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Molecular Biology and Transgenetics of Prion Diseases

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ABSTRACT: Considerable progress has been made deciphering the role of an abnormal isoform of the prion protein (PrP) in scrapie of animals and Gerstmann-Sträussler syndrome (GSS) of humans. Some transgenic (Tg) mouse (Mo) lines that carry and express a Syrian hamster (Ha) PrP gene developed scrapie 75 d after inoculation with Ha prions; non-Tg mice failed to show symptoms after >500 d. Brains of these infected Tg(HaPrP) mice featured protease-resistant $HaPrP^{Sc}$, amyloid plaques characteristic for Ha scrapie, and 10^9 ID_{50} units of Ha-specific prions upon bioassay. Studies on Syrian, Armenian, and Chinese hamsters suggest that the domain of the PrP molecule between codons 100 and 120 controls both the length of the incubation time and the deposition of PrP in amyloid plaques. Ataxic GSS in families shows genetic linkage to a mutation in the PrP gene, leading to the substitution of Leu for Pro at codon 102. Discovery of a point mutation in the Prp gene from humans with GSS established that GSS is unique among human diseases — it is both genetic and infectious. These results have revised thinking about sporadic Creutzfeldt-Jakob disease, suggesting it may arise from a somatic mutation. These findings combined with those from many other studies assert that PrP^{Sc} is a component of the transmissible particle, and the PrP amino acid sequence controls the neuropathology and species specificity of prion infectivity. The precise mechanism of PrP^{Sc} formation remains to be established. Attempts to demonstrate a scrapie-specific nucleic acid within highly purified preparations of prions have been unrewarding to date. Whether transmissible prions are composed only of PrP^{Sc} molecules or do they also contain a second component such as small polynucleotide remains uncertain.

KEY WORDS: scrapie, prion, prion protein (PrP), Creutzfeldt-Jakob disease, Gerstmann-Sträussler Scheinker syndrome.

I. INTRODUCTION

Seven diseases, four in animals and three in humans, are thought to be caused by prions. The animal prion diseases are scrapie, transmissible mink encephalopathy,[1] chronic wasting disease of mule deer and elk,[2] and bovine spongiform encephalopathy.[3,4] The human prion diseases are kuru,[5] Creutzfeldt-Jakob disease (CJD),[6] and Gerstmann-Sträussler-Scheinker syndrome (GSS),[7] which illustrate the transmissible, sporadic, and familial manifestations of these disorders.[8-11] Consistent with some of the observations on natural scrapie noted above, GSS and familial CJD are the only known human diseases that appear to be both inherited and transmissible.[8,12-15] Recent genetic linkage studies provide the best evidence, to date, that GSS is a genetic disorder in which infectious prions are produced.[8] An alternative hypothesis, for which there are few supporting data, is that prions are ubiquitous infectious pathogens causing CJD sporadically across the Earth at a rate of one case per million population and GSS in all individuals carrying a mutation in one allele of their prion protein (PrP) gene.[8,11,12,16,17] Our genetic linkage results with GSS raise the possibility that sporadic CJD arises from a somatic mutation.[8,10,16,18]

The unusual properties of the particle causing scrapie were first appreciated in the 1940s.[19] De-

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velopment of a bioassay for scrapie in mice by Chandler[20] led to the accumulation of additional data supporting the argument that the scrapie agent might not be a virus, bacterium, fungus, or parasite.[21,22] Radiobiological studies offered the most provocative observations suggesting that the scrapie agent is fundamentally different from all known infectious pathogens.[21,22] The unusual features of the scrapie agent led to many proposals concerning its possible molecular structure. Enriching fractions from rodent brain led to identification of a protein component.[23] Prior to demonstrating a protein component within the scrapie agent,[9,10] investigators suggested that the pathogen causing scrapie might be a replicating membrane fragment,[24,25] a viroid,[26] a carbohydrate moiety,[27,28] a protein,[29-32] or even a small virus.[33-36] Terms such as *unconventional virus* and *virino* were introduced to distinguish the scrapie agent from viruses.[27,28] Although the term *unconventional virus* has been used widely, some investigators believe it is counterproductive to adopt such terminology. In surveying the status of research for the decade 1969 to 1978, Ian Pattison[39] recently wrote "...with the causal agent of scrapie still obscure, and virologists as adamant as ever that theirs was the only worthwhile point of view. To explain findings that did not fit with a virus hypothesis, they had rechristened the causal agent an 'unconventional virus'. Use of this ingenious cover-up for uncertainty made 'virus' meaningless — for is not a cottage an unconventional castle?" Recently, unconventional viruses have been suggested to be mineral-protein complexes that multiply by crystallization and have been said to be similar to computer viruses.[40,41]

Virino was defined: "If the recent experimental results of Marsh and Malone are correct in implicating DNA as a necessary component of the infective unit of scrapie, then an appropriate name for this class of agent would be virinos, which (by analogy with neutrinos) are small, immunologically neutral particles with high penetration properties but needing special criteria to detect their presence".[42] Subsequent studies showed that the work of Marsh et al.[43] and Malone et al.[44] could not be confirmed.[45] The most recent definition of virino is "host proteins sequestering the agent genome which may code for no product other than copies of itself."[46]

While several studies raised the possibility that protein might be required for scrapie infectivity,[47-49] convincing data showing that a protein molecule is necessary for infectivity were obtained only after an effective protocol for partial purification of the scrapie agent was developed.[50] Attempts to use the same protocol to demonstrate that the scrapie agent contains a nucleic acid were unsuccessful. Five procedures that modify or hydrolyze nucleic acids failed to inactivate the scrapie agent, yet these procedures were capable of inactivating numerous viruses as well as viroids.[51,52] On the basis of these studies, the term *prion* was introduced in order to distinguish the class of particles causing scrapie from those responsible for viral illnesses.[51]

To avoid prejudging the structure of these infectious particles, prions were defined as "small **pro**teinaceous **in**fectious particles that resist inactivation by procedures which modify nucleic acids,"[51] and three hypothetical structures for the prion were proposed: (1) proteins surrounding a nucleic acid that encodes them (a virus), (2) protein surrounding a small noncoding polynucleotide, and (3) a proteinaceous particle devoid of nucleic acid. Data from many laboratories have established that scrapie is not caused by a virus. Although some investigators have chosen to redefine prions as infectious proteins,[53,54] we have avoided this oversimplification in order not to bias our experimental approaches.[55]

As described below, considerable data support the argument that a protein designated PrP^{Sc} is a major and necessary component of the infectious scrapie particle. Yet, unlike viral proteins, PrP^{Sc} is encoded by a chromosomal gene. Based on these observations, the definition of prions[10] can be extended and stated as "small proteinaceous infectious particles that resist inactivation by procedures modifying nucleic acids and contain an abnormal isoform of a cellular protein which is a major and necessary component."

II. SCRAPIE PRION PROTEIN

Enriching brain fractions for scrapie infectivity led to the discovery of PrP 27-30.[23] Development of a more rapid and economical bioassay[56,57] greatly facilitated purification of the

TABLE 1
Convergence of Experimental Results Arguing that PrP^{Sc} (or PrP 27-30) is a Major and Necessary Component of the Infectious Prion

Biochemistry
- Enriching brain fractions for scrapie infectivity using limited proteolysis, detergent extraction, differential centrifugation, and sedimentation through discontinuous sucrose gradients led to the discovery of PrP 27-30[23,58]
- Prion titers were found to be proportional to the *PrP 27-30 concentration*[59]
- Denaturation, hydrolysis, or selective modification of PrP 27-30 diminished prion titer[59,60]
- Scrapie infectivity and PrP 27-30 copartition into multiple forms — membranes, rods, spheres, DLPCs, and liposomes[23,61-63]

Immunology
- Immunoaffinity purification of scrapie infectivity was accomplished using PrP monoclonal antibodies[64]
- Rabbit antiserum raised against sodium dodecyl sulfate-polyacrylamide gel electrophoresis-purified PrP 27-30 neutralized scrapie infectivity in DLPCs[64,65]

Molecular Genetics
- Genetic linkage of the PrP gene to loci controlling the scrapie incubation time (*Prn-i* and *Sinc*);[66,67] Mice (*Prn-p*^{b}) with long incubation times exhibit amino acid substitutions of codons 108 and 189 of their PrP gene[68]
- Genetic linkage of an amino acid substitution (P→L) at codon 102 of the human PrP *gene with development of* GSS[8,69]
- Amino acid substitution (E→K) at codon 200 of the human PrP gene is associated with development of familial CJD in Libyan Jews and Slovakian Czechs[70-73]

Transgenetics
- Tg(HaPrP) mice incubation times, amyloid plaques, $HaPrP^{Sc}$, and scrapie infectivity characteristic of hamsters after inoculation with Ha prions[74,75]
- Prions synthesized by Tg(HaPrP) mice are species specific and reflect the genetic origin of the prion inoculum[75]
- Tg(GSSMoPrP$_{Leu101}$) mice expressing PrP leucine mutant of GSS develop spontaneous neurologic dysfunction, spongiform change, and gliosis[76]

Neuropathology
- PrP amyloid plaques are specific to prion diseases in animals and humans[77-79]
- Neuropathology in Tg(HaPrP) mice inoculated with Ha prions is similar to hamsters with scrapie, while those inoculated with Mo prions exhibit the neuropathology of mice with scrapie[75]
- Using a dot-blot assay for PrP^{Sc} (or PrP^{CJD}), there is an excellent correlation between prion diseases confirmed neuropathologically and the presence of protease-resistant PrP^{80}

Cell Biology
- Cultured cells infected with scrapie prions produce PrP^{Sc}[81,82]
- PrP^{Sc} is produced more slowly than PrP^{C} and is derived from a protease-sensitive precursor by posttranslational processing[83]
- PrP^{Sc} accumulates primarily inside of cells within cytoplasmic vesicles, while most of the PrP^{C} is transported to the external cell surface[82,84]

hamster scrapie agent.[23,58] PrP 27-30 migrates during SDS/PAGE as a broad band with an apparent M_r of 28,000 to 30,000.

There has been a remarkable convergence of experimental data that support the argument that PrP^{Sc} (or PrP 27-30) is a component of the infectious prion particle (Table 1). Biochemical purification and characterization studies, molecular cloning and genetic investigations, neuropathological studies in animals and humans, as well as immunoaffinity purification and neutralization all provide data supporting the contention that prions are composed largely, if not entirely, of PrP^{Sc} molecules. PrP 27-30 and the scrapie agent copurify[23,59,60,62] when using detergent extractions and limited proteolysis to promote aggregation of prions into amyloid rods, which are collected by centrifugation. PrP 27-30 is the most abundant macromolecule in purified preparations.[62] Immunoaffinity purification of scrapie prions was accomplished with PrP monoclonal antibody (mAb) coupled to Protein A-Sepharose. Dispersion of prions into detergent-lipid-protein complexes (DLPCs) was required before meaningful chromatography could be performed.[61,85] Copurification of scrapie prion infectivity and PrP^{Sc} was found in fractions eluted from a PrP mAb column.[64] Indeed, copurification by two

different procedures supports the argument that the molecular structure and properties of PrP^{Sc} (as well as PrP 27-30) and the infectious prion particles must be extremely similar. The PrP 27-30 concentration is proportional to the prion titer.[59] PrP^{Sc} is absent from normal uninfected animals.[86,87] Procedures that denature, hydrolyze, or selectively modify PrP 27-30 also diminish the prion titer.[59,60] The unusual kinetics of PrP 27-30 hydrolysis catalyzed by proteases were found to correlate with the diminution of prion titer. PrP 27-30 and scrapie infectivity copartition into many different forms — membranes, rods, spheres, DLPCs, and liposomes. These dramatically different physical forms all contain PrP 27-30 and high prion titers.[23,61,62,83,88-90] Rabbit antisera raised against PrP 27-30 purified by SDS/PAGE were found to neutralize scrapie infectivity in DLPCs.[64,65] However, no neutralization of prion infectivity was observed with prions aggregated in amyloid rods.[91,92]

The PrP gene (*Prn-p*) in mice is linked to a gene controlling scrapie incubation times (*Prn-i*).[66,67,93,94] Prolonged incubation periods are a cardinal feature of both scrapie and CJD. The preeminent role of PrP in the pathogenesis of scrapie has been made more compelling by the discovery of a correlation between PrP amino acid sequence and scrapie incubation times.[68] An amino acid substitution in the human PrP gene is linked genetically to the development of GSS.[9,69] GSS and familial CJD are the only known human diseases that are both genetic and infectious.

Recently, transgenic (Tg) mice expressing Syrian golden hamster (Ha) PrP^{C} have been produced.[74,75] The Tg(HaPrP) mice inoculated with Ha prions synthesize both $HaPrP^{Sc}$ and infectious Ha prions; these results assert that PrP^{Sc} is a necessary component of the infectious prion. Furthermore, the Tg(HaPrP) mice exhibit scrapie incubation times and neuropathology characteristic of hamsters. Numerous amyloid plaques containing HaPrP were found in the brains of the Tg(HaPrP) mice in a distribution similar to that found in hamsters.

Scrapie and CJD prion proteins have been identified only in tissues of animals and humans with transmissible neurodegenerative diseases and not in those with other disorders, such as murine systemic amyloidosis and human Alzheimer's disease, anoxic encephalopathy, or non-neurological disorders.[23,57,60,62,78-80,95-97]

Amyloid plaques in prion diseases were first identified in the brains of New Guinea natives dying of kuru.[98] Subsequently, they were reported in scrapie and chronic wasting disease of animals.[99-103] With antisera raised against PrP 27-30 isolated from scrapie-infected Ha brain, central nervous system amyloid plaques in experimental scrapie were shown to be composed of filaments containing PrP.[77,104] Similar studies with brain sections from humans dying of kuru, CJD, and GSS were also found to contain amyloid plaques that react with PrP antibodies.[78,79] To date, amyloid plaques reacting with PrP antibodies have been found only in prion diseases.

Cultured murine neuroblastoma cells have been infected with both scrapie and CJD prions.[81,105,106] Clones of the scrapie-infected cells were found to produce PrP^{Sc}, whereas clones showing no infectivity lacked PrP^{Sc}.[81] In scrapie-infected cultured cells, PrP^{C} is synthesized rapidly ($t_{1/2}$ <0.2 h)[107] and degraded with a $t_{1/2}$ ~5 h, while PrP^{Sc} is synthesized slowly ($t_{1/2}$ ~1-3 h) and does not appear to be degraded.[83] Most PrP^{C} is transported to the external cell surface, where it is bound by a glycosylphosphatidylinositol (GPI) anchor, but can be released by digestion with phsophatidylinositol-specific phospholipase C (PIPLC).[84] In contrast, PrP^{Sc} accumulates largely inside of cells, probably within the Golgi apparatus, and is not released from membranes by PIPLC.[82] The contrasting properties of PrP^{C} and PrP^{Sc} are listed in Table 2.

Many investigators have confirmed the presence of protease-resistant PrP in brains infected with the scrapie or CJD agent.[97,108-110] Although the amino acid sequence of PrP has been confirmed and there is agreement that PrP is glycosylated, some investigators have suggested that PrP 27-30 may not be a component of the scrapie agent.[111-115] One argument revolves around the inability of some investigators to detect PrP mRNA of PrP^{Sc} in spleens of scrapie-infected rodents;[116-118] however, investigators in several laboratories have clearly shown that both PrP mRNA and PrP^{Sc} are present in spleen tissue.[87,119-122] Indeed, recent studies have shown an excellent correlation between the concentra-

TABLE 2
Properties of Cellular and Scrapie PrP Isoforms

	PrP^{c}	PrP^{Sc}
Normal cells[a]	~1-5 μg/g	—
Scrapie-infected cells[a]	~1-5 μg/g	~5-20 μg/g
Purified prions	—	+[b]
Protease resistance	—	+[c]
Amyloid rods	—	+[d]
Subcellular localization	Cell surface	Primarily intracellular
PIPLC release from membranes	—	+
Synthesis ($t_{1/2}$)	<0.2 h	~1-3 h
Degradation ($t_{1/2}$)	~5 h	>>24 h

[a] Expressed as micrograms of protein per gram of brain.
[b] Copurification of PrP^{Sc} and prion infectivity demonstrated by two protocols: (1) detergent extraction, sedimentation protease digestion, and (2) PrP 27-30 monoclonal antibody affinity chromatography.
[c] Limited proteinase K digestion of $HaPrP^{Sc}$ produces PrP 27-30.
[d] After limited proteolysis of PrP^{Sc} to produce PrP 27-30 and detergent extraction, amyloid rods form; except for length, the rods are indistinguishable from amyloid filaments forming plaques.

tion of PrP mRNA in scrapie-infected rodent tissues and prion titer.[121] Another argument centers on experiments that demonstrate the loss of CJD infectivity in fractions following lectin chromatography. Neither denaturation of the PrP^{CJD} nor inactivation of CJD infectivity by immobilization on the lectin column was considered as an explanation.[123] Denaturation of PrP^{Sc} has been demonstrated to be accompanied by a loss of scrapie infectivity.[60,62] Still another argument involves the inability of some investigators to detect PrP 27-30 in Ha brains just prior to clinical signs of scrapie, even though high prion titers are found in this time.[112] Unfortunately, these investigators determined prion titers from homogenates but measured PrP 27-30 in partially purified fractions. We and other investigators have measured both prion titers and PrP 27-30 in crude extracts and have found an excellent correlation throughout the course of infection.[124,125] Indeed, no experimental studies have been reported where fractions with high levels of scrapie infectivity were found to contain less than one PrP^{Sc} (or PrP 27-30) molecule per infectious unit.

III. PURIFICATION OF INFECTIOUS SCRAPIE PARTICLES

Early attempts to purify the scrapie agent from sheep and goat tissues were frustrating and ineffective.[126] Equally frustrating were later studies using scrapie-infected mouse (Mo) brains.[49,126-128] Although these studies demonstrated the association of the scrapie agent with membranes and the resistance of scrapie infectivity to many detergents, no effective solubilization procedure was devised.[128,129] This association of scrapie infectivity with membrane-rich fractions led to an elaborate scheme embodying the structure and replication of the scrapie agent, which was called the *membrane hypothesis*.[24,25] So ineffective were detergents in separating scrapie infectivity from membranes that Hunter and co-workers concluded, "In terms of the membrane hypothesis the agent can be defined as the smallest piece of affected membrane which has full biological activity in the mouse assay system."[130] They also wrote, "Indeed, if the membrane hypothesis proves to be generally valid,

purification of the scrapie agent in the classical sense is impossible. Even if currently available methods of membrane fractionation are improved, the degree of concentration possible will be limited to one or two orders of magnitude by the sheer bulk of scrapie-affected membrane.''[131]

Subsequently, many other investigators tried to purify scrapie infectivity or to identify a subcellular fraction enriched for the agent. In general, the results of these studies were disappointing, and they served only to reinforce the view that scrapie agent purification was ''impossible''. A variety of protocols were used in attempts to purify the scrapie agent. One involved copurification of the scrapie agent and microsomes.[132] Another involved isolation of a ''membrane-free'' fraction after prolonged ultracentrifugation.[133] This fraction contained 1 to 10% of the scrapie agent and was precipitated with ammonium sulfate prior to SDS polyacrylamide gel electrophoresis (PAGE). After electrophoresis, prions were eluted from the gel in order to obtain a further purification.[44] Infectivity of a fraction eluted from a SDS-PAGE was diminished nearly 10^3 ID_{50} units by DNase digestion.[43,134] These results were puzzling since the residual SDS in the gel eluates was probably sufficient to inactivate the DNase.[135] By another procedure, DNase digestion of a fraction containing less than 0.01% of the initial scrapie infectivity from a hydroxyapatite column diminished scrapie agent titer by $10^{1.5}$ ID_{50} units. Although the results of these studies seemed encouraging initially, subsequent work failed to confirm the findings.[45]

Cho and co-workers attempted to isolate the scrapie agent using a variety of detergent extractions after vigorous homogenization.[136] Their protocol produced fractions with numerous particles measuring 14 nm in diameter.[137] After much interest, these particles were subsequently shown to be ferritin.[138]

Using equilibrium sucrose and sodium chloride density gradients, Siakotos and co-workers[139] attempted to purify the scrapie agent from murine brain. They suggested that there was a peak of infectivity at a sucrose density of 1.19 g/cm^3. However, multiple peaks of infectivity were found throughout the gradients, indicating considerable heterogeneity of the agent with respect to density. These results showed that density gradient centrifugation, when applied to crude suspensions of membranous material from brain, is probably not useful in isolating the scrapie agent. Other studies from the laboratory of Gajdusek and Gibbs demonstrated considerable heterogeneity of prions in metrizamide and cesium chloride density gradients.[140] None of the protocols described above led to the identification of a macromolecule within the scrapie prion.

IV. PrP 27-30 POLYMERS

Our own purification studies began with characterization of the sedimentation properties of scrapie infectivity assayed by end-point titrations in mice.[141] These studies established that the scrapie agent could exist as a continuum of sizes, and we postulated that this was due to hydrophobic interactions.[142] In contrast to Hunter and co-workers, we concluded that the monomeric scrapie agent must be quite small in the range predicted by Alper and her colleagues using ionizing radiation to inactivate the infectivity.[22] But like Hunter and colleagues,[129,138] we were unable to identify any ''magical'' detergent that completely dissociated the infectivity into a uniform species that could be readily purified.[56] Instead, we took advantage of (1) the protease resistance of the prion, (2) its ability to withstand repeated detergent extraction, and (3) its propensity to aggregate.[23,45,50]

Our purification studies were greatly accelerated by the development of a reliable incubation time bioassay[57] using Syrian hamsters.[143-145] A protocol was devised using agarose gel electrophoresis in the presence of sodium dodecyl sarcosinate (Sarkosyl) to isolate aggregated fractions from the top of the gel that were enriched for scrapie prions about 10^2-fold with respect to protein.[45,50] Those partially purified fractions could be inactivated by protease digestions, both as a function of protease concentration and the time of digestion; these observations provided the first convincing data for protein within the infectious particle.[50] As noted above, earlier studies had suggested the existence of such a protein.[47-49]

Subsequently, we replaced the electrophoretic step with a discontinuous sucrose gradient,[23]

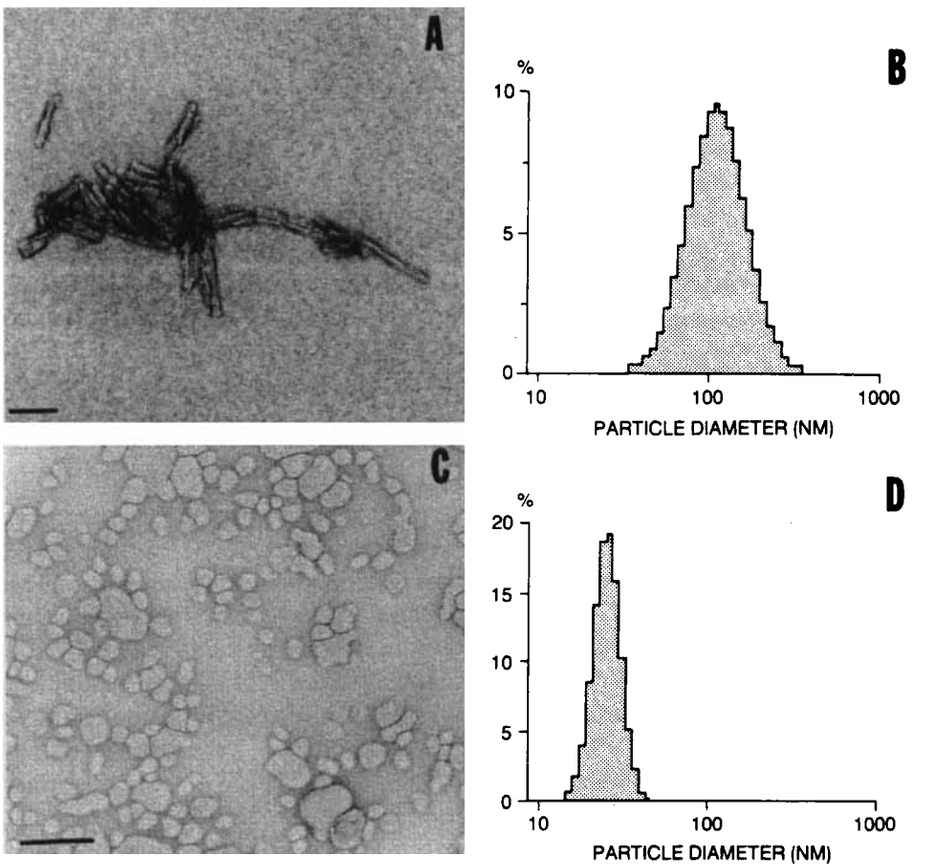


FIGURE 1. Electron microscopy and dynamic light scattering of prion rods and liposomes. (A) Typical prion rods negatively stained with uranyl formate. (Bar = 100 nm.) (B) Light-scattering profile of rods determined with laser particle analyzer (model NY, Coulter Electronics). (C) Negatively stained liposomes containing PrP 27-30. (D) Light-scattering profile of liposomes.

which allowed (1) enrichment of scrapie infectivity about 10^3-fold, (2) identification of the scrapie prion protein (PrP 27-30), and (3) large-scale purification.[62] Rapidly sedimenting fractions contained (1) high prion titers, (2) one protein PrP 27-30, and (3) numerous rod-shaped polymers (see Figure 1A).[23,62] These rods were (1) indistinguishable from purified amyloids ultrastructurally, (2) bound Congo red, and (3) exhibited green-gold birefringence when viewed under polarized light.[62] Diringer and co-workers modified and miniaturized our purification scheme and confirmed the unusual properties of PrP 27-30.[109] Based on a series of unsupported assumptions, these investigators claimed that the prion rods were identical to scrapie-associated fibrils (SAF) and that SAF are composed of PrP 27-30. They did not address the issue that SAF had been repeatedly distinguished from amyloid based on their ultrastructure and tinctorial properties. Despite claims to the contrary, there is no convincing study that reports the composition of SAF; whether or not authentic SAF contain PrP 27-30 or PrP^{Sc} is unknown.[146]

Recent studies have shown that prion rod formation requires both detergent extraction and limited proteolysis.[147] Membrane fractions isolated from scrapie-infected Ha brains in the presence of protease inhibitors failed to show any elongated structures upon detergent extraction. However, removal of the protease inhibitors followed by limited proteolysis led to prion rod formation upon detergent extraction. These results suggest that amyloid filaments in brains of ani-

mals and humans with prion diseases are composed of PrP^{Sc} (or PrP^{CJD}) molecules that are partially hydrolyzed. Our findings showing that limited proteolysis of PrP^{Sc} is required for polymerization into amyloid rods are of interest with respect to Alzheimer's disease, where cerebral amyloids in blood vessels and plaques are composed of a small β peptide derived by limited proteolysis from a much larger precursor protein.[148-150]

V. SOLUBILIZATION OF PRIONS

The availability of purified fractions containing prion rods allowed (1) characterization of many properties of prions, (2) determination of the N-terminal sequence of PrP 27-30 from which numerous molecular biology and genetics studies emerged, (3) study of the covalent structure of PrP 27-30, and (4) production of antibodies against PrP 27-30.

Numerous procedures were exmined for their effectiveness in disrupting the prion rods while preserving infectivity. These included detergents, salts, pH, and sonication, but none were successful.[60,89] On the other hand, denaturation with SDS and boiling, strong chaotropes, acid, and base disrupted prion rod structure but simultaneously reduced scrapie infectivity.[49,128,151]

Manuelidis and co-workers[114] have claimed solubilization of prions after detergent extraction of membranes followed by prolonged sonication and exposure to pH 8.9. Their criterion for solubilization was lack of sedimentation at 20,000 x g for 30 min. The supernatant fraction was reported to contain membrane fragments and CJD infectivity. We doubt that significant solubilization was achieved under these conditions.

Another protocol developed by Semancik, Marsh, and their co-workers involved prolonged ultracentrifugation, which yielded a membrane-free fraction.[133] They estimated the size of the membrane-free scrapie agent to be about 2 S. Unfortunately, the vast majority of scrapie infectivity was bound in the pellet, and it was unclear whether the supernatant infectivity represented contamination from the pellet.

The discovery of conditions for solubilization of nondenatured PrP 27-30 was an important advance.[61] Purified prion rods were mixed with a combination of detergent and phospholipid to form DLPCs upon sonication. The DLPCs could be centrifuged at 170,000 x g for 30 min with most of the PrP 27-30 and scrapie infectivity remaining in the supernatant fraction. Removal of detergent by dialysis produced closed liposomes. Scrapie infectivity generally increased 10- to 100-fold after dissociation of the rods into DLPCs or liposomes. Electron micrographs of the prion rods and liposomes are shown in Figure 1 (A and C). Laser light-scattering studies showed that the DLPCs have a mean diameter of 20 nm, while the rods have an effective diameter >100 nm (Figures 1B and 1D).

No difference in solubilization of prions was seen when Triton X-100, a nonionic detergent, was used in place of the anionic detergents cholate and Sarkosyl. Purified lecithin, which contains mostly phosphatidylcholine (PC), was used in most studies, but other phospholipids, including phosphatidylethanolamine, phosphatidylserine, and dolichol, were assessed for their ability to solubilize PrP 27-30 and to preserve prion titer.[85] No significant differences among the detergents and phospholipids examined were found.

Solubilization of scrapie prion infectivity bound to cellular membranes was accomplished by applying the same technique that was used to solubilize PrP 27-30 in rods. Microsomal membranes from scrapie-infected Ha brain were then extracted with a combination of Sarkosyl and lecithin, followed by DLPC formation upon sonication.[85] Dispersion of PrP^{Sc} into DLPCs preserved scrapie infectivity and allowed PrP^{Sc} to be fractionated and studied as a soluble molecule.[64] Another advantage of this protocol is that it avoids the aggregation of PrP^{Sc} and permits solubilization of prion infectivity directly from the membranes.

VI. IMMUNOAFFINITY PURIFICATION OF PRIONS

Having developed a protocol to disperse prion rods into DLPCs with full retention of infectivity and having adapted this protocol to permit DLPC formation directly from membranes, we sought to utilize this solubilization procedure in the pu-

rification of prion infectivity. We reasoned that PrP mAb-affinity chromatography would co-purify solubilized PrP^{Sc} and prion infectivity in PrP^{Sc} were a component of the infectious scrapie particle. On the other hand, immunoaffinity chromatography should separate PrP^{Sc} from infectivity if the two were unrelated, as suggested by some investigators.[111,114,117,123,152,153]

Microsomes were solubilized by a combination of Sarkosyl (2% w/v) and PC (5 mg/ml).[64] The DLPC formed by this procedure were subjected to ultracentrifugation, and the supernatant was applied to the PrP mAb affinity matrix. mAbs raised against PrP 27-30[154] were cross-linked to protein A-Sepharose in order to minimize the leakage of the antibodies. After an overnight incubation at 4°C, the immunoaffinity matrix was washed with buffers containing increasing concentrations of salt and different detergent, as well as 2% (w/v) PC. The PC was included in order to prevent the aggregation of PrP^{Sc} while bound to the matrix and during elution (Figure 2).

The elution of PrP from the matrix was accomplished with increasing concentrations of alkali. The pH of the eluate was increased progressively from 9.5 to 11.2. At pH 9.5, the first detectable PrP was eluted. Acid was also examined but it was not efficient in eluting the prion protein. Because alkali is known to inactivate scrapie infectivity,[151] samples were titrated immediately after elution to pH 7.

Selected fractions of the immunoaffinity purification procedure were analyzed by SDS-PAGE and bioassay (Figure 3). Most of the unbound PrP was eluted in the void volume; that not all of the PrP molecules are bound to the matrix may result from some PrP molecules possessing a configuration that is unfavorable for binding. No PrP was detected in subsequent washes prior to increasing the pH of the eluate buffer to 9.5. Approximately 10% of the PrP was recovered in the alkaline eluate; this represents ~20% of the PrP in the microsome fraction. An equal amount of PrP was found in the flow-through fraction. At least 20% of the unaccounted for PrP remained bound to the column and was eluted by additional washes with the pH-11.2 buffer. The extent of PrP purification was approximately 5700-fold, and the purity of the PrP, as judged by SDS-PAGE with silver staining, was excellent. Similar results were obtained when the IgG fraction of a polyclonal rabbit PrP 27-30 antiserum was coupled to protein A-Sepharose and used in place of the PrP mAb columns.

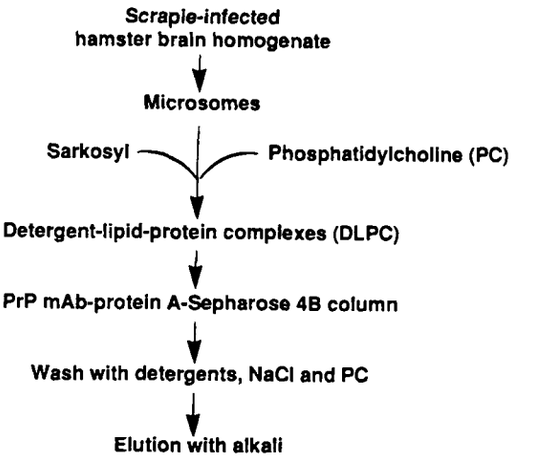


FIGURE 2. Protocol for immunoaffinity purification of PrP^{Sc} and scrapie prion infectivity. (From *Biochemical J.*, 266, 1, 1990. With permission.)

Aliquots of fractions from the PrP immunoaffinity column were inoculated into hamsters for the bioassay of scrapie prion titer. Those fractions that contained PrP^{Sc} also contained scrapie infectivity, while those fractions with no detectable PrP^{Sc} contained either low levels of scrapie prions or none (Figure 3A). Moreover, the amount of PrP^{Sc} recovered from the column was a function of the eluate pH and was roughly proportional to the prion titer. Although the specific infectivity (ID_{50} units/mg protein) increased by 4000-fold during purification, the ID_{50} units per microgram PrP remained constant. We recovered ~4% of the total infectivity in the pH-11.2 eluate; this corresponded to ~10% of infectivity found in the microsome fraction. An additional ~10% of the microsome infectivity was found in the flow-through fraction. What proportion of the unaccounted for infectivity remained bound to column and how much was inactivated during alkali elution is unknown. The imprecision of the animal bioassay[57] for scrapie infectivity complicated attempts to determine accurately the degree of purification and recovery for a particular step.

In order to establish the specificity of the immunoaffinity purification protocol, we con-

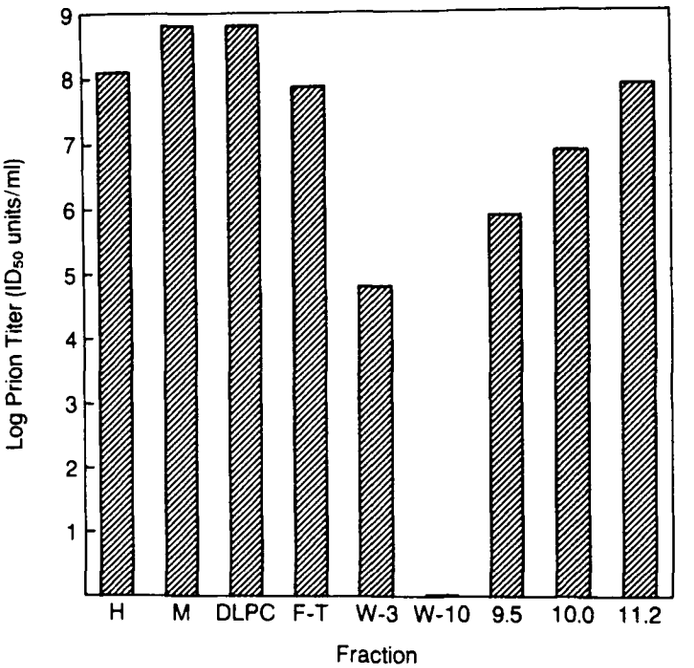


FIGURE 3. Immunoaffinity purification of prions. Microsomes isolated from 15 scrapie-infected Ha brains were solubilized by a combination of Sarkosyl and PC. The resulting DLPCs were incubated overnight at 4°C with (A) PrP mAb (13A5) or (B) polyclonal rabbit HIV-GP120 synthetic peptide antiserum coupled to protein A-Sepharose. The resin was then loaded into a column and washed sequentially with the following buffers: (1) 50 m*M* Tris-HCl (pH 8.2), 0.5 *M* NaCl, 1% (w/v) Sarkosyl, 2 mg/ml PC (4 washes); (2) 50 m*M* Tris-HCl, 0.5 *M* NaCl, 0.5% (w/v) Na deoxycholate, 1 mg/ml PC (3 washes). Each wash was equivalent to one column volume of 15 ml. The PrP^{Sc} bound to the resin was eluted by addition of alkali. Aliquots of selected fractions were analyzed by bioassays for scrapie infectivity. Fractions listed on the horizontal axis are H = homogenate; M = microsome, DLPC = detergent-lipid-protein complex; F-T = flow-through; W-3 = wash 3: W-10 = wash 10; 9.5 = pH 9.5 eluate; 10.0 = pH 10.0 eluate; 11.2 = pH 11.2 eluate. (From *Biochemical J.*, 266, 1, 1990. With permission.)

structed an immunoaffinity matrix using an unrelated antibody raised against a synthetic peptide of GP 120 of HIV. No PrP^{Sc} was eluted from the column, regardless of the buffer pH, establishing that the binding of the PrP^{Sc} to the column was not nonspecific. Aliquots of the fractions from this experiment were bioassayed in hamsters. Prion infectivity was recovered only in the flow through; none was found in fractions eluted with alkali (Figure 3B). Comparing the recoveries of prion infectivity eluted by alkali for the two columns revealed an impressive difference of $\sim 10^8$.

While PrP mAbs were incapable of neutralizing scrapie infectivity, we found that a polyclonal rabbit antiserum raised against SDS-PAGE-purified PrP 27-30 could neutralize scrapie infectivity.[64] Preimmune serum failed to alter prion titer, whereas increasing concentrations of immune serum caused a progressive decrease in titer. The neutralization of scrapie prion infec-

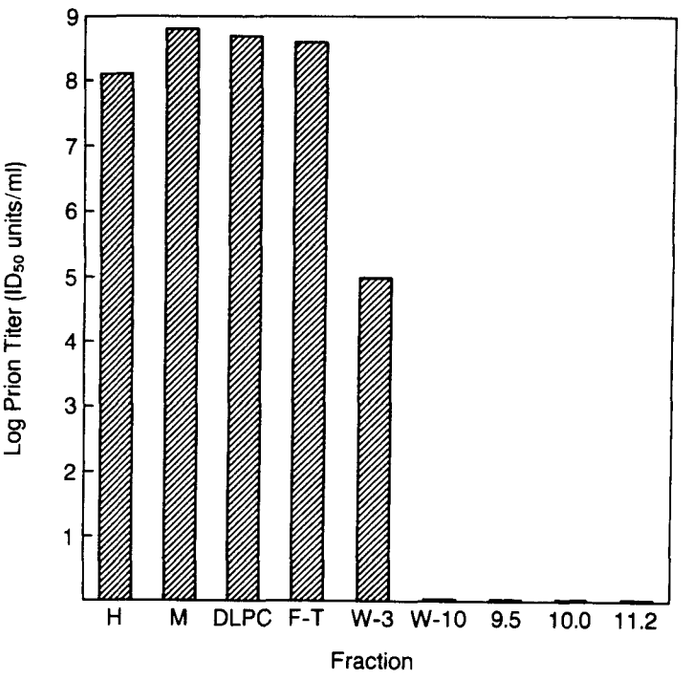


FIGURE 3B

tivity with PrP 27-30 polyclonal antiserum has been independently confirmed recently.[65]

The immunoaffinity purification and neutralization results provide direct immunological and chromatographic demonstrations of a link between PrP^{Sc} and prion infectivity. These experiments could not have been done without functional solubilization of scrapie prions into DLPCs.[61,85]

VII. STRUCTURE AND ORGANIZATION OF PrP GENES

Oligonucleotides corresponding to a portion of the N-terminal amino acid sequence of PrP 27-30[155] were synthesized and used to identify PrP cDNAs.[87,116] Southern blotting with PrP cDNA revealed a single-copy gene with the same restriction patterns as those in normal and scrapie-infected DNA from Ha brains. Unexpectedly, PrP mRNA was found at similar levels in both normal and scrapie-infected brains from hamsters and mice.[87,116,156,157]

Molecular cloning of the HaPrP cDNA led to elucidation of the organization and structure of the HaPrP gene; the entire open reading frame (ORF) or protein coding region is contained within a single exon (Figure 4).[158] The 5′ end of the PrP gene contains multiple G:C-rich initation sites. An intron of ~10 kb separates exons I and II. Six lines of evidence argue that the two PrP isoforms have the same amino acid sequence: (1) no evidence for rearrangement of the PrP gene in scrapie has been found.[87,158] (2) The organization of the PrP gene provides no possibility for alternative splicing within the ORF.[158] (3) Only one PrP mRNA of 2.1 kb has been detected, and its concentration does not change throughout the course of scrapie infection in Ha brain.[87] (4) ~70% of PrP^{Sc} and ~87% of PrP 27-30 have been sequenced by gas-phase protein sequencing.[159] These sequences correspond with the translated genomic DNA sequence. (5) Both scrapie brain Ha and Mo PrP cDNAs have translated ORF sequences that are identical with the corresponding translated genomic sequences.[68,87,158,160] (6) Both PrP isoforms have the same N-terminal amino acid sequence as determined by N-terminal gas-phase sequenc-

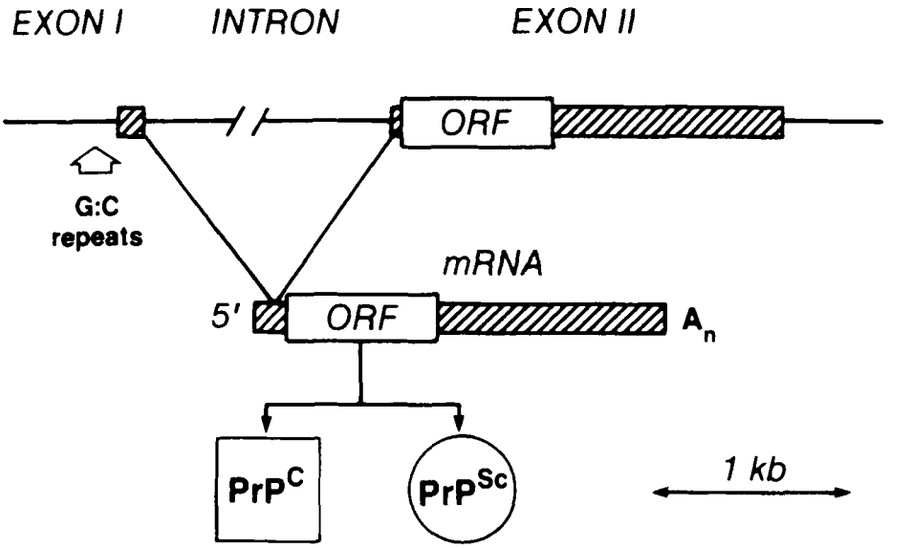


FIGURE 4. Organization and expression of the hamster PrP gene. The features presented were deduced from the nucleotide sequences of PrP genomic and cDNA clones. Untranslated regions of the mRNA are indicated by hatched boxes. An open reading frame or protein coding region is indicated by the open box. The diagonal lines show a splicing event that joins the 5′ leader sequences to the remainder of the coding sequences. (From the *N. Engl. J. Med.*, 317, 1571, 1987. With permission.)

ing.[161] Presumably, the difference in the properties of the two prion proteins is due to a post-translational event;[83,158] though seemingly remote, the possibility that differences in the amino acid sequences of the two PrP isoforms arising through RNA editing must continue to be considered.[162-165]

That PrP genes existed prior to the speciation of mammals is supported by the finding that human and Mo PrP genes are located on homologous chromosomes 20 and 2.[166] The ORF of PrP genes from humans,[167] Syrian hamsters,[158] Chinese hamsters,[168] Armenian hamsters,[168] mice,[68,160] rats,[169] and sheep[170] have been sequenced and all encode prion proteins of approximately 250 amino acids with N-terminal signal peptides (see Figure 5). Whether eukaryotes other than mammals have authentic PrP genes remains to be established.[171] All of the PrP ORFs encode C-terminal hydrophobic peptides, which are presumably removed upon GPI anchor addition, and all contain two concensus sites for Asn-linked glycosylation, as well as two cysteines within the C-terminal half of the molecule.[84,172] All of the ORFs also possess a series of Gly-rich repeats in the N-terminal portion of the PrP molecule.

VIII. TRANSCRIPTION OF PrP mRNA

Although PrP mRNA levels in rodents do not change throughout the course of scrapie infection,[87] recent investigations show that variations in PrP mRNA levels correlate with profound alterations in the length of the scrapie incubation time.[75] In both hamsters and mice, the highest PrP mRNA levels are found in brain;[87,116] the highest titers of prions are also found in brain.[144,173] *In situ* hybridization of normal and scrapie-infected Ha brains has shown that neurons contain the highest levels of PrP mRNA (~50 copies per cell); glial cells contain <3 mRNA copies per cell.[156] Recent *in situ* hybridization studies argue that PrP gene expression may be less selective.[174]

Although PrP mRNA levels are constant in adult rodents,[87] the expression of the PrP gene is developmentally regulated.[175] During the first 20 d after birth, both PrP and β-amyloid precursor

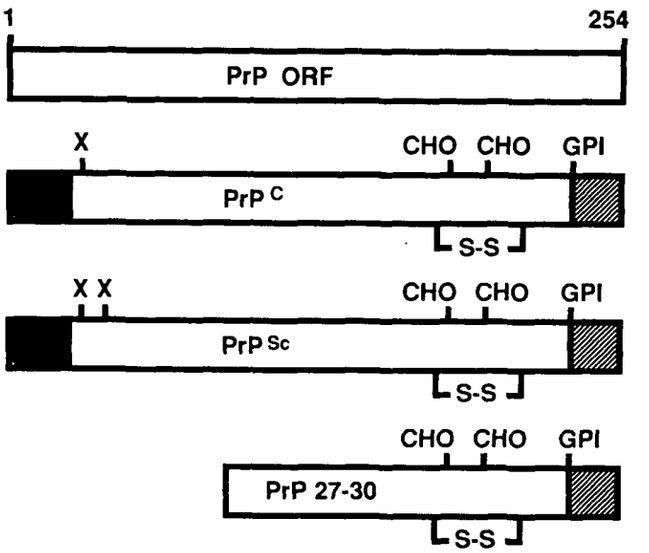


FIGURE 5. Prion protein structure. Hamster PrP gene encodes a protein of 254 amino acids. The N-terminal signal peptide of 22 amino acids (stippled pattern) is cleaved during maturation of PrP^{C} and PrP^{Sc}. Modified Arg residues (X) at codons 25 and 37 have been identified, as well as Asn-linked oligosaccharides (CHO) attached at codons 181 and 197 within a loop formed by a disulfide bond that joins Cys at codons 179 and 214. Upon removal of a C-terminal hydrophobic peptide of 23 amino acids (diagonal line pattern), a glycosyl phosphatidylinositol (GPI) anchor is added. PrP 27-30 is generated by limited digestion with proteinase K, during which the N-terminal 67 amino acids are hydrolyzed. (From *Annu. Rev. Microbiol.*, 43, 345, 1989. With permission.)

protein (β-APP) mRNAs increase in the neonatal Ha brain at different rates in various regions of the brain. In the septum, the kinetics of increase in PrP and β-APP mRNAs paralleled those of choline acetyltransferase. The steady-state level of both PrP and β-APP mRNAs, as well as the synthesis of choline acetyltransferase, are stimulated by intraventricular injections of nerve growth factor.[175]

IX. FUNCTION OF CELLULAR PRION PROTEIN

While the function of PrP^{C} is unknown, several observations suggest that it might be involved in cell recognition. PrP^{C} is bound to the external surface of cells by a GPI anchor.[84] The levels of PrP^{C} are highly regulated in the development of Ha brain and can be stimulated by nerve growth factor.[175,176] The Asn-linked oligosaccharides of PrP^{Sc}, and presumably PrP^{C}, contain the X-antigenic determinant [Galβ 1 → 4 (Fuc 1 → 3) GlcNAc] associated with cell surface molecules involved in recognition.[177] Recent studies on a protein exhibiting acetylcholine receptor-inducing activity (ARIA) have shown that a cognate cDNA encoding chicken ARIA protein possesses ~32% homology with MoPrP.[178] Whether $MoPrP^{C}$ functions like chicken ARIA remains to be established. Interestingly, the synthesis of PrP^{C} and choline acetyltransferase are coordinately controlled in the developing Ha brain.[175]

In scrapie, PrP^{C} may prevent an immune response to PrP^{Sc} and thus explain one of the most puzzling questions in research on scrapie and CJD. PrP^{C} may account for the lack of an immune

response to a lethal "slow infection" by rendering the host tolerant to the abnormal isoform (PrP^{Sc}).[51,87] The difficulties in raising antibodies to PrP 27-30 may be at least partly due to tolerance induced by PrP^{C}.[154]

Transgenic (Tg) mouse studies have given some insight into the replication of prions and the conversion of PrP^{C} or a precursor into PrP^{Sc}.[75] The results of these studies argue that PrP^{Sc} in the scrapie inoculum binds to homologous PrP^{C} to form a heterodimer that functions as a replication intermediate. Subsequently, the heterodimer is converted to a homodimer corresponding to the ionizing radiation target size of scrapie infectivity.[179] Dissociation of PrP^{Sc} homodimers to form PrP^{C}/PrP^{Sc} heterodimers, followed by conversion to homodimers, provides a mechanism for exponential replication of prions in accord with the observed kinetics of this process.[87,144]

Recent investigations using ligand blots with [^{125}I]-PrP 27-30 have identified several putative *P*rP *li*gands or *Plis* of M_r from 45, 66 and 110 kDa.[180] All of the Plis are acidic proteins with pIs between 4.5 and 5.0. The 45-kDa Pli (Pli 45) increased during scrapie infection and was found exclusively in brain; other Plis of higher M_r were found in systemic tissues. Amino acid sequencing and immunoblotting demonstrated that Pli 45 is glial fibrillary acidic protein (GFAP). Whether PrP^{Sc} binds to GFAP in the brains of animals with prion diseases remains to be established. It is unknown if the Plis detected by ligand blots participate in either the conversion of a PrP precursor into PrP^{Sc} or the initiation of scrapie infection. If the Plis identified by PrP 27-30 binding also bind PrP^{C}, then characterization of these molecules may offer some insight into the cellular function of PrP^{C}.

X. POSTTRANSLATIONAL MODIFICATIONS OF PRION PROTEINS

There is evidence for at least eight posttranslational modifications of PrP (Figure 5). The first modification to be identified was glycosylation; PrP 27-30 is a sialoglycoprotein.[155] All translated PrP gene ORFs sequenced to date contain two consensus sites for Asn-linked glycosylation. In Syrian HaPrP, these consensus sites are codons 181 and 197.[87,158] Both the cellular and scrapie PrP isoforms are resistant to digestion by endoglycosidase H but possess N-linked oligosaccharides that can be removed by digestion with peptide:N-glycosidase F.[181] Hydrazinolysis liberated ~2 mol of oligosaccharides per mole of PrP 27-30.[177] The released oligosaccharides were found to be a mixture of bi-, tri-, and tetra-antennary complex-type sugar chains with Man 1 → 6 (GlcNAcβ1 → 4) (Man 1 → 3) Manβ1 → 4GlcNAcβ1 → 4 (Fuc 1 → 6) GlcNAc as their cores. Variation is produced by combinations of the following oligosaccharides: Galβ1 → 4GlcNAcβ1 →, Galβ1 → 4 (Fuc 1 → 3) GlcNAcβ1 →, GlcNAcβ1 →, Neu5Ac → 2 → 3GAlβ1 → 4GlcNAcβ1, and Neu5Ac → 2 → 6Galβ1 → 4GlcNAcβ1 in their outer chains. Whether the structures of the Asn-linked oligosaccharides attached to PrP^{C} differ significantly from those of PrP^{Sc} remains to be determined.

Both mature PrP isoforms have two cysteine residues at codons 179 and 214 that are linked by an intramolecular disulfide bond.[161] This disulfide bond forms a loop that contains both Asn-linked oligosaccharides as well as a variant amino acid at codon 189 found in long incubation period mice (*Prn-p*^{b}).

A 22-amino-acid signal peptide is found at the N-terminus of PrP. Like all other signal peptides, it contains a hydrophobic core and a consensus cleavage site.[118,158,182] Cell-free translation studies have demonstrated the cleavage of the PrP signal peptide[183] and N-terminal amino acid sequencing of PrP^{C} and PrP^{Sc} confirm the cleavage.[110,161,184]

The C-terminal hydrophobic segment of 23 amino acids is removed from both PrP isoforms during maturation and a GPI anchor added.[63,84,185] Endopeptidase digestions combined with amino acid analysis and mass spectrometric studies have shown that the GPI anchor of PrP^{Sc} is attached to Ser_{231},[63,185] as previously predicted.[186] 10 to 15% of PrP 27-30 is truncated at the C-terminus ending at codon 228, which specifies a Gly.[185] Whether a portion of PrP^{C} is also truncated remains to be established.

Arg_{25} and Arg_{37} in PrP^{Sc} contain an unknown modification that prevents their detection by gas-phase sequencing using Edman degrada-

tion.[161,187] Arg_{25} of PrP^C contains a similar modification,[161] but whether Arg_{37} also contains this modification is uncertain. Arg_{48} of PrP^{Sc} does not contain the modification, since this Arg residue was detected.

XI. SEARCH FOR A SCRAPIE-SPECIFIC NUCLEIC ACID

The transfer of scrapie prion infectivity from rods to liposomes provided a new method by which we could search for a hidden or cryptic nucleic acid within the prion. Treatment of DLPCs as well as liposomes with nucleases or Zn^{2+} failed to alter scrapie infectivity.[61] In control experiments, nucleic acids were added to DLPCs at a concentration of one molecule per infectious unit. Under the conditions of our experiments, nucleases or Zn^{2+} degraded the exogeneously added DNA or RNA molecules to <1% of their initial concentration. In the experiments described above, nucleases were added after the DLPCs and liposomes were formed. In subsequent studies, the nucleases were present during the formation of the DLPCs.[85] As before, no change in prion titer was observed after prolonged incubations.

Irradiation at 254 nm of the DLPCs produced an inactivation curve that was virtually identical to that observed for the rods in the experiment reported here and earlier studies (Figure 6A).[85] The kinetics of inactivation of scrapie infectivity by ultraviolet irradiation yielded exponential survival curves characteristic of a single-hit process. From these ultraviolet inactivation curves, we estimated that the D_{37} values for the DLPCs and rods were 17 and 24 kJ/m^2, respectively. In another study, we reported a D_{37} value of 17 to 22 kJ/m^2 for purified prion rods.[188] The resistance of scrapie infectivity to inactivation by irradiation at 254 nm suggests that if prions contain a nucleic acid, it will be ~5 bases in length for a single-stranded molecule or 30 to 45 bp for a double-stranded molecule.[188] The D_{37} values for purified prion rods and DLPCs are in good agreement with values reported 2 decades earlier for murine scrapie agent in brain homogenates.[21]

Prolonged exposure of the rods and DLPCs to proteolytic digestion resulted in a significant decrease in scrapie infectivity.[85] Inactivation of prion rods and DLPCs by proteinase K was both a function of the time of digestion and the concentration of protease, as reported earlier for both partially purified[50] and more extensively purified samples.[59]

One explanation for the protease resistance of PrP 27-30 and prion infectivity has been the aggregation of PrP 27-30 into amyloid rods.[62] Dissociation of PrP 27-30 in DLPCs allowed us to test this hypothesis. The kinetics of scrapie infectivity loss as a function of proteinase K digestion were similar for both the DLPCs and rods.[85] After 24 h of digestion at 37°C with 100 μg/ml of proteinase K, the titer decreased by $>10^4$ ID_{50} units/ml.

These experiments, as well as studies on immunoaffinity-purified PrP^{Sc}, support the argument that resistance of PrP 27-30 to enzyme-catalyzed proteolysis is an intrinsic property of the scrapie PrP isoform and not merely a consequence of its polymerization into amyloid rods.

Early radiobiological investigations on the scrapie agent suggested that it might have a M_r of <150,000 Da and that it might not possess a nucleic acid.[21,22] While the results of these studies were often quoted, their implications have been frequently ignored and more recently their interpretation has been seriously questioned.[33,190-192] Prion rods and liposomes were irradiated with increasing doses up to 400 Mrad. The inactivation curves (Figure 6B) were virtually identical to those seen with microsomal fractions isolated from scrapie-infected Ha brain before and after extraction with detergent.[179] The survival curves for all forms were exponential over a range of more than 10^5 infectious units, which is characteristic of a single-hit process.

Results from 14 separate experiments with various types of preparations, each irradiated as a frozen solution, yielded a mean target size for the scrapie agent of 55,000 ± 9000 Da.[179] The early radiation studies,[22,193-195] corrected for temperature effects,[196,197] yielded target sizes between 60,000 and 150,000 Da. An equally important finding of our studies is that the target size for scrapie infectivity appears to be independent for the prion form.[179] Samples containing aggregates in the form of amyloid rods yielded an inactivation curve indistinguishable from those

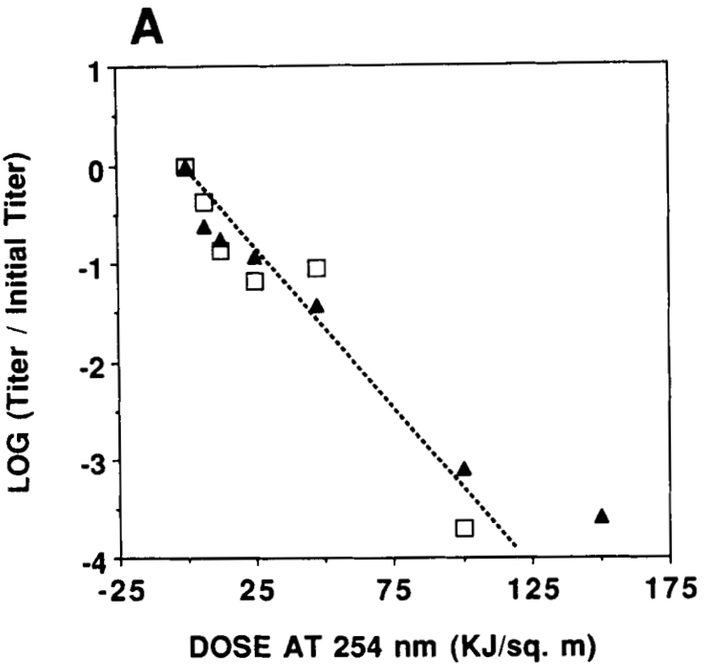
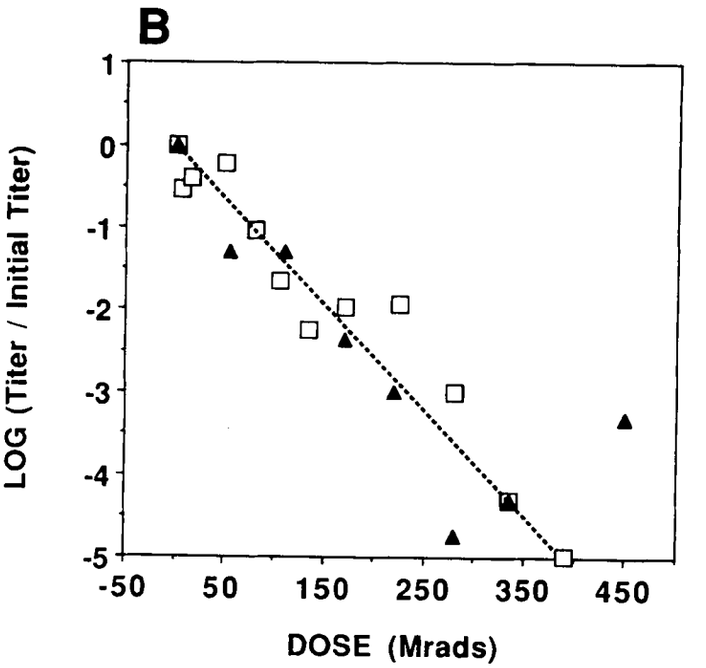


FIGURE 6. Inactivation by scrapie prion rods and liposomes by irradiation. Amyloid rods (□) containing ~30 μg/ml of PrP 27-30 or dispersed PrP 27-30 (▲) in 20 mg/ml PC as DLPCs were prepared as described previously.[61,62,86,188] After irradiation studies, samples were inoculated intracerebrally into Syrian hamsters for scrapie bioassay.[57] The log (titer of irradiated samples/titer of unirradiated samples) was plotted as a function of the irradiation dose. Curves were visually fitted. (A) Ultraviolet irradiation of prions. Fractions were irradiated with ultraviolet light with a 5 GE germicidal lamp for increasing periods of time. (B) Ionizing radiation of prions. All samples were frozen in ethanol dry ice baths prior to storage at −70°C. The samples were irradiated with 13 MV electrons at −135°C.[188,189] Controls receiving no irradiation were subjected to the same protocol. (From *Biochem. J.*, 266, 1, 1990. With permission.)

curves observed with microsomes, crude brain homogenates, or liposomes. Clearly the various forms of the scrapie prion do not influence its ionizing radiation target size.

Attempts to identify a scrapie-specific nucleic acid by molecular cloning have been unsuccessful, to date.[153,198-202] Despite considerable effort, no scrapie-specific nucleic acid has been identified.

Physicochemical studies of purified prion preparations have revealed a paucity of nucleic acid molecules. Purified sucrose gradient fractions containing prion rods were treated with Zn^{2+} and digested with nucleases to diminish contaminating cellular nucleic acids. The nucleic acids in phenol extracts were measured by silver staining after PAGE. No nucleic acid of >20 bases in length that is unique to scrapie-infected fractions could be identified at a particle-to-infectivity (P/I) ratio of 1 or greater. To explore the possibility that a scrapie-specific nucleic acid might be heterogeneous in length, the technique of return refocusing gel electrophoresis (RRGE) was developed. Nucleic acids of variable length in our purified fractions were reduced >90% by Bal 31 exonuclease digestion after dispersion of



the purified prion rods in DLPCs. Under these conditions, no change in scrapie prion titers was observed.[203]

XII. GENETIC LINKAGE AND TRANSGENETIC INVESTIGATIONS

The results of molecular genetic studies of scrapie in mice and GSS in humans have important implications for understanding the molecular structure of infectious prion particles. These studies constrain the possible structures of prions and demonstrate that the properties of prions are determined largely, if not entirely, by the structure of PrP^{Sc}.

Genetic linkage and PrP gene sequencing studies in humans and mice suggest that amino acid substitutions in PrP may modulate the development of prion diseases (Figure 7). One study demonstrated that GSS is an autosomal dominant disorder and that a Pro → Leu substitution at codon 102 of the PrP gene is linked to the development of GSS.[8,69] Earlier investigations showed genetic linkage between an incubation time gene (*Prn-i*) and the PrP gene in inbred mice.[66] Mice with long incubation times have PrP genes ($Prn\text{-}p^b$) with Leu → Phe and Thr → Val substitutions at codons 108 and 189, respectively.[68] Several discordant mice in genetic crosses raised the possibility that the PrP gene and *Prn-i* are separated but tightly linked,[93] but recombinant capture studies[204] and Tg Mo studies described below suggest the congruency of the *Prn-p* and *Prn-i* genes. Differences in murine PrP sequences produce distinct isolates of prions that exhibit significantly different scrapie incubation times in a single host.[205]

Investigations with Tg mice expressing foreign PrP genes have brought a wealth of new knowledge about prions.[74,75] Tg mice expressing both Syrian Ha and MoPrP genes, which encode proteins differing at 16 residues out of 254, were used to probe the mechanism of scrapie prion replication. Four Tg lines expressing HaPrP exhibited distinct incubation times ranging from 48 to 277 d after Ha prion incubation, which were inversely correlated with the steady-state levels of HaPrP mRNA and $HaPrP^C$. Bioassays of brain extracts from two scrapie-infected Tg lines showed that the prion inoculum dictates which prions are synthesized *de novo*, even though the cells express both PrP genes. Tg mice inoculated with Ha prions had $\sim 10^6$ ID_{50} units of Mo prions

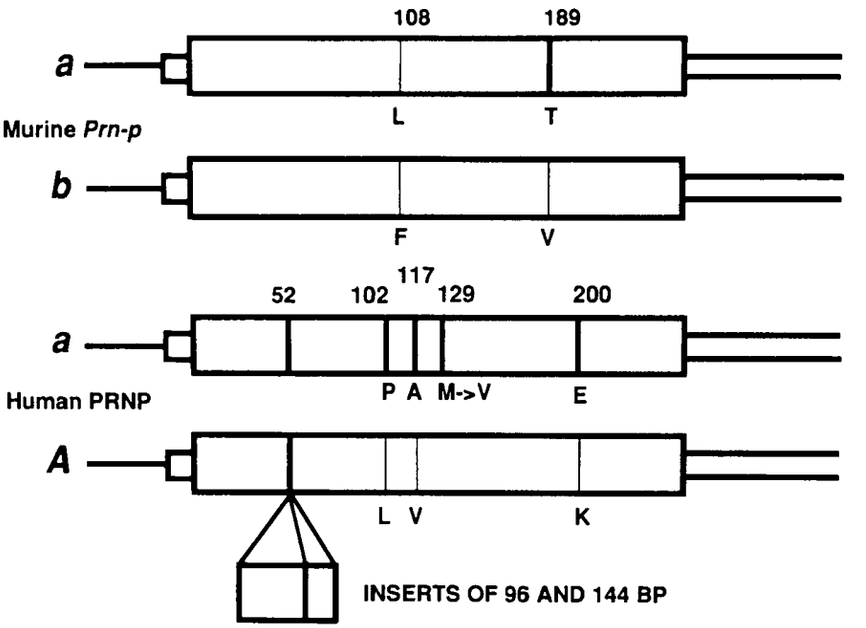


FIGURE 7. Genetic variations in the PrP gene linked to altered susceptibility phenotypes for prion diseases. Upper panel: a and b alleles of murine PrP gene (*Prn-p*) from NZW and I/Ln mice, respectively. Lower panel: a and A alleles of human PrP gene; the A allele is a composite of point mutations and insertions found in patients with Gerstmann-Sträussler syndrome or familial Creutzfeldt-Jakob disease.

and <10 units of Ha prions. Consistent with the analysis of prion synthesis, Tg mice inoculated with Ha prions exhibited neuropathologic changes characteristic of hamsters with scrapie, while Mo prions produced changes similar to those in non-Tg mice with scrapie. Our results argue that species specificity of scrapie prions resides in the primary structure of PrP and formation of infectious prions is initiated by a species-specific interaction between PrP^{Sc} in the inoculum and homologous PrP^{C}. These studies are the first demonstration of the synthesis of infectious scrapie prions programmed by a recombinant DNA molecule. Indeed, the use of Tg mice expressing foreign PrP genes should create a renaissance in prion research.

It now seems feasible to determine first the domains of PrP and later the precise amino acids that are required for the production of infectious scrapie prions, modulate the susceptibility of animals to prion diseases and influence scrapie incubation times. Deciphering how specific amino acid changes in PrP affect both the initiation of the disease and the course of CNS degeneration may have wider implications toward understanding a variety of neurodegenerative diseases, as noted above.

XIII. PRION DIVERSITY AND REPLICATION

Some investigators continue to argue that the scrapie and CJD agents must possess intrinsic nucleic acids that direct the synthesis of progeny, infectious particles.[54,111,114,115,123,153,202,206] Other workers, although suggesting that the agents may be devoid of nucleic acid, prefer to label them *viruses*.[40,65] Still other investigators argue that "strains" or scrapie agent demand a nucleic acid within the infectious particle.[46,207,208] Changes in the properties of scrapie-agent "strains" have

been reported, but the evidence that these changes are due to mutations within a putative scrapie-specific nucleic acid is not convincing.

To investigate the properties of prion inocula produced in mice expressing different prion proteins, scrapie infectivity was passaged in NZW (*Prn-p*^{a}), Swiss (*Prn-p*^{a}), I/Ln (*Prn-p*^{b}), and (NZW × I/Ln)F1 mice (Figure 7). NZW mice inoculated with prions containing PrP^{Sc}-A molecules exhibited significantly shorter incubation times than those inoculated with PrP^{Sc}-B prions.[205] Conversely, I/Ln mice inoculated with PrP^{Sc}-B prions exhibited a reduction of >90 d in their incubation times compared to those inoculated with PrP^{Sc}-A prions. Incubation times of I/Ln mice inoculated with isologous isolate were equal to or slightly less than those of F1 mice. In these studies, prolongation of scrapie incubation time by the I/Ln *Prn-i* allele behaves as a fully dominant trait for PrP^{Sc}-B prions, in contrast with apparent codominance following inoculation with PrP^{Sc}-A prions or PrP^{Sc}-A/B prions from F1 mice. These observations may begin to clarify the ''codominance'' observed by other investigators with a variety of scrapie ''strains'' in VM and IM mice (*Prn-p*^{b}).[38,207,208] Scrapie incubation times for (NZW × I/Ln)F1 mice were constant when inoculated with brain extracts of prions containing PrP^{Sc}-A, PrP^{Sc}-B, or PrP^{Sc}-A/B.

The foregoing results of experiments with prion isolates derived from *Prn-p*^{a} and *Prn-p*^{b} mice appear to reflect an inbred Mo strain or PrP allotype barrier defined by the *Prn* genotype.[205] The *Prn-p*^{a}/*Prn-p*^{b} allotype barrier may be analogous to the ''species barrier'' described by Pattison,[209] where a marked prolongation of the scrapie incubation time is observed upon interspecies passage, but the minimum incubation time is achieved upon a single intraspecies passage.[74,75,210] Mice inoculated with sheep scrapie isolate or human CJD have an exceptionally long incubation period, but in subsequent passage the incubation period rapidly shortens and becomes fixed.[38,211,212] Although Dickinson and coworkers[208,213] state that there was a gradual shortening after crossing a mouse strain or species barrier, their published data suggest that almost all of the change occurred in a single passage. Because all species susceptible to scrapie examined, to date encode distinct prion proteins,[68,158,160,167-170] we hypothesized that the PrP genotype may be responsible for the ''species barrier''.[18] In support of this hypothesis are studies with Tg(HaPrP) mice showing that the HaPrP transgene renders these mice susceptible to Ha prions.[74,75] In contrast, a few non-Tg mice inoculated with Ha prions develop scrapie in a stochastic process with incubation times >500 d.

The results of the studies in NZW, Swiss, and I/Ln mice argue that the prion gene complex (*Prn*) can exert two distinct influences on the scrapie incubation period. First, the *Prn-i* gene has a profound effect on the intrinsic length of the incubation period. Regardless of the source of the inoculum in our series of experiments, *Prn*^{b} acts to cause a dramatic prolongation of the interval between inoculation and illness, perhaps by controlling the rate of prion accumulation and consequent formation of pathological lesions. Second, the PrP^{Sc} allotype in the inoculum may influence the initiation of the ''infectious process'' through binding, entry, or some as yet undefined mechanism.

One interpretation of experiments showing that isologous prions cause scrapie more rapidly than allogeneic prions is that *Prn*^{a} and *Prn*^{b} mice select, from a mixture, different strains of prions that carry scrapie-specific nucleic acids. Dickinson, Kimberlin, and their co-workers have argued that the isolation of numerous ''strains'' of scrapie agent is sufficient evidence for the existence of a host-independent genome.[46,207,208,213] While the experiments described above do not address the basis for distinguishable behavior of some scrapie isolates upon repeated passage in the same host, they should force a careful reevaluation of the incubation time data used in defining the ''scrapie strains''. An alternative interpretation of the experiments demonstrating that isologous prions cause disease more rapidly than heterologous prions is that distinct prion isolates derive their different properties from different prion proteins (PrP^{Sc}-A and PrP^{Sc}-B). All of the data described above support this hypothesis, and there are no published experiments that militate it.

One possible explanation for the influence of PrP^{Sc} allotype is that PrP^{C} in concert with other cellular molecules forms a complex with PrP^{Sc} at some step during the initiation of scrapie in-

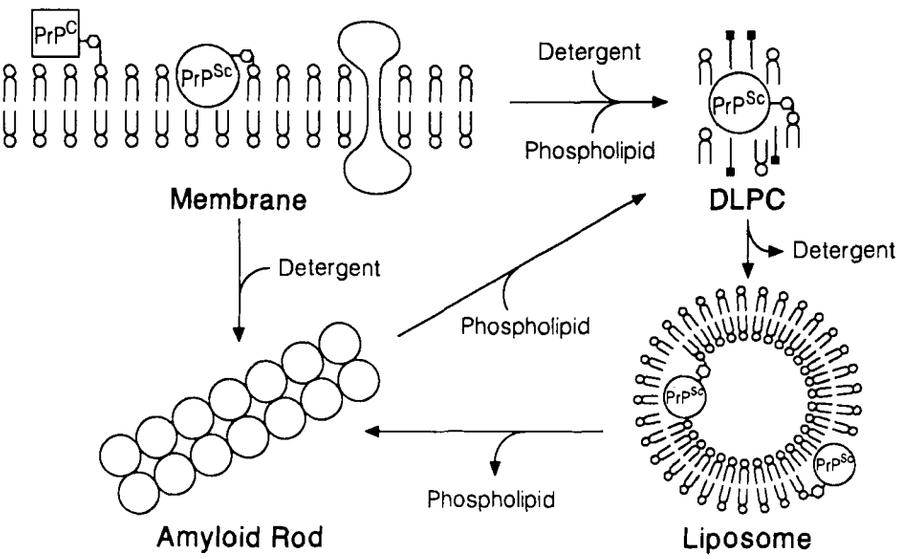


FIGURE 8. Interconversion of multiple prion forms. PrP^{Sc} is a membrane-bound protein that upon limited proteolysis generates PrP 27-30. Detergent extraction of membrane-bound PrP 27-30 produces amyloid rods that can be dispersed into DLPCs by the addition of phospholipids. DLPCs can be formed directly from membranes by the addition of detergent and phospholipid. Removal of detergents from DLPCs produces closed liposomes. Removal of phospholipid from liposomes by organic solvent extraction ($CHCl_3$:methanol) regenerated the rods. All of the prion forms shown here possess high levels of scrapie infectivity.[85] (From *Annu. Rev. Microbiol.*, 43, 345, 1989. With permission.)

fection. The binding of isologous prions in such a model would be more efficient than that of allogeneic prions. The results of transgenetic studies described above support this hypothesis. Tg(HaPrP) mice inoculated with Ha prions produced Ha prion infectivity and $HaPrP^{Sc}$. Those Tg mice inoculated with Mo prions synthesized only Mo prions and $MoPrP^{Sc}$. These results argue that PrP^{Sc} in the inoculum binds more efficiently to homologous PrP^{C}, leading to the *de novo* synthesis of homologous PrP^{Sc} molecules and scrapie infectivity. Binding of PrP^{Sc} to heterologous PrP^{C} is an inefficient process that does not lead to the synthesis of heterologous prions.

While our findings give new insight into the process of prion replication and argue persuasively that prions are not viruses, they do not resolve some issues involving different "strains" or isolates in an isologous host. While prion isolates with different incubation times in the same inbred host are more compelling as bona fide "strains", there are no biochemical or fluctuation test[214] data to equate the genesis of these scrapie prion isolates with nucleic acid mutation. Clearly, the molecular basis for genesis of scrapie "strains" is an unresolved issue.

XIV. MULTIPLE PHYSICAL FORMS OF PRIONS

Numerous studies have emphasized the association of scrapie infectivity with membranes.[48,127] Detergent extraction of membrane-containing fractions led to the production of infectious particles with a variety of sizes.[142] It is now known that prions may exist in multiple forms, all exhibiting high levels of infectivity (Figure 8). Interconversion of the multiple forms of prions depends upon the relative levels of protein, detergent, and lipid.[85] Rods measuring 10 to 20 nm in diameter and 100 to 200 nm in length (Figure 9A) were found in purified preparations of scrapie prions.[23,62,88,155] Although we reported previously that the rods were generated by detergent extraction of microsomal membranes from

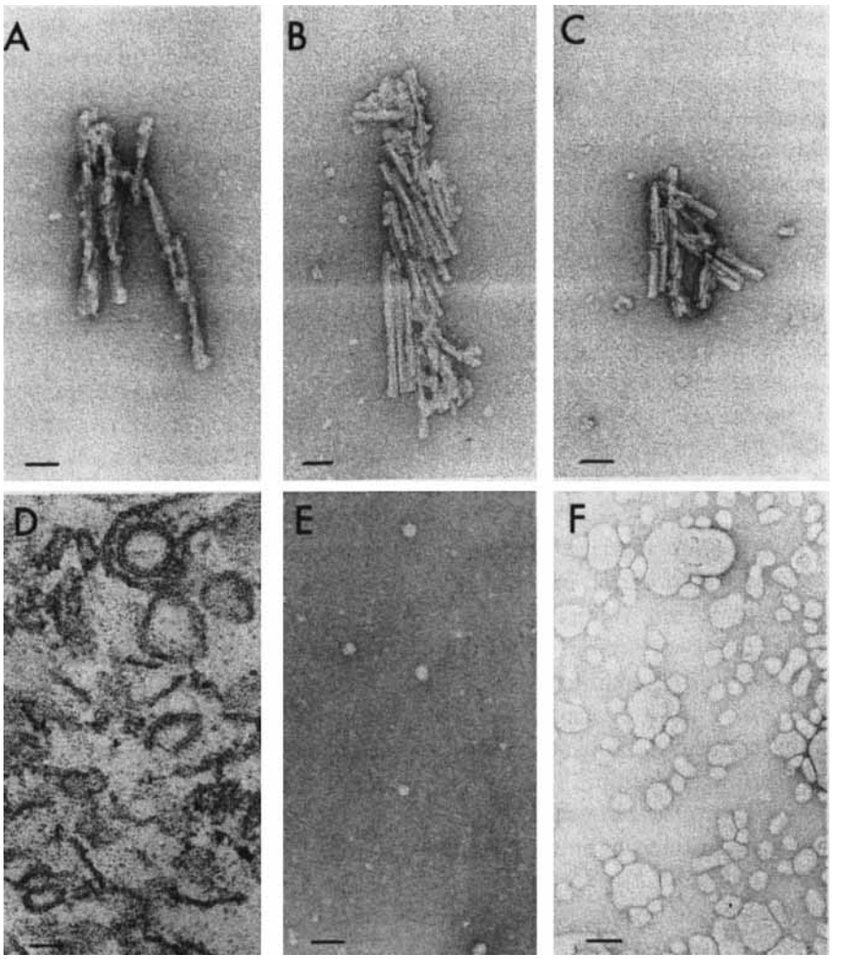


FIGURE 9. Ultrastructure of the multiple forms of hamster scrapie and human Creutzfeldt-Jakob disease prions. Panel A shows purified prion amyloid rods, which are generated upon detergent extraction of membranes from scrapie-infected brain. Panels B and C show human Creutzfeldt-Jakob disease prion rods in purified preparations from a patient who had a cerebellar syndrome on initial evaluation and later had a profound dementia (B), and from a patient who had a typical, rapidly progressive dementing illness of initial evaluation (C). Panel D shows microsomal membranes containing prions. Panel E shows spheres generated from extensive sonication of rods and isolated by sedimentation through a sucrose gradient, and Panel F shows infectious prion liposomes. All structures were negatively stained with uranyl formate and viewed in a JEOL 100B electron microscopy at 80 kev. Bar = 50 nm. (From *N. Engl. J. Med.*, 317, 1571, 1987. With permission.)

scrapie-infected brains (Figure 9D),[90] we now know that both detergent extraction and limited proteolysis are required.[147] Dispersion of the rods into DLPC was accomplished by the addition of nondenaturing detergent and phospholipid. Removal of detergent from the DLPC resulted in the formation of closed liposomes, and the subsequent removal of the phospholipid recreated the rods with full retention of scrapie prion infectivity. Dispersion of microsomal membranes in DLPC directly bypassing rod formation has also been accomplished.[64,85]

The rods that we observed in an earlier study presumably resulted from the aggregation of PrP

27-30 molecules that were produced by endogenous proteases.[90,147] While most of the PrP immunoreactive protein in these detergent-extracted microsomes migrated on Western blots with a M_r of 33 to 35 kDa, some PrP molecules of M_r 27 to 30 kDa were also detected. In reports on the N-terminal sequence of Ha and MoPrP^{Sc}, other investigators stated that their purified preparations contained numerous rod-like structures (inappropriately designated scrapie-associated fibrils, see below).[110,187] Presumably, the rods in these fractions were generated from partially hydrolyzed PrP^{Sc} molecules that were not sequenced. These investigators dissociated the rods by denaturation in SDS prior to isolating PrP^{Sc} by gel filtration.

Sonication of the prion rods reduced their mean length to 60 nm and produced many spherical particles, without altering infectivity titers (Figure 9E).[215] The rods were found to be dissociated under nondenaturing conditions, with a combination of cholate and phosphatidylcholine.[61] The resulting liposomes frequently showed a 10- to 50-fold increase in scrapie infectivity. Electron microscopy showed that the rods (Figure 9A) were completely disrupted upon the formation of liposomes (Figure 9F).

Although no unit morphologic structure could be identified for the rods, most have a relatively uniform diameter and often appear to be flattened cylinders (Figure 9A). Some of the rods have a twisted structure, suggesting that they might be composed of protofilaments, but no consistent substructure could be discerned. Similar rod-shaped particles were isolated from brain tissue from patients dying of CJD (Figures 9B and 9C).[95] The heterogeneous morphology of the prion rods and the lack of consistent substructure distinguish them from viruses.

On the other hand, the ultrastructure of the prion rods is indistinguishable from that of many purified amyloids.[62] Histochemical studies with Congo red dye have extended this analogy to purified preparations of prions,[62] as well as to scrapie-infected brain tissue in which amyloid plaques have been shown to stain with antibodies to PrP 27-30.[77,104] In addition, PrP 27-30 has been found to stain with periodic acid-Schiff reagent;[172] amyloid plaques in tissue sections readily bind this reagent.

Immunocytochemical studies with antibodies to PrP 27-30 have shown that filaments (~16 nm in diameter and up to 1500 nm in length) within amyloid plaques of scrapie-infected Ha brain are composed of prion proteins.[77] The prion filaments have a relatively uniform diameter, rarely show narrowings, and possess all the morphologic features of amyloids. Except for their length, the prion filaments appear to be identical ultrastructurally to the rods found in purified fractions of prions (Figure 9A).

Confusion about rod-shaped forms of prions has arisen due to premature and unsupported claims[146] that prion rods are identical to structures called scrapie-associated fibrils (SAF).[109] In crude extracts of scrapie-infected rodent brains, fibrillar structures were found and differentiated from amyloids by their well-defined morphology. Two types of fibrils, consisting of either two or four helically wound subfilaments, were described and labeled scrapie-associated fibrils types I and II, respectively.[216] The regular periodic crossing of the subfilaments, as well as the spaces of 4 to 6 nm between them, were used to distinguish SAF from amyloids and other filamentous structures.[206,216] While the composition of SAF types I and II remains to be established,[146] there is no convincing evidence to support the claim that SAF, as originally described, is composed of prion proteins.[10,62]

Ignoring the fine structure of SAF, some investigators have chosen to assign the term *scrapie-associated fibrils*[146] to virtually any elongated structure in fractions prepared from prion-infected brains.[109] This approach is misleading and has even prompted some investigators to report SAF accumulation in the brains of patients dying of AIDS[217] and stimulated others to claim significant nucleotide and amino acid sequence homology between PrP and HIV polymerase.[218] Analyses of the proposed homology argue persuasively that PrP and HIV polymerase are unrelated.[182,219,220] Of note, some investigators state that SAF exist within prion-infected brains.[152] This claim is not supported by any experimental data; to date, SAF have not been found *in situ*. Even if SAF are eventually shown to be composed of PrP molecules, the term *SAF* should be retained to describe the helically wound structures with regular periodicities that differ ultra-

structurally from both amyloids and the prion rods. Use of term *SAF* to describe almost any pleiomorphic structure in fractions from prion-infected brains serves no meaningful purpose.

The first scrapie-specific structures to be identified by electron microscopy were spherical particles within postsynaptic evaginations of scrapie-infected Mo brains.[221] Similar particles were found in scrapie-infected sheep brains[222] and brain tissue from patients dying of CJD.[223] Some investigators were unable to identify these scrapie-associated particles within infected Ha brains,[224] but others claim to have found them in Ha brain in the form of tubules.[225] However, the elongated shape of these tubules clearly distinguishes them from the scrapie-associated spherical particles. Similar tubules have been seen after touching electron microscopic grids to the surface of scrapie-infected brains.[226] Detergent treatments with SDS, as well as DNase digestions, reveal twisted filaments within the tubules that are thought to resemble SAF.[227] Whether the tubules contain aggregated arrays of PrP^{Sc} or PrP 27-30 molecules is unknown.

One recent study equates the prion rods to SAF and claims that these structures are found *in vivo* within the brains of scrapie-infected mice.[152] These investigations examined detergent extracts of subcellular fractions, but significant proteolysis of PrP^{Sc} is evident on Western blots. Certainly, prion rods are not found *in vivo*, except perhaps within extracellular amyloid deposits, since their formation requires both detergent and limited proteolysis *in vitro*.[147] Whether limited proteolysis is required for SAF formation has not been reported, although some investigators describe purified fractions prepared without digestion by exogenously added proteases that are said to contain both SAF and PrP^{Sc};[110,187] presumably, that portion of PrP that is PrP 27-30 was separated from PrP^{Sc} by size-exclusion chromatography (SEC) after SDS denaturation, but rod-shaped structures observed by electron microscopy are nevertheless composed of PrP 27-30. The same explanation seems applicable to our own earlier studies, where microsomal membranes prepared without protease inhibitors were thought to produce prion rods upon detergent extraction.[90]

The solubilization of prions using phospholipids has helped to clarify the issues concerning the heterogeneity of the infectious particle with respect to size, density, and charge. The apparent heterogeneity of prions is well documented and has plagued scrapie research over the last two decades.[228] The ability of prions to form rods, DLPCs, and liposomes, each having high prion titers, provided definitive evidence that prions may exist in multiple forms.[61] The results of such studies argue persuasively that hydrophobic interactions are responsible for the extreme apparent heterogeneity of prions, as suggested over a decade ago.[142,228]

Interconversion of the multiple forms of prions depicted in Figure 8 depends upon the relative levels of detergent and lipid.[85] As described above, both detergent extraction and limited proteolysis of PrP^{Sc} are required for amyloid rod formation. Dispersion of the rods into DLPCs was accomplished by the addition of detergent and phospholipid, while subsequent removal of detergent produced closed liposomes.[61] Removal of the phospholipid resulted in the reformation of rods with full retention of scrapie infectivity.[85] As illustrated, dispersion of microsomal membranes into DLPCs bypassed rod formation; this approach was used for the immunoaffinity purification studies.

The foregoing studies demonstrate that prions may exist in multiple forms and, to date, the only molecule identified in all of these forms is PrP^{Sc}. The rapid interconversion of various prion forms argues, but does *not* prove, that prions are likely to be composed of a single type of macromolecule. It is difficult to envision how a highly charged, water-soluble macromolecule, such as a nucleic acid, could partition with PrP^{Sc} throughout these interconversion steps and at the same time remain so highly protected that it has eluded all attempts at detection.

XV. CELL-FREE TRANSLATION AND CULTURED CELLS

The first recombinant PrP molecules were produced using cell-free translation systems.[183] Those studies indicated that the biogenesis of PrP^{C} is unusual and in one sense unprecedented. Synthesis of PrP in wheat germ of reticulocyte extracts in the presence of dog pancreas micro-

somal membranes produced PrP molecules with cleaved N-terminal signal peptides and Asn-linked oligosaccharides.[183,229] Two forms of PrP were identified: (1) the integral membrane form, which spans the membrane twice with both the N- and C-termini on the luminal side, and (2) the secretory form that is found unbound on the luminal side. Injections of PrP mRNA into oocytes produced the secretory form almost exclusively, which was secreted into the surrounding medium. Using chimeric proteins, the region of PrP that appears to control its topology has been identified. This region encompasses the N-terminus of PrP 27-30 and has been designated the stop-transfer effector or STE.[230,231]

While animal virology has scored many impressive advances using cultured cell systems over the past 4 decades, the study of prions in cultured cells has been disappointing until recently. Procedures for infecting cultured murine neuroblastoma cells with prions have been developed, and clones of infected cells have been isolated.[81,105,106,232] Scrapie-infected neuroblastoma clones were found to produce PrP^{Sc}, while uninfected clones were devoid of PrP^{Sc}.[81,82]

Pulse-chase experiments using [^{35}S]-methionine have shown that PrP^{C} is labeled rapidly and degraded with a $t_{1/2}$ of ~5 h in neuroblastoma cells.[83,107] Most of the PrP^{C} is found on the surface of neuroblastoma cells, and PIPLC digestion releases most of it.[84] In contrast, PrP^{Sc} accumulates slowly during the chase period and is thus derived from PrP^{C} or a precursor through a posttranslational process.[83] In contrast to PrP^{C}, PrP^{Sc} is found primarily within the cytoplasm of these cells[82] and has been localized to cytoplasmic vesicles by immunoelectron microscopy.

The expression of recombinant PrP driven by plasmid or viral vectors in cultured cells has yielded proteins resembling PrP^{C}. A variety of expression vector systems utilizing both plasmids and viruses have been examined for enhancing PrP biosynthesis. Plasmid vectors with retroviral, SV40, heat shock, BPV, and CMV promoters have been used.[233,234] Recombinant baculoviruses[234] and vaccinia viruses (VV)[235] have been constructed. Baculoviruses produce low levels of PrP in Spodoptera cells but high levels of PrP mRNA, suggesting translational block.[234] Not only has it not been possible to produce high levels of recombinant PrP^{C} to date, but multiple forms of PrP that do not migrate with authentic PrP^{C} on SDS-PAGE are often seen.

Recently, protease-resistant PrP molecules encoded by a recombinant expression vector have been produced in scrapie-infected murine neuroblastoma cells.[236,237] These candidates, recombinant PrP^{Sc} molecules, contain an epitope recognized by HaPrP mAbs allowing them to be differentiated from the $MoPrP^{Sc}$ produced. The chimeric Ha-MoPrP molecule, designated MHM2, was used in site-directed mutagenesis, where the two N-linked glycosylation sites were abolished. In scrapie-infected cells, unglycosylated protease-resistant PrP molecules were found, arguing that PrP^{Sc} formation does not require complex-type oligosaccharide addition. Protease-resistant PrP was also synthesized in scrapie-infected cells treated with tunicamycin that inhibits N-linked glycosylation.[236]

XVI. NATURAL SCRAPIE OF SHEEP AND GOATS

The history of investigations on scrapie dates back to 1732. The unique clinical features of the disease in sheep make it readily recognizable in written records in several languages under a variety of names. Several early British and French investigators attempted to transmit scrapie from affected sheep to healthy sheep by injection of various tissues and body fluids, and the French investigators Cuillé and Chelle succeeded in 1936 with an intraocular injection of spinal cord.[238] As described below, one group of investigators, focusing on the experimental transmissibility of the disease, viewed natural scrapie as a viral-like illness, while another group thought of it as a genetic disorder, which just happens to be experimentally transmissible.

Maternal (and lateral) contagious transmission of natural scrapie was first suggested by crosses of scrapied and scrapie-free Suffolk sheep performed by Dickinson and co-workers. Progeny of affected ewes rather than rams were about seven times more likely to develop the disease.[239] In a subsequent study of Suffolks crossed with Scottish blackface sheep, a similar but reduced tendency was again apparent; ratios of approxi-

mately 1.9:1 and 1.3:1 were obtained in experiments involving 54 and 66 offspring, respectively. A "background" scrapie incidence of up to 50% in the progeny of unaffected parents, interpreted in terms of lateral contagious transmission, was apparent in this later study.[240]

By performing crosses of Suffolk sheep, Parry reached quite a different conclusion. He deduced that scrapie was an autosomal recessive genetic disorder in which contagious spread of the infectious agent played little or no part. A tendency favoring maternal transmission in Parry's data[241] on twins born of scrapie-infected ewes was noted by Dickinson et al.,[239] although the numbers quoted for the progeny of unaffected ewes crossed with affected rams do not appear to correspond to the original data. However, the bulk of Parry's data on Suffolk sheep showed no overt tendency for ewes, rather than rams, to transmit the disease.[241] Parry's experiments also showed no evidence for contagious transmission, i.e., the scrapie-free animals (designated *proven white*) produced no affected offspring, even when crossed to scrapied animals.[242]

Dickinson's hypothesis of maternal transmission can be directly tested by bioassay for infectivity in scrapied ewes. In this respect, Hadlow and co-workers failed to detect infectious titre in the uterus (gravid or nongravid), ovary, or mammary gland of clinically affected Suffolk ewes.[243] Investigations by Pattison et al.[244,245] have been widely cited as indicating infectivity in the placentae of Swaledale ewes with scrapie. Unfortunately, negative controls were not described for these experiments and the incubation times in the inoculated recipients were scattered; an alternative explanation is that the observed instances of scrapie represent cross-contaminated inocula (however, see Pattison[39]).

Although the viewpoints espoused by Dickinson and Parry seemed irreconcilable at the time, demonstration that the major or sole component of the scrapie prion is host encoded (see below) suggests a resolution to the apparent conundrum of how a disease can be both genetic and infectious.

XVII. EXPERIMENTAL SCRAPIE OF SHEEP AND GOATS

It was clear from the studies of scrapie in different breeds of sheep naturally affected with the disease that the genetic background of the host played a major part in the course of the disease. Early work on the influence of the host in experimental disease was done in England by Gordon, who injected subcutaneously scrapie-infected brain extracts into 24 different breeds of British sheep. The published results showed incidences of disease ranging from 78% in Herdwicks and 72% in Dalesbreds to 0% in Dorset Downs. Subsequent studies revealed that the Dorset Downs were not fully resistant, but had a prolonged incubation period when compared to other breeds.[246] Despite these apparently clear results, the genetic analysis of scrapie susceptibility in sheep has been complicated by the possibility of maternal transmission of the disease.[240]

In 1961, Dickinson and associates began selecting two populations of Cheviot sheep, all of which were derived from a single foundation group presumed to be free from natural scrapie. One group was selected for increased incidence of scrapie following subcutaneous injection, while the other for decreased incidence of disease. The two lines differ by approximately 90% in their incidence of scrapie disease. Based on the response of each of these groups to either the subcutaneous or intracerebral inoculation of the SSBP/1 strain of the scrapie agent, the animals fell into a short incubation time (197 ± 7 d) group and a long incubation time (917 ± 90 d) group.[247]

When sheep with short incubation periods were mated with sheep with longer incubation periods, the results suggested that a single autosomal gene, which is dominant with respect to short incubation periods, controls the length of the incubation time. This gene was thought to have two alleles and these were designated SIP^{sA} and SIP^{pA}. It has been hypothesized that the action of SIP alleles may be to restrict the replication of certain "strains" or isolates while al-

lowing others to replicate. This hypothesis predicts a differential response to different scrapie isolates, which seems to be the case.[42]

Analysis of PrP gene polymorphisms in the two lines of Cheviot sheep suggest that the PrP gene may be linked genetically to the SIP gene. While the data suggest that sheep may be similar to mice where the PrP gene is linked to a gene controlling the length of the scrapie incubation time, additional samples are needed to clarify why 4 of 11 sheep thought to be homozygous for SIP^{sA} were heterozygous for PrP polymorphisms. Similarly, analysis of Suffolk and Ile-de-France sheep has revealed all four permutations of the EcoRI and HindIII polymorphisms.[248,249] As the chromosomal phase between these polymorphisms does not appear to be fixed with respect to each other, the probability of linkage disequilibrium between these particular restriction fragment length polymorphisms and SIP alleles seems remote, precluding accurate SIP genotyping outside of the Cheviot breed. A molecular cloning study of PrP gene clones sequenced from a Suffolk sheep genomic library reported two alleles.[250] A nucleotide change of G → A results in the substitution of Arg for Gln in PrP at codon 171.

The possibility of maternal transmission in the context of experimental scrapie was first addressed by Gordon.[246,251] In the first report, there was an incidence of ~8% (n = 123), and in the second, where both the ewes and the rams were inoculated, there was an incidence of ~5% (n = 63). Dickinson et al. reported one affected animal (n = 1) in an experiment involving implantation of a fertilized egg into an inoculated recipient, and two offspring scrapied (n = 4) in an experiment similar to Gordon's cited above, but using different breeds.[252] Incubation times for many of the lambs in these experiments[246,252] were short, implying that they did not represent cryptic cases of natural scrapie. In contrast to these reports, a comparable study[253] was essentially negative. Out of a total of 86 embryos transplanted from inoculated donors into free recipients or from free donors into inoculated recipients, none of the resulting offspring developed the disease within an observation period ≥5 years. Direct inoculation of control animals in these experiments produced a scrapie incidence >51%. Similarly, negative results were obtained for maternal transmission in experimental scrapie of goats.[254] Early reports[255,256] suggesting maternal transmission of scrapie in mice were strongly challenged by subsequent investigators.[257-259] A maternal effect is not apparent in the transmission of either familial or experimental CJD,[260-262] kuru,[263] or GSS.[7,8]

XVIII. BOVINE SPONGIFORM ENCEPHALOPATHY EPIDEMIC

"Mad" cows dying of bovine spongiform encephalopathy (BSE) present a major agricultural problem and may pose a potential public health dilemma. Since 1986, more than 30,000 cattle in Great Britain have been diagnosed with BSE.[264] Epidemiological studies suggest that the introduction of sheep offal into the diet of British cattle in 1981 may be the point source cause of BSE.[4,265,266] Transmission of BSE to mice and cattle,[267-269] symptoms confined to the CNS, spongiform change in the brain,[270] and protease-resistant PrP detected on immunoblots of affected brain extracts[3,271] all implicate scrapie prions in meat and bone-meal supplements derived from sheep carcasses.

Restriction on imports of British beef products by France and West Germany present a major problem for British agriculture.[272] The possibility of bovine to human transmission of prions has resulted in concern over the beef being served to school children in Great Britain. Fear has escalated with reports of more than 10 domestic cats dying of a spongiform encephalopathy, as well as the possibility that exotic ungulates in zoos may have developed CNS disorders by consuming prion-infected meat and bone meal.[273,274]

Numerous epidemiological studies have attempted to link the development of CJD in humans with the consumption of scrapie-infected sheep products.[275-279] Although such studies have failed to demonstrate a link between scrapie and CJD, the development of a related human disease, kuru, appears to be caused by consumption of prion-infected brains during ritualistic cannibalism.[37] Whether bovine prions will prove pathogenic for humans remains an unanswered question of extreme importance. Pertinent to the

question of whether consumption of bovine prions in beef products or bovine-derived pharmaceuticals will cause CNS dysfunction are the low efficiency of oral vertical transmission of prion infection, conflicting data about vertical transmission, and the "species barrier". Studies with Syrian hamsters have shown that oral transmission of experimental scrapie can be accomplished with regularity, but the oral route is 10^9 times less efficient than the intracerebral one; parental inoculation is 10^5 times less efficient than intracerebral inoculation.[280] Transmission between species is characterized by a stochastic process in which a few inoculated animals develop disease after extremely prolonged incubation periods.[209,281] On the next passage in the homologous host, development of disease is a nonstochastic process, with greatly shorter incubation times. Recent studies with Tg mice argue that the species barrier resides in the amino acid sequence of PrP.[74]

Whether BSE occurs naturally or is due exclusively to the oral consumption of sheep scrapie prions is unknown. Although the N-terminal sequence of PrP recovered from cattle brains is similar to that of HaPrP,[3] it will be important to learn about the PrP genes from a variety of cattle. Equally interesting will be the PrP sequences from many breeds of sheep, as well as mink, mule deer, and elk.

Whether cattle represent a dead-end host for prions as appears to be the case for mink developing transmissible mink encephalopathy after consumption of scrapie-infected sheep meat or the disease will spread horizontally among cattle is unknown. How scrapie spreads among sheep in flocks is unknown, and the controversy surrounding this issue reflects our ignorance about the origins, spread, and pathogenesis of natural scrapie.

XIX. NEURODEGENERATIVE DISEASES OF HUMANS

It is important to consider why experimental scrapie of rodents is such a valuable model for the investigation of central nervous system (CNS) degenerative diseases. The most common CNS degenerative disease is Alzheimer's disease (AD). By age 85, the prevalence of AD has been estimated to be about 1 in 4 people.[282,283] With the changing demography of our society, it is estimated that AD will grow to epidemic proportions over the next 2 decades.

AD and CJD share many clinical and pathologic features.[284-291] Both diseases are dementing disorders that occur later in life, but they differ with respect to their incidence and peak age of onset. AD is 5000 times more common than sporadic CJD. While the incidence of AD probably reaches a maximum around age 85, the maximal incidence of CJD occurs at around 60 years of age. On the other hand, well-documented cases of CJD in 80-year-old people have been recorded.[292,293] Although the duration of CJD is frequently less than 2 years, clinical courses as long as 5 years are well known. The progression of AD is frequently slower than CJD, with clinical courses of 5 to 10 years being common. While the multifocal and periodic discharges on the EEG are hallmarks of CJD, they have been seen in AD. The lack of specific blood or cerebrospinal fluid (CSF) laboratory tests for CJD and AD sometimes obscures the differential diagnosis. Long tract signs and frontal lobe release signs are common to both disorders, as are normal laboratory values for the blood and CSF. An afebrile course in both diseases in the rule.

At autopsy, both AD and CJD are characterized by a lack of gross changes. Histological examination shows changes primarily in the gray matter. Vacuolation of the neuropil is common and sometimes extreme in CJD, but there are exceptions, as noted above.[294] Vacuolation has also been observed in AD.[295] Amyloid plaques are sometimes seen in CJD and are now known to be composed of PrP molecules.[79,296,297] Amyloid plaques are the rule in AD and are now known to be composed primarily of β-peptide.[249,298] Neurofibrillary tangles (NFT) are usually found in AD,[299] but are infrequent in cases of CJD below age 65. We have found both PrP and β-amyloid plaques in older patients with CJD.[297] Some investigators have reported evidence for the coexistence of CJD and AD in the same patient.[157,287,300] Whether such cases indicate shared etiologic features between the two diseases or they only represent a coincidence is unknown. While PrP plaques are diagnostic of

prion diseases, β-plaques are not diagnostic of AD, since older, normal people also show accumulation of β-amyloid. It is noteworthy that the number of β-plaques has been said to correlate with the degree of dementia,[301-303] while the levels of β-amyloid peptide in serum and brain were found to be similar in AD to those of age-matched controls.[304] Some families with ~50% of their members afflicted with dementia were labeled familial AD.[305,306] Recent studies have revised this classification, since immunostaining shows these patients have PrP plaques without any β-amyloid deposits.[307,308] Patients in one of these families, the Indiana kindred, also have numerous NFT — perhaps providing a heuristic link between AD and the prion disorders.[307,309] PrP plaques and NFT have been found in the Indiana kindred with GSS.[310]

While CJD is transmissible to experimental animals, AD is generally *not* thought to be transmissible. Although there are two reports to the contrary claiming transmission of AD to animals, both reports emanate from laboratories working on CJD where contamination or sample mixup must be considered.[311,312]

About 90% of all CJD and AD cases are sporadic, while 10% are familial.[313] Molecular genetic analysis of humans with GSS and familial CJD reveal mutations in the PrP gene, leading to amino acid substitutions in PrP. Significant genetic linkage of PrP gene mutations with the development of GSS is discussed below.

The story surrounding familial AD is less clear. Some investigators have reported linkage to anonymous probes on chromosome 21, while others dispute conclusions that possibly reflect heterogeneity in the etiology of familial AD.[314-316] Of considerable interest is the identification of a point mutation in the β-APP gene, leading to the substitution of a Gln for Glu at residue 22 of the β-A4 peptide.[317,318] This mutation has been linked genetically to the development of a CNS Congophilic angiopathy.[319]

XX. INFECTIOUS, SPORADIC, AND GENETIC MANIFESTATIONS OF PRION DISEASES

The human prion diseases illustrate three different manifestations of CNS degeneration: slow infection, sporadic disease, and genetic disorder.[9,11] That the three human prion diseases can be transmitted to laboratory animals by inoculation is well documented.[5-7] Kuru is thought to have been spread exclusively through a slow infectious mechanism by means of ritualistic cannibalism.[37,263,320]

Although a few cases of CJD have been traced to inoculation with prions (e.g., injections of human growth hormone,[108,321,322] transplantation of corneas, and implantation of cerebral electrodes), most appear to be sporadic, despite considerable effort to implicate scrapie-infected sheep as an exogenous source.[276,278,279,323] It is possible, although unlikely, that sporadic CJD results from prions that are ubiquitous in humans but have a very low efficiency of infection. In hamsters, scrapie infection by the oral route has been found to be 10^9 times less efficient than intracerebral inoculation.[280]

Studies of GSS and familial CJD suggest that these disorders are inherited as autosomal dominant disorders.[8,262,324,325] The significance of this observation was uncertain until recently, when GSS was demonstrated to be a genetic disease and its development was linked to a PrP mutation.[8] Molecular cloning studies demonstrated a C-to-T substitution in the second position of codon 102, which probably results from deamination of a methylated cytosine situated 5′ to guanine.[326,327] This mutation creates a Dde 1 restriction site, which was used to demonstrate genetic linkage between the PrP codon 102 amino acid substitution (Leu → Pro) and the development of GSS. This mutation has been found in one American,[8] one British,[8] two Japanese,[15,328] and one German family,[14] all with ataxic GSS.

Tg mice harboring a mutant PrP gene encoding the Pro_{101} → Leu substitution (Pro_{101} in mice is homologous to Pro_{102} in humans) have been found to develop spontaneous neurologic dysfunction indistinguishable from experimental murine scrapie. The brains of these Tg(GSSMoPrP) mice were found to have widespread spongiform change and moderate gliosis.[76] Our results demonstrated that the Pro_{101} → Leu substitution in MoPrP is the cause of CNS degeneration in Tg(GSSPrP) mice and, by inference, argues that the Pro_{102} → Leu change in

HaPrP is the cause of GSS in humans. Indeed, this is the first human CNS degenerative disorder to be genetically modeled in experimental animals. About 10 to 15% percent of cases of CJD are familial, while most cases of GSS are inherited.[7,11,324] Familial CJD has been reported in families with amino acid substitutions or insertions in the PrP gene[12-14,329] (Figure 7). GSS and familial CJD are the only known human diseases that are both genetic and infectious.

A point mutation at PrP codon 200 resulting in the substitution of Lys for Glu has been found in a brother and sister of Polish descent, both having familial CJD.[14] Slovakian Czechs with familial CJD also have this mutation, as well as Libyan Jews.[70-72] Inserts in the Gly-Pro rich repeat region of PrP consisting of 144 bp have been reported in association with familial CJD.[12,13,329,330]

The genetic linkage of mutant PrP with GSS and the production of CNS degeneration in Tg(GSSPrP) mice expressing the Leu mutant of PrP constrain the possible structural models for prions. If prions contain a small, as yet undetected, nucleic acid, then this molecule must be widespread throughout the world to explain the incidence of sporadic CJD; yet it must segregate in rare GSS families with the PrP mutation. It is noteworthy that Huntington's disease, another autosomal dominant genetic disorder, does not manifest clinical illness until the fifth decade in most cases. The delayed onset of both Huntington's disease and GSS is not understood.

Molecular genetics of Israeli Jews of Libyan ancestry argue that the high incidence of CJD formerly attributed to the ingestion of lightly cooked lamb brain[331,332] and sheep eyeballs[333] is due to a PrP gene point mutation at codon 200, resulting in the substitution of a Lys for Glu. In one patient homozygous for the mutation, her clinical illness was similar to that seen in heterozygotes, arguing that familial CJD is a true autosomal dominant disorder,[72,73] like Huntington's disease.[334] These observations eliminate the possibility that familial CJD required a second event, such as familial retinoblastoma, where a "phenotypically dominant" disease is genotypically recessive.[335,336]

The genetic linkage and Tg Mo results also suggest a mechanism for sporadic CJD if prions contain only PrP^{Sc} (or PrP^{CJD}). A somatic mutation[335] in the PrP gene or even an RNA editing error[162-165] or translational error in a single cell might lead to the generation of PrP^{CJD} in that cell; prions would then spread to neighboring cells upon exit of the PrP^{CJD} molecules. Alternatively, wild-type PrP^{C} might be converted to PrP^{CJD} in a stochastic process as a rare event, which would have the same result as a somatic or germ-line mutation, that is, the initiation of a process in which PrP^{CJD} accumulates autocatalytically through a posttranslational mechanism.

XXI. PRIONS ARE NOVEL AND UNPRECEDENTED

The convergence of experimental results indicating a pivotal role for PrP^{Sc} (or PrP^{CJD}) in animal and human prion diseases is both impressive and persuasive. The wide variety of independent disciplines, including protein chemistry, molecular genetics, immunochemistry, neuropathology, and experimental neurology, employed to obtain these experimental data greatly strengthens the assertion that PrP^{Sc} has a central role in prion diseases.

That PrP is encoded by a cellular gene and not by a putative nucleic acid carried within the prion is a major feature that distinguishes prions from viruses. This discovery, coupled with recent genetic studies showing linkage between a PrP mutant and GSS, as well as the production of CNS degeneration in Tg mice expressing this PrP mutation, demand that scrapie and CJD no longer be considered virological disorders. Although prion diseases resemble viral illnesses in some respects, the structure, cell biology, and genetics of prions clearly separate them from viruses.

Whether prions are composed only of an abnormal isoform of the prion protein or they contain some additional molecule is uncertain. Many lines of evidence argue that PrP^{Sc} is the sole component of prions: (1) multiple forms of prions are infectious — membranes, rods, spheres, DLPC, and liposomes; (2) many attempts to demonstrate the dependence of scrapie infectivity on a nucleic acid have been unsuccessful; (3) the ionizing radiation target size of the prion is 55,000 Da; (4) PrP^{Sc} is encoded by a cellular gene; (5) mice with

short and long incubation times have different PrP genes that encode distinct prion proteins and produce prions with distinct properties; (6) GSS is linked to a mutation in the PrP gene; (7) in Tg(HaPrP) mice, all aspects of scrapie are controlled by the HaPrP transgene, including susceptibility, incubation time, and neuropathology; (8) the species-specific prion produced in Tg(HaPrP) mice is determined by the genetic origin of the inoculated prion, indicating that replication of prions must involve a specific interaction between inoculated PrP^{Sc} and homologous PrP^{C}; and (9) Tg(GSSMoPrP) mice spontaneously develop neurologic dysfunction and CNS degeneration similar to scrapie. The partitioning of infectivity in a wide variety of different forms suggests a single component but does not eliminate the possibility of a second macromolecule. Numerous attempts to inactivate scrapie prion infectivity by procedures that hydrolyze or modify nucleic acid have been consistently unsuccessful. Ultraviolet irradiation of membranes, rods, and DLPC suggests that if prions have an essential nucleic acid, then it will be <5 bases if single stranded or 30 to 45 bp if double stranded. Ionizing radiation studies give a target size too small to protect a large nucleic acid but do not rule out some other macromolecule.

Two arguments favor a second prion component. (1) Prion infectivity has not been reproducibly recovered from denatured samples after renaturation attempts. (2) Many "strains" of prions have been reported. The first argument raises the possibility of a second component, but it need not necessarily be a nucleic acid. The second argument focuses on prion diversity and offers a nucleic acid genome as the basis for this diversity.

Just as GSS and familial CJD are unprecedented human illnesses, since they are both genetic and infectious, prion particles seem equally novel. Learning the chemical mechanisms responsible for converting PrP^{C} or a precursor into PrP^{Sc} will be important. Elucidation of PrP^{C} function may open up new avenues of research into mechanisms of cell homeostasis, recognition, and possibly differentiation. Whether proteins other than PrP are converted from benign, cellular isoforms into malignant, pathogenic molecules resulting in degenerative diseases is unknown.

In summary, a wealth of recently acquired experimental data have established that prions are novel and unprecedented pathogens. The uniqueness of prions promises to open many new and unexpected avenues of research. Once the events in PrP^{Sc} formation are elucidated, then therapeutic and preventive strategies for prion disease can be formulated. Learning how to control effectively and possibly prevent scrapie and BSE are important goals. Equally important will be the search for other prion diseases, which should begin in earnest once the chemistry of PrP^{Sc} formation is elucidated. It seems likely that some of the pathogenic mechanisms operative in prion diseases will give new insights into CNS disorders of older people, such as AD, Parkinson's disease, and amyotrophic lateral sclerosis. Already familial AD pedigrees are being reclassified as prion diseases based upon the protein deposits within their amyloid plaques.[307,308]

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