and has been proven in subsequent publications to be affected by an inherited form of CJD (Meggendorfer 1930; Stender 1930; Jakob et al. 1950).

In 1973, Gajdusek, Gibbs, and their colleagues first demonstrated the transmissibility of inherited prion diseases to nonhuman primates (Roos et al. 1973). This finding followed earlier studies that reported the transmissibility of the sporadic form of CJD and kuru, a prion disease of the Fore tribe of New Guinea propagated through endocannibalism (Gajdusek et al. 1966; Gibbs et al. 1968). Subsequently, the experimental transmission of GSS was also achieved (Masters et al. 1981a).

The experimental transmission of familial CJD and GSS to animals by an infectious mechanism was a turning point in the history of human diseases because it presented the first example of a disease that was at the same time inherited and infectious. At that time, the transmissibility of familial CJD and GSS was interpreted within the framework of a viral illness. It was postulated that the affected subjects carried a genetically determined susceptibility to an infection that was acquired in early infancy or childhood (Masters et al. 1981b). Subsequent work, largely carried out by Prusiner and his colleagues, has provided a novel molecular basis for diseases that are inherited and infectious. A protein unique to the scrapie-infected hamster brain was discovered in fractions enriched for scrapie infectivity (Bolton et al. 1982; Prusiner 1982). Sequencing of the protein, designated prion protein (PrP), allowed the subsequent cloning of the PrP coding gene (Chesebro et al. 1985; Oesch et al. 1985). This work paved the way to the cloning and sequencing of the human PrP gene (*PRNP*) and the original discovery of mutations in subtypes of familial CJD and GSS (Owen et al. 1989; Hsiao et al. 1989). Analysis of genetic linkage, which was initially carried out in a subtype of GSS associated with a *PRNP* mutation at codon 102 and subsequently in other *PRNP* mutations, indicated that the mutations are likely to be the cause of GSS and other inherited prion diseases (Hsiao et al. 1989; Goldfarb et al. 1990; Dlouhy et al. 1992; Medori et al 1992; Poulter et al. 1992; Gabizon et al. 1993).

The discovery of *PRNP* mutations in familial prion diseases provided an easy explanation for the coexistence of genetic and infectious forms within the framework of the prion hypothesis (Prusiner 1989). This new

framework received additional support from the prduction of transgenic mice that developed spontaneously a prion disease when expressing mutant PrP, as well as from a wealth of other studies. However, some investigators interpret the *PRNP* mutations as affecting a viral receptor in a way that increases the susceptibility to infection by a highly ubiquitous virus (Chesebro 1997).

GENOTYPES AND PHENOTYPES

The genetic and physical map of the *PRNP* region on the short arm of chromosome 20, and diagrams of *PRNP* and normal or cellular PrP (PrP^C) are shown in Figure 1.

Currently, 22 mutations and seven polymorphisms have been identified in the PRNP gene (Fig. 2 and Table 1): (1) thirteen point mutations; (2) eight insertion mutations, which consist of additional 1, 2 and 4-9

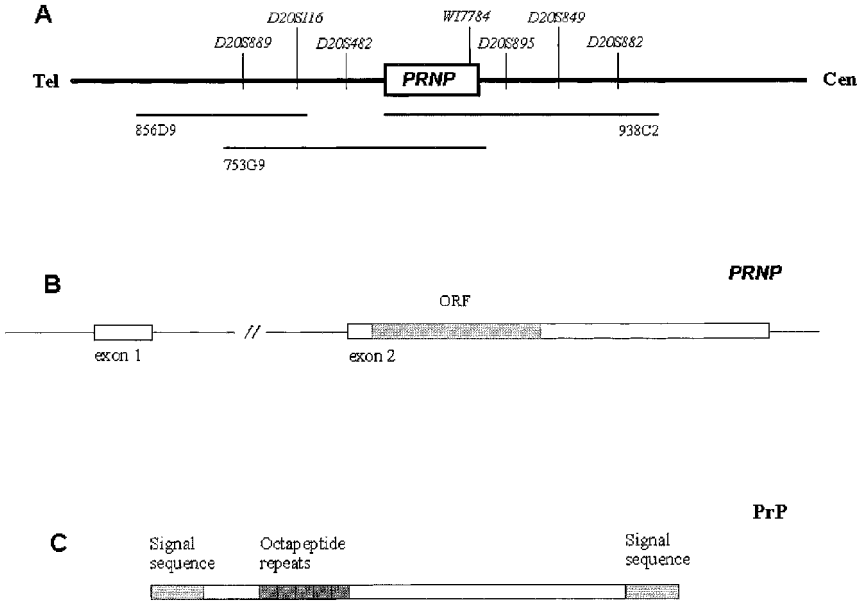


Figure 1 Genetic and physical maps of the PRNP region on chromosome 20p12-pter. (*A*) D20-S889, D20S116, D20S482, D20S895, D20S849, and D20S882 are genetic markers flanking the PRNP gene; 856D9, 753G8, and 938C2 are human YAC clones; W17784 is an expressed sequence tag located within the PRNP coding sequence in an overlapping region of YAC clones 753G8 and 938C2. (*B*) Schematic representation of the PRNP gene consisting of exons 1 and 2, with the open reading frame (ORF) located entirely within exon 2. The prion protein includes two signal sequences and a five-octapeptide repeat.

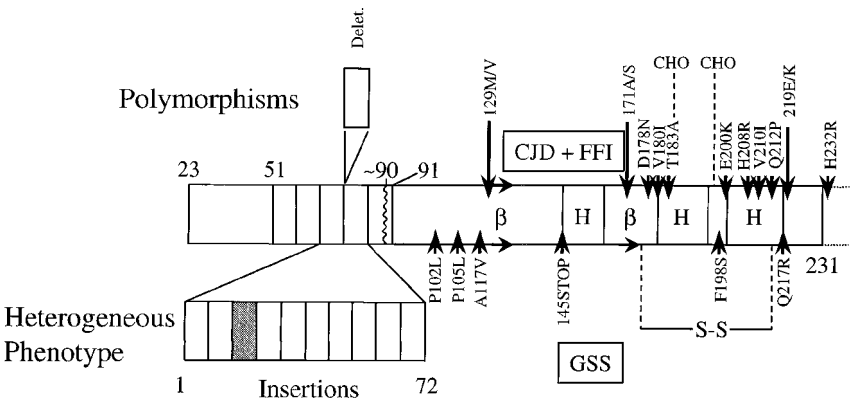


Figure 2 Diagram of PrP including mutations, polymorphisms, secondary structures, and posttranslational modifications. (β) β-sheet; (H) α-helix; (S-S) disulfide bond; (CHO) N-glycans. Mutations resulting in CJD or FFI phenotypes as well as the polymorphisms (*long arrows*) are indicated above the diagram. Mutations resulting in the heterogeneous and GSS phenotypes are indicated below. The pathogenic insertions reported to date include 1, 2, and 4–9 octapeptide repeats.

repeats of 24 base pairs (bp) located between codons 51 and 91; (3) one is a stop codon mutation at codon 145, which results in the premature termination of synthesis and yields a truncated PrP. Three of the seven polymorphisms located at codons 117, 124, and 161 do not result in amino acid substitutions (Wu et al. 1987; Prusiner 1997). The others include the methionine/valine (M/V) polymorphism at codon 129, a common polymorphism with a 0.62 prevalence of the methionine allele in a normal Caucasian population (Goldfarb et al. 1989; Palmer et al. 1991); the asparagine/serine (N/S) polymorphism at codon 171 (Samaia et al. 1997); the glutamic acid/lysine (E/K) polymorphism at codon 219, in which the lysine allele occurs with a 12% prevalence in the Japanese population (Kitamoto and Tateishi 1994; Barbanti et al. 1996); and the deletion of one 24-bp repeat that has been found in 1–2.5% of the population (Fig. 2) (Laplanche et al. 1990; Puckett et al. 1991; Vnencak-Jones and Phillips 1992; Palmer et al. 1993; Cervenakova et al. 1996). The polymorphisms at codon 129 and 219 have a dual effect: (1) On the mutant allele, they modify basic aspects of the disease phenotype; (2) on the normal allele, the 129 polymorphism has been shown to influence age at onset and duration of the disease. Because at least two polymorphisms modify basic aspects of the disease phenotype, it is appropriate to identify each *PRNP* genotype

associated with inherited prion diseases not only by the mutation but also by codons 129 and 219 (or other modifying polymorphic codons) present on the mutant allele; i.e., the haplotype. Twenty-six disease-associated *PRNP* haplotypes have been reported, which are associated with distinct phenotypes (Table 1).

CHARACTERISTICS OF THE PROTEASE-RESISTANT PRION PROTEIN

When PrP isolated from the brain of prion disease-affected subjects is treated with a protease, such as proteinase K (PK), the amino-terminal portion up to about residue 90 is digested while the remaining carboxy-terminal portion remains intact (McKinley et al. 1983). Thus, the PK-resistant fragment PrP^{res} migrates faster on gel than the full-length PrP (Fig. 3). This applies to each of the three PrP glycoforms, which include: (1) a highly glycosylated form that contains two complex glycan chains, (2) an intermediate form with only one complex chain, and (3) a form that is unglycosylated (Prusiner 1982; Chen et al. 1995; Petersen et al. 1996). The size of the PrP^{res} fragments and the ratio of the PrP^{res} forms that are differently glycosylated, also referred to as glycoforms, are other features that help in characterizing the various inherited prion diseases. The first observation that the PrP^{res} isolated from different human prion diseases could be distinguished on the basis of the size of the fragment generated by the PK digestion and the ratio of the glycoforms was made in 1992 and was further validated in 1994 (Fig. 3) (Medori et al. 1992; Monari et al. 1994). Since then it has been established that following deglycosylation to eliminate the heterogeneity generated by the presence of the glycoforms, the major PrP^{res} core fragments associated with human prion diseases exhibit only two principal sizes, which migrate on gel at 20–21 kD and 18–19 kD, respectively. These two PrP^{res} fragments are respectively identified as PrP^{res} type 1 and type 2 (Parchi et al. 1996c). PrP^{res} type 1 and type 2 may be further subdivided according to the pattern determined by the ratio of the three glycoforms. For example, the PrP^{res} associated with the insertion mutations is characterized by the predominance of the intermediate glycoform, whereas in the CJD linked to the E200K mutation and in fatal familial insomnia (FFI) the glycoform ratio is consistently dominated by the highly glycosylated form, whereas the unglycosylated form is markedly underrepresented (Monari et al. 1994; Parchi et al. 1996a). Therefore, it seems appropriate to characterize the inherited prion diseases not only according to the haplotype but also taking into consideration the type of PrP^{res} as determined by the size of the PrP^{res} fragment and the ratio of the three glycoforms.

Table 1 Genotype and phenotype of inherited prion diseases

Genotype	Onset (yrs)	Duration	• Clinical and •• Pathological features
Creutzfeldt-Jakob disease phenotype			
D178N-129V	26–56	9–51 months	• Dementia, ataxia, myoclonus, extrapyramidal and pyramidal signs •• Spongiosis, neuronal loss and astrogliosis in the cerebral cortex (most severe), striatum, and thalamus (least severe), while the cerebellum is spared
V180I-129M	66–85	1–2 years	• Similar to typical sCJD but with a slower progression •• Like typical sCJD
T183A-129M	45	4 years	• Personality changes followed by dementia and Parkinsonism •• Atrophy with spongiform degeneration in the cerebral cortex and, to a lesser extent, in the basal ganglia
E200K-129M	35–66	2–41 months	• Similar to typical sCJD; atypical signs such as supranuclear palsy and peripheral neuropathy in some cases •• Like typical sCJD
H208R-129M	60	7 months	• Like typical sCJD •• Like typical sCJD
V210I-129M	49–70	3–5 months	• Like typical sCJD •• Like typical sCJD
M232R-129M	55–70	4–24 months	• Like typical sCJD •• Like typical sCJD
Fatal familial insomnia phenotype			
D178N-129M	20–71	6–33 months	• Reduction of total sleep time, enacted dreams, sympathetic hyperactivity, myoclonus, ataxia; late dementia, pyramidal and extrapyramidal signs in the cases with a relatively long duration (>1year) •• Preferential thalamic and olivary atrophy; spongiform changes in the cerebral cortex in the subjects with a duration of symptoms longer than 1 year

Phenotype of Gerstmann-Sträussler-Scheinker disease and other amyloidoses

P102L-129M	30–62	1–10 years	• Slowly progressive cerebellar syndrome with late dementia, extrapyramidal and pyramidal signs; some cases (shorter duration) overlap with CJD •• PrP amyloid deposits in the cerebellum and, to a lesser extent, in the cerebrum; variable degree of spongiosis, neuronal loss, and astrogliosis; no NFT
P102L-129M-219K	31–34	4 years	• Differ from the above P102L form for the less prominent cerebellar signs •• Few PrP plaques in the cerebral and cerebellar cortices; no spongiosis
P102L-129V	33	12 years	• Seizures, numbness, gait difficulties, dysarthria, long tract signs; no dementia •• Widespread PrP plaques with no spongiosis
P105L-129V	40–50	6–12 years	• Spastic paraparesis progressing to quadriparesis; late dementia; no myoclonus and only mild cerebellar signs •• PrP amyloid deposits, neuronal loss, and gliosis in the cerebral cortex and, to a lesser extent, in the striatum and thalamus; no spongiform changes and NFT
A117V-129V	20–64	1–11 years	• Dementia, parkinsonism, pyramidal signs; occasional cerebellar signs •• Widespread PrP amyloid deposits in the cerebrum and, more rarely, in the cerebellum associated with variable degree of spongiform changes, neuronal loss, and astrogliosis; no NFT
Y145STOP-129M	38	21 years	• Slowly progressive dementia •• PrP amyloid deposits in the cerebral and cerebellar cortices associated with NFT in the neocortex, hippocampus, and subcortical nuclei; PrP amyloid angiopathy; no spongiosis
F198S-129V	34–71	3–11 years	• Like 102 GSS subtype, but with a more chronic course (no overlap with CJD) •• Like 102 GSS subtype but with more extensive PrP amyloid deposits, NFT in the cerebral cortex and subcortical nuclei, and inconspicuous spongiosis
Q212P	60	8 years	• Progressive ataxia, dysarthria with no dementia •• Prp plaque-like deposits neocortex and cerebellum; few PrP amyloid deposits; no NFT
Q217R-129V	62–66	5–6 years	• Slowly progressive dementia, cerebellar and extrapyramidal signs •• Like 198 GSS subtype but with the most severe lesions in the cerebral cortex, thalamus, and amygdala

(Continued on following page.)

Table 1 (Continued from previous pages.)

Genotype	Onset (yrs)	Duration	• Clinical and •• Pathological features
Heterogeneous phenotype: The insertional mutations			
Ins 24 bp-129M	73	4 months	• Like typical sCJD •• NA
ins 48 bp-129M	58	3 months	• Like typical sCJD •• Like typical sCJD
ins 96 bp-129M	56	2 months	• Like typical sCJD •• Like typical sCJD
ins 96 bp-129V	82	4 months	• Examined at terminal stage showed akinetic mutism, diffuse myoclonus, and pyramidal signs •• NA
ins 120 bp-129M	31, 45	5, 15 years	• Progressive dementia, myoclonus, cerebellar and extrapyramidal signs •• Spongiosis, gliosis, and neuronal loss (no information on topography, severity and presence of PrP deposits); CJD phenotype
ins 144 bp-129M	22–53	3 months–18 years	• Similar to 120-bp insertion subtype •• Most cases show a CJD phenotype with spongiosis, gliosis, and neuronal loss; one case had kuru-like plaques in the cerebellum; some cases show only mild aspecific gliosis and neuronal loss; PrP patches in the cerebellum
ins 168 bp-129M	23–35	7–13 years	• Similar to 120-bp insertion subtype •• Mild gliosis and neuronal loss, and no spongiosis in one case, CJD phenotype in another
ins 192 bp-129V	21–54	5 months–6 years	• Similar to 120-bp insertion subtype •• Spongiosis, gliosis, and neuronal loss, PrP multicentric amyloid plaques with widespread distribution; GSS-like phenotype
ins 216 bp-129M	32–55	2.5–>4 years	• Similar to 120-bp insertion subtype •• PrP amyloid plaques in the cerebellum, cerebral cortex, and striatum; no obvious neuronal loss, gliosis, or spongiosis; GSS-like phenotype

(NA) Not available; (PrP) prion protein; (NFT) neurofibrillary tangles.

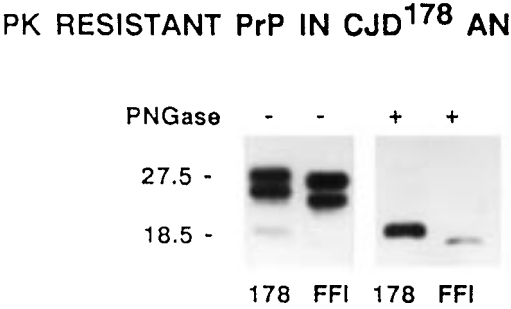


Figure 3 Immunoblot of PrP^{res} following treatment with proteinase K. The three bands in the left lane labeled 178 correspond to the diglycosylated, monoglycosylated, and the unglycosylated forms, respectively, which all comigrate as the unglycosylated form following PNGase treatment. Note the underrepresentation of the unglycosylated form (left 2 lanes) in both conditions but more pronounced in FFI.

The precise role of the PrP^{res} type in the phenotypic expression of the inherited prion diseases remains to be determined. The different size of the PrP^{res} fragments following treatment with exogenous PK has been shown to result from cleavage at different sites of the full-length PrP^{res} (Monari et al. 1994). This, in turn, is likely to be due to different conformations of PrP^{res} that expose distinct cleavage sites (Monari et al. 1994). The different ratio of the glycoforms may be due to a variety of causes, such as different topographic origin of the PrP^{res} within the brain in different prion diseases; preferential conversion of PrP^{C} to PrP^{res} of one glycoform over the others; or underrepresentation of one of the glycoforms due to the altered metabolism caused by the mutation, the underrepresented form becoming thus less available for conversion to PrP^{res} (also see below). Transmission of inherited and sporadic prion diseases to receptive animals has underlined the importance of the PrP^{res} typing (Telling et al. 1996). Transgenic mice expressing a chimeric wild-type human/mouse PrP^{C} develop a prion disease after the intracerebral inoculation with human brain homogenates carrying PrP^{res} of either type 1 or type 2 (Telling et al. 1996). The size of the PrP^{res} fragment expressed by the inoculated mice corresponds precisely to the PrP^{res} type present in the inoculum. Since the recipient mice did not carry any of the donor's *PRNP* mutations and were isogenic, this remarkable finding indicates that the distinct conformations associated with PrP^{res} type 1 and type 2 can be reproduced independently of the genetic information. However, the ratio of the glycoforms present in the recipient's PrP^{res} was different from that

of the donor's PrP^{res}, indicating that conformation and glycoform ratio are independently determined (Telling et al. 1996). The information necessary for the specification of the cleavage site determining the size of the PrP^{res} fragment is likely to be contained in the conformation of the donor's PrP^{res}, whereas the glycosylation is probably controlled by the host according to the cell population involved and glycoform convertibility, as mentioned above.

REFLECTIONS ON THE PATHOGENESIS OF INHERITED PRION DISEASES: THE CELL MODELS

Inherited prion diseases become symptomatic in the adult or at an advanced age. Therefore, the disease remains clinically silent for many years despite the presence of the mutant PrP (PrP^{M}). Presently, it is not known whether the extended presence of the abnormal PrP^{M} is sufficient to initiate the clinical disease and the conversion occurs subsequently, or if PrP^{res} is required for the expression of the disease phenotype.

The cell models have provided considerable information and insight on the effect that *PRNP* mutations have on the metabolism and physicochemical properties of PrP^{M}, including its convertibility to PrP^{res} (Table 2). Three major sets of studies have been carried out by three groups.

Meiner, Gabizon, and their coworkers (Meiner et al. 1992; Gabizon et al. 1996), using cultured fibroblasts from patients carrying the E200K mutation, observed that (1) the glycans of the "mature" mutant PrP are different from those of controls; (2) the mutant PrP recovered from the E200K fibroblasts is not protease resistant; and (3) the mature mutant PrP is more stable than that of the controls.

The characteristics of the mutant PrP have been studied in neuroblastoma cells transfected with the *PRNP* pathogenic mutations D178N, E200K, Q217R, F198S, and Y145STOP (Capellari et al. 1996; Petersen et al. 1996; Singh et al. 1997; Zanusso et al. 1997; S.I. Zaidi et al., unpubl.) and in Chinese hamster ovary cells carrying *PRNP* mutations corresponding to the human D178N, E200K, P102L, and six octapeptide insertion mutations (Lehmann and Harris 1995; 1996a,b). The studies of Petersen, Singh, and coworkers have shown shared metabolic changes as well as changes specific to each mutation. There are five major common changes: (1) Mutant PrP is unstable and may undergo degradation or aggregation. In particular, the unglycosylated form of mutant PrP is less stable than the mono and diglycosylated forms. Furthermore, when glycosylation is abolished with tunicamycin, all of the expressed mutant PrP

Table 2 Changes in PrP^{M} metabolism observed in cell models of inherited prion disease

Haplotype	System	Common	Specific
E200K–129M			
Meiner et al (1992) Gabizon et al. (1996)	fibroblast from patients	PrP^{M} releasable by PI-PLC and PK resistant in monocytes	abnormal glycosylation
Lehmann and Harris (1996a)	transfected CHO (mouse gene)	PrP^{res}-like (3.3 μg/ml)	NR
Capellari et al. (1996)	transfected neuroblastoma (human gene)	PrP^{res}-like underrepresentation of U form at cell surface	abnormal glycans at position 197 increased carboxy-terminal fragments
D178N–129M			
Lehmann and Harris (1996a)	transfected CHO (mouse gene)	PrP^{res}-like	NR
Petersen et al. (1996)	transfected neuroblastoma (human gene)	PrP^{res}-like underrepresentation of U form at cell surface	preferential degradation in ER
D178N–129V			
Petersen et al. (1996)	transfected neuroblastoma (human gene)	PrP^{res}-like underrepresentation of U form at cell surface	preferential degradation in a post-ER compartment
P102L–129M			
Lehmann and Harris (1996a)	transfected CHO (mouse gene)	PrP^{res}-like	NR
+6 Insertion–129M			
Lehmann and Harris (1996a) Lehmann and Harris (1995)	transfected CHO (mouse gene)	PrP^{res}-like abnormal membrane insertion	NR
Q217R–129V			
Singh et al. (1997)	transfected neuroblastoma (human gene)	PrP^{res}-like underrepresentation of U form at cell surface	formation of an additional fragment wt GPI anchor presence of two aberrant anchor-carrying fragments
F198S–129V			
S.I. Zaidi et al. (unpubl.)	transfected neuroblastoma (human gene)	PrP^{res} underrepresentation of U form at cell surface	hyperglycosylation
Y145STOP–129M			
Zanusso et al. (1997)	transfected neuroblastoma (human gene)		high instability of PrP^{M}, with preservation of signal peptide nuclear degradation through the proteosomal pathway

(NR) Not reported.

exhibits the same instability as the original unglycosylated form. This finding indicates that mutations destabilize PrP^M and that glycosylation partially corrects this effect. (2) Since PrP^M, especially the unglycosylated form, is less stable, it is transported to the plasma membrane in decreased amount. As a result, less PrP is present at the surface of mutant cells and the ratio of the three glycoforms is altered due to preferential underrepresentation of the unglycosylated form. (3) Overall, more PrP^M is recovered in the detergent insoluble (P2) fraction, showing that PrP^M has the tendency to aggregate. (4) A small fraction of PrP^M displays a mild resistance to digestion with PK (3.3 μg/ml for 5–10 min), an order of magnitude lower than that of the PrP^{res} present in the corresponding human disease (50 μg/ml for 30 min). (5) Most of these alterations are corrected by maintaining the mutant cells at 24ºC, indicating that they are secondary to the misfolding of the mutant PrP, which is known to be partially corrected at lower temperatures (Denning et al. 1992).

In addition, transfected neuroblastoma cells display PrP^M changes more specifically associated with the individual mutations. In some mutations, abnormal PrP^M fragments are formed in significant amounts. For example, a relatively stable 32-kD PrP^M fragment lacking the glycosyl phosphatidylinositol (GPI) anchor, as well as two unstable anchor-carrying fragments, have been found in cells transfected with the Q217R mutant construct and not with other constructs (Singh et al. 1997). Additionally, the Y145STOP mutation is associated with the corresponding truncated PrP^M fragment, which is extremely unstable even at 15ºC, suggesting a possible correlation between the extreme instability and the severity of this particular mutation (Zanusso et al. 1997). In the F198S mutation, the unglycosylated PrP^M is underrepresented not because it is degraded as in other mutations, but apparently because PrP^M is over-glycosylated (S.I. Zaidi et al., unpubl.). The E200K mutation is characterized by retarded and abnormal maturation of the N-glycans, resulting in altered gel mobility of the diglycosylated or mature PrP^M form (Capellari et al. 1996). Finally, in transfected cells carrying the D178N, 129M and the D178N, 129V haplotypes, corresponding to FFI and familial CJD linked to the D178N mutation, respectively, the instability of the PrP^M unglycosylated form is especially severe and results in an altered glycoform ratio at the cell surface. These changes are more conspicuous in the FFI than in the CJD^{178} cell model. Moreover, the unglycosylated form of PrP^M was also found to be preferentially underrepresented in the membrane fraction isolated from FFI brains, but not from brains of control subjects (Petersen et al. 1996). This finding suggests that the underrepresentation of the PrP^{res} unglycosylated form in FFI and CJD^{178} is due to the

relative unavailability of this form for conversion to PrP^{res} resulting from the mutation, rather than the preferential conversion of the highly glycosylated forms to PrP^{res} (Petersen et al. 1996; Parchi et al. 1998a).

Overall, comparable results have been obtained by Harris, Lehmann, and coworkers (Lehmann and Harris 1995, 1996a,b, 1997; Daude et al. 1997). These authors observed a number of characteristics shared by all these mutations, which include: (1) All PrP^{M} forms are in part hydrophobic, detergent-insoluble, and mildly protease resistant; (2) PrP^{M} acquires these characteristics through distinct and successive steps. A finding that is at variance with that of other studies is that the GPI anchor of the mutant PrP can be cleaved by PI-PLC, but PrP^{M} remains attached to the cell membrane, consistent with an abnormal PrP^{M}-membrane association (Lehmann and Harris 1995).

Despite some differences, the cell models demonstrate that *PRNP* mutations cause profound and early changes in the metabolism and physicochemical characteristics of PrP^{M}. These changes appear to result from an altered conformation that PrP^{M} acquires co-translationally. The altered conformation confers characteristics of increased hydrophobicity, aggregability, and resistance to proteases reminiscent of those of the PrP^{res}. Whether the "PrP^{res}-like" PrP^{M} is already pathogenic and capable of transmitting the disease, is the direct precursor of the pathogenic PrP^{res}, or is an irrelevant early by-product of the mutation and longer times are needed for the "real" mutant PrP^{res} to be formed, remains to be established.

CLASSIFICATION

Inherited prion diseases may be associated with a phenotype (1) that has the clinical and histopathological features of CJD, FFI, or GSS; (2) that blends the features of both CJD and GSS; or (3) that lacks distinctive histopathologic features (Table 1).

INHERITED PRION DISEASES WITH CJD PHENOTYPE

CJD with the E200K Mutation and the 129M Codon on the Mutant Allele ($CJD^{E200K,129M}$)

Epidemiology

The epidemiology of the $CJD^{E200K,\ 129M}$ is of particular interest. The largest cluster of $CJD^{E200K,\ 129M}$ occurs among Jews of Libyan origin (Kahana et al. 1974). In this community, the incidence of CJD is 100

times higher than the worldwide incidence (50 per million as opposed to 0.5 per million), the highest incidence of CJD in the world. Although early speculation attributed the high incidence to diet or other environmental factors, a series of epidemiologic studies done in Israel and other countries revealed the unusually high incidence of familial CJD in this community (Neugut et al. 1979), which indicated a genetic origin (Cathala et al. 1985; Radhakrishnan and Mousa 1988; Nisipeanu et al. 1990; Hsiao et al. 1991b; Zilber et al. 1991; Gabizon et al. 1992).

After the E200K mutation was discovered, new data were obtained regarding the penetrance and other genetic details of the disease, the biochemistry of the mutant and normal protein in these patients, and the transmission rate of prions from these patients to experimental animals. These discoveries strengthened the causative relationship between the E200K mutation and familial CJD, and began to suggest the modalities by which this pathogenic *PRNP* mutation causes a prion disease.

Genetics

DNA analysis of patients with familial CJD revealed the presence of the E200K mutation linked to the disease with a logarithm of odds (LOD) score exceeding 4.85, where a LOD score above 3 is considered significant evidence for linkage (Lathrop et al. 1984; Goldfarb et al. 1990; Chapman and Korczyn 1991; Hsiao et al. 1991b; Korczyn et al. 1991).

Three patients homozygous for the E200K mutation (due to consanguinity) have been identified, and two more are suspected according to offspring evaluation. The clinical course in two of the homozygous patients was similar to that of heterozygotes, whereas in a third patient the course was more protracted (Chapman et al. 1992; Gabizon et al. 1993). The finding that a second mutated allele does not worsen the clinical course of the disease supports the notion that $CJD^{E200K,\,129M}$ is a true dominant disease (Hsiao et al. 1991b).

The E200K mutation has been identified in other large clusters of patients with familial CJD in Slovakia and Chile (Goldfarb et al. 1990; Brown et al. 1992a), raising the possibility of a common founder for these three clusters. It has been suggested that the mutation originated in Spain and was dispersed by the expulsion of the Sephardic Jews to other parts of the world in 1492 (Goldfarb et al. 1991c).

A more likely explanation for the appearance of the E200K mutation in diverse genetic populations is that it occurred from a spontaneous deamination of deoxycytidine and the formation of deoxythymidine on the negative strand (Barker et al. 1984; Korczyn 1994); the process of

spontaneous deamination occurs frequently in various parts of mammalian DNA. This theory is supported by the identification of the E200K mutation in the presence, as well as in the absence, of a family history of CJD in the US (Bertoni et al. 1992), England (Collinge et al. 1993), France (Laplanche et al. 1994), and Japan, suggesting the occurrence of multiple independent mutations (Inoue et al. 1994).

In early genetic studies, presymptomatic mutation carriers above the mean age of onset of the familial CJD^{E200K,129M} were identified (Goldfarb et al. 1991c; Gabizon et al. 1993), giving the false impression that the penetrance of the disease is low and raising the possibility that other factors play a role in the pathogenesis of the disease. However, recent studies using life-table analysis have shown that the penetrance is age-dependent: 0.45 by age 60, 0.89 at 80, and 0.96 above 80 years (Chapman et al. 1994; Spudich et al. 1995). Such results argue that the E200K mutation is sufficient to cause familial CJD. Contrary to other inherited prion diseases, no correlation has been found between the M/V polymorphism at codon 129 and age of onset or disease duration in CJD$^{E200K,\ 129M}$ (Gabizon et al. 1993).

Clinical Manifestation

The CJD$^{E200K,\ 129M}$ resembles the typical form of the sporadic CJD (sCJD) and has a mean age at onset of 62 years with a range of 53–71, and a mean duration of less than a year with a range of 4.3–6 months (Kahana et al. 1991). According to another study, the mean age of onset is 55 ± 8 years and the mean duration 8 ± 18 months (Brown et al. 1991a).

As in sCJD, presenting signs include cognitive impairment and psychiatric changes (80–83% of patients), cerebellar signs (43–55%), visual signs (19%), and myoclonic jerks (12%) (Brown et al. 1991a; Kahana et al. 1991). Although in most of the patients the course of the disease is insidious, 10% of patients present with an acute, sudden onset. During the course of the disease, all patients develop dementia as well as other cognitive and psychiatric disturbances, 73% have myoclonus, 79% cerebellar signs, 40% seizures, while sensory and cranial nerve involvement is present in 24% (Brown et al. 1991a). A puzzling clinical feature of the CJD$^{E200K,\ 129M}$ is the involvement of the peripheral nervous system, which is rare in the course of sCJD (Lope et al. 1977; Schoene et al. 1981; Guiroy et al. 1989). The peripheral neuropathy is both motor and sensory and is often accompanied by protein elevation in the cerebrospinal fluid (Sadeh et al. 1990; Neufeld et al. 1992; Antonine et al. 1996; J.A. Mastrianni, pers. comm.).

Electrophysiological and neuroimaging findings are also similar to those of sCJD. The typical electroencephalogram (EEG) activity with periodic spike and wave (PSW) complexes is found in 74–76% of CJD$^{E200K,\ 129M}$, as compared with approximately 56% of sCJD (Brown et al. 1986, 1991a). Slowing of the background is found in all patients. In some of the CJD$^{E200K,\ 129M}$, the pattern seen in routine EEG is asymmetric and not strictly generalized (Neufeld et al. 1992). Computed tomography (CT) scans have shown brain atrophy in one third of the patients (Chapman et al. 1993). Brain single-photon emission computed tomography (SPECT) in one patient with a normal brain CT scan showed bilateral perfusion defects (Cohen et al. 1989). All these nonspecific diagnostic findings are similar to those seen in sCJD (Galvez and Cartier 1984).

Histopathology

The histologic changes associated with CJD$^{E200K,\ 129M}$ are very similar to those of the typical sCJD (Group 1 of Parchi et al. 1996c) and are invariably characterized by spongiosis, astrogliosis, and neuronal loss (Fig. 4). The severity of the astrogliosis and of the neuronal loss appears to be a function of the disease duration. These lesions are severe and widely dis-

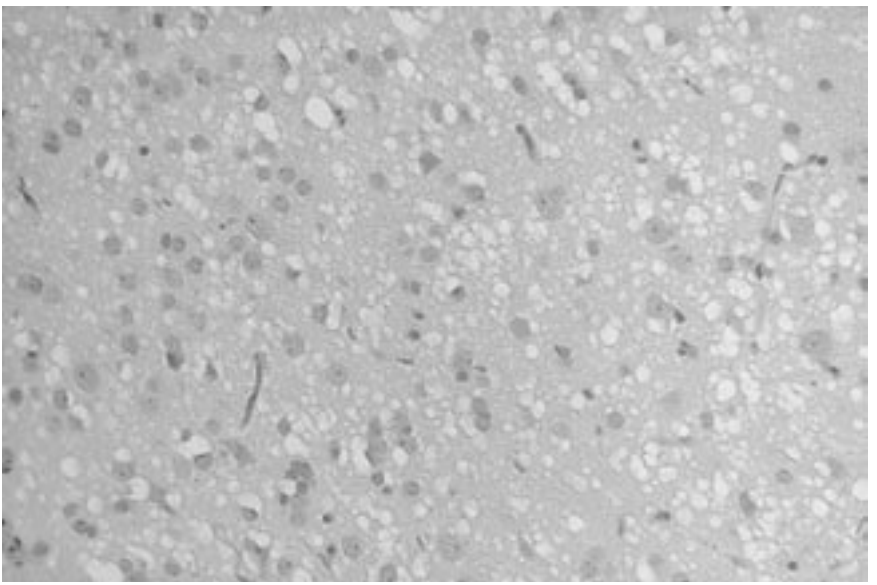


Figure 4 Typical histopathology of CJD $^{E200K,\ 129M}$. The cerebral cortex shows spongiform degeneration indistinguishable from that of the typical sporadic CJD (Group 1 of Parchi et al. 1996c). (Hematoxylin and eosin.)

tributed in the cerebral cortex. They are also present with decreasing severity in the striatum, diencephalon, and cerebellum. Immunostaining is consistently positive throughout the brain with the punctate or "synaptic" pattern and a severity that appears to be directly related to that of the histologic lesions (K. Young et al., unpubl.). In addition, punctate PrP immunostaining is also present in the substantia gelatinosa of the spinal cord. No PrP-positive amyloid or non-amyloid plaques are present. The peripheral neuropathy is both axonal and demyelinating (Chapman et al. 1993); the latter is characterized by segmental demyelination (Sadeh et al. 1990; Neufeld et al. 1992).

Characteristics and Allelic Origin of PrP^{res}

The availability of patients homozygous for the E200K mutation has provided the opportunity to examine whether the E200K PrP^{M} is formed as a soluble, nonpathogenic protein and is converted into mutant PrP^{res} during the long incubation period, or whether it is initially formed as mutant PrP^{res}. The indirect evidence for conversion during the incubation period includes the similarity in age of onset of $CJD^{E200K,\ 129M}$ and sCJD. A more direct finding is the absence of PrP^{res} in the brain biopsy of a presymptomatic homozygous carrier of the E200K mutation (R. Gabizon, unpubl.).

Studies have also been carried out to determine whether both the E200K PrP^{M} and the PrP^{C} expressed by the normal allele are converted into PrP^{res} and participate in the pathologic process, or if only the PrP^{M} is converted. PrP^{M} was found to be converted to PrP^{res}, whereas PrP^{C} did not acquire protease-resistance but became insoluble in detergents (Table 3). Since insolubility is also a characteristic of PrP^{res} (Meyer et al. 1986),

Table 3 Allelic origin of abnormal PrP in inherited prion diseases

Haplotype	Phenotype	PrP^{insol}	PrP^{res}	References
D178N, 129V	CJD	mutant	mutant	Chen et al. (1997)
D178N, 129M	FFI	mutant	mutant	Chen et al. (1997)
P102L, 129M	GSS	mutant	mutant	Parchi et al. (1998b)
F198S, 129V[a]	GSS	mutant	mutant	Tagliavini et al. (1994)
Q217R, 129V[a]	GSS	mutant	mutant	Tagliavini et al. (1994)
E 200K, 129M	CJD	wild-type and mutant	mutant	Gabizon et al. (1996)
V210I, 129M	CJD	wild-type and mutant	wild-type and mutant	Silvestrini et al. (1997)
Insertion, 129M	CJD	wild-type and mutant	wild-type and mutant	Chen et al. (1997)

[a]Only the amyloid subfraction was studied.

these findings raise the possibility that both (E200K) PrP^{M} and PrP^{C} participate in the pathogenesis of $CJD^{E200K, 129M}$.

The biochemical properties of the (E200K) PrP^{M} have been examined in brain samples, lymphocytes, and cultured fibroblasts of $CJD^{E200K, 129M}$ patients, and in brain samples of transgenic mice carrying the E200K mutation. In brains of subjects with $CJD^{E200K, 129M}$, the PrP^{res} has the gel migration pattern of the PrP^{res} type 1. $CJD^{E200K, 129M}$ migrates at 21 kD on gel and shows an underrepresentation of the unglycosylated form (Monari et al. 1994; Parchi et al. 1996a). This glycoform ratio is similar to that observed in FFI (see below) and in the new variant CJD (Monari et al 1994; Collinge et al. 1996). It was also shown that the highly glycosylated form of E200K PrP^{M} migrates faster on SDS-PAGE than does the corresponding PrP^{C} form (Gabizon et al. 1996), a difference that disappears following deglycosylation. This implies a difference in the glycosylation pattern between the E200K PrP^{M} and PrP^{C}, which is likely due to the proximity of the mutation to the glycosylation site at residue N197. This conclusion is also supported by the data obtained with the E200K cell transfectants (see above).

The amount of PrP, but not of PrP mRNA, was found to be higher in lymphocytes and fibroblasts from subjects with $CJD^{E200K, 129M}$ than in controls (Meiner et al. 1992), suggesting that PrP accumulates in $CJD^{E200K, 129M}$ patients, but the accumulation is not the result of accelerated synthesis but rather of a slower degradation process. This hypothesis is further supported by the slower degradation rate of the E200K PrP^{M} in fibroblasts from a $CJD^{E200K, 129M}$ homozygous patient compared to controls (Gabizon et al. 1996).

The PrP that accumulates in lymphocytes and fibroblasts of $CJD^{E200K, 129M}$ patients has been found to have the biochemical properties of PrP^{C} (Meiner et al. 1992). This finding is in disagreement with a recent study that E200K PrP^{M} expressed in transfected Chinese hamster ovary (CHO) cells is partially protease resistant, insoluble, and not released from the plasma membrane by phosphatidylinositol-specific phospholipase C as is the case for PrP^{res} (see above) (Lehmann and Harris 1996a,b).

Transmissibility

Intracerebral inoculation of $CJD^{E200K, 129M}$ brain homogenate has resulted in CJD transmission to apes after an incubation period of 6 years (Chapman et al. 1994). Studies with transgenic mice susceptible to human prions show transmission with specimens from familial $CJD^{E200K, 129M}$

patients (Telling et al. 1994). Two of three brain samples transmitted the disease following an incubation period of 170 days. The lack of transmission seen with the third sample may be attributed to codon 129 methionine/valine incompatibility (Telling et al. 1995). The precise risk of transmission of CJD from brain and other tissues, especially blood, from $CJD^{E200K,\ 129M}$ patients and healthy mutation carriers remains to be established.

CJD with the D178N Mutation and the 129V Codon on the Mutant Allele ($CJD^{D178N,\ 129V}$)

Epidemiology

Currently, ten apparently unrelated kindreds are known (Parchi et al. 1996b) which include five American, three French, one Finnish, and one German. Of the five American kindreds, three, of Hungarian-Rumanian, Dutch, and French-Canadian origins, respectively, have been repeatedly published, often with the appellation of Day (Friede and DeJong 1964; May et al. 1968; Masters et al. 1981b; Brown et al. 1991a; Fink et al. 1991; Nieto et al. 1991; Goldfarb et al. 1992b,c; Medori et al. 1992), Kui (Masters et al. 1981b; Brown et al. 1991a; Nieto et al. 1991; Goldfarb et al. 1992b,c), and LaP families (Masters et al. 1981b; Goldfarb et al. 1992b), and one is unpublished (P. Parchi and P. Gambetti, unpubl.). Of the three French kindreds, two commonly identified as Wui and Bel or AB families have been published (Buge et al. 1978; Guidon 1978; Masters et al. 1981b; Vallat et al. 1983; Brown et al. 1991a,b; Nieto et al. 1991; Goldfarb et al. 1992b,c; Medori et al. 1992) and one is unpublished (L.G. Goldfarb, unpubl.). The Finnish kindred has been known for several years (Haltia et al. 1979; Masters et al. 1981b; Kovanen and Haltia 1988; Brown et al. 1991a; Goldfarb et al. 1991a, 1992b,c; Medori et al. 1992) and the German kindred is the above-cited Baker family, which was originally reported in 1930 but has been recently shown to have the CJD^{178}, 129V haplotype (Meggendorfer 1930; Brown et al. 1994a; Kretzschmar et al. 1995).

PRNP Genotype and Genetic Linkage

$CJD^{D178N,\ 129V}$ shares the D178N *PRNP* mutation with FFI. The genotypic difference between the two diseases resides in the codon 129 located on the mutant allele, which encodes valine in the $CJD^{D178N,\ 129V}$ and methionine in FFI (Goldfarb et al. 1992c). Moreover, the alternative presence of the methionine or valine codons at position 129 on the normal allele

results in two patient subpopulations that are valine homozygous and valine/methionine heterozygous, respectively (Goldfarb et al. 1992c). Genetic linkage tested in two informative kindreds (Finnish and Day) provided a LOD score of 5.30, supporting the hypothesis that the D178N, 129V haplotype is the cause of the disease (Goldfarb et al. 1992b).

Clinical Features

The mean age at onset of the disease is 39 ± 8 years (range: 26–47) for the 129 valine homozygous, and 49 ± 4 years (p <00.1) (range: 45–56) for the heterozygous patients. The mean durations are 14 ± 4 months (range: 9–18) for the homozygous and 27± 14 months (p <00.5) (range: 7–51) for the heterozygous patients (Kirschbaum 1968; Goldfarb et al. 1992c; Kretzschmar et al. 1995). Clinical signs are fairly consistent and apparently independent of the zygosity at codon 129. Presentation is characterized by cognitive impairment, especially memory decrease, often associated with depression, irritability, and abnormal behavior. Ataxia, speech impairments with dysarthria and aphasia, tremor, and myoclonus appear during the course of the disease. Electroencephalographic examination invariably reveals generalized slow-wave activity without periodic complexes.

Histopathology

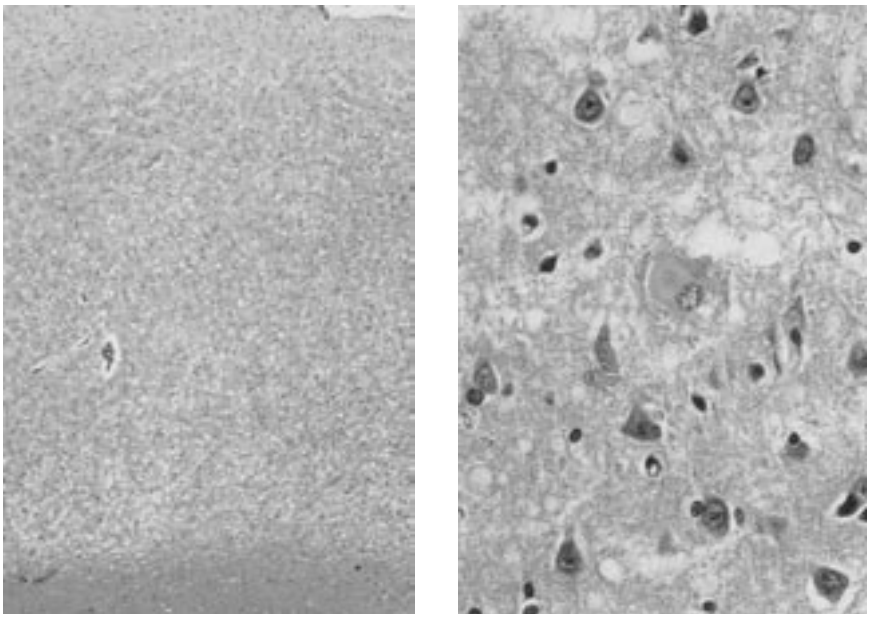
There is apparently no difference between codon 129 homozygous and heterozygous subjects; however, the number of subjects examined at autopsy in which codon 129 is known is limited. The common changes of this familial CJD subtype are spongiosis associated with prominent gliosis, often in the form of gemistocytic astrocytes, and variable degrees of neuronal loss (Fig. 5) (Parchi et al. 1996b). Enlarged or ballooned neurons may be present, which contain argyrophilic and Lewy body-like inclusions, that immunoreact with antibodies to neurofilaments (Fig. 5) (P. Parchi and P. Gambetti, unpubl.). The topography of these lesions is consistent and fairly distinctive. The involvement of the cerebral cortex is widespread, but frontal and temporal cortices are generally more severely affected than the occipital cortex. Within the hippocampal region, these changes are especially prominent in the subiculum and entorhinal cortex, whereas spongiosis is often present in the fascia dentata. Among the subcortical structures, the putamen and the caudate nucleus show severe spongiosis with variable degrees of gliosis; the thalamus is minimally or moderately affected with spongiosis and gliosis, whereas the cerebellum is spared, and minimal or no pathology is seen in the brain stem. The immunostaining pattern is

punctate, and its intensity matches the severity of the histopathology. However, the cerebellum shows minimal but definite immunostaining despite the lack of structural changes. No PrP-positive deposits, either in the form of amyloid or non-amyloid plaques, are present.

Characteristics and Allelic Origin of the PrP^{res}

All seven cases of $CJD^{D178N,\ 129V}$ examined to date have been associated with PrP^{res} that in the unglycosylated form has a gel mobility of 20–21 kD, corresponding to the PrP^{res} identified as type 1 (Monari et al. 1994; Parchi et al. 1996a). The glycosylation pattern (Fig. 3) is characterized by a marked underrepresentation of the unglycosylated form, which accounts for approximately 20% of the total, whereas the intermediate and highly glycosylated forms are similar in amount, and each accounts for approximately 40% of the total (Parchi et al. 1996a, 1998a).

Determination of the allelic origin has shown that both detergent-insoluble, i.e., aggregated, PrP and PrP^{res} derive exclusively from the mutant PrP in brains of $CJD^{D178N,\ 129V}$ subjects either homozygous or heterozygous at *PRNP* codon 129 (Table 3) (Chen et al. 1997). Therefore,



.*Fgure 5* Typical histopathology of CJD $^{D178N,\ 120V}$. Severe spongiosis with disorganization of the cytoarchitecture (*left*) and occasional neuronal Lewy body-like inclusions or ballooned neurons (*right*) are found in the cerebral cortex. (Hematoxylin and eosin).

there appears to be no direct participation in the disease process of the PrP^{C} expressed by the normal allele. This finding has implications for the mechanism(s) regulating the disease duration. The role of codon 129 in modulating the disease duration was previously explained as the result of the easier conversion of PrP^{C} to PrP^{res} in 129 homozygotes than in 129 heterozygotes due to the higher homology of mutant PrP and PrP^{C} in the 129 homozygotes (Palmer and Collinge 1992). The lack of wild-type PrP^{res} in the brain of subjects with $CJD^{D178N,\ 129V}$ suggests that the mechanism causing a shorter disease duration in the 129 homozygous subjects must be different in this familial CJD subtype.

Animal Transmissibility

$CJD^{D178N,\ 129V}$ has been transmitted to squirrel monkeys with brain tissue of seven out of ten subjects from five of six kindreds (Brown et al. 1992b, 1994b). Squirrel monkeys are homozygous for methionine at codon 129 (Schatz et al. 1995). Transmission to transgenic mice expressing the human/mouse chimera PrP (Telling et al. 1996) has failed. However, the recipient animals carried the methionine codon at position 128 corresponding to the position 129 in the human *PRNP*, which may be incompatible with the disease transmission with PrP^{res} containing valine at position 129.

CJD with the V210I Mutation and the 129M Codon on the Mutant Allele ($CJD^{V210I,\ 129M}$)

Epidemiology

The V210I mutation has been reported in ten affected subjects (Pocchiari et al. 1993; Ripoll et al. 1993; Furukawa et al. 1996; Parchi et al. 1996a; Shyu et al. 1996). Only two of these affected subjects belong to the same kindred (Pocchiari et al. 1993; Ripoll et al. 1993); the others are either the only affected member of kindreds in which other members carry the V210I mutation but are asymptomatic despite their advanced age, or are from uninformative families. These findings indicate that the V210I mutation has low penetrance, as reported for some of the insertion mutations (Capellari et al. 1997).

Clinical Features

The age at onset reported in nine subjects varies between 48 and 70 years of age. Although the number of subjects available is small, the polymorphism at codon 129 and a 24-bp deletion polymorphism present on the non-mutant allele in one case appear to influence the age at onset

(Pocchiari et al. 1993). In the four subjects 129M/M homozygous with no deletion, the disease started at a mean age of 54 years, and in the three subjects who are either 129M/V heterozygous or 129M/M homozygous but with the 24-bp deletion, the disease started at the mean age of 68 years. Therefore, the subjects carrying the V210I mutation that are heterozygous at codon 129 or carry the 24-bp deletion appear to have a later onset of the disease. This has also been observed in the 144 insertion, D178N, and F198S mutations (Dlouhy et al. 1992; Goldfarb et al. 1992a,c). The disease duration varies between 3 and 5 months (mean 4 months) ($n = 4$), comparable to the duration of the typical sCJD, and does not seem to be affected by the heterozygosity at the polymorphisms. However, one subject is still alive 2 years after the onset of the disease (Shyu et al. 1996). Adequate clinical data are available only in five subjects (Pocchiari et al. 1993; Ripoll et al. 1993; Furukawa et al. 1996). The presentation is reported to include memory, behavioral and gait disturbances, sudden sensory and motor hemiparesis, clumsiness, dystonic movements, and dysarthria. Subsequent common signs are myoclonus, dysarthria, mutism, and cerebellar signs. The EEG shows the typical PSW complexes in all four subjects examined (Pocchiari et al. 1993; Furukawa et al. 1996). Serial magnetic resonance imaging (MRI) in one case revealed increased signal intensity in the basal ganglia and thalamus in T2-weighted image and proton density as well as severe brain atrophy. Diffuse white matter degeneration was present in the later stages (Shyu et al. 1996).

Histopathology

The only two cases examined at autopsy revealed spongiosis and gliosis of cerebral cortex and molecular layer of the cerebellum (Ripoll et al. 1993; Pocchiari et al. 1993).

Characteristics and Allelic Origin of the Proteinase K-resistant PrP (PrP^{res})

On gel electrophoresis, the unglycosylated PrP^{res} migrates at 20–21 kD corresponding to the PrP^{res} type 1 (Parchi et al. 1996a,c). The ratio of the three PrP^{res} glycoforms is similar to that of the PrP^{res} associated with sporadic CJD group 1, which shows the relative dominance of the intermediate glycoform while the other two forms are less represented (Parchi et al. 1996a,c). Detailed studies have demonstrated that in V210I CJD, PrP^{res} is formed by both the mutant and normal PrP as it is believed to occur in the inherited prion diseases with 5 or 6 insertion mutations (Table 3) (Chen et al. 1997; Silvestrini et al. 1997).

CJD with the V180I Mutation and the 129M Codon on the Mutant Allele ($CJD^{V180I, 129M}$)

Four cases, which are all Japanese (one of which was not confirmed by histological examination), are known (Hitoshi et al. 1993; Kitamoto and Tateishi 1994). Three subjects are methionine homozygous and one is M/V heterozygous at codon 129. One has a double mutation, V180I and M232R, on different alleles. None of the subjects has a family history of CJD. The age at onset of the cases with the V180I mutation only varies between 66 and 81 years of age and the duration between 1 and 2 years. The only subject examined in detail presented with cognitive impairment, especially memory decrease, followed by akinetic mutism, pyramidal and extrapyramidal signs, and myoclonus. None of the patients demonstrated PSW in the EEG. Typical spongiform degeneration is present in the cerebral cortex, basal ganglia, and thalamus. Neuronal loss and astrocytosis were observed in the cerebral cortex. PrP immunostaining of the gray matter was weak and diffuse. The phenotype of the subject carrying the V180I and the M232R mutations is described with the phenotype of the $CJD^{M232R, 129M}$ (see below).

CJD with the T183A Mutation and the 129M Codon on the Mutant Allele ($CJD^{T183A, 129M}$)

This haplotype has been reported in one Brazilian kindred of Spanish and Italian origin with nine affected members (Nitrini et al. 1997). Mean age at onset is 48 years (range 37–49 years); mean duration is 4 years (range 2–9 years). The codon 129 haplotype was only determined in two subjects; it is unclear whether codon 129 modifies the duration of the disease. Nonetheless, the disease had a course of 2 years in the homozygous subject and 9 in the heterozygous. The clinical presentation includes personality changes associated with memory impairment in half of the cases. The predominant subsequent signs include rapidly progressive dementia, aggressive behavior, hyperorality, verbal stereotypes, and often parkinsonian signs. In the one case examined, the EEG failed to demonstrate PSW complexes even in advanced stages of the disease and when the patient exhibited myoclonus. The histologic examination carried out in four subjects showed widespread spongiosis and atrophy in the cerebral cortex with more severe involvement of frontal and temporal lobes. The basal ganglia are variably involved, whereas the hippocampus, thalamus, brain stem, and cerebellum are preserved. Immunostaining for PrP is only detected in the putamen, where it has a plaque-like pattern, and in the molecular layer of the cerebellum, where it is diffuse and punctate. The

PrP^{res} gel pattern has not been examined. The T183A mutation abolishes the N-linked glycosylation site present at codon 181. Studies on CV-1 (green monkey kidney) transfected cells carrying the mutation show that the T183A mutant PrP accumulates in the endoplasmic reticulum (ER)-proximal Golgi and does not reach the cell surface (Rogers et al. 1990). In CHO cells PrP^{M} was shown to become weakly PK resistant (Lehman and Harris 1997).

CJD with the R208H Mutation and the 129M Codon on the Mutant Allele ($CJD^{R208H, 129M}$)

Only one affected subject from a kindred carrying the mutation, but negative for history of neurodegenerative disease, has been reported (Mastrianni et al. 1996). In this subject, the disease phenotype appears to be very similar to that of the typical sporadic CJD. Clinically, the patient presented at the age of 60 years with sensory and motor difficulties with ataxia of the right leg, which improved after physical therapy. It is not clear, however, whether these signs are part of the main disease. Two and a half years later, the patient showed cognitive impairment, including forgetfulness, anomia, and dyscalculia. He became paranoid with hallucinations and delusions, developed an ataxic gait and myoclonus, his speech showed perseverations with minimal output, and he died 7 months after the onset of dementia. The EEG showed typical PSW complexes. The MRI was consistent with the atrophy of the left sensory-motor cortex. This finding was confirmed by positron-emission tomography (PET) imaging, which demonstrated hypometabolism of the left hemisphere, especially in the parietal region. The histopathology, apparently limited to the frontal cortex, is characterized by marked spongiosis and gliosis with the punctate pattern of PrP immunostaining. The PrP^{res} migration pattern following gel electrophoresis was consistent with the PrP^{res} type 1 (Parchi et al. 1996a,c).

CJD with the M232R Mutation and the 129M Codon on the Mutant Allele ($CJD^{M232R, 129M}$)

The M232R mutation has been observed in eight Japanese subjects (Hoque et al. 1996). It has been found to be associated with the V180I mutation located on the other allele in one subject (also see above) (Hitoshi et al. 1993), whereas another subject was heterozygous at codon 219, the site of a rare glutamine/lysine polymorphism (Barbanti et al. 1996), with the lysine codon on the normal allele (Kitamoto and Tateishi 1994). A third subject was M/V heterozygous at codon 129; the valine

codon, which is extremely rare in the Japanese population (Doh-ura et al. 1989), was on the normal allele (Hoque et al. 1996). The remaining mutant subjects were M/M homozygous (Kitamoto and Tateishi 1994; Hoque et al. 1996). In all of these cases, the family history was negative for neurodegenerative diseases. Clinical and pathologic findings have been reported in detail in three cases (Hoque et al. 1996). Age at onset varies between 55 and 70 years, duration between 4 and 24 months. Common presenting signs are memory and gait disturbances, which progress and are associated with myoclonus and mutism at more advanced stages. EEG is typical, with PSW complexes observed in all cases. CT scan shows widespread cerebral atrophy; PET, carried out in one case, showed decreased cerebral blood flow (Hoque et al. 1996). The histopathology is characterized by widespread spongiosis, gliosis, and neuronal loss of variable degree, regardless of the disease duration. The thalamus shows the most severe spongiosis, especially in the medio-dorsal nucleus. Neuronal loss is severe in the basal ganglia, and the spongiosis is minimal. The cerebral cortex shows moderate spongiosis, and the gliosis and neuronal loss may be severe. All three lesions are present in the brain stem; the cerebellum lacks spongiosis but shows neuronal loss and gliosis (Hoque et al. 1996). The PrP immunoreactivity is widespread in cerebrum and spinal cord with more intense immunostaining in the cerebral cortex, especially the hippocampus, and is of the punctate type with no plaque-like PrP deposits (Hoque et al. 1996). The M232R mutation is of particular interest because it affects a PrP residue that is supposed to be removed posttranslationally when the glycolipid anchor is attached (Stahl et al. 1990). Therefore, it is likely that the arginine replacing the methionine in the M232R mutant PrP is not cleaved by the anchoring process and alters the conformation of the mutant PrP. The $CJD^{M232R,\ 129M}$ has been transmitted to mice with brain tissue suspensions, but not with suspensions from lymph nodes and spleen, and the resulting number of symptomatic animals, incubation times, and pathology were similar to those observed following transmission of sCJD (Hoque et al. 1996). In the subject with the V180I and M232R double mutation who was M/M homozygous at codon 129, the disease started at 84 years of age and lasted 1 year (Hitoshi et al. 1993). The onset was characterized by cognitive impairment with memory deficit and discalculia. At midcourse, the dementia worsened and was accompanied by akinetic mutism. Motor signs were not detected with the exception of an increase in the deep tendon reflexes and myoclonus. No PSW complexes were detected in several EEG examinations. Serial MRI showed slight atrophy of cerebral white matter and basal ganglia and an abnormally high signal, which was first

detected in the temporal cortex and extended into the fronto-parietal-occipital cortex during the first 4 months of illness. PET revealed markedly decreased glucose metabolism involving most of the brain, with relative preservation of sensory-motor and occipital cortices, thalamus, and cerebellum (Hitoshi et al. 1993). Histologic examination was limited to the parietal lobe and demonstrated cortical spongiosis and diffuse immunostaining. The gel pattern of the PrP^{res} is unusual because it apparently lacks the high-molecular-weight glycoform (Hitoshi et al. 1993).

Fatal Familial Insomnia (D178N, 129M)

Epidemiology

Currently, we are aware of 24 families that carry the FFI mutation. In addition, there are at least 2 subjects in which the FFI mutation has been found but the families are either uninformative or have not yet been described (Table 4). To date, the FFI haplotype appears to be the third most common after the E200K-129M and P102L-129M haplotypes. In Germany, the FFI haplotype has been found to be the most common in a survey conducted between 1993 and 1997 (Kretzschmar et al. 1998).

Table 4 FFI kindreds

	Kindreds	Origin	Subjects (no.)[a]
Montagna et al. (1998)	2 (1)[b]	Italian	10
	2	French	4
Brown et al. (1998)	2	American/English	6[c]
	1	American/unknown	3
	2	Australian/Irish	3
	1	Japanese	1
Kretzschmar et al. (1998)	7 (1)[b]	German	6
Budka et al. (1998)	1	Austrian	4
Will et al. (1998)	2	British	2
Pocchiari et al. (1998) and Padovani et al. (1998)	1		2[d]
Goldfarb et al. (1992c)	1	American/German	2
G. Green and K. Berry (unpubl.)	1	Canadian/Chinese	1
Rossi et al. (1998)	1	Italian	1
Totals	24 (2)[b]		45

[a]Subjects with known PRNP 129 polymorphism used for disease duration analysis.
[b]Mutant subjects with no familial history of FFI.
[c]Cases 1 and 2 and III-5; IV-7; IV-12; and V-14.
[d]Case 2, and 4 from FFI 1.

Clinical Phenotype

Several aspects of the phenotype are slightly different in patients who are homozygous or heterozygous at codon 129 of *PRNP* (Montagna et al. 1998). In the 45 subjects in which the codon 129 is known (Table 4), 30 are methionine homozygous and 15 are methionine/valine heterozygous. The disease begins at a mean age of 49 years (range 20–71 years) and is not significantly different in homozygous and heterozygous subjects. In contrast, the mean disease duration is significantly shorter in the homozygous, 12 ± 4 months, than in the heterozygous subjects, 21 ± 15 ($p < 0.004$), but the ranges, 6–21 and 7–33 months, respectively, show considerable overlap. Recently, the clinical phenotype has been carefully analyzed in 14 clinically well-documented patients, 8 of which were homozygous and 6 heterozygous at codon 129 (Montagna et al. 1998). Most clinical symptoms and signs were comparable among the different patients. They comprised sleep-wake and vigilance disturbances (insomnia, oneiric stuporous episodes with hallucinosis and episodic confusion), altered autonomic functions (systemic hypertension, irregular breathing, diaphoresis, pyrexia, impotence), and somatomotor manifestations (diplopia, dysarthria, dysphagia, ataxia/abasia, dysmetria, spontaneous and evoked myoclonus, spasticity); tonic-clonic seizures were observed in some cases. Analysis of clinical symptoms and signs according to the codon 129 polymorphism showed that the 129 homozygous patients presented prominent oneiric episodes with hallucinations and episodic confusion as well as spontaneous and evoked myoclonus and more evident autonomic alterations (irregular breathing, hypertension) at the onset of the disease. In contrast, these features occurred later in the 129 heterozygous patients who instead presented with ataxia and dysarthria; these somatomotor disturbances worsened subsequently and were always more severe in these patients. Tonic-clonic seizures were more frequent among the 129 heterozygotes.

Routine EEGs did not show specific alterations in any of the patients at onset. With the progression of the disease, especially in the terminal stages, the background EEG activity progressively changed to a monomorphic flat activity. At advanced stages, bursts of repetitive diffuse 1–2 Hz periodic sharp waves associated with myoclonus may appear in cases of long duration.

On 24-hour video polysomnographic recordings, two patterns emerged. In the 129 homozygous subjects, normal sleep was absent from the onset of the disease and was associated with normal wakefulness activity; the latter was, however, interrupted by periods during which patients were unresponsive and manifested peculiar motor activity in the form of

rapid twitching of one or more limbs, utterance of words or sentences, and finalistic complex gestures that corresponded to an oneiric content. When fully awake and in contact, patients complained of increasing generalized fatigue and would try unsuccessfully to fall asleep. Such behavior explains why these patients have been defined as "sleepy." Non-REM sleep was abolished and only brief REM sleep episodes occurred, often in clusters and associated with oneiric behavior. REM sleep could present a normal pattern, with desynchronized low-voltage θ activity on EEG in the presence of physiological electromyograph (EMG) atonia. However, short, 10–20-second REM sleep could abnormally emerge directly from wakefulness and recur periodically, every 10–30 seconds. REM sleep could otherwise be present with persistent axial tone, and flow into a complex behavior characterized by the absence of physiological muscle atonia, increase in muscle tone, and intensified myoclonic jerks, during which the patients enacted their dream. This state resembled the REM sleep behavior disorder, or status dissociatus, seen in some other neurodegenerative diseases. Spectral analysis performed on the 24-hour EEG recordings confirmed that in these cases there was nearly complete, or complete, absence of slow-wave sleep.

In 129 heterozygous patients, 24-hour recordings at the onset of the disease were characterized by a relative preservation of the cyclic structure of nocturnal sleep and by the persistence of slow (<4 Hz) EEG activity, typical of slow-wave sleep. However, REM sleep often showed the characteristic lack of physiological muscle atonia, and oneiric activity was present, although less prominent.

In all patients, blood pressure (BP), heart rate (HR), and norepinephrine (NE) resting plasma levels were higher than in normal controls comparable for age and sex. These data, along with tests for autonomic functions, indicated sympathetic overactivity in all patients.

Circadian studies of BP and HR rhythms showed that the nocturnal blood pressure fall was lost early on in the disease, while the physiological bradycardia was still preserved and the rhythmic component persisted, although with a reduced amplitude and shifted phase, even in the absence of recorded sleep. Rhythmicity was abolished in the terminal stages only. Body core temperature was likewise persistently elevated.

Circadian catecholamine rhythms were essentially preserved in the early stages of FFI, but increasing mean plasma levels and decreasing circadian amplitudes marked the progression of the disease, with total loss of rhythms in the terminal stages. Cortisol levels were high, whereas adrenocorticotropic hormone (ACTH) remained at normal levels, suggesting a condition of hypercortisolism added to a functional dysregula-

tion of the hypothalamic-pituitary-adrenal axis. Melatonin levels showed a gradual decrease in circadian amplitude and a shift in phase until an eventual complete rhythm loss. Somatotrophin also showed a reduced or even absent rhythmicity, which, however, paralleled the loss of deep sleep, whereas prolactin rhythmicity remained unaltered.

Social behavior of the patients remained normal; cooperation was good and personal relationships were maintained until late in the disease. Cognitive functions also remained normal, with good results on IQ tests until these could no longer be performed. The main disturbances consisted of altered vigilance with frequent and spontaneous intrusions of dream-like states into normal wakefulness (oneiric stupors). Attention was especially impaired, in the early stages, even more than vigilance. Tests showed also altered encoding, defective manipulation of information, and incorrect ordering of events (chronoataraxis). Therefore, working memory was particularly impaired, whereas semantic, retrograde, and procedural memory remained unaffected. There was no significant difference in neuropsychological tests between 129 homozygote and 129 heterozygote patients.

Given the normal cognitive tests, preserved behavior, and the prominent vigilance alterations, our patients did not fulfill standard criteria for the diagnosis of dementias. Thus, we argue that FFI should not be defined as a dementing, but rather as a confuso-oneiric illness.

Neuroradiological and PET Studies

Standard brain CT and MRI disclosed cerebral and cerebellar atrophy and ventricular dilatation only in those patients with a prolonged course. PET studies with (18F)-FDG performed in seven patients showed marked reduction of glucose utilization in the thalamus and, to a lower degree, in the cingular cortex in all cases. Glucose hypometabolism was also found in the basal and lateral frontal and middle and inferior temporal cortices, and the caudate nuclei, in six patients. The 129 homozygote cases had less metabolic involvement, restricted to the thalamus, basal frontal and cingular cortex, whereas in the 129 heterozygote subjects, severe hypometabolism also affected the hippocampus, putamen, and caudate. Since the latter were examined at later stages, the more widespread involvement may reflect diffusion of the disease process with time.

Histopathology

The hallmark is loss of neurons and astrogliosis in the thalamus, which is present in all subjects, independent of the disease duration (Fig. 6)

(Manetto et al. 1992; Parchi et al. 1995). The medio-dorsal and anterior ventral thalamic nuclei are invariably and severely affected, whereas the involvement of other thalamic nuclei varies (Fig. 6A,B). The inferior olives also show neuronal loss and gliosis in most cases. In contrast, the pathology of the cerebral cortex varies in proportion to the disease duration and is more severe in the limbic lobe than in the neocortex (Fig. 6C) (Manetto et al. 1992; Parchi et al. 1995). The entorhinal cortex and, to a lesser extent the piriform and paraolfactory cortices, show spongiosis and astrogliosis in most subjects. Instead, the neocortex is virtually spared in the subjects with a disease duration of less than 1 year but focally affected by spongiosis and gliosis in those with a course between 12 and 20 months and diffusely involved in subjects with a disease duration of more than 20 months (Fig. 6D). In addition, the frontal, temporal, and parietal lobes are affected more severely than the occipital lobe. The other structures are virtually normal or show mild focal pathology. Overall, the thalamus is more severely and consistently involved than any other brain region. Therefore, on the basis of the pathology, FFI can be defined as a preferential thalamic degeneration. Criteria to diagnose FFI are listed in Table 5.

Genotype–Phenotype Correlation

The heterogeneity in clinical and histopathologic features that has been noted in FFI-affected subjects is consistent with the existence of two slightly different phenotypes that are determined by the genotype at codon 129 of the normal allele (Goldfarb et al. 1992c). FFI patients M/M

Table 5 FFI diagnostic criteria

1. Autosomal dominant disease, onset at adult age, and duration of several months to several years
2. Presence of untreatable insomnia, dysautonomia, memory impairment, ataxia and/or myoclonus, pyramidal and extrapyramidal signs
3. Decrease or loss of sleep-related EEG activities
4. Preferential hypometabolism in the thalamic region using $^{[18F]}$PET
5. Preferential thalamic atrophy by pathologic examination
6. 129^{Met}, 178^{Asn} haplotype
7. PrP^{res} of type 2

Any combination of two of the criteria 1–5 makes the diagnosis of FFI highly probable. Criterion 6 in combination with any one of the other criteria, or criterion 7 combined with criteria 2–6, makes the diagnosis definitive.

at codon 129 manifest, on average, a more rapid course and prominent sleep and autonomic disturbances whereas signs of motor and cognitive dysfunction are mild. In contrast, the subjects M/V at codon 129 have a

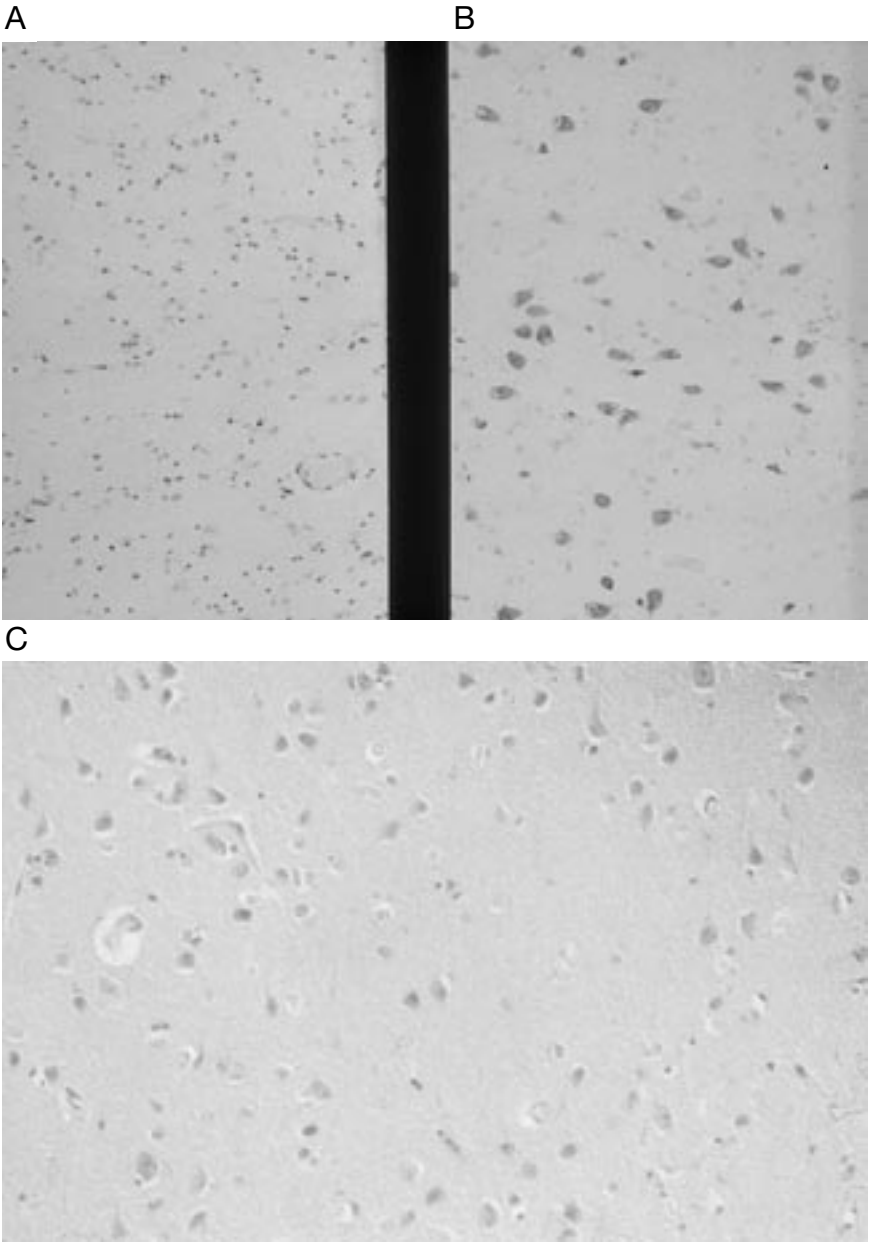


Figure 6 (*See facing page for legend.*)

more chronic course and manifest motor signs as a prominent clinical feature at onset, whereas sleep disturbances and autonomic signs are less severe. In addition, signs of cortical involvement also appear in the heterozygous subjects, although late in the course of the disease. As noted above, the histopathology varies as a function of the disease duration, which, in turn, is a function of the 129M/V polymorphism. Overall, the thalamus is similarly affected in all subjects, or slightly more in the 129 M/M subjects, but the cerebral cortex is generally more severely involved in the 129 M/V subjects.

Prion Protein in FFI and CJD[178]

The PrP^{res} fragments associated with FFI and CJD[178] differ both in the size of the core protein and in the ratio of the three PrP^{res} glycoforms (Fig. 3) (Monari et al. 1994). FFI PrP^{res} has been classified as type 2. Strong evidence indicates that the size variation is the result of different degrees

D

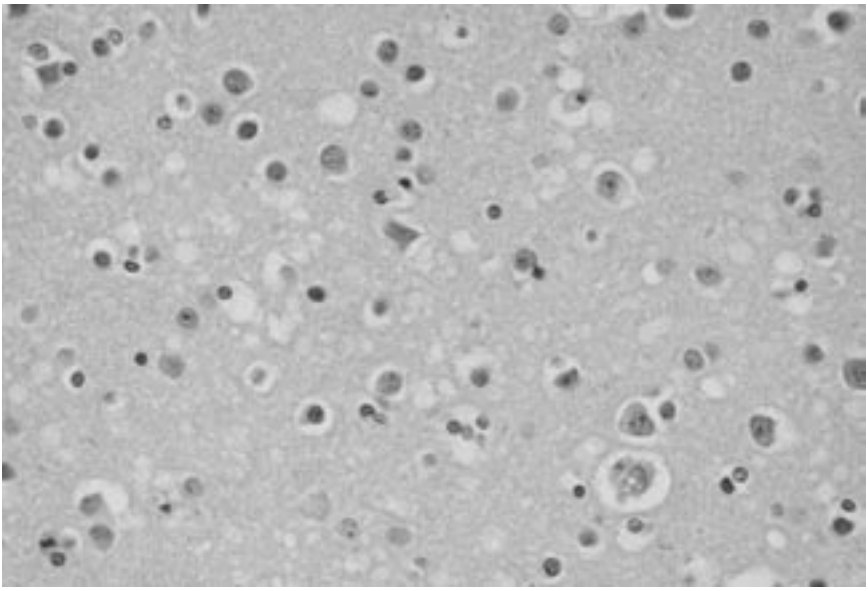


Figure 6 (*continued*) Histopathology of FFI. (*A*) Severe loss of neurons and gliosis in the dorso-medial thalamic nucleus. (*B*) Control. (*C*) Lack of spongiosis and gliosis in the cerebral cortex of an FFI subject homozygous for methionine at codon 129 with a disease course of 7 months (GFAP immunostaining). (*D*) Moderate spongiform degeneration in an FFI 129 heterozygous subject with a 25-month disease course. (Hematoxylin and eosin.)

of amino-terminal trimming by proteases (Bessen and Marsh 1994; Monari et al. 1994) which, in turn, is likely to result from a different conformation of the PrP^{res}. The distinctive glycoform ratio of FFI and CJD^{178} PrP^{res} is the result of the underrepresentation of the mutant unglycosylated form prior to the conversion to the PrP^{res} isoform, probably due to the pronounced instability of this D178N mutant glycoform (Petersen et al. 1996). These findings are consistent with the conclusion that the PrP^{res} associated with FFI and CJD^{178} have different protein conformations and/or distinct ligand-binding interactions. Therefore, the FFI and CJD^{178} phenotypes are likely to be determined by codon 129 on the mutant allele which, coupled with the D178N mutation, results in the expression of PrP^{res} with distinct biophysical characteristics.

Allelic Origin of PrP^{res} in Inherited Prion Diseases

As described above for the $CJD^{178,129V}$, both insoluble PrP and PrP^{res} derive exclusively from the PrP^{M} in FFI, regardless of whether the affected subjects are homozygous or heterozygous at codon 129 (Table 3) (Chen et al. 1997). Whether the low amount of the PrP^{res} found in FFI is due to the monoallelic origin of PrP^{res} or to other mechanisms remains to be determined. The transmissibility, to animals not carrying the FFI haplotype, of diseases such as FFI in which only PrP^{M} becomes PrP^{res} is puzzling. It is unclear how PrP^{C} can be converted in the recipient animal following inoculation of FFI PrP^{res}, while conversion does not occur spontaneously in FFI patients. The high local concentration of PrP^{res} achieved by the animal inoculation might overcome the barrier that blocks the conversion of PrP^{C} in FFI.

Transmissibility of FFI

Transgenic mice expressing a chimeric human/mouse PrP^{C} develop a prion disease 200 days after the intracerebral inoculation with a homogenate from FFI brains (Telling et al. 1996). The pathology as well as the presence of PrP^{res} is predominant in the thalamus. Transmission experiments (Collinge et al. 1995; Tateishi et al. 1995) not only demonstrate that FFI shares transmissibility with other prion diseases, but also provide important clues concerning the mechanisms of PrP^{C}-PrP^{res} conversion. It has been demonstrated that the size of the PrP^{res} fragment expressed by the inoculated transgenic mice is identical in electrophoretic mobility to the PrP^{res} fragment present in the inoculum from subjects

with FFI. Replication of the PrP^{res} fragment size was also observed in the transmission of sporadic and inherited CJD associated with PrP^{res} type 1. Since the recipient mice did not carry any of the donor's *PRNP* mutations and all had the same genetic background, this remarkable finding indicates that the distinct conformations associated with PrP^{res} type 1 and type 2 can be reproduced independently of the genetic information, probably on the basis of information contained in the conformation of the donor's PrP^{res}. This mechanism explains prion strain diversity by a mechanism that does not require the participation of nucleic acids.

FFI and Its Relationship with Thalamic Dementia: Does a Sporadic Form of FFI Exist?

The concept of thalamic dementia or thalamic degeneration was introduced by Stern in 1939 when he described a subject with severe cognitive impairment of approximately 6 months' duration, which at autopsy showed only severe atrophy of ventral anterior and medial dorsal thalamic nuclei and a variable degree of astrogliosis in the cerebral cortex (Stern 1939). Thalamic degeneration has been subsequently classified by Martin (1970) into three groups: (1) the thalamic degeneration associated with multisystem atrophies, (2) the so-called preferential thalamic degeneration, and (3) the thalamic form of CJD. Groups 1 and 2 included familial and sporadic forms, whereas thalamic CJD was described only in sporadic cases. The nosologic position of preferential thalamic degeneration and in particular its delineation from thalamic CJD, however, has always been unclear. The reclassification of the Stern's case as a thalamic form of CJD (Petersen et al. 1992) is an example of the confusion that has surrounded the definition of conditions characterized by preferential thalamic atrophy. After the description of FFI, it was shown that previously reported familial cases of preferential thalamic degeneration or dementia have the same *PRNP* D178N, 129M haplotype as FFI. Therefore, the condition previously identified as familial thalamic atrophy or dementia and FFI are one and the same disease (Kirschbaum 1968; Petersen et al. 1992; Monari et al. 1994). In contrast, no *PRNP* mutations are found in multiple system atrophy with thalamic degeneration (Kirschbaum 1968).

In contrast to the familial forms, however, only one of the sporadic cases of preferential thalamic degeneration has been analyzed for the presence of PrP^{res} (Mizusawa et al. 1988). Clinically and histopathologically, this case is similar to that of Stern (1939); however, insomnia was reported by clinical observation. Immunoblotting demonstrated the pres-

ence of PrP^{res} type 2, as found in FFI, in the cerebral cortex (P. Parchi and P. Gambetti, unpubl.). Sequencing ruled out the presence of the D178N mutation (Mizusawa et al. 1988). The presence of PrP^{res}, insomnia, dysautonomia, and endocrine disturbances must be proven in more cases in order to definitely clarify the nosology of the sporadic form of preferential thalamic degeneration and decide whether these cases should now be classified as the thalamic form of sporadic CJD or as the sporadic form of fatal insomnia.

INHERITED PRION DISEASE WITH VARIABLE PHENOTYPE: THE INSERTIONAL MUTATIONS

Epidemiology and Genetics

The wild-type *PRNP* gene has five repeating sequences between codons 51 and 91 of which the first comprises 27 bp, the other four have 24 bp, and they code for a P(H/Q)GGG(G/-)WGQ nona/octapeptide. Owen et al. (1989, 1990, 1992) were the first to report a six 24-bp extra repeat insert in a British family with atypical dementia. Several additional families with one to nine 24-bp extra repeat insertions were subsequently reported in the US, several European countries, and Japan (Goldfarb et al. 1991b, 1992a, 1993, 1996; Collinge et al. 1992; Poulter et al. 1992; Duchen et al. 1993; Isozaki et al. 1994; Mizushima et al. 1994; Cervenakova et al. 1995; Krasemann et al. 1995; Laplanche et al. 1995; Nicholl et al. 1995; Oda et al. 1995; van Gool et al. 1995; Campbell et al. 1996; Cochran et al. 1996; Capellari et al. 1997). Altogether, at the time of this writing, 20 families with 95 affected members have been clinically characterized, and 24 subjects were studied neuropathologically (Tables 2 and 6).

Each family carried a unique allele, which was most likely generated by unequal crossover and differed by the number of the repeats as well as the order and the composition of the inserted repeats (Table 7). Moreover, in 16 families, the repeat expansion was coupled with the methionine codon at position 129, whereas in 4 other families the inserts were found on the 129 valine allele (Table 6).

Clinical Features

The disease phenotype associated with the insertion mutations is highly variable. For example, the age at onset ranges between 21 and 82 years and the disease duration between 2 months and 18 years (Table 6). The

Table 6 Affected subjects carrying the octapeptide repeat

Extrarepeat/ Codon 129	No. of families	No. of patients	Age at onset Mean (years) ± S.D.	Duration of Illness Mean (months) ± S.D.	References
+1/Met	1	1	73	4	Laplanche et al. (1995)
+2/Met	1	1	58	3	Goldfarb et al. (1993)
+4/Met	3	2	59	31	Goldfarb et al. (1991b); Campbell et al. (1996); Isozaki et al. (1994)
+5/Met	2	6	40 ± 6	85 ± 17	Goldfarb et al. (1991b); Cochran et al. (1996)
+6/Met	5	64	35 ± 7	82 ± 34	Owen et al. (1990); Cervenakova et al. (1995); Oda et al. (1995); Nicholl et al. (1995); Capellari et al. (1997)
+7/Met	2	4	28 ± 3	114 ± 21	Goldfarb et al. (1991b); Mizushima et al. (1994)
+9/Met	2	2	42	27	Krasemann et al. (1995); Duchen et al. (1993); Owen et al. (1992)
+4/Val	1	1	82	4	Laplanche et al. (1995)
+5/Val	1	2	46	4	Cervenakova et al. (1995)
+8/Val	2	12	43 ± 10	43 ± 27	Goldfarb et al. (1992a); van Gool et al. (1995)
Total	20	95			

clinical and pathologic features include the typical CJD phenotype, a phenotype more consistent with GSS, and conditions of several-year duration lacking distinctive histopathology (Table 2) (Cochran et al. 1996; Capellari et al. 1997). This phenotypic heterogeneity appears to be related, at least in part, to the heterogeneity of the genotypes associated with these diseases, since it is markedly reduced when insertional mutations are grouped according to size (Table 8). Thus, the examination of the clinical features of patients with four or fewer octapeptide insertions, or 24–96 bp, shows that the mean age at onset is 68 years and the duration is 10 months. Approximately two-thirds of these patients have a clinical phenotype indistinguishable from that of sCJD group 1, i.e., rapidly progressive dementia often associated with ataxia and visual disturbances,

Table 7 Reported alleles with number and sequence of the extra 24-bp repeat in *PRNP*

Repeats (No.)	Sequence	Ethnic origin	Reference
5 (wild-type)	R1,R2,R2,R3,R4		
+1 insertion	R1,R2,R2,**R2**,R3,R4	French	Laplanche et al. (1995)
+2	R1,R2,R2,**R2a**,**R2a**,R3,R4	American	Goldfarb et al. (1993)
+4	R1,R2,R2,**R3**,**R2**,**R3**,**R2**,R3,R4	American	Goldfarb et al. (1991b)
+4	R1,R2,R2,**R2**,**R2**,**R2**,**R2**,R3,R4	British	Campbell et al. (1996)
+4	R1,R2,R2,**R3**,**R2**,**R2**,**R2**,R3,R4	French	Laplanche et al. (1995)
+4	R1,R2,R2,**R2**,**R2**,**R2**,**R2**,R3,R4	Japanese	Isozaki et al. (1994)
+5	R1,R2,R2,**R3**,**R2**,**R3g**,**R2**,**R2**,R3,R4	American	Goldfarb et al. (1991b)
+5	R1,R2,R2,**R2a**,**R2**,**R2a**,**R2**,**R2**,R3,R4	American	Cervenakova et al. (1995)
+5	R1,R2,R2,**R3**,**R2**,**R2**,**R2**,**R2**,R3,R4	American	Cochran et al. (1996)
+6	R1,R2,R2,**R2**,**R3**,**R2**,**R3g**,**R2**,**R2**,R3,R4	British	Owen et al. (1992)
+6	R1,R2,R2,**R2**,**R2**,**R2**,**R2**,**R2**,**R3g**,R3,R4	American	Cervenakova et al. (1995)
+6	R1,R2,R2,**R3g**,**R2**,**R2**,**R3g**,**R2**,**R2**,R3,R4	Japanese	Oda et al. (1995)
+6	R1,R2,R2,**R3**,**R2**,**R3g**,**R2**,**R3g**,**R2**,R3,R4	British	Nicholl et al. (1995)
+6	R1,R2,R2,**R2**,**R2**,**R2**,**R2**,**R2**,**R2**,R3,R4	Basque	Capellari et al. (1997)
+7	R1,R2,**R2c**,**R3**,**R2**,**R3**,**R2**,**R3**,**R2**,**R3g**,R3,R4	American	Goldfarb et al. (1991b)
+7	NA	Japanese	Mizushima et al. (1994)
+8	R1,R2,R2,R3,**R2**,**R2**,**R2**,**R2**,**R2**,**R2**,**R2**,**R2a**,R4	French	Goldfarb et al. (1992a)
+8	R1,R2,R2,**R3g**,**R3**,**R2**,**R2**,**R2**,**R2**,**R2**,**R2**,R3,R4	Dutch	van Gool et al. (1995)
+9	R1,R2,R2,**R3**,**R2**,**R3g**,**R2a**,**R2**,**R2**,**R2**,**R3g**,**R2**,R3,R4	British	Owen et al. (1992)
+9	R1,R2,R2,**R3**,**R2**,**R3**,**R3g**,**R2**,**R2a**,**R2**,**R3**,**R2**,R3,R4	German	Krasemann et al. (1995)

*Each repeat (R) is a variant 24-bp sequence, for codes see Goldfarb et al. (1991b). Extra repeats in bold. (NA) Not available.

myoclonus and PSW on the EEG (Capellari et al. 1997). The penetrance is low in these subjects, so the familial incidence of the disease may be undetectable. In contrast, the mean age at onset of the patients with five or more octapeptide inserts, 120–216 bp, is 36 years with a mean disease duration of 6 years (Tables 6 and 8). Moreover, fewer than 10% of these patients present the typical CJD clinical phenotype, whereas the vast majority present a slowly progressive syndrome characterized by the presence of mental deterioration, cerebellar and extrapyramidal signs often lacking the PSW complexes at EEG examination. In conclusion, these observations clearly show that the clinical phenotype, especially the age at onset and the duration of the disease, is related to the number of repeats. A CJD-like clinical phenotype is associated in the majority of cases with four or fewer extra repeats, whereas a GSS-like syndrome is observed in most cases carrying five or more additional repeats (Table 8). The age at onset seems to correlate inversely, and the duration of disease directly, with the number of repeats (Table 6). Similar data concerning disease onset and duration have been obtained with the trinucleotide repeat expansion recently characterized in Huntington's disease and in other neurologic disorders (La Spada et al. 1994). Huntington's alleles are highly unstable when transmitted from parents to children, especially through male meioses (La Spada et al. 1994). This is not the case with the PRNP repeat expansion. The inserted sequences were exactly the same in the descendants of different lines in the six-generation British family (Poulter et al. 1992), as well as in four smaller families (Goldfarb et al. 1991b, 1992a, 1993; Oda et al. 1995). The anticipation phenomenon that is very characteristic of the trinucleotide repeat expansion disorders is absent in the repeat-expansion families with spongiform encephalopathy.

As in other inherited prion diseases, codon 129, either on the mutant

Table 8 Familial prion diseases with insertions: General phenotypic features

Overall marked heterogeneity:
Clinical features:
Subjects with <4 octapeptide insert have low penetrance and often the CJD phenotype
Subjects with >4 octapeptide insert have high penetrance and often the GSS phenotype; the CJD phenotype is uncommon
Pathological features:
Are variable and include CJD, GSS, mixed and nonspecific phenotypes
Presence of non-amyloid (Congo Red negative) elongated PrP deposits in the cerebellum molecular layer by immunostaining is highly distinctive

or on the normal allele, may influence the effects of PRNP mutation (Goldfarb et al. 1992c). In the large British family with the 6 extra repeat expansion, the age at death in M/M patients is significantly lower than in M/V heterozygotes, suggesting a protective role of valine at position 129 (Poulter et al. 1992). The four and five extra repeat insertions have been reported to occur coupled with either the M or V at codon 129. Although the number of patients is low, the two patients with five extra repeats coupled with 129V codon had a markedly longer disease duration as compared to the six patients having the same size expansion coupled with the 129M codon, but the age at onset was similar in the two groups (Table 6). In contrast, two patients with four extra repeats coupled with the 129M codon had a markedly lower age at onset than that of two patients in which the four extra repeats were coupled with the 129V codon. The clinical phenotype was CJD-like in the patients with either four or five extra repeats but a short disease duration, and GSS-like in those in which the course of the disease was long. Therefore, codon 129 coupled with the mutant codon influences the characteristics of the phenotypes.

Histopathology

The pathological features mirror fairly closely the distribution of the CJD-like and GSS-like clinical phenotypes (Table 8). Almost 90% (13 of 15) of the subjects with 6 or fewer octapeptide inserts, which have undergone autopsy examination, show histopathological changes consistent with CJD, even if the majority of these cases have a disease duration of more than one year. These changes include spongiform degeneration or status spongiosus, astrogliosis, and neuronal loss in different combinations, but lack of PrP amyloid plaques outside the granular cell layer of the cerebellum. In contrast, the great majority (6 of 7) of autopsied subjects with 7 or more octapeptide inserts show a different histopathologic phenotype. Five of these cases, along with one carrying a six octapeptide insertion, have a histopathology consistent with that of GSS. In addition to various degrees of spongiosis, gliosis, and neuronal loss, PrP amyloid plaques, often multicentric, are also present in the molecular layer of the cerebellum and frequently in the cerebral gray matter, a distribution not seen in CJD. Rarely (1 subject from this group and 1 with 6 octapeptide repeat insertion of a total of 22 autopsied cases), there are minimal or not distinctive histologic changes such as astrogliosis and neuronal loss without spongiosis or PrP amyloid plaques. In conclusion, the CJD-like histopathologic phenotype is very common in subjects with up to 6 octapeptide insertion mutations. The GSS-like phenotype is common in

association with 8 and 9 octapeptide insertion mutations, and significant amyloid formation only occurs in association with 6 or more octapeptide repeats. Although the CJD and the GSS histopathological phenotypes are more often associated with a disease duration of less and more than 1 year, respectively, this is far from being the rule since, as mentioned above, the CJD histopathology may be associated with a course of more than 1 year and PrP amyloid plaques were present in a subject with a 3-month disease duration. Finally, a distinctive feature is the presence of linear granular deposits with PrP immunoreactivity, located in the molecular layer of the cerebellum (Fig. 7).

Characteristics and Allelic Origin of PrP^{res}

The pattern of the gel migration and the glycoform ratios of PrP^{res} have been examined in cases with four, five, and six extra insertions (Parchi et al. 1996a; Cochran et al. 1996; Capellari et al. 1997). The unglycosylated PK-treated PrP^{res} from subjects with four and six extra insertions migrates on gels at 20–21 kD, corresponding to the PrP^{res} type 1, whereas the corresponding PrP^{res} from the subject with the five extra insertion migrates at 18-

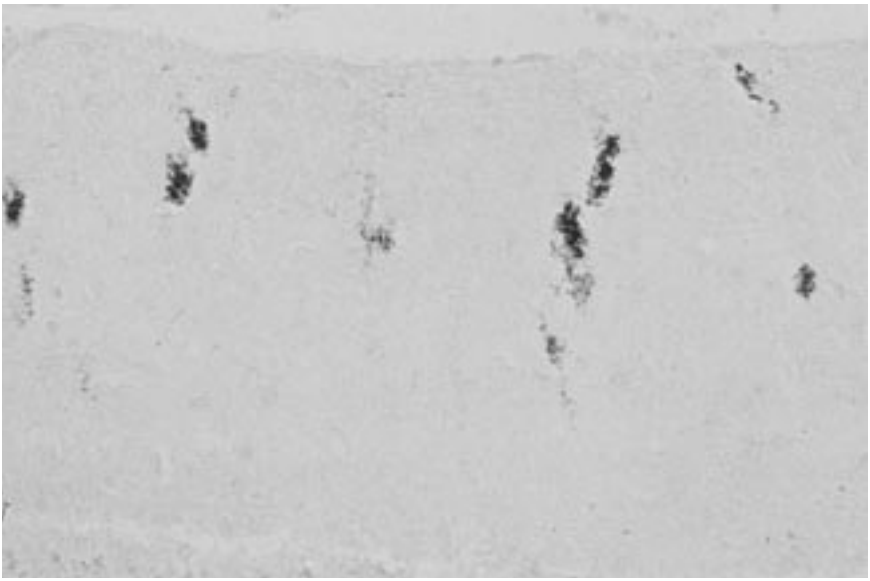


Figure 7 Typical PrP immunostaining of cerebellar cortex in insertion mutation. The molecular layer of the cerebellum shows granular linear aggregates of PrP immunoreactivity. (Immunostaining with the monoclonal antibody 3F4 to PrP.)

19 kD, as the PrP^{res} type 2. The ratio of the glycoforms is similar in the three insertion mutations and is characterized by the predominance of the intermediate glycoform, whereas the highly glycosylated and unglycosylated forms are less abundant and similarly represented as in the sporadic form of CJD (Parchi et al. 1996a,c).

The amount and physicochemical characteristics of PrP^{M} and PrP^{C} expressed by the mutant and normal alleles have been examined in brain tissues from subjects carrying five and six extra insertions (Chen et al. 1997). PrP^{M} and PrP^{C} were differentiated because of the different size resulting in different gel mobility. The amount of PrP^{M} was approximately 40% lower than PrP^{C} with either insertion, and approximately 90% of the PrP^{M} is insoluble in detergents, an indication that it is aggregated. In contrast, only 50% of the total PrP^{C} was detergent-insoluble. Since the detergent-insoluble fraction was resistant to PK digestion, it was concluded that in the insertional mutations with five and six extra repeats, both PrP^{M} and PrP^{C} are converted to PrP^{res}.

Transmissibility to Animals

Brain suspensions from three of four subjects with five, seven, and eight extra repeats transmitted the disease to primates after intracerebral inoculation (Brown et al. 1994b).

INHERITED PRION DISEASES WITH GERSTMANN-STRÄUSSLER-SCHEINKER DISEASE PHENOTYPE

GSS is a chronic hereditary autosomal dominant cerebellar syndrome accompanied by pyramidal signs and cognitive decline, which may evolve into severe dementia (Ghetti et al. 1995). Amyotrophy and parkinsonian signs may be present early or late in the course of the disease. The characteristic pathological phenotype is the presence of PrP-amyloid plaques in the cerebellar cortex in association with pyramidal tract degeneration. In addition, specific features such as spongiform changes, neurofibrillary tangles (NFT), Lewy bodies, and a combination of them may be differentiating the various GSS haplotypes (Ghetti et al. 1995, 1996a,b; Mirra et al. 1997). The *PRNP* mutations associated with GSS are shown in Table 1. In view of the consistent presence of PrP amyloid deposits in GSS and other hereditary prion disease variants, the term hereditary prion protein amyloidosis has also been introduced to include forms with extensive PrP amyloid deposition in disorders that have a phenotype different from

GSS. This nomenclature would allow us to include in this group the forms referred to as "inherited prion diseases with variable phenotypes" and the variant characterized clinically by severe dementia and pathologically by the presence of a prion protein cerebral amyloid angiopathy (CAA) and NFT. The latter is a rare entity, referred to as PrP-CAA (Ghetti et al. 1996c).

In GSS the symptomatology occurs in the third to seventh decades; the mean duration of illness is 5 years. The incidence of GSS is believed to be less than two per hundred million; however, it is probably underestimated, since GSS may present as a syndrome mimicking spinocerebellar degeneration, olivopontocerebellar atrophy, spastic paraparesis, Parkinsonism, and dementia (Ghetti et al. 1995).

Although the neuropathologic diagnosis of GSS is based on the presence of PrP-amyloid deposits, their distribution and extent differ widely between families. Amyloid is accompanied by glial proliferation and by loss of neuronal processes and perikarya, leading to variable degrees of atrophy of the affected regions. The clinical phenotypes are associated with mutations of *PRNP*, allelic polymorphism, PrP^{res} characteristics, and possibly with environmental and tissue-specific factors.

GSS with the P102L Mutation and the 129M Codon on the Mutant Allele (GSSP102L, 129M)

Epidemiology

This mutation is the most common in GSS (Young et al. 1995), and more than 30 families have been reported. The P102L mutation has been found in Japan (Doh-ura et al. 1989), Britain (Hsiao et al. 1989), Austria (Hainfellner et al. 1995), Germany (Goldgaber et al. 1989), Italy (Kretzschmar et al. 1992), Israel (Ashkenazi Jew) (Goldhammer et al. 1993), France (Laplanche et al. 1994), Mexico (E. Alonso et al., unpubl.), Canada (Young et al. 1995), and the US (Hsiao et al. 1989; Young et al. 1995). The P102L mutation was the first point mutation described in a prion disease, and genetic linkage of the mutation to the disease has been shown (Hsiao et al. 1989; Speer et al. 1991).

Genetics

A CCG to CTG mutation at codon 102 causes a leucine (L) for proline (P) substitution. The mutation has high penetrance; in fact, individuals carrying the mutant allele develop the disease between the fourth and the seventh decade. The P102L *PRNP* mutation is also shared by two recent-

ly described rare GSS phenotypes. The genotypic differences between the three diseases reside in the codon 129 and in the codon 219 located on the mutant allele. The former encodes methionine or valine, the latter encodes glutamic acid or lysine. The P102L mutation has most likely occurred more than once, since it has been seen in families of different ethnic groups.

Clinical Manifestations of GSS P102L 129M

The clinical phenotype is characterized by a progressive cerebellar syndrome with ataxia, dysarthria, and incoordination of saccadic movements. Pyramidal and pseudobulbar signs are also seen. Mental and behavioral deterioration leading to dementia or akinetic mutism occur in the advanced stages of disease. The age at onset of clinical signs occurs in the fourth to sixth decades of life, and the duration of the disease ranges from a few months to six years. Considerable intrafamilial phenotypic variability may be observed (Adam et al. 1982; Ghetti et al. 1995; Hainfellner et al. 1995; Young et al. 1995). Myoclonus and PSW in the EEG, a finding of diagnostic relevance in CJD, occurs in some of the GSS P102L patients. In some instances, the disease presents a rapid course of 5–9 months with a clinical picture indistinguishable from that of CJD (Barbanti et al. 1994).

Histopathology

Neuropathologically, deposits of fibrillar and nonfibrillar PrP in the cerebral and cerebellar parenchyma against a background of variable spongiform changes are consistently found (Figs. 8–11) (Adam et al. 1982; Hainfellner et al. 1995; Masters et al. 1981a; Piccardo et al. 1995). Spongiform changes, neuronal loss, and astrocytosis vary in severity even among patients of the same kindred and are most severe when the course of the illness is rapid (Adam et al. 1982; Barbanti et al. 1994; Hainfellner et al. 1995).

Characteristics and Allelic Origin of PrP^{res}

Recent studies have demonstrated the presence of two major PrP^{res} fragments in brain of GSS P102L 129M (Parchi et al. 1998b; Piccardo et al. 1998b). One has a M_r of approximately 21 kD and the other of approximately 8 kD; both are also present in intact tissue. The 21-kD fragment is similar to the PrP^{res} type 1 described in sporadic CJD (Parchi et al. 1996a). However, the ratio of the three major glycoforms of PrP^{res} is significantly different and shows, in the GSS P102P 129M, a pattern in which the diglycosylated form is the most, and the unglycosylated form

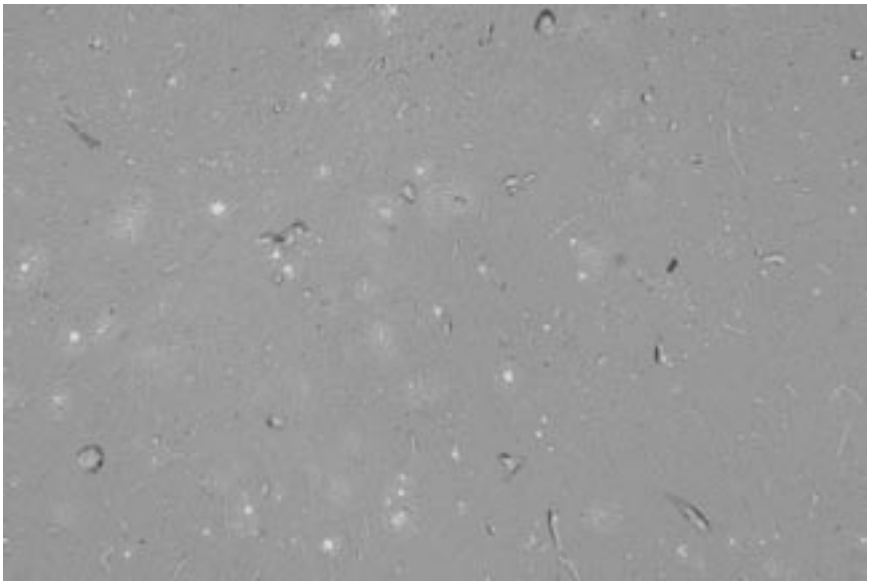


Figure 8 Cerebral cortex of a patient with GSS P102L showing multiple amyloid deposits. Note that some deposits show strong fluorescence while others are less bright and not well circumscribed. Also note that some amyloid deposits are multicentric with a larger core at the center and multiple brightly fluorescent or diffuse deposits at the periphery. Thioflavin S method.

the least, represented form. The 8-kD fragment is similar to those described in other variants of GSS (Piccardo et al. 1998b). Sequencing and mass spectrometry have shown that the amino terminus of the 21-kD fragment begins at residues 78 and 82, whereas the amino terminus of the 8-kD fragment begins at residues 78, 80, and 82 and the carboxyl terminus ends at a position spanning residues 147–153 (Parchi et al. 1998a). Therefore, the 21-kD and 8-kD fragments differ only in the carboxyl terminus, since the former has an intact carboxyl terminus of PrP^{res}, and the latter is truncated at both amino- and carboxy-terminal ends. Both fragments derive exclusively from the mutant allele as demonstrated by the consistent presence of the mutated L residue at position 102. It was also shown that the presence of the 21-kD form correlates with the presence of spongiform degeneration and "synaptic" pattern of PrP deposition, whereas the 8-kD fragment is found in brain regions showing PrP-positive multicentric amyloid deposits (Parchi et al. 1998a; Piccardo et al. 1998b). These data further indicate that the neuropathology of prion diseases largely depends on the type of PrP^{res} fragment that forms in vivo.

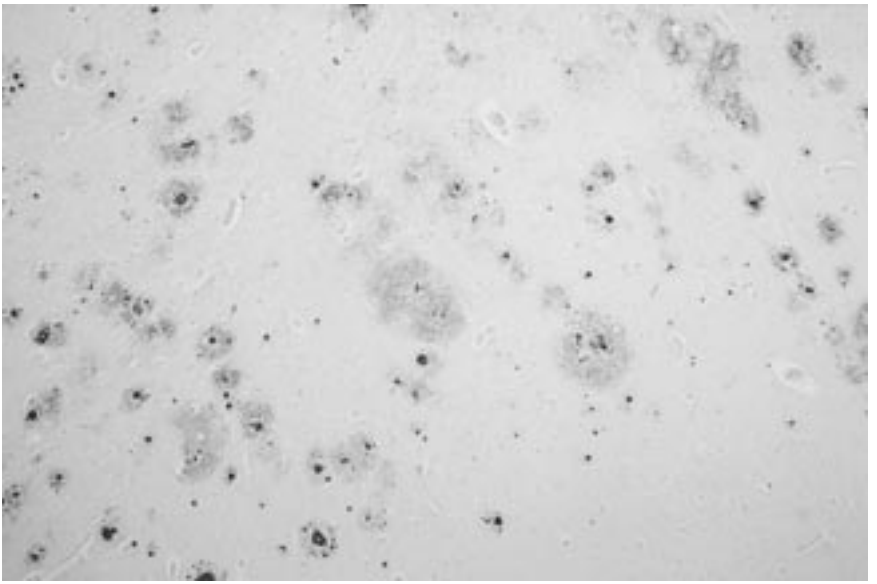


Figure 9 Cerebral cortex of a patient with GSS P102L 129M showing multiple immunopositive deposits. Note that some deposits show strong immunolabeling, whereas others show diffuse immunolabeling. Also note that some of the strongly immunolabeled deposits are multicentric with a larger core at the center and multiple diffuse deposits at the periphery. Immunohistochemistry using anti-PrP serum raised against a synthetic peptide homologous to residue 90-102 of the amino acid sequence deduced from human PrP.

Because the formation of PrP^{res} fragments of 8 kD with ragged amino and carboxyl termini appear to be shared by most GSS subtypes, it may represent a molecular marker for this disorder (Piccardo et al. 1996, 1998b; Parchi et al. 1998).

Transmissibility

Masters showed that the inoculation into nonhuman primates of brain homogenates obtained from GSS patients induced a spongiform encephalopathy in the recipient animals (Masters et al. 1981a). Some of the patients from whom transmission was obtained had the P102L mutation. One of the patients was the member of the W family reported by Rosenthal et al. (1976). Subsequently, intracerebral inoculation into marmosets from another patient of the W family induced a spongiform encephalopathy indistinguishable from that seen in marmosets inoculated with brain tissue from a case of CJD (Baker et al. 1990). Transmission experiments from P102L GSS patients to mice resulted also in the devel-

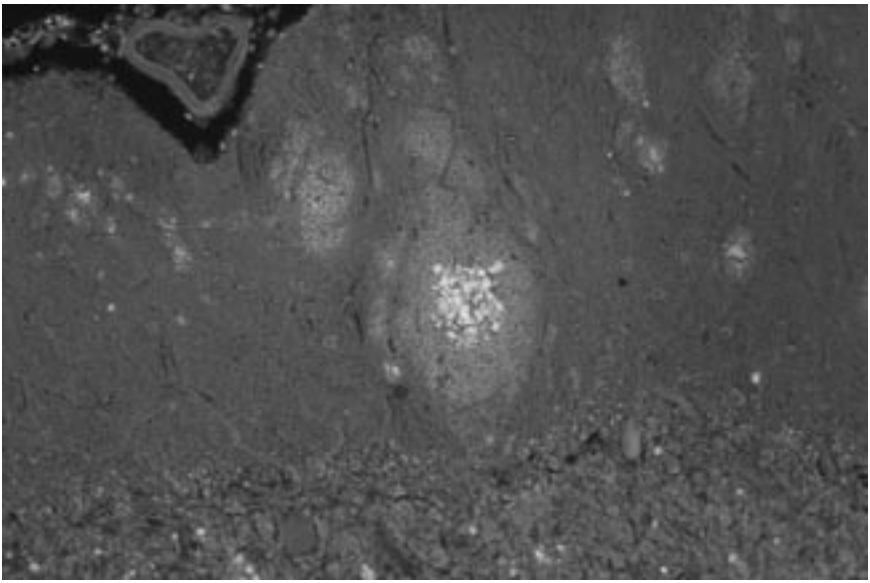


Figure 10 Cerebellar cortex of a patient with GSS P102L 129M showing multiple amyloid deposits. Note that some deposits show strong fluorescence, whereas others are less bright and not well circumscribed. Also note that the amyloid deposits are multicentric in the molecular layer. Deposits in the granule cell layer are small and unicentric. Thioflavin S method.

opment of spongiform degeneration (Manuelidis et al. 1987; Tateishi et al. 1996). It is significant that the donor tissue is characterized by the presence of PrP amyloid and severe spongiform changes, whereas the recipient primates and rodents develop a rapidly progressing disease with severe spongiform degeneration but not PrP amyloid deposition. Transmission is not consistently obtained from P102L patients.

GSS with the P102L Mutation and the 129M and the 219K Codons on the Mutant Allele (GSSP102L, 129M, 219K)

Epidemiology

Only one family has been reported.

Genetics

The P102L mutation was detected in coupling with methionine at residue 129 and lysine at residue 219.

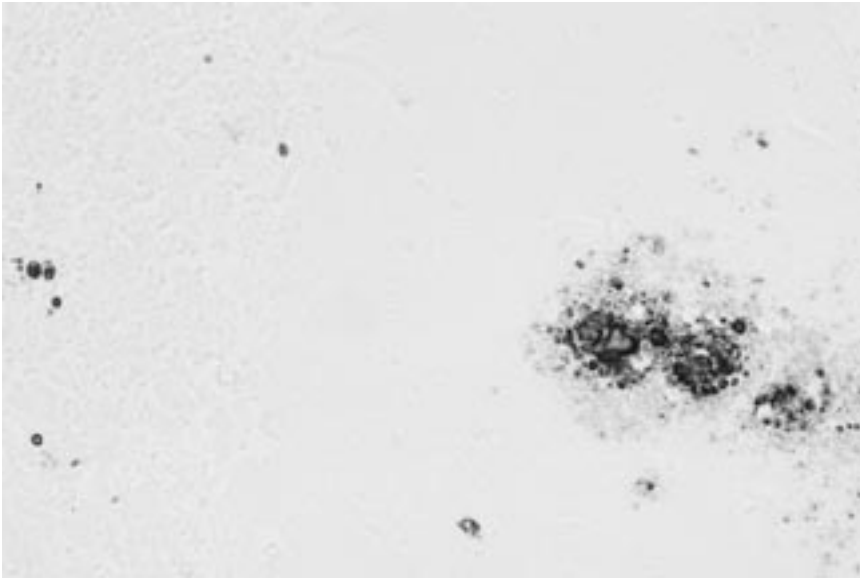


Figure 11 Cerebellar cortex of a patient with GSS P102L 129M showing multiple immunopositive deposits in the molecular and granule cell layer. The deposits in the molecular layer are significantly larger than those in the granule cell layer. Immunohistochemistry using anti-PrP serum raised against a synthetic peptide homologous to residue 90-102 of human PrP.

Clinical Manifestations

Symptomatic subjects of this family had either dementia or cerebellar signs.

Histopathology

Neuropathologic studies demonstrated mild PrP deposition in the cerebral and cerebellar cortex and basal ganglia with no amyloid or spongiform changes (Furukawa et al. 1995).

Characteristics and Allele Origin of PrP^{res}

No biochemical data are available.

Transmissibility

No data are available.

GSS with the P102L Mutation and the 129V Codon on the Mutant Allele (GSSP102L, 129V)

Epidemiology

There are two known cases of P102L GSS disease with homozygosity for valine at codon 129, most likely from different families (Telling et al. 1995; Young et al. 1997).

Genetics

One patient, homozygous for valine (GTG) at codon 129, had the P102L mutation, CCG to CTG, on one allele. This patient, therefore, had the *PRNP* P102L mutation in coupling with valine at residue 129. This coupling probably arose by a cytosine-to-thymine mutation occurring at codon 102 on a 129 valine allele, since cytosine to thymine is the most frequent point mutation. However, we cannot rule out the possibility that the methionine to valine mutation (adenine to guanine) occurred on a P102L allele.

Clinical Manifestations

The clinical course in the patient with the P102L-V129 mutation was significantly different from that observed in GSS P102L-M129 in that the presentation was with seizures, the patient had long tract signs, dementia was not part of the clinical phenotype, and the duration of 12 years was unusually long. Typically, in GSS P102L-M129 the clinical onset is with cerebellar signs, seizures are generally not observed, dementia is frequently seen, and the duration is of approximately 5 years.

Histopathology

The neuropathologic findings in this patient differ from those frequently seen in P102L-M129 in view of the absence of spongiform changes. The involvement of the corticospinal, spinocerebellar, and gracile tracts, as well as the presence of PrP deposits in the substantia gelatinosa, may be correlated with the severe postural and sensory abnormalities observed in this patient (Young et al. 1997).

Characteristics and Allelic Origin of PrP^{res}

No biochemical data are available.

Transmissibility

No data are available.

GSS with the P105L Mutation and the 129V Codon on the Mutant Allele (GSSP105L, 129V)

Epidemiology

At least four families have been reported in Japan. Recently, another patient has been reported, but it is not clear whether this patient belongs to a previously reported family (Yamazaki et al. 1997).

Genetics

A proline (CCA) to leucine (CTA) substitution at codon 105 on a Val-129 allele has been found in patients with hereditary spastic paraparesis from four Japanese families (Nakazato et al. 1991; Amano et al. 1992; Terao et al. 1992; Kitamoto et al. 1993b,c; Yamada et al. 1993). However, it is not known whether the mutation has occurred more than once.

Clinical Manifestations

Clinically, spastic gait, hyperreflexia, and Babinski sign dominate the picture in the initial stages (Kitamoto et al. 1993c; Yamada et al. 1993). Extrapyramidal signs such as fine finger tremor and rigidity of limbs may be seen. Paraparesis progresses to tetraparesis and is accompanied by emotional incontinence and dementia. Myoclonus, PSW in EEG, or severe cerebellar signs have not been reported. The onset of the clinical signs is in the fourth and fifth decades of life, the duration of the disease ranges from 6 to 12 years.

Histopathology

Neuropathologically, PrP deposits in the neocortex, especially the motor area, striatum, and thalamus, are found. Multicentric PrP-amyloid plaques and diffuse deposits are present in superficial and deep layers of the neocortex, respectively, in association with neuronal loss and astrocytosis. NFT are occasionally seen, but not spongiform changes. In the case recently reported, a 57-year-old woman with dementia, gait disturbance, but not spastic paraparesis, numerous NFT were seen in the cerebral cortex and several subcortical nuclei (Yamazaki et al. 1997). In most cases, amyloid plaques are rare in the cerebellum, and axonal losses occur in the pyramidal tracts (Nakazato et al. 1991; Amano et al. 1992; Terao et al. 1992; Kitamoto et al. 1993c; Yamada et al. 1993).

Characteristics and Allelic Origin of PrP^{res}

No biochemical data are available.

Transmissibility

No data are available.

GSS with the A117V Mutation and the 129V Codon on the Mutant Allele (GSSA117V, 129V)

Epidemiology

The mutation has been described in five families: a French (Alsatian) family and a British family, as well as two US kindreds, one being of German origin (Doh-ura et al. 1989; Hsiao et al. 1991a; Mastrianni et al. 1995). Recently, another US family has been identified (Ghetti et al. 1998).

Genetics

The alanine (A) to valine (V) substitution at residue 117 results from a cytosine-to-thymidine mutation in the second position of the codon. The mutant codon also contains the noncoding, or "silent," adenine-to-guanine mutation in the third position, so that the codon change from wild-type is GCA to GTG. These families may share a common founder, since the GCG codon at 117 is a rare polymorphism. However, the GCG variant contains a CpG dinucleotide, so this mutation could also have arisen more than once.

Clinical Manifestations

The clinical phenotypes are presenile dementia in the Alsatian family (Warter et al. 1981; Tranchant et al. 1991, 1992; Mohr et al. 1994); presenile dementia, pyramidal signs, and Parkinsonism in the US-German kindred (Nochlin et al. 1989; Hsiao et al. 1992); and severe ataxia, dysarthria, mild Parkinsonism, and dementia in a patient from the family reported by Mastrianni et al. (1995). In the Alsatian and in the US-German families, the age at the onset of the clinical signs is 19–64 and 23–58 years, respectively (Nochlin et al. 1989; Tranchant et al. 1992), and the duration of the disease ranges from 1 to 11 and 2 to 6 years, respectively. The clinical phenotype in the Alsatian family is variable (Mohr et al. 1994); although the three patients studied in the first and second generations of the published pedigree exhibited dementia as the main clinical symptom, affected subjects from subsequent generations showed the association of dementia, pyramidal and pseudobulbar signs with ataxia, extrapyramidal symptoms, amyotrophy, myoclonus, and tonic-clonic seizures. EEG did not show PSW.

Histopathology

Neuropathologically, there are PrP-amyloid deposits and PrP deposits without the tinctorial properties of amyloid that are widespread throughout the cerebrum, but rare or absent in the cerebellum of subjects with dementia alone (Warter et al. 1981; Nochlin et al. 1989; Tranchant et al. 1991; Mohr et al. 1994; Mastrianni et al. 1995). Numerous PrP-amyloid deposits in the cerebral cortex, basal ganglia, and thalamus as well as in the cerebellum were found in three patients from the Alsatian family (Mohr et al. 1994), who had died at 24, 39, and 73 years. In the latter, who survived 9 years after the onset of the clinical signs, NFT were numerous in the cerebral cortex, in contrast with the rare deposits of Aβ. Spongiform changes are variable in severity and extent. In patients of the US-German family, PrP-amyloid deposits are prominent in the cerebral cortex and striatum, but not in the cerebellum; NFT are occasionally found. In the family reported by Mastrianni et al. (1995), there is a conspicuous deposition of PrP amyloid in the cerebellum.

Characteristics of PrP^{res} and Allelic Origin of PrP Amyloid

Recently, it has been shown that distinctive PrP isoforms are seen in GSS A117V patients with and without NFT (Lievens et al. 1998; Piccardo et al. 1998b). In all cases, low-molecular-weight PrP peptides of about 7–8 kD were detected using antibody 3F4 directed to the mid region of PrP. The smallest amyloid subunit corresponds to peptides spanning residues 58–150 and 81–150. These peptides originated from the mutant allele (Tagliavini et al. 1995).

Transmissibility

Transmission from one case was tested in mice and was negative (Tateishi et al. 1996).

GSS with the F198S Mutation and the 129V Codon on the Mutant Allele (GSSF198S, 129V)

Epidemiology

Two families have been reported in the US (Farlow et al. 1989; Ghetti et al. 1989, 1995; Hsiao et al. 1992; Mirra et al. 1997).

Genetics

A phenylalanine (TTC) to serine (TCC) substitution at codon 198 on a Val-129 allele has been described in patients from two US families of Caucasian

ethnic origin (Dlouhy et al. 1992; Ghetti et al. 1992, 1995; Hsiao et al. 1992; Mirro et al. 1997).

Clinical Manifestations

The clinical phenotype is characterized by cognitive, cerebellar, and pyramidal signs. The main symptoms are gradual loss of short-term memory and progressive clumsiness in walking, bradykinesia, rigidity, dysarthria, and dementia. Signs of cognitive impairment and eye-movement abnormalities may be detected by specific tests before clinical onset of symptoms. Psychotic depression has been seen in several patients; tremor is mild or absent. Symptoms may progress slowly over 5 years or rapidly over as little as 1 year. The age at onset of clinical signs is 40–71 years; patients homozygous for valine at codon 129 have clinical signs more than 10 years earlier, on average, than heterozygous patients (Dlouhy et al. 1992). The duration of the disease ranges from 2 to 12 years (Farlow et al. 1989; Ghetti et al. 1992, 1995).

Histopathology

The neuropathologic phenotype is characterized by presence of severe PrP deposition and amyloid formation in the cerebral and cerebellar parenchyma as well as neurofibrillary lesions in the cerebral gray matter (Ghetti et al. 1989, 1992, 1995; Giaccone et al. 1990, 1992; Tagliavini et al. 1993). In the twelve patients and the 1 nonsymptomatic carrier studied, unicentric and multicentric PrP-amyloid deposits are distributed throughout the gray structures of the cerebrum, cerebellum, and midbrain. Amyloid deposition is severe in frontal, insular, temporal, and parietal cortex; the highest concentration of deposits is in layers one, four, five, and six. A moderate involvement is seen in the hippocampus, where plaques occur predominantly within the stratum lacunosum-moleculare of the CA1 sector and subiculum. PrP deposits are numerous in the claustrum; the caudate nucleus; putamen; the anterior, dorsomedial, ventrolateral, and lateral dorsal nuclei of the thalamus; the cerebellar molecular layer; the mesencephalic tegmentum; the substantia nigra; and the periaqueductal gray matter; however, the degree of amyloid formation in these areas varies. Amyloid deposits are surrounded by astrocytes, astrocytic processes, and microglial cells. In the neocortex, many amyloid cores are associated with abnormal neurites, so that when these lesions are analyzed with classic stains, they are morphologically similar to neuritic plaques of Alzheimer's disease (AD) (Ghetti et al. 1989, 1992). The neurites immunoreact with antibodies to tau (τ), ubiquitin, and amino-

and carboxy-terminal domains of the β-amyloid precursor protein (βAPP). The accumulation of βAPP in nerve cell processes is not associated with extracellular deposition of Aβ, except in older patients, where Aβ immunoreactivity also may be observed around PrP-amyloid deposits (Bugiani et al. 1993; Ghetti et al. 1995).

NFT and neuropil threads are found in large number in the neocortex and in the subcortical gray matter. Cortical regions particularly affected are the frontal, cingulate, parietal, insular, and parahippocampal cortex. In the remaining cortical regions, NFT are present but less numerous. Of the subcortical gray areas, the caudate nucleus, putamen, nucleus basalis, midbrain and pontine nuclei, substantia nigra, griseum centrale, and locus coeruleus show a variable degree of involvement.

Moderate to severe cerebral and cerebellar atrophy, nerve cell loss, and gliosis are found in the neocortex, striatum, red nucleus, substantia nigra, cerebellum, locus coeruleus, and inferior olivary nucleus, and iron deposition is found in the globus pallidus, striatum, red nucleus, and substantia nigra. Spongiform changes are inconspicuous.

A neurologically asymptomatic subject, who had the F198S mutation and was heterozygous at codon 129, died at the age of 42 of an unrelated cause. In this subject, PrP-amyloid deposits were already numerous in the cerebellar cortex but rare in the cerebral cortex.

Characteristics of PrP^{res}

PK digestion of the brain extracts generated three prominent broad bands of about 27–29, 18–19, and 8 kD, and a weaker but sharp band at 33 kD, as detected with antibody 3F4 (Piccardo et al. 1996). The latter band (33 kD) may be attributed to PrP and/or to cross-reactivity of antibody 3F4 with residual PK. The stoichiometry among the PrP species differed from that of the undigested peptides for a notable increase in the signal of the low-molecular-weight band. No PrP signal was observed in PK-treated and non-treated brain extracts when antibody 3F4 was absorbed against a PrP peptide spanning residues 102–114.

N-Deglycosylation of non PK-treated extracts with PNGase F resulted in disappearance of the 33–35-kD band accompanied by an increased signal of the 28–30-kD band (Piccardo et al. 1996). The 28–30-kD band, seen with antibody 3F4, is consistent with the molecular weight of deglycosylated full-length PrP, as shown by a similar species detected with antibodies raised against synthetic peptides homologous to residues 23–40 and 220–231 of human PrP (PrP23-40 and PrP220-231) (Piccardo et al. 1996). In non PK-treated brain extracts, the electrophoretic mobility of the 19–20 and 9-kD bands was not modified by deglycosylation.

The combination of PK and enzymatic deglycosylation with PNGase F treatment generated a pattern similar to that of PK treatment alone with prominent fragments at about 27–29, 18–19, and 8 kD. These PrP fragments were immunoreactive with antibody 3F4 and with antisera AS 6800 (raised against a synthetic peptide homologous to residues 89–104 of human PrP), but not with PrP23-40 and PrP220-231.

To investigate the sensitivity of PrP to PK, samples from the cerebellum were exposed to PK under nondenaturing and denaturing conditions. Under nondenaturing conditions, the major PrP isoforms present in GSS-IK retained partial PK resistance, the intensity of the signal after 4 hours being similar to that observed after 1 hour of enzyme treatment. Conversely, denatured PrP was completely degraded after a 30-minute digestion with PK at 37°C in the presence of sodium dodecyl sulfate (Piccardo et al. 1996).

PK-resistant PrP fragments of similar electrophoretic mobility were seen in all brain regions examined (frontal cortex, caudate nucleus, and cerebellum) of two IK patients analyzed. In semi-quantitative experiments (similar amounts of total protein loaded), comparable signals were observed in samples from the cerebellum and caudate nucleus, two areas that have a high and a low amount of amyloid, respectively (Piccardo et al. 1996).

In immunoblot studies, the strongest signal was obtained from tissue corresponding to a patient who had the longest clinical course of the disease (12 years). To follow up on this observation, we also studied PK-resistant PrP obtained from the cerebella of five additional patients of the IK, who had a duration of clinical signs varying from 2 to 7 years. Similar electrophoretic patterns were observed, and high amounts of PrP were present in all cases, regardless of the duration of disease. Nevertheless, in repeated experiments, the patient with 12 years' duration of clinical signs always showed the most intense signal.

PrP was localized in the microsomal fraction. Sarkosyl-soluble and PK-sensitive PrP isoforms from this fraction were seen as prominent bands of approximately 33–35 kD in both control (familial AD) and GSS-IK (Piccardo et al. 1996). In addition, Sarkosyl-insoluble PrP was present as four major PrP species of approximately 33–35, 28–30, 19–20, and 9 kD (Piccardo et al. 1996). PK digestion of these samples generated three prominent bands of 27–29, 18–19, and 8 kD in GSS-IK, comparable to the PK-resistant species present in brain homogenates (Piccardo et al. 1996).

Characteristics and Allelic Origin of PrP Amyloid

The biochemical composition of PrP amyloid was first determined in brain tissue samples obtained from patients of the Indiana kindred

(Tagliavini et al. 1991, 1994) carrying the F198S mutation in coupling with 129V (Dlouhy et al. 1992; Hsiao et al. 1992). Amyloid cores were isolated by a procedure combining buffer extraction, sieving, collagenase digestion, and sucrose gradient centrifugation. Proteins were extracted from amyloid fibrils with formic acid, purified by gel filtration chromatography and reverse-phase high performance liquid chromatography (HPLC), analyzed by SDS-PAGE and immunoblot, and sequenced. The amyloid preparations contained two major peptides of 11 kD and 7 kD spanning residues 58–150 and 81–150 of PrP, respectively. The amyloid peptides had ragged amino and carboxyl termini.

The finding that the amyloid protein was an amino- and carboxy-terminal truncated fragment of PrP was verified by immunostaining brain sections with antisera raised against synthetic peptides homologous to residues 23–40, 90–102, 127–147, and 220–231 of human PrP. The amyloid cores were strongly immunoreactive with antibodies that recognized epitopes located in the mid-region of the molecule, whereas only the periphery of the cores was immunostained by antibodies to amino- or carboxy-terminal domains. In addition, antisera to the amino and carboxy termini of PrP labeled large areas of the neuropil that did not possess the tinctorial and optical properties of amyloid. Immunogold electron microscopy showed that antibodies to the mid-region of PrP decorated fibrils of amyloid cores, whereas antisera to amino- and carboxy-terminal epitopes labeled amorphous material at the periphery of the cores or dispersed in the neuropil. These data suggest that amyloid deposition in GSS is accompanied by accumulation of PrP peptides without amyloid characteristics (Giaccone et al. 1992).

In GSS-IK, the amyloid protein does not include the region containing the amino acid substitution. To establish whether amyloid peptides originate from mutant protein alone or from both mutant and wild-type PrP, we analyzed patients heterozygous M/V at codon 129 and used 129V as a marker for protein from the mutant allele. Amino acid sequencing and electrospray mass spectrometry of peptides generated by digestion of the amyloid protein with endoproteinase Lys-C showed that the samples contained only peptides with 129V, suggesting that only mutant PrP was involved in amyloid formation (Tagliavini et al. 1994).

Paired Helical Filament Characteristics

Paired helical filament-enriched fractions obtained from the neocortex of IK patients contained SDS-soluble τ isoforms with electrophoretic mobility and an immunochemical profile corresponding to the isoforms extracted from the brain of patients with AD. These proteins migrate between 60 and

68 kD, immunoreact with antibodies to the amino and carboxyl termini of τ, and require dephosphorylation to be accessible to the antibody Tau-1. Thus, the immunocytochemical findings are consistent with those of the Western blot analysis showing that significant similarity exists between GSS-IK and AD as to the Alz50, T46, and Tau-1 immunostaining of NFT (Tagliavini et al. 1993).

Transmissibility

In the case of the Indiana kindred, brain tissue and buffy coat from one affected individual were inoculated into hamsters in two experiments in the laboratory of Drs. E. and L. Manuelidis. No pathologic changes were observed in the primary transmission attempt, nor in the second and third serial passage (Dr. L. Manuelidis, pers. comm.). Tissue homogenates from another F198S patient have been inoculated into hamsters and mice and no transmission has occurred (Ghetti et al. 1992; Hsiao et al. 1992). Amyloid-enriched fractions and tissue homogenates from IK patients have been inoculated into marmosets and no transmission has occurred 30 months after inoculation (H.F. Baker, pers. comm.). Studies are in progress in Dr. Collinge's laboratory to determine whether transgenic mice containing a normal human *PRNP* gene develop a prion disease following the inoculation of tissue homogenates from IK patients.

GSS with the Q212P Mutation and the 129M Codon on the Mutant Allele (GSSQ212P, 129M)

Epidemiology

Only one family with this mutation is known at this time (Young et al. 1998).

Genetics

A glutamine (CAG) to proline (CCG) mutation has been found in one GSS patient who had no family history of neurologic disease. The mutation was not found in 100 control subjects (Young et al. 1998).

Clinical Manifestations

Onset occurred at age 60 with gradual development of incoordination and slurring of speech. Three years after onset, the patient was found to have normal mental status, dysarthria, and ataxia. His condition began to progress more rapidly and approximately 6 years after onset the patient entered a nursing home where he remained until his death, 8 years after onset. At time of death, the patient was still mentally competent.

Histopathology

Amyloid deposition was mild throughout the central nervous system. Among GSS variants, this appears to be the form with the least amount of amyloid deposits. The cerebellum was significantly less affected than in all other variants. Immunopositive PrP deposits were present in the cerebellum where they were more numerous than in the neocortex and striatum. There was axonal degeneration in the anterior and lateral corticospinal tracts throughout the spinal cord.

Characteristics and Allelic Origin of PrP^{res}

Proteinase K digestion of brain homogenates resulted in a poorly defined smear in the 25–35-kD region and two major bands of 18–19 and 10 kD (Piccardo et al. 1998a).

Transmissibility

No data are available.

GSS with the Q217R Mutation and the 129V Codon on the Mutant Allele (GSSQ217R, 129V)

Epidemiology

One family of Swedish origin has been reported (Ghetti et al. 1995).

Genetics

A glutamine (CAG) to arginine (CGG) substitution (Hsiao et al. 1992) on a Val-129 allele has been described in two patients from an American family of Swedish origin.

Clinical Manifestations

The clinical phenotype is characterized by gradual memory loss, progressive gait disturbances, Parkinsonism, and dementia. The age at onset of clinical signs is 62–66 years. The duration of the disease is 5–6 years (Ghetti et al. 1994). The neurologic signs may be preceded by episodes of mania or depression that respond to antidepressant medications, lithium, and neuroleptics.

Histopathology

Neuropathologically there are PrP-amyloid deposits in the cerebrum and cerebellum and abundant NFT in the cerebral cortex and several subcor-

tical gray structures. The neocortex, amygdala, substantia innominata, and thalamus are severely involved.

Characteristics and Allelic Origin of PrP^{res}

PrP^{res} is essentially similar to that observed in F198S (Piccardo et al. 1998b).

Transmissibility

Tissue homogenates from one A217G patient have been inoculated into hamsters and mice and no transmission has occurred (Hsiao et al. 1992).

Cerebral Amyloid Angiopathy (PrP-CAA) with the Y145Stop Mutation and the 129M Codon on the Mutant Allele (PrP-CAAY145Stop, 129M)

Epidemiology

Only one case has been reported in the literature.

Genetics

A tyrosine (TAT) to stop codon (TAG) substitution on a 129M allele (Ghetti et al. 1996c) has been found in a Japanese patient with a clinical diagnosis of Alzheimer's disease (Kitamoto et al. 1993a).

Clinical Manifestations

The clinical phenotype is characterized by memory disturbance, disorientation, and a progressive, severe dementia. The EEG did not show PSW. The age at the onset of the clinical signs was 38 years, and the duration of the disease was 21 years.

Histopathology

Neuropathologically, diffuse atrophy of the cerebrum and dilation of the lateral ventricles were present. Neuronal loss and gliosis were severe, but no spongiform changes were observed. There were PrP-amyloid deposits in the walls of small and medium-sized parenchymal and leptomeningeal blood vessels and in the perivascular neuropil as well as neurofibrillary lesions in the cerebral gray matter. The NFT are composed of paired helical filaments with a periodicity of 70–80 nm and were decorated with monoclonal antibodies recognizing abnormal phosphorylation.

Characteristics and Allelic Origin of PrP^{res}

PrP^{res} was not detected.

Transmissibility

No data are available.

CONCLUSIONS

Inherited human prion diseases have contributed a wealth of information applicable to the understanding of all forms of prion diseases because the presence of the pathogenic mutation offers a firm etiological starting point and lends itself remarkably well to disease modeling. Since many inherited prion diseases have a high degree of penetrance, the mutation must provide conditions that are sufficient to develop and express the disease phenotype with the apparent contribution of age-related events. Therefore, a detailed and accurate knowledge of the metabolism and physicochemical properties of the PrP^{M} should provide a key to the understanding of the conditions that inevitably lead to the conversion of PrP^{M} into PrP^{res}, or to other forms of pathogenic PrP^{M}, and hence, the onset of the clinical disease. All the evidence available to date indicates that the events underlying the pathogenesis of the sporadic form of CJD, by far the most common form, are similar to those of the inherited forms, although the starting point would be stochastic rather than by the presence of a germ-line mutation.

The direct studies of the human diseases have provided important, basic information. They have demonstrated, at least in the diseases which have been studied, that the mutation is linked to the disease phenotype. This finding indicates that the mutation in *PRNP* is the cause of the disease and that *PRNP* is, therefore, the causative, rather than the predisposing gene. The presence of PrP^{res} and/or PrP amyloid has been demonstrated in all cases examined to date. The physicochemical characteristics of PrP^{res} and PrP amyloid associated with the inherited form are similar to those of the PrP^{res} and PrP amyloid present in sCJD and in the CJD acquired by infection. These data argue that the pathogenic mechanisms are similar in the three forms of prion diseases. The study of the genotype/phenotype correlations has revealed a wide spectrum of phenotypes in the inherited prion diseases and has provided persuasive and novel

examples of the mechanisms by which the mutant *PRNP* can modulate the disease phenotype.

The cell models of the inherited prion diseases have revealed that *PRNP* mutations introduce common and specific changes in the metabolism of PrP^{M}. All mutations examined to date destabilize PrP^{M}, which results in aggregation and abnormal processing. On the other hand, the cell models have highlighted the large spectrum of specific changes introduced in the PrP^{M} metabolism by the individual mutations. For example, the glycan of the 197 N-glycosylation site is altered in the E200K PrP^{M} but not in the D178N PrP^{M}; the Y145Stop PrP^{M} but not the Q217R PrP^{M} is degraded in part through the proteosomal pathway although the pathological phenotype is characterized by accumulation of PrP amyloid in both of these diseases. Clearly, diverse mechanisms are involved in the pathogenesis of the inherited prion diseases.

Several animal models of inherited prion diseases have been developed, but to date they have been largely used to characterize the phenotype and the transmissibility of the disease expressed, and studies aimed at elucidating the pathogenesis are still few. Therefore, although the effects of the various mutations on the metabolism of the respective PrP^{M} are becoming increasingly clear, several important issues remain to be clarified. The first issue concerns the actual spectrum of the inherited prion diseases. Are all inherited prion diseases based on the conversion of PrP^{M} into PrP^{res}, or are some of these diseases based on other mechanisms, such as mistargeting of PrP^{M}, within the cell, as a recent study indicates (Hegde et al 1998)? Are all inherited prion diseases transmissible to the appropriate recipient or are some non-transmittable like the other neurodegenerative diseases? Since inherited prion diseases become symptomatic in the adult or advanced age despite the congenital presence of the mutation, another issue concerns the events that trigger the clinical onset of the disease after many symptom-free years and the role of aging in these events. Finally, and most importantly, we must learn how to prevent the clinical onset of the inherited prion diseases and extend the symptom-free interval indefinitely.

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Neuropathology of Prion Diseases

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The discovery of mechanisms of nerve cell dysfunction, degeneration, and death in prion diseases acquired by infection, in dominantly inherited forms, and in sporadic (idiopathic) forms has gone hand in hand with the discovery of the prion and how prions are propagated. The generation of PrP-specific antibodies led to our first immunohistochemical study, in which we discovered that the amyloid plaques in experimental scrapie in Syrian hamsters contain protease-resistant PrP (Fig. 1A) (Bendheim et al. 1984; DeArmond et al. 1985). That finding convinced us that we had a unique opportunity to obtain a better understanding of the pathogenesis of scrapie in animals and the related human disorders that include sporadic, iatrogenic, and familial Creutzfeldt-Jakob disease (CJD), and the rare familial disorder Gerstmann-Sträussler-Scheinker syndrome (GSS). The overall objective was to test the hypothesis that PrP^{Sc} accumulation in the brain causes the clinically relevant neuronal dysfunction, vacuolation, and death that are the characteristics of prion diseases. The results of many studies have led to the unifying hypothesis that neuronal degeneration in spontaneous, infectious, and genetic prion diseases and the propagation of prions in those diseases are both related exclusively to abnormalities of PrP.

Multiple investigators in Great Britain, Europe, Japan, and the US have contributed to our understanding of the mechanisms of CNS degeneration peculiar to prion diseases. We have attempted to identify and acknowledge their many contributions; however, the number of prion disease investigators is increasing logarithmically and, therefore, inevitably we will have missed some and apologize to them for that oversight.

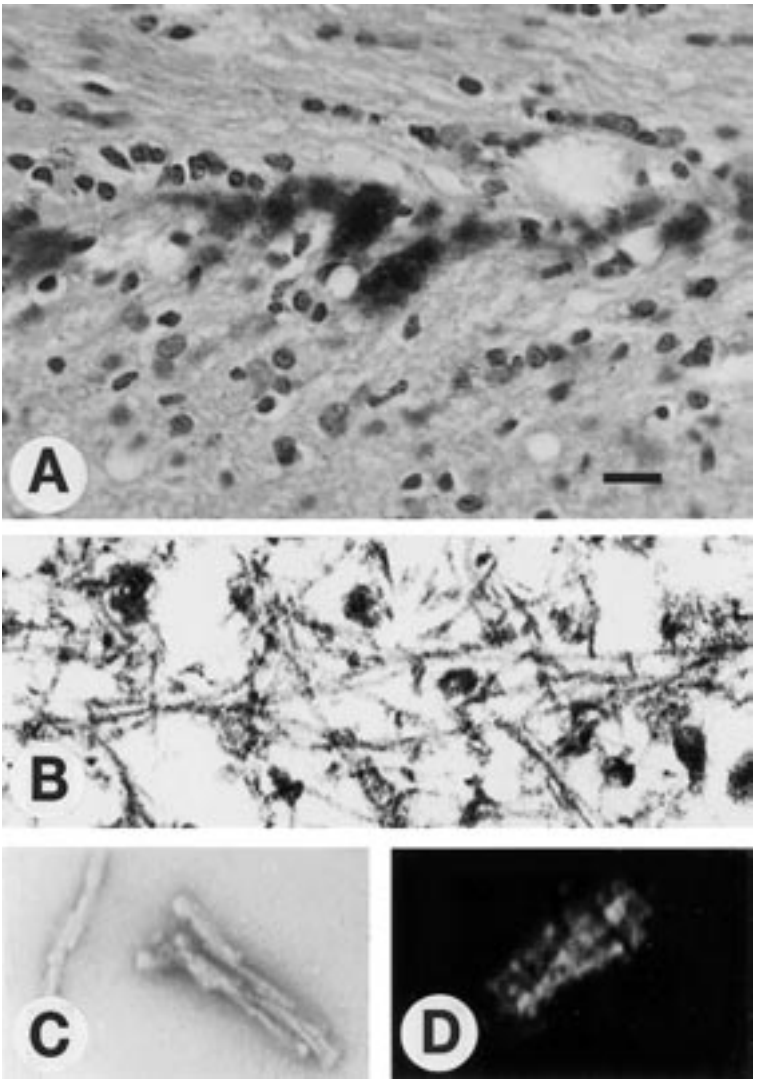


Figure 1 Comparison of PrP amyloid plaques depositied in situ with prion rods formed in vitro from Syrian hamsters inoculated with the Sc237 scrapie prion strain. (*A*) PrP amyloid plaques in the hippocampus are immunopositive with the first generation of PrP-specific antibodies in formalin-fixed, paraffin-embedded tissue sections (Bendheim et al. 1984). (*B*) Transmission electron microscopy of the PrP plaques shows loose aggregates of straight, unbranched 8- to 16-nm-wide amyloid filaments (DeArmond et al. 1985). (*C*) Electron microscopy of negatively stained, whole-mounted PrP 27-30 prion rods: individual rods of the aggregate are 10–16 nm wide (Barry et al. 1985; DeArmond et al. 1985). (*D*) Light microscopy of prion rods show they bind Congo Red dye, which displays green-gold birefringence in polarized light. Bar in *A*, 25 μm.

THE ACCUMULATION OF PrP^{Sc} CAUSES THE NEUROPATHOLOGIC CHANGES CHARACTERISTIC OF PRION DISEASES

The neuropathologic features characteristic of prion diseases include spongiform (vacuolar) degeneration of the brain parenchyma, nerve cell degeneration and death, variable reactive astrocytic gliosis, and variable amyloid plaque formation. The discoveries that there is a single copy PrP gene, designated Prnp in animals and PRNP in humans, and that there are two PrP conformers (Cohen et al. 1994; James et al. 1997), a constitutively expressed, nonpathogenic conformer (PrP^{C}) and a pathogenic conformer in prion diseases (PrP^{Sc}), argued that PrP^{Sc} accumulation, rather than a putative slow virus, was the cause of neuronal degeneration.

Amyloid plaques

Kuru, CJD, and GSS are, along with Alzheimer's disease, Down syndrome, pathological aging, and cerebral amyloid angiopathy, included among the cerebral amyloidoses. Amyloid is defined as an extracellular mass of protein that has the tinctorial and histochemical properties of starch (e.g., amyloid means starch-like) (Glenner 1980; Glenner et al. 1986). The starch-like characteristics include strong staining by the periodic acid Schiff (PAS) reaction that is most probably due to the high glycosaminoglycan content of all amyloids (Snow et al. 1987, 1989, 1990). In hematoxylin and eosin stained tissue sections, it often appears as homogeneous, glass-like masses that stain pale pink. However, the characteristic of amyloids that distinguishes them from other extracellular masses is their affinity for Congo Red dye, which displays green-gold birefringence when viewed with polarized light. The birefringence of the Congo Red reaction indicates that the protein component of amyloids has a high β-sheet content. Electron microscopy reveals that the major protein component of amyloid is polymerized into aggregates of straight unbranched filaments. Although all amyloids have similar light and electron microscopic appearances, there are in reality multiple forms, each defined by its main protein component. The most common form of amyloidosis is caused by polymerization of the Aβ42 peptide fragment that forms as the result of aberrant degradation of the β-amyloid precursor protein during pathologic aging, Alzheimer's disease, and Down syndrome. The main protein component of the amyloid in prion diseases is composed of protease-resistant PrP peptides.

The first evidence that PrP^{Sc} causes the neuropathologic changes in prion diseases was finding that amyloid plaques in scrapie-infected animals and in human prion diseases (CJD, kuru, and GSS) are composed of

protease-resistant PrP (Figs. 1, 2, 3) and not of the Aβ42 peptide (Bendheim et al. 1984; DeArmond et al. 1985; Kitamoto et al. 1986; Snow et al. 1989; DeArmond and Prusiner 1995). Amyloid plaques in prion diseases were relatively easy to immunostain with PrP-specific antibodies by routine immunohistochemical techniques. The intensity of immunostaining of PrP amyloid, like Aβ amyloid of Alzheimer's disease, could be increased by pretreatment of the tissue section with formic acid (Fig. 2B) (Kitamoto et al. 1987). PrP immunohistochemistry for electron microscopy indicated that the amyloid plaques in scrapie-infected Syrian hamsters have a filamentous ultrastructure like other amyloids (Fig. 1B) and that the filaments contain protease-resistant PrP (DeArmond et al. 1985). These discoveries were the first to link the neuropathologic features of prion diseases with the prion particle, because Prusiner and colleagues had found earlier that purified prions tend to form into amyloid rods in vitro (Fig. 1C) (Prusiner et al. 1983; Prusiner 1984). During the process of purifying prions from scrapie-infected brain, it was found that exposure of 33–35-kD PrP^{Sc} to proteinase K (PK) digested 87 amino acids from its amino-

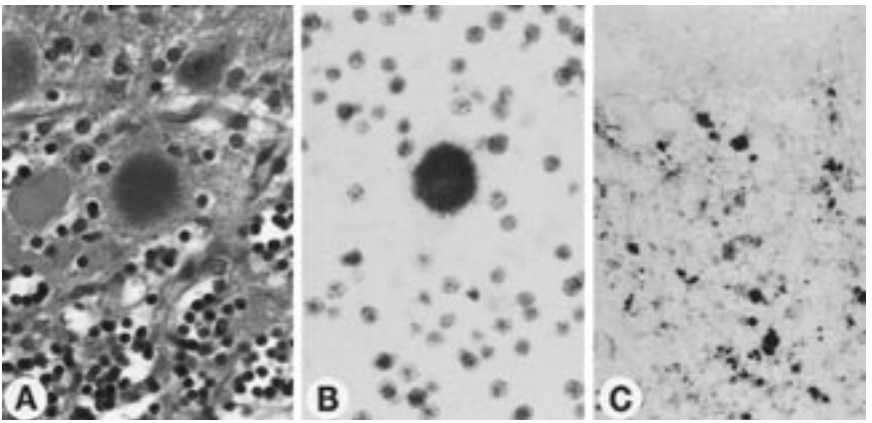


Figure 2 Typical kuru-type amyloid plaque in the cerebellar granule cell layer from a case of kuru can also be found in 5–10% of CJD cases, are abundant in nvCJD, and are mixed with other morphologic amyloid plaque subtypes in GSS. (*A*) A single kuru plaque stained by the PAS method is about the same size as the Purkinje cells at the top of the micrograph, 15–20 μm in diameter. (*B*) The kuru plaque is selectively stained by PrP immunohistochemistry following pretreatment of the section with formic acid. (*C*) PrP immunohistochemistry by the hydrolytic autoclaving method reveals numerous, non-amyloid PrP plaques and smaller punctate deposits in kuru as well as in some CJD cases. Magnification of *C* is one-half that of *A* and *B*.

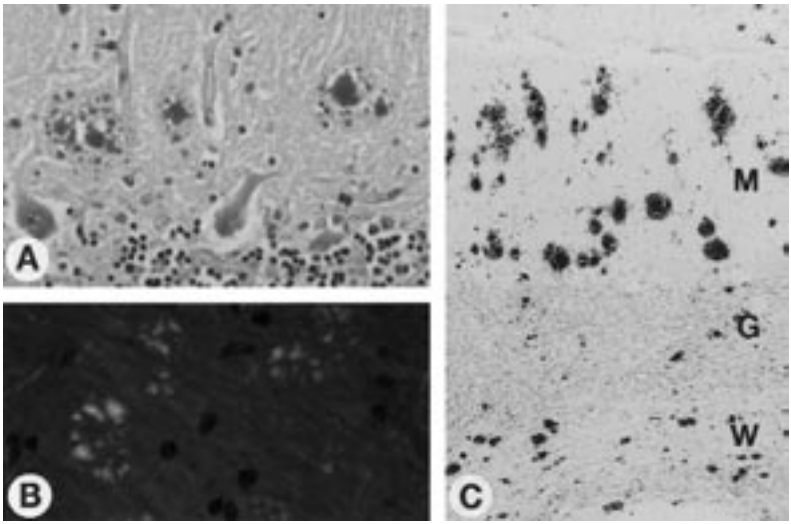


Figure 3 Multicentric GSS-type amyloid plaques in the cerebellum from a case of GSS(P102L). (*A*) PAS stain of multicentric plaques shows a central core of amyloid 15–30 μm in diameter surrounded by multiple smaller satellite amyloid deposits. (*B*) The GSS plaques bind Congo Red dye, which displays green-gold birefringence in polarized light. (*C*) PrP immunohistochemistry by the hydrolytic autoclaving method results in strong immunostaining of the multicentric plaques and also reveals numerous large and small primitive plaques that do not stain by the PAS method or bind Congo Red dye. The largest of these plaques are located in the molecular layer of the cerebellar cortex and can be 100 μm in length. Smaller PrP plaques are located in the granule cell layer and in the white matter. Magnification in *C* is half that in *A* and *B*. (W) Cerebellar white matter; (M) molecular layer; (G) granular layer.

terminal region leaving PrP 27-30 (Fig. 4), which is as infectious as PrP^{Sc} and has a propensity to polymerize into amyloid rod-like structures. These so-called "prion rods" were subsequently found to be an "artifact" of the procedure used to purify prions, because both PK digestion and detergents were required for their formation (McKinley et al. 1991a). Prion rods bind Congo Red dye and display green-gold birefringence in polarized light like purified filaments from tissue amyloids (Fig. 1D) (Prusiner et al. 1983). The greater propensity of PrP 27-30 to form into amyloid rods compared to full-length PrP^{Sc} may be due to its higher β-sheet content or because digestion of a large portion of the amino terminus eliminated any steric hindrance to polymerization. Several groups

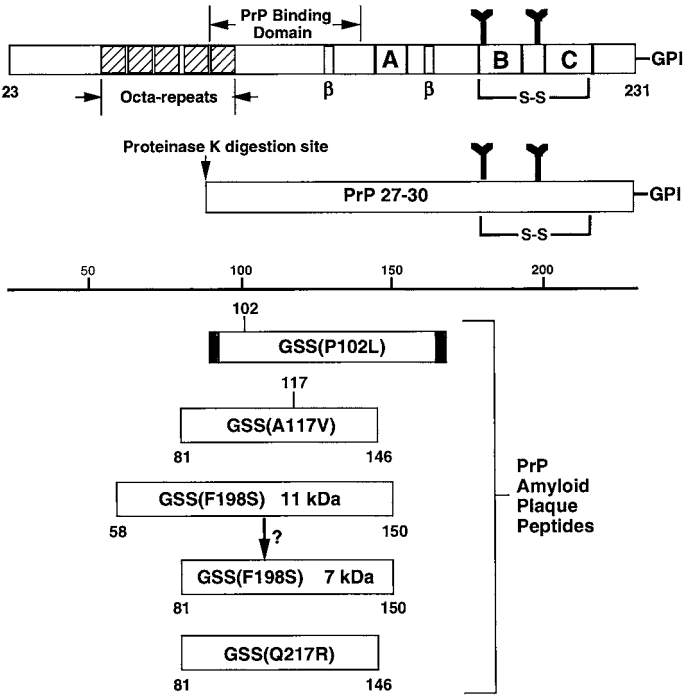


Figure 4 Amyloidosis in prion diseases requires truncation of the PrP molecule to highly amyloidogenic peptides. (Adapted, in part, from Ghetti et al. 1996a.)

have found that PrP^{Sc} is 43% β-sheet and 30% α-helix, whereas PrP^{C} is 3% β-sheet and 42% α-helix (Pan et al. 1993; Safar et al. 1993). PrP 27-30 has an even higher β-sheet content (54%) and lower α-helix content (21%) than PrP^{Sc} (Caughey et al. 1991; Gasset et al. 1993).

Amyloidosis In Vivo Involves Truncation of Pathogenic PrP

Amino-terminal truncation of PrP^{Sc} by limited digestion with PK yields PrP 27-30 that is amyloidogenic in vitro (Fig. 1C,D) (McKinley et al. 1991a). Truncation of the PrP also features in GSS syndromes, which, by definition, require dominant inheritance and the presence of large numbers of PrP amyloid plaques in the brain. The amino acid sequence of PrP in GSS amyloid was first determined for the Indiana kindred-type of GSS (Tagliavini and Pilleri 1983; Tagliavini et al. 1994) in which a mutation at codon 198 results in a substitution of a serine for phenylalanine, GSS(F198S) (Dlouhy et al. 1992; Hsiao et al. 1992). Amyloid cores were

purified from brain, proteins were extracted from amyloid fibrils with formic acid, and the resulting peptides were sequenced. Two major peptides with ragged amino and carboxyl termini were obtained of about 11 and about 7 kD comprising PrP residues 58–150 and 81–150, respectively (Fig. 4). Immunohistochemistry of the GSS(F198S) with antisera to synthetic PrP peptides verified the presence of the approximately 11- and approximately 7-kD peptides in the plaques in situ. Immunohistochemistry with antibodies specific for the amino and carboxyl termini of PrP immunostained amorphous material at the periphery of the plaques and in the neuropil away from the plaques, suggesting that full-length PrP exists in those locations. To determine whether or not amyloidogenic peptides were derived from wild-type PrP^C or mutant PrP, Val-129 was used as a marker for mutant PrP peptides in patients whose wild-type PrP^C was Met-129. Amino acid sequencing and electrospray mass spectrometry of PrP amyloid peptides indicated they contained only 129V, arguing they were derived exclusively from mutant PrP(F198S). Ghetti et al. (1996a) used the same biochemical techniques to isolate and determine the size of peptide fragments from the amyloid in GSS(A117V) and GSS(Q217R). In both cases, mutated PrP was truncated to about 7-kD peptides spanning residues 81 to 146 (Fig. 4). Limited information was obtained about the amyloid of GSS(P102L). Western analysis of amyloid-enriched fractions showed two PrP immunoreactive bands at 25–30 kD and 15–20 kD. Amino acid sequence analysis of the high-performance liquid chromatography (HPLC)-purified peptides showed they contained the leucine substitution at residue 102, indicating that the amyloid was composed of mutant PrP(P102L). The residues included in the shorter PrP(P102L) peptide have been estimated by immunohistochemistry with antibodies to synthetic PrP peptides. It appears to extend from near residue 90 to near 165 (Fig. 4). A fifth mutation leading to a Gerstmann-Sträussler-Scheinker syndrome, GSS(Y145Stop), results in formation of a stop-codon at codon 145 and the synthesis of a truncated form of amyloidogenic PrP (Kitamoto et al. 1993a).

The mutant amino acid substitutions that cause GSS-like cerebral amyloidosis result in both amino- and carboxy-terminal truncation of PrP that yields highly amyloidogenic peptides. All of these peptides contain the putative PrP^{Sc} to PrP^C binding domain that studies of the host species barrier to prion transmission in transgenic (Tg) mice suggest resides between residues 90 and 140 (Scott et al. 1993). The propensity of those peptides to polymerize into amyloid filaments testifies to this domain's ability to bind with peptides containing the homologous sequence and also to the tendency for this binding to form highly β-sheeted polymers.

Non-amyloid PrP^{Sc} Accumulation Causes Neuronal Dysfunction and Degeneration

Except for the several genetically distinct forms of GSS in which large amounts of PrP amyloid plaques are deposited throughout the brain, most prion diseases in animals and humans are associated with few or no PrP amyloid plaque deposits. For example, only 5–10% of cases of the most common form of human prion disease, sporadic CJD, contain mature amyloid plaques. It seems very unlikely, therefore, that cerebral amyloidosis in prion diseases accounts for nerve cell dysfunction and degeneration.

The location of a non-amyloid form of PrP^{Sc} or, for that matter, the location of nonpathogenic PrP^{C} in the brain parenchyma, and their association with spongiform degeneration and reactive astrocytic gliosis has been a more complex problem than localizing PrP in amyloid plaques. There are several reasons for this difficulty: First, no PrP-specific antibodies can distinguish PrP^{C} and PrP^{Sc}. Second, PrP^{Sc} is poorly antigenic in its native conformation: It requires denaturation to make its antigenic sites available to antibodies (Serban et al. 1990). Third, because both PrP^{C} and PrP^{Sc} exist together in the brain in prion diseases, PrP^{C} must be eliminated before PrP^{Sc} can be localized by immunohistochemical techniques. Because of these problems, in our first attempts to determine whether or not there is a correlation between sites of PrP^{Sc} accumulation and sites of spongiform degeneration and reactive astrocytic gliosis, we compared our neuropathologic and immunohistochemical results with biochemical measurements of PrP^{Sc} in homogenates of unfixed, dissected brain regions (DeArmond et al. 1987). The results showed for the first time that both spongiform degeneration and reactive astrocytic gliosis colocalize with sites of PrP^{Sc} accumulation in the brain.

Progress became possible with the development of two sensitive immunohistologic techniques that differentiate PrP^{C} and PrP^{Sc} in tissue sections. The histoblot technique combines the morphologic advantages of histologic sectioning with the neurochemical advantages of dot-blotting to distinguish PrP^{C} from PrP^{Sc} on the basis of their differential susceptibility to PK digestion and their relative reactivity with PrP antibodies in their natural and denatured states (Taraboulos et al. 1992b). The second advance, developed in Tatieshi's laboratory, has been named hydrolytic autoclaving (Muramoto et al. 1992). Aldehyde-fixed, paraffin-embedded tissue sections are autoclaved in dilute hydrochloric acid prior to immunohistochemical staining. This technique takes advantage of the relative resistance of PrP^{Sc} to acid hydrolysis compared to PrP^{C}. There are advantages and disadvantages to both techniques. Histoblots allow quan-

titation and subregional localization of both PrP^{C} and PrP^{Sc} in serial sections of the CNS but do not show cellular localization. Hydrolytic autoclaving is less sensitive and appears to be selective for PrP^{Sc} based on comparison with histoblot analysis but, importantly, reveals the cellular localization of PrP^{Sc} (DeArmond and Prusiner 1995).

Histoblots are made by transferring (blotting) frozen sections of unfixed brain tissue to nitrocellulose paper where PrP^{C} can be completely eliminated with certainty by PK digestion (Taraboulos et al. 1992b). The undigested portion of PrP^{Sc}, PrP 27-30, which is poorly antigenic in its native configuration, is then denatured with 4 M guanidinium (Gdn), which greatly enhances binding of PrP-specific antibodies. The result is an intense immunohistochemical signal with little or no background that reveals the neuroanatomic location of PrP^{Sc} and is quantifiable. Because PrP^{Sc} in its native state shows little or no reaction with the current PrP-specific antibodies, the location of PrP^{C} can be determined in histoblots not treated with PK or Gdn. Histoblotting has verified the earlier biochemical and immunohistochemical studies of PrP^{Sc}, which showed that PrP^{Sc} deposition precedes the development of histopathology and that spongiform degeneration and reactive astrocytic gliosis colocalize precisely with PrP^{Sc}. An example of the precision of the relationship between sites of PrP^{Sc} deposition and pathologic changes was seen in the cerebral cortex of Syrian hamsters inoculated with two different scrapie strains (Fig. 5) (Hecker et al. 1992). With the Sc237 prion strain, PrP^{Sc} accumulation tends to remain localized to the deeper layers of the cerebral cortex; spongiform degeneration and reactive astrocytic gliosis are localized to the same region. In contrast, with the 139H strain, PrP^{Sc} is distributed to all layers of the cerebral cortex as are the pathologic changes. Similar results were obtained by Bruce and her colleagues (Bruce et al. 1989) using standard immunohistochemical methods.

Subsequently, we examined the temporal pattern of PrP^{Sc} accumulation by measuring its concentration in dissected brain regions throughout the course of scrapie in Syrian hamsters (Jendroska et al. 1991). Animals were inoculated intrathalamically with the Sc237 strain of scrapie agent. Four relevant observations were made: First, PrP^{Sc} accumulates exponentially in many, but not all, brain regions beginning with the site of inoculation in the thalamus (Fig. 6). Second, the pattern of spread of PrP^{Sc} from brain region to brain region was stereotypic and suggested that it occurred largely by slow axonal transport; however, spread to septum also appeared to occur by diffusion of PrP^{Sc} in the extracellular space of the CNS. The latter form of intracerebral spread has not been identified in other animal

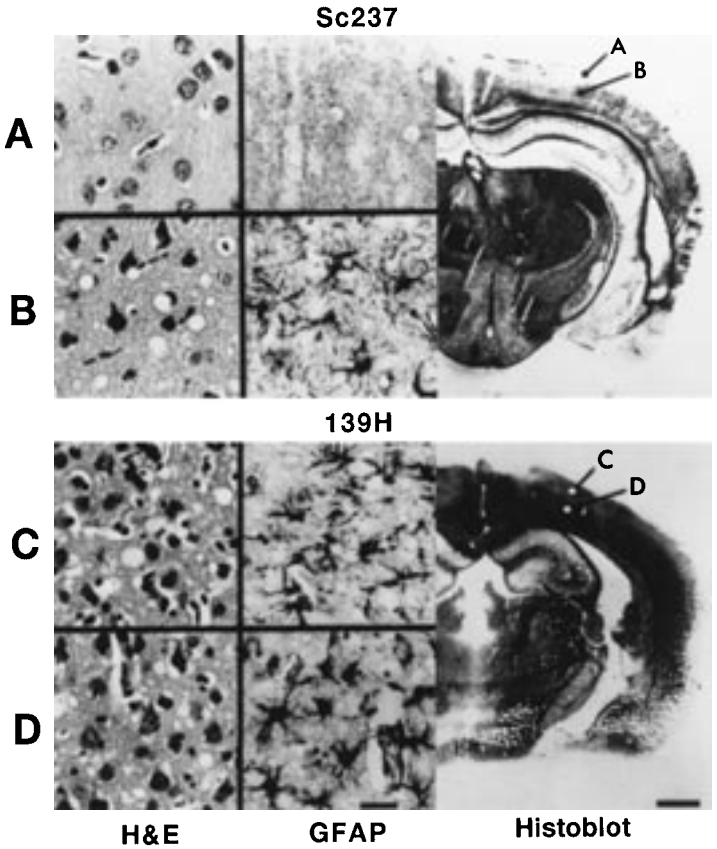


Figure 5 There is a precise colocalization of vacuolar degeneration of the brain parenchyma and reactive astrocytic gliosis with sites of PrP^{Sc} deposition. Syrian hamsters were inoculated intracerebrally with either the Sc237 or 139H strain of scrapie prions. Incubation times (post-inoculation interval to clinical signs) were about 65 and 160 days, respectively. Histoblot analysis showed that PrPSc was more widely distributed and more intensely immunostained with 139H than Sc237. Correlation of the neuropathologic changes with sites of PrP^{Sc} deposition are made for the cerebral cortex. (*A*) With Sc237, there is little or no PrP^{Sc} immunoreactivity in the outer half of the cerebral cortex and (*B*) moderate PrP^{Sc} immunoreactivity in the inner half. No vacuolation (H&E stain) or reactive astrocytic gliosis (GFAP immunostain) is located in the outer cortex, whereas mild to moderate changes are present in the inner cortex. With 139H, there is intense PrP^{Sc} immunoreactivtiy in both the outer and inner neocortex that is associated with vacuolation and reactive astrocytic gliosis in both locations. Bar on histblot, 1 mm; bar on histological section, 50 μm (Reprinted, with permission, from Hecker et al. 1992.)

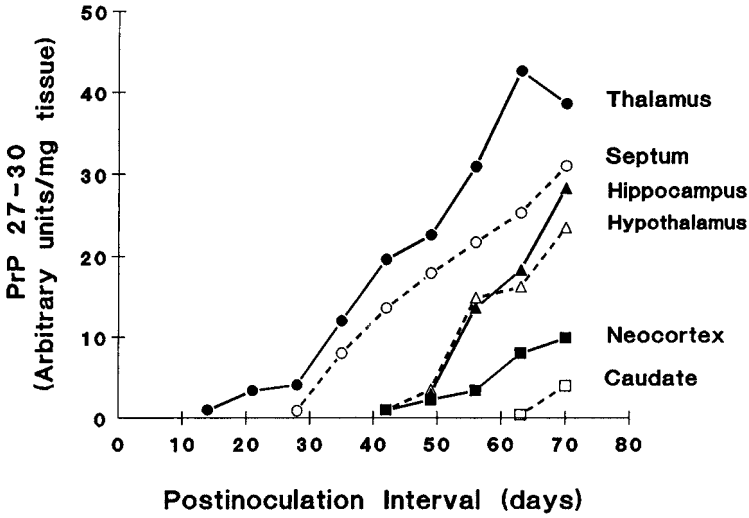


Figure 6 Kinetics of PrP 27-30 accumulation during the course of scrapie in Syrian hamsters inoculated intrathalamically with Sc237 prions. PrP 27-30 is the proteinase K digestion product of PrP^{Sc}. Homogenates of dissected brain regions were treated with proteinase K, and the relative amount of PrP 27-30 was determined by Western analysis. Clinical signs of scrapie presented about 65 days post-inoculation. (Reprinted, with permission, from Jendroska et al. 1991.)

species or with other strains of scrapie agent. Third, spongiform degeneration and reactive astrocytic gliosis developed 1–2 weeks after the start of PrP^{Sc} accumulation in a region. Fourth, total brain PrP^{Sc}, based on the sum of PrP^{Sc} levels in each brain region, was directly proportional to the whole-brain scrapie infectivity titer measured throughout the course of scrapie (Fig. 7).

From these initial studies, a unifying prion disease hypothesis resulted, which proposed that propagation of prions and the development of neuropathologic changes are both linked to the formation of PrP^{Sc}.

NEUROPATHOLOGIC CHARACTERISTICS OF PRION DISEASES

Prion diseases in animals and humans can be divided into three broad categories based on their neuropathological features: (1) those characterized by vacuolar (spongiform) degeneration of the brain, little or no PrP amyloid plaque formation, and accumulation of protease-resistant PrP; (2) those with abundant amyloid plaque formation, variable amounts of vac-

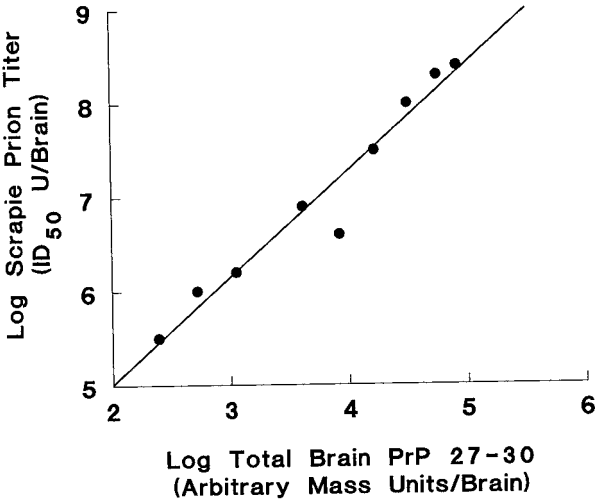


Figure 7 Whole-brain Sc237 strain infectivity titer is directly proportional to the brain concentration of PrP 27-30 throughout the course of scrapie in Syrian hamsters. (Reprinted, with permission, from Jendroska et al. 1991.)

uolar degeneration, and abnormal PrP accumulation that is relatively protease sensitive; and (3) those that are characterized by intense vacuolar degeneration, abundant PrP amyloid plaque formation, and protease-resistant, abnormal PrP.

The first category includes the vast majority of prion diseases, including scrapie in sheep and rodents; bovine spongiform encephalopathy (BSE); kuru; sporadic, familial, and iatrogenic CJD; and fatal familial insomnia (FFI). The striking similarity of the neuropathologic changes in these disorders prompted the veterinary neuropathologist, William Hadlow, to propose that scrapie of sheep and kuru of the Fore people of New Guinea were homologous diseases and that kuru could be transmitted to animals by inoculation of brain homogenates into laboratory animals (Hadlow 1959) in the same way that sheep scrapie had been experimentally transmitted to goats 20 years earlier by inoculation of spinal cord homogenates (Cuillé and Chelle 1939). The striking resemblance of the neuropathologic changes in kuru and in CJD was recognized by Klatzo (Klatzo et al. 1959). By 1966 and 1968, respectively, brain homogenates from kuru and CJD patients were successfully transmitted to nonhuman primates (Gajdusek et al. 1966; Gibbs et al. 1968). Successful transmission of familial CJD and GSS to nonhuman primates was first reported in 1973 and 1981, respectively (Roos et al. 1973; Masters et al. 1981b). A mutation of the PRNP gene was

genetically linked to FFI in 1992 (Medori et al. 1992a,b): It was successfully transmitted to rodents in 1995 (Tateishi et al. 1995). A summary of the successful transmissions of human neurodegenerative diseases to primates by the National Institutes of Health was published (Brown et al. 1994).

The second neuropathologic category of prion disease appears to be confined largely to humans. The only prion diseases included in this category are the six dominantly inherited syndromes designated GSS. GSS was first transmitted to nonhuman primates in 1981 (Masters et al. 1981a), which was the first evidence that it might be related to CJD. Although PrP^{CJD} is resistant to PK digestion in sporadic, iatrogenic, and familial CJD and in FFI, mutated PrP in GSS cases is relatively senstive to protease digestion (Hsiao et al. 1989b, 1991b; Hegde et al. 1998).

In 1996, a new variant of CJD, designated nvCJD, was discovered in Great Britain that is characterized by abundant PrP amyloid, like GSS, intense vacuolation and no PRNP gene mutation, like sporadic and iatrogenic CJD, and protease-resistant PrP^{CJD} (Will et al. 1996). In GSS syndromes, nonconservative amino acid substitutions in the PrP molecule appear to favor abnormal degradation into highly amyloidogenic PrP peptides (Fig. 4). nvCJD appears to be caused by infection with a new strain of prions, possibly from BSE. If indeed this is the case, this new prion strain is highly amyloidogenic. In the context of our current understanding of the propagation of prion strains in a host and of PrP amyloidosis, it creates a β-sheeted conformation in the host's PrP that results in degradation to highly amyloidogenic PrP peptides.

The first two categories of prion diseases also differ in the potency of their prions. In the National Institutes of Health series, laboratory transmission rates were highest for iatrogenic CJD (100%), less for sporadic CJD (90%), and least for familial prion diseases (68%) (Brown et al. 1994). Of the familial prion diseases, the highest transmission rate occurred with familial CJD(E200K) (85%), whereas GSS(P102L) had a relatively low rate of 38%.

The following clinical and neuropathologic descriptions of human prion diseases have been condensed from other more detailed accounts (see DeArmond and Prusiner 1996, 1997, 1998).

Kuru

Kuru is a neurologic disorder confined to the aboriginal Fore people of New Guinea and was one of several CNS diseases discovered in the Western Pacific after World War II. The combined anthropologic-epidemi-

ologic-neurologic-neuropathologic investigation of kuru by Australians and Americans and the subsequent laboratory transmission, first of kuru and then of CJD to nonhuman primates, was begun in 1957 when Zigas and Gajdusek began investigations of kuru among the Fore people of the Eastern Highlands of New Guinea (Zigas and Gajdusek 1957). By 1959, the clinical and pathologic features of kuru were published (Gajdusek and Zigas 1959; Klatzo et al. 1959). The physicians who examined victims of kuru noted progressive cerebellar degeneration with ataxia and tremor, and dementia in some cases during the later stages. Investigations of the Fore peoples by anthropologists suggested that kuru had begun between 1900 and 1920 (Glasse and Lindenbaum 1992) as a case of sporadic CJD. Alpers has convincing epidemiologic evidence that ritualistic cannibalism was the mode of transmission (Alpers 1992). His conclusion was based on three observations: according to the rules of cannibalism among the Fore, women and children consumed all body parts, including brain, whereas the men only consumed skeletal muscle; kuru mainly affected adult women, children, and adolescents of both sexes; and the incidence of kuru among children declined with the end of cannibalism. According to the anthropologists (Glasse and Lindenbaum 1992), little of the corpse was discarded: even bones were pulverized, cooked, and eaten with green vegetables.

Neuropathology

Except for atrophy of the vermis and flocculonodular lobes of the cerebellum, the brain was grossly normal. Microscopically, the most severe pathologic changes were located in the cerebellum with loss of granule cells, loss of Purkinje cells, fusiform swelling of the proximal portion of many remaining Purkinje cell axons (torpedoes), and intense Bergmann radial gliosis consistent with the clinical features dominated by ataxia. The changes in the cerebral cortex consisted of slight spongiform degeneration in the neuropil between nerve cell bodies in most regions. These lesions were most severe in paramedian cerebral cortical region (cingulate gyrus, subiculum, entorhinal area, parahippocampal gyrus, and superior frontal gyrus), the putamen and caudate nucleus, and in the anterior and medial nuclei of the thalamus (Kakulas et al. 1967). Beck and Daniel were particularly impressed by the severe degeneration in the olivo-ponto-cerebellar system in addition to marked cerebellar vermal atrophy (Beck and Daniel 1979). In the caudate nucleus and putamen, many of the remaining neurons contained intracytoplasmic vacuoles. Finally, amyloid plaques consisting of spherical deposits with radiating spicules at their periphery (spiked-ball plaque) were found in 75% of cases (see Fig. 2). The largest

number of plaques was located in the granule cell layer of the cerebellar cortex. They are PAS positive (Fig. 2A) and bind Congo Red dye, which shows green birefringence in polarized light, as do all amyloids. Similar amyloid plaques are found in some cases of CJD, GSS, and scrapie, where most neuropathologists continue to refer to them as kuru-plaques.

Creutzfeldt-Jakob Disease

CJD is a neurodegenerative syndrome caused by accumulation of protease-resistant PrP^{CJD} in the brain, similar to the accumulation of PrP^{Sc} in scrapie, and is distinguished by a specific set of clinical and neuropathologic features. About 10% of CJD cases are caused by one of the several inherited PRNP mutations. In these cases, accumulation of mutated PrP, which is most probably expressed in most if not all nerve cells, causes spongiform degeneration and the spontaneous formation of prions. The great majority of CJD cases, accounting for 85–90% of all human prion diseases, are sporadic with no evidence of an infectious or genetic etiology. There are reasons to believe that sporadic CJD, in some cases, may be caused by an age-related spontaneous PRNP mutation in a single cell and, in others, by aberrant metabolism of PrP^{C} and its spontaneous conversion to PrP^{CJD} in a "thermodynamically unlucky" victim. Sporadic CJD has a worldwide incidence of about 1 per million per year (Masters et al. 1979; Brown et al. 1987), which may represent the probability that sufficient numbers of PrP molecules spontaneously cross the energy barrier that separates the normal α-helical conformation and the pathogenic β-sheeted conformation. Males and females are affected in equal numbers. The peak age of onset is around 60 years with a wide range of up to 90 years; a small number of sporadic CJD cases have been reported in individuals under the age of 20 years (Monreal et al. 1981; Brown 1985; Packer et al. 1980; Berman et al. 1988). Finally, a small proportion of CJD cases are acquired by infection. The vast majority of the latter were transmitted through medical procedures, thus the designation of iatrogenic CJD. Contaminated intracerebral electroencephalogram (EEG) electrodes, human growth hormone (HGH) preparations, human pituitary gonaodotropin preparations, dura mater grafts, and corneas have caused iatrogenic CJD (for review, see DeArmond and Prusiner 1996, 1997). The epidemiologic evidence indicates that nvCJD is a new form of CJD also acquired by infection; however, the origin of the infectious prions is still being debated (see below) (Will et al. 1996). Iatrogenic and nvCJD can often be distinguished from sporadic and familial CJD cases by the age of

onset. Therefore, the occurrence of CJD in more than 55 patients ranging in age from 10 to 41 years who had received HGH suggested an iatrogenic etiology (Brown 1985; Buchanan et al. 1991; Fradkin et al. 1991; Brown et al. 1992). The early onset of CJD in these patients clearly differentiates them from cases of sporadic CJD and most forms of familial CJD. The risk of CJD among those who have received HGH is estimated to be about 1 per 200, whereas the risk of CJD in the general population under 40 years of age is about 1 per 20 million (Fradkin et al. 1991). All of these patients received injections of HGH every 2–4 days for 4–12 years (Gibbs et al. 1985; Koch et al. 1985; Powell-Jackson et al. 1985; Titner et al. 1986; Croxson et al. 1988; Marzewski et al. 1988; New et al. 1988; Anderson et al. 1990; Billette de Villemeur et al. 1991; Macario et al. 1991; Ellis et al. 1992). Interestingly, most of the patients presented with cerebellar syndromes that progressed over time periods varying from 6 to 18 months. Some patients became demented during the terminal phase of their illnesses. The clinical courses of some patients with dementia occurring late resemble kuru more than ataxic CJD in some respects (Prusiner et al. 1982). Assuming these patients developed CJD from injections of prion-contaminated HGH preparations, the possible incubation periods range from 4 to 30 years (Brown et al. 1992). These incubation times are not surprising, given that some cases of kuru have incubation periods of two to three decades (Gajdusek et al. 1977; Prusiner et al. 1982; Klitzman et al. 1984). Many patients received several common lots of HGH at various times during their prolonged therapies, but no single lot was administered to all the American patients. An aliquot of one lot of HGH has been reported to transmit CNS disease to a squirrel monkey after a prolonged incubation period (Gibbs et al. 1993).

As a general rule, all forms of CJD present with a relatively rapid course leading to death within 4–12 months from start of signs and symptoms; however, cases lasting 2–5 years are not uncommon (Masters and Richardson 1978; Malamud 1979). Familial CJD cases tend to present at an earlier age than sporadic CJD. For example, CJD(E200K) has an onset at 55 ± 8 years of age, which progresses to death in an average of 8 months, and CJD(D178N, V129) has on onset at 46 ± 7 years of age with death occurring in an average of 22 months (Brown et al. 1991). CJD linked to octapeptide inserts presents at 23–35 years of age and are followed by a particularly long progression to death of 4–13 years. Multiple mutations of the PRNP gene have been genetically linked to familial CJD syndromes. The penetrance is close to 100% in familial prion diseases, but it is age dependent. For CJD(E200K), penetrance is as low as 1% for carriers of the mutation at age 40 and close to 100% at age 80

(Rosenmann et al. 1997). Penetrance was found to be related to expression levels of both mutant PrP(E200K)mRNA and mutant PrP(E200K) protein. They were lower than expression levels of the wild-type PrPmRNA and PrP^C in healthy carriers, whereas most of the clinically ill E200K patients expressed equal levels of mutant and wild-type proteins.

Clinical features that suggest the diagnosis of CJD are often preceded by a prodromal period in which nonspecific clinical signs occur, including fatigue, sleep disturbances, memory disturbances, behavioral changes, vertigo, and ataxia. However, the most characteristic clinical features include rapid progression of mental deterioration with dementia, myoclonus, a broad spectrum of motor disturbances (extrapyramidal, cerebellar, pyramidal, and/or anterior horn cell), and an EEG showing periodic short wave activity. When the quartet of dementia, myoclonus, periodic EEG activity, and rapid progression are seen in a patient, the diagnosis of CJD is relatively certain. In contrast to kuru, in which the clinical and neuropathologic features were relatively uniform, sporadic CJD is characterized by diversity of clinical features and an equally diverse distribution of neuropathology. About 15% of sporadic CJD cases are similar to kuru, with the development of ataxia as an early sign followed by dementia (Brown et al. 1984). The majority of the patients who developed CJD after intramuscular pituitary growth hormone injections presented with cerebellar syndromes that progressed over 6–18 months. CJD subtypes with visual disturbances and severe occipital cortex pathology have been designated the Heidenhain variant.

Neuropathology

The gross appearance of the brain in CJD is variable and not diagnostic. In some cases, no recognizable abnormalities are seen, whereas others show varying degrees of cerebral cortical, striatal, and/or cerebellar atrophy with brain weights as low as 850 g (Malamud 1979).

Spongiform degeneration and reactive astrocytic gliosis in CJD exhibit a wide spectrum of intensity and distribution (Beck and Daniel 1979). Some investigators divided the neuropathology of CJD into 16 subtypes (Beck et al. 1969) whereas others (Malamud 1979) preferred to subclassify CJD into six clinico-neuropathologic categories: cortical, corticostriatal with or without visual loss, corticostriatocerebellar, corticospinal, and corticonigral. Richardson and Masters (1995) recently reviewed the clinical spectrum of human prion diseases and emphasized the Heidenhain variants with visual symptoms, striatal variants resembling Huntington's disease, thalamic variants including FFI, cerebellar

variants that resemble kuru, variants with oculomotor disturbances that can resemble progressive supranuclear palsy, panencephalopathic variants with involvement of white matter, and variants with demyelinative peripheral neuropathy.

The hallmarks of CJD are spongiform degeneration of neurons and their processes, neuronal loss, intense reactive astrocytosis, and amyloid plaque formation; however, these vary considerably from case to case. In some patients, the only histologic clue is delicate vacuolization of the gray matter with minimal or no detectable nerve cell loss or reactive astrocytosis (Fig. 8A). The vacuoles that comprise spongiform degeneration are intracellular and located in the neuropil between nerve cell bodies. They should not be confused with spaces around nerve cell bodies and blood vessels, which are artifacts. The vacuoles tend to be round to oval and vary in diameter from 5 to 25 μm in most cases, but can be as large as 50–60 μm in others (Fig. 8B). Masters and Richardson (1978) designated the late stage of disease "status spongiosis" to distinguish it from the former patterns, which they designated "spongiform degeneration." Status spongiosis is characterized by massive loss of neurons and gray matter neuropil, development of numerous extracellular microcysts, as large as 100 μm in the parenchyma, and a dense meshwork of reactive astrocytic processes surrounding the spaces (Fig. 9).

In CJD, spongiform degeneration may be found in the neocortex, subiculum of the hippocampus, putamen, caudate nucleus, thalamus, and molecular layer of the cerebellar cortex. It is usually minimal or absent from the globus pallidus, Ammon's horn, and dentate gyrus of the hippocampus, brain stem, and spinal cord (Masters and Richardson 1978; Beck and Daniel 1979; Malamud 1979); however, we have found cases of CJD with extensive spongiform degeneration of the hippocampus. Spongiform degeneration of the cerebral cortex occurs in virtually all cases, regardless of the clinical presentation. In the cerebral cortex, the amount of vacuolation can vary considerably from region to region even

Figure 8 Vacuolar degeneration of the gray matter is characteristic of scrapie and CJD syndromes and less so of GSS syndromes. These vacuoles are in neuronal processes including dentrites, distal axons, and presynaptic boutons. Reactive astrocytic gliosis is variable. (*A*) The most common form of vacuolation in the cerebral cortex in a case of CJD consists of vacuoles 5–20 μm in diameter between nerve cell bodies (H&E stain). (*B*) A less common form of vacuolation in a CJD case includes larger vacuoles up to 50–70 μm in diameter (H&E stain). (*C*) Intense reactive astrocytic gliosis in a different case of CJD (GFAP immunostain). All photomicrographs are at the same magnification.

within the same cortical section. It can be diffusely distributed to all cortical layers or may have a pseudolaminar appearance, the latter primarily in the deep cortical layers. Vacuolation of nerve cell bodies has also been

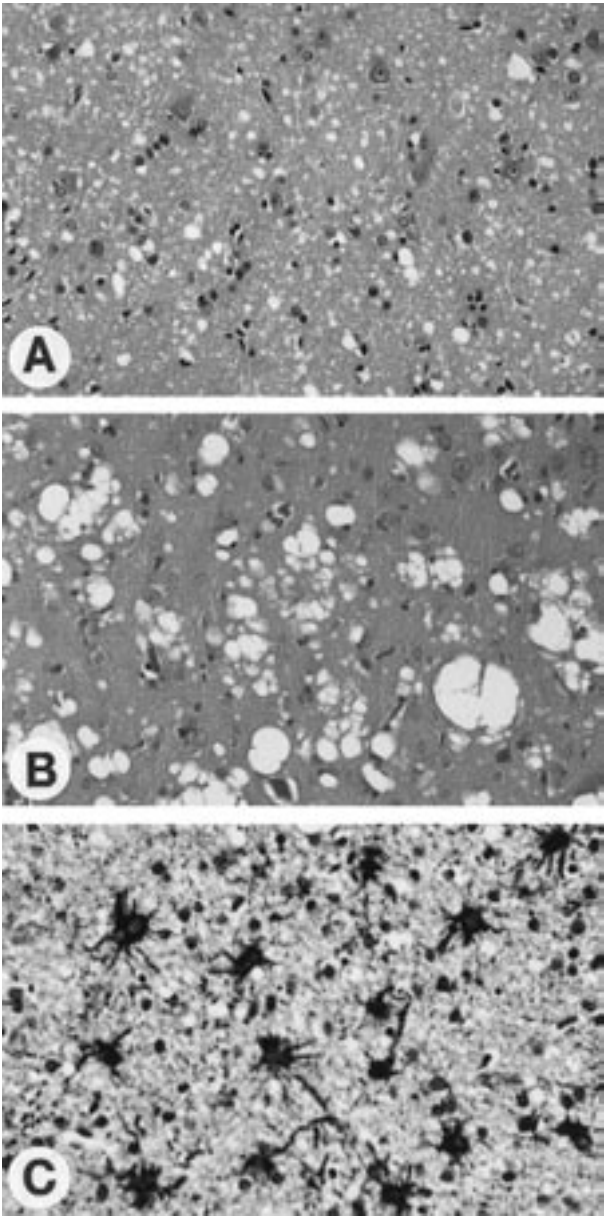


Figure 8 (See facing page for legend.)

reported in some cases of CJD; however, it is more characteristic of natural scrapie in sheep and of kuru.

As shown by electron microscopy, spongiform degeneration consists of focal swelling of neuritic processes, both axonal and dendritic, and synapses with loss of internal organelles and accumulation of lacy abnormal membranes (Lampert et al. 1972; Chou et al. 1980; Beck et al. 1982). The relative disappearance of spongiform degeneration reported by Masters and Richardson (1978) with progressive nerve cell loss is consistent with the hypothesis that vacuolation is mostly confined to nerve

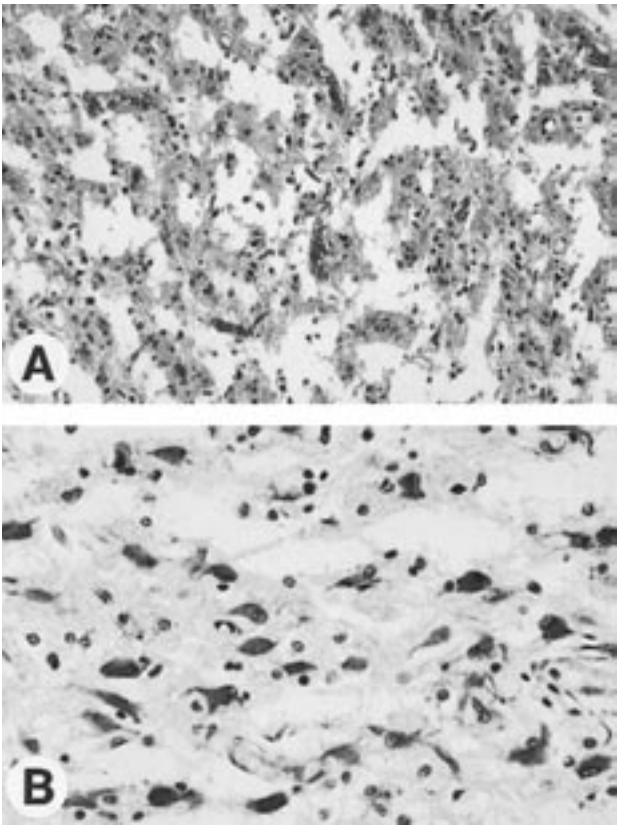


Figure 9 Status spongiosis of the cerebral cortex in a CJD case follows severe loss of neurons. It consists of microcysts in the extracellular space that are surrounded by reactive astrocytes and their processes. (*A*) Low-magnification view of the cerebral cortex showing microcysts (H&E). (*B*) Higher magnification view of the large reactive astrocytes in the walls of the microcysts in the same case (GFAP immunohistochemistry with hematoxylin counterstain). The magnification in *A* is half that in *B*.

cell processes. In status spongiosis, "vacuolation" is primarily extracellular in the form of microcysts.

Reactive astrocytic gliosis in CJD (Figs. 8C and 9B) and in scrapie has been described as more intense than one would expect based on the amount of nerve cell loss (Dormont et al. 1981; Mackenzie 1983). Nevertheless, Masters and Richardson (1978) found that the degree of reactive astrocytosis correlated very well with the degree of nerve cell loss. PrP 27-30 in the form of prion rods has been found to stimulate astrocyte proliferation in vitro (DeArmond et al. 1992) and may contribute to the very intense astrocytic reaction in prion diseases.

Generally, lesions in the white matter in CJD, as well as in other prion diseases, are secondary to neuronal loss. However, a vacuolar myelopathy is found in some CJD cases, particularly in Japan (Tateishi et al. 1981). Vacuolar myelopathy is a characteristic of prion disease in mice inoculated with Mo(RML) or the Chandler scrapie prion isolates (Carlson et al. 1986; Prusiner et al. 1990) and with human CJD prions (Liberski et al. 1989). Ultrastructurally, the vacuoles are within the myelin sheath, and occasionally intra-axonal.

Kuru-type amyloid plaques are found in only 5 to 10% of CJD cases, in association with MV genotype at codon 129 in the PrP genes (Porschl et al. 1996). They are immunopositive with PrP antibodies, but are negative with antibodies to β-amyloid (Snow et al. 1989, 1990). The plaques in CJD consist of discrete eosinophilic, periodic-acid Schiff (PAS)-positive spherical masses with radiating amyloid spicules at their periphery (Fig. 2A). In kuru and CJD, they are found most often in the granule cell layer of the cerebellar cortex but can occasionally be seen in other brain regions, including the cerebral cortex, basal ganglia, and thalamus (Klatzo et al. 1959). Most, but not all, patients in whom ataxia is prominent have kuru-type plaques in their cerebellum (Pearlman et al. 1988). These patients exhibit a protracted clinical course that may last up to 3 years. Amyloid angiopathy has also been found in some cases of CJD; however, this vascular amyloid is composed of the Aβ peptide and not of PrP (Tateishi et al. 1992).

It is possible that more cases of CJD with PrP plaques will be found as a result of an improved technique, using hydrolytic autoclaving, for their detection by immunohistochemistry (Muramoto et al. 1992). Prior to immunostaining, glass-mounted histologic sections of brain are immersed in 3 mM HCl (effective range, 1–30 mM) and autoclaved for 10 minutes. This not only results in intense staining of kuru plaques, but it also reveals numerous primitive PrP plaques (Fig. 2C). Primitive PrP plaques stain poorly by the PAS reaction and do not generally show green birefringence with Congo Red dye.

New Variant CJD

There is a substantial degree of concern in Great Britain that some humans have contracted a prion disease as the result of ingesting meats infected with BSE. There are three main reasons for suspecting an infectious etiology and BSE in particular: (1) nvCJD appears to be a new form of prion disease with unique clinical and neuropathological features and does not resemble cases of sporadic or iatrogenic CJD found in young or old patients. (2) The majority of the victims were from Great Britain and a couple from France; therefore, they were all geographically in the same region as the BSE epidemic. (3) Known incubation periods for other forms of CJD acquired by infection are well within a range that would link nvCJD with BSE. To date (April 1999), there have been 40 cases of nvCJD identified (22 female, 18 male) with a mean age of death of 30 years (range 18–53 years) and median duration of illness of 13 months (range 7–38 months). All cases that have been tested are methionine homozygotes at codon 129 in the PRNP. Sporadic CJD cases are extremely rare under the age of 40 years and have a mean age of presentation of around 63 years, with a duration of illness of less than 6 months in the majority of cases (Will et al. 1996; nathanson, et al., this volume). None of the patients were related or had known pathogenic mutations of the PRNP gene. The clinical presentations were atypical for sporadic CJD, since the disease began with sensory or psychiatric disturbances rather than dementia or motor abnormalities. In addition, the neuropathologic features in all of the cases were identical. Moreover, they were markedly different from other cases of sporadic, iatrogenic, or familial CJD in elderly or young patients (Monreal et al. 1981; Koch et al. 1985; Kulczycki et al. 1991; Kitamoto et al. 1992; Billette de Villemeur et al. 1994).

James Ironside and Jeanne Bell, the neuropathologists in the UK National CJD Surveillance Unit who reviewed all of the nvCJD cases (Ironside 1996; Will et al. 1996), emphasize that the most remarkable feature of nvCJD is the massive amount of protease-resistant PrP in the form of innumerable mature, PAS-reactive, PrP amyloid plaques, many with the features of kuru plaques, and the massive number of PrP primitive plaque-like deposits that are PAS nonreactive (Fig. 10). The number of PrP plaque deposits rivals that seen in the many GSS cases; but, it should be emphasized, this disease is classified as CJD because it is not dominantly inherited, because of the intense spongiform degeneration, and because of the presence of protease resistant PrP. Many of the mature PrP amyloid plaques are distinctive because they are located at the center of vacuoles, particularly in the cerebral cortex (Fig. 10A). These have been

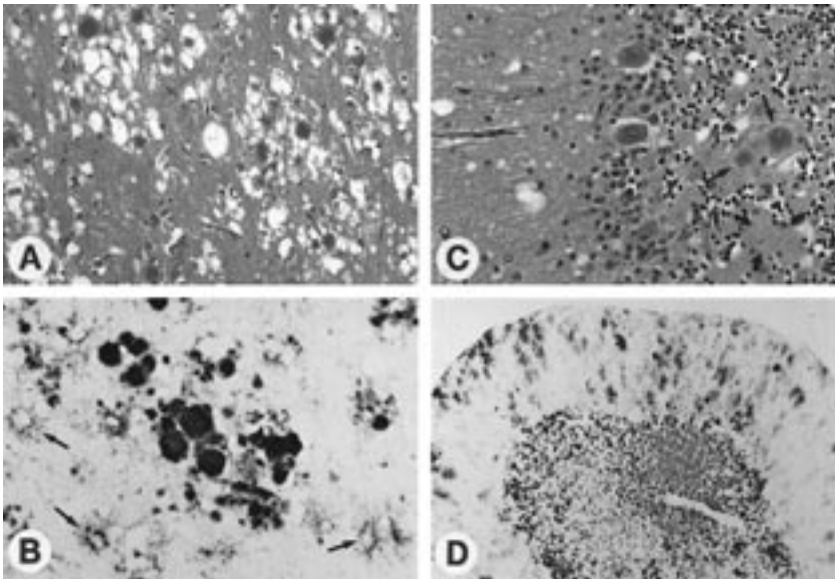


Figure 10 Main neuropathologic features of nvCJD. (*A*) Numerous 15–30-μm florid amyloid plaques and severe vacuolar degeneration in the cerebral cortex (PAS stain, 20× objective). (*B*) Some of the florid plaques in the cerebral cortex are arranged in clusters; in addition, some neurons and the proximal portions of their processes (*arrows*) are encrusted with PrP^{CJD} (PrP immunohistochemistry, hydrolytic autoclaving method, 40× objective). (*C*) Kuru plaques (*straight arrows*) and primitive plaques (*curved arrows*) in the granule cell layer and vacuolation of the molecular layer of the cerebellum (H&E, 40× objective). (*D*) Abundant PrP^{CJD} deposition in the granule cell and molecular layer of the cerebellar cortex (PrP immunohistochemistry, hydrolytic autoclaving method, 10× objective).

designated "florid plaques." Another distinctive feature is the clustering of mature and immature plaques, particularly well seen by PrP immunohistochemistry using the hydrolytic autoclaving technique (Fig. 10B). There were multiple less well-defined protease-resistant PrP deposits scattered throughout the gray matter. Some of the latter surround nerve cell bodies and proximal portions of neuritic processes (Fig. 10B). Although all regions of the cerebral cortex contain PrP plaques, the greatest concentrations appear to occur in the occipital lobe and cerebellum. In the cerebellum, typical kuru-type plaques can be found in the granule cell layer (Fig. 10C) and occasionally in the molecular layer of the cerebellar cortex. However, the number of kuru plaques seen in H&E and PAS stains

are relatively small compared to the uniquely massive deposits of PrP^{CJD} in the molecular layer and granule cell layer (Fig. 10D). Many of these are PAS nonreactive and do not have the classic appearance of amyloid, particularly in the molecular layer. The amount of PrP plaque deposition in nvCJD is far more extensive than in typical sporadic CJD or HGH iatrogenic CJD (Ironside 1996). Furthermore, the nvCJD plaques do not resemble plaques found in GSS.

The most intense spongiform degeneration in nvCJD occurs in the basal ganglia and thalamus with particularly marked astrocytic gliosis in the latter. The massive accumulation of protease-resistant PrP in the cerebellum is associated with severe spongiform degeneration, neuronal loss (Fig. 10C), and reactive astrocytic gliosis.

Another major difference between nvCJD and sporadic CJD is the presence of disease-associated PrP outside the central nervous system. This has been detected both by immunoctyochemistry and Western blotting in lymphoid tissues including the tonsil, lymph nodes, spleen, and appendix (Hilton et al. 1998; Hill et al. 1999). In one case, PrP immunoreactivity was detected in follicular dendritic cells in the appendix, which had been removed 8 months before the onset of neurological disease (Hilton et al. 1998). This observation has given rise to concerns that lymphocytes may be involved in the transport of disease-associated PrP, which in turn has given rise to concerns over the potential safety of blood and blood products in the UK.

The hypothesis that nvCJD is causally related to BSE has received some support from observations of experimental BSE transmission in macaque monkeys (Lasmézas et al. 1996), which showed neuropathological changes similar to those in nvCJD (Fig. 11). Subsequent strain typing studies (Bruce et al. 1997) indicated that the BSE and nvCJD agents are indistinguishable on primary transmission into syngenic mice. Additional biochemical studies have demonstrated that the predominant PrP isoform in nvCJD and BSE carries a characteristic glycosylation pattern, although this is not entirely specific for nvCJD and has been reported in other human prion diseases including FFI (Hill et al. 1997). At present, it is difficult to predict the likely numbers of future nvCJD cases. One of the major determinants of any likely epidemic is the incubation period of the disease, which at present is uncertain, and thus it is not possible to predict with confidence future disease trends (Cousens et al. 1997).

Comparative pathology of BSE in animals and humans has indicated that BSE prions are not amyloidogenic when transmitted among cattle (Wells and Wilesmith 1995) and do not cause amyloidosis in Tg mice expressing bovine Bo PrP^{C}, designated $Tg(BoPrP)Prn^{0/0}$ mice (Scott et al.

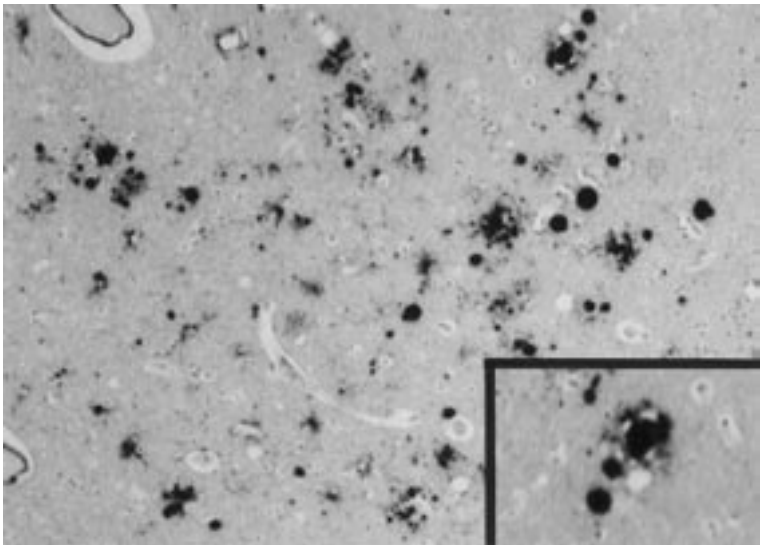


Figure 11 Cerebral cortex of a macaque monkey following inoculation with BSE prions contains numerous protease-resistant PrP plaques that vary in diameter from 5 to 25 μm. *Inset*: Several plaques are within or surrounded by vacuoles, similar to the florid plaques in nvCJD. Magnification of inset is twice that of the main photomicrograph. (This figure was adapted from a 35-mm transparency generously provided by Dr. James Ironside, Neuropathology Laboratory, CJD Surveillance Unit, University of Edinburgh, Edinburgh, United Kingdom.)

1997). Interestingly, the confinement of PrP accumulation to the brain stem and thalamus was similar in cattle and in Tg(BoPrP)/Prnp$^{0/0}$ mice. Furthermore, BSE-related diseases in felines and other zoo ungulates (Kirkwood and Cunningham 1994; Wyatt et al. 1991; Willoughby et al. 1992) have indicated that amyloid plaque formation is not a characteristic feature of these diseases. However, the pathological phenotype of prior diseases is dependent on both host and agent characteristics, and further studies are required to address the interesting question of amyloid plaque formation in nvCJD.

Fatal Familial Insomnia (D178N, M129)

This dominantly inherited form of prion disease is an example from nature of the relationship between the amino acid sequence of PrP and selective targeting of neurons for degeneration. Specifically, in FFI, a

D178N mutation occurs on an allele that encodes a methionine at position 129, and the major pathology is limited to the thalamus. In contrast, in familial CJD(D178N, V129) in which a valine is encoded at 129 on the mutated allele, the pathologic changes are widespread, but do not include the thalamus (Goldfarb et al. 1992). FFI was recently transmitted to rodents (Tateishi et al. 1995).

The clinical and neuropathologic data have been obtained from studies of two large kindreds with FFI (Manetto et al. 1992; Medori et al. 1992a,b). The age of onset is between 35 and 61 years. FFI progresses relatively rapidly over a period of 7–36 months. The primary sleep/wake disturbances are progressive insomnia followed by complex hallucinations and then stupor and coma. In addition, there are autonomic disturbances including hyperhidrosis, pyrexia, tachycardia, hypertension, and irregular breathing. The principal motor findings are ataxia, spontaneous and evoked myoclonus, dysarthria, and pyramidal signs. In contrast, patients with familial CJD(D178N, V129) present with dementia but not insomnia.

Neuropathology

Pierluigi Gambetti at Case Western Reserve University has made all of the original neuropathologic observations of FFI and has recently reviewed the clinical, neuropathologic, and molecular differences between FFI and CJD(D178N, V129) (Gambetti et al. 1995). In FFI, the most severe neuropathology occurs in the anterior ventral and medio-dorsal nuclei of the thalamus, where there is over 50% loss of neurons with reactive astrocytosis. The cerebral cortex is little affected, with minimal to mild patchy astrocytosis. Mild spongiosis of the cerebral cortex was largely confined to layers 2–4 in one case. In the cerebellum, there is swelling of the proximal axon (torpedoes) of many Purkinje cells and mild loss of Purkinje cells and granule cells. There are no amyloid plaques. There is a greater than 50% loss of neurons from the inferior olives.

FFI patients who are homozygous at codon 129 for methionine have a shorter duration of disease (7–18 months) in contrast to heterozygotes (20–35 months). There is thalamic and olivary atrophy in both. However, there is more widespread spongiosis of the cerebral cortex in homozygotes, whereas spongiosis and gliosis are occasional and focal in the entorhinal cortex and absent from the neocortex in heterozygotes.

Protease-resistant PrP^{CJD} is detected in both FFI and CJD(D178N, V129). In FFI of short duration, significant amounts of PrP^{CJD} are detected in many brain regions that lack pathologic changes, consistent with the hypothesis that abnormal PrP accumulation precedes and causes neuronal

dysfunction and neuropathology. There appears to be a greater accumulation of PrP^{CJD} in the brain stem of homozygous patients compared to heterozygous, and this may explain in part the shorter time to death in the former.

Gerstmann-Sträussler-Scheinker Syndromes

GSS is one of the rarest neurodegenerative diseases known with an incidence of 2–5 individuals per 100 million (Hsiao and Prusiner 1991). The diagnosis of GSS requires dominant inheritance, a mixture of cognitive and motor disturbances, and widespread deposition of PrP amyloid plaques. Like CJD, GSS is not a single disease. There are six dominantly inherited disorders to which this name has been given, each with a different clinical presentation, different neuropathologic features, and linkage to a different mutation of the PRNP gene. A codon 102 mutation resulting in a leucine for proline substitution (Hsiao et al. 1989b) is linked to the original GSS family described by Gerstmann, Sträussler, and Scheinker, GSS(P102L) (Kretzschmar et al. 1991). Of the more than 20 known pathogenic PRNP mutations causing familial prion diseases, 5 mutations, in addition to the P102L, are associated with deposition of numerous PrP amyloid plaques: GSS(P105L) (Nakazato et al. 1991; Kitamoto et al. 1993b; Yamada et al. 1993), GSS(A117V) (Doh-ura et al. 1989; Nochlin et al. 1989; Mastrianni et al. 1995), GSS(Y145Stop), GSS(F198S) (Ghetti et al. 1989, 1995; Hsiao et al. 1992), and GSS(Q217R) (Hsiao et al. 1992). Three generalities can be made about GSS syndromes based on combined neurohistological, immunohistochemic, biochemical, and molecular genetic analysis (Ghetti et al. 1996a). (1) The clinical features associated with each mutation are different. (2) Each mutation is associated with different morphologic subtypes of PrP plaques. (3) Some mutations are associated with significant neurofibrillary tangle degeneration of neurons and neuritic plaque formation similar to Alzheimer's disease.

GSS(P102L)

This primarily ataxic form of GSS was the first to be described by the neurologists Gerstmann and Sträussler and the neuropathologist Scheinker in 1936 (Gerstmann et al. 1936). Seitelberger (1981b) defined it as "spinocerebellar ataxia with dementia and plaque-like deposits." The P102L mutation has been found in about 32 families in Austria, Great Britain, Canada, the US, France, Germany, Italy, Israel, and Japan (Doh-ura et al. 1989; Goldgaber et al. 1989; Hsiao et al. 1989a; Kretzschmar et

al. 1992; Goldhammer et al. 1993). Three out of 7 GSS cases have been transmitted to nonhuman primates (Masters et al. 1981a).

Neuropathology. The pathologic hallmark of ataxic GSS is the multicentric amyloid plaque (GSS plaque). These are most numerous in the molecular layer of the cerebellar cortex but are also found in large numbers in the cerebral cortex (Fig. 3A). The GSS plaque consists of a larger central mass of amyloid surrounded by smaller satellite deposits. They bind Congo Red dye, which displays green birefringence in polarized light (Fig. 3B). The molecular layer of the cerebellum in some cases contains numerous "amorphous" or "primitive" plaques that can be 150–200 μm in diameter (Fig. 3C) (Kuzuhara et al. 1983). The latter do not fulfill the criteria for mature amyloid, since they are weakly PAS-positive and rarely show green birefringence with the Congo Red stain. Unicentric kuru-type plaques can also be found. The GSS plaques, kuru plaques, and primitive plaques immunostain specifically with PrP antibodies (Kitamoto et al. 1986; Roberts et al. 1986; Snow et al. 1989; DeArmond and Prusiner 1995); the reaction can be augmented by the HCl-autoclave technique (Fig. 3C) (Muramoto et al. 1992). White matter degeneration resembling that of other system degenerations, such as Friedreich's ataxia, is a prominent feature in most cases (Seitelberger 1981a). Neuronal loss is scattered throughout the brain and spinal cord. Spongiform degeneration is variable in degree and extent and can be difficult to detect. Neurofibrillary tangles, if present, are usually found in numbers and in locations consistent with the patient's age.

Tg(GSS-P101L) Mice

Tg mouse lines expressing a mutant mouse PrP that carries the codon 102 mutation linked to GSS in humans, designated Tg(MoPrP-P101L), have verified that this PrP amino acid sequence is amyloidogenic and causes a GSS syndrome (Hsiao et al. 1990). Codon 101 in mice is analogous to codon 102 in humans. Tg mouse lines expressing high (H) levels of the transgene product developed a spontaneous neurodegenerative disorder with formation of numerous PrP immunopositive amyloid plaques in multiple brain regions similar to those found in human GSS (Fig. 12). Thus, the characteristic neuropathologic features of GSS were duplicated in Tg(MoPrP-P101L)-H mice. Tg mice expressing low levels of MoPrP-P101L, Tg(MoPrP-P101L)-L mice, did not develop a neurodegenerative disease spontaneously. Disease in high expressors of the muPrP resulted in the formation of prions (Hsiao et al. 1994). Brain homogenates from

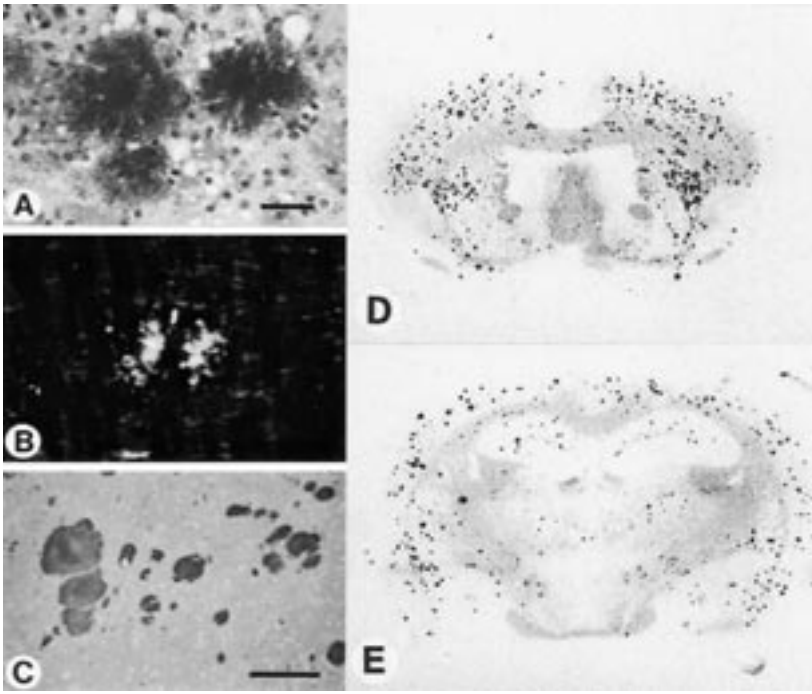


Figure 12 Numerous GSS-type amyloid plaques develop spontaneously throughout the brain in Tg(GSS-P101L) mice. The majority of the plaques are PAS positive (*A*) and bind Congo Red dye that displays green-gold birefringence in polarized light (*B*) and, therefore, fulfill the criteria for amyloid. (*C*) The plaques are PrP immunopositive by the hydrolytic autoclaving method. The histoblot method shows numerous PrP amyloid plaques in both gray and white matter: level of the head of the caudate nucleus (*D*) and level of the hippocampus and thalamus (*E*). Bar in *A*, 50 μm and in *C*, 100 μm. (Adapted from Hsiao et al. 1994.)

spontaneously ill Tg(MoPrP-P101L)-H mice transmitted to 7% of Syrian hamsters into which it was inoculated and to 40% of Tg(MoPrP-P101L)-L mice. Low rates of transmission of GSS to nonhuman primates were also found for the human disorder: in the National Institutes of Health series (Brown et al. 1994), the rate of transmission to primates was highest (85%) for CJD(E200K), 70% for CJD(D178N,V129), and relatively unsuccessful (38%) for GSS(P102L). In a subsequent study of transmission of mutant prions derived from Tg(MoPrP-P101L)-H mice, it was discovered that success rate and incubation time were influenced greatly by wild-type $MoPrP^{C}$ (Telling et al. 1996a). Thus, when low levels of

MoPrP-P101L were expressed in combination with wild-type $MoPrP^C$, incubation times with mutant prions were about 350 days. In contrast, when MoPrP-P101L was expressed by itself, in the absence of $MoPrP^C$, all of the Tg mice expressing low levels of MoPrP-P101L became ill at about 200 days. These results argue that $MoPrP^C$ can have an inhibitory effect on the interaction of an infecting prion with a transgene, presumably by competition of $MoPrP^C$ and MoPrP-P101L for protein X. In these studies, successful transmission of MoPrP-P101L prions was 100%, possibly because the Tg mice were constructed differently (modified ILn/J mouse cosmid in this study versus cosSHa.Tet vector in the earlier study).

GSS (A117V)

Originally classified as CJD, it was later reclassified GSS because of the number and location of PrP immunopositive amyloid plaques (Nochlin et al. 1989). The A117V mutation was first described in a French family (Doh-ura et al. 1989) and then in an American family of German descent (Hsiao et al. 1991a). The main clinical features include progressive dementia usually associated with dysarthria, rigidity, tremor, and hyperreflexia. Masked facies in several individuals plus tremor and rigidity suggests Parkinsonism. Ataxia and myoclonus are uncommon.

Neuropathology. The most striking neuropathologic feature is widespread amyloid plaque formation, of which four morphological types have been described (Nochlin et al. 1989). First, there are multiple multicentric GSS-type plaques consisting of 4 to more than 10 amyloid masses. The entire cluster of amyloid ranges from 150 to 500 μm in diameter. These are located in the neocortex, hippocampus, caudate nucleus, and putamen. Some are also present in the subcortical white matter. Second, there are typical kuru-type amyloid plaques. These are 20 to 70 μm in diameter and located principally in the white matter. Third, there are 50 to 150 μm unicentric amyloid plaques without radiating spicules at their periphery that resemble the cores of the neuritic plaques of Alzheimer's disease; however, they do not have a halo of dystrophic neurites surrounding them. These are found in the neocortex, hippocampus, caudate nucleus, and putamen. Fourth, there are "amorphous" or "primitive" plaques that are as large as 250 μm in greatest dimension, similar to those seen in some ataxic-GSS cases, which are located in the deep cerebral cortical layers and in the molecular layer of the cerebellar cortex (Fig. 13) (Tranchant et al. 1992; Mastrianni et al. 1995). All of the plaques immunostain specifically with PrP antibodies but not with Aβ antibodies. Spongiform degeneration is not found in cor-

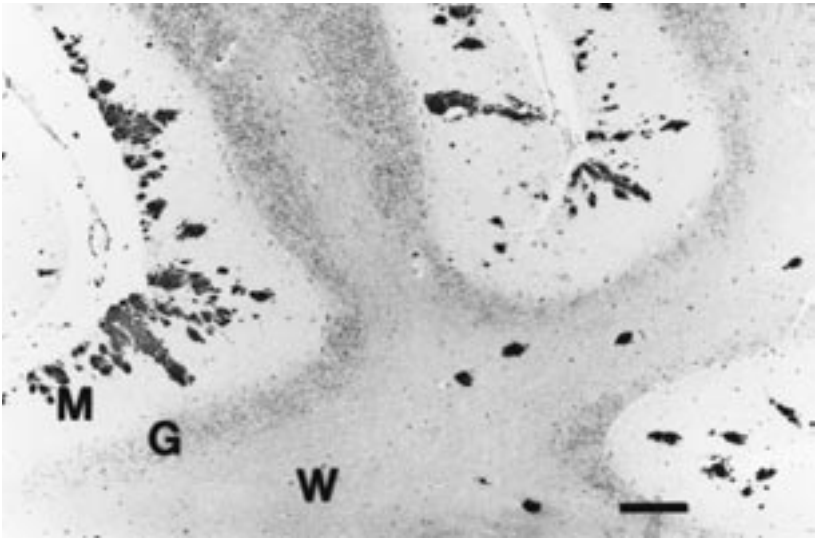


Figure 13 In GSS(A117V), numerous large primitive PrP plaques, 150–200 μm in longest dimension, deposit in the molecular layer of the cerebellar cortex. PrP deposits can also be seen in the white matter. PrP immunohistochemistry by the hydrolytic autoclaving technique with hematoxylin counterstain. (W) White matter; (M) molecular layer; (G) granule cell layer. Bar, 200 μm.

tical or subcortical gray matter. Astrocytosis is primarily associated with amyloid plaques in the neocortex. The caudate nucleus, putamen, globus pallidus, and thalamus show severe neuronal loss with astrocytosis. There is mild neuronal loss in the substantia nigra.

GSS(F198S) with Neurofibrillary Tangles

The clinical features of this familial prion disorder are ataxia, Parkinsonism, and dementia (Farlow et al. 1989). Individuals carrying the mutation are normal until their mid-30s to early 60s. The presenting symptoms are gradual loss of short-term memory and progressive clumsiness, which may be exaggerated when the individual is under stress or tired. The symptoms can progress rapidly over a period of a year or slowly over 5 years. Rigidity and bradykinesia generally occur late in the disease, at which time dementia also worsens. There is little or no tremor. The rigidity and bradykinesia improves with L-dopa analogs. Without treatment of the Parkinsonism, the patient dies within 1 year, and with treatment, succumbs to intercurrent illness in 2–3 years. The onset of dis-

ease is about 10 years earlier in patients who are homozygous for the valine polymorphism at codon 129 compared to patients with Val/Met heterozygosity (Dlouhy et al. 1992).

Neuropathology. Bernardino Ghetti has fully described the neurohistopathologic features of this unique prion disorder (Ghetti et al. 1989). The neuropathologic changes in GSS(F198S) are remarkable because there are numerous neuritic plaques and intraneuronal neurofibrillary tangles of the type characteristic of Alzheimer's disease. Like Alzheimer's disease, the neuritic plaque consists of an amyloid core surrounded by τ-protein immunopositive dystrophic neurites; however, the amyloid core in GSS(F198S) is PrP immunopositive and Aβ peptide negative (Fig. 14). Like other PrP amyloids, it is composed of highly truncated PrP peptides (Fig. 4). As with other forms of GSS, large numbers of multicentric GSS-type and unicentric plaques are located throughout the cerebral and cerebellar cortex. In older patients, Aβ immunoreactivity could be found at the periphery of the PrP amyloid deposits (Bugiani et al. 1993). Nerve cell loss occurs in almost all cerebral cortical regions, but is most severe in the cerebellar cortex, where there is marked loss of Purkinje cells. Neuronal loss is also prominent in many subcortical nuclei including the substantia nigra, red nucleus, inferior olive, and dentate nucleus. Spongiform degeneration is minimal or focal.

Other GSS Syndromes with Neurofibrillary Tangles

Neurofibrillary degeneration significantly more severe than that consistent with aging occurs with other PRNP mutations causing GSS-like cerebral amyloidosis. Large numbers of neurofibrillary tangles are found in cortical and subcortical regions in patients with the Q217R (Hsiao et al. 1992) and the Y145Stop (Kitamoto et al. 1993a) mutations in addition to the F198S mutation. Smaller numbers are found in some patients with the P105L (Kitamoto et al. 1993b; Yamada et al. 1993) and A117V mutations (Mohr et al. 1994; Ghetti et al. 1995). In Q217R patients, Aβ immunoreactivity was also found in the periphery of PrP amyloid plaques (Ikeda et al. 1992). Numerous neurofibrillary tangles and neuropil threads are found in GSS (Y145Stop); however, no Aβ immunoreactivity was found to be associated with the PrP amyloid plaques. The neurofibrillary tangles in GSS syndromes are composed of phosphorylated τ-protein as they are in Alzheimer's disease (Ghetti et al. 1996a). The mechanisms of neuritic plaque formation and neurofibrillary degeneration of neurons in GSS and Alzheimer's disease are unknown. However, because a single protein

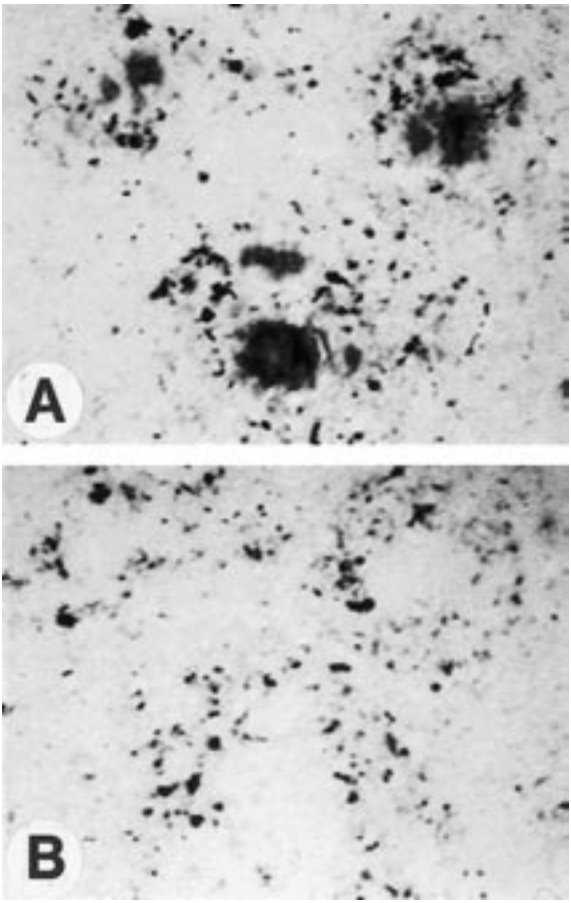


Figure 14 In GSS(F198S), neuritic plaques form that are similar to those in Alzheimer's disease except that the amyloid cores contain PrP peptides and do not contain the Aβ peptide. (*A*) Double immunoperoxidase immunohistochemistry shows that the amyloid cores of neuritic plaques contain PrP (*brown* reaction product) and the dystrophic neurites contain τ-protein (*blue* reaction product). (*B*) The cores of neuritic plaques do not immunostain for the Aβ peptide by double immunostaining. (This figure was adapted from a 35-mm transparency generously provided by Dr. Bernardino Ghetti, Indiana University, Indianapolis and was previously published [by permission of Oxford University Press] in DeArmond and Prusiner 1997.)

abnormality is the cause of GSS, whereas multiple protein abnormalities cause Alzheimer's disease, it is possible that an understanding of the mechanisms neuritic plaque and neurofibrillary tangle formation will emerge first from investigations of GSS.

GSS(Y145Stop): GSS with Prominent Vascular Amyloid

This mutation at codon 145 results in the formation of a TAG stop codon and the synthesis of a highly truncated form of mutant PrP that has a methionine at residue 129 (Kitamoto et al. 1993a; Ghetti et al. 1996b). A Japanese patient with this form of GSS presented with memory disturbance and disorientation at age 38 that progressed over a 21-year period to severe dementia.

Neuropathology. The most striking characteristic of this disorder is the predominance of 10–20 μm spherical PrP amyloid deposits that coat small and medium-sized vessels in the brain parenchyma and in the leptomeninges. Electron microscopy of these plaques shows amyloid fibrils radiating away from the vessel wall (Ghetti et al. 1996a). There was no spongiform degeneration, probably due to the severe neuronal loss with accompanying severe astrogliosis, cerebral atrophy, and hydrocephalus ex vacuo. Some remaining neurons contained τ-protein-immunopositive neurofibrillary tangles.

DIAGNOSTIC PROCEDURES FOR PRION DISEASES

Consensus criteria for the diagnosis of prion diseases have been established (Kretzschmar et al. 1996). A diagnosis of "probable CJD" can be made by biopsy or at autopsy if the quartet of clinical signs and symptoms are present and spongiform degeneration of gray matter is found in the absence of other confounding pathology. In the National Institutes of Health series, 52 of 55 autopsy-verified cases of CJD (95%) showed the characteristic spongiform degeneration of cerebral cortex in surgical biopsy specimens (Brown et al. 1994). The "definitive diagnosis" of a human prion disease requires that one of the four following additional criteria be met: presence of PrP amyloid plaques, transmission of spongiform encephalopathy to animals, presence of PrP^{Sc} or PrP^{CJD}, or presence of a pathogenic PrP gene mutation.

PrP Amyloid Plaques

Finding kuru-type "spiked ball" amyloid plaques in the cerebellum is diagnostic of CJD (Fig. 2); however, they are found in only 5–10% of CJD cases. Numerous PrP immunoreactive amyloid and/or preamyloid plaques in dominantly inherited neurodegenerative diseases are diagnostic of a GSS syndrome (Figs. 3, 10, 14).

Presence of PrP^{Sc} or PrP^{CJD}

Proteinase-resistant PrP^{Sc} is only found in animal prion diseases, and PrP^{CJD} is only found in human prion diseases. PrP^{CJD} has been detected by Western transfer (Bockman et al. 1985; Brown et al. 1986), dot-blot analysis of homogenates of cerebral cortex from CJD and GSS brains (Serban et al. 1990), peroxidase immunohistochemistry with PrP-specific antibodies on tissues pretreated by the hydrolytic autoclaving technique (Muramoto et al. 1992), and the histoblot procedure (Taraboulos et al. 1992b).

In scrapie, PrP^{Sc} may be found in both gray and white matter; in contrast, in CJD, PrP^{CJD} is confined to the gray matter. PrP^{CJD} is distributed uniformly in all layers of the neocortex in most cases, but it may be con-

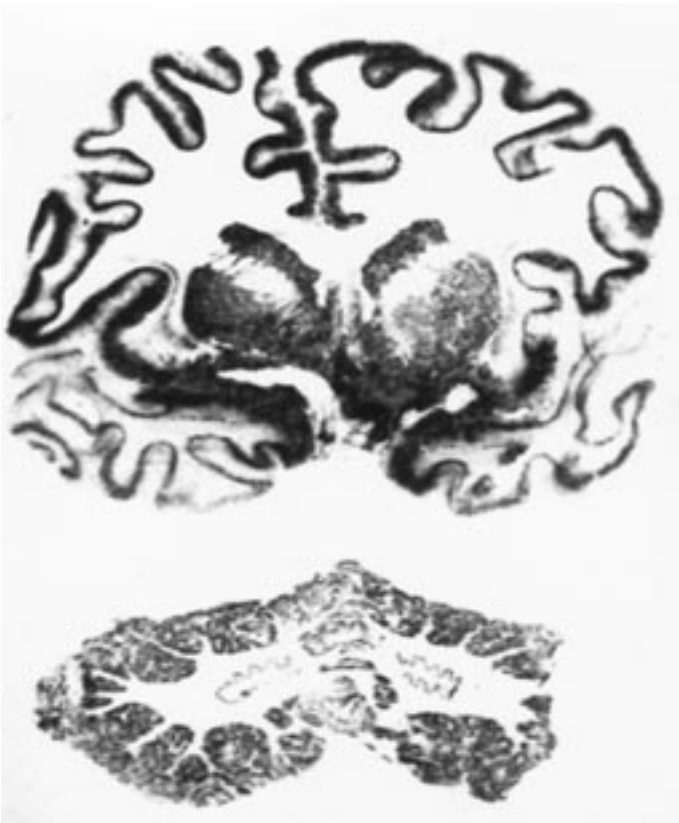


Figure 15 In CJD, histoblot analysis for PrP^{CJD} shows that it accumulates in multiple brain regions. Depicted is accumulation in the cerebral cortex, basal ganglia, and cerebellum. White matter is negative.

fined to deeper cortical layers, particularly in ataxic forms of CJD. In the terminal stages of CJD, PrP^{CJD} is found in most brain regions including the cerebral cortex, basal ganglia, thalamus, brain stem, cerebellum, deep cerebellar nuclei, and spinal cord (Fig. 15). Non-amyloid protease-resistant PrP is not uniformly distributed in the gray matter neuropil in GSS cases as it is in CJD; rather, in GSS, when it occurs, it often deposits focally in primitive plaque-like masses (Fig. 13).

Although histoblots reveal the regional distribution of PrP^{Sc} and PrP^{CJD}, the cellular compartments in which they are located are not well defined. In retrospect, our earliest modified PrP immunohistochemical techniques accurately revealed the cellular distribution of PrP^{Sc} in scrapie-infected brain tissue (Fig. 16). PrP^{Sc} was localized to presynaptic bouton-like structures in and around nerve cell bodies. It was also located within some nerve cell bodies where it appeared as granular deposits, suggesting localization to endosomes and/or lysosomes. Today, we rou-

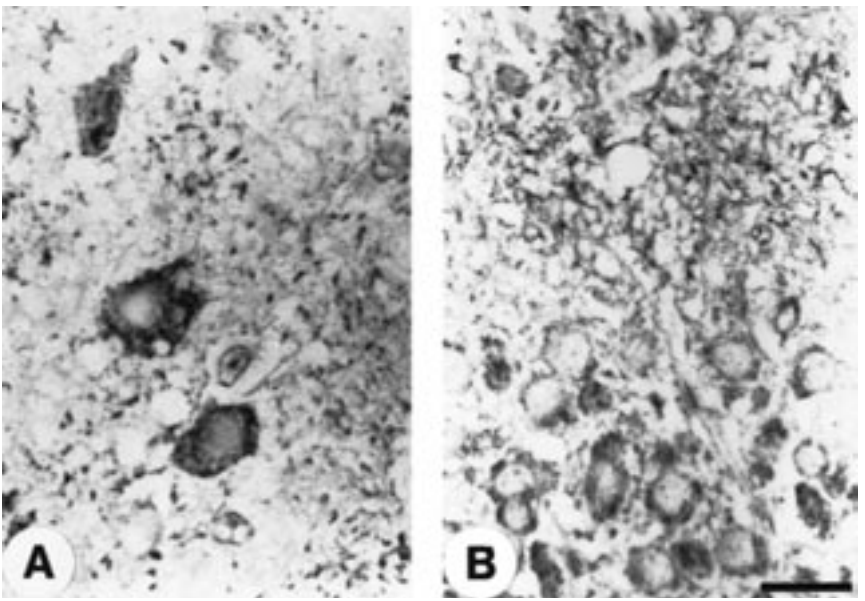


Figure 16 PrP^{Sc} is localized to synapse-like structures in the neuropil adjacent to nerve cell bodies and appears as granular deposits within some nerve cell bodies in Syrian hamsters inoculated with the Sc237 strain of prions. (*A*) Red nucleus. (*B*) CA3 region of the hippocampus. McLean's periodate-lysine-paraformaldehyde fixation and glycomethacrylate embedding. Bar, 20 μm. (Reprinted, with permission, from DeArmond et al. 1987.)

tinely use the hydrolytic autoclaving technique to localize PrP^{Sc} in animal and PrP^{CJD} in human aldehyde-fixed, paraffin-embedded brain sections (Muramoto et al. 1992). In human brain sections, immunostaining for PrP^{CJD} is confined to the gray matter as it is in histoblots (Fig. 17A). In our experience, the intensity of immunostaining varies considerably in formalin-fixed tissue sections even with hydrolytic autoclaving, whereas the staining in histoblots is more reproducible. Hydrolytic autoclaving reveals two immunostaining patterns in the cerebral cortex: first, a diffuse or synaptic pattern that consists of granular immunostaining of the gray matter resembling that seen with synaptophysin antibodies (Fig. 17B) and second, a coarse or primitive plaque-like pattern that is often particularly intense around vacuoles in the neuropil (Fig. 17C). Direct evidence that PrP^{Sc} and PrP^{CJD} accumulate in synaptic boutons is the report that PrP^{CJD} is identified by biochemical analysis of purified synaptosomes from CJD cases (Kitamoto et al. 1992).

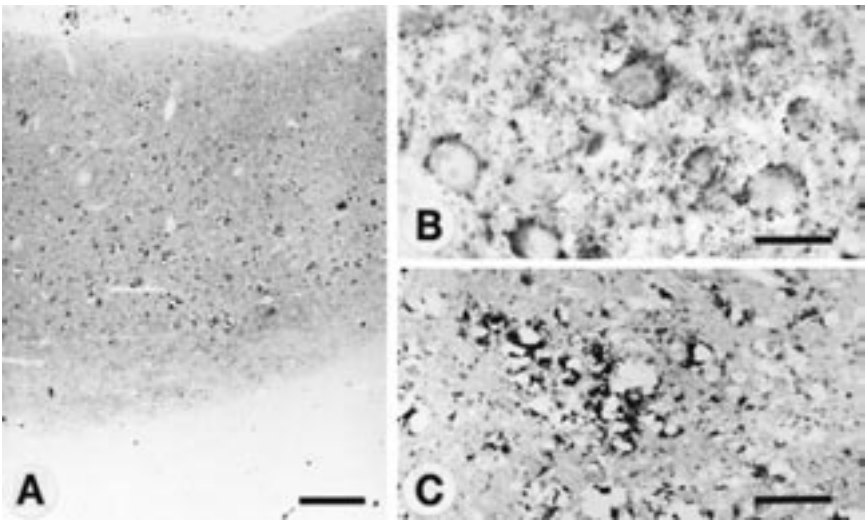
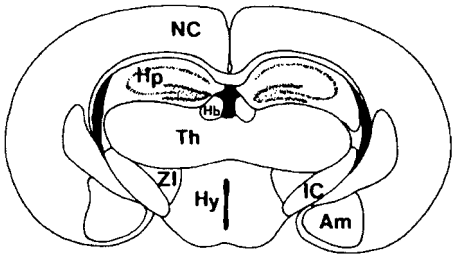


Figure 17 CJD: PrP immunohistochemistry of formalin-fixed, paraffin-embedded tissue sections by hydrolytic autoclaving technique. (*A*) PrP^{CJD} is deposited throughout the entire thickness of the cerebral cortex. (*B*) Higher magnification shows the characteristic synaptic pattern of PrP^{CJD} deposition at the periphery of nerve cell bodies and in the neuropil between nerve cell bodies. (*C*) Coarse masses of PrP^{CJD} surround many vacuoles in some CJD cases, but not all. Bar in *A*, 150 μm, in *B*, 25 μm, and in *C*, 50 μm. (Adapted from DeArmond and Prusiner 1995.)

Transmission to Animals

In the past, the definitive diagnosis of sporadic, iatrogenic, or familial CJD required transmission to nonhuman primates. The incubation times for transmission of human prion diseases to primates ranges from 17 months for chimpanzees to 64 months for rhesus monkeys (Brown et al. 1994). Tg(MHu2M) mice, which express a chimeric Mo/HuPrP^{C}, are a more practical, efficient, and less expensive bioassay for this purpose, since CJD incubation times have been about 200 days (6–7 months) (Telling et al. 1994). In addition to their diagnostic usefulness, results being obtained from these mice are increasing our understanding of human prion strains and the prion disease phenotype.

Different reproducible patterns of PrP^{Sc} accumulation have been found in clinically ill Tg(MHu2M)/Prnp$^{0/0}$ mice following intracerebral inoculation with CJD prions from familial CJD(E200K) and FFI(D178N, *cis*-129M) (Telling et al. 1996b). The most intense PrP^{Sc} signal in mice-inoculated FFI prions was localized to thalamic nuclei with smaller amounts deposited in the thalamocortical pathway termination regions of the deeper layers of the frontal cortex (Fig. 18A). There was little or no PrP^{Sc} deposition in the brain stem (not shown). Remarkably, the thalamus is the site of the most severe neuropathologic changes in patients with FFI(D178N, *cis*-129M). In contrast, PrP^{Sc} accumulation in mice inoculated with familial CJD(E200K) prions was deposited in many brain regions including the thalamus, the hypothalamus, the entire thickness of the cerebral cortex, the striatum, and spinal cord, similar to the wide distribution of neuropathologic changes in the human disease counterpart (Fig. 18B). The PrP^{Sc} accumulation pattern following inoculation with sporadic CJD(M/M129) was intermediate with more regions involved than with FFI(D178N, *cis*-129M), but less than with familial CJD(E200K) (not shown). These data support the hypotheses that there are multiple strains of human prions, as there are for scrapie, and their properties remain relatively unchanged following passage from human to the Tg(MHu2M)/Prnp$^{0/0}$ mouse. We have not yet had a chance to analyze histoblots from subsequent passages of prions within Tg(MHu2M)/Prnp$^{0/0}$ mice to determine whether or not the PrP^{Sc} distribution phenotype remains unchanged. We suspect that it will remain unchanged because the properties and the characteristics of the PrP^{CJD}s in human brains and the respective PrP^{Sc}s from Tg(MHu2M)/Prnp$^{0/0}$ brains have remained unchanged after passaging from humans to Tg(MHu2M)/Prnp$^{0/0}$ mice and on subsequent serial passages in Tg(MHu2M)/Prnp$^{0/0}$ mice. Thus, proteinase K-resistant PrP from multiple sporadic and familial CJD cases, including familial



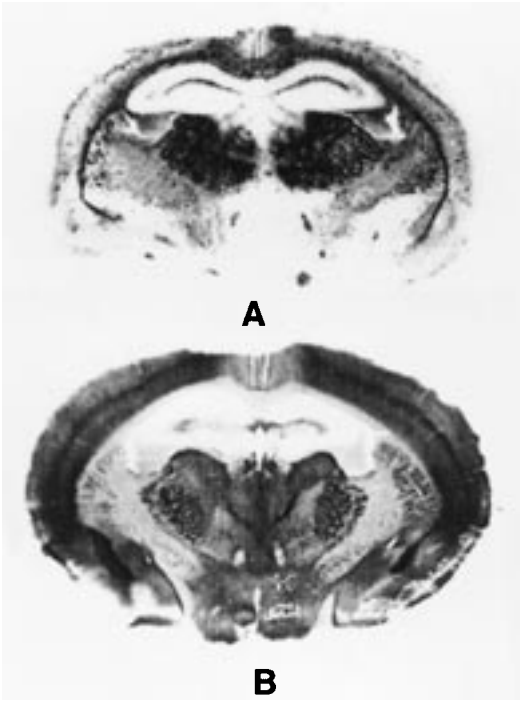


Figure 18 Histoblots of coronal sections through the thalamus and hippocampus of Tg(MHu2M) mouse brains inoculated with brain homogenates from patients with either (*A*) fatal familial insomnia or (*B*) familial CJD(E200K), show markedly different PrP^{Sc} accumulation patterns.

CJD(D178N, *cis*-129V), migrate as PrP immunopositive bands at 29, 27, and 21 kD and from FFI cases at 29 and 26 kD (Medori et al. 1992b; Monari et al. 1994). N-deglycosylation of the proteinase K-digested samples yield single PrP immunoreactive bands at 19 kD for FFI(D178N, *cis*-129M) and 21 kD for sporadic CJD and familial CJD(D178N, *cis*-129V).

The respective PrP^{Sc}s had the identical properties after serial transmission in Tg(MHu2M)/$Prnp^{0/0}$ mice. These results argue that distinct protease-resistant PrP glycoforms and conformers are formed in FFI(D178N, *cis*-129M), sporadic CJD, and CJD(D178N, *cis*-129V), and that those PrP properties are replicated precisely during interspecies passaging (e.g., humans to mice) and are, therefore, characteristic of prion strains. The distinct glycoforms probably originate from the population of neurons synthesizing the PrP^{C} that is converted to nascent PrP^{Sc} (see below); however, protease-resistant PrP's oligosaccharides do not appear to encode prion strain characteristics (DeArmond et al. 1997). The strain of a prion is most likely encoded in the conformation PrP, since that alone would account for differential digestibility of deglycosylated PrP comprising different prion strains (Telling et al. 1996b).

Mutations of the PrP Gene

If patients with a CNS disorder have a mutation in the PRNP gene that results in an amino acid substitution that is not a polymorphism, such as found at codons 129 and 219, then the diagnosis of prion disease is probably secure. If the mutation is one of the five known pathogenic mutations of the PRNP gene for which genetic linkage has been established, then the diagnosis is clearly secure. If the mutation is one of those already described for a prion disease pedigree, but for which genetic linkage has not yet been established, then the likelihood of it being pathogenic is increased. If a new mutation is found, then at least 100 people of similar ethnic background should be screened for the mutation to determine whether it is a polymorphism or a pathogenic change.

MECHANISMS OF NERVE CELL DEGENERATION (PATHOGENESIS)

The most characteristic and clinically relevant neuropathologic feature of prion diseases is vacuolar degeneration of neurons and their processes, particularly in synaptic regions, and its association with failure of synaptic transmission and nerve cell death. A second characteristic is that different populations of neurons are targeted for degeneration as a function of the strain of prion infecting an animal or specific PrP gene mutations. The reproducibility of the neuroanatomic distribution of nerve cell degeneration with each prion strain and each mutation of the PrP gene implies that each neuron population responds to these etiological factors in different ways. Although the clinical–neuropathologic features of sporadic CJD are diverse, the primarily cortical–striatal variant predominates. This

raises the possibility that neurons in those regions are more inherently suceptible to spontaneous conversion of PrP^{C} to PrP^{Sc}, or those regions are susceptible because they have a larger number of neurons than other brain regions and, therefore, a greater chance for spontaneous conversion.

The existence of two markedly different categories of prion disease, those that resemble scrapie and CJD and those that resemble GSS, has raised the possibility that PrP can cause nerve cell degeneration by two distinctly different mechanisms. Recent discoveries suggest that there are indeed two different mechanisms and that they are related to two different topologies of the PrP molecule: glycolipid-anchored versus transmembrane.

Glycolipid-anchored PrP

Prusiner's original studies of prion diseases focused on the composition of the scrapie agent and the mechanism of its propagation once it was found to be composed mostly, if not exclusively, of PrP^{Sc}. The results of those studies showed that nascent PrP^{Sc} is formed from PrP^{C} attached to the outer surface of the plasma membrane by a glycolipid anchor. Presumably, although not yet proven, most of the resulting nascent PrP^{Sc} is also glycolipid-anchored. This mechanism of formation of pathogenic PrP accounts for the propagation of prions and the neuropathologic changes in prion diseases acquired by infection and, most likely, also for prion diseases arising from spontaneous conversion of PrP^{C} to nascent PrP^{Sc}; e.g., cases of sporadic CJD, and in those forms of genetically determined prion diseases that result in CJD-like neuropathologic features. Common to these forms of prion disease is the accumulation of protease-resistant PrP^{Sc} or PrP^{CJD} in the brain, little amyloid plaque formation (except for nvCJD), and the propagation of highly infectious prions.

Transmembrane PrP

A second PrP mechanism of nerve cell degeneration was recently identified and may underlie the pathogenesis of some GSS syndromes. The Lingappa laboratory showed that expression of a doubly mutated PrP in transgenic mice, that results in the substitution of a KH to II at residues 110 and 111, causes PrP to adopt a transmembrane topography rather than the usual glycolipid-anchored topography (Hegde et al. 1998). These animals develop a spontaneous neurodegenerative disease characterized by spongiform degeneration and reactive astrocytic gliosis that resembles scrapie, but with no PrP plaques. PK digestion of brain homogenates from these animals showed that the mutated PrP molecules are significantly less

resistant to PK digestion than PrP^{Sc}, but more resistant than PrP^{C}, and that PK digestion results in the formation of highly truncated PrPs. That study also found that the mutated PrP from a case of GSS(A117V), with abundant PrP plaques, had similar PK properties. Interestingly, GSS(P102L) is also characterized by PK-sensitive PrP (Hsiao et al. 1989b, 1990). It is becoming evident that PrP can be made in more than one topographical form, glycolipid-anchored or transmembrane, and that the proportion of PrP molecules that are in one topography or the other depends in part on the amino acid sequence (Hegde et al. 1998).

MECHANISM OF NERVE CELL DEGENERATION BY CONVERSION OF GLYCOLIPID-ANCHORED PrP^{C} TO NASCENT PrP^{Sc}

The Conversion of PrP^{C} to Nascent PrP^{Sc}, Not Just the Presence of PrP^{Sc}, Is a Requirement for Neurodegeneration

The mechanisms of cell dysfunction in prion diseases must be placed into the context of what is known about the conversion of PrP^{C} to PrP^{Sc} and the subsequent accumulation of PrP^{Sc} in and around cells, because they are the driving forces that generate the neuropathologic abnormalities. The evidence that this is the case comes from studies of scrapie-infected cell lines in culture and from studies of PrP knockout mice.

Most of our knowledge of the metabolism and trafficking of PrP^{C} and PrP^{Sc} comes from investigations in the mouse N2a neuroblastoma cell line. Once infected with scrapie prions, the resulting ScN2a cell line retains infectivity chronically and on all subsequent passages in culture (Butler et al. 1988). PrP^{C} is constitutively expressed in both N2a and ScN2a cells. Ninety percent of the cell's PrP^{C} becomes attached to the outer surface of the cells by its GPI anchor following its synthesis and passage through the Golgi complex (Borchelt et al. 1990). Like other GPI-anchored proteins, PrP^{C} re-enters the cell through cholesterol-rich caveolae (Ying et al. 1992). About 10% of the endocytosed PrP^{C} is degraded with each endocytic cycle. The remainder is recycled to the cell surface along with other proteins and lipids of the endosome. In pulse-chase experiments, PrP^{C} in uninfected cells is rapidly labeled by a radioactive amino acid tracer and appears to be completely degraded with loss of the tracer in about 6 hours (Caughey et al. 1989; Borchelt et al. 1990). In scrapie-infected ScN2a cells, labeling of PrP^{Sc} is delayed by about 1 hour after the pulse and increases to a maximum during the time period when the PrP^{C} pool loses its tracer (Borchelt et al. 1990). Over the next 24 hours, there is little or no loss of tracer from the PrP^{Sc} pool. These

results argue that PrP^{Sc} is derived from PrP^{C}, but, unlike PrP^{C}, PrP^{Sc} is not degraded and accumulates in the cell. The PrP^{C} precursor pool from which PrP^{Sc} is formed must reach the cell surface since blocking PrP^{C} export from the ER/Golgi complex to the plasma membrane inhibits formation of PrP^{Sc} (Taraboulos et al. 1992a) and since exposure of scrapie-infected cells to phosphatidylinositol-specific phospholipase C (PIPLC), which releases PrP^{C} from the cell surface, also inhibits formation of PrP^{Sc} (Caughey and Raymond 1991). Details of where PrP^{Sc} becomes distributed in cells are still being determined. Immunoelectron microscopy indicated that PrP^{Sc} accumulates in secondary lysosomes (McKinley et al. 1991b). More recently, we found that a large proportion of nascent PrP^{Sc} accumulates in caveolae-like domains (CLDs) (Vey et al. 1996). Eighty to ninety percent of plasma membrane PrP^{C} is also localized to CLD regions (Vey et al. 1996; Wu et al. 1997). The only other location in the cell culture definitely known to contain PrP^{Sc} is the conditioned culture medium, which appears to accumulate PrP^{Sc} as a result of excretion from infected cells or as a result of death of infected cells. The site where exogenous PrP^{Sc} comes into contact with PrP^{C} and where PrP^{C} is converted to nascent PrP^{Sc} is not known; however, the colocalization of PrP^{Sc} and PrP^{C} in CLDs suggests that they may be the site of conversion.

Mice in which the PrP gene has been knocked out, $Prnp^{0/0}$ mice (Büeler et al. 1992), have yielded strong support for the concept that PrP^{Sc} is derived from PrP^{C}, since no PrP^{Sc} is formed, no prions propagated, nor neuropathologic changes developed in these mice. Both acute and chronic exposure of the brain in PrP knockout mice to prions has failed to propagate prions or to cause neuropathologic changes (Büeler et al. 1992, 1993; Prusiner et al. 1993; Brandner et al. 1996). These findings indicate that exogenously derived PrP^{Sc} by itself is not pathogenic; rather, in order for PrP^{Sc} to cause neuronal degeneration, it must be derived from PrP^{C}. It is likely that when nascent PrP^{Sc} is derived from glycolipid-anchored PrP^{C}, it enters a cellular compartment where it can disrupt functions that PrP^{Sc} in the extracellular space cannot. It is possible that PrP^{Sc} must be anchored to membranes by a glycolipid anchor and that it can only do so if it is derived from glycolipid-anchored PrP^{C}.

The Prion Disease Phenotype Is a Function of Both the Infecting Prion Strain and the Host's Response to the Prion Strain

One of the most fundamental principles of microbiology is that the infectious disease phenotype is determined both by the infectious agent and by

the host's response to it. Thus, conventional agents such as bacteria, viruses, fungi, and parasites attach to specific intracellular and extracellular regions to which they have become adapted for nourishment and proliferation. The host has evolved different immune and inflammatory responses to each pathogenic organism. For example, the host responds to pyogenic bacteria in the subarachnoid space, such as *Neisseria meningitidis*, by an intense neutrophil reaction that, in combination with proliferation of bacteria, can lead to brain swelling, venous infarcts, and cerebral herniation that cause death in hours to days. In contrast, the host response to *Mycobacterium tuberculosis* in the subarachnoid space is a slowly evolving lymphocytic–histiocytic–fibroblastic reaction that leads to obstructive hydrocephalus, arteritis, and arterial strokes that cause death in weeks to months.

The prion/host response complex that generates the clinical and neuropathologic features in prion diseases does not appear to involve a host immune response. To date, the only host factor known to influence the prion disease phenotype is the PrP expressed by the host. The parameters of the prion disease phenotype most commonly used to identify and classify prion strains are the host species barrier to infection, incubation time, the intensity and distribution of neuropathologic spongiform degeneration, and whether or not amyloid plaques form. Our understanding of the roles played by the prion and by the host in modulating these disease parameters have emerged slowly over the past 15 years in parallel with the discovery of how prions propagate. By viewing prion diseases from the perspective of the neuropathologic changes, we have learned that host-determined variations in PrP^{C} play as important a role as the conformation of PrP^{Sc} comprising an infecting prion. The role of PrP^{C} in prion biology cannot be overemphasized, particularly for understanding the mechanism of prion strain-determined variations in the disease phenotype. Indeed, the critics of the protein-only hypothesis often argue that a protein cannot encode all of the information necessary to account for all of the prion strain-specific variations in the disease phenotype; in this regard, they may be correct. However, these critics do not differentiate between the coding of strain information in the prion and the combination of prion and host factors that actually generate the disease phenotype.

Our current understanding of the formation of PrP^{Sc} by neurons is that it involves four factors: First, neurons must come into contact with prions, in which strain properties are encoded in the conformation of their PrP^{Sc} (Telling et al. 1996b). Second, a prion's PrP^{Sc} must physically interact with the host's PrP^{C} on the neuron's surface (Scott et al. 1989, 1993;

Prusiner et al. 1990; Cohen et al. 1994; Telling et al. 1995). Third, the prion strain-specific conformation of PrP^{Sc} acts as a template for the refolding of PrP^{C} into an exact duplicate conformation (Cohen et al. 1994). Fourth, an additional cellular mechanism, provisionally designated "protein X," appears to bind to the host's PrP^{C} and facilitate its unfolding and refolding into nascent PrP^{Sc} (Telling et al. 1995; Kaneko et al. 1997b). These events result in an exponential accumulation of PrP^{Sc} in and around neurons (Jendroska et al. 1991). That PrP^{Sc} generated by neurons is sufficient to cause nerve cell degeneration has been verified by neuron-specific expression of $ShaPrP^{C}$ in Tg mice under the control of a neuron-specific enolase promoter and infected with hamster-adapted prions (Race et al. 1995). This is also consistent with the observation that neurons express the highest PrPmRNA levels significantly higher than glial or endothelial cells in the CNS (Kretzschmar et al. 1986; Jendroska et al. 1991).

The prion attribute that gives it strain properties is the conformation of its PrP^{Sc}, which appears to remain constant during multiple passages in host animals (Telling et al. 1996b). From the perspective of the host animal, when one factors in host-determined variations in the level of expression of PrP^{C}, whether one or two PrP^{C} allotypes (or more in some transgenic mice) are expressed in a host, the level of expression of each PrP^{C} allotype, and nerve-cell-specific variations in the conformation of PrP^{C} (see below), there are more than sufficient prion and host variations to account for all of the known strain-determined variations in the prion disease phenotype. Other host factors could also influence the disease phenotype, such as the rate of clearance of nascent PrP^{Sc} from the brain, the degree of reactive astrocytic gliosis, and possible immune responses (Raeber et al. 1997).

The preeminent roles played by both the prion and the host's PrP^{C} have been highlighted in studies of each component of the prion disease phenotype.

The Host Species Barrier Phenotype

The failure of transmission or relatively low success rate of transmission of a prion strain to a host were found to be due in part to a large variation in the amino acid sequence of an infecting prion's PrP^{Sc} and the host's PrP^{C}, particularly between residues 90 and 120. The failure of transmission of hamster prions to mice could be overcome by expressing hamster PrP^{C} in Tg mice (Scott et al. 1989; Prusiner et al. 1990) or by expressing

chimeric mouse–hamster–mouse PrP^C constructs in which the region between residues 90–120 in the mouse PrP^C molecule was replaced with the hamster amino acid sequence (Scott et al. 1993).

Scrapie Incubation Time Phenotype

Over 25 years ago, Dickinson and his colleagues found that short and long scrapie incubation times in inbred mouse strains are determined by the strain of scrapie agent and by a single host gene (Dickinson et al. 1968; Dickinson and Meikle 1971). Following the discovery of PrP, it was found that the scrapie incubation time gene and the Prnp gene are the same (Carlson et al. 1986, 1988). Inbred mouse strains have one of two Prnp alleles that express two $MoPrP^C$ allotypes which differ by two amino acids at codons 108 and 189 (Westaway et al. 1987). These are designated $MoPrP^C$-A and $MoPrP^C$-B. Inoculation of a mouse prion composed of a single $MoPrP^{Sc}$ allotype into congenic and transgenic mice expressing different numbers and combinations of $MoPrP^C$-A and $MoPrP^C$-B showed that short and long incubation times were dependent on whether the amino acid sequence of the PrP^{Sc} of the infecting prion was the same or different from the $MoPrP^C$ allotype expressed and, furthermore, that long and short incubation times were shortened proportional to the number of PrP^C-A and PrP^C-B allotypes expressed (Carlson et al. 1994). Thus, scrapie incubation time is a function of the PrP^{Sc} comprising the prion, the amino acid sequence of the host's PrP^C, and the expression level of PrP^C allotypes.

The PrP Amyloid Plaque Phenotype

The two factors that determine whether or not PrP amyloid is formed in prion diseases are the strain of infecting prion and the amino acid sequence of PrP synthesized by the host.

That the strain of scrapie agent can determine whether or not amyloid forms was discovered over 25 years ago by neuropathologic analysis of scrapie-infected mice (Fraser and Bruce 1973, 1983; Bruce et al. 1976). Most mouse-adapted scrapie prion strains are not amyloidogenic; a few strains, such as the 87V, are highly amyloidogenic. Our current understanding of this phenomenon is that an amyloidogenic conformation of an infecting PrP^{Sc} is imposed on a host's PrP^C and that conformation favors truncation of nascent PrP^{Sc} into peptides that readily polymerize into amyloid filaments (Fig. 4).

Two lines of evidence indicate that the amino acid sequence of PrP expressed by an animal plays a role in amyloidogenesis. The first is based

on genetic linkage of PRNP mutations in humans to the GSS phenotype for which there are six amyloidogenic at codons 102, 105, 117, 145, 198, and 217. Some of the non-amyloidogenic mutations genetically linked to CJD-like syndromes occur at codons 178, 180, 200, 208, 210, and 232. Intense spontaneous PrP amyloidogenesis in Tg(GSS-P101L) mice expressing MoPrP with the mutation homologous to GSS(P102L) supports the view that some PrP sequences are amyloidogenic (Fig. 12). A second line of evidence argues that the amino acid sequence of the PrP expressed by the host plays a role in amyloidogenesis independent of the strain of prion or the host species in which it is expressed. For example, Sc237 prions derived from Syrian hamster brain result in the formation of mature kuru-type PrP amyloid plaques in Chinese hamster (CHa) brain, primitive PrP amyloid plaques in Syrian hamster (SHa) brain, and no amyloid plaques in Armenian hamster (AHa) brain (Lowenstein et al. 1990). AHa and CHa PrP^{C} differ by three amino acids at residues 103, 108, and 112, whereas SHa PrP^{C} differs from CHa by only one amino acid at residue 112. In another unpublished study from our laboratory, mouse-adapted RML prions serially passaged in CD1 mice, RML(CD1) prions, failed to produce amyloid plaques (Fig. 19A). However, when RML(CD1) prions were inoculated into Tg(MoPrP-P101L)-L, which expresses the amyloidogenic GSS-like muPrP, but do not spontaneously develop neurodegeneration or amyloid, multiple mature kuru-type PrP amyloid

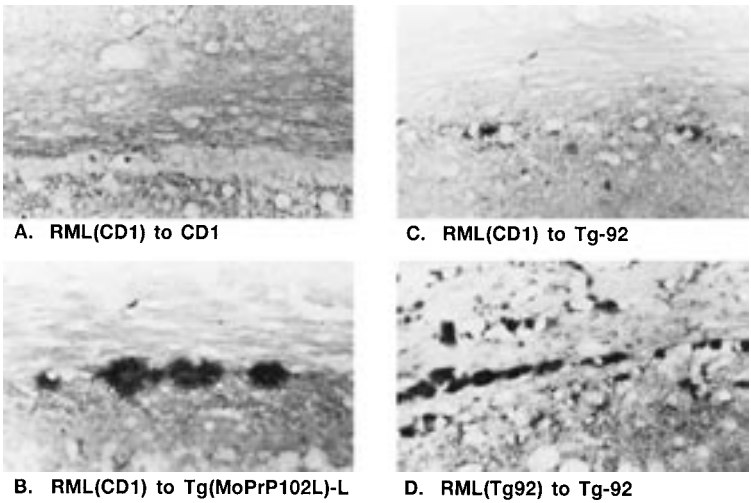


Figure 19 PrP amyloidosis in scrapie is in part a function of the amino acid sequence of the PrP^{C} converted to nascent PrP^{Sc}. See text for explanation.

plaques were formed (Fig. 19B). When RML(CD1) was inoculated into Tg92 mice that express a chimeric mouse–Syrian hamster–mouse PrP^{C}, designated MH2M-PrP (Scott et al. 1993), primitive PrP amyloid plaques formed (Fig. 19C). The prions resulting from the latter transmission, RML(Tg92) prions, produced an even larger number of primitive PrP plaques when serially transmitted to Tg92 mice (Fig. 19D), possibly reflecting the combination of a difference in the properties of the infecting RML(Tg92) prion, composed of MH2M-PrP^{Sc} rather than wild-type MoPrP^{Sc} when RML was derived from CD1 mice, and reflecting the amyloidogenic potential of MH2M-PrP^{Sc} that was formed in Tg92.

Of interest to this discussion, kuru-type amyloid plaques occur in only about 5–10% of sporadic CJD cases. Whether these plaques develop may be genetically linked to the valine polymorphism at codon 129 (de Silva et al. 1994).

The Vacuolation Histogram Phenotype

The distribution and intensity of spongiform (vacuolar) degeneration, usually displayed in histograms and designated the "lesion profile," has been the most commonly used neuropathologic measure to differentiate one prion strain from another (Fraser and Dickinson 1968, 1973; Fraser 1979; Bruce et al. 1991). The mechanism of differential "targeting" of neurons by prion strains for vacuolar degeneration is not yet completely understood. However, PrP immunohistological data reviewed above revealed a precise correlation between sites of vacuolar degeneration and sites of PrP^{Sc} accumulation. That correlation argues that the neuroanatomic pattern of PrP^{Sc} accumulation, like the pattern of vacuolation, is itself a characteristic of prion strains (Fig. 5).

The PrP^{Sc} Distribution Phenotype

With the discovery that the neuropathologic changes in prion diseases colocalize precisely with sites of PrP^{Sc} accumulation (DeArmond et al. 1987), it was not surprising to find that the distribution of PrP^{Sc} in the brain is also characteristic of prion strains (Figs.18, 20) (Bruce et al. 1989; Hecker et al. 1992; DeArmond et al. 1993). Although vacuolation and reactive astrocytic gliosis occur only at sites where PrP^{Sc} accumulates, the converse is not true: PrP^{Sc} accumulates in some brain regions without obvious neuropathologic changes. One explanation for the latter is that neuropathologic changes require a finite amount of time to develop following local accumulation of PrP^{Sc} (Jendroska et al. 1991). Another

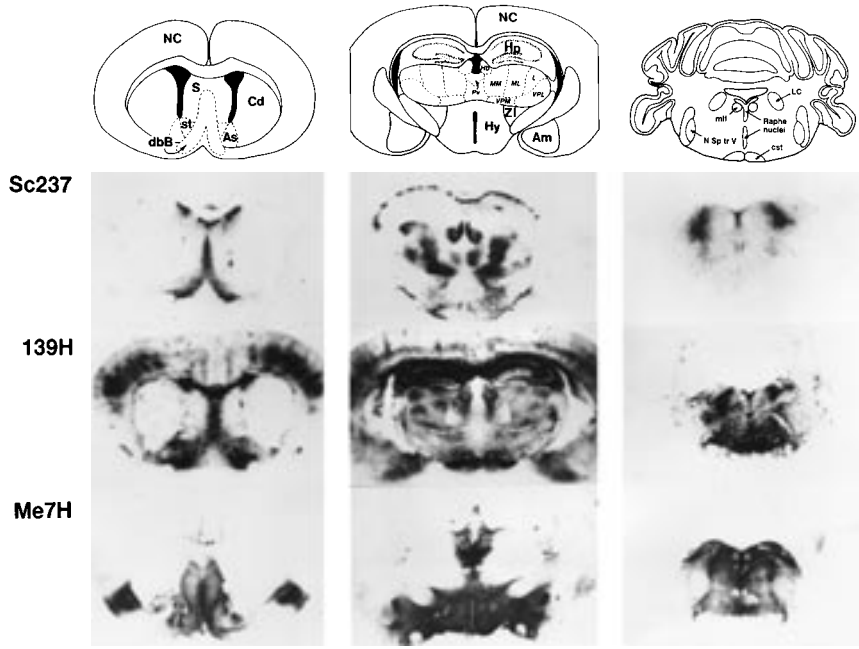


Figure 20 Differential targeting of neurons for conversion of PrP^{C} to PrP^{Sc} by prion strains. Tg(SHaPrP) mice were inoculated intrathalamically with either the Sc237, 139H, or Me7H prion strains obtained from Syrian hamster brains. (Am) Amygdala; (As) accumbens septi; (Cd) caudate nucleus; (cst) corticospinal tract; (dbB) diagonal band of Broca; (Hp) hippocampus; (Hy) hypothalamus; (LC) locus ceruleus; (mlf) medial longitudinal fasiculus; (NC) neocortex; (N Sp tr V) nucleus of the spinal tract of the trigeminal nerve; (S) septal nuclei; (st) interstitial nucleus of the stria terminalis; (ZI) zona incerta. Thalamic nuclei are in italics.

possibility is that vacuolation of neuronal processes does not develop in some neuronal populations in response to PrP^{Sc} deposition. A further feature of the PrP^{Sc} deposition phenotype is that, although the distribution of PrP^{Sc} is different for each strain of prions, many of the same brain regions accumulate PrP^{Sc} with each prion strain (Fig. 20). Specifically, the brain stem tegmentum accumulates PrP^{Sc} with many prion strains. Analysis of this overlap phenomenon is hampered at this time because it is not known whether PrP^{Sc} accumulation in a region, such as the brain stem, is due to local conversion of PrP^{C} to PrP^{Sc} by brain stem nerve cells or whether PrP^{Sc} is transported to the brain stem by anterograde and/or retrograde axonal transport from many distant sites.

Although the mechanism of differential targeting of neurons by prion strains is not understood, analysis of this phenomenon must involve cell-specific mechanisms that influence the interaction of a prion's PrP^{Sc} with the neurons PrP^{C} or that influence the mechanism that duplicates the conformation of PrP^{Sc} in the cell's PrP^{C}. Analysis must also take into account the early investigations of scrapie within inbred mouse strains, which have shown that the vacuolar degeneration lesion profile varies as a function of the scrapie strain, the route of infection of prions, and the mouse genotype (Fraser and Dickinson 1968, 1973; Fraser 1979, Fraser 1993).

PrP^{C} Glycotypes and the Lesion Profile

Where our early studies showed that the distribution of PrP^{Sc} was markedly different in animals of the same genotype inoculated with different prion strains (Fig. 20), more recently, we found that the converse is also true. Thus, the pattern of PrP^{Sc} accumulation in three different inbred mouse strains was different in response to intrathalamic inoculation of a single scrapie prion strain, the RML (Rocky Mountain Laboratory) scrapie isolate (DeArmond et al. 1997). RML prions inoculated into C57BL, CD-1, and FVB mice, all of which carry the *Prnp*^{a} gene, exhibited different patterns of PrP^{Sc} deposition (Fig. 21). C57BL mice were distinguished by little or no immunostaining in the hippocampus and neocortex and relatively weak immunostaining in other brain regions. The most intense signals were found in the thalamus and brain stem. In contrast, PrP^{Sc} was uniformly and widely distributed throughout the cerebrum and brain stem in FVB mice. PrP^{Sc} immunostaining in CD-1 mice was intermediate, in that it was more widely distributed in the brain compared to C57BL, but weak or absent in some regions such as the hippocampus, amygdala, and hypothalamus. Common to all three mouse strains was the presence of immunostaining in the brain stem tegmentum and the absence of immunostaining in the cerebellar cortex.

Because each of these mouse strains is homozygous for the *Prnp*^{a} gene and, therefore, each expresses PrP^{C}-A with the same amino acid sequence, and because all the mice were inoculated with the RML prion strain passaged through CD-1 mice, finding different patterns of PrP^{Sc} accumulation argues that a non-*Prnp* gene(s) of the host modifies the PrP^{Sc} deposition phenotype. One set of non-Prnp factors that have the potential to influence rate of synthesis, rate of degradation, and post-translational modification of PrP^{C} are the series of enzymatic steps in the endoplasmic reticulum and Golgi apparatus which attach and modify the

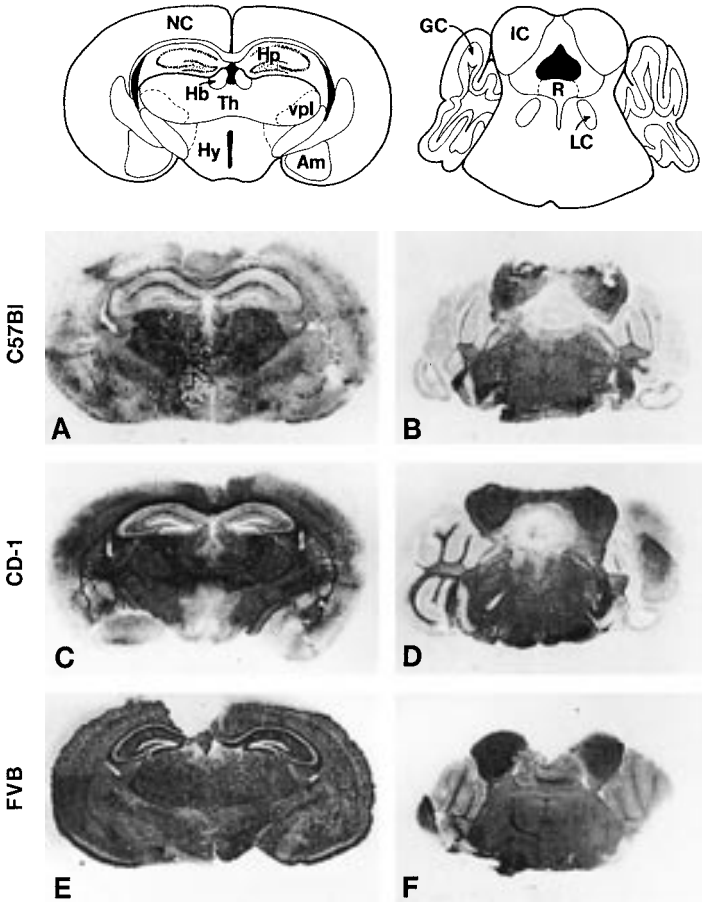


Figure 21 Differential targeting of neurons for conversion of PrP^{C} to PrP^{Sc}: influence of the strain of animal. A single mouse prion strain, RML(CD1), from a single animal source (CD1 mice) was inoculated intrathalamically into three inbred mouse strains and resulted in three different PrP^{Sc} deposition patterns. (Am) Amygdala; (GC) granule cell layer of the cerebellar cortex; (Hb) habenula; (Hp) hippocampus; (Hy) hypothalamus; (IC) inferior colliculus; (LC) locus ceruleus; (NC) neocortex; (R) dorsal nucleus of the raphe; (vpl) ventral posterior lateral nucleus of the thalamus; (Th) thalamus.

two asparagine-linked oligosaccharides of the PrP molecule at asparagine 181 and 197 (Endo et al. 1989). Neuron-specific glycosylation of PrP^{C} might underlie selective targeting of neurons because experience with other proteins indicates that asparagine-linked oligosaccharides influence

protein conformation and protein/protein interactions (Rademacher et al. 1988; O'Connor and Imperiali 1996). Cell-specific differences in asparagine-linked glycosylation are also well known for other cellular proteins (Rademacher et al. 1988). The fidelity of cell-specific glycosylation patterns is such that it is referred to as a cell's "glycotype." The possibility that each neuron population synthesizes a different PrP^C glycotype is supported by the evidence that the brain contains more than 400 different PrP^C glycoforms (Endo et al. 1989).

To test whether asparagine-linked glycosylation of PrP^C influences the prion disease phenotype, we mutated its threonine residues to alanine within the NXT consensus sites (Taraboulos et al. 1990). Single and double glycosylation site mutations were made in PrP constructs encoding Syrian hamster PrP, and these were expressed in Tg mice deficient for MoPrP ($Prnp^{0/0}$) (DeArmond et al. 1997). Wild-type $SHaPrP^C$ was uniformly distributed in the neuropil of all gray matter regions in Tg mice, but was absent from cell bodies of neurons and from white matter. In contrast, mutations of one or both glycosylation sites had a profound effect on the neuroanatomical distribution of $SHaPrP^C$. Mutations of the first glycosylation site alone or in combination with mutation of the second site resulted in low levels of mu-$SHaPrP^C$, accumulation of mu-$SHaPrP^C$ in nerve cell bodies, and little mu-$SHaPrP^C$ in the gray matter neuropil. When the second site alone was mutated, the levels of mu-$SHaPrP^C$(T199A) were the same as for wild-type $SHaPrP^C$; however, it was distributed to all neuronal compartments including the nerve cell body, dendritic tree, and axons of the white matter. These results suggest that the oligosaccharide at residue 181 plays a particularly important role in the trafficking, sorting, and stability of PrP^C.

Two Syrian hamster prion strains, Sc237 and 139H, were inoculated intracerebrally into the Tg mice expressing wild-type and single or doubly deglycosylated $SHaPrP^C$ (DeArmond et al. 1997). No Tg mice expressing mu-$SHaPrP^C$ were affected in which the oligosaccharide at Asn-181 was deleted either because of low levels of mu-$SHaPrP^C$ and/or because these mu-$PrPSHa^C$s did not appear to be transported out of the nerve cell body. Tg mouse lines that expressed mu-$SHaPrP^C$(T199A) in which the oligosaccharide at Asn-181 was in place but the second at Asn-197 was deleted, Tg(SHaPrP-T199A)/$Prnp^{0/0}$ mice, did develop scrapie following inoculation with prions; however, the disease phenotype was profoundly different from that in Tg mice expressing wild-type $SHaPrP^C$, Tg(SHaPrP)7/$Prnp^{0/0}$ mice. First, whereas Tg(SHaPrP)7/$Prnp^{0/0}$ mice became scrapie sick with both Sc237 and 139H prions at 44 and 47 days post-inoculation, respectively, Tg(SHaPrP-T199A)/$Prnp^{0/0}$ mice only

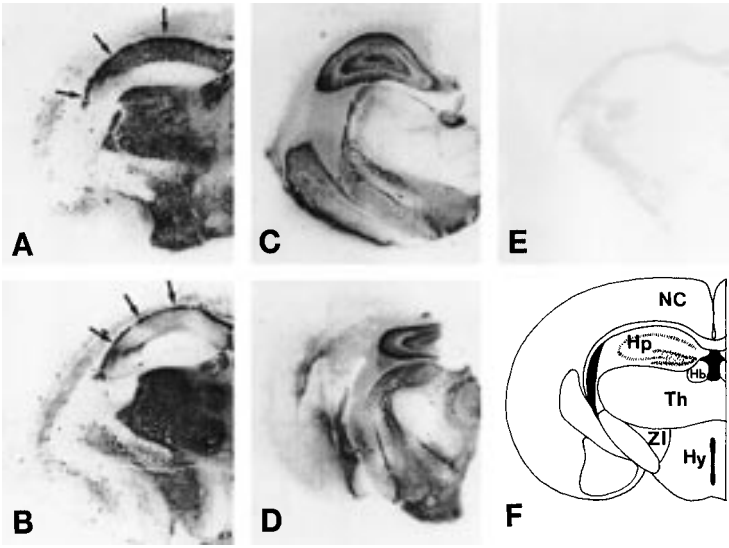


Figure 22 Deletion of the second asparagine-linked oligosaccharide at residue 197 from $SHaPrP^{C}$ by mutation of the asparagine consensus sequence at codon 199 profoundly alters the PrP^{Sc} accumulation pattern in response to inoculation with Sc237 prions passaged in Syrian hamsters: (*A*,*B*) Pattern of wild-type $SHaPrP^{Sc}$ accumulation in two Tg mouse lines expressing wild-type $SHaPrP^{C}$ and (*C*,*D*) pattern of deglycosylated mutant $SHaPrP^{Sc}$-T199A accumulation in two Tg mouse lines expressing mutant $SHaPrP^{C}$-T199A. (*E*) Inoculation of Tg mouse lines expressing deglycosylation mutant $SHaPrP^{C}$-T199A failed to develop scrapie or to accumulate protease-resistant PrP. (*F*) (Hb) Habenula; (Hp) hippocampus; (Hy) hypothalamus; (NC) neocortex; (ZI) zona incerta.

became scrapie sick with the Sc237 strain and with an incubation time of about 550 days. Second, whereas Sc237 prions resulted in the formation of PrP amyloid plaques in $Tg(SHaPrP)7/Prnp^{0/0}$ mice, no amyloid plaques formed in $Tg(SHaPrP\text{-}T199A)/Prnp^{0/0}$ mice (Fig. 22). Third, the neuroanatomic distribution of $SHaPrP^{Sc}$ was markedly different. For example, there was intense immunostaining for $SHaPrP^{Sc}$ in the thalamus of $Tg(SHaPrP)7/Prnp^{0/0}$ mice but little or none in the thalamus of $Tg(SHaPrP\text{-}T199A)/Prnp^{0/0}$ mice (Fig. 22).

We are currently testing the possibility that each nerve cell population synthesizes a different set of PrP^{C} glycoforms; that is, that each neuron population has its own PrP^{C} glycotype. Preliminary data suggests there is brain-regional heterogeneity in the glycosylation of PrP^{C} (DeArmond et al. 1997). This conclusion is based on 2-dimensional gel analysis of wild-

type and single and double deglycosylated SHaPrP^{C} in hippocampal and cerebellar homogenates, which showed that the number of charge isomers is directly proportional to the amount of asparagine-linked glycosylation. The charge isomers are probably due to sialic acid residues in the oligosaccharide trees, which are variably sialated (Endo et al. 1989). The number and the pI of the charge isomers were different in the hippocampus and cerebellum for wild-type and mutated SHaPrP^{C}s.

These results are remarkable because they suggest that variations in the glycosylation of PrP^{C} can influence the host species barrier, incubation time, amyloidogenesis, the sites of PrP^{Sc} accumulation, and therefore, the sites of vacuolar degeneration. How might oligosaccharides attached to PrP^{C} have such a profound effect on the rate and sites of conversion of PrP^{C} to PrP^{Sc}? We hypothesize that the oligosaccharides attached to PrP^{C} modify the conformation of and/or stabilize its PrP^{Sc}-binding domain in a way that modulates the rate of conversion into nascent PrP^{Sc}. This kinetic view of PrP^{Sc} formation is consistent with the hypothesis that some PrP^{C} molecules are more readily converted into PrP^{Sc} than others (Eigen 1996). Moreover, brain-region variations in the composition and structure of the two asparagine-linked oligosaccharides might also modulate the amount of reactive PrP^{C} in the cellular compartment, where it interacts with PrP^{Sc} through level of expression and differential trafficking of PrP glycotypes.

Molecular and Cellular Mechanisms of Vacuolar Degeneration

The principal neuropathologic change common to all prion diseases is vacuolation of nerve cell processes (spongiform degeneration) (Masters and Richardson 1978; Beck and Daniel 1987). Vacuolation occurs primarily in the region of synapses and is characterized by focal neuritic swelling, loss of internal organelles, and accumulation of abnormal membranes (Lampert et al. 1972; Chou et al. 1980). Spongiform degeneration suggests focal abnormalities of the plasma membrane with failure of electrolyte and water homeostasis. Regions of synapses might be particularly vulnerable because of their high density of neurotransmitter receptors and ion channels dedicated to information transfer between neurons.

There are only a few plausible hypotheses backed by data concerning mechanisms of vacuolar degeneration as a result of PrP^{Sc} deposition in the brain. In one scenario, PrP^{Sc} is thought to accumulate in lysosomes and trigger dysfunction as these aberrantly folded molecules are released into the cytosol (Laszlo et al. 1992). The lysosome hypothesis was originally

proposed because PrP^{Sc} could only be localized with certainty in lysosomes and in the adjacent cytosol by immunogold electron microscopy of a scrapie-infected cell line (McKinley et al. 1991b). A more likely scenario is emerging from recent biochemical measurements of PrP^{Sc} in cell fractions derived from scrapie-infected cell lines. These indicate that PrP^{Sc} is formed from PrP^{C} in CLDs of the plasma membrane where it accumulates, as well as in lysosomes (Gorodinsky and Harris 1995; Taraboulos et al. 1995; Vey et al. 1996; Kaneko et al. 1997a). Accumulation of PrP^{Sc} in CLDs may generate aberrant responses to stimuli mediated through signal transduction systems (Anderson 1993). Accumulation of PrP^{Sc} at or near the surface of a cell may account for a number of plasma membrane abnormalities that have been detected, including decreased plasma membrane fluidity, decreased binding affinity of bradykinin (Bk) receptors, decreased Bk-stimulated inositol triphosphate (IP_3) response, and decreased Bk-stimulated intracellular calcium response (Kristensson et al. 1993; DeArmond et al. 1996; Wong et al. 1996). In fact, focal vacuolar degeneration of neurons in prion diseases might be best explained by focal plasma membrane abnormalities controlling transynaptic signaling and water and electrolyte balance. A third scenario that could result either from abnormal plasma membrane signaling or from accumulation of PrP^{Sc} in several subcellular compartments is a crippled stress response triggered by malfolded, undegradable PrP (Kenward et al. 1994; Tatzelt et al. 1995).

SUMMARY

The discovery of the mechanisms of nerve cell degeneration in prion diseases has gone hand in hand with the discovery of prions and how they replicate. Viewing prion diseases from the perspective of the neuropathologic changes has become one of the strongest arguments for the protein-only hypothesis because immunohistochemical and neuropathologic analyses have shown there are indeed two forms of PrP in the brain, that the protease-resistant form of PrP increases exponentially and is conveyed from one brain region to another largely by axonal transport, and that nerve cell degeneration and amyloidosis in prion diseases are caused by accumulation of abnormal PrP. Furthermore, extensive neuropathologic analysis by both light and electron microscopy of all forms of prion disease have failed to reveal any contribution to the pathogenesis by a conventional pathogen, nor do prion diseases resemble any form of infection with a conventional pathogen. Viewing prion diseases from the perspec-

tive of the neuropathologic changes has also emphasized the importance of host-determined factors that influence the prion disease phenotype, particularly host-determined variations in PrP^C that appear to have a profound effect on all of the parameters of the disease phenotype used to define prion strains. Finally, one of the strongest arguments in favor of the protein-only hypothesis is that it explains how prion diseases can be sporadic and dominantly inherited and also transmissible.

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Some Strategies and Methods for the Study of Prions

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The discovery of the prion protein (PrP) by enriching fractions prepared from scrapie-infected hamster brains transformed research on the prion diseases. Prior to identification of PrP 27-30, the protease-resistant core of the scrapie prion protein (PrP^{Sc}), almost all studies of prions required bioassays. With the isolation of PrP 27-30 came the ability to apply the tools of molecular cloning, genetics, immunology, cell biology, and structural biology.

Many different areas of the prion diseases can now be investigated using a wide variety of approaches. Recombinant PrP expressed in bacteria or mammalian cells can be isolated in large quantities and used for structural studies (Mehlhorn et al. 1996; Blochberger et al. 1997). The biology of PrP^{Sc} formation as well as the molecular pathogenesis of prion diseases can be studied in cultured cells and transgenic (Tg) mice. Similarly, potential therapeutics might be evaluated by measuring the inhibition of PrP^{Sc} formation in scrapie-infected cultured cells or Tg mice. The biology of prions is sufficiently advanced that there are now three reasonably rational approaches available for the development of effective therapies and preventive measures in humans. First, drugs that alter the conformation of PrP^{Sc} and allow the cell to degrade it might prove to be efficacious. Second, drugs that block the formation of nascent PrP^{Sc} by interfering with the binding of PrP^{C} to PrP^{Sc} might also prove

effective. Third, pharmacotherapeutics that disrupt the binding of PrP^{C} to protein X may prove the most effective of all the approaches currently available (Kaneko et al. 1997c). In domestic animals, PrP transgenes that act as dominant negatives and prevent prion disease might be superior to ablation of the PrP, but resolution of such issues requires further experimentation. Besides developing effective therapies, deciphering the structural changes that accompany the transformation of PrP^{C} into PrP^{Sc} is particularly challenging.

Immunoassays for PrP^{Sc} show great promise as tools for rapid diagnosis of prion disease as well as screening for subclinical cases in humans and domestic animals. Because the particle to infectivity ratio for prions is about 10^5 PrP^{Sc} molecules per ID_{50} unit, it may be possible to develop an immunoassay that is 10- to 100-fold more sensitive than animal bioassays.

In this chapter, we attempt to give the reader an overview of the application of some modern technologies that have been adapted to prion research. Although we cover such applications rather selectively, we do try to point out where such studies are described in more detail in this monograph as well as in other sources. In addition, we describe a series of rather extensive studies that were mounted to search for a scrapie-specific nucleic acid. Although these studies were unsuccessful, we believe that it is important to summarize the extent and thoroughness of that effort.

PRION PROTEINS

The only known essential component of the infectious prion particle is the scrapie PrP isoform (PrP^{Sc}). The apparent molecular mass (M_r) of the protein is 33–35 kD. PrP^{Sc} is a sialoglycoprotein that also contains a GPI anchor; it has a single disulfide bond. Secondary structure analysis by optical spectroscopy suggests that approximately half of the protein is composed of β sheets.

In homogenates of scrapie-infected tissues, PrP^{Sc} is sedimented by centrifugation at 100,000*g* for 1 hour at 4ºC and, thus, it is labeled as insoluble. Many attempts to solubilize PrP^{Sc} with detergents, organic solvents, and various salts under nondenaturing conditions where prion infectivity is preserved have been unsuccessful to date. Although PrP^{Sc} seems to complex readily with lipids, no other proteins are found to be consistently associated with PrP^{Sc} in fractions prepared by repeated detergent and salt extraction followed by differential centrifugation.

By electron microscopy, purified PrP^{Sc} has no recognizable quaternary structure, but PrP 27-30, the amino-terminally truncated fragment of PrP^{Sc}, does polymerize into rod-shaped structures with the ultrastructural, tinctorial, and spectroscopic properties of amyloid (see Chapter 2, Fig. 3). By electron microscopy (EM) both PrP^{C} and PrP^{Sc} appeared as amorphous aggregates when dried onto grids; the presence of these proteins was confirmed by immunogold labeling with an α-PrP 3F4 monoclonal antibody. In contrast, polymers of PrP 27-30 were visualized as rod-shaped amyloids (Prusiner et al. 1983). Amyloid polymers composed of PrP 27-30 have a higher proportion of low frequency (LF) β-sheet than PrP^{Sc}; the LF β-sheet characteristic of the intermolecular bonding associated with polymer formation is more pronounced for PrP 27-30.

That purified PrP 27-30 assembles into amyloid suggested that the amyloid plaques previously reported in kuru, Creutzfeldt-Jakob disease (CJD), and scrapie were composed of the same protein that was the major component of the infectious prion particle (Chapter 2). PrP 27-30 is formed from PrP^{Sc} after limited proteinase K digestion; no loss of infectivity occurs during this period of proteolysis (McKinley et al. 1983; Prusiner et al. 1983; Meyer et al. 1986). In contrast, prolonged proteolysis hydrolyzed PrP 27-30 and diminished prion titers as determined by bioassay (Fig. 1). The extreme resistance of both PrP 27-30 and prion infectivity to proteolytic digestion, followed by concomitant decreases in both, argued persuasively that PrP 27-30 was a component of the infectious particle.

Once the amino-terminal amino acid sequence of PrP 27-30 was determined (Prusiner et al. 1984a), isocoding sets of oligonucleotides were synthesized and used to retrieve cognate cDNA clones from banks prepared from the $poly(A)^{+}$ RNA of hamster and mouse brains (Chesebro et al. 1985; Oesch et al. 1985). The finding of mRNA encoding PrP in the brains of uninfected, control animals led to two different conclusions: in one case, PrP was dismissed as irrelevant to scrapie (Chesebro et al. 1985), and in the other, it prompted the discovery of PrP^{C} (Oesch et al. 1985).

PURIFICATION OF PRIONS

Purification of PrP^{Sc} and PrP 27-30 is accomplished by taking advantage of the insolubility of these proteins in nondenaturing detergents (Prusiner et al. 1982a, 1983; Diringer et al. 1983; Hope et al. 1986; Bolton et al. 1987; Turk et al. 1988). Repeated detergent extractions of prion-infected

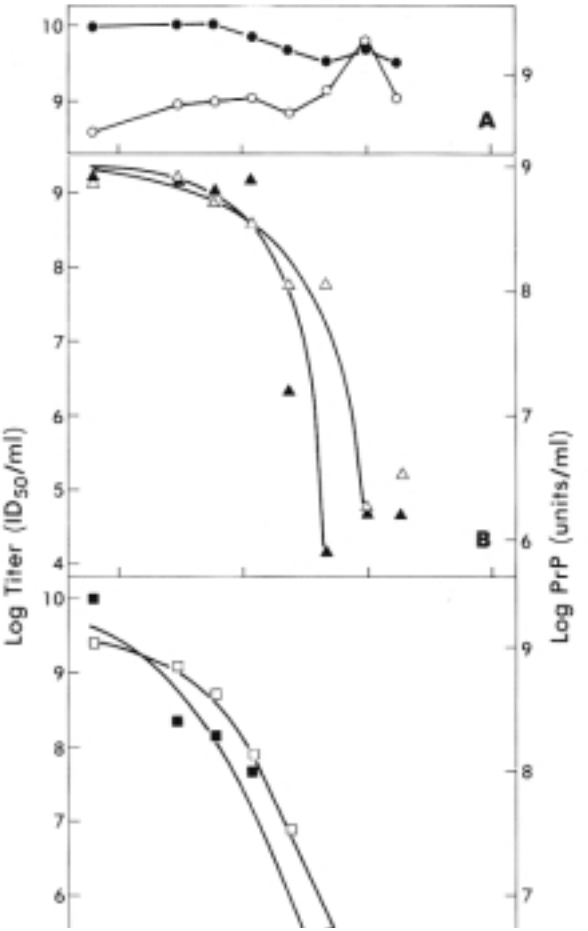

Figure 1 Kinetics of PrP 27-30 degradation and prion inactivation by proteolytic digestion. Concentration of PrP 27-30 was determined by densitometry and prion titer by bioassay. (*A*) Control. (*B*) Samples were digested with 100 μg/ml proteinase K for the times denoted on the *x*-axis. (*C*) Digested with 100 μg/ml proteinase K. Filled symbols represent the log PrP 27-30 and open symbols designate the log prion titer. (Reprinted, with permission, from McKinley et al. 1983 [copyright Cell Press].)

brain tissue in the presence or absence of salt is frequently used to isolate both PrP^{Sc} and PrP 27-30. Preparations of PrP 27-30 are generally more homogeneous than those of PrP^{Sc} since limited digestion with proteinase K removes most protein contaminants. Alternatively, PrP^{Sc} has been isolated by immunoaffinity chromatography after dispersion into detergent–lipid–protein complexes (DLPCs) (Gabizon et al. 1988b), but such

a procedure has not been adapted to the large-scale preparations due to the large amount of purified phospholipid required.

Enriching Fractions for Prion Infectivity

The development of an effective protocol for enriching rodent brain fractions for scrapie prion infectivity led to the discovery of PrP 27-30. Several general principles proved useful in developing a purification scheme for prions. First, prions are stable in a variety of detergent solutions that do not denature proteins; the infectivity is unstable in detergent solutions that denature proteins, such as SDS (Millson and Manning 1979; Prusiner et al. 1980b). Second, prion infectivity can be separated from cellular components by detergent extractions followed by differential centrifugation or precipitation (Prusiner et al. 1977, 1980b). Although ultracentrifugation is useful in sedimenting prions, it is cumbersome on a large scale; at an early step in our large-scale purification we substituted precipitation by polyethylene glycol (PEG) (Prusiner et al. 1982b, 1983). Third, for many years prion infectivity in crude extracts had been found to be resistant to nucleases and even protease digestions (Hunter 1979; Prusiner et al. 1980b). Both of these hydrolytic enzymes proved to be valuable in purification. Omission of either micrococcal nuclease or proteinase K resulted in a diminished extent of purification. More specifically, omission of the proteinase K digestion diminished the purification by the sucrose gradient procedure by a factor of 10 (Prusiner et al. 1982b). The enzyme digestions were carried out at 4ºC because warming prions to 37ºC caused increased aggregation (Prusiner et al. 1978).

After proteolytic digestion, some of the small peptides and oligonucleotides could be removed by ammonium sulfate precipitation (Prusiner et al. 1980b). This was performed in the presence of cholate, since other investigators studying membrane-bound enzymes had shown that effective precipitation of hydrophobic proteins with ammonium sulfate required cholate (Tzagoloff and Penefsky 1971). In one study on the scrapie agent, ammonium sulfate was used in the absence of cholate, but precipitation of the agent was nonselective (Malone et al. 1979).

Using sodium dodecyl sarcosinate (Sarkosyl) gel electrophoresis, the digested peptides and polynucleotides were separated from aggregates (Prusiner et al. 1980a). Although the method was cumbersome, it did provide sufficient purification to allow us to show convincingly that infectivity depended on a protein(s) using protease digestion and reversible chemical modification with diethylpyrocarbonate (DEP) (McKinley et al. 1981; Prusiner et al. 1981). Without the purification provided by Sarkosyl

gel electrophoresis, neither protease digestion nor chemical modification with DEP was found to diminish the titer of prions (Prusiner et al. 1981).

The major drawback of gel electrophoresis for purifying the prion was the limited amount of material that could be processed. Only 3–4 ml of detergent-extracted, enzyme-digested material could be loaded on a preparative electrophoresis apparatus at any one time; electrophoresis for 6–8 hours was necessary (Prusiner et al. 1981). This was followed by a cumbersome procedure in which electroelution of the infectious agent from the top of the gel was performed.

Because the size of our preparations was severely limited by using Sarkosyl gel electrophoresis, we developed an alternative purification scheme using sucrose gradient sedimentation (Prusiner et al. 1982b). This procedure takes advantage of the hydrophobicity of the scrapie prions. An enzyme-digested ammonium-sulfate-precipitated fraction was mixed with Triton X-100 and SDS prior to rate-zonal sedimentation through a sucrose step gradient containing no detergent. Presumably, the large forms of the prions are present either as aggregates that were not dissociated in the Triton X-100/SDS or as aggregates that are formed as the prions enter the gradient. Both of these possibilities appear to occur. The behavior of prions under these conditions seems to be analogous to that of the calcium ATPase, where this procedure was first used (Warren et al. 1974a,b). Omitting the Triton X-100/SDS mixture or substituting octylglucoside or Triton X-100 alone resulted in virtually no purification of the prion (Prusiner et al. 1982a).

The development of the discontinuous sucrose gradient method provided another major step in the level of purification as well as characterization of prions. Preparations were of sufficient purity to allow identification of a unique protein (PrP) within these preparations (Bolton et al. 1982; Prusiner et al. 1982a). Although the purification was substantially increased over that obtained with Sarkosyl gel electrophoresis, and the amount of material that could be processed in a single centrifugation was 10-fold greater than that achieved with Sarkosyl gel electrophoresis, the quantities of purified fractions were still insufficient to extend purification and to provide a sufficient amount for characterization.

To overcome this problem, a large-scale purification procedure was developed (Prusiner et al. 1983). Not only did the large-scale purification protocol increase the amount of purified prions, but equally important, it yielded fractions that contained significantly fewer contaminants compared to preparations derived with smaller-scale protocols. The zonal rotor sucrose gradient centrifugation used in the large-scale protocol gave

increased resolution of the particles being separated due to its configuration and long path length, especially when compared to gradient centrifugation in a reorienting vertical rotor (Anderson 1962). In addition, dynamic loading and edge-unloading of the rotor probably also helped to maximize the resolution of purification procedure.

In our early studies on scrapie, we reported that prions could exist in multiple molecular forms, with small forms having a sedimentation coefficient of 40S or less, as well as a succession of larger forms; in fact, some larger aggregates had sedimentation coefficients of more than 10,000S (Prusiner et al. 1978, 1980b). All these data were derived from studies in which the infectivity of gradient fractions was determined by endpoint titrations in mice. Subsequent studies showed that scrapie prions aggregate into rods after exposure to limited proteolysis and that these rods form large arrays or clusters of varying size and shape (Prusiner et al. 1983; McKinley et al. 1991). The aggregation of prions into polymeric structures of varying size and shape made purification extraordinarily difficult.

Large-scale Purification of PrP 27-30

To prepare sufficient amounts of purified fractions containing scrapie prions for a variety of studies, it was necessary to scale up the purification protocol described above. An outline of this large-scale protocol is depicted in Figure 2. We have adapted this large-scale protocol for purification of PrP^{Sc} or PrP 27-30 from as few as three scrapie-infected brains from Syrian hamsters.

Typically, brains are taken from Syrian hamsters that were inoculated intracerebrally 75 days earlier with about 10^7 ID_{50} units of Sc237 scrapie prions. Hamsters are anesthetized with CO_2 and sacrificed by cervical dislocation. The brains are frozen in liquid nitrogen and stored at –70ºC. For each preparation, 900–1000 brains are thawed. Ten liters of a 10%(w/v) homogenate is made by suspending the brains in 0.32 M sucrose and disrupting the tissue with a Brinkmann Polytron equipped with a PT45 generator set at 8 for 2 minutes in a Baker Sterilgard biosafety hood. The homogenization vessel is a stainless steel flask with a capacity of 13.5 liters. The vessel measures 31.8 cm high and 26.7 cm in diameter. The lid is fastened to the cylinder with three screws with wing nuts. An O ring around the edge of the cylinder provides a gas-tight seal for the lid. The center of the lid has an opening 4.45 cm in diameter for the PT45 probe to be inserted. This opening is made nearly gas-tight with a gasket. The homogenization vessel is kept in an ice bath during use. The prepa-

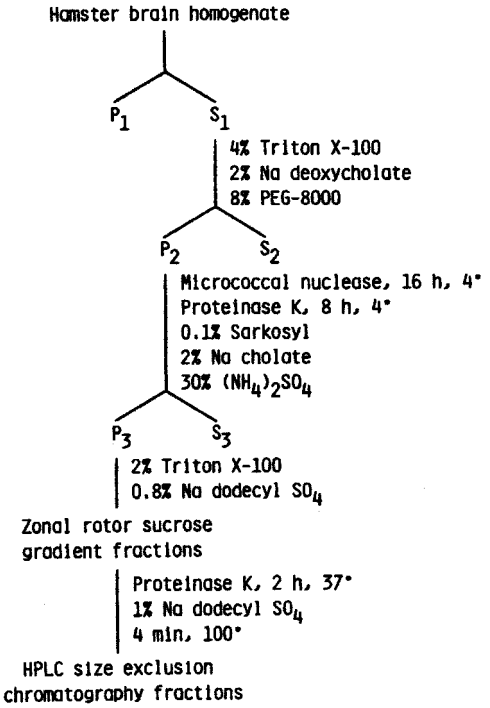

Figure 2 Scheme for large-scale purification of PrP 27-30. (P_n) Pellet, (S_n) supernatant. (Reprinted, with permission, from Prusiner et al. 1984a [copyright Cell Press].)

ration is kept at 4ºC throughout the purification. All solutions are purged with argon to remove any oxygen remaining after evacuation.

The homogenate is clarified by continuous-flow centrifugation at 10,000 rpm using a Beckman JCF-Z rotor equipped with a large pellet core. The centrifugation is performed in a Beckman J21-C centrifuge housed in a Baker biosafety cabinet. The homogenate is pumped through the rotor at 180 ml/min. To the supernatant (S_1), 1 mM EDTA and 1 mM DTT are added, to which Triton X-100 and DOC are then added at detergent:protein(w/w) ratios of 4:1 and 2:1, respectively. Once the volume of the S_1 with the detergent is determined, PEG-8000 cocktail is added to the extract to a final concentration of 0.03 M Tris-OAc, 0.1 M KCl, 20% glycerol, and 8% PEG-8000. The detergent extract is precipitated by adding the PEG-8000 cocktail and stirred at 4ºC for 15 minutes.

The pellet (P_2) is collected by continuous-flow centrifugation at 15,000 rpm using the JCF-Z rotor equipped with a standard pellet core. The fraction S_1 is pumped through the rotor at 160 ml/min. Fraction P_2 is resuspended in 20 mM Tris-OAc (pH 8.3) containing 0.2% (v/v) Triton X-100 and 1 mM DTT to give a protein concentration of 10 mg/ml. It is digested with 12.5 units/ml of micrococcal nuclease in the presence of 2 mM $CaCl_2$ for 12 hours at 4ºC followed by 100 µg/ml proteinase K for 8 hours at 4ºC in the presence of 2 mM EDTA and 0.2% (w/v) Sarkosyl. The digestion was terminated with 0.1 mM PMSF. The digest is precipitated with $(NH_4)_2SO_4$ (ultrapure) at 30% saturation in the presence of 2% (w/v) sodium cholate. This is centrifuged in a Beckman type 19 rotor in a Beckman L5-65 ultracentrifuge housed in a Baker biosafety cabinet at 19,000 rpm for 1 hour at 4ºC. The pellet (P_3) is adjusted to 0.825 mg/ml, and 20% Triton X-100 and 8% SDS (specially pure) are added to a final concentration of 2% Triton X-100 and 0.8% SDS. The protein concentration is reduced to 0.75 mg/ml. Typical preparations yield approximately 500 ml of the P_3 fraction.

A Beckman Ti-l5 zonal rotor with a B-29 liner is filled through the edge at a pump speed of 25 ml/min while rotating at 2000 rpm. The centrifugation is performed in a Beckman L5-65 ultracentrifuge housed in a Baker biosafety cabinet. The rotor is loaded with 600 ml of 25% sucrose in 20 mM Tris-OAc, 1 mM EDTA (pH 8.3) at 4ºC, followed by 800 ml of 56% sucrose in 20 mM Tris-OAC, 1 mM EDTA (pH 8.3) at 4ºC. Each zonal centrifugation uses 140 ml of the P_3 sample, which is 10% of the total rotor volume. Sucrose is added to 6% to fraction P_3 prior to loading it into the center of the rotor while displacing 56% sucrose through the outer edge. The pump speed is now 15 ml/min. An overlay of 320 ml of 20 mM Tris-OAc, 1 mM EDTA (pH 8.3) follows the sample through the center line. The rotor is accelerated to 5000 rpm and then capped. The speed is increased to 32,000 rpm for a period of 14.5 hours. Fractions of 40 ml each are collected from the edge by replacing the rotor volume with 20 mM Tris-OAc, 1 mM EDTA (pH 8.3) at 4ºC. All fractions are kept on ice. Sulfobetaine 3-14 is added at a final concentration of 0.05% to each fraction. All fractions are frozen in a dry ice and ethanol bath and stored at –70ºC.

Each zonal gradient yields two or three fractions of 40 ml each that have specific infectivities of $10^{10.5}$ to 10^{11} ID_{50}/mg protein representing a purification of 3000- to 10,000-fold over the homogenate. As much as 95% of the infectivity loaded onto the gradient is recovered in the two or three fractions containing the highest titers (Fig. 3). The overall recovery

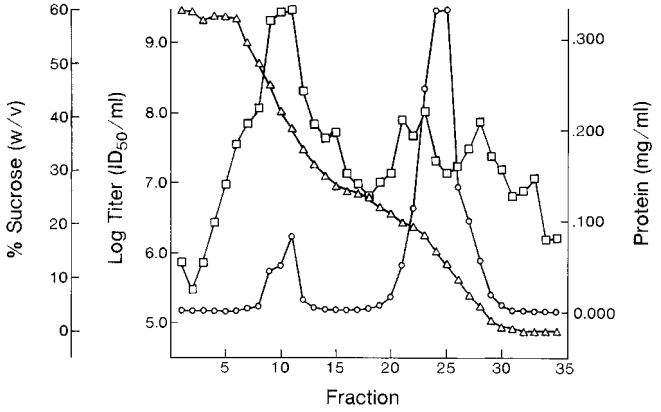

Figure 3 Discontinuous sucrose gradient fractionation of prion infectivity using a zonal rotor. The gradient was centrifuged at 32,000 rpm for 14.5 hr at 4ºC. The gradient was fractionated from the outer edge of the Ti15 zonal rotor equipped with a B-29 liner. Sucrose concentration (*triangles*), protein concentration (*circles*), and scrapie prion titer (*squares*) are shown. Fraction 1 was from the outer edge of the rotor. Each fraction was 40 ml. (Reprinted, with permission, from Prusiner et al. 1983 [copyright Cell Press].)

of scrapie infectivity from this protocol is generally greater than 20%. Disaggregation of scrapie prions during the purification procedure may contribute to this high level of recovery.

Purification of PrP^C

Purification of PrP^C was modified to avoid using low pH, urea, and SDS-PAGE, all of which might denature PrP^C (Pan et al. 1993; Pergami et al. 1996). Such procedures had been used in earlier studies of PrP^C (Turk et al. 1988; Pan et al. 1992). Typically, a crude microsomal fraction was prepared from 100 normal Syrian hamster brains, extracted with Zwittergent 3-12 (ZW) and centrifuged at 100,000*g* for 1 hour. The supernatant was applied to an IMAC-Cu^{++} column that was washed with 5 volumes of 0.015 M imidazole/0.15 M NaCl/10 mM sodium phosphate, pH 7.0/0.2% ZW. PrP^C was eluted by increasing the imidazole to 0.15 M. The pH of the eluate was adjusted to 6.4 with 2 M HCl prior to loading onto a SP cation-exchange column (1.5 x 3 cm), equilibrated with 0.15 M NaCl/20 mM MES, pH 6.4/0.2% ZW. The column was washed with 20 mM MOPS, pH 7.0/0.2% ZW (buffer A), containing initially 0.15 M NaCl and then 0.25

M NaCl. PrP^C was eluted with buffer A containing 0.5 M NaCl. The eluate was concentrated and desalted on an IMAC-Co^{++} column (1 x 2.5 cm), from which PrP^C was eluted with 0.1 M imidazole in buffer B: 0.15 M NaCl/20 mM MOPS, pH 7.5/0.2% ZW and applied to a wheat germ agglutinin (WGA)-Sepharose column (1 x 6.5 cm) equilibrated in the same buffer. The flow rate was 0.3 ml/min during loading, then 0.75 ml/min. After washing with 10 volumes of buffer B, PrP^C was eluted by 0.05 M N-acetyl glucosamine in buffer B. Purified PrP^C fractions were pooled and concentrated with a Centricon-30. N-Acetyl glucosamine was removed by three washes in 0.15 M NaCl/10 mM sodium phosphate, pH 7.5/0.12% ZW (PBSZ), and samples were stored at –20ºC.

The final step in the PrP^C purification utilized lectin chromatography, based on earlier observations that PrP 27-30 binds to WGA (Haraguchi et al. 1989). The extent of enrichment relative to other proteins was evaluated by silver-stained SDS-PAGE (Pan et al. 1993).

The amide I′ band of the Fourier transform infrared (FTIR) spectrum of purified PrP^C from pool 2 showed a symmetrical peak with a maximum at 1653 cm^{-1} (Fig. 4, solid line). Such spectra are characteristic of proteins

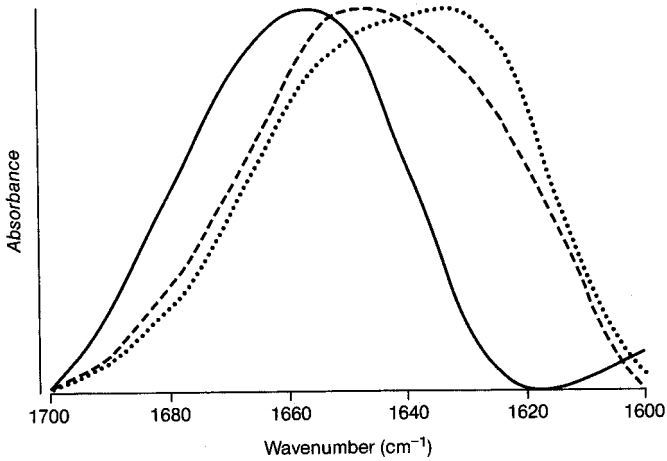

Figure 4 Fourier transform infrared spectroscopy of prion proteins. The amide I′ band (1700–1600 cm^{-1}) of transmission FTIR spectra of PrP^C (*solid line*), PrP^{Sc} (*dashed line*), and PrP 27-30 (*dotted line*). These proteins were suspended in a buffer in D_2O containing 0.15 M NaCl/10 mM sodium phosphate, pD 7.5 (uncorrected)/0.12% ZW. The spectra are scaled independently to be full scale on the ordinate axis (absorbance). (Reprinted, with permission, from Pan et al. 1993 [copyright National Academy of Sciences].)

Table 1 Secondary structural elements of PrP^C, PrP^{Sc}, and PrP 27-30

	Content of secondary structure, %										
	PrP						PrP 27-30				
	FTIR		predicted[a]					predicted[a]			
Structure	PrP^C	PrP^{Sc}	native	α/α	α/β	β/β	FTIR	native	α/α	α/β	β/β
α-Helix	42	30	14	31	27	0	21	20	45	39	0
β-Sheet	3	43	6	0	1	24	54	8	0	1	30
Turn	32	11					9				
Coil	23	16					16				

[a]Predicted values were calculated by using a neural network program (Presnell et al. 1993).

For PrP^C and PrP^{Sc} residues 23-231 were used in the calculation; for PrP 27-30 residues 90-231 were used. (Reprinted, with permission, from Pan et al. 1993 [copyright National Academy of Sciences].)

with high α-helix content. In contrast, the FTIR spectra of PrP^{Sc} (Fig. 4, dashed line) and PrP 27-30 (Fig. 4, dotted line) showed patterns that are characteristic of proteins with high β-sheet content. Deconvolution of the PrP^C spectrum gave an estimate of 42% α-helical content and only 3% β-sheet, whereas the PrP^{Sc} and PrP 27-30 spectra revealed 43% and 54% β-sheet, respectively (Table 1). The secondary structure of PrP 27-30 determined here by transmission FTIR is in very good agreement with data from attenuated total reflection of thin films (Gasset et al. 1993).

Although the FTIR spectrum for PrP^C with a peak at 1653 cm^{-1} is indicative of a secondary structure with a high α-helix content, we sought confirmation of this interpretation by circular dichroism (CD) spectroscopy, a technique that gives an unambiguous signal for proteins with a large α-helical component. The CD spectrum of PrP^C shows a minimum at 208 nm and a shoulder at 222 nm, clearly indicating that the protein contains one or more α-helices (Fig. 5). For estimating the α-helical content of proteins, far-UV data is thought to be preferable; however, the α-helical content can be determined from the ellipticity at 222 nm. At the 222-nm minimum, the rotational strength of an amino acid polymer composed completely of α-helices varies depending on the helix length (Yang et al. 1986). The ellipticity data in Figure 5 rely on protein concentrations determined by amino acid analyses. Assuming an average helix length of 14 residues, this gives an α-helix content of approximately 36% for PrP^C.

Expression and Purification of Recombinant PrP

DNA constructs encoding Syrian hamster (SHa) PrPs of varying lengths were inserted into expression vectors that were transfected into

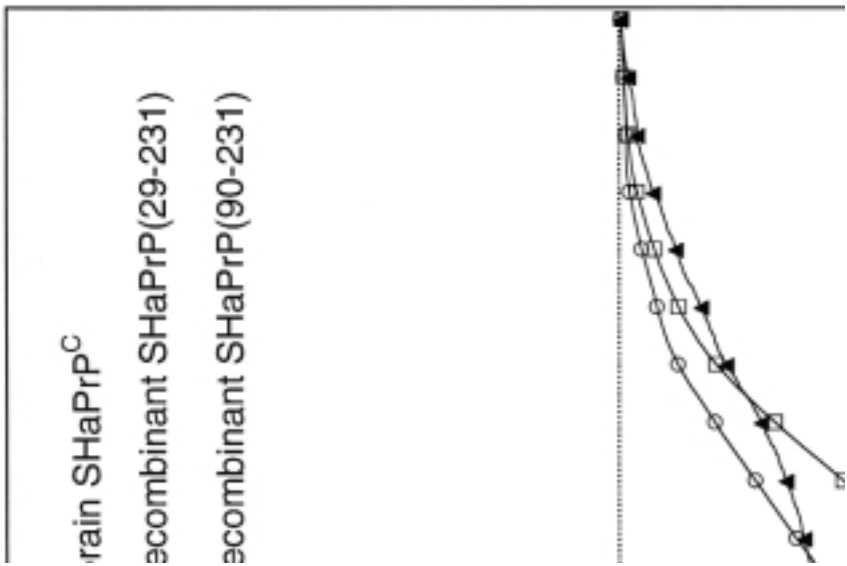

Figure 5 Circular dichroism spectra of PrP^C, rSHaPrP(90-231), and rSHaPrP(29-231). Purified PrP^C was prepared in a solution of 0.15 M NaCl/10 mM sodium phosphate, pH 7.5/0.12% ZW. The ordinate axis (ellipticity θ) was calibrated from amino acid analyses (Pan et al. 1993; Mehlhorn et al. 1996; Donne et al. 1997).

Escherichia coli deficient for proteases (Mehlhorn et al. 1996; Donne et al. 1997). Maximum expression was obtained with SHaPrP containing residues 90–231 corresponding to the sequence of PrP 27-30 and residues 29–231 corresponding to full-length PrP except for the amino-terminal six residues. Approximately 30 mg of recombinant (r) SHaPrP(90-231) and SHaPrP(29-231) could be obtained from each liter of *E. coli* culture. Disruption of the bacteria using a microfluidizer gave the highest yields of rSHaPrP, which had been targeted to the periplasmic space where it was deposited into refractile bodies. After solubilization of rPrP in 8 M GdnHCl, rSHaPrP was purified by size-exclusion chromatography and reversed-phase chromatography. The primary structure of rPrP was determined by Edman sequencing and mass spectrometry, and secondary structure by CD and FTIR spectroscopy. When rSHaPrP was purified under reducing conditions, it had a high β-sheet content and relatively low solubility similar to PrP^{Sc}, particularly at pH values greater than 7. Refolding of rSHaPrP by oxidation to form a disulfide bond between the two cysteine residues of this polypeptide produced a soluble protein with a high α-helical content similar to PrP^C. These multiple conformations of rSHaPrP are reminiscent of the structural plurali-

ty that characterizes the naturally occurring PrP isoforms. The high levels of purified rSHaPrPs have allowed determination of their molecular structures by solution nuclear magnetic resonance (NMR) (Donne et al. 1997; James et al. 1997).

Mouse PrP has also been expressed in *E. coli* and purified for structural studies (Hornemann et al. 1997; Riek et al. 1997). The expression of full-length MoPrP(23-231) was recovered from intracellular inclusion bodies and refolded by procedures similar to those described above. A carboxy-terminal fragment of MoPrP comprising 111 residues was expressed in *E. coli* and targeted to the periplasmic space, where it remained soluble and could be purified without prior denaturation (Hornemann and Glockshuber 1996).

Refolding of Recombinant PrPs

Several related strategies were employed for refolding the lyophilized rSHaPrPs obtained from the reverse-phase chromatography (RPC) fractions (Mehlhorn et al. 1996). Recombinant SHaPrP in the oxidized form was solubilized at 3–10 mg/ml in 6 M GdnHCl/50 mM Tris, pH 8, then rapidly diluted into either (1) 20 mM Tris, pH 8; (2) 20 mM sodium phosphate, pH 7.2; or (3) 20 mM sodium acetate, pH 5, to a final protein concentration of 100 μg/ml. The resulting solutions were incubated at 10–25ºC for 2–12 hours, then the insoluble material was separated by centrifugation, leaving α-helical rSHaPrP in solution, as determined by CD. The yield of soluble α-rPrP as measured by bicinchoninic acid (BCA) assay (Pierce) was ≥85%. For further physical studies, this α-rSHaPrP (Fig. 5) was dialyzed against a particular buffer and concentrated by Centriplus 10.

In another protocol that also yields α-rPrP, the lyophilized, oxidized fractions were dissolved in a small volume of water, assayed for protein content, and then made up to 1 mg/ml in 8 M GdnHCl (Zhang et al. 1997). This fraction was then diluted into 10 volumes of 20 mM Tris acetate, pH 8.0, with 5 mm EDTA. The resulting solution was dialyzed against 50 mM HEPES, pH 7.0, with 0.005% thimerosal, and the protein was further purified by cation exchange chromatography with a HiLoad SP Sepharose Fast Flow 26/10 column using a gradient of 0–0.4 M LiCl at 4 ml/min, monitoring UV absorption at 280 nm. Fractions containing the protein were dialyzed against 20 mM sodium acetate, pH 5.5, with 0.005% thimerosal. It was noted that over time, α-rPrP refolded by the first protocol described above underwent a degree of proteolysis; in material refolded by the second method, degradation was inhibited by the presence

of thimerosal, even when the rPrP solution was maintained at 37ºC for 10 days.

Lyophilized RPC fractions containing freshly purified rSHaPrP obtained under reducing conditions were dissolved in 20 mM MES, pH 6.5, to give β-rSHaPrP, as established by CD and FTIR (Zhang et al. 1997). These fractions could be concentrated to 10 mg/ml and appeared to remain in solution over an extended period of time without precipitating. At higher pH (7.5), the solubility was reduced substantially. The solubility at pH 6.5 was investigated by ultracentrifugation at 100,000*g* for 1 hour. The oxidation state of the cysteines was determined by carboxymethylation with iodoacetic acid, before and after reduction with dithiothreitol (DTT) (Mehlhorn et al. 1996)

Dispersion of Prions into Liposomes

The formation of PrP amyloid in vitro requires limited proteolysis in the presence of detergent (McKinley et al. 1991); these rod-shaped structures are often referred to as prion rods. Thus, PrP amyloid composed of PrP 27-30 in fractions enriched for scrapie infectivity are largely, if not entirely, artifacts of purification protocol. Although the insolubility of prion rods facilitated purification of infectious prions, it prevented many studies of the molecular structure of infectious prion particle. The discovery of conditions for solubilization of nondenatured PrP 27-30 and PrP^{Sc} with lipids and detergents to form detergent-lipid-protein complexes (DLPC) and liposomes was therefore an important advance (Fig. 6) (Gabizon et al. 1987, 1988b). The DLPCs could be centrifuged at 170,000*g* for 30 minutes with most of the PrP 27-30 and scrapie infectivity remaining in the supernatant fraction. In fact, scrapie infectivity generally increased 10- to 100-fold after dissociation of the prion rods into DLPCs or liposomes.

Subsequently, a protocol was developed for transfer of PrP^{Sc} from microsomal/synaptosomal membranes into DLPCs with preservation of infectivity and allowed fractionation of PrP^{Sc} as a soluble molecule (Gabizon et al. 1988a,b). The microsomes were solubilized by a combination of Sarkosyl (2%, w/v) and phosphatidylcholine (5 mg/ml). The resulting DLPCs were subjected to ultracentrifugation and the supernatant was applied to PrP monoclonal antibody affinity matrix. Monoclonal antibodies raised against purified PrP 27-30 were cross-linked to protein-A-Sepharose in order to minimize the leakage of the antibodies (Barry and Prusiner 1986). After the overnight incubation at 4ºC, the immunoaffinity matrix was washed with buffers containing increasing concentrations of salt, followed by progressive increments of

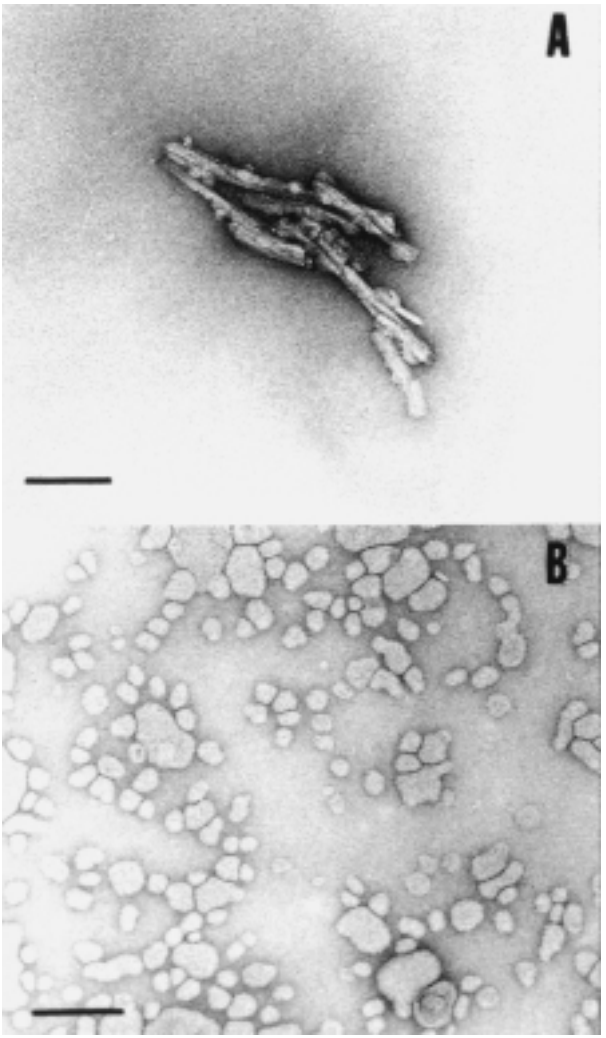

Figure 6 Electron micrographs of negatively stained and immunogold-labeled prion rods containing PrP 27-30 dispersed into DLPCs. (*A*) Typical prion rods negatively stained with uranyl formate. (Bar, 100 nm.) (*B*) Negatively stained liposomes containing PrP 27-30. (Reprinted, with permission, from Gabizon et al. 1987.)

pH. Fractions eluted from the column were screened for both PrP and infectivity. Those fractions that contained PrP^{Sc} also carried infectivity, whereas those fractions with no detectable PrP^{Sc} contained either low levels of scrapie prions or none. Moreover, the amount of PrP^{Sc} recovered

from the column roughly correlated with infectivity. These results demonstrated a clear correlation between PrP^{Sc} and infectivity as reported earlier using other procedures (see Fig. 1).

The transfer of scrapie infectivity from prion rods to liposomes also provided a new method by which we could search for hidden or cryptic nucleic acid within the prion as described in detail below. Treatment of DLPCs as well as liposomes with nucleases or Zn^{++} failed to alter scrapie infectivity (Gabizon et al. 1987). In subsequent studies, nucleases were added during the formation of the DLPCs. As before, no change in infectivity was observed after prolonged incubations.

In additional experiments with irradiation of DLPC-incorporated prions, UV light at 254 nm produced an inactivation curve virtually identical to that observed for the prion rods in the experiments reported earlier (Gabizon et al. 1988c). The kinetics of inactivation of scrapie infectivity by UV irradiation yielded exponential survival curves characteristic of a single-hit process. We estimated the D_{37} values 17–24 J/m^2 for purified prion rods and DLPCs; the values are in good agreement with those reported two decades earlier for murine scrapie agent in brain homogenate (Alper et al. 1967).

Although an early radiobiological investigation on the scrapie agent suggested M_r of about 150,000 (Alper et al. 1966, 1978), the implication that the scrapie pathogen might not possess a nucleic acid was frequently ignored. We irradiated prion rods and liposomes in 14 different preparations with increasing doses up to 400 Mrad. The results yielded a mean target size for the scrapie agent of 55,000 ± 9,000 D (Fig. 7) (Bellinger-Kawahara et al. 1988). An equally important conclusion from those studies is that the target size for the prion infectivity appears to be independent of the physical form of the prion. Samples containing aggregates in the form of amyloid rods, microsomes, crude homogenates, or liposomes had the same target size.

The solubilization of prions with phospholipids helped to clarify the issues concerning heterogeneity of the infectious particle with respect to size, density, and charge. The interconversion of prion rods, DLPCs, and liposomes, all with high levels of infectivity, argued that prions are likely to be composed of a single hydrophobic macromolecule, i.e., PrP^{Sc}. All attempts to reveal a putative hidden or cryptic nucleic acid upon transfer of scrapie infectivity in DLPCs have been unsuccessful.

DLPC and liposome technology has found the most recent application in the development of recombinant antibody fragments (rFabs) against PrP (Chapter 16). In our initial studies, $Prnp^{0/0}$ mice were immunized with purified mouse prion rods (Prusiner et al. 1993b); from these

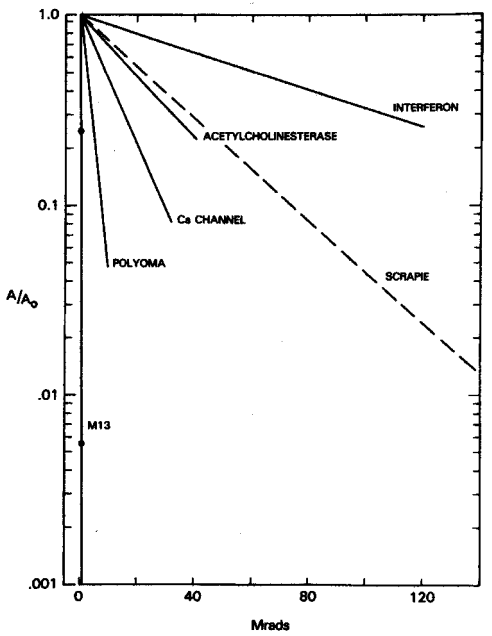

Figure 7 Ionizing radiation target size of prions. Radiation inactivation curves of several different biological activities: enzyme reactions, receptors, and viral infectivity. Data are adjusted to a common irradiation temperature of –135ºC (Kempner and Haigler 1982). The double-stranded DNA polyomavirus data are from Latarjet et al. (1967). Diltiazem binding to muscle Ca channel gave a target size of 130 kD (Goll et al. 1984b); acetylcholinesterase size was 66 kD (Goll et al. 1984a); leukocyte interferon displayed a 20-kD target (Pestka et al. 1983). M13 target size >1000 kD. M13 was prepared and assayed as described previously (Bellinger-Kawahara et al. 1987a). (Reprinted, with permission, from Bellinger-Kawahara et al. 1988.)

mice, a panel of monoclonal Fab fragments were obtained corresponding to five major specificities (Williamson et al. 1996). Disappointingly, however, PrP 27-30 was not detected by any of these antibodies prior to treatment with GdnSCN. An explanation for this finding was in the physicochemical properties and antigenicity of PrP 27-30 in prion rods. Purified PrP 27-30 polymerizes into rod-like structures of 100–200 nm in length that are composed of as many as 1000 PrP 27-30 molecules (Prusiner et al. 1983; McKinley et al. 1991). This aggregation apparently reduced the effective epitope concentrations, thereby hindering efficient immunization and selection of specific antibody phage. It has been previously shown that epitope concentration is a critical factor in effective selection

from antibody phage display libraries (Parren et al. 1996). In a recent study (Peretz et al. 1997), it was possible to overcome the problem of cryptic epitopes in PrP 27-30 within prion rods by immunizing $Prnp^{0/0}$ mice with a PrP 27-30 incorporated into liposomes (Gabizon et al. 1987) and rescuing the corresponding rFabs. $Prnp^{0/0}$ mice immunized with dispersed PrP 27-30 were used to generate a new panel of recombinant antibodies, some of which reacted with nondenatured PrP^{Sc} and PrP 27-30.

IMMUNOASSAYS OF PrP ISOFORMS

For many years, investigators thought that scrapie and the related human prion diseases kuru and Creutzfeldt-Jakob disease (CJD) were caused by slow viruses (Gajdusek 1977; Parry 1983; Pattison 1988). Among the most puzzling features of these transmissible diseases was the lack of any detectable immune response to the inoculated infectious particles (Chandler 1959; Clarke and Haig 1966; Tsukamoto et al. 1985). Neither scrapie-specific antibodies nor a cellular immune response was found. In addition, prion-mediated neurodegeneration does not activate the viral surveillance system, since there is no evidence of induction of interferon (Katz et al. 1968; Field et al. 1969; Worthington 1972).

Once the protease-resistant core of PrP^{Sc}, designated PrP 27-30, was discovered in fractions highly enriched for scrapie, antisera were generated in rabbits (Bendheim et al. 1984; Bode et al. 1985). High-titer, specific polyclonal antibodies became an extremely important tool for the mapping and characterization of PrP by cellular or molecular methods. Initial attempts to raise such sera were formidable tasks (Bendheim et al. 1984; Bode et al. 1985), in large part because it was difficult to purify sufficient quantities of PrP 27-30 from scrapie-infected hamster brains for immunogen, as well as due to the low antigenicity of prion rods.

Because of the general lack of antibodies that distinguish PrP^{Sc} from PrP^{C}, with one possible exception (Korth et al. 1997), current detection systems often utilize either the proteinase K resistance or insolubility of the PrP^{Sc} isoform. After limited digestion with proteinase K, PrP 27-30 has been detected by Western blots (Bendheim et al. 1984), dot blots (Serban et al. 1990), and histoblots (Taraboulos et al. 1992). The foregoing immunoassays are relatively insensitive compared to bioassays. Additionally, their use in some transgenic models of prion diseases is limited because the presence of intermediate conformations of PrP leads to seemingly ambiguous results (Hsiao et al. 1990; Scott et al. 1997b). Superior assays for PrP^{Sc} are likely to be based on conformationally sensitive antibodies able to distinguish

PrP^{C} from PrP^{Sc} or on conformationally sensitive procedures (Safar et al. 1998).

The production of anti-PrP polyclonal antisera, monoclonal antibodies, and recombinant antibody fragments (Fabs) is described in Chapter 16. In addition, the production and use of monospecific antisera directed against synthetic PrP peptides are described. The practical applications of rapid, sensitive immunoassays for PrP^{Sc} are discussed in Chapter 17.

Conformation-dependent Immunoassay for PrP^{Sc}

Dot-blotting studies showed that in contrast to PrP^{C}, the immunoreactivity of PrP^{Sc} was greatly enhanced by denaturation (Serban et al. 1990). Subsequently, studies with anti-PrP monoclonal antibodies (MAbs) and recombinant Fabs showed that transformation of PrP^{C} into PrP^{Sc} is accompanied by the burying of epitopes near the amino terminus of PrP, whereas carboxy-terminal alepitopes remain exposed (Peretz et al. 1997).

Recombinant SHaPrP(90-231) could be refolded into proteins with predominantly α-helical or β-sheet structures and yielded purified proteins that share some structural features with PrP^{C} and PrP^{Sc}, respectively (Mehlhorn et al. 1996; Zhang et al. 1997). To measure the binding of antibodies to PrP, we adapted a direct ELISA-formatted, dissociation-enhanced time-resolved fluorescence (TRF) detection system (Hemmilä and Harju 1995). The sensitivity limit of detecting denatured SHaPrP(90-231) with europium (Eu)-labeled anti-PrP 3F4 MAb IgG (Kascsak et al. 1987) was less than or equal to 5 pg/ml with a dynamic range of over 5 orders of magnitude (data not shown).

Since the immunoreactivity of this MAb is high with PrP^{Sc} after the protein has been denatured but weak when PrP^{Sc} is folded into its native, infectious state, the immunoreactivity of 3F4 MAb was investigated using 5% brain homogenate obtained from PrP-deficient ($Prnp^{0/0}$) mice (Büeler et al. 1992) and spiked with purified recombinant SHaPrP(90-231) in either an α-helical or β-sheet conformation. The conformation of SHaPrP(90-231) after refolding was verified by CD spectroscopy (Fig. 8A). SHaPrP(90-231) was unfolded by treatment with 4 M GdnHCl for 5 minutes at 80ºC, diluted 20-fold, and stored for 16 hours at 5ºC before its conformation was determined by CD spectroscopy. The results (Fig. 8A) confirmed that the protein did not refold under the conditions used for crosslinking to the activated plastic ELISA plate.

When Eu-labeled 3F4 IgG was used in the presence of 5% $Prnp^{0/0}$ mouse brain homogenate, the detection limit of denatured SHaPrP(90-231) was less than or equal to 2 ng/ml, and the dynamic range was over 3

orders of magnitude. The input/output calibration for denatured SHaPrP(90-231) in both α-helical and β-sheet conformations and with purified infectious PrP^{Sc} gave sensitivity and linearity within the same range (data not shown). The interassay variation coefficient (100*SD/average) was within the assay range of less than or equal to 6.6%.

The data obtained with SHaPrP(90-231) in an α-helical conformation (Fig. 8B) indicated a relatively small difference between signals of α-helical and denatured proteins. In contrast, the reactivity with the native β-

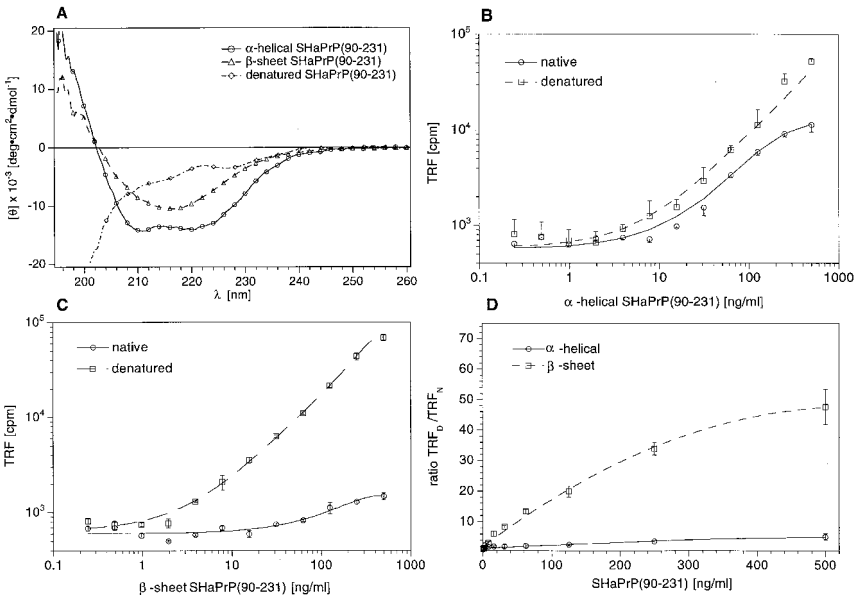

Figure 8 Development of a conformation-dependent immunoassay for recombinant SHaPrP(90-231) purified from *E. coli* and folded into different conformations. (*A*) Conformation of recombinant SHaPrP(90-231) as determined by CD spectroscopy. (*B*) Calibration of conformation-dependent immunoassay with recombinant SHaPrP(90-231) in α-helical conformation and (*C*) in β-sheet conformation. (*D*) Ratios between signals of denatured/native recombinant SHaPrP(90-231) in α-helical and β-sheet conformations. Two major bands in the CD spectrum with minima at 208 and 222 nm indicate an α-helical conformation; a single negative band with minimum at 217 nm is characteristic of predominantly β-sheet conformation; a negative trough toward 197 nm documents random-coil conformation. The calibration of the conformation-dependent immunoassay was performed in the presence of 5% (w/v) $Prnp^{0/0}$ mouse brain homogenate. The data points and bars represent average ± S.E.M. obtained from four independent measurements. (Reprinted, with permission, from Safar et al. 1998 [copyright *Nature Medicine*].)

sheet form of SHaPrP(90-231) only marginally exceeded the background (Fig. 8C). When the results were expressed as a ratio between binding of 3F4 MAb to native versus denatured conformations of PrP, the ratio for α-helical SHaPrP(90-231) was less than or equal to 5, and for the β-sheet conformation, it was greater than 5 (Fig. 8D). Thus, the ratio of 3F4 MAb binding to denatured/native PrP was a sensitive indicator of the original native PrP conformation where a value greater than 5 in the presence of *Prnp*$^{0/0}$ brain homogenate indicated the presence of β-sheet conformers of recombinant SHaPrP(90-231).

Measurement of SHaPrP^{Sc} in Brain Homogenates

Fluorescence signals for native and denatured samples of normal SHa brain homogenate containing PrP^{C} were similar and the ratio of antibody binding to denatured/native PrP was less than or equal to 1.8 (Fig. 9A,B). In contrast, the ratio for a serial dilution of scrapie-infected SHa brain homogenate containing a mixture of PrP^{C} and PrP^{Sc} was greater than 2 (Safar et al. 1998). In general, the data on serially diluted normal and scrapie-infected SHa brain homogenate followed the pattern described for α-helix and β-sheet conformations of recombinant PrP(90-231). A ratio

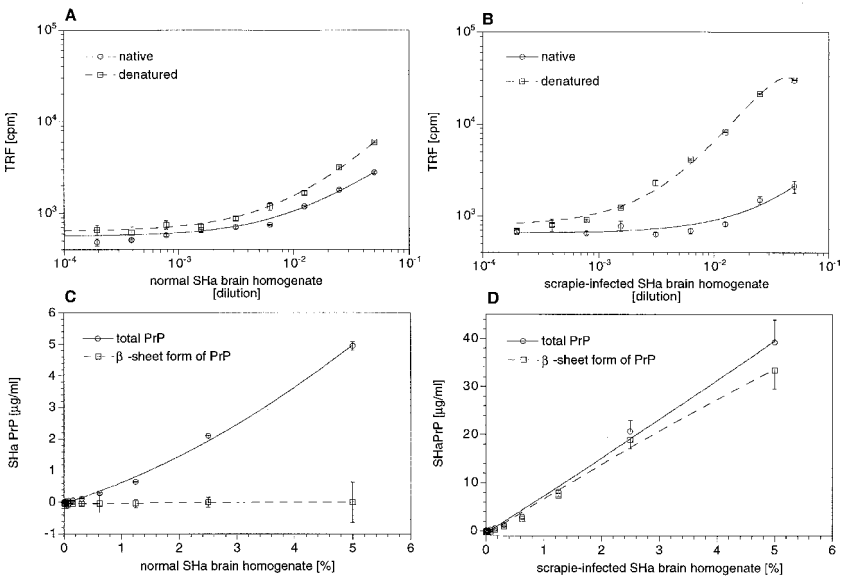

Figure 9 (See facing page for legend.)

of antibody binding to denatured/native PrP greater than 1.8 indicated the presence of SHaPrP^{Sc}.

From the signal of denatured and native samples developed with Eu-labeled 3F4 IgG, the concentration of PrP^{Sc} may be directly calculated by formula 1 (Fig. 9C and 9D). As expected, the normal brain homogenate contained only PrP^{C} in an α-helical conformation. In contrast, the data show that an approximately 5-fold increase of total PrP in the brains of scrapie-infected Syrian hamsters was caused by accumulation of PrP^{Sc} in a β-sheet conformation (Fig. 9D).

In a typical experiment, the monoclonal IgG was purified from ascitic fluid by protein A chromatography on a Pharmacia FPLC column and labeled with europium-chelate of N-(p-isothiocyanatobenzyl)-diethylene-

Figure 9 Conformation-dependent immunoassays of PrPs in homogenates prepared from SHa brains. (*A*) PrP^{C} in normal SHa brain homogenates. (*B*) PrP^{C} and PrP^{Sc} in homogenates from the brains of Syrian hamsters exhibiting signs of CNS dysfunction ~70 days after inoculation with Sc237 prions. (*C*) Total amount of PrP in normal SHa brain homogenates and the amount of PrP in the β-sheet. (*D*) Total amount of PrP in homogenates from the brains of Syrian hamsters inoculated with Sc237 prions and the amount of PrP in the β-sheet. The samples of 5% brain homogenates obtained from normal or scrapie-infected Syrian hamsters were serially diluted into 5% *Prnp*$^{0/0}$ mouse homogenate. Data points and bars represent average ± S.E.M. obtained from four independent measurements. The concentration of SHaPrP on the ordinate was calculated from formula 1 below. The ratio between binding of Eu-labeled IgG to PrP protein in native and denatured conformations measured by TRF was designated TRF_D/TRF_N. A mathematical model was developed to calculate the content of β-sheet-structured PrP in an unknown sample directly:

$$[PrP_\beta] \sim \Delta\, TRF_\beta = TRF_D - (TRF_N * f_\alpha) \quad (1)$$

In Equation 1, an excess of antibody binding to PrP protein in the transition from native to denatured state over that expected for α-helical conformation is directly proportional to the amount of PrP protein in β-sheet conformation. Symbols in the equations are as follows: ($\Delta\, TRF_\beta$) change in the time-resolved fluorescence of PrP in β-sheet conformation during the transition from the native to denatured state; (TRF_D) time-resolved fluorescence of PrP in the denatured state; (TRF_N) time-resolved fluorescence of PrP in the native conformation; (fα) correlation coefficient for dependency of TRF_D on TRF_N obtained with standard recombinant α-helical SHaPrP(90-231) or purified Syrian hamster PrP^{C}. The coefficient was obtained by fitting the plot of antibody binding to denatured/native protein by the nonlinear least-squares regression program. (Reprinted, with permission, from Safar et al. 1998 [copyright *Nature Medicine*].)

triamine-N^1,N^2,N^3,N^3-tetraacetic acid (DTTA) at pH 9.6 for 16 hours at room temperature according to manufacturer's protocols (Wallac Inc, Turku, Finland). The final Eu/IgG molar ratio was 13. Immunoassay for PrP^{Sc} by dissociation-enhanced TRF was performed by dividing each sample into two aliquots: (1) untreated and designated native and (2) mixed to a final concentration of 4 M GdnHCl, heated for 5 minutes at 80ºC, and designated denatured. Both samples were immediately diluted 20-fold with H_2O containing protease inhibitors (5 mM PMSF; aprotinin and leupeptin, 4 μg/ml each), and aliquots were loaded on a 96-well polystyrene plate that was previously activated for 1 hour with 0.2% glutaraldehyde in PBS, pH 7.4. The plates were incubated overnight at 5ºC and then blocked with TBS, pH 7.8 containing 0.5% BSA (w/v) and 6% sorbitol (w/v) for 2 hours at room temperature. In the next step, they were washed three times with TBS, pH 7.8, containing 0.05% (v/v) of Tween 20 and incubated at room temperature for 2 hours with europium-labeled antibodies listed above. The plates were developed after seven washing steps in enhancement solution provided by the europium label supplier (Wallac Inc, Turku, Finland), and the signal was counted on a DELFIA 1234 (Wallac Inc, Turku, Finland) fluorometer.

Evidence for Different Conformations of PrP^{Sc} in Eight Prion Strains

Using the highly sensitive conformation-dependent immunoassay for measurement of PrP^{Sc} in tissue homogenates, we examined eight different prion strains passaged in Syrian hamsters (Safar et al. 1998). Brains from Syrian hamsters were collected when the animals displayed signs of neurologic dysfunction; the incubation times for the prion strains varied from 70 to 320 days (Fig. 10A). Most of the PrP in the brains of Syrian hamsters with signs of neurologic disease was PrP^{Sc}, as defined by the β-sheet conformation. The level of PrP^{Sc} in the brains of these clinically ill animals exceeded that of PrP^{C} by 3- to 10-fold. The highest levels of PrP^{Sc} were found in the brains of Syrian hamsters infected with the Me7-H strain; in contrast, the lowest levels were found in the brains of Syrian hamsters inoculated with the SHa(Me7) strain (Fig. 10A). Interestingly, the Me7-H and SHa(Me7) strains, which were both derived from Me7 passaged in mice, possessed similar denatured/native PrP ratios, but they accumulated PrP^{Sc} to quite different levels (Fig. 10A). The highest denatured/native PrP ratio of all tested strains was SHa(RML). Interestingly, there seemed to be no relationship between incubation time and either the concentrations of PrP^{Sc} or the ratio of denatured/native PrP.

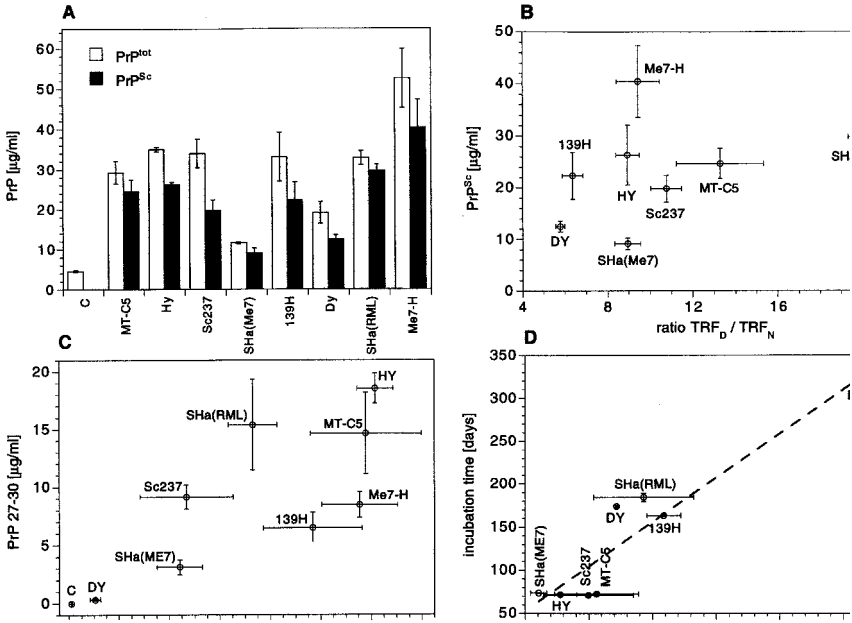

Figure 10 Eight prion strains distinguished by the conformation-dependent immunoassay. (*A*) Concentration of total PrP and PrP^{Sc}. Data represent the average ± S.E.M. obtained from three different brains of LVG/LAK Syrian hamsters infected with different prion strains and measured in three independent experiments. (*B*) Ratio of antibody binding to denatured/native PrP and a function of concentration of PrP^{Sc} in the brains of Syrian hamsters infected with different prion strains. Concentration of PrP^{Sc} (Eq. 1) and the ratio of antibody binding to denatured/native PrP were measured by the conformation-dependent immunoassay. (*C*) Brain homogenates of Syrian hamsters inoculated with different scrapie strains and uninoculated controls (*C*) were digested with proteinase K before the conformation-dependent immunoassay. (*D*) Incubation time plotted as a function of the concentration of the proteinase-K-sensitive fraction of PrP^{Sc} ([PrP^{Sc}]–[PrP 27-30]). (Reprinted, with permission, from Safar et al. 1998 [copyright Nature Medicine].)

The apparent independence of the ratio of denatured to native PrP from the concentration of PrP^{Sc} became apparent after plotting both parameters in a single plot (Fig. 10B). Each strain occupied a unique position, indicating differences in the conformation of accumulated PrP. Because the PrP^{C} concentration in each strain was less than or equal to 5 µg/ml and the PrP ratio for PrP^{C} was less than or equal to 1.8, the expected impact of the presence of PrP^{C} on the final PrP ratio was less than or equal to 15%.

Since only the most tightly folded conformers of PrP^{Sc} are likely to be protease resistant, we digested each of the brain homogenates with proteinase K prior to measuring the ratio of denatured to native PrP. As shown, the positions of many of the strains changed when the protease-sensitive conformers of PrP^{Sc} were enzymatically hydrolyzed (Fig. 10C). Most notable was the DY strain, which was readily detectable before limited proteolysis by immunoassay (Fig. 10B) but became almost undetectable after digestion (Fig. 10C), in accord with earlier Western blot studies (Bessen and Marsh 1992, 1994). Equally important, strains such as Sc237 and HY were marginally separated prior to proteinase K digestion (Fig. 10B) but became quite distinct afterwards (Fig. 10C). These findings argue that Sc237 and HY are distinct strains even though they exhibit similar incubation times of about 70 days when passaged in Syrian hamsters (Scott et al. 1997a). It is noteworthy that limited proteolysis of PrP^{Sc} from Sc237- and HY-infected brains produced PrP 27-30 proteins that were indistinguishable by migration in SDS-PAGE as detected by Western immunoblotting (Scott et al. 1997a).

In contrast to the lack of any correlation with incubation times noted above, an excellent correlation was found when the proteinase K-sensitive fraction of PrP^{Sc} ([PrP^{Sc}]–[PrP 27-30]) was plotted as a function of the incubation time for all eight prion strains (Fig. 10D) (Safar et al. 1998). The proteinase K-sensitive fraction of PrP^{Sc} can be considered a surrogate for PrP^{Sc} clearance. Since the binding of PrP^{C} or a metastable intermediate PrP* to protein X seems to be the rate-limiting step in prion replication (Kaneko et al. 1997c), it follows that the different incubation times of various prion strains should arise predominantly from distinct rates of PrP^{Sc} clearance rather than from the different rates of PrP^{Sc} formation. In accordance with the excellent correlation between protein K-sensitive PrP^{Sc} and incubation times, prion strains that seem to be readily cleared have prolonged incubation times, whereas those that are poorly cleared display abbreviated incubation periods. However, it is important to recognize that proteinase K sensitivity is an imperfect model for in vivo clearance and that only one strain with a long incubation time has been studied.

Equilibrium Dissociation and Unfolding of PrPs by GdnHCl

To extend the foregoing studies on the Sc237 and HY strains, equilibrium dissociation and unfolding of the PrP^{Sc} molecules in guanidine hydrochloride (GdnHCl) were monitored by Eu-labeled 3F4 IgG (Fig. 11) (Safar et al. 1998). Additionally, data for PrP^{C} and the DY strain are

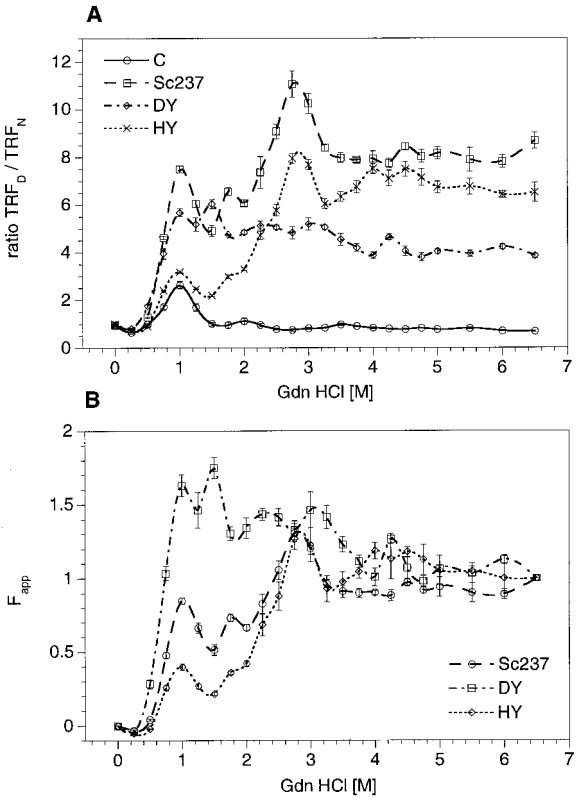

Figure 11 Equilibrium dissociation and unfolding of PrP^C and PrP^{Sc} in three prion strains. Ratio of antibody binding to denatured/native PrP and apparent fractional change of unfolding of prion proteins during equilibrium dissociation and unfolding. (*A*) The brains of uninoculated controls (C) and Syrian hamsters infected with Sc237, DY, and HY strains of prions were analyzed by the conformation-dependent immunoassay; the ratio of antibody binding to denatured/native PrP is plotted as a function of the GdnHCl concentration. (*B*) The apparent fractional change unfolding (F_{app}) of PrP^{Sc} from the Sc237, DY, and HY strains. The points and bars represent the average ± S.E.M. obtained from four different measurements. (Reprinted, with permission, from Safar et al. 1998 [copyright *Nature Medicine*].)

depicted. After equilibrium unfolding, the proteins were rapidly diluted to the same protein and GdnHCl concentrations and then cross-linked to the glutaraldehyde-activated plate. The binding of 3F4 IgG was expressed as the ratio of antibody binding to denatured/native PrP (Fig. 11A) or as the fractional change in the transition from the native (~0) to fully unfolded

(~1) state (Fig. 11B). The PrP ratios increased with increasing GdnHCl concentrations, indicating continued unfolding. The unfolding patterns of the ratio of antibody binding to denatured/native PrP for PrP^C in normal brain were clearly different from the unfolding patterns for the PrPs in scrapie-infected brains. Each strain had a unique ratio of antibody binding to denatured/native PrP and, as a result, a unique unfolding pattern. The peaks indicated the presence of PrP conformers with a higher affinity than at the next higher or lower GdnHCl concentrations. The largest interstrain differences were at ~1 M, ~3 M, and 6.5 M GdnHCl (Fig. 11A). The ratios of antibody binding to denatured/native PrP for different prion strains at 6.5 M GdnHCl, where all PrPs were unfolded (Safar et al. 1993; Hornemann et al. 1997; Zhang et al. 1997), were close to the values obtained with samples exposed to 4 M GdnHCl and 80ºC for 5 minutes (Fig. 11A).

We also compared the relative abundance of different PrP conformers in Sc237-, DY-, and HY-infected brains during the transition from the folded (native, ~0) to unfolded state (denatured, ~1) (Fig. 11B). The data expressed as fractional change (F_{app}) allowed for the comparison of samples with different PrP concentrations. The largest interstrain differences were at ~1 M GdnHCl; the highest fraction of conformers that were sensitive to such low concentrations of GdnHCl was found in SHa brains infected with the DY strain.

Selective Precipitation of PrP^{Sc} by NaPTA

Once the utility of our conformation-dependent immunoassay for PrP^{Sc} became clear, we sought to increase both sensitivity and specificity by enriching for PrP^{Sc} prior to immunoassay. Although we could readily detect PrP^{Sc} when the concentration of PrP^{Sc} was equal to or exceeded that of PrP^C, we encountered difficulty with measuring PrP^{Sc} when its level was 1% or less than that of PrP^C. After testing a number of precipitation procedures, we found that at neutral pH in the presence of Mg^{++}, Na phosphotungstate (NaPTA) formed complexes with oligomers and polymers of infectious PrP^{Sc} and PrP 27-30 but not with PrP^C (data not shown) (Safar et al. 1998). The resulting dense aggregates were then collected by a single 30-minute low-speed centrifugation. The pellet contained about 99% of the prions but less than 1% of the other proteins. PrP 27-30 formed rod-like structures that could be detected directly by electron microscopy without further processing. The morphology of these rod-like structures resembled in some respects the rods found in highly

purified preparations of PrP 27-30 (Prusiner et al. 1983).

After the selective precipitation of PrP^{Sc} by NaPTA was incorporated into the conformation-dependent immunoassay for PrP^{Sc}, it became possible to measure the low concentrations of PrP^{Sc} in brain homogenates from prion-infected animals. Scrapie-infected SHa brain homogenate was serially diluted into normal, uninfected SHa brain homogenate in order to simulate in vivo conditions where the levels of PrP^{Sc} would be low early after infection was initiated. The resulting samples contained different levels of infectivity and PrP^{Sc} in the presence of a constant level of PrP^{C}. The level of PrP^{Sc} could be measured as a function of the ratio of antibody binding to denatured/native PrP over approximately a 10^5-fold range. After scrapie-infected homogenates were diluted about 10^{-5}, the ratio of denatured to native PrP approached 1.8, which is what was found when PrP^{C} alone was measured (Fig. 12A). Above a value of 1.8, the ratio of antibody binding to denatured/native PrP reliably indicated the presence of increasing quantities of PrP^{Sc}.

The application of formula 1 (Fig. 9) allowed direct calculation of the concentration of PrP^{Sc} in a mixture of scrapie-infected and normal brain homogenates (Fig. 12B). The sensitivity limit by phosphotungstate (PTA) precipitation followed by immunoassay is less than or equal to 1 ng/ml of PrP^{Sc} in the presence of a 3000-fold excess of PrP^{C}. Assuming an average prion titer of 10^8 ID_{50}/ml for Sc237-inoculated SHa brain homogenate, the sensitivity limit of PrP^{Sc} detection corresponds to an infectivity titer of about 10^3 ID_{50}/ml.

In a typical experiment, brain homogenate (5% [w/v]) containing 2% Sarkosyl was mixed with stock solution containing 4% sodium phosphotungstate (NaPTA) and 170 mM $MgCl_2$, pH 7.4, to obtain a final concentration of 0.2–0.3% NaPTA. Generally, 1-ml samples were incubated for 16 h at 37ºC on a rocking platform and centrifuged at 14,000*g* in a tabletop centrifuge (Eppendorf) for 30 minutes at room temperature. The optional treatment with 25 μg/ml of proteinase K for 1 hour at 37ºC was performed before or after precipitation. The pellet was resuspended in H_2O containing protease inhibitors (0.5 mM PMSF; aprotinin and leupeptin, 2 μg/ml each) and assayed by the conformation-dependent immunoassay as described above.

SEARCH FOR NUCLEIC ACIDS IN PRIONS

Three approaches have been used to search for a scrapie-specific nucleic acid component within the infectious prion particle causing scrapie and CJD. These include (1) inactivation of prions by procedures that modify

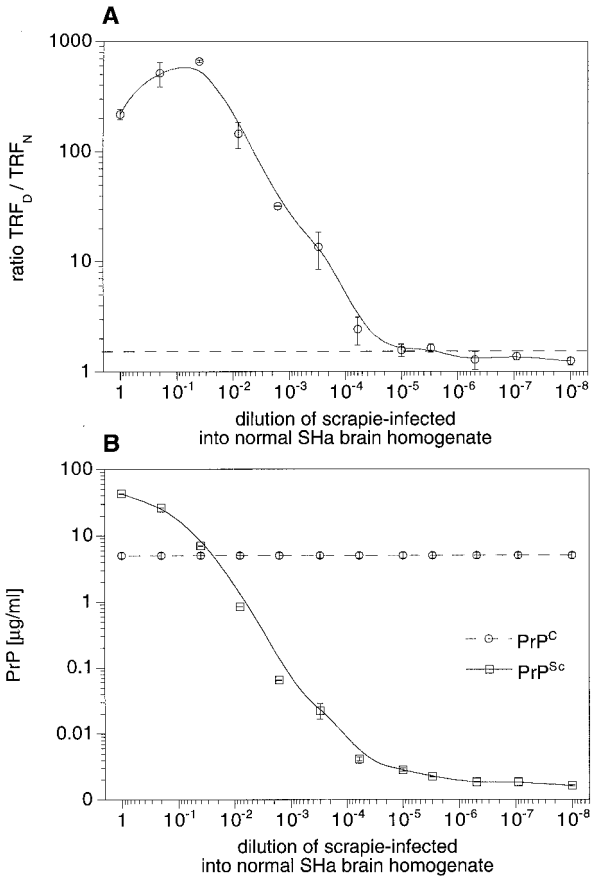

Figure 12 Dynamic range and sensitivity of the ratio of antibody binding to PrP in denatured and native states. Homogenates (5%) prepared from the brains of Syrian hamsters exhibiting signs of CNS dysfunction ~70 days after inoculation with Sc237 prions were serially diluted into 5% normal SHa brain homogenate, and the presence of PrP^{Sc} was measured after NaPTA precipitation by the conformation-dependent immunoassay. (*A*) The ratio of the fluorescence signals of denatured and native aliquots plotted as a function of the dilution of scrapie-infected brain homogenate. (*B*) The absolute amount of PrP^C and PrP^{Sc} was calculated from Equation 1 and plotted as a function of the dilution of scrapie-infected brain homogenate. The data points and bars represent average ± S.E.M. obtained from four independent measurements. (Reprinted, with permission, from Safar et al. 1998 [copyright *Nature Medicine*].)

or hydrolyze nucleic acids, (2) characterization of nucleic acids in fractions enriched for prion infectivity, and (3) search for a nucleic acid unique to scrapie infectious preparations.

The resistance of prions to inactivation by physical, chemical, and enzymatic procedures that modify nucleic acids is an important argument in support of the proposition that prions are devoid of nucleic acid. Prions resist inactivation by procedures that hydrolyze, modify, or shear nucleic acids but are inactivated by procedures that denature or modify proteins (Prusiner 1982). Similar results were obtained with crude brain homogenates (Alper et al. 1966, 1967), purified prion preparations (Prusiner 1982; Bellinger-Kawahara et al. 1987b), prion liposomes (Gabizon et al. 1988c), and prions from cultured neuroblastoma cells (Neary et al. 1991). The search for a scrapie-specific nucleic acid utilized gel electrophoresis, ultracentrifugation, and differential hybridization employing cDNA libraries, as well as molecular cloning and PCR to search for foreign sequences. Some aspects of the search for a scrapie-specific nucleic acid have assumed that such a polynucleotide would be protected by a protein coat, as is the case for viruses. Such a hypothetical nucleic acid was assumed to possess physical properties similar to those exhibited by known nucleic acids.

To assess the unprecedented possibility that scrapie-specific nucleic acids are heterogeneous in size, the use of return refocusing gel electrophoresis (RRGE) was developed. Studies described below demonstrate that small nucleic acids up to several hundred nucleotides in length withstand harsh procedures that hydrolyze nucleic acids in prion preparations. Although the variety of procedures used in these studies argue that a nucleic acid component within the prion particle is highly unlikely, they do not exclude such a molecule.

Nucleic Acids in Fractions Enriched for Prion Infectivity

In some studies, nucleic acids were reported to be found only in fractions prepared from scrapie-infected animals. By end-labeling and gel electrophoresis (Dees et al. 1985; German et al. 1985; Castle et al. 1987) and by differential hybridization of a cDNA library (Duguid et al. 1988; Aiken et al. 1989; Duguid and Dinauer 1989), differences in the patterns of nucleic acids from infected and noninfected tissue were found. In other studies, copurification of nucleic acids and scrapie infectivity was described. The studies include analysis of nucleic acids in ultracentrifugation (Sklaviadis et al. 1989), detection of retroviral and polyadenylated RNA by PCR technique (Akowitz et al. 1990; Murdoch et al. 1990), identification of mitochondrial DNA by hybridization (Aiken et al. 1990), and reports on ssDNA in electron microscopy (Narang et al. 1988; Narang

1990). These results were interpreted as evidence indicative of a scrapie-specific nucleic acid.

The detection of a nucleic acid under these circumstances does not mean that it is an essential component of the infectious prion particle. In no case reported to date has a convincing correlation between a scrapie-specific nucleic acid and prion infectivity been demonstrated. The ultracentrifugation studies mentioned above were carried out after disaggregation of CJD infectious material by detergent treatment and yielded a PrP peak sedimenting at 6S to 9S that was well separated from the infectious fractions. This was interpreted as an argument against PrP being the major component of infectivity. However, in similar disaggregation experiments (Riesner et al. 1996), we found that the PrP separated from infectivity had changed drastically in its conformation; thus, the findings supported the prion hypothesis rather than falsified it. When residual nucleic acids in highly purified prions were analyzed by molecular cloning, no evidence for a scrapie-specific nucleic acid could be found (Oesch et al. 1988).

Search for a Nucleic Acid Unique to Scrapie-infected Preparations

Because of the many unsuccessful attempts to identify a nucleic acid component required for scrapie infectivity, we set out to develop an experimental paradigm that would offer the possibility of definitely excluding a nucleic acid if prions are devoid of such molecules (Meyer et al. 1991). We assumed that one infectious unit must contain at least one nucleic acid molecule if it is to be essential for infectivity. In other words, the particle (nucleic acid molecule)-to-infectivity ratio (P/I) has to be at least unity. Nucleic acids with a P/I smaller than unity would be excluded as scrapie-specific. Because the properties of the hypothetical scrapie-specific nucleic acid are unknown, we considered that such a molecule might be either DNA or RNA; single or double stranded; circular or linear; as well as capped, chemically modified, or covalently bound to proteins. In addition, we entertained the remote possibility that a scrapie-specific polynucleotide might be heterogeneous in size. Since high levels of scrapie infectivity have been found only in animals, it was not possible to incorporate radiolabeled nucleotides into the putative scrapie nucleic acid. Cell culture systems producing high levels of prions would obviate the problem of radiolabeling, but such cultures are not available.

A general method for the analysis of all the types of nucleic acids listed above is PAGE combined with silver staining. Depending on the size of

the polynucleotide, 20–200 pg of nucleic acid per gel band can be detected. For example, 170 pg would be contained in 3×10^9 units of infectious prions if the hypothetical nucleic acid were 100 nucleotides in length and the P/I ratio were unity. Obviously, studies on a smaller scrapie-specific nucleic acid would require higher titers, and studies on a larger nucleic acid would need lower titers to yield the same mass of nucleic acid. The sensitivity of silver staining after conventional PAGE decreases substantially if the nucleic acid is heterogeneous in size. To be able to detect heterogeneous nucleic acids, the method of RRGE was developed.

Search for Homogeneous Nucleic Acids by PAGE

Scrapie prions were purified from brains of Syrian hamsters using a discontinuous sucrose gradient (Prusiner et al. 1983). The infectious prions consisted mainly of rod-shaped aggregates composed largely of PrP 27-30. The prion rods were precipitated from the sucrose gradient fractions with ethanol and submitted to a nucleic acid degradation protocol using DNase and Zn^{++}. No significant changes in prion titer were detected after digestion with DNase and exposure to Zn^{++} ions, in agreement with earlier observations (Prusiner 1982). The samples were disaggregated and deproteinized by boiling in 2% SDS, digested with proteinase K, and extracted once with phenol and once with phenol:chloroform (1:1), or in later experiments, twice with phenol:chloroform:isoamyl alcohol (50:48:2) and precipitated with ethanol.

PAGE analysis of the DNase-digested and Zn^{++} treated prions showed some background smearing as well as distinct bands migrating near the dye front (Fig. 13a, lane 2). Omission of DNase digestion and Zn^{++} hydrolysis resulted in a prion fraction with a large number of silver-stainable bands throughout the lane, in addition to the rapidly migrating molecules (Fig. 13a, lane 1). The size of the rapidly migrating molecules was estimated to range between 8 and 15 bases, as judged by the mobility of control oligonucleotides (Fig. 13a, lane 3). From analyses of samples after additional treatments with DNase, Zn^{++} ions, and NaOH prior to electrophoresis, as well as phenol-sulfuric acid measurements, we concluded that the rapidly migrating bands observed on PAGE are likely to be either complex asparagine-linked oligosaccharides released from PrP 27-30 during proteinase K digestion or noncovalently bound sugar polymers that purified with the prion rods. Control nucleic acids were analyzed in the same way as infectious scrapie prions. In Figure 13b, lane 1 demonstrates that 3×10^{10} molecules of each of the control nucleic acids

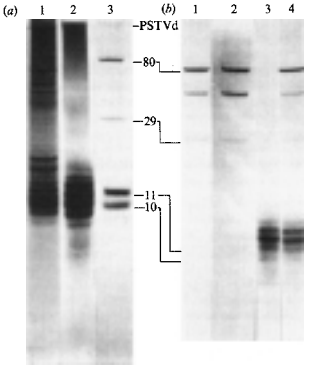

Figure 13 Analysis of nucleic acids in prion rod fractions by 20% PAGE before and after DNase and Zn^{++} treatment. (*a*) Nucleic acids without and with DNase and Zn^{++} treatment. (Lane *1*) Prion rods not treated with DNase and Zn^{++}; (lane *2*) prion rods treated with DNase and Zn^{++}; (lane *3*) marker nucleic acids: circular potato spindle tuber viroid (PSTVd, 300 pg), tRNA (80 nt, 1 ng), oligo DNA (29 nt, 200 pg), oligo RNA (11 nt, 3 ng), oligo RNA (10 nt, 2 ng). (*b*) Comparable amounts of control nucleic acids (number of molecules) and prions (ID_{50} units), 3×10^{10} molecules of control nucleic acids and 1.2×10^{10} ID_{50} units of prions recovered from sucrose gradients were analyzed. (Lane *1*) Control nucleic acid: tRNA (80 nt, 1.4 ng), oligo RNA (11 nt, 0.19 ng), oligo RNA (10 nt, 0.17 ng); (lane *2*) control nucleic acids as in lane 1 but treated by the deproteinization procedure; (lane *3*) prions treated with DNase and Zn^{++} and by deproteinization; (lane *4*) control nucleic acids as in lane *1* added to prions after DNase and Zn^{++} treatment but before deproteinization. The bands of 10 and 11 nt are weak in the photographic reproduction but were clearly seen in the original gel. (Reprinted, with permission, from Meyer et al. 1991.)

were readily detected, and lane 2 shows that this amount could be recovered using the protocol described (Meyer et al. 1991). If the control nucleic acids were hydrolyzed with DNase I and Zn^{++}, no silver-stained bands were detected (data not shown). Silver staining of prions after PAGE showed no signal above 20 nucleotides (Fig. 13b, lane 3). If the control nucleic acid molecules (see also lanes 1 and 2) were added to the scrapie sample after DNase and Zn^{++} treatment but before destroying the infectivity with SDS and boiling (Fig. 13b, lane 4), all of them were visible except those of 10–11 nucleotides in length, because these were hidden by the non-nucleic acid molecules purifying with the prions.

The prion infectivity was determined to be 1.2×10^{10} ID_{50} units immediately prior to deproteinization. Since 170 pg of nucleic acid in a

single band was assumed to be the limit of detection, 1.2×10^{10} nucleic acid molecules of at least 25 nucleotides in length would have been detected. The imprecision of the bioassay required that we qualify our conclusion, since prion titer determinations frequently vary by a factor of 10. Because the analysis utilized 20% polyacrylamide gels, only nucleic acids greater than 300 nucleotides in length could be detected. Larger nucleic acids were assessed using a modified procedure.

The Method of RRGE for the Analysis of Heterogeneous Nucleic Acids

Although it was remote and unprecedented, we considered the possibility that prions contain nucleic acid molecules of non-uniform length. In such a case, the nucleic acids would migrate during PAGE as many bands, and each band might be below the threshold for detection or not resolved from neighboring bands, resulting in a smear of staining. The smear could represent a scrapie-specific nucleic acid of non-uniform length or could be unspecific background hiding a weak specific band.

With the method of RRGE, nucleic acids were separated from other molecules staining with silver and focused into one sharp band. Using RRGE (Fig. 14), the heterogeneous nucleic acids were detected with a sensitivity close to that attained with a homogeneous nucleic acid.

In an initial set of experiments, a 15% polyacrylamide gel matrix was used for RRGE, but this restricted our analyses to polynucleotides smaller than 200 bases. In a subsequent study (Kellings et al. 1992), the size range of the polynucleotides analyzed was extended to 1100 bases by using a 9% polyacrylamide gel matrix. To obtain a quantitative estimate of the amount of nucleic acid present in a band, known amounts of reference nucleic acids were analyzed simultaneously in adjacent slots.

Detection of Heterogeneous Nucleic Acids in Purified Prion Preparations

Prion samples that were prepared by a protocol similar to that used for the studies described above (Fig. 13) were evaluated by RRGE (Fig. 15). After the first electrophoresis, only a faintly stained smear in gel sections *a* and *b* as well as a ladder of silver-stained bands comigrating with oligonucleotides ranging from 4 to 12 bases was visible. After RRGE, distinct silver-stained bands were visualized from pieces *a* and *b* and weaker bands from pieces *c* to *e*. The total amount of heterogeneous nucleic acid in the prion sample was estimated to be about 20 ng. It

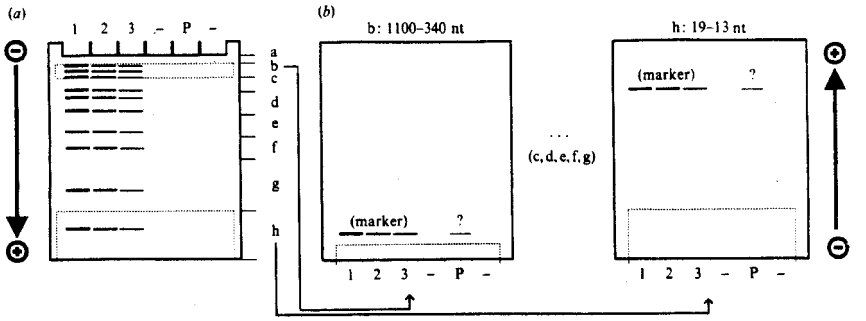

Figure 14 Scheme of return refocusing gel electrophoresis (RRGE). After conventional PAGE (e.g., 100 min at 150 V) as a first step, heterogeneous nucleic acids are dispersed over the whole length of the lane (lane *P* in *a*). The lane is cut into a few segments (*a–h*), each corresponding to a well-defined range of M_r. The segments are repolymerized into the bottom of new gel matrices (*b*) and second electrophoresis (250 V) is performed with reversed polarity so that the nucleic acids migrate into the new gel matrix. Because all nucleic acids in a gel segment begin migration from the same position at the beginning of the first PAGE, they meet again after reversal of the polarity of the second electrophoresis if the second run is stopped at a definite time. By adding SDS to the second PAGE, the focusing effect still works for nucleic acids while other substances such as proteins and polysaccharides remain dispersed. This is a significant advantage since proteins, like nucleic acids, stain with silver. The times of refocusing of different gel segments are chosen optimal for the different segments (between 42 and 48 min). The unknown nucleic acid of the prion sample is determined by comparison with the nucleic acid markers of known concentrations (markers 1, 2, 3). Only the two gel segments b and h are given as an example for the refocusing step; gel segment a is not used for refocusing. (Reprinted, with permission, from Kellings et al. 1992.)

should be noted that with the method of normal PAGE (Fig. 13), nucleic acids could not be detected in these samples.

Nuclease digestion studies were carried out prior to RRGE in order to confirm the nucleic acid nature of the bands and to differentiate between RNA and DNA. The RNA and DNA detected differed in size distribution: Nucleic acids below 50 nucleotides were mainly RNA, and longer molecules were primarily DNA. The ladder in the range of 4–12 bases was not composed of nucleic acids (Meyer et al. 1991).

During the course of investigations by RRGE, procedures were developed for dispersing prion rods into detergent–lipid–protein complexes (DLPCs) and liposomes with retention of infectivity (Gabizon et al. 1987, 1988c). We thought that nucleic acids, which were possibly protected

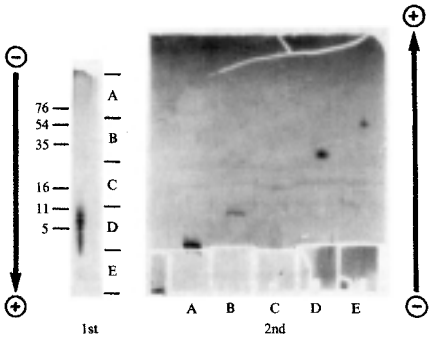

Figure 15 Detection of heterogeneous nucleic acids in prion rods by RRGE. Prion rods were treated with DNase and Zn^{++} and deproteinized prior to RRGE. In this RRGE, the sample contained $10^{7.3}$ ID_{50} units as measured by bioassay prior to boiling in SDS. The separation run is indicated as "1st." Positions of marker oligonucleotides are indicated at the left. An identical gel lane but without staining was cut into gel segments (A to E) and analyzed in the refocusing run, indicated "2nd." Marker nucleic acids as depicted in the scheme of Fig. 14 were not analyzed in this experiment. The total amount of nucleic acid was estimated as 20 ng. (Reprinted, with permission, from Meyer et al. 1991.)

from degradation by inclusion within the rod-shaped aggregrates, might become accessible to nucleases upon formation of the DLPCs.

To purify the prion samples further with retention of infectivity, they were dispersed into DLPCs composed of sodium cholate and egg lecithin (Gabizon et al. 1988c; Meyer et al. 1991). The DLPCs were digested either with a mixture of DNase, Ba313, and RNase A or a mixture of micrococcal nuclease, alkaline phosphatase, RNase A, and phosphodiesterase. A 10-fold reduction in nucleic acid content of the prion preparations was observed after DLPC formation followed by nuclease digestion.

Ratio of Nucleic Acid Molecules per Infectious Unit

Scrapie infectivity was monitored by incubation time interval bioassays (Prusiner et al. 1982b) at each step in the preparation. Separation of the prion rods from the sucrose by ethanol precipitation used for discontinuous gradient centrifugation resulted in a loss of infectivity of 1–3 orders of magnitude; this was probably due to aggregation as well as denaturation (Meyer et al. 1991). Dispersion of ethanol-precipitated prion rods into DLPCs frequently increased the titer more than 10-fold.

On the basis of the amount of nucleic acid estimated from RRGE and the titers of the prion fractions prior to boiling in SDS, the P/I ratio of

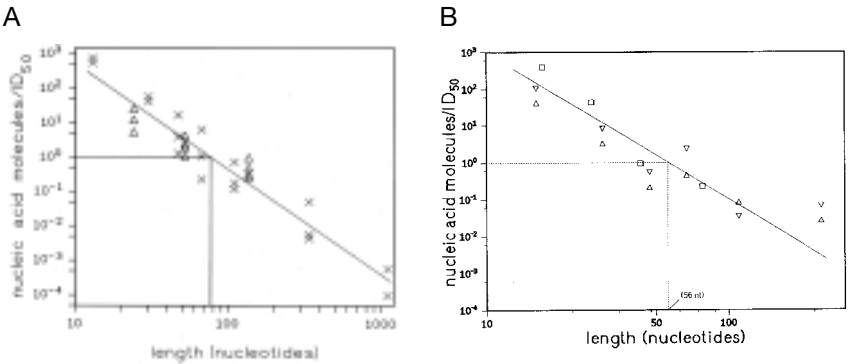

Figure 16 Relationship of the ratio of nucleic acid molecules/ID_{50} (P/I) to average length of nucleic acid species from determinations of the kind illustrated in Fig. 14 on five independent prion samples in *A* and three independent prion samples in *B*. The relationship is linear over the size range of 10–1000 nucleotides with an intercept of about 76 nt (*A*) and 50 nt (*B*) for a P/I of unity. Only smaller nucleic acids have P/I >1. In *A*, data Δ were taken from Meyer et al. (1991), x from Kellings et al. (1992). The data in *B* are unpublished (K. Kellings et al., cf. text). The relationships were calculated as follows, using a fragment containing 450 pg of nucleic acids in the size range of 54–79 nt as an example. Assuming a continuous distribution of different sizes, the 26 species in this class will have an average MW of 22×10^3 and there will be:

$$450 \times 10^{-12}\ \text{g} \times \frac{6 \times 10^{23}\ \text{mole}}{26 \times 22 \times 10^3\ \text{g}} 47 \times 10^8\ \text{mole}$$

of a particular size in this ensemble. Since the starting sample contained $10^{8.7}$ (5×10^8) ID_{50}, the P/I is approximately 1 for a hypothetical discrete scrapie-specific nucleic acid in the ensemble. (*A,* modified, with permission, from Riesner 1991.)

nucleic acid molecules to ID_{50} units was calculated. If the nucleic acids that were detected are related to scrapie infectivity, then one of two alternative paradigms must apply. First, a putative scrapie-specific nucleic acid of uniform length might be hidden among an ensemble of background nucleic acids. Such a scrapie-specific nucleic acid would not have been detected by PAGE (see Fig. 13), even if it were present in sufficient amounts. Second, a scrapie-specific polynucleotide might be heterogeneous in length. In Figure 16, the numbers of nucleic acid molecules per 1 ID_{50} unit are plotted as a function of their length as estimated from the individual gel sections. In these plots, the calculation was based on the first paradigm, i.e., a well-defined scrapie-specific nucleic acid among the het-

erogeneous background nucleic acids. In Figure 16A, all published data (Meyer et al. 1991; Kellings et al. 1992) are depicted. If the scrapie-specific nucleic acid were longer than 76 nucleotides, the P/I ratio would fall below unity. In Figure 16B, more recent data are presented where the steps of sucrose gradient centrifugation and ethanol precipitation of the prion rods, i.e., steps in which infectivity was lost occasionally, were replaced by ultrafiltration and high-speed centrifugation (Kellings 1995; K. Kellings et al., in prep.). This improved protocol gave an even shorter estimate of the maximum length of a hypothetical scrapie-specific nucleic acid; at a P/I ratio of 1, such a polynucleotide would contain 50 nucleotides.

Conclusions about a hypothetical, scrapie-specific nucleic acid must be considered with respect to a particular case. As described above, the first case envisions a hypothetical scrapie-specific nucleic acid that is a well-defined molecular species hidden in the smear of heterogeneous nucleic acids, which represent preparative impurities in the sample. In this case, molecules longer than about 50 nucleotides were not present in concentrations above one molecule per infectious unit. If we assume an order of magnitude error in the bioassay and a factor of 2 in the nucleic acid determination, the limit would be 110 instead of 50 nucleotides chain length. Although larger nucleic acids can be excluded as scrapie-specific candidates, the smaller ones cannot, but no functional significance can be assigned to any of these oligonucleotides.

The second case considers a hypothetical scrapie-specific nucleic acid that is heterogeneous in length, where more of the detected nucleic acids might function in scrapie agent propagation. Under these circumstances, either all of the polynucleotides or a particular subset must be considered. If all nucleic acids of chain length longer than 240 nucleotides are considered, there is less than one such molecule per infectious unit. We emphasize that a hypothetical scrapie-specific polynucleotide of heterogeneous length is an extremely remote assumption that is unprecedented in biology. Thus, whatever the structure of a hypothetical scrapie genome may be, it would have to be constructed of molecules smaller than 240 nucleotides.

Since nucleic acids smaller than 50 nucleotides with a P/I ratio of greater than 1 have been detected in highly purified prion preparations, such oligonucleotides cannot be excluded as an essential component of the prion on the basis of these measurements. However, we have no corroborating evidence to suggest that such molecules have any functional importance in the maintenance or propagation of scrapie infectivity. Such

oligonucleotides may simply reflect the decreasing efficiency of nuclease degradation with the decreasing size of the nucleic acid. In this scenario, such nucleic acids are merely impurities in the highly purified preparations without any functional relevance for scrapie infectivity. It is worth noting that purified prion preparations tend to protect nucleic acids against degradation. In the absence of any evidence to suggest a scrapie-specific nucleic acid, further attempts to evaluate such oligonucleotides seem unlikely to be productive.

If a nucleic acid of 50 nucleotides or less were essential for infectivity, it could not function as an mRNA encoding either PrP or another polypeptide, but rather, it might possess some regulatory function. Small regulatory nucleic acids, in most cases RNAs, might act as ribozymes, primers for replication or transcription, antisense RNA for inhibition of transcription, guide RNA for editing, or as RNA that forms splicing sites. In any of these scenarios, the genetic information would exert a regulatory influence on the host cell. Whether such a hypothetical oligonucleotide is responsible for prion strains has been considered, but there are no data for such molecules and the diversity of prion strains is now known to be enciphered in the conformation of PrP^{Sc} (Chapter 8).

COMPLEXES OF PrP^{C} AND PrP^{Sc}

Studies with Tg mice demonstrated that the cellular isoform (PrP^{C}) must interact with PrP^{Sc} during its conversion into nascent PrP^{Sc} (Prusiner et al. 1990; Scott et al. 1993). Our attempts to reproduce the formation of PrP^{Sc} in vitro using full-length PrP^{C} and PrP^{Sc} molecules mixed in equimolar amounts were unsuccessful (Raeber et al. 1992). Subsequent studies using a large excess of PrP^{Sc} defined conditions under which PrP^{C} binds to PrP^{Sc}, as demonstrated by the resistance of the PrP^{C} to proteolysis (Kocisko et al. 1994, 1995; Bessen et al. 1995). Since nondenaturing conditions for the release of PrP^{C} bound to PrP^{Sc} have not been identified, it has not been possible to determine whether the conformation of PrP^{C} was altered when the PrP^{C}/PrP^{Sc} complex was formed (Kaneko et al. 1995).

Although some investigators have reported the in vitro formation of PrP^{Sc} by mixing a 50- to 700-fold excess of PrP^{Sc} with ^{35}S-labeled PrP^{C}, their conclusions assume that protease-resistant PrP^{C} is equivalent to PrP^{Sc} (Kocisko et al. 1994; Bossers et al. 1997; DebBurman et al. 1997; Raymond et al. 1997). Interestingly, the binding of PrP^{C} to PrP^{Sc} was found to be dependent on the same residues (Kocisko et al. 1995) that render Tg(MH2M) mice susceptible to SHa prions (Scott et al. 1993), and it seems to be strain-dependent (Bessen et al. 1995).

Since a small fraction of PrP^C acquired protease resistance when incubated alone, and a much larger fraction showed resistance when incubated with synthetic PrP peptides, we revisited the possibility that PrP^{Sc} mixed with PrP^C might render it protease-resistant (Kaneko et al. 1995). Other investigators reported that PrP^{Sc} denatured in 3 M GdnHCl undergoes renaturation and renders PrP^C resistant to proteolysis within 2 minutes of mixing (Kocisko et al. 1994). Since numerous attempts to renature prion infectivity from both Gdn and urea had failed (Prusiner et al. 1993a), we investigated the effect of 3 M GdnHCl on PrP^{Sc}. As before, we were unable to demonstrate renaturation of PrP^{Sc} that had been denatured in 3 M GdnHCl and then diluted 4- to 10-fold prior to limited protease digestion and SDS-PAGE. Of note, when the dilution was carried out in the same tube to which the 3 M GdnHCl had been added, we did see protease-resistant PrP (data not shown). This was never seen when the tubes were changed, and we surmise that this was due to residual undenatured PrP^{Sc} bound to the walls of the tube.

When we mixed PrP^{Sc} that had been denatured in 3 M GdnHCl and then diluted in buffer to give a final concentration of 0.3–2 M GdnHCl with PrP^C, no protease-resistant ^{35}S-labeled PrP^C could be detected. However, mixing undenatured PrP^{Sc} with PrP^C did produce protease-resistant ^{35}S-labeled PrP^C (Fig. 17B, lanes 2, 7–10). As reported by other workers (Kocisko et al. 1994), a 50-fold excess of PrP^{Sc} was required to produce protease-resistant ^{35}S-labeled PrP^C, whereas a 10-fold excess of PrP^{Sc} did not work. The presence of 0.3 M GdnHCl in the reaction mixture seems to be essential, since its removal by methanol precipitation prior to mixing prevented complex formation. Although about 50% of the ^{35}S-labeled PrP^C was recovered in complexes sedimented at 100,000*g* for 1 hour, only 10–15% was protease-resistant.

The interaction between PrP^C and PrP^{Sc} was found to be inhibited by the α-PrP 3F4 monoclonal antibody but not by 13A5 (data not shown). This difference between the two MAbs might indicate a critical role for the PrP residues in the vicinity of the 3F4 epitope, which is at the amino terminus of the putative H1 region, or might reflect a difference in the avidity of the two MAbs. PrP^C-II truncated at the amino terminus and lacking the 3F4 epitope did not exhibit protease resistance after exposure to PrP^{Sc} (data not shown), supporting the notion that the putative H1 region, in which the 3F4 epitope lies, is particularly significant (Haraguchi et al. 1989; Pan et al. 1992; Chen et al. 1995).

We estimate that 10–15% of PrP^C mixed with PrP^{Sc} acquired protease resistance after 48 hours in contrast to PrP^C mixed with the SHaPrP(90-145[A117V]) peptide, where about 50% of PrP^C demonstrated protease

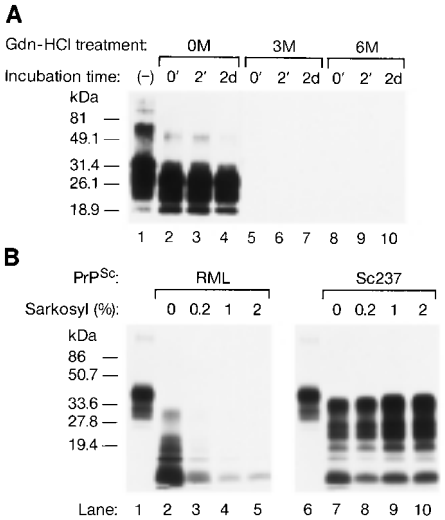

Figure 17 Incubation of PrP^{Sc} with GdnHCl or PrP^{C}. (*A*) $SHaPrP^{Sc}$ was incubated with 0, 3, or 6 M GdnHCl for 0 min, 2 min, or 48 hr at 37ºC followed by digestion with proteinase K for 1 hr. Samples were analyzed by SDS-PAGE and Western blotting using the α-PrP 3F4 MAb. (Lane *1*) Sample was not digested with proteinase K. (Lanes *2*, *5*, *8*) Samples were digested with proteinase K at time 0; (lanes *3*, *6*, *9*) at 2 min; (lanes *4*, *7*, *10*) at 48 hr. (Lanes *2–4)* Samples incubated without GdnHCl; (lanes *5–7)* samples with 3 M GdnHCl; (lanes *8–10)* samples with 6 M GdnHCl. Equal amounts of protein were applied to each lane. *(B)* ^{35}S-labeled $SHaPrP^{C}$ incubated for 48 hr with unlabeled $MoPrP^{Sc}$ (lanes *1–5)* or $SHaPrP^{Sc}$ (lanes *6–10*). (Lanes *1* and *6*) Samples were not digested with proteinase K; (lanes *2* and *7*) samples were digested but in the absence of Sarkosyl. (Lanes *3* and *8*) Samples were exposed to 0.2% Sarkosyl and proteinase K for 1 hr; to 1% or 2% Sarkosyl in lanes *4* and *9*, and lanes *5* and *10*, respectively. Lanes *1* and *6* have 15% of the PrP^{C} in lanes *2–5* and *7*–10. (Reprinted, with permission, from Kaneko et al. 1995 [copyright National Academy of Sciences].)

resistance. After incubation with SHaPrP(90-145) for 1 hour, about 35% of the ^{35}S-labeled PrP^{C} that exhibited protease resistance at 48 hours was present; by 24 hours, about 75% of the PrP^{C} was protease-resistant. Although it has been reported that protease-resistant ^{35}S-labeled PrP^{C} was generated within 2 minutes after mixing with a 50-fold excess of unlabeled PrP^{Sc}, we were unable to reproduce this finding (Kocisko et al. 1994).

When we mixed $MoPrP^{Sc}$ with $SHaPrP^{C}$, relatively little protease-resistant PrP^{C} was formed (Fig. 17B, lanes 1 and 2), and addition of Sarkosyl rendered the complex sensitive to proteolysis (lanes 3–5). In contrast, the ^{35}S-labeled $PrP^{C}/SHaPrP^{Sc}$ complex was resistant to proteol-

ysis (Fig. 17B, lanes 6–10), even when exposed to 2% Sarkosyl for 48 hours prior to digestion. These results are consistent with the finding that SHaPrP(90-145) mixed with SHaPrP^{C} produced protease-resistant protein whereas MoPrP(90-145) mixed with SHaPrP^{C} did not.

Attempts to Disrupt PrP^{C}/PrP^{Sc} Complexes

Although addition of Sarkosyl to the PrP^{C}/peptide complexes or PrP^{C} alone abolished protease resistance, this was not the case for the PrP^{C}/PrP^{Sc} complexes. Additional attempts to disrupt the PrP^{C}/PrP^{Sc} complexes under conditions likely to preserve scrapie prion infectivity utilized detergents such as NP-40, Tween-20, Zwittergent 3-12, and sodium deoxycholate, alone or in combination with phospholipids to form detergent–lipid–protein complexes (Gabizon et al. 1988b). We also investigated the possibility of disrupting these complexes using the α-PrP 3F4 MAb and synthetic peptides containing residues 109–122 or 90–145. Although the MAb and peptides were added to the complexes in molar ratios of 1000:1, they were unable to dissociate the ^{35}S-labeled PrP^{C} from PrP^{Sc}. Addition of a 10-fold excess of cold PrP^{C} from Chinese hamster ovary (CHO) cells also failed to displace the ^{35}S-labeled PrP^{C} from the complex. These results prevented us from determining whether PrP^{C} had acquired protease resistance or displayed this property because it was bound to PrP^{Sc}.

As described above, attempts to separate ^{35}S-labeled PrP^{C} from PrP^{Sc} under conditions where scrapie infectivity is preserved were unsuccessful using a variety of detergents, α-PrP MAbs, DLPCs, and synthetic peptides (Kaneko et al. 1995, 1997b). Until such conditions are identified, we cannot determine whether PrP^{C} has been converted into PrP^{Sc} or is only tightly bound. The experiments presented here with PrP peptides that bind to PrP^{C} and render it protease-resistant argue that the latter possibility is more likely to be correct, since Sarkosyl disrupted the PrP^{C}/peptide complex and made PrP^{C} sensitive to protease.

Complexes of PrP^{C} and Synthetic PrP Peptides

Identifying conditions for the in vitro conversion of PrP^{C} into PrP^{Sc} is an important avenue of investigation (Raeber et al. 1992; Kocisko et al. 1994; Kaneko et al. 1995). If conditions can be found whereby PrP^{C} is converted into PrP^{Sc} and scrapie infectivity is acquired as measured by bioassay, then the issue of whether prions contain any molecule besides PrP^{Sc} can be answered. The production of infectivity in vitro in the

absence of PrP^{Sc} would argue convincingly that prions are composed only of PrP^{Sc} molecules. Since one infectious unit of prions as assayed in Syrian hamsters requires more than 10^5 PrP^{Sc} molecules, it is difficult by chemical analyses to eliminate the possibility that prions contain a small accessory molecule (Prusiner et al. 1982a; Stahl et al. 1993). On the other hand, an enlarging body of information militates against a second molecule, particularly since the information for prion strains now seems to be enciphered within the conformation of PrP^{Sc} (Bessen and Marsh 1994; Telling et al. 1996).

Despite an extended search for a chemical modification that might distinguish PrP^{Sc} from PrP^{C}, none has been found (Stahl et al. 1993). Whether it is possible to convert PrP^{C} into PrP^{Sc} in vitro in the absence of additional macromolecules that are likely to feature in the in vivo formation of PrP^{Sc} remains to be established. The conversion of PrP^{C} into PrP^{Sc} is a posttranslational process that seems likely to occur within caveolae-like domains (CLDs) that are rich in glycosphingolipids and cholesterol (Gorodinsky and Harris 1995; Taraboulos et al. 1995; Vey et al. 1996; Kaneko et al. 1997a; Naslavsky et al. 1997; Chapter 9). Thus, the process of PrP^{Sc} formation seems to be localized to a particular subcellular compartment; this specific localization makes it likely that auxiliary molecules participate in the conversion of PrP^{C} into PrP^{Sc}. The results of some transgenetic studies also argue that auxiliary molecules are required for PrP^{Sc} formation (Telling et al. 1995). Since such a molecule is likely to be a protein, we have provisionally designated it "protein X" (Kaneko et al. 1997c). Because a profound conformational change occurs during the conversion of PrP^{C} into PrP^{Sc} (Caughey et al. 1991; Pan et al. 1993; Safar et al. 1993), it seems reasonable to postulate that another protein might function as a molecular chaperone to facilitate the unfolding of PrP^{C} and its refolding into PrP^{Sc}.

Complexes of $SHaPrP^{C}$ and synthetic SHaPrP peptides were found to mimic many of the characteristics of PrP^{Sc} (Kaneko et al. 1995, 1997b). Either PrP^{C} expressed in CHO cells or a carboxy-terminal fragment of 142 residues of rSHaPrP(90-231) produced in *E. coli* was mixed with an excess of a synthetic 56-amino-acid peptide, denoted PrP(90-145). Complex formation required PrP^{C} or rSHaPrP to be destabilized by GdnHCl or urea and PrP(90-145) to be in a coil conformation; it was enhanced by an acidic environment, salt, and detergent. When PrP(90-145) was in a β-sheet conformation, no complexes were formed. Although complex formation was rapid, acquisition of protease resistance was a slow process. Amorphous aggregates with a PrP^{C}/PrP(90-145) ratio of 1:1 were formed in phosphate buffer, whereas fibrils with a diameter

of about 10 nm and a PrP^C/PrP(90-145) ratio of 1:5 were formed in Tris buffer. The complexes were stable only in the presence of excess peptide in either the coil or β-sheet conformation; they dissociated rapidly after centrifugation and resuspension in buffer without peptide. Neither a peptide having a similar hydrophobicity profile/charge distribution to PrP(90-145) nor a scrambled version, denoted hPrP(90-145) and sPrP(90-145), respectively, was able to induce complex formation. Although hPrP(90-145) could stabilize the PrP^C/PrP(90-145) complexes, sPrP(90-145) could not.

The in vitro formation of PrP^C/peptide complexes required an acidic environment, and the nature of the complex could be markedly altered by the composition of the buffers. The stoichiometry of the PrP^C/peptide complexes formed in PNS buffer (20 mM NaH_2PO_4, pH 4.3/130 mM NaCl/0.05% Sarkosyl) was approximately 1:1, whereas complexes formed in TN (10 mM Tris-HCl, pH 7.0/130 mM NaCl with a pH of 4–5 due to the acidity of the synthetic peptide) buffer possessed a stoichiometry of 1:5. The complexes in PNS buffer exhibited no discernable ultrastructure, whereas those in TN buffer polymerized into relatively uniform fibrils with a diameter of about 10 nm (Kaneko et al. 1995, 1997b). Another important factor in the formation of PrP^C/peptide complexes is the presence of detergent; in PNS buffer, 0.02–0.05% Sarkosyl was optimal. Whether detergent micelles function to facilitate complex formation by providing a surface for maximal interaction of peptides with PrP^C remains to be established (Egan et al. 1976).

Although the PrP^C/peptide complexes mimic many features of PrP^{Sc}, the reversibility of complex formation contrasts with the stability of PrP^{Sc}. Whereas the SHaPrP^C/peptide complexes readily dissociated in the absence of excess peptide, some peptides not capable of forming complexes were found to be capable of maintaining them after they were formed. The SHaPrP(90-145) peptide in the β-sheet conformation, the MoPrP(90-145) mouse peptide, and the hydrophobic hSHaPrP(90-145) were as effective in maintaining the SHaPrP^C/peptide complexes as SHaPrP(90-145) in the coil form, whereas only the coil conformation was capable of inducing the complexes. These findings argue for an equilibrium process in which the PrP(90-145) peptide is converted into a β-sheet conformation as the complexes form. The reversibility of these complexes contends that PrP^C has not been converted into PrP^{Sc}, a conclusion that is supported by the lack of scrapie infectivity registered by bioassay for the complexes, to date. It is of interest that denatured PrP 27-30, which lost much of its β-sheet structure following denaturation, could not be renatured into a form that polymerized into prion rods or was associated

with infectivity, even though the β-sheet content was increased by exposure to acetonitrile (Riesner et al. 1996).

Our results demonstrate that peptides bind rapidly to PrP^C or rSHaPrP, rendering these proteins insoluble as determined by sedimentation assays. In contrast, the acquisition of protease resistance by these complexes required as much as 2 days. The slow development of protease resistance is similar to the results of kinetic studies on the formation of PrP^{Sc} in scrapie-infected neuroblastoma cells, in which $t_{1/2}$ was found to be about 15 hours (Borchelt et al. 1990, 1992). The interaction between PrP^C and peptide is an equilibrium process since removal of the excess unbound peptide in the solution causes the complexes to dissociate. Thus, not only the conformational change itself, but also aggregation, may contribute to the protease resistance. Formation of extensively visible aggregates was not associated with any increase in the extent of the protease resistance, despite the observation of a time-dependent increase in β-sheet content that was proportional to the extent of the protease resistance.

These results contrast with the report of protease resistance being acquired by radiolabeled PrP^C within 2 minutes of mixing with a 50-fold excess of unlabeled PrP^{Sc} (Kocisko et al. 1994). It is noteworthy that we were unable to reproduce the rapid development of protease-resistance for labeled PrP^C mixed with PrP^{Sc} (Kaneko et al. 1995). Instead, we found the kinetics of formation of PrP^C/PrP^{Sc} complexes to be similar to those of PrP^C/peptide complexes.

Studies of PrP^C/peptide complexes may provide insights into how PrP^C interacts with PrP^{Sc} during the formation of a nascent PrP^{Sc} molecule and into the process by which PrP^C is converted into PrP^{Sc} (Kaneko et al. 1995, 1997b). Although many properties of the PrP^C/peptide complexes, such as insolubility, protease resistance, high β-sheet content, and formation of 10-nm fibrils, resemble those of PrP^{Sc} clearly they are not equivalent. Although synthetic peptides alone and in combination with prion proteins have been useful in dissecting some features of PrP structure and the transitions that PrP molecules can undergo, we remain cautious not to overreach in our analogies regarding the conversion of PrP^C into PrP^{Sc}. Whether mutant PrP^C such as that used to construct Tg mice will be a useful substrate for the in vitro generation of prion infectivity remains uncertain. Clearly, in vitro production of infectivity from components, each of which alone is devoid of infectivity, is most desirable. If prion infectivity can be generated from rPrP or a similar molecule, then defining the events involved in PrP conversion should be greatly facilitated. Deciphering the mechanism by which PrP peptides bind to PrP^C will be of considerable interest with respect to how the two molecules are

aligned within the complex they form. In addition, elucidating the mechanism by which the PrP^{C}/peptide complexes acquire protease resistance should help advance our understanding of the molecular events that feature in PrP^{Sc} formation.

PROTEIN X

PrP^{C} interaction with PrP^{Sc} during the formation of nascent PrP^{Sc} was surmised from Tg mouse studies where mice expressing a SHaPrP transgene were susceptible to SHa prions (Prusiner et al. 1990). When similar Tg mice were produced expressing HuPrP, no transmission of Hu prions was found. However, mice expressing a chimeric Hu/Mo PrP transgene denoted MHu2M were susceptible to Hu prions. In addition, we found that Tg mice expressing HuPrP did become susceptible to Hu prions when they were crossed with PrP-deficient ($Prnp^{0/0}$) mice. These data together argued that it is likely that a molecule other than PrP is involved in the formation of PrP^{Sc}. We assumed that this molecule is a protein and designated it "protein X" (Telling et al. 1995). On the basis of the results with the MHu2M transgene and earlier studies showing that the amino terminus of PrP is not required for PrP^{Sc} formation (Rogers et al. 1993), we surmised that the binding of PrP^{C} to protein X is likely to occur through specific side chains of amino acids located at the carboxyl terminus of PrP^{C}.

Studies in Transgenic Mice

To explain the results on the transmission of Hu prions from the brains of CJD patients to Tg mice, we suggested that a macromolecule provisionally designated protein X participates in the conversion of PrP^{C} into PrP^{Sc} (Telling et al. 1995). In those studies, Hu prions did not transmit disease to Tg(HuPrP)$Prnp^{+/+}$ mice coexpressing Hu and $MoPrP^{C}$ but did transmit to Tg(MHu2M)$Prnp^{+/+}$ mice coexpressing MHu2M PrP^{C} and $MoPrP^{C}$ (Telling et al. 1994). Subsequently, transmission of Hu prions to Tg mice expressing $HuPrP^{C}$ was achieved when the mice were crossed onto a $Prnp^{0/0}$ background. These findings were interpreted in terms of $MoPrP^{C}$ binding to Mo protein X more avidly than $HuPrP^{C}$ and thus inhibiting the conversion of $HuPrP^{C}$ into PrP^{Sc} (Telling et al. 1995). $MoPrP^{C}$ binding to Mo protein X was similar to that of MHu2M PrP^{C}, and thus, $MoPrP^{C}$ did not inhibit appreciably the conversion of MHu2M PrP^{C} into PrP^{Sc}. An alternative interpretation of these results was that the carboxyl terminus of $MoPrP^{C}$ bound to $HuPrP^{Sc}$ more avidly than $HuPrP^{C}$. In this scenario, heterologous PrP^{C} binds to PrP^{Sc} more avidly than does homologous

PrP^C; yet, homotypic interactions seem to govern conversion of PrP^C into PrP^{Sc} whenever this has been studied (Prusiner et al. 1990; Scott et al. 1993).

Studies in ScN2a Cells in Culture

In the studies with ScN2a cells, the results seemed most readily interpreted in terms of the binding of PrP^C to protein X. If we tried to explain the results in terms of PrP^C binding to PrP^{Sc}, then we had to postulate that the carboxyl terminus of $MoPrP^C$ binds more avidly to $MoPrP^{Sc}$ than does that of MHMHuA PrP^C. In other words, homologous PrP^C binds to PrP^{Sc} more avidly than does heterologous PrP^C; however, this is antithetical to the alternative interpretation offered above where heterologous PrP^C binds to PrP^{Sc} more avidly than does homologous PrP^C (Telling et al. 1995). On this basis, we argue that the data presented here in concert with the earlier results, build a convincing edifice for the existence of protein X.

Three chimeric constructs, denoted as MHMHuA (Mo residues 214, 218, and 219 replaced with Hu), MHMHuB (Mo residues 226, 227, 228, and 230 replaced with Hu), and MHMHu(A/B) (combined replacements), were transfected transiently into ScN2a cells (Table 2). Neither MHMHu(A/B) nor MHMHuA was converted into PrP^{Sc} as judged by the acquisition of protease resistance (Fig. 18B). In contrast, MHMHuB was converted into PrP^{Sc} as efficiently as the control MHM2. We interpreted these results as indicating that Mo protein X did not bind to MHMHu(A/B) or MHMHuA but did recognize MHMHuB and MHM2, both of which were converted into PrP^{Sc}. The mutant PrP molecules were all expressed at about the same level (Fig. 18A,D), and no inhibition of wt $MoPrP^{Sc}$ formation could be detected (Fig. 18C.F). Having identified the HuA region that prevents conversion of modified PrP^C into PrP^{Sc}, we produced additional constructs with Mo residues 214, 218, and 219 replaced by their Hu counterparts. To test the replacement of these residues either alone or in combination, we generated five constructs and expressed them in ScN2a cells. Substitution of Hu residue 218 abolished PrP^{Sc} (Fig. 18E, lanes 8, 9, and 11), whereas substitution of Hu residue 219 was not inhibitory (Fig. 18E, lane 10). Substitution of Hu residue 214 was partially inhibitory (Fig. 18E, lanes 7). To examine the specificity of amino acid substitutions at position 218, we introduced seven artificial mutations: lysine, isoleucine, alanine, tryptophan, proline, phenylalanine, arginine, or histidine (Table 2).

Since only a minority of the ScN2a cells express the mutant PrPs in these transient transfection experiments, we could not assess the effect of

Table 2 Mutations in epitope-tagged MHM2 PrP inhibit PrP^{Sc} formation in ScN2a cells

Mo codon number	PrP residue[a]				Mutant MHM2	Type of inhibition of PrP^{Sc} formation
	mouse	human	Syr. hamster	sheep		
167	Q	E	Q	Q/R	R	2
					E	1
169	S	S	N	S	N	none
170	N	N/S	N	N	S	none
171	Q	Q	Q	Q	R	2
209	V	V	V	V	K	1
210	E	E	E	E	K	none
211	Q	Q	Q	Q	K	none
214	V	I	T	I	I	2
					K	1
					E	2
					A	1
					W	2
					P	1
215	T	T	T	T	Q	none
216	Q	Q	Q	Q	R	3
218	Q	E/K	Q	Q	E	1
					K	2
					I	1
					A	2
					W	2
					P	1
					F	1
					R	2
					H	2
219	K	R	K	R	R	none
221	S	S	S	S	A	none
222	Q	Q	Q	Q	K	none

[a]Multiple residues at a particular position indicate naturally occurring polymorphisms.

expressing mutant PrP on conversion of wild-type MoPrP into PrP^{Sc} (Fig. 18C,F). To measure the influence of mutant PrP on the conversion of wt PrP into PrP^{Sc}, we performed cotransfection studies. These results argue that the MHM2 PrP mutants that carry lysine, alanine, tryptophan, arginine, or histidine at residue 218 bind to protein X with a greater affinity than does wt MHM2 with glutamine at 218 (Table 2). These findings also contend that the two polymorphic Hu residues, glutamic acid and lysine, interact very differently with Mo protein X. Mutant MHM2 PrP(E218)

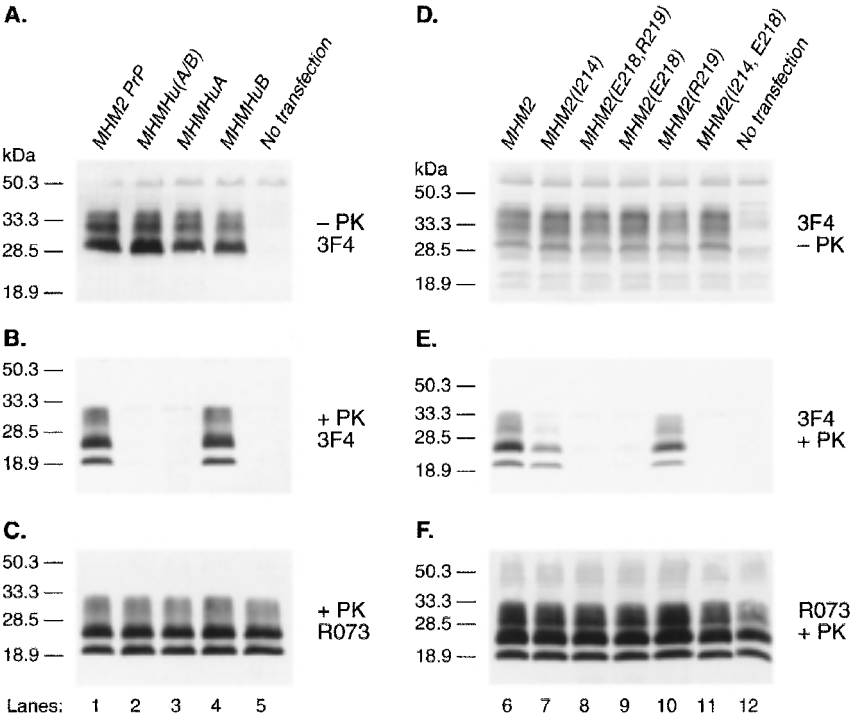

Figure 18 Characterization of the binding site for protein X. Western blot of each MHM2-chimeric construct expressed in ScN2a cells is shown. (*A, B, C*) Lane *1*, MHM2 PrP; lane *2*, MHMHu(A/B); lane *3*, MHMHuA; lane *4*, MHMHuB; lane *5*, untransfected control ScN2a cells. (*D, E, F*) Lane *6*, MHM2 PrP; lane *7*, MHM2(I214); lane *8*, MHM2(E218,R219); lane *9*, MHM2(E218); lane *10*, MHM2(R219); lane *11*, MHM2(I214,E218); lane *12*, untransfected control ScN2a cells. Panels *A* and *D* demonstrate the expression of each chimeric MHM2 PrP construct: 40 µl of undigested cell lysates was applied to each lane and MHM2 PrP was detected by staining with α-PrP 3F4 MAb. Panels *B* and *E* demonstrate the conversion of chimeric MHM2 PrP^{C} into PrP^{Sc} and were stained with α-PrP 3F4 MAb. Panels *C* and *F* show endogenous $MoPrP^{Sc}$ as well as chimeric constructs detected with α-PrP RO73 rabbit antiserum. In panels *B–C* and *E–F*, 500 µl of cell lysate was digested with proteinase K (20 µl/ml) at 37ºC for 1 hr followed by centrifugation at 100,000*g* for 1 hr and the loading of the resuspended pellet onto the gel. (Reprinted, with permission from Kaneko et al. 1997c [copyright National Academy of Sciences].)

binds more weakly to Mo protein X than does wt MHM2 PrP(Q218), which results in MHM2 PrP(E218) not being converted into PrP^{Sc} and no inhibition of the conversion of wt MHM2 PrP^{C} into PrP^{Sc}. In contrast, mutant MHM2 PrP(K218) binds more tightly to Mo protein X than does

wt MHM2 PrP(Q218), which results in both MHM2 PrP(K218) not being converted into PrP^{Sc} and inhibition of the conversion of wt MHM2 PrP^{C} into PrP^{Sc}.

A HuPrP polymorphism at codon 219, which corresponds to MoPrP codon 218, has been reported in the Japanese population (Kitamoto and Tateishi 1994); about 12% of the people carry the lysine allele instead of Glu. To date, the lysine allele has not been found in 50 autopsied CJD cases in Japan (Shibuya et al. 1998). This is a highly significant finding (Fisher's exact test, $p = 0.00005$) that suggests $HuPrP^{C}$(K219) acts as a dominant negative in preventing CJD. In view of the results presented here with MHM2 PrP(K219), it seems likely that the K219 allele prevents CJD through the high avidity of $HuPrP^{C}$(K219) for protein X. The high-affinity binding of $HuPrP^{C}$(K219) to protein X prevents $HuPrP^{C}$(K219) from being converted into PrP^{Sc}, and it prevents $HuPrP^{C}$(E219) from interacting with protein X. The latter mode of action of $HuPrP^{C}$(K219) in patients heterozygous for the polymorphism would explain the dominant negative effect of the K219 substitution. When we introduced the K218 mutation into MHM2 PrP expressed in ScN2a cells, the recombinant protein was not converted into PrP^{Sc}, and it inhibited the conversion of wt MHM2 PrP into PrP^{Sc}.

The NMR structure of SHa rPrP(90-231) shows a loop composed of residues 165–171 immediately adjacent to the protein-X-binding site on the carboxy-terminal helix, which raises the possibility that one or more of these residues also participate in the binding to protein X. To explore this possibility, we constructed mutants MHM2 PrP(Q167R), MHM2 (Q167E), MHM2 PrP(S169N), MHM2 PrP(N170S), and MHM2 PrP(Q171R) and transfected the DNAs into ScN2a cells (Table 2). MHM2 PrP(N170S) is equivalent to human polymorphism N171S (Fink et al. 1994). These findings argue that Q167 and Q171 in MoPrP form a discontinuous epitope with V214 and Q218 to which protein X binds.

In sheep, the substitution of a basic residue at position 171 probably prevents scrapie through a dominant negative mechanism similar to that postulated for a basic residue at 219 protecting humans from CJD. With few exceptions, only sheep that are Q/Q at 171 develop scrapie; sheep that are Q/R or R/R are resistant (Hunter et al. 1993, 1997a,b; Goldmann et al. 1994; Westaway et al. 1994; Belt et al. 1995; Clousard et al. 1995; Ikeda et al. 1995; O'Rourke et al. 1997). These findings suggest that R171 creates a PrP^{C} molecule in sheep that acts as a dominant negative in preventing PrP^{Sc} formation. When we introduced the Q167R or Q171R mutation into MHM2 PrP expressed in ScN2a cells, the recombinant protein was not converted into PrP^{Sc}, and it inhibited the conversion of wt

Table 3 Protein X-mediated mechanisms of inhibition of PrP^{Sc} formation.

Type of inhibition	Example	Putative mechanism	Relative affinity for protein X[a]
1	$HuPrP^{C}$(E219) binding to Mo protein X inhibited by $MoPrP^{C}$	competitive	low
2	$HuPrP^{C}$(K219) prevents $MoPrP^{C}$ binding to protein X	noncompetitive	high
3	$SHaPrP^{C}$ binds to protein X and is not released by $MoPrP^{Sc}$	noncompetitive	similar

[a]Affinity is relative to that of $MoPrP^{C}$ for Mo protein X.

MHM2 PrP into PrP^{Sc}. Q167R and Q171 in MoPrP correspond to Q171 and Q175 in ShaPrP, respectively.

In our studies, we were able to distinguish three classes of inhibition of PrP^{Sc} formation, designated as types 1, 2, and 3 (Table 3). Type 1 inhibition is illustrated by the competition between $MoPrP^{C}$ and $HuPrP^{C}$(E219) for binding to Mo protein X. In the absence of $MoPrP^{C}$, $HuPrP^{C}$(E219) is converted into PrP^{Sc} (Telling et al. 1995). The mutant MHM2 PrP(E218) was not converted into PrP^{Sc} in ScN2a cells and did not prevent conversion of wt MHM2 PrP^{C} into PrP^{Sc}. Type 2 inhibition appears to be noncompetitive and is depicted by MHM2 PrP(K218), which binds to protein X in ScN2a cells and prevents conversion of wt MHM2 PrP^{C} into PrP^{Sc}. The binding is sufficiently tight that MHM2 PrP(K218) is also not converted into PrP^{Sc}. Type 3 inhibition is also noncompetitive with respect to protein X but occurs through a different mechanism. This case is demonstrated by $SHaPrP^{C}$, which binds to Mo protein X but is not released by interacting with $MoPrP^{Sc}$. In Tg(SHaPrP) mice, $SHaPrP^{C}$ is converted into PrP^{Sc} in the presence or absence of $MoPrP^{C}$ when the animals are inoculated with SHa prions (Prusiner et al. 1990, 1993a; Büeler et al. 1993).

As our understanding of prion propagation increases, it should be possible to design effective therapeutics. Since people at risk for inherited prion diseases can now be identified decades before neurologic dysfunction is evident, the development of an effective therapy for these fully penetrant disorders is imperative (Chapman et al. 1994; Spudich et al. 1995). Although we have no way of predicting the number of individuals who may develop neurologic dysfunction from bovine prions in the future

(Cousens et al. 1997), seeking an effective therapy now seems most prudent. Interfering with the conversion of PrP^{C} into PrP^{Sc} would seem to be the most attractive therapeutic target (Cohen et al. 1994). Either stabilizing the structure of PrP^{C} by binding a drug or modifying the action of protein X which might function as a molecular chaperone are reasonable strategies. Whether it is more efficacious to design a drug that binds to PrP^{C} at the protein-X-binding site or one that mimics the structure of PrP^{C} with basic polymorphic residues that seem to prevent scrapie and CJD remains to be determined. Since PrP^{Sc} formation seems limited to CLDs, drugs designed to inhibit this process need not penetrate the cytosol of cells, but they do need to enter the CNS. Alternatively, drugs that destabilize the structure of PrP^{Sc} might also be possible to construct.

The production of domestic animals that do not replicate prions may also prove to be important with respect to preventing prion disease. Sheep encoding the R/R polymorphism at position 171 seem resistant to scrapie (Hunter et al. 1993, 1997a,b; Goldmann et al. 1994; Westaway et al. 1994; Belt et al. 1995; Clousard et al. 1995; Ikeda et al. 1995; O'Rourke et al. 1997); presumably, this was the genetic basis of Parry's scrapie eradication program in Great Britain 40 years ago (Parry 1962, 1983). A more effective approach using dominant negatives for producing prion-resistant domestic animals, including sheep and cattle, is probably the expression of PrP transgenes encoding K219 or R171 or possibly both basic residues. Such an approach can be readily evaluated in Tg mice and, once shown to be effective, it can be instituted by artificial insemination of sperm from males homozygous for the transgene.

CONCLUDING REMARKS

Although knowledge of prions and the diseases that they cause has increased dramatically over the past 15 years since the discovery of PrP 27-30 was reported, much more remains to be learned. Although exhaustive searches for a scrapie-specific nucleic acid have failed to identify any candidates, many new questions have emerged. For example, little information is available about the tertiary structure of PrP^{Sc} (Chapters 5, 16) and much is unknown about the cell biology of PrP^{Sc} formation (Chapter 9). The identification of protein X, which is thought to function as a molecular chaperone in PrP^{Sc} formation, is another important goal.

The conformation-dependent immunoassay for PrP^{Sc} should find wide application in human and veterinary medicine as well as in agriculture (Chapter 17). It seems likely that many features of the pathogenesis of prion diseases will be uncovered by the availability of a sensitive, high-

throughput assay for PrP^{Sc}. If variant Creutzfeldt-Jakob disease (vCJD) is shown to be caused by bovine prions, then massive screening of cattle throughout the world for prions is likely to become mandatory. Anti-PrP antibodies with high affinity for denatured PrP^{Sc} will become an important tool in the identification of animals and possibly humans with asymptomatic prion infections.

As more is learned about the formation of prions, new approaches to the development of effective pharmacotherapeutics should emerge. At present, a prime candidate for therapeutic intervention is the binding of PrP^{C} to protein X. Whether peptidomimetic drugs that bind to protein X, similar to dominant negative forms of PrP^{C}, can be developed with meaningful therapeutic efficacy remains to be determined (Kaneko et al. 1997c).

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Antibodies as Tools to Probe Prion Protein Biology

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Prion diseases are neurodegenerative disorders afflicting animals and humans that are characterized by progressive neurodysfunction and lead invariably to death. Prion diseases have been shown to be inheritable due to mutations in the chromosomal gene encoding the prion protein (PrP), to arise sporadically at low frequencies, or to be acquired through the transmission of infectious prion particles. These features are highly unusual in combination and hint at the unorthodox biological and physico-chemical properties of prion proteins.

Early transmission studies provided clues that the infectious prion agent possessed properties not normally associated with a viral pathogen (Alper et al. 1966; Lartajet et al. 1970). It was some years later, however, before progressive enrichment of infectivity from fractions of diseased hamster brain first identified PrP as an integral component of the infectious scrapie agent (Prusiner 1982; Prusiner et al. 1982). Further investigation of prion disorders has unveiled a unique and unexpected molecular pathology, in which the central event is the aberrant metabolism of the ubiquitous cellular prion protein (PrP^C), the physiological function of which remains poorly understood (Brown et al. 1997; Martins et al. 1997; Rieger et al. 1997; Weiss et al. 1997).

PrP^C is a glycosyl-phosphatidylinositol (GPI) anchored protein composed of 209 amino acids and expressed on a wide variety of tissue types, particularly neurons. However, the disease state is typified by an accu-

mulation in the brain of an abnormal protease-resistant isoform of PrP^{C}, designated PrP^{Sc}. Intensive scrutiny from diverse experimental perspectives has been directed toward unraveling the molecular events governing de novo PrP^{Sc} formation and deciphering a mechanism for prion replication. These studies have acquired greater urgency in light of recent evidence linking the incidence of "new variant" (nv) CJD cases in the UK and France with ingestion of prion-contaminated beef (Hill et al. 1997).

CHANGING CONFORMATIONS: HOW DOES PrP^{Sc} DIFFER FROM PrP^{C}?

Studies in animal models revealed PrP^{C} and PrP^{Sc} to be encoded by the same single-copy host-cell chromosomal gene and therefore to possess identical primary structure (Oesch et al. 1985). In addition, no chemical difference between the two molecular species has yet been identified (Stahl et al. 1993). However, with few exceptions, protease resistance has proven a defining characteristic of infectious PrP and has been widely employed in the purification and detection of PrP^{Sc}. In contrast, PrP^{C} is completely degraded following treatment with proteinase K. Protein sequencing experiments have revealed that the protease-resistant infective core of PrP^{Sc} (PrP 27-30) lacks the first 66 amino-terminal amino acids of the full-length protein. Other properties also distinguish the two forms of PrP. Whereas PrP^{C} is a soluble protein, its PrP^{Sc} counterpart is poorly soluble (Meyer et al. 1986) and exhibits a pronounced propensity for aggregation. Purified PrP 27-30 aggregates into insoluble rod-shape polymers with the ultrastructural and tinctorial properties of amyloid (Prusiner et al. 1982, 1983). Studies of PrP metabolism in normal and scrapie-infected cells have shown PrP^{C} to be synthesized and degraded rapidly, with a half-life on the cell surface of approximately 6 hours (Caughey et al. 1989). PrP^{Sc}, on the other hand, is synthesized slowly in a posttranslational pathway (Borchelt et al. 1990; Caughey and Raymond 1991) and is deposited mostly within cells (McKinley et al. 1991).

Divergent Secondary Structure Content of PrP^{C} and PrP^{Sc}

In the absence of posttranslational chemical modifications, differences in the conformations adopted by the two PrP forms were postulated to account for their divergent properties. This suspicion was borne out when studies with Fourier transform infrared (FTIR) spectroscopy and circular dichroism (CD), employed to estimate the secondary structure composi-

tion of purified PrP^{C} and PrP^{Sc}, indicated that PrP^{C} has a high α-helical content (measured at 42%) and a paucity of β-sheet structure (3%) (Pan et al. 1993). In marked contrast, PrP^{Sc} (PrP 27-30) was estimated to possess a β-sheet content of 43%, with 30% α-helix (Pan et al. 1993; Safar et al. 1993). These data clearly highlight the grossly disparate conformations of the two PrP isoforms. Moreover, reduction of β-sheet content in PrP 27-30 following denaturation is commensurate with lower scrapie infectivity in the treated preparations (Gasset et al. 1993).

NMR Spectroscopy

Recently, four different recombinant PrP (rPrP) molecules have been expressed in *Escherichia coli* and utilized experimentally for nuclear magnetic resonance (NMR) structure determination. The recombinant molecules, corresponding to MoPrP(121-231) (Riek et al. 1996), SHaPrP(90-231) (Mehlhorn et al. 1996; James et al. 1997), MoPrP(23-231) (Riek et al. 1997), and SHaPrP(29-231) (Donne et al. 1997), were folded into conformations sharing similar secondary structure content (i.e., high α-helix, low β-sheet) to that found in PrP^{C}. Three α-helices, designated A, B, and C, have been assigned for each of the four recombinant molecules. In SHaPrP(29-231), helix A spans residues 144–156, with helices B and C containing residues 172–193 and 200–227, respectively (Donne et al. 1997). Two short β-strands were reported for MoPrP(121-231) and SHaPrP(90-231), corresponding to residues 128–131 and 161–164. In SHaPrP(29-231) the amino-terminal β-strand was not seen. These regions of limited β-structure have been suggested as nucleation points for more extensive β-sheet folding, which manifests as PrP^{C} is converted to PrP^{Sc}. The NMR studies performed to date clearly indicate residues 130–231 constitute a structured 3-helix core of PrP. Remarkably, under the experimental conditions employed by Donne et al. (1997) and Riek et al. (1997), amino acids amino-terminal of residue 124 were shown to be highly flexible in full-length rPrP and to lack any secondary structure arrangements. Whether this portion of PrP is similarly disordered in the physiological environment is uncertain, particularly in light of recent reports describing the copper-binding properties of this region of the protein (Brown et al. 1997).

In light of NMR studies with rPrP molecules, attention has focused on the structural characteristics of PrP^{Sc} and how this molecule differs from PrP^{C}-like arrangements. This information is important for our understanding of the conformational conversion of PrP^{C} and the acquisi-

tion of infectivity. Thus far, the physical properties of PrP^{Sc} have hampered these avenues of investigation. However, antibodies, which can function as sensitive probes of protein structure, offer a means to identify both shared and unique conformational motifs of PrP^{C} and PrP^{Sc}.

PrP-SPECIFIC ANTIBODIES

Intriguingly, natural scrapie infection fails to induce either the humoral or cellular arm of the immune system. Mechanisms of immune tolerance are invoked to prevent immune reactivity with normal cellular PrP. In all probability, given that PrP^{C} is expressed on the surface of many different tissues, including T lymphocytes, PrP^{C}-reactive T cells will be clonally deleted during their development in the thymus. More surprisingly, however, given the clear conformational differences observed between PrP^{C} and PrP^{Sc}, immune surveillance in the infected host appears not to view scrapie particles as foreign. Why PrP^{Sc} is immunologically inert remains uncertain, although it has been suggested that a lack of nonspecific immune mediators, such as interferons and interleukins, in response to scrapie infection contributes to immune passivity (Berg 1994).

Nevertheless, antibody reagents recognizing distinct PrP isoforms have many applications in prion research; for example, in immunohistochemistry of PrP in situ, for PrP purification and structural characterization, and for early clinical diagnosis of prion diseases both in animals and humans. Below we examine the production of, and applications for, currently available polyclonal and monoclonal antibodies recognizing PrP.

Polyclonal PrP-specific Antisera

Immune tolerance to prion proteins has restricted the production of PrP-specific polyclonal and monoclonal antibodies in the laboratory. Until purified prion preparations became available, extensive efforts to raise PrP-reactive antibodies had proven unsuccessful (Kasper et al. 1981). Subsequently, large quantities of purified PrP 27-30, recovered from the brains of scrapie-infected rodents, were used as an antigen for the production of PrP-specific antisera in rabbits (Bendheim et al. 1984; Barry et al. 1985; Bode et al. 1985; Takahashi et al. 1986). Immune rabbit serum was initially shown to react specifically with PrP 27-30 in dot immunoblot and Western blot assays. Later, immunoelectron microscopy experiments provided direct evidence that PrP 27-30 was a structural component of prion rods (Barry et al. 1985).

Following the generation of antisera to PrP 27-30, investigators immunized rabbits with a number of PrP synthetic peptides corresponding to amino acid residues 90–101, 142–174, and 220–233. Typically, antisera raised against synthetic polypeptides were more reactive with the peptide than with PrP 27-30. However, these antibody reagents were able to decorate purified prion rods and stain amyloid plaques in scrapie-infected hamster brain, provided the materials were first denatured in guanidinium thiocyanate (GdnSCN) (Barry et al. 1986; Wiley et al. 1987). Taken together, these findings demonstrated that following treatment with denaturant, PrP 27-30 and its non-proteinase-treated precursor PrP 33-35^{Sc} shared primary sequence with PrP^{C}.

Monoclonal PrP-specific Antibodies

Once it became apparent that PrP^{C} and PrP^{Sc} were conformational isoforms of the same protein, the generation of monospecific immunologic probes became increasingly important. Attempts to generate PrP-reactive polyclonal reagents clearly demonstrated the difficulty in producing specific antibodies in a host immunized with homologous PrP. However, PrP primary sequence variation between species presented the opportunity to generate monoclonal antibodies (MAbs) against a limited number of species-specific PrP epitopes containing sites of amino acid variation. Operationally, only two high-affinity MAbs have been raised in this way. The antibodies, designated 13A5 and 3F4, have been generated from mice immunized with large amounts of purified SHaPrP 27-30 (Barry and Prusiner 1986; Kascsak et al. 1987). The antibodies bound SHa- but not MoPrP. Because the antibodies only reacted with denatured PrP 27-30, investigators concluded that the binding epitopes were likely to be determined by one or more of eight differences in the primary sequence comprising the protease-resistant core of SHa- and MoPrP, rather than species-specific conformational traits.

The epitopes of the two antibodies were mapped using different strategies. Synthetic peptides were used to localize the binding epitope of MAb 3F4 to within 10 amino acid residues (Bolton et al. 1991). Linear peptide containing SHaPrP sequence between residues 105 and 114 efficiently inhibited antibody binding to full-length PrP antigen, whereas peptide composed of the corresponding Mo sequence did not. Within this region, MoPrP sequence differs from that of SHaPrP at positions 109 (Leu_{Mo}–Met_{SHa}) and 112 (Val_{Mo}–Met_{SHa}). Thus, the species-specific reactivity of 3F4 was shown to be largely determined by the presence of methionine residues at positions 109 and 112. This conclusion was sup-

ported by the finding that 3F4 also recognizes human PrP, the sequence of which is completely homologous to that of SHaPrP between residues 105 and 114. In addition, bovine, rat, sheep, and rabbit PrP, which do not contain methionine residues at amino acid positions equivalent to 109 and 112 of SHaPrP, are not recognized by 3F4.

The close similarity between the amino acid sequences of SHa- and MoPrP made it possible to determine the epitope specificity of the MAbs by creating a series of chimeric Mo-/SHaPrP molecules (Rogers et al. 1991). Chimeric constructs, in which portions of SHaPrP sequence were inserted into a MoPrP genetic background, were prepared in vaccinia virus, and the engineered proteins were transiently expressed in tissue culture. The resulting PrP molecules were composed of a MoPrP backbone into which SHaPrP-specific amino acid substitutions had been introduced. The chimeric PrP molecules were blotted onto nitrocellulose and probed with 3F4 and 13A5. These experiments confirmed the critical importance of the methionine residues at positions 109 and 112 in SHaPrP to the binding epitope of 3F4. MAb 13A5 recognized a chimeric protein containing a single SHaPrP-specific amino acid substitution at position 138 (Ile–Met). The importance of this residue was confirmed when this antibody failed to react with Hu-, Chinese Ha-, Armenian Ha-, and rat PrP which contain either isoleucine or leucine in this position. The species-specific reactivity of these antibody reagents has proven of substantial value in studies of transgenic mice expressing SHaPrP.

Monoclonal Antibodies Generated from $Prnp^{0/0}$ Mice

In normal mice, mechanisms of immune tolerance effectively restrict the anti-PrP antibody response to a small number of non-murine PrP epitopes. An opportunity to access a wider spectrum of PrP-reactive MAbs arose with the production of mice homozygous for PrP gene knockout ($Prnp^{0/0}$) (Büeler et al. 1993). Following immunization with PrP preparations, $Prnp^{0/0}$ mice produce antisera reacting strongly with PrP^{C} from several species, including mouse (Büeler et al. 1992; Prusiner et al. 1993).

Recombinant Antibodies Recovered following Immunization with Prion Rods

The first PrP-specific MAbs recovered from immunized $Prnp^{0/0}$ mice were isolated via antigen-based selection from antibody Fab libraries expressed on the surface of M13 phage (Williamson et al. 1996). Initially, IgG1κ and IgG2bκ Fab libraries were prepared from spleen, lymph node,

and bone marrow tissues taken from a mouse immunized and boosted three times with sucrose-gradient-purified Mo prion rods composed of MoPrP 27-30 (Huse et al. 1989; Barbas and Lerner 1991; Burton and Barbas 1994; Williamson et al. 1996). Antisera prepared from the immunized mouse were able to recognize PrP^{Sc} only following treatment with GdnSCN. This finding reflected earlier studies in which rabbits were immunized with similar PrP antigenic preparations. Moreover, the PrP-specific reactivity of the polyclonal serum IgG of the donor mouse was mirrored by the monoclonal Fab fragments isolated from the phage libraries. No specific Fab phage were recovered when the libraries were panned against prion rods coated onto enzyme-linked immunosorbent assay (ELISA) wells. However, panning against denaturant-treated PrP 27-30, recombinant PrP (90-231), and detergent-dispersed PrP 27-30 yielded a panel of five distinct monoclonal antibodies (Williamson et al. 1996).

Since the highest serum antibody titers of the donor mouse were found against MoPrP 27-30 pretreated with GdnSCN, the phage libraries were panned against this PrP antigen presentation. These experiments yielded a large number of PrP-specific Fab clones that contained closely related heavy-chain amino acid sequences, consistent with somatic variation of a dominant B-cell clone. The prototypic member of this family was then used to mask the denaturant-treated MoPrP 27-30 panning antigen (Ditzel et al. 1995), redirecting phage selection to alternative PrP epitopes. Fab clones recovered from this panning experiment contained two novel heavy-chain amino acid sequences. In a third experiment, the libraries were panned against recombinant SHaPrP(90-231), yielding an additional antibody. A fifth Fab clone was derived by panning against MoPrP 27-30 dispersed in detergent, incorporated into liposomes, biotinylated, and immobilized onto streptavidin-coated ELISA wells.

Recombinant Antibodies Recovered following Immunization with PrP Liposomes

All of the recombinant MAbs generated following immunization with prion 27-30 rods bound to PrP 27-30 only when the antigen was pretreated with denaturant. The lack of an antibody response to antigenic determinants specific to PrP^{Sc} may have resulted from the loss of specific epitopes following oligomerization of purified PrP 27-30. However, it has proven possible to solubilize PrP 27-30 in detergent and incorporate the disaggregated preparations into liposomes (Gabizon et al. 1987; Gabizon and Prusiner 1990) without any diminution of conformational integrity (as measured by scrapie infectivity) (Masters et al. 1981). This finding

indicated large PrP polymers are not required for infectivity and provided an opportunity to access epitopes that may be concealed in prion amyloid rods. Once incorporated into liposomes, the dispersed PrP is exposed on the surface and is available for biotin labeling, which facilitates its capture onto streptavidin-coated ELISA wells for library panning.

Mice immunized with liposomes containing SHaPrP 27-30 were split into two groups. The first group received an immunization and two boosts. The second group, in an attempt to increase the diversity of the anti-PrP antibody repertoire, received an immunization and only a single boost. Phage-antibody libraries of the IgG1κ, IgG2aκ, and IgG2bκ subclasses were subsequently independently prepared from animals in each immunization group. Panning experiments performed against recombinant PrP(90-231) and against dispersed and biotinylated SHaPrP 27-30 bound to ELISA wells led to the generation of 14 additional PrP-specific recombinant antibodies (Williamson et al. 1998).

Panning experiments performed against recombinant SHaPrP(90-231) yielded a large number of closely related sequences. A second set of sequence-related Fabs was recovered by panning against dispersed SHaPrP 27-30. On a number of occasions, Fabs with similar sequence were recovered by panning against both SHaPrP(90-231) and SHaPrP 27-30. To generate greater diversity, the PrP antigens were first masked with Fabs rescued from the preliminary panning experiments, then re-presented to the libraries. Amino acid sequences of ELISA-reactive Fab clones taken from these experiments contained several additional Fabs with novel heavy-chain amino acid sequences.

Epitope Mapping

The epitope specificities of the recombinant antibodies listed in Table 1 were defined using several complementary strategies. Antibody reactivity was measured against a series of synthetic peptides representing residues 90–231 of SHaPrP. Twenty-seven peptides of 15 residues in length and overlapping by 5 residues at the amino terminus were individually coated onto ELISA plates to determine the reactivity of each antibody. The experiments demonstrated that a subset of the antibodies recognized three distinct and apparently linear PrP epitopes. One set of Fabs bound two overlapping peptides toward the amino terminus of PrP (90-231), suggesting an epitope region (designated epitope region I) was located between residues 95 and 104. One Fab (R72) bound two peptides corresponding to residues 152–164 (designated epitope region II). A third

Table 1 Reactivity of recombinant monoclonal Fabs against different antigenic presentations of PrP

Recombinant Fab	Epitope	Cell-surface PrP^C	Recombinant PrP^C	PrP^{Sc} (PrP 27-30)
D4 (R10, D13)	I	+	+	–
R72	II	–	+	+/–
R1 (R2, R5, D2, D7)	III	+	+	+
PrP1	conf	+	+	–
PrP3	conf	+/–	+	–
PrP28	conf	+	+	–
PrP28 DLPC	conf	+	+	–
PrP34	conf	+	+	–
R23	conf	+	+	–
R25	conf	+	+	–
R40	conf	+	+	–
D14	conf	+	+	–
D18	conf	+	+	–

Strong reactivity is denoted by (+), weak reactivity (+/–), and no reactivity (–). The epitope reactivity of the antibodies is given as conformational (conf) or linear (I, II, III).

epitope region (designated epitope region III), recognized by another set of Fabs, was identified toward the carboxyl terminus of the protein. Fabs recognizing this epitope region bound a peptide containing residues 220–231, but not a peptide containing residues 215–229. The remaining antibodies did not recognize any of the synthetic peptides in ELISA and were therefore likely to recognize discontinuous epitopes that may be formed from secondary and possibly tertiary structural elements of PrP.

In a parallel approach to identify PrP epitopes, random PrP fragment libraries were displayed on the surface of filamentous phage (Petersen et al. 1995). Two separate libraries were constructed. In the first, PrP sequence was tethered via its amino terminus to the phage coat protein 3 via equal proportions of linkers encoding either a glycine-glycine or proline-proline spacer sequence. In the second library, PrP sequence was linked to the phage coat via a cysteine residue, with a second cysteine residue flanking the carboxy-terminal end of the PrP fragment, affording the possibility of a constrained loop structure being formed through disulfide bridge formation. Both of the fragment libraries were panned individually over each recombinant Fab coated onto ELISA wells. Following specific enrichment over sequential rounds of panning, the encoded PrP

fragments of a representative population of phagemid clones were determined by DNA sequencing. Alignment of these sequences identified a core sequence common to each clone, which is likely to approximate the epitope of the antibody.

The PrP fragment libraries were subjected to three rounds of panning against recombinant Fabs binding epitope regions I, II, or III. Alignments of sequences derived from each panning correlated well with the synthetic peptide studies and further refined the location of the linear epitopes. In epitope region I, the sequence recognized was assigned to residues 96–105 for Fab R10, residues 96–102 for Fab D4, and residues 96–106 for Fab D13 (Table 1). The location of epitope region II, bound by Fab R72, was shown to lie between residues 152 and 163, and epitope region III recognized by Fabs R1, R2, R5, and D2 (Table 1) was shown to be composed of residues 225–231, adjacent to the GPI anchor. However, Fab D7, which bound to peptide 220-231 in ELISA, did not enrich for any consensus sequence from either of the PrP fragment libraries. In addition, even if five rounds of panning were performed, only R40 and D14 (Table 1) of the recombinant Fabs that did not react with short peptides specifically enriched phage from the PrP fragment library. Fab R40 isolated phage exclusively from the cysteine library that contained amino acid sequence between 138 and 155, with a minimum consensus sequence of 17 amino acids between residues 137 and 153. Fab D18 enriched for phage bearing PrP sequence containing residues 133 through 157.

A competition ELISA technique was used to determine whether any of the Fabs would specifically bind to PrP determinants in solution. The concentration of competing antigen required to inhibit 50% of the Fab binding to SHaPrP(90-231) on the plate (IC_{50}) was determined. Two Fabs, possessing divergent heavy-chain amino acid sequences but both binding similar if not identical epitopes between residues 96 and 104, were competed effectively with synthetic peptides corresponding to residues 90–104 and 95–109. Fabs recognizing epitope region III (residues 225–231) were efficiently competed by peptides containing amino acids 220–231 and 225–231. Interestingly, the Fab recognizing epitope region II was efficiently competed with a peptide containing residues 152–163 (the region identified as the binding epitope by the fragment libraries), but did not bind at all to recombinant SHaPrP (90-231) in solution. This epitope was, however, bound tightly when SHaPrP(90-231) was coated directly onto ELISA wells, indicating that the epitope is normally either partially or completely inaccessible but becomes exposed when PrP is coated onto ELISA plates.

Discontinuous Epitopes

Antibodies recognizing discontinuous (nonlinear) epitopes of PrP were tested for binding to longer synthetic peptides that could adopt secondary structure arrangements approximating those found in the full-length protein (Riek et al. 1996; James et al. 1997). A series of longer peptides corresponding to SHaPrP sequence between amino acids 90–145, 121–167, 147–167, 141–178, 159–201, 178–231 (containing protected cysteine side chains and therefore unable to form the disulfide bridge normally found between cysteine 179 and cysteine 214 in intact PrP), and 174–231 were used in the binding studies. None of the recombinant Fabs was able to bind well to any of these peptides in a direct binding or competition ELISA, although recombinant SHaPrP(90-231) and SHaPrP(29-231) in conformations shown by CD analysis to be rich in α-helix (Zhang et al. 1997) were bound tightly (data not shown). Similarly, in a competitive ELISA, with the exception of a single Fab (R40), none of the Fabs was competed by the longer peptides. This antibody was partially competed with a peptide containing residues 127–167, which includes the region of sequence identified by this Fab from the protein fragment libraries. It therefore seems probable that the discontinuous epitopes of PrP recognized by the antibodies may only be fully formed in the intact PrP(90-231) molecule.

Species Reactivity

Recombinant antibodies were reacted in ELISA with SHa-, Mo-, bovine, and human PrP. Fabs binding epitope region I reacted very strongly with SHa- and MoPrP, but had only very weak reactivity with bovine and human PrP. When amino acid sequences from the different species are examined in the region of epitope I, the only variation occurs at position 97, which is an asparagine residue in SHa- and MoPrP, but serine in human PrP and glycine in bovine PrP. The results suggest that the amino acid at position 97 makes direct contact with the group I antibodies. Epitope region II, recognized by Fab R72, is invariant across the species examined here and, predictably, this antibody bound very strongly to all the PrP samples in ELISA. In contrast, residues 225–231 that comprise epitope region III exhibit considerable diversity across different species. Fabs recognizing this region of PrP predictably bind to SHa- and MoPrP, which contain identical sequences between residues 225 and 231, but not to PrP from the other species tested, which contain markedly different sequences in this region.

Fab R23, recognizing a discontinuous PrP epitope, reacted well with SHa- and human PrP but did not recognize Mo or bovine PrP. This finding is consistent with the finding that R23 was able to bind to cell-surface SHaPrP, but not MoPrP expressed on the surface of N2a cells. Another Fab binding a nonlinear epitope (Fab R40) recognized all but bovine PrP. The amino acid sequence of bovine PrP contains a glutamine residue at position 186, which is unique within the species studied here, implying that this region of the molecule is important for antibody binding. However, panning the PrP fragment library against R40 yielded a consensus sequence including residues between 138 and 153. A synthetic peptide corresponding to this sequence was prohibitively insoluble for study in competition ELISA; however, peptide spanning residues 127–167 afforded partial inhibition of antibody binding. Taken together, the data suggest that the complete epitope of this antibody may be composed of two regions that are distant in the amino acid sequence but proximate in the folded PrP molecule.

Comparing Epitope Presentation in PrP^C and PrP^{Sc}

Each novel recombinant anti-PrP Fab, together with the hybridoma-derived antibody 3F4, was characterized for its ability to recognize cellular and infectious forms of PrP in immunoprecipitation, flow cytometry, and histoblot assays and in ELISA (Table 1). Importantly, the antibodies provided the first opportunity to probe the gross conformation of PrP^C in its native state on the cell surface. Flow cytometry measurements indicated that, with the exception of two Fabs (PrP3rPrP and R72), all of the antibodies recognized PrP^C in its native environment on the surface of either the mouse neuroblastoma line N2a (Klebe and Ruddle 1969), or CHO cells expressing SHaPrP. Similarly, again with the exception of Fab R72, all of the antibodies also efficiently immunoprecipitated $SHaPrP^C$ from transfected CHO cells. The antibodies were assessed for their ability to recognize PrP^C in situ in a histoblotting assay using cryostat sections of brain from normal and scrapie-infected mice (Taraboulos et al. 1992). All of the antibodies were able to recognize PrP^C in normal and diseased brain sections, either before or after treatment with GdnSCN.

In ELISA, all PrP-specific Fabs, binding to both linear and discontinuous epitopes, and 3F4, reacted strongly with recombinant PrP(90-231) and PrP(29-231) refolded into conformations with low β-sheet and high α-helical content. Since the antibodies (apart from R72) also bind cell-surface PrP^C, it is likely that the conformations of the recombinant molecules closely approximate the native molecule. These data provide

important evidence that the NMR structure solved for rPrP is equivalent to that adopted by the native molecule. In addition, all of the antibodies have been shown to bind well to PrP 27-30 prion rods following incubation with denaturing agents. Thus, following denaturation in GdnSCN, presumably to a partially unfolded or random coil state, PrP does not refold into the infective form, but into a PrP^{C}-like conformation.

A very different pattern of immunoreactivity emerged when the antibodies were tested for their ability to recognize infectious PrP by immunoprecipitation and ELISA reactivity of PrP 27-30 dispersed into liposomes (Figs. 1 and 2). In both of these assays only antibodies recognizing epitope region III, located at the carboxyl terminus of the primary sequence, were able to bind nondenatured PrP 27-30.

Mapping the Conformational Change as PrP^{Sc} Is Formed from PrP^{C}

The findings described above indicate that an epitope region at the carboxyl terminus of PrP is exposed in both PrP^{C} and PrP 27-30. In contrast, epitopes lying toward the amino terminus of the protein, that is epitope I and the 3F4 epitope, were found to be largely cryptic in nondenatured PrP 27-30, but were exposed in PrP^{C}. These observations provide a structural map of the conformational transition that features in the conversion of PrP^{C} into PrP^{Sc} and suggest that the carboxy-terminal portion of PrP^{C}, which contains a highly ordered structural core containing α-helices B and C (Donne et al. 1997), remains relatively unaltered as PrP^{C} is converted to PrP^{Sc}, whereas the amino-terminal portion of the molecule undergoes extensive conformational rearrangement in which epitopes in the amino terminus are either altered or buried in PrP^{Sc}. Figures 3 and 4 superimpose epitopes I, II, and III on the solution structure of recombinant PrP.

Findings from other studies are in agreement with the conclusion that the amino-terminal portion of the prion protein is a region of considerable conformational flexibility. For example, synthetic peptide corresponding to residues 90–145 of the PrP molecule exhibits considerable conformational plasticity in spectrophotometric studies, consistent with the formation of β-sheet arrangements (Zhang et al. 1995). In addition, protein-engineering studies in which portions of sequence between residues 90 and 120 are deleted demonstrate that the amino-terminal region of PrP 27-30 is critical to the acquisition of protease resistance (Muramoto et al. 1997). Finally, the NMR structures reported for recombinant PrP with low β-sheet/high α-helical content all find the amino terminus of PrP to be

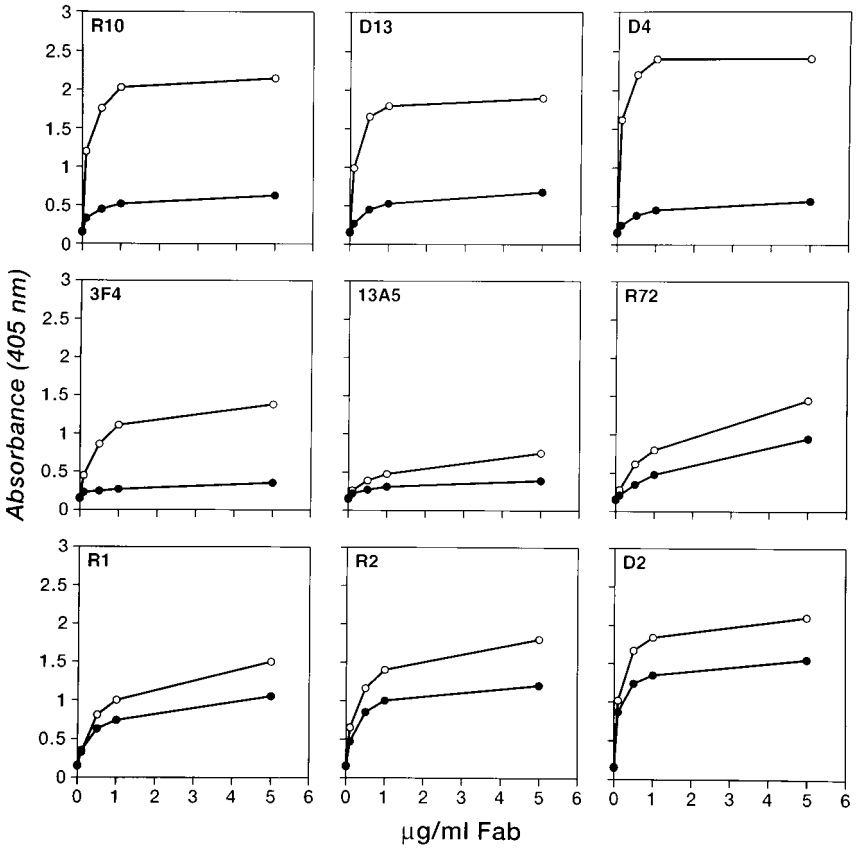

Figure 1 Epitope presentation of denaturant-treated and untreated PrP 27-30. SHaPrP 27-30 solubilized into liposomes was bound to ELISA wells, and antibody binding was measured against untreated SHaPrP 27-30 (*filled circles*) or SHaPrP 27-30 treated with GdnSCN (*open circles*).

highly disordered (Donne et al. 1997; James et al. 1997; Riek et al. 1997). Together, these studies argue that the major conformational changes required for the formation of PrP^{Sc} probably occur in the amino-terminal portion of the protein.

DNA Immunization of $Prnp^{0/0}$ Mice

PrP-specific MAbs have also been raised in knockout mice using traditional hybridoma fusion technology (Krasemann et al. 1996). In this

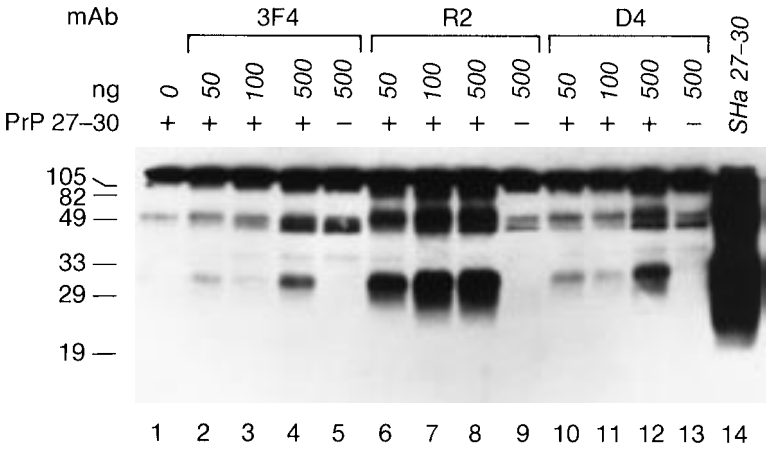

Figure 2 Immunoprecipitation of SHaPrP 27-30. The ability of an Fab (R2) binding epitope group III, an Fab (D4) binding epitope group I, as well as the 3F4 MAb (binding to an epitope slightly carboxy-terminal of epitope I) to immunoprecipitate SHaPrP 27-30 dispersed and incorporated into liposomes was determined.

study, mice were immunized by intramuscular injection of DNA plasmids containing sequence coding for different HuPrPs, including mutants associated with CJD, Gerstmann-Sträussler-Scheinker syndrome (GSS), and fatal familial insomnia (FFI). Following primary inoculation with plasmid, mice were boosted with either the corresponding DNA or recombinant live-attenuated Semliki Forest Virus particles containing the HuPrP open reading frame.

A total of 274 hybridomas were generated. Of these, 46 were shown in Western blot, immunoprecipitation, and immunofluorescence assays to secrete PrP-specific MAbs. The antibodies were then screened against a panel of overlapping synthetic peptides, representing the entire sequence of HuPrP. Almost all of the antibodies were mapped to a total of four linear epitopes. Three other antibodies were reactive with PrP in immunofluorescence, but did not bind to any of the overlapping peptides. These antibodies likely recognize more complex epitopes.

A Putative PrP^{Sc}-specific Monoclonal IgM Antibody

Korth et al. (1997) prepared hybridoma fusions from PrP-null mice immunized with full-length proteinase-K-sensitive recombinant bovine

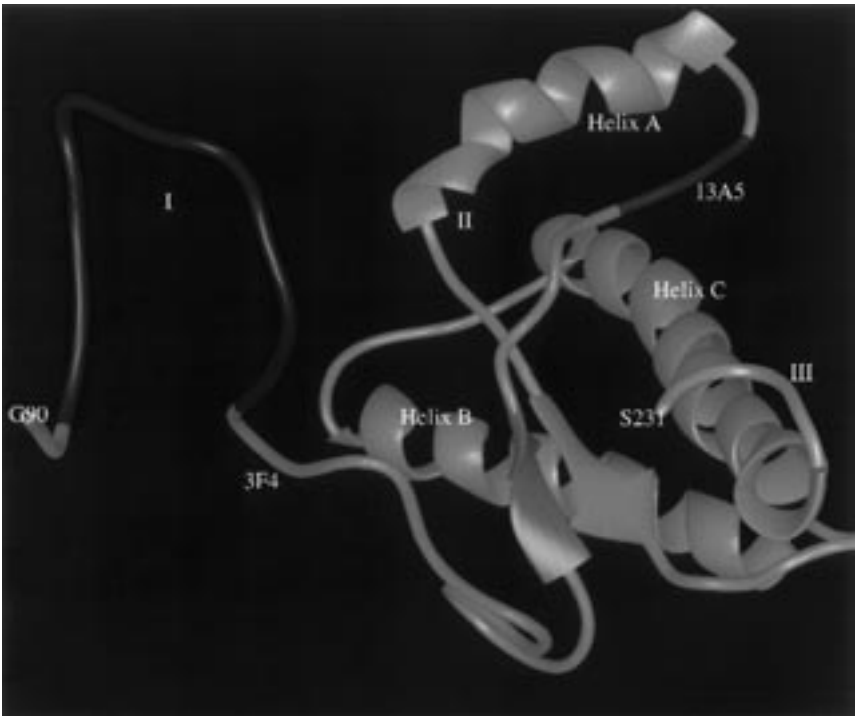

Figure 3 Epitope superimposition on the solution structure of recombinant PrP(90-231). Epitope region I (SHaPrP amino acids 95-104) is shown in *red*, epitope region II (residues 152–164) is shown in *cyan*, and epitope region III (residues 225–231) is shown in *gold*. The epitopes recognized by the hybridoma-derived antibodies 3F4 and 13A5 (directed to residues 109–112 and 138–141, respectively) are highlighted in *pink* and *magenta*. The α-helices are labeled A, B, and C and are shown in *gray*. The S2 β-sheet corresponds to residues 129–131 and is shown in *green*. (Adapted from James et al. 1997.)

PrP. Two PrP-reactive MAbs were recovered from the fusion, and their binding epitopes were mapped with synthetic peptides. The first MAb recognized a single linear epitope of PrP between amino acid residues 144 and 152. The second MAb (15B3), notably of the IgM class, apparently recognized three distinct linear peptide sequences corresponding to amino acids 142–148, 162–170, and 214–226.

MAb 15B3 was tested in immunoprecipitation assays using brain homogenates from normal and bovine spongiform encephalopathy (BSE)-infected cattle. PrP was precipitated only from the brains of BSE-infected animals, either before or after the homogenate was treated with

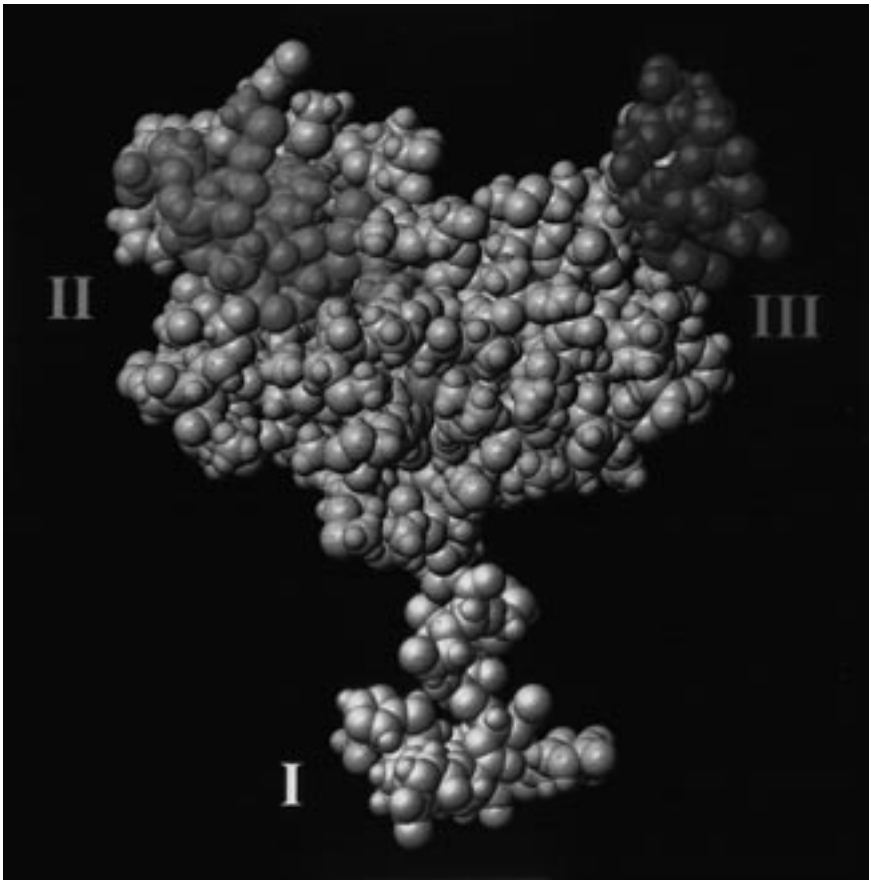

Figure 4 Epitope regions I, II, and III, defined by recombinant antibodies, are shown superimposed onto the NMR structure of a recombinant PrP^{c} molecule (Donne et al. 1997). Conversion of Prp^{c} and PrP^{Sc} appears to involve conformational changes in the epitope I region.

proteinase K. This finding indicated that the antibody recognized disease-specific forms of PrP and that the amino-terminal portion of the protein, between amino acids 23 and 90, was not required for antibody binding. Interestingly, since not all the immunoprecipitated PrP was found to be proteinase resistant, the antibody may bind to multiple disease-specific forms of PrP, possibly including conformations that occur as folding intermediates in the generation of fully proteinase-resistant PrP (Daude et al. 1997).

CRYSTALLOGRAPHIC STUDIES OF ANTIBODY/PrP COMPLEXES

NMR studies have indicated that in solution, in the absence of copper, the amino-terminal portion (residues 23–125) of recombinant PrP is highly disordered and devoid of secondary structure arrangements (Donne et al. 1997; Riek et al. 1997). An alternative approach to elicit information about the conformational properties of this portion of PrP is via its interaction with antibody Fab fragments (Z. Kanyo, unpubl.). The 3F4 antibody (Kascsak et al. 1987) has been shown to bind peptides containing amino acid positions 104–113 of SHa- and HuPrP (KPKTNMKHMA) with nanomolar affinities (Bolton et al. 1991). Protein crystals of enzymatically prepared 3F4 as well as 3F4 bound to SHa 104–113 were grown and their structures solved using X-ray crystallographic methods (MIP). As has been the case in some (Churchill et al. 1994; Ghiara et al. 1994), but not all (Stanfield et al. 1990; Wien et al. 1995), comparable systems, the conformation of the peptide when complexed with 3F4 Fab may bear strong similarity to its corresponding region in SHaPrP.

The structure of the 3F4-peptide complex is shown in Figure 5. The Fab is represented by a semi-transparent van der Waals surface highlighting the considerable surface complementarity between the peptide structure and the Fab-binding surface. This association seems largely driven by removal of the hydrophobic residues M109 and M112 from the solvent. This observation readily explains why the reactivity of 3F4 is restricted to SHa- and HuPrP, which contain methionine residues in these positions (Bolton et al. 1991). However, there are numerous hydrogen bonds and salt links between the peptide and the antibody contributing to the binding energetics. Additionally, several hydrogen bonds are found within the peptide itself, the strongest of which are delineated in Figure 5. These hydrogen bonds allow the peptide to adopt a structure very different from that seen for this region (Donne et al. 1997; Riek et al. 1997). NMR studies of recombinant PrP were performed under non-physiological pH conditions that would be likely to disrupt the His-110 to Lys-106 backbone hydrogen bond that acts to stabilize the turn structure observed in the peptide. The ability of 3F4 to bind PrP^C on the cell surface, as determined by indirect immunofluorescence, indicates that the peptide conformation observed in the crystal structure is equivalent to that found in natively folded PrP^C.

The structure of this complex provides a wealth of data not only on structural features seen in PrP^C, features not yet available from studies on the intact molecule, but also on the larger questions of antibody affinity and specificity. A detailed analysis of the complex has yielded several

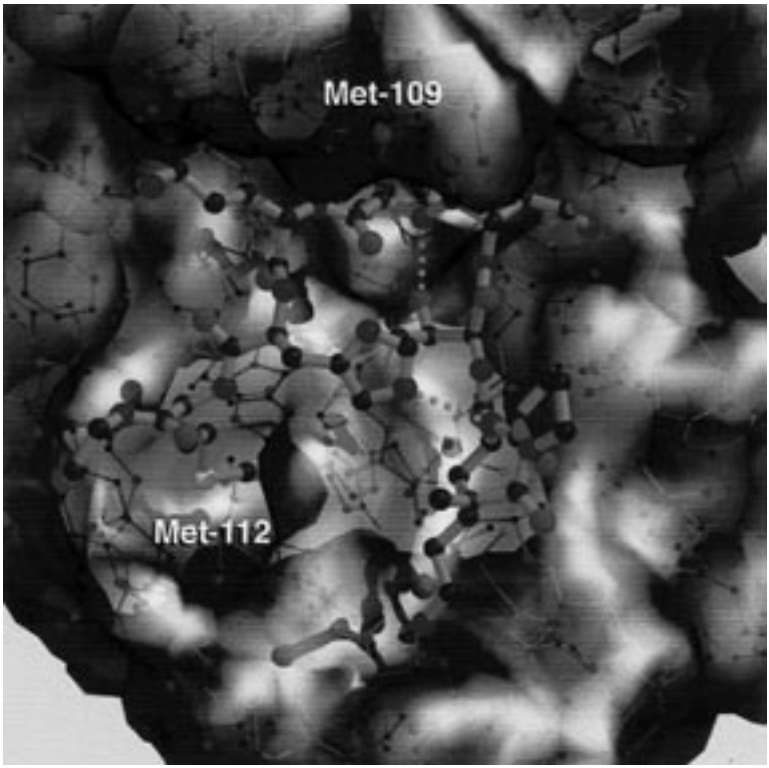

Figure 5 The crystal structure of the 3F4-SHaPrP (104-113) peptide complex. The binding clefts for Met-109 and Met-112 are clearly visible. Intra-peptide hydrogen bonds are shown as *green* spheres.

sites on the Fab which, if changed, might evolve the binding affinity or specificity of the Fab.

IMMUNOPATHOLOGY OF PrP IN SITU

The distribution of PrP in scrapie-infected brains is of considerable pathologic interest. PrP^{Sc} is readily detected immunologically in situ in extracellular amyloid plaques (DeArmond et al. 1985; Rubenstein et al. 1987). However, when tissue samples were probed using both polyclonal and monoclonal antibody reagents in traditional immunohistochemical procedures, the resolution of differential localization of PrP isoforms in infected brain tissues was disappointing (DeArmond et al. 1987).

In the absence of a scrapie-specific antibody, specific detection of PrP^{Sc} was achieved by limited proteolysis, to remove PrP^{C}, and denatura-

tion with GdnSCN, to improve immunoreactivity (Serban et al. 1990; Taraboulos et al. 1990). These procedures have proven unsuitable for standard histologic sections on glass slides. To increase the sensitivity and resolution of the immunostaining, an alternative methodology was developed (Taraboulos et al. 1992). Here "histoblots" of cryostat sections of snap-frozen, unfixed brain were transferred to nitrocellulose membranes and lysed in situ. PrP^{Sc} distribution within the histoblots of scrapie-infected tissue was determined by immunostaining after proteinase K digestion and denaturation in GdnSCN. The distribution of PrP^{C} was also detected in normal and infected brains using this methodology.

PrP immunostaining in histoblots yielded high signal-to-background ratios, was quantifiable by densitometry, and could be readily examined in detail under the microscope, allowing the distribution of PrP in specific structures to be determined.

PrP IMMUNODIAGNOSTICS

Increasing concern over the presence of infectious prion particles in the human food chain, and the likelihood of prolonged asymptomatic incubation periods in infected individuals, have potentially serious implications for transfusion and transplant medicine. Sensitive and reliable diagnostic tools to detect prion diseases in living hosts would contribute greatly to the control of infection in both humans and domestic livestock. Animal bioassays have been used, but they are very costly and involve long incubation times.

The most promising specific diagnostic marker of prion disease is the presence of abnormal PrP conformers in infected tissues. A system capable of efficiently detecting these abnormal PrP species may provide a robust platform for the development of a reliable diagnostic test. Such a system should incorporate a high throughput design, be able to distinguish between cellular and abnormal forms of PrP, and detect abnormal PrP when it is present at very low concentrations (such as are likely to be encountered in non-central nervous system tissues of the infected host [Kitamoto et al. 1989]). Immunoassays utilizing PrP-specific MAb reagents provide a starting point for the development of such a system. Previously, antibody reagents had been capable of recognizing PrP 27-30 following denaturation in immunoblotting assays. Unfortunately, these assays fell well short of the sensitivities required of a clinically useful test. However, recent reports describing the generation of MAbs recognizing epitopes found in nondenatured PrP 27-30 (Korth et al. 1997; Peretz et al. 1997) provide the opportunity to specifically select for abnormal forms of

PrP, thereby dramatically increasing the effective concentration of these PrP species and the sensitivity of the assay. In addition, experimental protocols developed for the improvement of antibody affinity in vitro offer the possibility to evolve antibody-binding constants toward the picomolar range (Yang et al. 1995; Barbas and Burton 1996). Such improvements would be expected to yield substantial increases in the sensitivity of antibody-based PrP diagnostic tests.

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Biosafety Issues in Prion Diseases

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Biosafety relevant to prions has been addressed in several guidelines, and recommendations have been published by health authorities in an attempt to limit the potential risk associated with prion contamination in laboratory studies, as well as in foods and medicinal products. Unfortunately, these issues often are not considered within the context of prion pathobiology and epidemiology. Instead, prion diseases are treated as viral-like diseases, and this can result in erroneous assumptions and misguided regulations.

Of the many distinctive features that separate prion diseases from viral, bacterial, fungal, and parasitic disorders, the most remarkable is that prion diseases can be manifest as infectious, inherited, and sporadic illnesses. Yet in all three manifestations of prion disease, infectious prions are generated in the brains of afflicted individuals, and these prions are composed of PrP^{Sc} molecules with the amino acid sequence encoded by the PrP gene of the affected host. When prions are passaged into the brain of a different host species, a "species barrier" related primarily to interspecies differences in PrP sequences is responsible for inefficient infection (Pattison 1965; Scott et al. 1989; Telling et al. 1995). If interspecies transmission does occur, then the prions generated in the brain of the alternate host carry the amino acid sequence encoded by the PrP gene of that particular species and not the PrP sequence found in the original inoculum. In other words, in interspecies infection, such as from sheep to cattle or from cattle to humans, the prions that replicate in the host brain are not the same as those that initiated replication. This scenario is pro-

foundly different from what happens during a viral infection. Although it is not the intent of this chapter to explore the molecular pathogenesis of the prion diseases (Chapters 4 and 8), a basic understanding of this area of biology and medicine is essential to the formulation of sound, intelligent, and effective risk assessment and management. If health authorities evaluate prion-related risk from the perspective of the virologist and ignore the unique properties peculiar to prions, they are doomed to make uninformed and perhaps harmful decisions.

There has been much public and professional concern over the potential transmission of scrapie and especially bovine spongiform encephalopathy (BSE) prions to humans and animals through consumption of contaminated foodstuffs. Prions in meat and bone meal (MBM), most likely derived from scrapied sheep offal, are believed to be the cause of the BSE epidemic in the UK (Chapter 11) (Wilesmith et al. 1991), and the transmission of a similar disease to cats is suspected to be due to prion-contaminated cat food (Wyatt et al. 1991). More recently, the announcement of a probable link between BSE and Creutzfeldt-Jakob disease (CJD) by UK authorities (Will et al. 1996) has initiated a profound reassessment of European Community policy on prion-associated risks to the human population. In addition, a further hazard to human health could reside in the administration of biological as well as medicinal products derived from or associated with human or animal tissues potentially contaminated with prions. In France, public and political reaction to the rising number of CJD cases related to administration of human growth hormone extracted from the pituitary glands of cadavers with undiagnosed CJD has regulatory authorities in a quandary as to what to do with respect to products derived from human tissues. These concerns have spread to questions regarding the safety of human blood and blood products as well.

All these fears and anxieties have given rise to a number of scenarios and forecasts, ranging from the cavalier to the apocalyptic, with attendant protective guidelines and measures. In this chapter, we strive to sort out the truth from the myth, the legitimate from the unreasonable, and to provide a factual basis for concerns, as well as an informed rationale for actions to be implemented. We also explore the potential consequences of underreaction as well as overconcern with respect to these issues.

BIOSAFETY LEVEL CLASSIFICATION

Human prions and those propagated in apes and monkeys are considered Biosafety Level 2 or 3 pathogens, depending on the studies being con-

ducted (Safar et al. 1999). BSE prions are likewise considered Risk Group 2 or 3 pathogens due to the possibility that BSE prions have been transmitted to humans in Great Britain and France (Chapter 12) (Will et al. 1996).

All other animal prions are considered Biosafety Level 2 pathogens. Thus, based on our current understanding of prion biology described above, once human prions are passaged in mice and mouse PrP^{Sc} is produced, these prions should be considered Biosafety Level 2 prions, although the human prions are Biosafety Level 3 under most experimental conditions. An exception to this statement occurs with mice expressing human or chimeric human/mouse transgenes. These animals produce human prions when infected with human prions and should be treated as Biosafety Level 2 or 3. The mechanism of prion spread among sheep and goats developing natural scrapie is unknown (Dickinson et al. 1974; Foster et al. 1992). Chronic wasting disease (CWD), transmissible mink encephalopathy (TME), bovine spongiform encephalopathy (BSE), feline spongiform encephalopathy (FSE), and exotic ungulate encephalopathy (EUE) are all thought to occur after the consumption of prion-infected foods (Gajdusek 1991; Marsh 1992; Collinge and Palmer 1997; Prusiner 1997).

Physical Properties of Prions

The smallest infectious prion particle is probably a dimer of PrP^{Sc}. This estimate is consistent with an ionizing radiation target size of 55 ± 9 kD (Bellinger-Kawahara et al. 1988). Therefore, prions may not be retained by most of the filters that efficiently eliminate bacteria and viruses. Additionally, prions aggregate into particles of nonuniform size and cannot be solubilized by detergents, except under denaturing conditions where infectivity is lost (Gabizon and Prusiner 1990; Safar et al. 1990). Prions resist inactivation by nucleases (Bellinger-Kawahara et al. 1987b), UV-irradiation at 254 nm (Alper et al. 1967; Bellinger-Kawahara et al. 1987a) and treatment with psoralens (McKinley et al. 1983), divalent cations, metal ion chelators, acids (between pH 3 and 7), hydroxylamine, formalin, boiling, or proteases (Prusiner 1982; Brown et al. 1990).

Care of Patients

In the care of patients dying of human prion disease, those precautions used for patients with AIDS or hepatitis are certainly adequate. In contrast to these viral illnesses, the human prion diseases are not communicable

or contagious (Ridley and Baker 1993). There is no evidence of contact or aerosol transmission of prions from one human to another. However, they are infectious under some circumstances, such as in the case of ritualistic cannibalism in New Guinea causing kuru, the administration of prion-contaminated growth hormone causing iatrogenic CJD, and the transplantation of prion-contaminated dura mater grafts (Gajdusek 1977; CDC 1997; Public Health Service 1997). Familial CJD, Gerstmann-Sträussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) are all dominantly inherited prion diseases; five different mutations of the PrP gene have been shown to be genetically linked to the development of inherited prion disease. Prions from many cases of inherited prion disease have been transmitted to apes, monkeys, and mice carrying human PrP transgenes (Brown et al. 1994a; Telling et al. 1995, 1996).

Surgical Procedures

Surgical procedures on patients carrying the diagnosis of prion disease should be minimized. It is thought that CJD has been spread from a CJD patient who underwent neurosurgical procedures to two other patients shortly thereafter in the same operating theater (Brown et al. 1992). Although there is no documentation of the transmission of prions to humans through droplets of blood or cerebrospinal fluid (CSF) or by exposure to intact skin or gastric and mucous membranes, the theoretical risk of such occurrences cannot be ruled out definitively. Sterilization of the instruments and the operating room should be performed in accord with recommendations described below.

Because it is important to establish a "definitive" diagnosis of a human prion disease and to distinguish between sporadic or familial cases and those acquired by infection as a result of medical procedures or from prion-contaminated food products, unfixed brain tissue should be obtained. For all cases of suspected human prion disease, a minimum of 1 cm^3 of unfixed cerebral cortex should be part of any biopsy. This specimen should be bisected from the cortical surface through to the underlying white matter with one half of the specimen formalin-fixed and the other half frozen.

Autopsies

Routine autopsies and the processing of small amounts of formalin-fixed tissues containing human prions require Biosafety Level 2 precautions. At

autopsy, the entire brain is collected and cut into coronal sections about 1.5 inches (~4 cm) thick; small blocks of tissue can easily be removed from each coronal section and placed in fixative for subsequent histopathologic analyses. Each coronal section is immediately heat-sealed in a heavy duty plastic bag. The outside of this bag is assumed to be contaminated with prions and other pathogens. With fresh gloves or with the help of an assistant with uncontaminated gloves, the bag containing the specimen is placed into another plastic bag that does not have a contaminated outer surface. The samples are then frozen on dry ice or placed directly in a –70ºC freezer for storage. At the very minimum, a coronal section of cerebral hemisphere containing the thalamus and a section of the cerebellar hemisphere and brain stem should be taken and frozen.

The absence of any known effective treatment for prion disease demands caution in the manipulation of potentially infectious diseases. The highest concentrations of prions are in the central nervous system and its coverings. On the basis of animal studies, it is possible that high concentrations of prions may also be found in spleen, thymus, lymph nodes, and lung. The main precaution to be taken when working with prion-infected or contaminated material is to avoid puncture of the skin, eyes, and mucous membranes (Ridley and Baker 1993). The prosector should wear cut-resistant gloves if possible. If accidental contamination of skin occurs, the area is swabbed with 1 N sodium hydroxide for 5 minutes and then washed with copious amounts of water. Tables 1-4 provide guidelines to reduce the chance of skin punctures, contamination from aerosols, and contamination of operating room and morgue surfaces and instruments. Unfixed samples of brain, spinal cord, and other tissues containing human prions should be processed with extreme care at Biosafety Level 3.

Bovine Spongiform Encephalopathy

The risk of infection for humans by BSE prions is unclear. Perhaps, the most prudent approach is to study BSE prions in a Biosafety Level 2 or 3 facility depending on the studies to be performed, as noted above for human prions.

Experimental Rodent Prion Diseases

Mice and hamsters are the experimental animals of choice for all studies of prion disease. With the development of transgenic mice that are high-

Table 1 Autopsies of patients with suspected prion disease: Standard precautions

1. Attendance is limited to three staff including at least one experienced pathologist. One of the staff avoids direct contact with the deceased but assists with handling of instruments and specimen containers.
2. Standard autopsy attire is mandatory. However, a disposable, waterproof gown is worn in place of a cloth gown.
   a. Cut-resistant gloves are worn underneath two pairs of surgical gloves, or chain mail gloves are worn between two pairs of surgical gloves.
   b. Aerosols are mainly created during opening of the skull with a Stryker saw. The personal protective equipment approved at the University of California at San Francisco for protection from aerosols is an AIR-MATE® HEPA 12-Powered Air-Purifying Respiratory System (RACAL Health and Safety, Inc., Frederick, Maryland). This unit consists of the following parts:
      i. Polycoated Tyvek head cover
      ii. HEPA filter unit containing battery pack, belt, and HEPA filter
      iii. AC adapter/charger
      iv. Airflow indicator
      v. Breathing tube assembly
   c. The above respirator system can be worn throughout the autopsy; however, when no aerosols are being generated, such as when the brain is being removed or when organ samples are being removed in situ, we often switch to a surgical mask with a wraparound splash guard transparent visor.
3. To reduce contamination of the autopsy suite:
   a. The autopsy table is covered with an absorbent sheet that has a waterproof backing.
   b. Contaminated instruments are placed on an absorbent pad.
   c. The brain is removed with the head in a plastic bag to reduce aerosolization.
   d. The brain can be placed into a container with a plastic bag liner for weighing.
   e. The brain is placed onto a cutting board and appropriate samples are dissected for snap freezing (see Table 3).
   f. The brain or organs to be fixed are immediately placed into a container with 10% neutral buffered formalin.
   g. In most cases of suspected prion disease, the autopsy can be limited to examination of the brain only. In cases requiring a full autopsy, consideration should be given to examining and sampling of thoracic and abdominal organs in situ.

Table 2 Autopsy suite decontamination procedures

1. Instruments (open box locks and jaws) and saw blades are placed into a large stainless steel dish, soaked for 1 hr in 2 N sodium hydroxide[a] or 2 hr in 1 N sodium hydroxide, and/or autoclaved at 134ºC (gravity displacement steam autoclaving for 1 hr; porous load steam autoclaving for one 18-min cycle at 30 lb psi or six 3-min cycles at 30 lb psi).
2. The Stryker saw is cleaned by repeated wiping with 2 N sodium hydroxide solution, followed by 1 N HCl and water.
3. The absorbent table cover and instrument pads, disposable clothing, etc. are double-bagged in appropriate infectious waste bags for incineration.
4. Any suspected areas of contamination of the autopsy table or room are decontaminated by repeated wetting over 1 hr with 2 N sodium hydroxide, followed by 1 N HCl and rinsing well with water.

[a]5% Sodium hypochlorite solution at least 20,000 ppm free chloride for 2 hr, or 96% formic acid may substitute but will corrode the stainless steel.

ly susceptible to human prions, the use of apes and monkeys is rarely needed. The highest titers of prions (~10^9 ID_{50}/g) are found in the brain and spinal cord of laboratory rodents infected with adapted strains of pri-

Table 3 Brain-cutting procedures

1. After adequate formaldehyde fixation (at least 10–14 days), the brain is examined and cut on a table covered with an absorbent pad with an impermeable backing.
2. Samples for histology are placed in cassettes labeled with "CJD precautions." For laboratories that do not have embedding and staining equipment or microtome dedicated to infectious diseases including CJD, blocks of formalin-fixed tissue can be placed in 95–100% formic acid for 1 hr, followed by fresh 10% neutral buffered formalin solution for at least 48 hr (Brown et al. 1990). The tissue block is then embedded in paraffin as usual. Standard neurohistological or immunohistochemical techniques are not obviously affected by formic acid treatment; however, in our experience, tissue sections are brittle and crack during sectioning.
3. All instruments and surfaces coming in contact with the tissue are decontaminated as described in Table 2.
4. Tissue remnants, cutting debris, and contaminated formaldehyde solution should be discarded within a plastic container as infectious hospital waste for eventual incineration.

Table 4 Tissue preparation

1. Histology technicians should wear gloves, apron, laboratory coat, and eye protection.
2. Adequate fixation of small tissue samples (e.g., biopsies) from a patient with suspected prion disease is followed by post-fixation in 96–100% formic acid for 1 hr, followed by 48 hr in fresh 10% formalin.
3. Liquid waste is collected in a 4-liter waste bottle containing 600 ml 6 N sodium hydroxide and diluted to a final volume of 4 liters to maintain the optimal concentration for disinfection. Gloves, embedding molds, and all handling materials are disposed in a "biohazard" waste receptacle for incineration.
4. Tissue cassettes are processed manually to prevent contamination of tissue processors.
5. Tissues are embedded in a disposable embedding mold. If used, forceps are decontaminated.
6. In preparing sections, gloves are worn and section waste is collected and disposed in a biohazard waste receptacle. The knife stage is wiped with 1–2 N NaOH. The used knife is immediately discarded in a "biohazard sharps" receptacle. Slides are labeled with "CJD Precautions." The sectioned block is sealed with paraffin.
7. Routine staining:
   a. Slides are processed manually.
   b. Reagents are prepared in 100-ml disposable specimen cups.
   c. After placing the coverslip on, slides are decontaminated by soaking them for 1 hr in 2 N NaOH.
   d. Slides are labeled as "Infectious-CJD."
8. Other suggestions:
   a. Use disposable specimen cups or slide mailers for reagents.
   b. Process slides for immunocytochemistry in disposable petri dishes.
   c. Decontaminate equipment as described above.

ons (Eklund et al. 1967; Prusiner et al. 1980); lower titers (~10^6 ID_{50}/g) are present in the spleen and lymphoreticular system (Kimberlin 1976; Prusiner et al. 1978).

Inactivation of Prions

Prion infectivity can be diminished by prolonged digestion with proteases and other treatments such as boiling in SDS. Sterilization of rodent brain extracts with high titers of prions requires autoclaving at 132ºC for 4.5 hours; denaturing organic solvents such as phenol (1:1), chaotropic

agents such as guanidine isocyanate or hydrochloride (>4 M, or alkali such as NaOH for 24 hours (Prusiner et al. 1984, 1993; Taylor et al. 1995, 1997).

With the exceptions noted above, Biosafety Level 2 practices and facilities are recommended for all activities utilizing known or potentially infectious tissues and fluids containing nonhuman prions from naturally or experimentally infected animals. Although there is no evidence to suggest that aerosol transmission occurs in the natural disease, it is prudent to avoid the generation of aerosols or droplets during the manipulation of tissues or fluids and during the necropsy of experimental animals. It is further strongly recommended that gloves be worn for activities that provide the opportunity for skin contact with infectious tissues and fluids. Formaldehyde-fixed and paraffin-embedded tissues, especially of the brain, remain infectious. Some investigators recommend that formalin-fixed tissues from suspected cases of prion disease be immersed for 30 minutes in 96% formic acid or phenol before embedding in paraffin and histopathologic processing (Brown et al. 1990), but such treatment may alter some immunohistochemical reactions.

Prions are characterized by extreme resistance to conventional inactivation procedures including irradiation, boiling, dry heat, and chemicals (formalin, betapropiolactone, alcohols). However, they are inactivated by 1 N NaOH, 4.0 M guanidinium hydrochloride or isocyanate, sodium hypochlorite (≥2% free chlorine concentration), and steam autoclaving at 132ºC for 4.5 hours (Prusiner et al. 1984, 1993; Taylor et al. 1995, 1997). It is recommended that dry waste be autoclaved at 132ºC for 4.5 hours or incinerated. Large volumes of infectious liquid waste containing high titers of prions can be completely sterilized by treatment with 1 N NaOH (final concentration) followed by autoclaving at 132ºC for 4.5 hours. Disposable plasticware, which can be discarded as a dry waste, is highly recommended. Because the paraformaldehyde vaporization procedure does not diminish prion titers, the biosafety hoods must be decontaminated with 1 N NaOH, followed by 1 N HCl, and rinsed with water; hepa filters should be autoclaved and incinerated.

HUMAN-TO-HUMAN TRANSMISSION OF PRIONS

Iatrogenic CJD

Since the extinction of kuru, an infectious human prion disorder transmitted by the now abolished practice of ritualistic cannibalism among the Fore population of Papua New Guinea (Alpers 1987), all currently known

cases of infectious, human-to-human transmission of prions are iatrogenic CJD (iCJD). Iatrogenic CJD accounts for less than 1% of total CJD worldwide and always involves transmission of prions originating from the central nervous system (CNS), where there is potential for high levels of infectivity, or from tissues intimately associated with the CNS, such as the cornea, pituitary gland, and dura mater (Brown et al. 1992). No case of iCJD in which the source of infection was established to be peripheral tissue or blood has ever been reported.

The current status of iatrogenic CJD with respect to source of contamination, numbers of cases, and mode of transmission is summarized in Table 5. New cases continue to appear, particularly in relation to administration of human growth hormone (HGH) extracted from cadaver pituitary glands in France (52 cases identified thus far) and to dura mater transplantation in Japan, where more than 50 cases have been reported.

. An intriguing susceptibility factor associated with iCJD appears to involve a polymorphism at codon 129 of the PrP gene (Chapter 12). In the Caucasian population, three amino acid phenotypes have been identified at this codon: heterozygous methionine/valine (Met/Val) in approximately half of all subjects tested, homozygous Met/Met in roughly 40%, and homozygous Val/Val in about 10% (Owen et al. 1990; Collinge et al. 1991; Laplanche 1996). In iCJD, however, only about 10% of all studied cases are heterozygous Met/Val, with an overrepresentation of homozygous Met/Met (~60%) and Val/Val (~30%), suggesting that homozygosity at this site may predispose an individual to the acquisition of disease following exposure to exogenous human prions (Collinge et al. 1991; Brown et al. 1994b; Deslys et al. 1996; Laplanche 1996). In sCJD, there is an even greater overrepresentation of homozygous Met/Met (~80%) with a representative proportion of Val/Val (~10%) but an under-repre-

Table 5 Current status of iatrogenic CJD worldwide

Tissue source of contamination	Number of cases	Mode of transmission
Brain	4	neurosurgical procedures
Brain	2	implantation of stereotactic EEG electrodes
Eye	3	corneal transplantation
Dura mater	80	dura mater transplantation
Pituitary gland	102	parenteral growth hormone therapy
Pituitary gland	4	parenteral gondotrophin therapy

sentation of heterozygous Met/Val (~10%) (Palmer et al. 1991; Laplanche 1996; Parchi et al. 1996). Whether these differences at codon 129 truly predispose to heightened susceptibility to acquired CJD, or whether their role involves modulation of the duration of incubation periods, remains to be determined. The situation bears close watching, however, particularly in France, where recent cases of iatrogenic, HGH-related CJD in Met/Val heterozygotes could be of ominous portent (Deslys et al. 1998). If heterozygosity at codon 129 was not truly protective but only conferred prolonged incubation periods, an already dramatic situation (France has more than half the total cases of HGH-related CJD worldwide) could represent only the tip of the iceberg.

CJD and Human Blood

The possibility of transmission of prions from human to human by blood transfusion or through administration of blood components or plasma derivatives has been a cause for concern among health professionals throughout the world. Despite no confirmed example of the transmission of prions from a human blood donor who later developed CJD to a recipient, many investigators still think that such transmissions are possible (Manuelidis 1994; Brown 1995; Ricketts et al. 1997). When a recipient of a blood transfusion, blood components, or plasma derivatives has developed CJD, the first question that must be asked is whether this is a case of sporadic or iatrogenic CJD. The difficulty comes in distinguishing iatrogenic from sporadic CJD. In both iatrogenic and sporadic CJD, wild-type PrP^{Sc} is usually found in the brains of the patients, which is a unique feature of prion disease. If CJD were caused by a virus, then the virus isolated from the blood or blood product that was given to the patient could be compared to that recovered from the patient. In prion diseases, only PrP^{Sc} can be compared to that recovered from the patient, but the detection of PrP^{Sc} in blood from a CJD patient has never been accomplished. Whether such an approach will prove feasible using transgenic mice and conformation-dependent immunoassays is unclear.

The application of transgenic mice and conformation-dependent immunoassays to the field of prion biology is so new that many "applied studies" have not been done. Previous investigations with non-transgenic rodents have often not employed experimental designs utilizing adequate controls and safeguards against potential contamination. Such controls are essential in the study of prion diseases because of the extraordinary resistance of prions to standard decontamination procedures.

Experimental Data

Although there is no convincing evidence demonstrating the presence of CJD prion infectivity in human blood, prion infectivity in blood or blood constituents has been reported in multiple studies with experimentally infected laboratory rodents (Clarke and Haig 1967; Dickinson and Fraser 1969; Manuelidis et al. 1978; Kuroda et al. 1983; Diringer 1984; Casaccia et al. 1989). These studies must be considered in the context of the experimental design where the animals were inoculated with exogenous prions, either peripherally or intracerebrally, and part of the inoculum probably found its way across disrupted blood vessels into the bloodstream during the inoculation procedure. Infectivity levels in these rodent blood samples, as estimated by the incubation periods in bioassay animals, were ~10^2 ID_{50} per ml, compared to 10^6–10^7 ID_{50} per gram of brain tissue (Brown 1995).

It must be stressed that in the vast majority of CJD cases, i.e., sporadic and familial CJD, which account for more than 99% of the total number of CJD cases worldwide, no penetrating inoculation occurs as in experimental prion disease in laboratory animals. Moreover, it is now believed that both sporadic and familial CJD result from a spontaneous conversion of PrP^C into PrP^{Sc}. This being the case, it can be argued that there is no reason to suspect passage of infectivity into the bloodstream, except possibly in late stages of disease when there may be breakdown of the blood brain barrier. Iatrogenic CJD, which represents less than 1% of total CJD and in which inoculation of exogenous prions via injection or surgical procedure is documented, may pose a greater potential risk of blood-borne prions. Current blood donor selection criteria as discussed below exclude virtually all donors at risk for iCJD. We have deliberately avoided exploring the potential implications of new variant CJD (nvCJD) on the blood supply here but discuss this issue separately at the end of this chapter.

To this date, there have been no systematic, well-controlled studies of the potential for transmission of human prions from blood of CJD patients using highly susceptible assay animals. With the advent of transgenic mice carrying a chimeric human/mouse PrP gene (Telling et al. 1994) or the human PrP gene on an ablated background (Telling et al. 1995), this issue can be addressed for the first time. Unfortunately, we can refer presently only to putative isolations of prion infectivity from the blood of five patients clinically diagnosed with CJD, using less susceptible laboratory rodent hosts (guinea pigs, hamsters, or mice). Two patients were reported by one laboratory (Manuelidis et al. 1985) and the remaining

three by three different laboratories (Table 6). (Tateishi 1985; Deslys et al. 1994; Tamai et al. 1992) The work of Manuelidis et al. (1985) deserves special mention here since it is frequently cited as the first demonstration of prion infectivity in human blood. As pointed out by Brown (1995), the low transmission rate and very high non-CJD experimental mortality in these studies are disquieting and invoke other, more plausible interpretations, i.e., intercurrent illness or laboratory cross-contamination. These suspicions were confirmed when the same authors later reported transmission of a spongiform encephalopathy to hamsters from buffy coats of Alzheimer's disease patients and non-affected relatives (Manuelidis et al. 1988) and subsequently from 26 of 30 neurologically healthy control subjects (Manuelidis and Manuelidis 1993). When the National Institutes of Health (NIH) attempted to verify the results of these latter two studies in duplicate experiments, the results proved to be nonreproducible (Godec et al. 1991, 1994). A review of the data of Tateishi (1985), Tamai et al. (1992), and Deslys et al. (1994) raises similar concerns of artifactual, laboratory cross-contamination as suggested by one or more of the following problems: incubation times in animals inoculated with blood and urine (Tateishi 1985) and plasma (Tamai et al. 1992) comparable to those in animals inoculated with brain from the same patient; irreproducibility in a subsequent experiment (Doi 1991 re: Tateishi 1985; Brown 1995); very long incubation periods; and very high unexplained mortality in both inoculated and control groups (Deslys et al. 1994).

In contrast to these anecdotal, putatively positive but controversial isolations of prion infectivity from human blood using relatively unsusceptible rodent hosts presenting significant species barriers are the uniformly negative results obtained using more susceptible nonhuman primates. Attempts at the NIH to transmit disease from the blood of 13 CJD patients to different primate species, including chimpanzees, by using multiple routes of inoculation were all unsuccessful (Brown et al. 1994a).

In conclusion, a review of the published experimental data does not unearth any convincing evidence to support the current perception that human blood may contain prions. This does not mean that the notion of potential prion infectivity in human blood can be dismissed entirely, but only that it is not supported by any of the foregoing transmission studies, many of which have been flawed. The consistent detection of prions in rodent lymphoid tissues (Eklund et al. 1967; Dickinson and Fraser 1969; Lavelle et al. 1972; Kimberlin 1976; Prusiner et al. 1978; Kitamoto et al. 1991; Muramoto et al. 1993; O'Rourke et al. 1994; Fraser et al. 1996; Brown et al. 1996; Klein et al. 1997) may give cause to suspect the presence of prions in blood carried by circulating lymphocytes during some

Table 6 Putative isolations of prion infectivity from blood of CJD patients

Source of isolation	Number of CJD cases	Assay animals	Reference	Causes for concern
Buffy coat	2/2 (sporadic)	guinea pigs, hamsters	Manuelidis et al. (1985)	lack of controls low transmission rates high unexplained mortality aberrant results in subsequent experiments irreproducibility of subsequent experiments
Whole blood, urine	1/3 (sporadic)	mice	Tateishi (1985)	low transmission rates improbable incubation times (comparable to those obtained with brain) irreproducibility in subsequent experiments
Concentrated plasma[a]	1/1 (sporadic)	mice	Tamai et al. (1992)	low transmission rate improbable incubation time (comparable to that obtained with brain)
Buffy coat	1/1 (HGH-iatrogenic)	hamsters	Deslys et al. (1994)	low transmission rate very long incubation period very long unexplained mortality in both control and inoculated animals

[a]Unconcentrated plasma and white blood cells from the same patient failed to transmit disease.

phase of the illness. However, the relevance of such findings in experimentally inoculated rodents to CJD in humans remains unclear. In any event, the apparent difficulty in demonstrating prions in human blood argues that if they are present, they exist at low concentrations.

At present, we have no knowledge of the titers and distribution of prions in human lymphoid tissues. Moreover, we currently have no means of identifying asymptomatic individuals who are destined to develop sporadic CJD. Without such diagnostic tools, even the detection and measurement of prions in blood of symptomatic patients with sporadic CJD are problematic. We describe these problems not to paint a hopeless picture, but to delineate where additional research needs to focus in order to gain urgently needed information. At the moment, no systematic, well-controlled transmission study has been performed in which blood drawn from symptomatic sporadic CJD patients was bioassayed in transgenic mice that are highly susceptible to human prions. The need for such a study is crucial.

Epidemiologic Data

Available epidemiologic data do not suggest prion contamination of blood, blood components, or plasma derivatives. In fact, these data argue against transmission of CJD through blood.

Epidemiologic analysis of this issue is based on five different types of studies: (1) case reports, (2) case control studies, (3) routine surveillance, (4) cohort investigation of recipients from CJD donors, and (5) special population studies.

A survey of case reports attempting to link CJD to blood transfusion or administration of blood products has failed to reveal any convincing instances of prion transmission via blood. The utility of case reports in identifying causative links between CJD cases and therapeutic products or procedures should not be underestimated since case reports provided the initial warnings of the implications of HGH treatment (CDC 1985) and dura mater grafts (Prichard et al. 1987; CDC 1989, 1997) in CJD transmission.

Case control studies of CJD conducted in the US (Davanipour et al. 1985), the UK (Esmonde et al. 1993), and Japan (Kondo and Kuroina 1982) have addressed the issue of whether receiving blood is a risk factor for CJD. In all of these investigations, there is no evidence of a higher proportion of CJD cases having a history of blood transfusion than that of control subjects. More recent, unpublished, ongoing case control studies in the US (CDC 1996; L.B. Schonberger, pers. comm.) and the UK (R.G.

Will, pers. comm.) continue to show that blood exposure is not a risk factor for CJD. There are no significant differences between CJD and control populations with respect to receiving or donating blood.

Routine surveillance studies of the epidemiology of CJD have been conducted in many countries and even repeated in some (Brown et al. 1987; Holman et al. 1995; Will 1996; Ruffie et al. 1997). With the exception of the UK, where nvCJD has slightly altered the traditional epidemiologic pattern of CJD (Chapter 12), the disease continues to have a similar and constant incidence (approximately 1 case per million population per year) and age distribution (peak in the 60s to 70s, rare under 30), despite increasing use of blood transfusion and plasma proteins.

Perhaps the most compelling evidence against transmission of CJD through blood or blood products is provided from cohort studies of recipients from CJD donors and from special population studies, including patients suffering from hemophilia A, hemophilia B, β-thalassemia, and sickle cell disease, and those receiving large quantities of cryoprecipitate. Retrospective, follow-up studies with the goal of tracing all recipients of blood or blood products from CJD donors have been conducted in Germany (Heye et al. 1994) and are ongoing in the US, involving the American Red Cross, the American Association of Blood Banks, the New York Blood Center, and the Centers for Disease Control and Prevention (CDC) (Evatt et al. 1998), and in the UK (R.G. Will, pers. comm.). Data from all these studies appear to indicate that despite identification of large numbers of recipients of potentially tainted blood products (up to 196 investigated recipients of blood products from 15 CJD donors in the American study), none of the identified recipients developed CJD.

In studies from the CDC, no patients afflicted with hemophilia A, hemophilia B, β-thalassemia, or sickle cell anemia have contracted CJD in the United States over a 17-year period (L.B. Schonberger, pers. comm.). Since clotting factor concentrates for hemophiliacs can come from pools of 20,000–30,000 donors, hemophiliacs should have been exposed to prions if they were present in blood at appreciable levels. The CDC currently is actively seeking cases of CJD in hemophiliacs; yet, they have seen no evidence of CJD in the hemophilia community despite increased surveillance since 1994. In another CDC study of 101 patients who received over 238,000 units of cryoprecipitate over a 17-year period, no cases of CJD were found. Moreover, 76 of these cryoprecipitate users were still alive a minimum of 12 years later, and 3 had received at least one unit from a known CJD donor.

In conclusion, the data from epidemiologic studies conducted in several parts of the world argue that CJD prions are not transmitted from

human to human through blood or blood derivatives. Although it has been stated that epidemiologic methods are limited in the detection of rare events such as CJD (Ricketts et al. 1997), the multiple studies currently under way in many countries should give additional information about the risk of transmitting CJD through blood or blood products.

Regulatory Policies

The prevailing view among regulatory authorities with respect to the risk of transmitting CJD prions through blood and blood products is that not enough is known about the potential of individuals in an asymptomatic phase of CJD to carry infectious prions in their blood. Thus, in the absence of a specific and sensitive diagnostic test, it is feared that such "carriers" could disseminate infection by blood donation while escaping detection until the onset of symptoms and signs of CJD. Therefore, regulatory groups have decided that transmission of CJD through blood constitutes a "theoretical risk" and that appropriate measures must be taken to preclude the occurrence of blood-related CJD in the future. Their actions toward that objective include the requirement of screening from the blood donor population all individuals considered theoretically at risk of carrying infectious prions, essentially those at risk for inherited (family history of neurodegenerative disease) or iatrogenic (history of having received cadaver pituitary-extracted HGH or a dura mater graft) CJD.

In addition, validation of separation and fractionation procedures for removal or carryover of prion infectivity has been recommended. Such studies are designed to address two essential questions with respect to the prion safety of blood products: Where does prion infectivity segregate during fractionation, and what clearance factor or safety margin is achieved in fractionation? One set of such studies has been recently performed in which blood was taken from mice or hamsters with experimentally acquired prion disease and subjected to separation and fractionation procedures used for human blood. Bioassays showed prion infectivity to be present in many plasma fractions (P. Brown and R.G. Rohwer, pers. comm.). The results of these currently unpublished but frequently presented studies have stirred considerable concern about the safety of plasma and certain plasma fractions, although they are of uncertain relevance and raise many questions as to study design and interpretation.

The design of proper validation studies is difficult in many respects: first is the choice of the prion itself, second is the choice of the tissue in which the prion was replicated, and third is the choice of the prion preparation. Since prions do not behave as ideal particles in either crude or

purified preparations, it is difficult to choose an appropriate inoculum. These issues raise a number of perplexing questions. If one chooses to do spiking studies, what does one use as starting material, especially when prion infectivity cannot be found in human blood? Is the correct approach to spiking experiments the addition of prion-infected mouse or hamster brain to human whole blood or plasma? What is the validity of using mouse or hamster prions that could have different separation and removal/inactivation profiles from human prions? What is the relevance of using brain homogenates for spiking blood or plasma? Could the association of infectivity with brain tissue affect partitioning or adsorptions?

In our opinion, the use of brain and lymphoid tissues from transgenic mice expressing human PrP on the null ($Prnp^{0/0}$) background provides the best source of inocula for spiking experiments. Such prions can be readily bioassayed in mice expressing chimeric human/mouse PrP transgenes (Telling et al. 1994, 1995) (Chapter 8). Moreover, new highly sensitive immunoassays can be used for many aspects of these studies (Chapter 15).

Regulatory policies, with respect to donor exclusion criteria and process validation, are relatively uniform from country to country. They diverge, however, with regard to required actions upon discovery of a blood donor who subsequently develops CJD. In the US, following a special Food and Drug Administration (FDA) advisory committee meeting in 1995, the FDA decided to recall all lots of any blood/plasma product emanating from a single donation or from a pool involving a donor who was diagnosed with CJD after giving blood. The same year, the Committee for Proprietary Medicinal Products (CPMP) in Europe ruled against recall, considering CJD a rare disease and the risk of transmission via blood products purely theoretical. However, France in 1996 and Italy in 1997 decided to break with the CPMP position and ruled in favor of recall. Finally, Japan also decided to adopt the US policy in 1997, ruling in favor of recall. The CPMP modified its own position in 1998, when it ruled to withdraw plasma-derived medicinal products if a donor to a plasma pool was subsequently diagnosed as having nvCJD, at the same time reaffirming its position that recall is not justified in the case of a sporadic or familial CJD donor (CPMP 1998). More recently, the FDA has revised its recall policy along the same lines, now requiring recall only of products in which a nvCJD donor was implicated (announcement at the DHHS Blood Safety and Availability Committee Meeting held August 27, 1998).

When considered in terms of a risk/benefit scenario, it is legitimate to question the judiciousness of recall policies such as those implemented in the US and elsewhere. Since the available data offer no support for a risk of transmission of CJD through blood or blood products, the effi-

cacy of such measures in preventing CJD is questionable, and the benefit for the patients who use these products is unclear. A clear risk for users of plasma-derived medicinal products is that of shortages of potentially life-saving therapeutics secondary to CJD withdrawals. At the time of writing, scarcity of intravenous immunoglobulin (IVIG) in the United States is thought to be largely related to CJD-initiated withdrawals. IVIG is often a lifesaving product for patients with immune deficiency disorders, and its availability is an issue of considerable concern.

Since 1995, identification of donors who subsequently developed CJD appears to account for about 50% of all withdrawals of plasma derivatives in the US by the FDA (Mark Weinstein, pers. comm.). The potentially damaging financial impact of CJD withdrawals on the blood products industry also cannot be ignored. According to the American Red Cross, CJD withdrawals of its products have resulted in up to $120,000,000 in lost revenues over the last 2–3 years. This in turn impacts negatively on research and development activities and ultimately on product supply and costs. There is no telling what future consequences might result from currently implemented recall policies, but these effects may be far-reaching, even touching industries outside the realm of the producers of plasma derivatives. A recall involving albumin, for instance, which is used as an excipient in numerous vaccines and recombinant proteins, could cause major supply difficulties for these essential products as well.

In conclusion, the "theoretical risk" of human-to-human prion transmission through blood is under continuing review by worldwide regulatory authorities. Efforts have been made to assure the safety of the blood supply and of plasma derivatives through the application of donor exclusion criteria, the recommendation to perform process validation studies, and the implementation of recall policies. Whether any of these measures will be successful in reducing the theoretical risk is unknown. Although the use of appropriate selection and exclusion criteria is beyond reproach, the scientific relevance of validation studies as they are currently being conducted is questionable. A reevaluation of recall policies involving CJD donors, taking into account the closely intertwined issues of safety and availability, might prove beneficial in averting potentially serious consequences in the future.

RISK OF ANIMAL-TO-HUMAN TRANSMISSION OF PRIONS

BSE is the most worrisome of all the animal prion diseases from a biosafety standpoint. BSE is widely thought to be a manmade epidemic,

caused by a form of industrial cannibalism in which cattle were fed meat and bone meal (MBM) produced through faulty industrial processes from prion-contaminated cattle and sheep offal (Wilesmith et al. 1991; Anderson et al. 1996; Prusiner 1997; Chapter 11). Epidemiologically, BSE has shown a disquieting propensity to cross species barriers through oral consumption of prion-contaminated bovine foodstuffs (Kirkwood et al. 1990; Wyatt et al. 1991; Willoughby et al. 1992), and this propensity has been confirmed experimentally in laboratory transmission studies employing several routes of inoculation, including the oral route (Barlow and Middleton 1990; Dawson et al. 1990; Fraser et al. 1992; Baker et al. 1993; Foster et al. 1996; Lasmézas et al. 1996). Most alarmingly, there is now some epidemiologic and experimental basis for concern that BSE may be responsible for a new variant form of Creutzfeldt-Jakob disease (nvCJD) primarily in the UK (Collinge et al. 1996; Lasmézas et al. 1996; Will et al. 1996; Bruce et al. 1997; Hill et al. 1997b; Zeidler et al. 1997; Chapter 12), although definitive evidence for this is still lacking.

Against this backdrop, considerable concern has erupted in Europe over the safety of not only foodstuffs, but also pharmaceutical and biological products either derived from bovine source materials (active ingredients) or manufactured with bovine raw materials used as reagents in production. Additionally, there is concern about products that contain bovine components as excipients in final formulations or as constituents in the ingested product covering (capsule material). The major categories of "at-risk" products include recombinant proteins, vaccines, and gene therapy products produced in cultured cell systems using bovine-derived factors, as well as drugs that employ tallow or gelatin products as binders. A nonexhaustive list of bovine derivatives in pharmaceuticals is provided in Table 7. The pervasiveness of bovine-derived products on our planet is remarkable.

Regulatory Policies

In 1991, the European Union (EU) issued CPMP Guideline III/3298/91 (CPMP 1991), which recommended four measures for minimizing the risk of transmitting animal prions via medicinal products: (1) transparent traceability of the origin of source animals from low-risk regions for BSE, (2) preferential use of younger animals, (3) avoidance of tissues from high and medium infectivity categories as source materials (Table 8), and (4) process validation studies with implementation of removal and inactivation procedures within processes where feasible.

Table 7 Bovine derivatives in pharmaceuticals

Active ingredients		Raw materials in manufacture		Ingested covering		Excipients	
bovine derivative	bovine source material	bovine derivative	bovine source material	bovine derivative	bovine source material	bovine derivative	bovine source material
Aprotinin	lung	albumin	serum	gelatin	bone	gelatin	bone
Gelatin	bone/hide	amicase	milk (casein)			lactose	milk
Glucagon	pancreas	brain-heart infusion	brain and heart			Mg stearate	tallow
Heparin	intestine	fetal calf serum	serum			polysorbate	tallow
Insulin	pancreas	glycerol	tallow				
Surfactant	lung	liver infusion	liver				
		meat extract	carcass				
		newborn calf serum	serum				
		pepticase	milk (casein)				
		peptone	muscle				
		polysorbate	tallow				
		primatone	blood/spleen				
		trypsin	pancreas				
		tryptone	milk				

Table 8 Categories of infectivity in bovine tissues and body fluids

CATEGORY I High infectivity	→	brain, spinal cord, (eye)
CATEGORY II Medium infectivity	→	spleen, tonsil, lymph nodes, ileum, proximal colon, cerebrospinal fluid, pituitary gland, adrenal gland, (dura mater, pineal gland, placenta, distal colon)
CATEGORY III Low infectivity	→	peripheral nerves, nasal mucosa, thymus, bone marrow, liver, lung, pancreas
CATEGORY IV No detectable infectivity	→	skeletal muscle, heart, mammary gland, milk, blood clot, serum, feces, kidney, thyroid, salivary gland, saliva, ovary, uterus, testis, seminal vesicle, fetal tissue, (colostrum, bile, bone, cartilaginous tissue, connective tissue, hair, skin, urine)

Tissues in parentheses were not titrated in the original studies (Hadlow et al. 1980, 1982).

Based on relative scrapie infectivity of tissues and body fluids from naturally infected Suffolk sheep and goats with clinical scrapie.

The above recommendations invite a number of comments. First of all, they do not propose any quantitative method for prion risk assessment with respect to pharmaceuticals. Second, the hierarchy of risk levels from different tissues contains no numerical range of infectivity titers for each of the four categories. Moreover, the whole concept of high-to-low risk levels from various tissues is based on transmission experiments from scrapied sheep and goats to wild-type mice (Hadlow et al. 1980, 1982), which might bear little relation to the tissue distribution of prions in infected cattle. Third, the guidelines give no clue as to the potential impact of subclinical disease. Fourth, the models generally employed for process validation and for determination of removal and inactivation capacity of certain procedures involve spiking materials with titered mouse or hamster brain homogenates. In such studies, downstream products are tested by intracerebral inoculation into wild-type mice or hamsters; such a system may or may not reflect the behavior of bovine prions under similar conditions.

In parallel with the human prion studies noted above, we believe that the use of brain and lymphoid tissues from transgenic mice expressing bovine PrP on the null ($Prnp^{0/0}$) background provides the best source of

inocula. Such prions can be readily bioassayed in mice expressing bovine PrP transgenes (Scott et al. 1997) (Chapter 8). Moreover, highly sensitive immunoassays can be used in many aspects of these studies as well (Chapter 15).

In 1997, under intense political pressure from the EU Parliament, the European Commission (EC) issued a decision (EC 1997) that (1) defined "specified risk materials" (SRM) as the skull and its contents (including the brain and eyes); tonsils and spinal cord from bovine, ovine, and caprine animals aged over 12 months, or from ovine and caprine animals with an erupted permanent incisor tooth; and the spleen from ovines and caprines and (2) banned use of said SRM for any purpose and under any circumstances, with the exception of research. This decision also prohibited the use of vertebral column of bovine, ovine, and caprine animals for the production of mechanically recovered meat. It further banned import into the European Community of any "medical, pharmaceutical or cosmetic products, or their starting materials or intermediate products," unless accompanied by a declaration signed by the "competent authority of the country of production" stating the product neither contains nor is derived from SRM.

As currently defined, SRM as such are used in relatively few pharmaceutical products. However, materials such as tallow and gelatin, derivatives of which are found in most tablets and capsules as excipients and/or ingested covering, can be considered to be derived from starting materials that may have come into contact with SRM, and thus classified as SRM-derived, would be affected by EC Decision 97/534/EC. If implemented in its current form, the decision might force roughly 80% of pharmaceuticals off the European market, creating shortages in essential medicines and imposing alternative formulations for a host of medicinal products with unforeseen, potentially deleterious consequences.

Because of the potentially adverse implications of the EC decision and in the face of serious concerns expressed by the pharmaceutical industry, by the manufacturers of gelatin and tallow derivatives, and by the EU's own pharmaceutical regulatory agency (the European Agency for the Evaluation of Medicinal Products) in a recent report (CPMP 1997), implementation of the decision, which was to have come into effect in January 1998, has been repeatedly deferred. As of this writing, numerous amendments and revisions have been proposed and are under discussion, and implementation of a final decision does not seem achievable before 1999. Of note, one of the proposed revisions would actually expand the SRM list, thereby creating an even greater impact on pharmaceutical products affected by the decision.

In the US, the FDA has provided guidance through a series of letters to manufacturers and importers of FDA-regulated products containing or manufactured with bovine derivatives from countries reporting BSE cases. The US Department of Agriculture (USDA) began identifying BSE-stricken countries in 1991 and has prohibited the importation of livestock and bovine-derived products for animal use from any country declaring BSE. In addition, the USDA has monitored the US cattle population by examining "fallen cattle" for BSE. Of the approximately 5000 cases examined to date, no cases of BSE have been found (L. Detwiler, pers. comm.). As a measure designed to prevent BSE in the US, the USDA recently initiated a "mammalian"-to-ruminant feed ban although porcine MBM may still be fed to cattle and cattle MBM to hogs. The ban also does not cover refeeding of bovine material back to cattle through plate waste and chicken feces.

A notice in the Federal Register of August 29, 1994 summarized FDA's position and recommendations to reduce any potential BSE risk in bovine-derived products (USDHHS 1994). In essence, FDA requested that bovine-derived materials originating from animals born or living in BSE countries not be used in the manufacture of FDA-regulated products intended for humans. Although the FDA did not object to the use of bovine-derived materials from BSE countries in the manufacture of pharmaceutical grade gelatin, it did consider it prudent to source from BSE-free countries. That position was based on an assessment that the manufacturing conditions for gelatin were likely to remove/inactivate prion infectivity sufficiently to obviate any risk of BSE prion transmission. Since studies with bovine prions were not used in making such an assessment, it is unclear whether the conclusions are warranted.

The safety of gelatin from BSE-stricken countries was revisited in 1997, following concerns raised by the Transmissible Spongiform Encephalopathy Advisory Committee (TSEAC), resulting in the FDA's first BSE-related "Guidance for Industry" document, announced in the Federal Register of October 7, 1997 (USDHHS 1997) and approved by the TSEAC in April 1998. This guidance document recommends that manufacturers determine the tissue, species, and country of origin of gelatin raw materials and exclude gelatin from bones and hides of cattle sourced from BSE countries or from countries of unknown/dubious BSE status for use in injectable, implantable, or ophthalmic products. It allows oral and cosmetic use of gelatin from bones of cattle from BSE countries with the condition that the raw material is sourced from BSE-free herds and that the heads, spines, and spinal cords are removed at slaughter. Bovine gelatin sourced in the US or other non-BSE countries is uncondi-

tionally authorized, and pig-skin gelatin is permitted if uncontaminated with bovine materials from BSE-stricken or BSE-unknown countries.

MONITORING ANIMALS FOR PRIONS

The specified offals bans instituted in Great Britain in 1988 and 1989 (Chapter 11) were based largely on data derived from studies using Swiss mice for the bioassay of sheep prions. The legislation made two assumptions: (1) the distribution of prions in cattle with BSE is the same as that in sheep with scrapie and (2) bioassays of non-CNS sheep prions in mice yielded reliable data. Although we are unaware of any comparative study of the distribution of prions in sheep and cattle, there are studies that allow for a comparison of prion titers in mice and Syrian hamsters. Such quantitative data show clear differences between these rodent hosts (Kimberlin and Walker 1977). Equally disturbing is the bioassay of tissues with low titers in non-transgenic mice. Typically, Swiss mice require an incubation time of about 500 days before showing signs of scrapie when inoculated with a 1% brain homogenate from a sheep with scrapie (Hadlow et al. 1982). Greatly prolonged incubation times have also been recorded for BSE prions with a variety of mouse strains (Fraser et al. 1992; Bruce et al. 1997; Lasmézas et al. 1997). An ongoing comparative study of BSE prions in bovine brain measured by endpoint titrations in cattle and C57BL mice suggests that the titer of the bovine prions is more than 1000-fold higher in cattle than in mice (J. Wilesmith, pers. comm.). The implications for accurate assessment of prions in peripheral tissues where the titers are likely to be much lower are disconcerting.

The advent of transgenic mice expressing bovine PrP but not endogenous mouse PrP ($Prnp^{0/0}$), which are highly susceptible to BSE prions with abbreviated incubation times (Scott et al. 1997), should provide considerable information that has been lacking to date. Endpoint titrations of homogenates derived from numerous tissues of BSE-afflicted cattle using Tg(BoPrP)$Prnp^{0/0}$ mice will be important. Such studies will allow the development of standard, titered bovine inocula as well as incubation time assays. The results of such studies will not only be important in assessing the safety of the food supply, but they should also pave the way for process validation and prion removal studies in the pharmaceutical industry.

The availability of Tg(BoPrP)$Prnp^{0/0}$ mice also provides the basis for calibrating sensitive immunoassays (Chapter 15). Such immunoassays may find widespread use in certifying that cattle and other domestic animals are free of prions. Interestingly, 2 asymptomatic cattle in Switzerland have been identified in a survey of 1761 supposedly healthy

cattle that were slaughtered (MacKenzie 1998). The PrP^{Sc} in the brain stems of the 2 animals was detected by Western blotting, which is considerably less sensitive than the conformation-dependent immunoassay (Chapter 15). How many cattle would have been found using the conformation-dependent immunoassay is of considerable interest and may have important implications for the safety of the food supply.

Although USDA surveys of "fallen cattle" have failed to reveal any cases of BSE in the US as noted above, this surveillance is far too limited and is not directed toward identifying asymptomatic cases. It is notable that a TME outbreak in Stettsonville, Wisconsin in 1985 is thought to have been due to a sporadic case of BSE in a fallen cow (Marsh et al. 1991). Since the incubation period for BSE is 3–4 years (Chapter 11), most infected cattle will never show neurologic deficits because the vast majority of cattle in the US are slaughtered by 1 year of age. Such animals might harbor significant titers of prions but not show any signs of CNS dysfunction or histologic evidence of spongiform degeneration. We argue that the availability of highly sensitive methods for detection of PrP^{Sc} in brain stems and other tissues of cattle ought to be employed routinely in slaughterhouses worldwide to ensure that humans are not infected by bovine prions.

NEW VARIANT CREUTZFELDT-JAKOB DISEASE

The importance of nvCJD (Chapter 12) with respect to biosafety is that it may represent the first example of the transmission of animal prions to humans. Once kuru was experimentally transmitted to apes (Gajdusek et al. 1966), the search for an animal reservoir intensified, but none was found. Eventually, ritualistic cannibalism became accepted as the mode of kuru transmission (Gajdusek 1977).

The transmission of sporadic CJD to apes (Gibbs et al. 1968) stimulated a 25-year search for a relationship between scrapie in sheep and CJD in humans, but none was identified. Exhaustive epidemiologic studies were conducted in the hope of identifying scrapie prions as the cause of CJD (Malmgren et al. 1979; Will and Matthews 1984; Brown et al. 1987; Cousens et al. 1990). When nvCJD was first reported, the differences in one or more of the seven amino acid side chains that distinguish bovine from sheep PrP were hypothesized to be the reason that bovine, but not sheep, prions may have been transmitted to humans. Subsequently, the BSE strain of bovine prions has also been offered as a factor in their possible permissivity in humans.

Have Bovine Prions Been Transmitted to Humans?

Although the origin of nvCJD has not been established, there is mounting evidence that it is due to the transmission of bovine prions to humans. This conclusion is based on multiple lines of inquiry: (1) the spatial-temporal clustering of nvCJD (Will et al. 1996; Zeidler et al. 1997), (2) the successful transmission of BSE to macaques with induction of PrP plaques similar to those seen in nvCJD (Lasmézas et al. 1996), (3) the similarity of the glycosylation pattern of the disease-related isoform of PrP in nvCJD to that noted in mice, domestic cats, and macaques infected with BSE prions (Collinge et al. 1996), and (4) experimental BSE transmission studies in mice which suggest that nvCJD and BSE represent the same prion strain (Bruce et al. 1997).

Taken together, these observations build a formidable case for a causal link between BSE and nvCJD, but they do not provide "proof." A number of enigmas still surround nvCJD and seek an adequate explanation. Why should the disease strike young people predominantly, with a majority in their teens and twenties? Why was there not a particularly evocative dietary history for any of the patients? Why does the disease incidence appear to be stagnating rather than increasing? What is the point of analyzing PrP glycoforms in attempting to link BSE and nvCJD, since PrP^{Sc} is formed after glycosylation of PrP^{C}? Why does it remain consistently difficult to infect mice expressing only human PrP or chimeric human/mouse PrP transgenes with BSE prions?

It is not our intention to dismiss the notion that nvCJD may be human BSE. Much evidence suggests that this is indeed the case. Although some questions may be resolved using new experimental systems, only the passage of time and the shape of the incidence curve of nvCJD in humans will allow a definitive judgment on this issue.

Whatever the origin of nvCJD, there is little doubt that nvCJD is different from sporadic or inherited CJD. That being the case, nvCJD must be considered separately with respect to biosafety issues. There appears to be evidence for a higher frequency of lymphoid (particularly tonsillar) involvement in nvCJD (Hill et al. 1997a), which raises the potential risk of blood infectivity carried by circulating lymphocytes. The implication that lymphoid tissue is involved during the preclinical incubation period of nvCJD was raised by the recent, retrospective demonstration of PrP immunoreactivity in lymphoid tissue in an appendix taken from a patient whose appendectomy preceeded clinical onset of nvCJD by at least a year (Hilton et al. 1998). If nvCJD proves to be peripherally acquired and not a sporadic illness, then it may be reasonable to fear a hematogenous phase

at some point in the evolution of the disease. Since we have no way of knowing or even estimating the number of individuals potentially harboring nvCJD prions, we cannot make the same assumptions in risk assessment and management that we do with sporadic CJD. Within the context of these considerations, the European CPMP and American FDA positions on nvCJD and plasma derivatives advocating precautionary withdrawal of batches of plasma derivatives if a pool donor was subsequently diagnosed as having nvCJD seem prudent.

CONCLUDING REMARKS

From this review of some of the biosafety issues relevant to prion-related risk, whether cattle-to-human via BSE-contaminated bovine products or human-to-human through CJD-contaminated blood, it becomes readily apparent that existing information is generally insufficient. This situation squeezes regulatory authorities into the uncomfortable position of having to recommend highly conservative, precautionary measures aimed at precluding a "theoretical risk." Some of these measures are eminently reasonable and would have been taken whatever the state of the knowledge. Others invite scrutiny and court the danger of provoking medical product shortages while pursuing a theoretical risk.

One of the most frustrating aspects of trying to deal with prion risk assessment and management has been the lack of rapid and sensitive methods for detection of human and animal prions. The need for such methods is imperative if we are to minimize the risk of prion contamination to public health. Animal bioassays have been widely used and have generated much of the data upon which we currently rely, but they are precluded from routine monitoring because of the prolonged incubation times that are required. Currently available immunoassays, including Western blotting, lack sufficient sensitivity. At best, they can detect PrP^{Sc} in brain from clinically ill persons or animals when levels of PrP^{Sc} are as high or higher than those of PrP^{C}. The key for a diagnostic test is to be able to detect low levels of PrP^{Sc} not only in brain, but also in peripheral tissues or fluids of asymptomatic subjects, in which PrP^{Sc} may represent less than 0.00001% of the total PrP present.

Fortunately, recent advances have been made on both fronts. First, the development of transgenic mice that are highly susceptible to human (Telling et al. 1994, 1995) and bovine (Scott et al. 1997) prions with abbreviated incubation periods have given us precious new tools for the confirmation of prion disease and a better understanding of species barriers, strain characteristics, tissue distribution, and levels of infectivity at

various disease stages. Such transgenic mice should be the models of choice for process validation and prion removal studies in the pharmaceutical industry.

Second, rapid, highly sensitive conformation-dependent immunoassays have recently been developed that take advantage of differences between predominantly α-helical PrP^{C} and PrP^{Sc} which has a high β-sheet content (Chapter 15). Such immunoassays may, for the first time, provide a highly sensitive and extremely rapid alternative test for prion infectivity, which would find practical application in blood donor testing, plasma pool screening, and slaughterhouse and live animal testing, as well as validation of key removal steps in manufacturing processes.

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