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Isolation of a cDNA clone encoding the leader peptide of prion protein and expression of the homologous gene in various tissues

(scrapie/scrapie-associated fibril protein/membrane protein/prion hypothesis)

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ABSTRACT **We have isolated a hamster cDNA clone representing the coding sequences for the entire precursor of prion protein (PrP) 27–30. This clone encodes a protein of 254 residues and contains an in-frame ATG codon 42 bases upstream from the one previously reported. Analysis of the predicted amino acid sequence suggests that the PrP precursor protein contains an amino-terminal signal sequence, and a membrane-spanning domain in the carboxyl terminus. Cleavage of the signal peptide would produce a mature protein of 232 amino acids. Sequences homologous to the hamster PrP cDNA were detected in hamster, mouse, sheep, human, and rabbit genomes. A related 2.5-kilobase transcript was present in the brain of normal and scrapie-infected rodents. Two homologous transcripts, 2.5 and 1.1 kilobases, were detected in the lung and heart of normal animals. No PrP mRNA was detected in spleen stroma, a tissue known to contain high titers of scrapie. Antisera raised to the 27- to 30-kDa polypeptide detected the PrP in both normal and infected brains but failed to detect this protein in either normal or infected spleens. Homologous mRNA species were detected in human, sheep, and rabbit brain, even though the latter is resistant to scrapie infection. Our data suggest that PrP is not a necessary component of the infectious agent.**

Scrapie is a progressive degenerative nervous system disorder occurring naturally in sheep and goats. It is considered a prototype of the unconventional slow virus infections of humans: Creutzfeldt–Jakob disease, kuru, and Gerstmann–Straussler syndrome (1, 2).

Transmission of scrapie to mice and hamsters has shown that high titers of the agent are present in the brain, spleen, and lymph glands of the infected animals (3, 4). Infectivity in brain homogenates is resistant to a variety of treatments expected to hydrolyze or modify nucleic acids but is sensitive to several protein-specific reagents (5–7). These molecular properties of the scrapie agent, and the inability to detect the presence of any scrapie-specific nucleic acid in infectious preparations, prompted the introduction of the term "prion" to denote a small proteinaceous infectious particle that resists inactivation by procedures that modify nucleic acids (8).

Recent studies have shown that scrapie infectivity is associated with amyloid-like fibrils, called scrapie-associated fibrils (SAF) (9, 10) or prion rods (11). These structures are observed exclusively in the unconventional slow virus diseases and are identified by their unique morphology (12). A polypeptide designated prion protein (PrP) 27–30 (13) or SAF protein (10), with an apparent size of 27–30 kDa, was found in the infectious fractions, and it was suggested that this protein polymerizes to form the SAF (10, 11). PrP 27–30 has been proposed as the major structural component of the scrapie agent (14). Recently, it has been shown that PrP 27–30 is encoded by a host gene, which is expressed at similar levels in normal and scrapie-infected brains. The partial nucleotide sequence of the mRNA encoding the PrP 27–30 precursor protein was also described (15). Immunological evidence suggests that PrP 27–30 derives from the proteolytic cleavage of a larger precursor, PrP 33–35 (15, 16).

Here we report the isolation and characterization of a cDNA clone encoding the entire PrP 27–30 precursor protein. Our results show that there is an in-frame ATG codon 42 nucleotides upstream from the methionine codon previously reported as the start signal for the PrP (15). Studies on the expression of the *PrP* gene in various tissues argue against the theory that PrP in itself is infectious or that this protein is necessary for scrapie replication.

MATERIALS AND METHODS

Inoculation of Animals. Hamsters of the LVG strain (16) were infected intracerebrally with a 1% brain homogenate of the 263K scrapie agent, and sacrificed between 35 and 60 days later. C57BL mice were sacrificed between 80 and 120 days after infection with a 1% homogenate of the ME7 scrapie strain. Infected mice showed the first clinical symptoms ≈135 days post-infection and died 25 days later. Controls were inoculated with a 1% normal brain homogenate.

Oligonucleotide Synthesis and Labeling. A 44-nucleotide probe 5′ GGCTTGTTCCACTGGTTGTGGGTGCCGC-CGCCCTGGCCCCATGG 3′ was chemically synthesized by Applied Biosystems (Foster City, CA). It was designed on the basis of the partial amino acid sequence Pro-Trp-Gly-Gln-Gly-Gly-Gly-Thr-His-Asn-Gln-Trp-Asn-Lys-Pro of PrP 27–30 (17), the eukaryotic codon usage frequencies (18), the relative stability of G·T versus A·C mismatches, and the infrequency of the dinucleotide CpG in vertebrate genes (19). The oligonucleotide was labeled to a specific activity of 4×10^8 cpm/μg using [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq; ICN) and T4 polynucleotide kinase (20).

Construction of the Hamster Brain cDNA Library. Hamsters were sacrificed 40 days after inoculation, and their brains were homogenized in 5 vol of 4 M guanidinium isothiocyanate. Total RNA was prepared from the brain homogenates (21) and poly(A)$^+$ mRNA was selected on an oligo(dT) column (22). cDNA was prepared from the poly(A)$^+$ RNA and then ligated to *Eco*RI-digested λgt11 DNA (23). The ligation mixture was packaged into the λ *in vitro* packaging system (Bethesda Research Laboratories) and used to infect *Escherichia coli* strain Y1088. Duplicate nitrocellulose filters were prepared from each plate. Hybridization with the 44-mer probe and washing of the filters were

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Abbreviations: PrP, prion protein; SAF, scrapie-associated fibrils; bp, base pair(s); kb, kilobase(s).

performed as described (24). Positive clones were grown on *E. coli* strain Y1088 and phage DNA was isolated (20). cDNA inserts were recovered by *Eco*RI digestion.

DNA Sequence Analysis. A 974-base-pair (bp) *Eco*RI/*Acc* I cDNA fragment containing the coding sequences of PrP was subcloned into pJV102. This plasmid is a derivative of pUC18 containing the M13 origin of replication (J. Van Arsdell and M.A.I., unpublished observations). DNA sequence was determined on the single-stranded template (25). Both strands of the cDNA fragment were sequenced.

Southern Transfer Analysis. High molecular weight DNA was prepared from hamster, mouse, sheep, and rabbit brains (20). Human placental DNA was obtained from Sigma. Samples of 10 μg DNA were digested with *Eco*RI restriction endonuclease. The digested samples were electrophoresed on a 1% agarose gel and then transferred to nitrocellulose filters (26). Filters were prehybridized as described (20). Clone XIV DNA was nick-translated to a specific activity of 1×10^8 cpm/μg (20) and then added to the prehybridized nitrocellulose filter. Filters were washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 60°C and autoradiographed for 5 days at −70°C with an intensifying screen.

RNA Blot Analysis. Human brain frontal cortex was obtained 5 hr postmortem from a medical examiner case of a 70-year-old female. Frozen rabbit brains were obtained from Pel-Freeze. Sheep cortex was obtained from a normal adult Suffolk sheep. Stroma tissue was isolated from mouse spleen by removing the pulp cells as described (27). All tissues were quickly frozen and total RNA was prepared from the removed tissues (21). Poly(A)$^+$ mRNA was isolated from total RNA (22) and stored in liquid nitrogen. After thawing, mRNA samples were ethanol-precipitated and then redissolved in a buffer containing 20 mM morpholinopropanesulfonic acid (pH 7.0), 2 mM EDTA, 50% formamide, and 6% formaldehyde. The samples were heated at 68°C for 5 min and electrophoresed through a 1% agarose gel in 20 mM morpholinopropanesulfonic acid, pH 7.0/1 mM EDTA. The electrophoresed RNA was transferred to a nitrocellulose filter (28) and baked at 80°C in a vacuum for 2 hr. Filters were prehybridized in 0.75 M NaCl/75 mM sodium citrate/0.5% bovine serum albumin/0.5% polyvinyl pyrrolidone/0.5% Ficoll/sonicated salmon sperm DNA (100 μg/ml)/0.1% sodium pyrophosphate at 65°C except the human, sheep, and rabbit samples, which were prehybridized at 60°C. Hybridization in the presence of the nick-translated probe was performed under the same conditions as prehybridization. The filters were washed in 15 mM NaCl/1.5 mM sodium citrate/0.1% $NaDodSO_4$ at 55°C and then autoradiographed.

Immunoblots. Antisera to the three polypeptides (28, 24, and 21 kDa) isolated from ME7-infected mice (29) were prepared in rabbit (16). Subcellular fractionation of spleen and brain material was performed as detailed (12). Proteins were electrophoresed in 15% gels (30) and then transferred to nitrocellulose filters (31). After washings, goat anti-rabbit IgG conjugated with alkaline phosphatase and then substrate (nitroblue tetrazolium) were applied.

RESULTS

A cDNA library was constructed in the expression vector λgt11 from poly(A)$^+$ mRNA isolated from scrapie-infected hamster brain. The library was screened with a 44-base-long oligodeoxynucleotide synthesized on the basis of the published NH_2-terminal amino acid sequence of PrP 27–30. About 3×10^5 recombinant phage plaques were screened and 6 positively hybridizing clones were detected. These clones were rescreened with plasmid pHaPrP, which contains a cDNA insert of the PrP mRNA (kindly provided to us by S. Prusiner) and one positive clone (termed clone XIV) was identified. The cDNA insert contained in clone XIV was 2.2 kilobases (kb) long, ≈0.25 kb longer than the cDNA present in clone pHaPrP (15). The restriction maps of both cDNA inserts were almost identical. The 2.2-kb insert of clone XIV was cut with *Acc* I to yield a 974-base-long fragment which contains the coding sequence of PrP (15). This fragment was inserted into vector pJV102 and sequenced. Our data revealed an additional 79 bp upstream from the 5′ end of clone pHaPrP (Fig. 1). The sequence of the remaining 895 nucleotides was identical to the corresponding 5′ end sequence present in clone pHaPrP. An open reading frame extends from nucleotide 2 to nucleotide 808 and is followed by the TGA stop codon. The first in-frame ATG start codon is at position 47, 42 bases upstream from the ATG codon previously thought to be the start signal for PrP 33–35 (15).

The sequence CCATC flanking the ATG codon at position 47 is homologous to the highly conserved consensus sequence that flanks most of the functional initiation sites in eukaryotic mRNAs: $CC^{A}_{G}CC$ (32). A hydropathy plot of the deduced amino acid sequence obtained by the method of Kyte and Doolittle (33) is presented in Fig. 2. Regions comprising 19 or more amino acids with hydropathy averages >1.6 are usually tightly associated with lipids (33). The COOH-terminal stretch of 22 amino acids predicted from clone XIV displays a hydropathy value of 2.7, and therefore it is most probably used to anchor PrP 33–35 in the membrane. Region b (Fig. 2) has a hydropathy value of 1.4 and

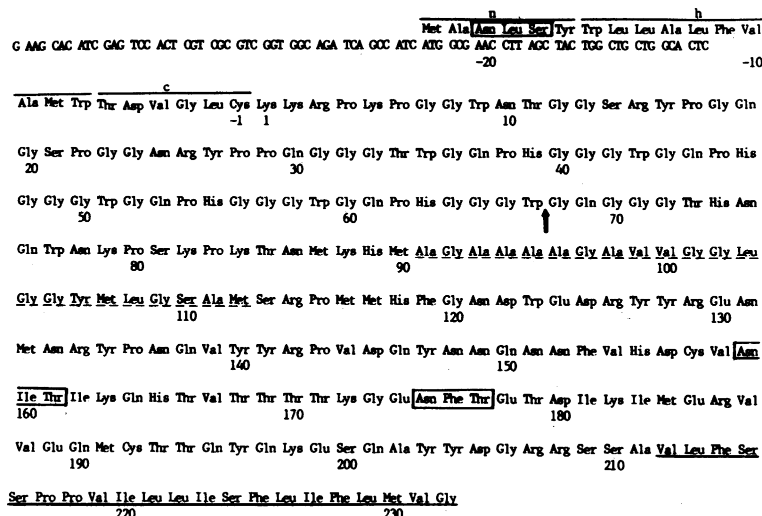

FIG. 1. Amino acid sequence of the leader peptide and mature PrP 33–35 protein derived from the nucleotide sequence of clone XIV. Only the first 79 nucleotides at the 5′ end of the 974-bp *Eco*RI/*Acc* I DNA fragment present in clone XIV are shown. The remaining sequence of this fragment is identical to the 5′ sequence present in clone pHaPrP (15). The untranslated region downstream of the *Acc* I site of clone XIV was not sequenced. The amino acids of the leader peptide are indicated by negative numbers. The NH_2-terminal, hydrophobic, and COOH-terminal regions of the signal peptide are indicated by n, h, and c respectively. Potential *N*-linked glycosylation sites are boxed. The proposed transmembrane region of PrP 33–35 is underlined with a solid line. The hydrophobic region presumed to be buried within the protein is underlined with a broken line. Arrow indicates the NH_2-terminus of PrP 27–30 (for details see text).

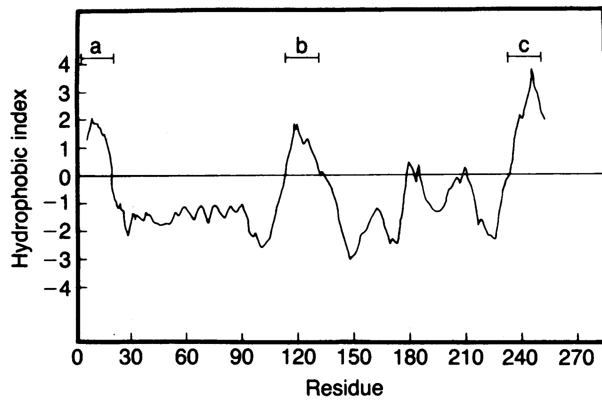

FIG. 2. Hydropathy plot of the PrP 33–35 precursor amino acid sequence. Hydropathy values (33) for a window of 11 amino acids residues were averaged, assigned to the middle residue of the span, and plotted with respect to position along the amino acid sequence. a, Leader peptide sequence; b, hydrophobic sequences that are probably passing through the interior of the protein; c, transmembrane sequence. Residue counting begins at the initiator methionine.

probably represents sequences passing through the interior of the protein (33). The stretch of hydrophobic amino acids at the NH_2-terminus resembles a signal peptide. There are three potential *N*-linked glycosylation sites (Asn-X-Thr/Ser) at positions 3, 181, and 197 (Fig. 1).

Southern Blot Analysis. Chromosomal DNA from hamsters, mice, rabbits, sheep, and humans was digested with restriction endonuclease *Eco*RI and analyzed by Southern blotting using the nick-translated cDNA clone as a probe. It can be seen from Fig. 3 that sequences homologous to the *PrP* gene were present in all genomes tested. The restriction pattern of hamster DNA consisted of two bands of ≈3.8 and ≈2.8 kb. Human DNA yielded one fragment of ≈15 kb, and three smaller ones of 4.4 kb, 3.6 kb, and 2.2 kb. These results differ from those published recently in which *Eco*RI-digested human and hamster DNA yielded only one band of 15 kb and 3.4 kb, respectively (15). The mouse DNA yielded one band at 2.6 kb. Three hybridizing fragments of 9.0 kb, 4.7 kb, and 2.4 kb were present in sheep DNA. One band of 3.0 kb was also detected in the DNA isolated from rabbit (Fig. 3). Since the hybridizations were carried out under conditions of high stringency for all samples, a high degree of homology must exist among the related *PrP* genomic sequences.

RNA Blots. It is known that in scrapie-infected hamsters and mice, the brain contains the highest amounts of infectivity (3). High amounts of infectivity were also found in spleen and other lymphoid tissues of infected animals. In contrast, little or no infectivity has been found to accumulate in the lung (3). Recently, the distribution of the scrapie agent between the pulp and stroma fractions of mouse spleens was determined, and the infectivity concentration in stroma was ≈10 times greater than in pulp (27). We examined the levels of expression of the *PrP* gene in the brain, spleen, spleen stroma, lung, and heart of hamsters and mice by RNA blot analysis by using the DNA insert of clone XIV as probe. In agreement with recently published results (15, 34), we detected a hybridizing mRNA species of ≈2.5 kb, which was present at the same level in control and scrapie-infected brains (data not shown). Two hybridizing transcripts of 2.5 and 1.1 kb were present in the lung and heart of hamsters and in the lung of mice (Fig. 4, lanes b, d, and h). The two mRNAs may differ in the lengths of their 3′ and 5′ untranslated sequences, as described for other transcripts (35), or, alternatively, these mRNAs could be generated by variable splicing of a primary transcript from the same gene. At this point, it is not known whether both mRNAs encode PrP 33–35 and why the smaller transcript is not present in the brain. PrP mRNA was not found in the spleen stroma fraction or whole spleen of normal or scrapie-infected mice (lanes g, i, and j). A signal could be detected in the mouse brain lane when the filter was autoradiographed for 2% of the time needed to obtain the signals shown in Fig. 4. Since no signal was seen in the spleen or spleen stroma preparations, the concentration of PrP mRNA in these preparations must be < 1/50th the concentration in the brain. Likewise, PrP mRNA was not observed in the spleen of scrapie-infected or normal hamsters (lanes c and e). Acridine orange staining of the agarose gels did not show any significant degradation of the spleen or spleen stroma mRNA preparations, and a strong signal was obtained when these mRNAs were probed with rat β-actin cDNA (data not shown). Transcripts homologous to PrP mRNA were detected in human, sheep, and rabbit brains (Fig. 5). The related human and rabbit mRNA species were ≈3.0 kb and ≈2.5 kb, respectively. In contrast, the homologous sheep brain mRNA was much larger (≈5.0 kb). A 1.1-kb transcript was also detected in the rabbit brain (Fig. 5).

Immunoblots. In immunoblot analyses of spleen preparations using an antiserum raised to SAF protein (16), we were unable to detect any PrP 33–35 in either normal or scrapie-infected mouse spleens (Fig. 5 *Right*, lanes c and d). This polypeptide was detected in both normal and scrapie-infected mouse brains, with the latter containing higher levels of PrP 33–35 than the former (Fig. 5 *Right*, lanes a and b). PrP 33–35 was also detected in crude 20% brain homogenates prepared in 10% sarcosyl. However, this protein was not detected in similarly prepared spleen homogenates (data not shown). Antigenically related polypeptides in the 21- to 30-kDa region

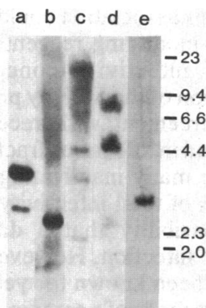

FIG. 3. Genomic Southern blot analysis of hamster (lane a), mouse (lane b), human (lane c), sheep (lane d), and rabbit (lane e) DNA. λ DNA digested with *Hin*dIII was used as a marker. Numbers on right represent kb.

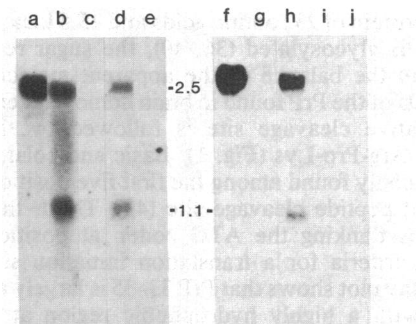

FIG. 4. RNA blot analysis of poly(A)$^+$ RNA isolated from various hamster and mouse tissues. RNA (10 μg) was electrophoresed through a 1% agarose gel. RNA was transferred to a nitrocellulose filter and hybridized as described. Hamster RNA was isolated from normal brain (lane a), lung (lane b), spleen (lane c), and heart (lane d), or from scrapie-infected spleen (lane e). Mouse RNA was isolated from normal brain (lane f), spleen (lane g), and lung (lane h), or from scrapie-infected spleen stroma (lane i) and spleen (lane j). Numbers represent kb.

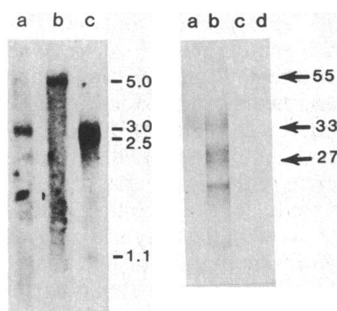

FIG. 5. (*Left*) RNA blot analysis of poly(A)$^+$ RNA isolated from human (lane a), sheep (lane b), and rabbit (lane c) brains. Numbers represent kb. (*Right*) Immunoblots of mouse brain and spleen extracts prepared from normal (lane a) or scrapie-infected (lane b) brain, and from normal (lane c) or scrapie-infected (lane d) spleen. The antigenically related band at 55 kDa in the infected sample has been observed before (16, 36). We do not know the relationship, if any, between this band and the PrP protein. Numbers represent kDa.

were observed in infected spleens occasionally, and only in mice sacrificed during the clinical stage of the disease—i.e., ≈70 days after infectivity peaks (37) (data not shown). These polypeptides are probably transported to the spleen from the brain, where they are seen even in the absence of proteinase K treatment (Fig. 5 *Right*, lane b), presumably deriving from a modification of PrP 33–35.

DISCUSSION

Our results show that scrapie PrP 27–30 is encoded by the 2.2-kb cDNA insert cloned in phage XIV. An open reading frame extends from nucleotide 2 to 808, with the first in-frame start codon at position 47. Initiation of translation at this ATG codon would produce a protein of 254 amino acids, 14 amino acids longer than previously reported (15). At the 5′ end, this cDNA insert contains an additional 79 bases, which are absent from the cDNA insert cloned in pHaPrP (15). Since the insert of clone XIV is ≈0.25 kb longer than the one present in pHaPrP, we conclude that the former must contain a longer poly(A) tail. Several lines of evidence suggest that translation initiates at position 47. First, analysis of the predicted amino acid sequences suggests the presence of a leader peptide at the amino terminus of the precursor PrP. The predicted leader peptide contains the n, h, and c regions that characterize signal sequences (38). Second, application of the von Heijne rules (39) predicts that the signal peptide would be cleaved after cysteine residue 22 to produce a mature protein of 232 amino acids and 25.5 kDa. Considering that PrP is glycosylated (36, 40), the sugar residues could contribute the balance of the apparent molecular mass of 33–35 kDa of the PrP found in brain homogenates (15, 16, 36). The putative cleavage site is followed by the sequence Lys-Lys-Arg-Pro-Lys (Fig. 1). Basic and polar amino acids are commonly found among the first five positions following the signal peptide cleavage site (41). Third, the nucleotide sequences flanking the ATG codon at position 47 match Kozak's criteria for a translation initiation site (32). The hydropathy plot shows that PrP 33–35 is largely a hydrophilic protein with a highly hydrophobic region at the COOH-terminus (Fig. 2). This region is probably used to permanently anchor PrP 33–35 in the membrane with the rest of the polypeptide projecting into the cytoplasm. The published NH_2-terminal amino acid sequence of PrP 27–30 (17) corresponds to residues 68–82. Therefore, PrP 27–30 must derive from the 25.5-kDa polypeptide by proteolytic cleavage after amino acid 67 (Fig. 1). In agreement with this conclusion, PrP 33–35 has been shown to share antigenic determinants with PrP 27–30 (15, 16). Moreover, sequencing of PrP 33–35 revealed a stretch of 22 residues perfectly matching a 22-amino acid segment predicted from our cDNA clone (D. C. Bolton and P. E. Bendheim, personal communication).

Three models proposed for the scrapie agent have attracted most attention recently: the prion, virino, and virus models (42). According to the prion model, the agent is a small infectious particle of ≈50 kDa, which most probably consists solely of protein (8, 11). In this theory, PrP 27–30 has been proposed as the major structural component of scrapie (14). According to the virino model, the scrapie agent consists of a small noncoding nucleic acid surrounded by host protein. Since PrP is a host protein, it could serve as the protein component of the virino. Protein components of conventional viruses are encoded by the virus genome, so that PrP does not fit this requirement.

In the present study, findings on the expression of the *PrP* gene argue against the concept that PrP protein is necessary for the replication of the agent. Outside the brain, high amounts of infectivity have been found in the spleen and other lymphoid tissues (3, 37). Mouse spleen stroma cells have been reported to contain 10 times higher concentrations of agent than splenic pulp cells (27). Despite this, we were unable to detect any PrP mRNA in whole spleen or in fractions enriched for spleen stroma cells. Other investigators also failed to detect PrP mRNA in whole mouse spleen extracts (34). Moreover, PrP 33–35 was not detected in normal or scrapie-infected spleen. Since high titers of the agent can accumulate in the spleen several weeks before its appearance in the brain (3), it seems unlikely that infectivity is transported to the spleen from the brain. Therefore, our results suggest that the agent can replicate in the absence of the PrP protein. SAF have been observed in spleen extracts prepared from infected mice at the clinical stage (12). Our data suggest that these structures or their monomers are produced elsewhere, most probably in the brain. Additional data presented here support the contention that the protein itself is not the infectious agent: (*i*) a gene homologous to hamster *PrP* gene was present not only in those species that are susceptible to scrapie-like agents—i.e., sheep, humans, hamsters, and mice, but also in rabbits, a species that is resistant to these agents (43); (*ii*) the *PrP* gene is expressed in the brain of all mammalian species examined regardless of their susceptibility to scrapie-like agents; (*iii*) the gene is expressed in mouse lungs, a tissue that does not support scrapie replication; and (*iv*) we and others (15, 34) found no difference in the level of expression of the *PrP* gene in the brain of normal and infected animals.

A more likely explanation of our findings is that due to scrapie infection, PrP protein is abnormally processed in the brain. As a result, PrP 33–35 or its degradation product PrP 27–30 aggregates and forms the amyloid-like SAF. Furthermore, it is possible that the scrapie agent, which is hydrophobic and displays "sticky" properties, adheres to the SAF or that infectivity is entrapped during the polymerization of PrP into the SAF. This association could "protect" the agent against nucleic acid-modifying reagents and would explain the destruction of infectivity concomitantly with the proteolytic degradation of the SAF by proteinase K (14). This explanation is in agreement with recent reports that PrP 27–30 was not detected in infectious fractions (44, 45) and that scrapie resistance to many inactivating agents is limited to small subpopulations of total infectivity (46). Our results do not exclude the possibility that a different protein(s) is necessary for scrapie infection. However, since well-defined scrapie strains have been known for years (47), it is reasonable to assume the presence of a scrapie-specific nucleic acid. The existence of this putative nucleic acid remains to be demonstrated.

Scrapie has been proposed as a model for Alzheimer disease (48). Although the etiologies of these diseases almost

certainly differ, the mechanism of induction of each disease may be related to the accumulation and abnormal processing of host proteins in the brain and to the presence of amyloid-like structures that characterize both conditions. In this context, it would be interesting to know the biological significance of PrP 33–35. The evolutionary conservation of the corresponding gene implies that the protein plays an important biological role. Its abnormal processing in the infected brain could be harmful to the cell. The availability of a cDNA clone encoding the complete protein should help in defining the biological and pathological importance of this protein.

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