Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 6372–6376, September 1986
Biochemistry

Molecular cloning and complete sequence of prion protein cDNA from mouse brain infected with the scrapie agent

(amyloid protein/spongiform encephalopathy/Alzheimer disease/hamster prion/polyprotein)

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Communicated by Bernard N. Fields, May 19, 1986

ABSTRACT **The prion protein (PrP) is a scrapie-associated fibril protein that accumulates in the brains of hamsters and mice infected with the scrapie agent, and also in the brains of persons affected with kuru or Creutzfeldt–Jakob disease. It has been previously proposed that PrP could be either the primary transmissible agent of scrapie or a secondary component involved in the pathogenesis of scrapie. At present, the second possibility seems more likely, for the PrP-specific mRNA is present in both infected and uninfected brains. We have isolated and sequenced the complete PrP-specific cDNA from mRNA isolated from infected mouse brains. Comparison of the mouse PrP with the hamster PrP reveals a high homology in the amino acid sequence and the presence of a conserved octapeptide repeated four times, whose function is unknown at present. Structural features are discussed and compared with other proteins. Except for its homology with the hamster PrP, mouse PrP has no significant homology to any known protein sequence, including neurofilaments, neuropeptides, and amyloid proteins of Alzheimer disease. Some features of the PrP, however, are similar to structures found in aggregating proteins, such as the wheat glutenin, keratin, and collagen.**

Scrapie is a progressive fatal neurologic disease that occurs naturally in sheep and goats (1). In the laboratory, the disease has been successfully transmitted to other animals, including mice (2), hamsters (3), mink (4), and, more recently, nonhuman primates (5). The unusual biophysical properties of the scrapie agent and the characteristic neurohistologic changes of scrapie are identical in certain respects with those of transmissible spongiform encephalopathies in humans, such as kuru (6), Creutzfeldt–Jakob disease (7), and some cases of Gerstmann–Straussler syndrome (8). Thus, all these diseases are believed to be caused by similar unconventional agents. The scrapie infectious agent has not yet been identified, although a 27- to 30-kDa protein, named prion protein (PrP) (9) or scrapie-associated fibril protein (10), seems to copurify with infectivity obtained from hamster brains. PrP appears to be assembled into large fibril protein structures that can be visualized by electron microscopy (11). These structures may be either the infectious agent itself (12) or a pathologic by-product that accumulates secondary to the infection (10).

PrP from hamster was isolated and a region of 15 amino acids was sequenced (13). Based on the reverse translation of a portion of this sequence, two identical 20-mer oligonucleotides were independently synthesized and used to isolate PrP-specific cDNA prepared from brains of hamsters and mice infected with the scrapie agent (13, 14). RNA blot experiments have led to the conclusion that the *PrP* gene is transcribed in both infected and uninfected brains in equal amounts and at lower levels in some non-neural tissues (13, 14). Differences in the regulation of expression at the post-translational level could not be ruled out, however. At present, the function of this gene product is unknown. We report here the cDNA sequence of the PrP mRNA from brains of mice infected with the scrapie agent. The deduced amino acid sequence of the complete mouse PrP allowed comparison with the partial hamster PrP sequence and sequences of proteins present in amyloid deposits and other proteins associated with Alzheimer disease. Unique features of both the nucleotide and amino acid sequences are discussed.

MATERIALS AND METHODS

Techniques used for the isolation and identification of PrP-specific cDNA cloned into the *Eco*RI site of λgt11 have been described (14). The inserted cDNA was subcloned into pEMBL8. Recombinant pEMBL8 plasmids were digested with various restriction endonucleases (Bethesda Research Laboratories) to establish a restriction map. The DNA sequence was determined by both the Maxam and Gilbert chemical cleavage method (15) and the dideoxy-chain termination method (16) after subcloning the DNA insert into M13 mp18 and mp19 (17) and producing sequential series of overlapping clones as described (18), using The Cyclone Subcloning System (IBI). The universal 17-base primer and the nucleotides were purchased from Bethesda Research Laboratories and P-L Biochemicals. Klenow fragment (Lyphozyme), T4 DNA ligase, T4 polynucleotide kinase, and the M13 cloning vectors were purchased from Bethesda Research Laboratories; calf intestine alkaline phosphatase was from Boehringer Mannheim. Base-modifying chemicals came from Kodak (dimethylsulfate, hydrazine, and piperidine) and EM Science (formic acid). Radiochemicals were supplied by ICN (crude [γ-^{32}P]ATP, 7000 Ci/mmol; 1 Ci = 37 GBq) and New England Nuclear ([α-^{32}P]dGTP, 800 Ci/mmol). Protein structure analyses were carried out by the methods of Kyte and Doolittle (19) for hydrophobicity and Garnier *et al.* (20) for the secondary structure.

RESULTS

cDNA Analysis. Two different mouse PrP-specific cDNA clones were isolated by using the PrP-specific 20-mer oligonucleotide probe. One of these clones (clone 9) has been described (14). This clone contains a 1.7-kilobase (kb) fragment consisting of two segments (0.8 and 0.9 kb) joined by an *Eco*RI site (Fig. 1). Our DNA sequence data show that the 0.9-kb segment (subclone 9-13) contains a large open reading frame that includes the PrP amino acid sequence used to design the oligonucleotide probe. The 0.8-kb segment (subclones 9-9) contains a large poly(dA) segment corre-

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Abbreviations: PrP, prion protein; kb, kilobase(s).

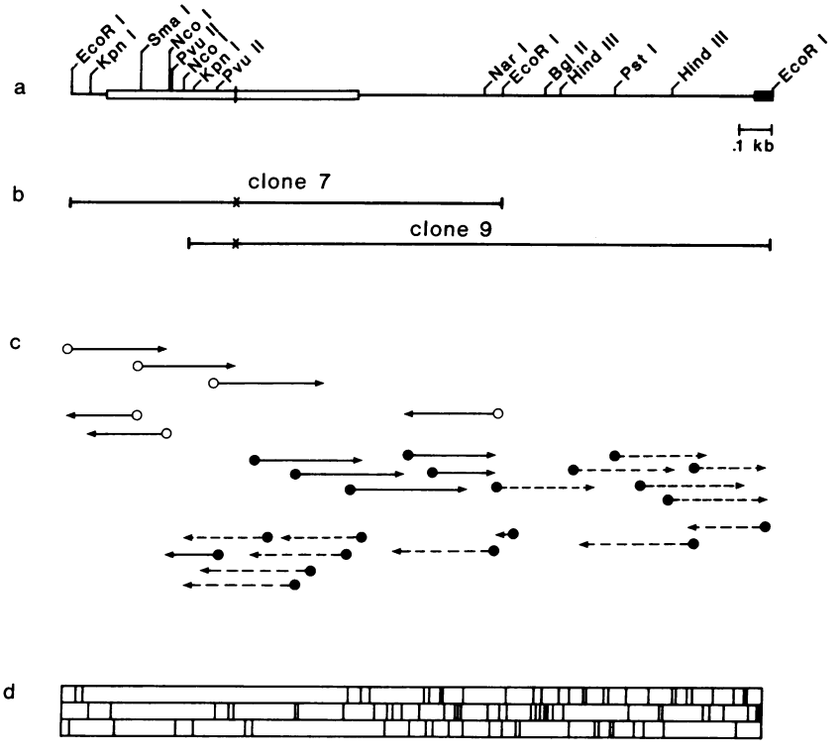

FIG. 1. Physical map of the PrP cDNA from scrapie-infected mouse brain. (*a*) Restriction map of the 2.1-kilobase-pair cDNA. The coding region is represented by the open box. The poly(A) tail is represented by the closed box. (*b*) The two lines represent the location and size of the cDNA inserts from the two clones used for the determination of the consensus sequence. The single nucleotide difference in the overlapping segments of the two clones is represented by asterisks. (*c*) Sequencing strategy: The direction and length of each sequence determination is indicated by the arrows. Solid arrows and broken arrows show sequence determination from clone 7 and clone 9, respectively. Maxam and Gilbert sequencing method was used for segments indicated by open circles and the dideoxy-chain termination method was used for segments indicated by closed circles. (*d*) Open reading frames in the three forward directions. Vertical lines represent stop codons.

sponding to the 3′ end of the mRNA. The second clone (clone 7) is 1.3 kb long and contains the entire 0.9-kb segment of clone 9 plus an additional 350 bases at its 5′ end that contains a leader sequence and the protein initiation site. The DNA sequencing strategy and a selected restriction map representing the entire mouse PrP cDNA are shown in Fig. 1. The DNA was sequenced in both directions by either the Maxam and Gilbert method, the dideoxy-chain termination method, or both. Each nucleotide was determined an average of 3.8 times.

The sense strand consensus sequence of the entire mouse PrP cDNA (Fig. 2) was compiled by evaluating both cDNA clones. The 0.9-kb overlapping segments in the two clones differ by only one nucleotide. Clone 7 contains an adenosine residue in position 496, whereas clone 9 contains a guanosine residue in the same position. This difference at the first position of the codon would cause an amino acid change from methionine to valine.

The complete cDNA is 2151 nucleotides long, including 54 deoxyadenosine residues corresponding to the 3′-terminal poly(A) tail of the mRNA. The cDNA contains a 5′ end noncoding leader sequence of 99 nucleotides, followed by 762 nucleotides coding for the PrP, and a heteropolymeric 3′ end noncoding sequence of 1236 nucleotides. The overall G+C content of 49.2% is very similar to that of the hamster PrP cDNA (49.1%) (13). There is a nearly equal distribution of all four nucleotides in the sense strand (25.7% for A, 24.5% for C, 24.7% for G, and 25.1% for T). The open reading frame from position 100–861 codes for a putative protein of 254 amino acids (including the amino-terminal methionine), which has a calculated molecular weight of 27,981 (see Fig. 2). The ATG at position 100–102 is most likely the initiation codon because it is the first ATG in the cDNA and it is surrounded by a typical eukaryotic translational initiation sequence—i.e., C ANN ATG G, where N can be any nucleotide, but is quite often a C residue (21). This sequence matches perfectly the sequence at the first ATG site in the mouse PrP cDNA sequence—i.e., C ATC ATG G. Furthermore, the PrP could not initiate upstream of this site, since two stop codons are found in the same reading frame. A 1.2-kb 3′ end noncoding region begins at residue 862 and contains 74 stop codons in the three forward reading frames (Fig. 1). At 21 residues from the poly(A) tail, the sequence ATTAAA is located. This is a functional analog of the 3′ end signal sequence AATAAA that directs endonucleolytic cleavage of the mRNA precursor prior to polyadenylylation (22, 23).

Deduced Amino Acid Analysis. The NH_2 terminus of the PrP is composed of mainly hydrophobic amino acids in a 20-amino acid region predicted to be in a partially α-helical partially β-pleated sheet conformation (Fig. 3); however, none of these amino acids fully satisfies von Heijne's rule for being the COOH-terminal amino acid residue of a possible signal peptide (24, 25). With this absence of a signal peptide cleavage site, it seems unlikely that this hydrophobic region has a protein export function. Structurally similar hydrophobic regions are also found in the central part and at the COOH terminus of the PrP (Fig. 3). These regions may play a role in anchoring the protein in the membrane rather than in an export or secretion process. Indeed, the PrP seems to be a membrane-associated protein (12). On the other hand, the hydrophobic regions may be involved in the assembly of the PrP into polymers through hydrophobic interactions. The predicted isoelectric point of the nascent protein is likely to be higher than 7, since the number of basic amino acids (n = 22) is significantly higher than the number of acidic amino acids (n = 15), but post-translational modifications, such as glycosylation, might significantly modify the isoelectric point (26). Hamster PrP has been shown to be a glycoprotein (26) and two glycosylation sites have been suggested (27). Indeed, inspection of the hamster and mouse amino acid sequences reveals two potential *N*-glycosylation sites between the second and third cysteines at amino acid position 180 and 196—i.e., the sequence Asn-X-Thr (28), where X stands for either Ile or Phe in PrP. The entire protein contains three cysteine residues at amino acid positions 22, 178, and 213, potentially involved in disulfide bridges.

A striking feature of this protein is that 13% of the residues are in four direct tandem repeats of eight amino acids—i.e., the octapeptide Trp-Gly-Gln-Pro-His-Gly-Gly-Ser/Gly, from

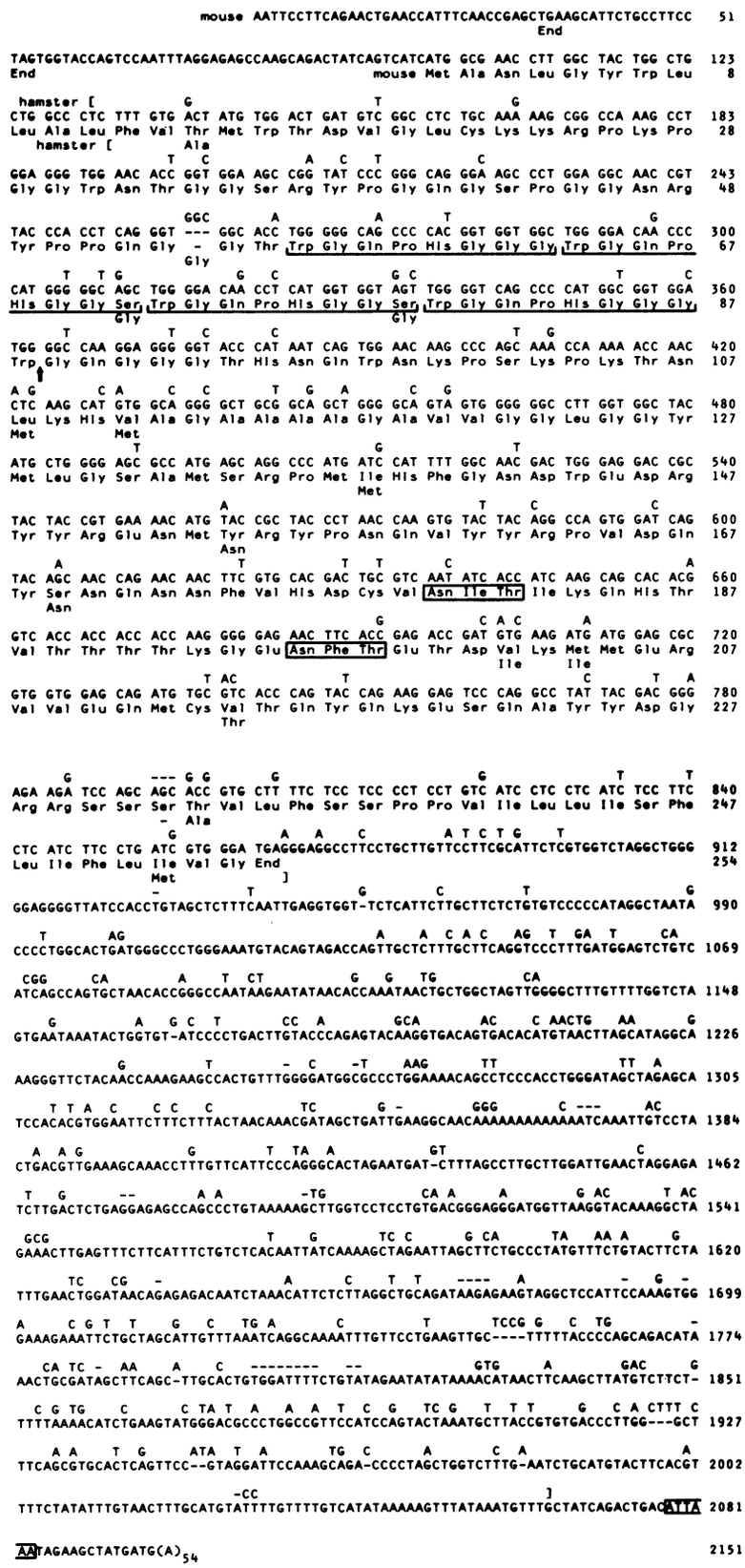

FIG. 2. Complete nucleotide and deduced amino acid sequences of the PrP cDNA from scrapie-infected mouse brain compared with the hamster PrP partial sequences. The mouse PrP nucleotide and amino acid sequences are numbered on the right. The beginning and the end of the hamster PrP cDNA and amino acid sequence (13) are indicated by brackets. Only differences in the hamster sequence are shown. End, stop codon; a dash indicates that the residue is not present in the compared sequence. The repeated octapeptides are underlined and the polyadenylylation signal is shown in the box. Arrow indicates the protease cleavage site for the hamster PrP. Glycosylation sites in the mouse PrP are shown in boxes.

position 56 to 87. In addition, remnants of this sequence are found on either side of the four repeats (Fig. 2). The suggested secondary structure of these repeats is composed exclusively of random coils and β-turns (Fig. 3). Identical repeats are also found in the hamster PrP sequence (see Fig. 2).

Comparison of Mouse and Hamster PrP Sequences. A comparison of the nucleotide and amino acid sequences between the complete mouse and the partial hamster cDNA and PrP is shown in Fig. 2. The published hamster cDNA sequence appears to be incomplete at both ends as it lacks the translational initiation site at the 5′ end, the first 11 codons, and the polyadenylylation signal at the 3′ end, as concluded by analogy to the mouse PrP sequence. The overall homology of the comparable sequences at the nucleotide level is 82%,

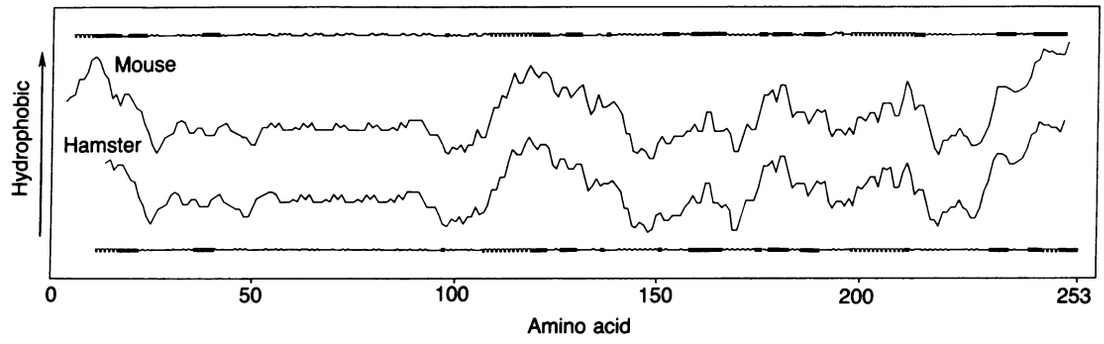

FIG. 3. Comparison of the hydropathy and predicted secondary structure profiles for mouse and hamster PrP. Secondary structures are represented by ····· for α-helix, — for β-sheets, ---- for turns, and — for random coils.

whereas, in the coding region, the cDNA homology is 89%. Interestingly, the 3′ end noncoding regions share less homology (76%) than the coding regions, except for the last 102 nucleotides of the hamster cDNA, where the homology increases to 92%. This striking homology at the 3′ end of the mRNA may indicate that this sequence plays a role in processing the mRNA (29, 30). This is consistent with the conservation of the sequences around the polyadenylylation signal site of other hamster and mouse genes, such as the αA crystallin gene (31). The fact that this conserved region in the PrP cDNA is different from the one in the αA crystallin cDNA might reflect some specificity in the expression of each of these genes.

When the coding regions of mouse and hamster PrP are compared, 60 of the 72 mismatches occur at the third position of the codons and only 3 of those 60 confer an amino acid substitution, whereas 10 nucleotide mismatches occur at the first position of the codons and all except 1 confer an amino acid substitution. Interestingly, of the 13 amino acid substitutions, 5 are due to two-point mutations per codon. All three cysteine residues and both potential glycosylation sites are conserved. The four tandem octapeptide repeats are also conserved, except that the last glycine residue in the second and third repeats is replaced by a serine residue in the mouse PrP. When compared with the hamster sequence, the mouse PrP is missing a complete codon for a glycine residue at cDNA position 258, whereas the hamster PrP is missing a complete codon for serine at cDNA positions 793–795. However, none of these differences changes the secondary structure or the hydrophobicity profile significantly (Fig. 3), nor should any of them interfere with or alter the protein folding. Thus, the tertiary structure of mouse and hamster PrP should be nearly identical.

DISCUSSION

PrP genes are present and transcribed in brains of both normal animals and those infected with the scrapie agent (13, 14). The gene product may have a unique functional role in brain, since transcription is greatly increased in brain relative to other tissues (14). However, no functional role for this protein in healthy animals is known. The scrapie-associated PrP appears to have a unique primary structure with no significant homology to any other known protein, including the mammalian neurofilament protein (32), which is an aggregating neuron-specific cytoskeletal element. However, the presence of four octapeptide repeats in the PrP resembles somewhat the structure of polyproteins, precursors for neuroendocrine peptides (33). The major difference between the repeating peptides in PrP and the neuroendocrine peptides found in polyproteins is the absence of basic amino acids flanking the octapeptides in PrP. These basic residues are important for post-translational cleavage of the neuroendocrine peptides. If the PrP is a polyprotein, an alternative proteolytic cleavage mechanism would be required.

Recently, the sequence of a wheat high molecular weight glutenin also revealed a series of similar repeated β-turn structures, rich in glycine, proline, and glutamine residues, which could be responsible for the formation of aggregates from monomers (34). Glycine-rich repeats in a β-turn conformation were also found in keratin (35) and type I collagen (36), which also assemble into filaments. By analogy to these proteins, it is possible that the repeated octapeptides in PrP may be involved in the polymerization of the PrP into rod-shaped scrapie-associated fibrils.

The NH_2-terminal amino acid sequence of the hamster PrP from infectious fractions (13) indicated that some proteolytic cleavage of PrP occurred after the four octapeptide repeats, as shown by the arrow in Fig. 2. Infectivity is not inactivated by this proteolytic cleavage (37), suggesting that, if PrP is the infectious agent, only the COOH-terminal part is needed for infection.

The presence and transcription of the *PrP* gene in uninfected brains is puzzling if PrP represents an infectious entity. On the other hand, if PrP is the transmissible agent that causes scrapie, the protein in infected tissues must differ in some subtle but important way from the PrP in normal tissues. The two PrP cDNA clones isolated from infected mouse brain indeed showed a single nucleotide difference, resulting in an amino acid difference. However, we have concluded that this discrepancy between the two clones probably represents an error in reverse transcription rather than a punctual difference in the mRNA for infectious and noninfectious PrP, since 1 error per 900 nucleotides in the reverse transcription of the mRNA is consistent with the infidelity rate of the avian myeloblastosis virus reverse transcriptase (38). It seems likely that the mRNA contains an adenosine rather than a guanosine residue at position 496, since the change from adenosine to guanosine generally occurs more frequently in reverse transcription than a change from guanosine to adenosine. Furthermore, hamster PrP cDNA also contains an adenosine in this position.

Adaptation of a mouse-passaged strain of the scrapie agent to hamsters occurred after three serial passages in hamsters over the course of 20 months (3). This switch in species specificity probably involved mutational changes in the structure of genes encoding the agent. These changes would not be expected to mimic the evolutionary differences observed in normal endogenous genes of mouse and hamster. Sequence differences of homologous genes in relationship to evolutionary divergence between two species can be analyzed in the open reading frame section of the gene by calculating the frequency of base changes located in the third position of codons giving synonymous amino acids (39, 40). The evolutionary rate observed for these bases is similar to the variation of bases in 5′ portion of the 3′ noncoding region of the mRNA, which has the least amount of selective pressure. The frequency of synonymous changes between the hamster and mouse *PrP* genes was 57/254 = 0.22. The

minimum and maximum frequency of variation in the noncoding region from position 912 to 1927, as calculated by the method described by Miyata *et al.* (39, 40), was found to be 0.19–0.23. Thus, both the coding and noncoding portions of mouse and hamster *PrP* genes were evolving at a similar rate, as has been found for several other genes (39, 40). For both the coding and noncoding portions of the *PrP* gene, the calculated time of evolutionary divergence between hamster and mouse is $\approx 26 \times 10^6$ years, according to the formula proposed by Miyata *et al.* (39, 40). The same calculation applied to the noncoding sequence of αA crystallin genes in mouse and hamster (31) gives about the same time of divergence. This divergence time for the *PrP* and αA crystallin genes is in relatively good agreement with the paleontologically deduced divergence time and argues against the possibility that the *PrP* gene could have been introduced into the hamster genome from the mouse by horizontal gene transfer (41). The difference between the mouse and hamster PrP mRNAs thus appears to be due to evolutionary divergence rather than virus strain adaptation through a few hamster passages.

Although much of the pathology of mouse-adapted scrapie and related human diseases differs from that of Alzheimer disease, amyloid deposits occur in the brain tissue in both disease groups. This similarity in amino acid composition and the presence of β-pleated sheets may explain some of the amyloid-like characteristics of the proteins found in deposits in the two disease groups (42, 43). However, antiserum raised against hamster PrP does not cross-react with these Alzheimer-specific proteins, whereas it does cross-react with preparations of brain tissue from patients with Creutzfeldt–Jakob disease, kuru, and Gerstmann–Straussler syndrome (44). Moreover, no significant sequence homology exists between either the mouse or hamster PrP and the paired helical filaments composing the intracellular neurofibrillary tangles or the extracellular amyloid plaque core proteins of Alzheimer disease (45, 46). In spite of differences in the proteins contained in the amyloids of these two disease groups, the accumulation of such insoluble protein deposits might cause subsequent similar pathogenic effects. Indeed, an example of this possibility might be the recent observation that transcription of the *Scr-1* gene was found to be increased in brains of mice infected with the scrapie agent and in brain tissues of persons with Alzheimer disease, as well as other dementias, and multiple sclerosis (47).

We thank Dr. William Hadlow for helpful suggestions and critical reading of the manuscript; Sylvia Perryman, Jane Nishio, and Kathy Wehrly for excellent technical assistance; Gary Hettrick and Robert Evans for graphics; and Susan Smaus, Helen Blahnik, Betty Kester, and Susan Smith for their help in preparing the manuscript.

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