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Propagation of Prion Strains through Specific Conformers of the Prion Protein

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Two prion strains with identical incubation periods in mice exhibited distinct incubation periods and different neuropathological profiles upon serial transmission to transgenic mice expressing chimeric Syrian hamster/mouse (MH2M) prion protein (PrP) genes [Tg(MH2M) mice] and subsequent transmission to Syrian hamsters. After transmission to Syrian hamsters, the Me7 strain was indistinguishable from the previously established Syrian hamster strain Sc237, despite having been derived from an independent ancestral source. This apparent convergence suggests that prion diversity may be limited. The Me7 mouse strain could also be transmitted directly to Syrian hamsters, but when derived in this way, its properties were distinct from those of Me7 passaged through Tg(MH2M) mice. The Me7 strain did not appear permanently altered in either case, since the original incubation period could be restored by effectively reversing the series of passages. Prion diversity enciphered in the conformation of the scrapie isoform of PrP (PrP^{Sc}) (G. C. Telling et al., Science 274:2079–2082, 1996) seems to be limited by the sequence of the PrP substrates serially converted into PrP^{Sc}, while prions are propagated through interactions between the cellular and scrapie isoforms of PrP.

Prion multiplication is accompanied by a radical change in the conformation of the prion protein (PrP). Spectroscopic studies showed that the cellular isoform (PrP^{C}) is rich in α-helices and contains little β-sheet, whereas the scrapie isoform (PrP^{Sc}) is rich in β-sheet and contains substantially less α-helix (53, 57, 67). A mechanism for prion multiplication in which a specific conformational change is induced in PrP^{C} following interaction with PrP^{Sc} appears to explain best the available data (24).

Prion strains with distinct biological properties have long been recognized since the identification of two strains of scrapie in goats, described originally as "scratching" and "drowsy" (56). Subsequent studies have detailed the appearance of numerous prion strains following serial passage in rodents (10, 13, 20–22, 85), and studies of scrapie in inbred mice demonstrated the existence of a genetic locus, *Sinc*, which profoundly influenced the scrapie incubation period of mouse (Mo) prion strains (27, 29). Subsequent molecular genetic studies showed that the *Sinc* locus was probably synonymous with the scrapie incubation period determinant *Prn-i* and that both were linked to the structural gene for PrP designated *Prnp* in mice (18, 38). Mice with different alleles of the *Sinc*/*Prn-i* gene encoded PrP proteins which differ at two amino acid residues; these *Prnp* alleles were designated $Prnp^{a}$ and $Prnp^{b}$ (82). Whereas previous genetic investigations had described the overdominance of the "long" incubation period allele (27), studies using transgenic mice with supernumerary copies of murine PrP genes demonstrated that the lengthening of incubation time in mice with the long allele reflects a relative reduction in the level of expression of the short allele (17, 83). These findings argue that the scrapie incubation time gene (*Sinc* or *Prn-i*) is congruent with the PrP gene.

While previous comparisons of prion strains did not reveal any biochemical or physical differences in PrP^{Sc} (36), an altered sensitivity to cleavage with protease was evident in PrP^{Sc} when two prion strains isolated from mink with transmissible encephalopathy were compared (6). One strain (HY) produced hyperactivity in Syrian hamsters, and the other (DY) was manifest as a drowsy syndrome like the scrapie strains first seen in goats (5, 6). PrP^{Sc} produced by the DY prions showed diminished resistance to proteinase K digestion and truncation of the N terminus compared to HY and many other strains (7), providing evidence for the hypothesis that different strains might represent different conformers of PrP^{Sc} (58). It is noteworthy that this altered sensitivity to protease displayed by the DY strain in vivo was demonstrated in vitro when partially denatured, radiolabelled PrP^{C} was bound to PrP^{Sc} (4), although it was not possible to demonstrate the propagation of infectious prions using this system.

While the unusual properties of DY were provocative and are consistent with a mechanism whereby prion diversity is enciphered in PrP conformation, all other prion strains that we had studied in rodents were indistinguishable from the HY strain with respect to sensitivity to protease cleavage (41, 73). The studies reported here also support the notion that the biochemical properties of DY are not exhibited by other rodent strains. Since DY appeared to be an isolated case among rodent prion strains, with obscure origins, it could be argued that the unusual properties of the DY strain provided little insight into the mechanism of prion diversity.

Compelling support for the notion that PrP^{Sc} tertiary structure enciphers the information for each strain emerged with the transmission of two different inherited human (Hu) prion diseases to mice expressing chimeric Hu/Mo PrP transgenes. In fatal familial insomnia, the protease-resistant fragment of PrP^{Sc} after deglycosylation has a molecular mass of 19 kDa, whereas that from other inherited and sporadic prion diseases is 21 kDa (51, 54). Extracts from the brains of patients with fatal familial insomnia transmitted disease to mice expressing a chimeric human (Hu)/Mo (MHu2M) PrP gene about 200 days

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after inoculation and induced formation of the 19-kDa PrP^{Sc}, whereas familial Creutzfeldt-Jakob disease (CJD) (E200K) and sporadic CJD produced the 21-kDa PrP^{Sc} in these mice (78). These findings argue that PrP^{Sc} acts as a template for the conversion of PrP^{C} into nascent PrP^{Sc}. Imparting the size of the protease-resistant fragment of PrP^{Sc} through conformational templating provides a mechanism for the propagation of both prion strains. These studies and those of DY (7) suggest that propagation of distinct prion strains must occur through specific conformers of the prion protein.

Resolution of the detailed mechanism of prion diversity will require the determination of the complete molecular structure of at least two distinct prion strains. Despite the fact that many previous studies on scrapie strains have exploited the mouse as a host, almost all studies on the molecular structure of scrapie prions conducted to date have used Syrian hamsters in preference to other hosts such as mice. This results from the well-recognized advantages of hamsters over mice in the large-scale preparation and purification of scrapie prions (63). Although illuminating, previous attempts to study hamster prion strains by physical and biochemical means (35) were hampered by problems of stability when large-scale preparations of prions were attempted (34a) as well as by the limited availability of distinct SHa prion strains.

Faced with problems of prion strain stability, we decided to exploit the ability of transgenic (Tg) mice expressing the chimeric MH2M PrP gene [Tg(MH2M) mice] to facilitate transmission of established Mo prion strains into Syrian hamsters (70, 71). With Tg(MH2M) mice, we sought to expand the available spectrum of SHa prion isolates and to avoid problems with strain stability of long-incubation-period isolates during amplification prior to inoculation of large populations of Syrian hamsters for preparative purposes.

In the course of our studies, we found that strain characteristics depend little, if at all, on the original ancestral source. Instead, we found a completely different strain can be established in the same host, starting from the same source inoculum, if we included a single intermediate passage in a host with a distinct but closely related PrP sequence. In previous studies, in which distinct isolates were passaged between closely related species of hamsters (36) or between Tg(MH2M) mice, Syrian hamsters, and normal mice (70), we showed that strain properties may be restored by reversing the passage history. Since the Me7 isolate could be passaged directly to Syrian hamsters, and the original strain could also be restored by reversing this sequence of transmissions (44), the dramatically different properties of the SHa(Me7) and Me7-H strains argue that prion diversity enciphered in the conformation of PrP^{Sc} is limited by the primary structure of PrP and that differences in the sequence of PrP can force prion strains to change during propagation, presumably by restricting the number of available conformers. We also show that two prion isolates with virtually indistinguishable characteristics may be generated from completely different ancestral sources (66), providing further evidence that prion diversity is limited to a finite and probably highly restricted number of conformations of PrP^{Sc} that can be adopted by the sequence of PrP encoded by the host.

MATERIALS AND METHODS

Scrapie prion isolates. The Chandler isolate (20) was originally provided by W. Hadlow and was propagated in Swiss mice at the Rocky Mountain Laboratory and subsequently in this laboratory in Swiss CD-1 mice obtained from Charles River Laboratories. We designated this isolate RML (for Rocky Mountain Laboratory). The Sc237 hamster was originally obtained from Richard Marsh (49) and was passaged repeatedly in golden Syrian hamsters (LVG:Lak) purchased from Charles River Laboratories. This strain appears indistinguishable from strain 263K (43). The Me7 and 22A mouse strains as well as the Me7-H hamster isolate were a generous gift of Richard Kimberlin and Richard Carp. The DY hamster strain was kindly provided by Richard Marsh (48).

Determination of scrapie incubation periods. Mice were inoculated intracerebrally with 30 μl of a 1% brain homogenate containing scrapie prions, using a 27-gauge disposable hypodermic syringe needle inserted into the right parietal lobe. Hamsters received 50 μl. Diagnoses of scrapie in hamsters, mice, and Tg mice have been extensively described elsewhere (18, 60, 64). Once clinical signs were detected, the animals were inspected daily and sacrificed when death was clearly imminent. A representative fraction of brains was removed for histological analysis to confirm the diagnosis of scrapie.

Chimeric PrP genes and Tg mice. The construction of Tg mice with chimeric PrP genes has been described previously (70, 71). Determinations of transgene copy number and evaluation of transgene-derived PrP expression were accomplished as previously reported (70, 71).

Preparation of brain homogenates and microsomal fractions. Homogenates (10% [wt/vol]) of rodent brains were prepared by repeated extrusion (four to six times) through a 16-gauge, then an 18-gauge, and finally a 22-gauge syringe needle in phosphate-buffered saline. Connective tissue and large particulate matter were allowed to settle out and were avoided prior to use. Microsomal membranes were prepared as described previously (5).

Protease digestion. Brain homogenates or enriched microsomal membrane fractions were adjusted to 1 mg of total protein ml^{-1} in 25 mM Tris-Cl (pH 7.4)–150 mM NaCl–2% Sarkosyl. Proteinase K was added to a final concentration of 100 μg ml^{-1} and incubated for various times as indicated in the figure legends, and the reaction was terminated by addition of phenylmethylsulfonyl fluoride to 1 mM. An equal volume of 2× sodium dodecyl sulfate sample buffer was added before heating to 100°C for 5 min. Each lane was loaded with 10 μg of total protein as measured prior to digestion.

Detergent solubility assay. Brains from either normal or scrapie-infected mice were homogenized in 10 volumes of buffer A (1% [wt/vol] Triton X-100 and 1% [wt/vol] sodium deoxycholate in phosphate-buffered saline) by successive extrusions through 16-, 18-, and 20-gauge needles. The lysate was centrifuged at 15,000 × *g* for 20 min at 4°C, and the supernatant and pellets then examined by Western blotting (76a).

Immunoblotting. Western immunoblot analyses were performed essentially as previously described (1, 2, 52, 80) except that an enhanced chemiluminescence detection method (Amersham, Arlington Heights, Ill.) was used. Protein assays were performed by using bicinchoninic acid assay reagents obtained from Bio-Rad Laboratories.

Neuropathology. Brain tissue was immersion fixed in 10% buffered formalin solution after the animals were sacrificed. The brains were embedded in paraffin, and histological sections were prepared and stained with hematoxylin and eosin for evaluation of spongiform degeneration. Peroxidase immunohistochemistry with antibodies to glial fibrillary acidic protein was used to evaluate the degree of reactive astrocytic gliosis. Vacuolation scores were determined in 8-μm-thick hematoxylin-eosin-stained sections. The score represents the area occupied by vacuoles in a high-power field: 0, no vacuoles; 3, questionable prion disease related vacuolation; 5, mild vacuolation; 20, moderate; 70, severe.

Histoblots for PrP^{Sc}. Histoblotting was performed as described previously (74). Animals were sacrificed by asphyxiation with CO_2. The brain was removed rapidly and frozen in powdered dry ice. Cryostat sections 10 μm thick were cut, mounted on glass, thawed, and pressed to a nitrocellulose membrane wetted in lysis buffer containing 0.5% Nonidet P-40, 0.05% sodium deoxycholate, 100 mM NaCl, 10 mM EDTA, and 10 mM Tris-HCl (pH 7.8) (74). The slide was pressed to the nitrocellulose for 25 s and checked for complete transfer. To eliminate PrP^{C} from the section, the membranes were air dried, rehydrated for 1 h in Tris-buffered saline containing 0.05% Tween 20, 100 mM NaCl, and 10 mM Tris-HCl (pH 7.8) (TBST), and exposed for 18 h at 37°C to proteinase K (400 μg/ml) in a buffer containing 0.5% Brij 35, 100 mM NaCl, and 10 mM Tris-HCl (pH 7.8). To terminate the reaction, the blots were rinsed three times in TBST and immersed for 30 min in TBST containing 3 mM phenylmethylsulfonyl fluoride. To enhance the immunostaining of PrP^{Sc}, the histoblots were exposed to 3 M guanidinium isothiocyanate for 10 min at room temperature in 20 mM Tris-HCl (pH 7.8) and rinsed three times with TBST before immunostaining.

RESULTS

Prion transmission into Tg(MH2M) mice expressing chimeric PrP. When the Me7 and RML Mo prion strains were passaged in CD-1, C57BL, or FVB mice, the incubation times for both strains were ~150 days (data not shown); however, passage of the two strains through Tg(MH2M)92/FVB mice produced very different incubation times of 262 and 144 days, respectively (Tables 1 and 2; Fig. 1 and 2). Subsequent passage of these chimeric prions produced in the Tg(MH2M)92/FVB mice yielded several unexpected results. First passage of Me7 and RML chimeric prions in the Tg(MH2M)92/FVB mice gave substantially different incubation times of 88 and 124 days, respectively. Me7 also produced shorter incubation times than

TABLE 1. Nomenclature and characteristics of mice, hamsters, and Tg mouse lines

Animal line designation	Expressed PrP^C molecules	PrP transgene expression (fold)[a]	Source[b]
Non-Tg mice			
CD-1	Mo[c]		Charles River Laboratories
C57BL	Mo		Charles River Laboratories
FVB	Mo		Charles River Laboratories
Tg(MH2M) mice			
Tg(MH2M)455/FVB	Mo, MH2M	1–2	UCSF
Tg(MH2M)92/FVB	Mo, MH2M	4–8	UCSF
Tg(MH2M)229/FVB	Mo, MH2M	16–32	UCSF
Tg(MH2M)$Prnp^{0/0}$ mice			
Tg(MH2M)455/$Prnp^{0/0}$	MH2M	1–2	UCSF
Tg(MH2M)92/$Prnp^{0/0}$	MH2M	4–8	UCSF
Tg(MH2M)229/$Prnp^{0/0}$	MH2M	16–32	UCSF
Hamsters			
Syrian golden	SHa		Charles River Laboratories

[a] Level of PrP transgene expression in brain was measured by serial dilution of the samples followed by dot immunoblotting. Each sample was compared to PrP^C in non-Tg mouse brain.
[b] Animals used in this study were either purchased from Charles River Laboratories or produced in our facility at the University of California, San Francisco (UCSF).
[c] Refers to PrP^C-A.

RML prions in Tg(MH2M)FVB mice expressing high levels of chimeric PrP but not in mice expressing the transgene at low levels. While Tg(MH2M)92/FVB mice express chimeric PrP at 4- to 8-fold above wild-type (wt) MoPrP, the Tg(MH2M)455/FVB and Tg(MH2M)229/FVB lines express chimeric PrP at 1- to 2- and 16- to 32-fold, respectively (Table 1). These three different Tg lines permitted us to evaluate the relationship between incubation times and the levels of PrP expression.

An inverse relationship between chimeric PrP transgene expression and incubation period was observed for each prion strain that mirrored results reported previously for the transmission of SHa prions in Tg(SHaPrP) mice (64). But as noted above, this relationship between two strains was not always maintained as in the case of Tg(MH2M)455/FVB mice expressing low levels of chimeric PrP. Chimeric Me7 and RML prions inoculated into these mice gave incubation times of 187 and 132 days, respectively.

Finally, subsequent transmission of both chimeric strains, i.e., passaged in Tg(MH2M)92/FVB mice, in homologous Tg mice shortened the incubation times but to a different extent for each strain. The incubation time for chimeric Me7 prions in Tg(MH2M)92/FVB mice was drastically reduced, from 262 to 88 days (Fig. 1), whereas the incubation time for chimeric RML prions was only modestly decreased, from 144 to 124 days (Fig. 2).

Prion transmission into Tg(MH2M) mice deficient for MoPrP. The analysis of chimeric prions produced in Tg(MH2M)FVB mice was complicated by the fact that these mice express both chimeric and wt PrP^C. The availability of mice in which the MoPrP gene had been disrupted by homologous recombination ($Prnp^{0/0}$) (15) offered the opportunity to generate mice expressing only chimeric PrP. By crossing $Prnp^{0/0}$ mice with Tg(MH2M)FVB mice, we derived the Tg(MH2M)92/$Prnp^{0/0}$ and Tg(MH2M)229/$Prnp^{0/0}$ lines (Table 1).

Elimination of endogenous MoPrP led to a reduction of incubation period with the chimeric Me7 and RML prion isolates, but the extent of this decrease in the length of the incubation time was dependent on both the level of transgene expression and the prion strain. For example, Tg(MH2M)229/FVB mice inoculated with chimeric Me7 prions exhibited an incubation period of 74 days, whereas the Tg(MH2M)229/$Prnp^{0/0}$ mice showed an incubation time of 50 days (Fig. 1). In contrast, the effect of *Prnp* ablation for chimeric RML prions was minimal, with only a 6-day reduction, from 87 to 81 days, in Tg(MH2M)229/$Prnp^{0/0}$ mice (Fig. 2). When Tg(MH2M)92/FVB mice were inoculated with chimeric Me7 prions, they exhibited an incubation period of 88 days, whereas the Tg(MH2M)92/$Prnp^{0/0}$ mice showed an incubation time of 65 days (Fig. 1). For chimeric RML prions, the reduction in incubation time was of a similar magnitude for Tg(MH2M)92/$Prnp^{0/0}$ mice from 124 to 94 days (Fig. 2).

Transmission of chimeric Me7 and RML prions to Syrian hamsters. When Syrian hamsters were inoculated with chimeric Me7 prions, signs of neurologic dysfunction developed 136 days postinoculation (Fig. 3A). Subsequent passage of Me7 prions in Syrian hamsters, to produce the isolate which we term SHa(Me7), gave an incubation time of 78 days. This incubation period in hamsters following passage of Me7 through Tg(MH2M)92/FVB mice is indistinguishable from that which we routinely obtain with the Sc237 isolate (Table 2). Our findings contrast dramatically with those for Me7 prions transmitted directly from C57BL mice to Syrian hamsters, where the incubation time is ~300 days (Fig. 3A); this isolate was termed Me7-H (44, 46).

Extracts of the brains of Tg(MH2M)92/FVB mice infected with Mo(RML) prions were also transmitted to Syrian hamsters with an incubation time of 216 days (70). On second and third passages, SHa(RML) displayed incubation times of 175 and 180 days. The incubation time for SHa(RML) is similar to that reported for 139H, which was derived by passage of 139A prions in Syrian hamsters (36, 46). Both the 139A and RML strains are derived from the Chandler isolate (Table 2) (20).

Neuropathology of prion strains in Syrian hamsters. The distribution of vacuolation in the brains of inbred mice has been used frequently to characterize strains of prions and to differentiate one strain from another (13, 30, 32, 33). Based on the results of these earlier studies, we performed a detailed neuropathological analysis of brains of Syrian hamsters inoculated with the SHa(RML), SHa(Me7), and Me7-H strains, using as a control the SHa isolate Sc237. The hamsters were sacrificed after clinical signs of central nervous system dysfunction appeared, and their brains were removed. Quantitative analysis of the regional distribution of spongiform degeneration in 10 brain regions was performed (Fig. 4). The distribution of lesions observed with the Me7-H strain was different from those found with the other three strains. The most prominent differences were observed in the cortex and cingulate

TABLE 2. Nomenclature and characteristics of prion strains

Prion strain	Animal source for this study	Original source	Reference
Me7	C57BL mouse	Suffolk sheep	27
RML	CD-1 mouse	Cheviot sheep	20
Me7-H	Syrian hamster	Suffolk sheep	46
Sc237	Syrian hamster	Cheviot sheep	49
DY	Syrian hamster	Mink	48
SHa(Me7)	Tg(MH2M) mouse	Suffolk sheep	This study
SHa(RML)	Tg(MH2M) mouse	Cheviot sheep	70

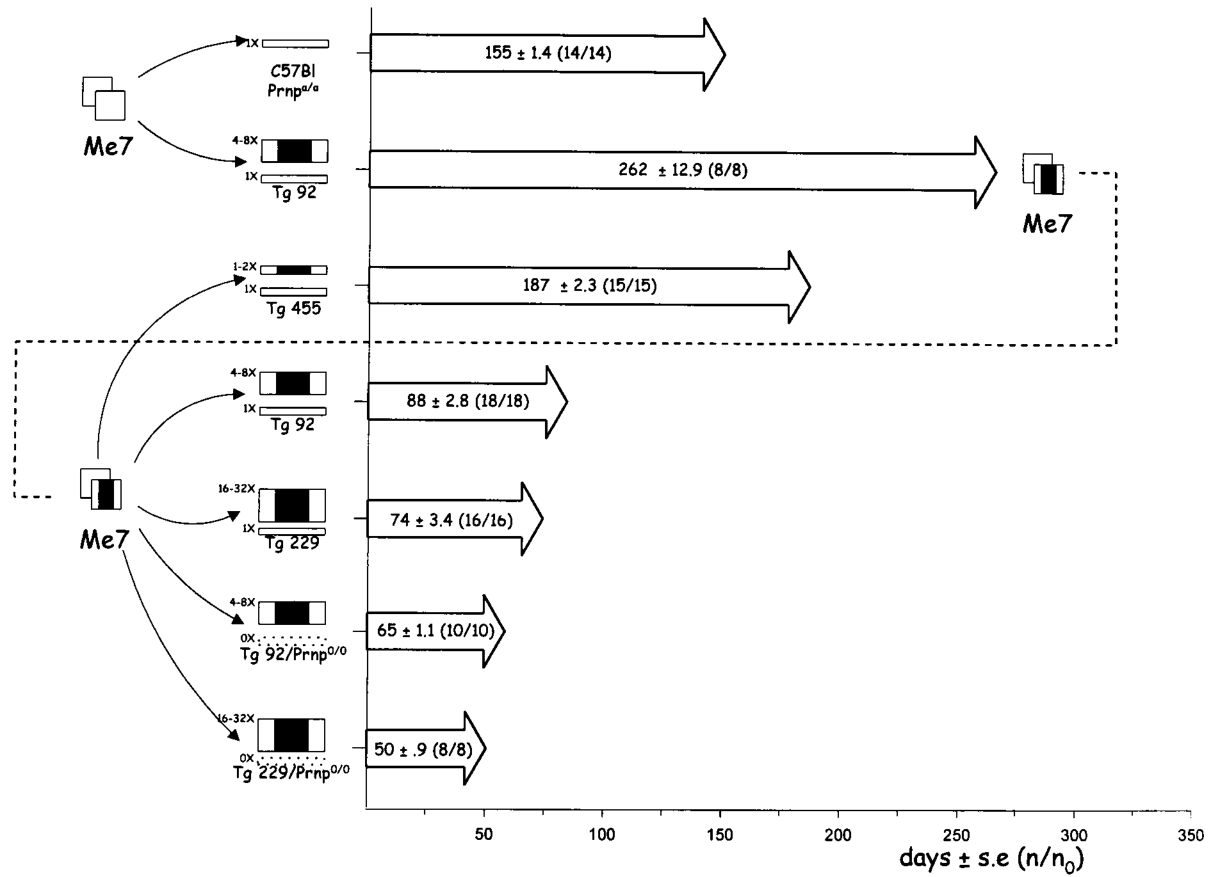

FIG. 1. Passage history of Me7 strain in Tg(MH2M) mice. The first column represents the inocula used. The icon summarizes the most recent passage history. A white square represents previous passage in a Prnp$^{a/a}$ mouse; a black square represents a previous passage in Syrian hamsters; passage through Tg(MH2M) mice is represented by a white square with a black vertical bar. The most recent passage is at the front. The second column represents the host animal used. The wide bars represent the PrP genes expressed in the host animals, and the color scheme corresponds to that described for passage history. The approximate level of expression of the transgene, as well of the endogenous MoPrP alleles, is depicted by the height of the bar and the text to the left of the icon. A disrupted endogenous MoPrP gene is depicted by a dotted line. The third column shows the incubation period relative to onset of symptoms as arrows of various lengths relative to the scale at the bottom. The incubation period in days, together with the standard error and the ratio of number of sick animals to total surviving animals, is shown within the arrow. In some cases where a brain from an inoculation was used in a subsequent experiment, it is depicted next to the arrow and linked to the appropriate experiment by a dotted line. Serially passaged Me7 was inoculated into Tg(MH2M)92 mice and non-Tg Prnp$^{a/a}$ mice. Following the initial passage in Tg(MH2M), the chimeric MH2M(Me7) was inoculated into several Tg(MH2M) lines, Tg(MH2M)455, Tg(MH2M)92, and Tg(MH2M)229, which express various amounts of MH2MPrP (Table 1). In addition, we inoculated Tg(MH2M)92 and Tg(MH2M)229 mice in which the transgene arrays were crossed into a background lacking endogenous MoPrP. In each case, two independent brains were used in two separate experiments.

gyrus, which were severely affected with Me7-H and mildly affected with SHa(Me7) or Sc237. The white matter also appeared almost completely unaffected with SHa(Me7) or Sc237, in contrast to Me7-H. In fact, apart from a slight difference in the white matter of SHa(RML), the SHa(Me7), SHa(RML), and Sc237 strains are virtually indistinguishable based on profiles of vacuolation; yet the 180-day incubation time of SHa(RML) clearly distinguishes it from SHa(Me7) and Sc237, both of which have much shorter incubation times (Fig. 3). That the SHa(Me7) and Sc237 strains produced similar patterns of spongiform degeneration is noteworthy since these two strains have very different origins (Table 2).

Since early studies established that PrP^{Sc} deposition precedes spongiform degeneration and astrocytic gliosis (36, 39), we compared the patterns of PrP^{Sc} accumulation in the brains of Syrian hamsters inoculated with SHa(Me7), Me7-H, Sc237, or SHa(RML) prions. Whereas SHa(Me7) and Sc237 showed only slight differences in the intensity and distribution of the PrP^{Sc} signal, Me7-H and SHa(RML) were markedly different (Fig. 5). That the distribution of PrP^{Sc} in the brains of hamsters inoculated with the Me7-H strain was different from those found with the other three strains is in accord with the neuropathologic analysis described above.

Convergence of prion strains. The similar incubation times, vacuolation profiles, and patterns of PrP^{Sc} deposition in Syrian hamsters inoculated with either the SHa(Me7) or Sc237 strain raised the possibility that these two strains represent an example of strain convergence. That the SHa(Me7) and Sc237 strains have markedly different origins and passage histories yet possess such similar properties in hamsters prompted further comparisons. The incubation periods for these two prion strains were indistinguishable when passaged in C57BL mice, Syrian hamsters, or Tg(MH2M)Prnp$^{0/0}$ lines expressing different levels of chimeric PrP (Fig. 6). In this experiment, we used mice in which the transgene arrays had been crossed into a mouse background devoid of endogenous MoPrP. In each of the experiments shown, the strain was first passaged in the homologous host prior to measurement of the incubation time (Fig. 6). In every instance, Me7 and Sc237 yielded similar incubation periods (Fig. 6). To confirm the identity of Mo-

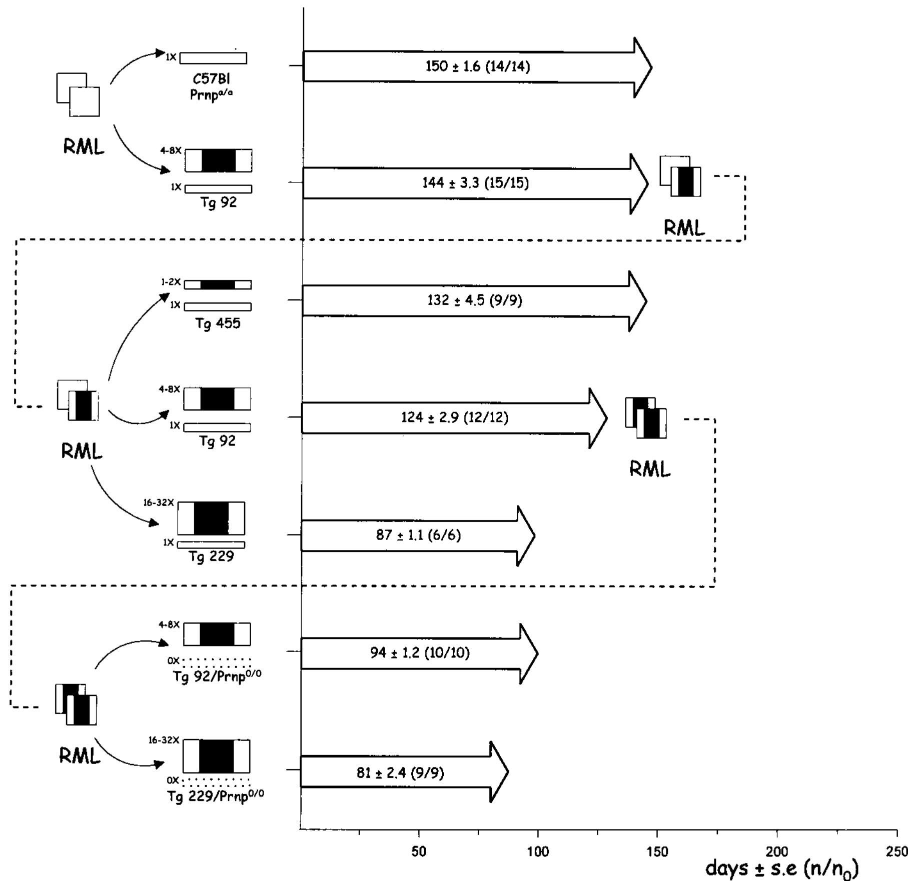

FIG. 2. Passage history of RML strain in Tg(MH2M) mice. The schematic is as described in the legend to Fig. 1. Serially passaged RML was inoculated into Tg(MH2M)92 mice and non-Tg $Prnp^{a/a}$ mice. Following the initial passage in Tg(MH2M), the chimeric MH2M(RML) was inoculated into several Tg(MH2M) lines, Tg(MH2M)455, Tg(MH2M)92, and Tg(MH2M)229, which express various amounts of MH2MPrP. Following the second passage in Tg(MH2M)92 mice, we inoculated Tg(MH2M)92 and Tg(MH2M)229 mice in which the transgene arrays were crossed into a background lacking endogenous MoPrP.

passaged Sc237 and Me7, we also performed a detailed neuropathological analysis of the distribution of vacuolation in C57BL mice infected with either Mo(Sc237) or Me7. The distribution of vacuolation was similar for both strains in C57BL mice (data not shown).

The indistinguishable properties of the Me7 and Sc237 strains following passage into mice, Tg(MH2M) mice, and Syrian hamsters (Fig. 4 to 6) make it highly unlikely that they represent different strains with fortuitously similar properties, since these similarities are propagated between different species in an identical manner (Fig. 6). Of note, we have also attempted to passage the SHa(Me7) strain into C57BL mice. These mice are refractory to transmission of Sc237 prions (69) and have not shown signs of disease in excess of 600 days following inoculation with SHa(Me7). In contrast, SHa(Me7) prions could be passaged into Tg(MH2M) mice and then subsequently into C57BL mice (data not shown).

Strain-specific properties of PrP^{Sc}. Although the relative resistance of prions to digestion by proteases facilitated discovery of the N-terminally truncated fragment of PrP^{Sc} designated PrP 27-30 (8, 59), this property of prions is not an obligatory feature of infectivity (37, 77). In Syrian hamsters, prions passaged from mink produced two different strains, designated HY and DY. The HY strain seems to be similar to Sc237 and SHa(Me7), but the DY strain is unique in that it shows very modest resistance to protease digestion (6). Furthermore, the DY strain exhibits greatly prolonged incubation times. Because of the interesting characteristics of the DY strain, we examined whether the prolonged incubation times displayed by either the SHa(RML) or Me7-H strain in hamsters were accompanied by a diminution in the protease resistance of PrP^{Sc}. The protease resistance of PrP^{Sc} was found to be independent of the incubation time of the particular prion strain which was inoculated into Syrian hamsters. PrP^{Sc} molecules in homogenates prepared from the brains of hamsters inoculated with Sc237, SHa(RML), Me7-H, or SHa(Me7) prions all exhibited a marked resistance to digestion by proteinase K (100 μg/ml). In contrast, PrP^{Sc} produced by the DY strain was digested in less than 1 h under these conditions (Fig. 7A). Intriguingly, when enriched microsomal membrane preparations were treated with proteinase K under identical conditions, a significantly higher level of protease-resistant PrP was noted in animals infected with DY (Fig. 7B), perhaps reflecting an increased recovery of DY prions relative to Sc237 prions in these fractions (Fig. 7B).

While the properties of the DY strain seem to be anomalous, they are nevertheless of interest. Other studies of the DY prions suggested that PrP^{Sc} sediments less rapidly than PrP^{Sc} produced by the HY strain (6). Based on these findings with the DY strain, we examined whether the prolonged incubation times displayed by either the SHa(RML) or Me7-H strain in hamsters were accompanied by a diminution in the sedimentation of PrP^{Sc}. Brain homogenates were detergent extracted, insoluble proteins were recovered by sedimentation at

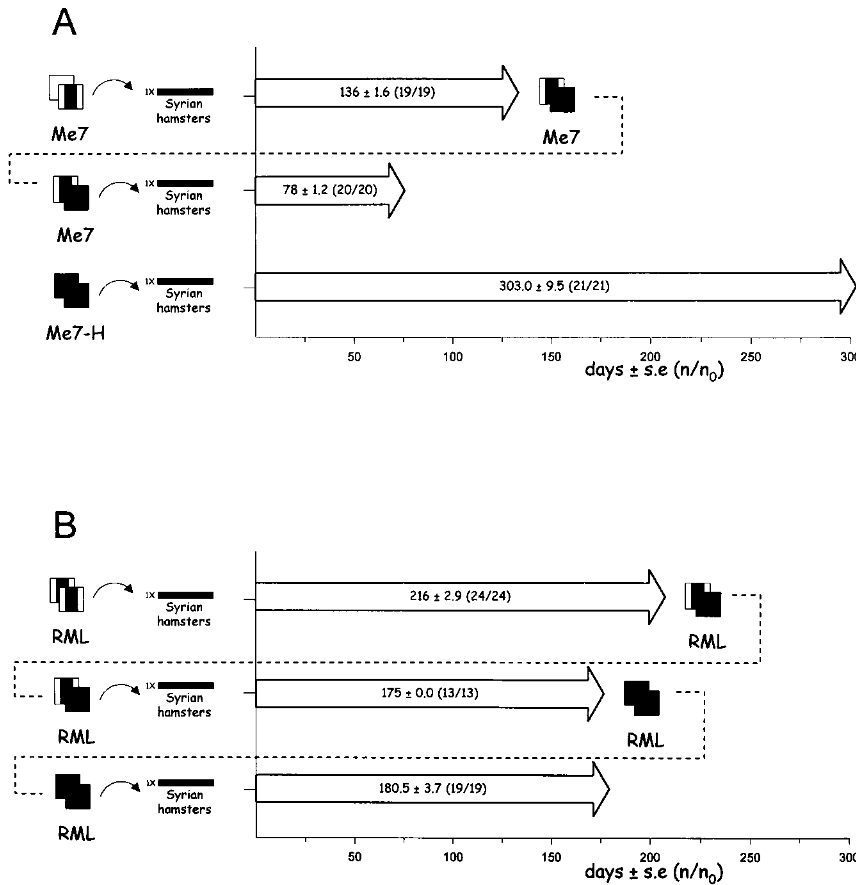

FIG. 3. Transmission of Me7, Me7-H, and RML strains in Syrian hamsters. The schematic is as described in the legend to Fig. 1. (A) MH2M(Me7) prions were obtained as depicted in Fig. 1 and used to inoculate Syrian hamsters. Two independently isolated brains were taken and passaged a second time in Syrian hamsters. In a parallel study, Syrian hamsters were inoculated with Me7-H which had been serially passaged in Syrian hamsters (B) MH2M(RML) prions were obtained as depicted in Fig. 2 and used to inoculate Syrian hamsters. Two independently isolated brains were taken and passaged a second time in Syrian hamsters. A subsequent passage in Syrian hamsters was also performed using two independently derived brains to confirm the stability of the strain properties.

$15{,}000 \times g$, and the partitioning of PrP into the soluble and insoluble fractions was monitored by Western blotting (Fig. 7C). Brain homogenates of hamsters infected with both Sc237 and DY contained similar large amounts of PrP in the detergent-insoluble fraction, whereas most of the PrP was found in the detergent-soluble fraction in uninfected hamsters (50). Our finding that the accumulation of insoluble PrP in brains of Syrian hamsters infected with the DY strain was similar to that found in those infected with Sc237 (Fig. 7C) contrasts with previous reports (5–7). Our results suggest that the level of PrP^{Sc} in the brains of DY-infected hamsters is similar to that of PrP^{Sc} in the brains of Sc237-infected animals. Of note are earlier studies with SHa strains where 139H prions presenting prolonged incubation times in hamsters exhibited comparable titers of infectious prions and similar levels of PrP^{Sc} in brain compared to short incubation period Sc237 prions (36).

Although some investigators have argued that changes in the relative levels of di-, mono-, and unglycosylated PrP^{Sc} are strain specific, we have not noted any such variation between the strains described in this report (Fig. 7).

DISCUSSION

The properties of any given prion isolate are determined, at least in part, by the sequence of PrP encoded within both the inoculated recipient and donor host animals (64, 69, 70, 79). Since prion strain characteristics seem to be enciphered in the conformation of PrP^{Sc} (78), we would expect that the sequence of PrP must in turn restrict the presumably limited set of conformations of PrP^{Sc} which may be supported. In the event that a new host encodes a PrP which cannot adopt the correct conformation, the closest available isomer would be selected, thereby prolonging the incubation period for primary transmission in the new host, due to the increased height of the activation energy barrier for prion formation. Upon subsequent passage to a third host with yet another PrP sequence, this process would be repeated. The final strain obtained might differ substantially from the starting prion strain with respect to the conformation of PrP^{Sc}. Passage of the same original strain through alternative hosts encoding PrPs of different sequences might generate distinct conformers even though the PrP encoded in the subsequent host is identical. Two of our findings, that prion diversity appears to be restricted and that prion characteristics change depending on the sequence of PrP encoded by the host during multiple serial transmissions, are entirely consistent with this model for propagation of prion strains. A detailed analysis of the effect of variations in PrP sequence on prion strain properties following serial transmission may allow the identification of structural features involved in maintaining prion diversity.

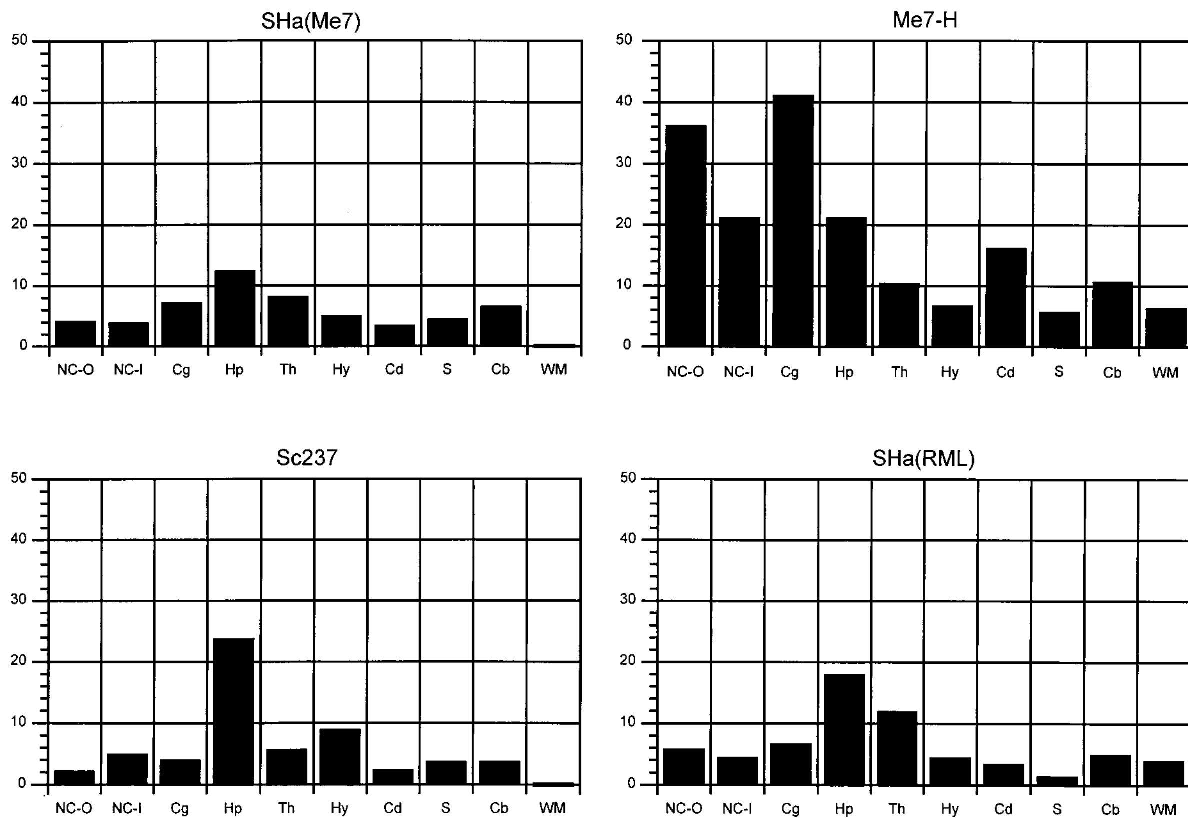

FIG. 4. Neuropathological characterization of hamster prion strains, expressed as vacuolation scores (area occupied by vacuoles) in the grey and white matter in Syrian hamsters infected with SHa(Me7), Me7-H, Sc237, and SHa(RML) prions. NC-O, outer half of neocortex; NC-I, inner half of neocortex; Cg, cingulate gyrus; Hp, hippocampus; Th, thalamus; Hy, hypothalamus; Cd, caudate nucleus; S, septum; Cb, cerebellar cortex; WM, white matter.

Two distinct prion strains with similar incubation times in mice. In most $Prnp^{a/a}$ mice, the two mouse prion strains designated Me7 and RML exhibit similar incubation times of ~150 days. When we used Tg(MH2M) mice expressing the chimeric PrP gene to transmit these strains into hamsters efficiently, we found that the properties of these strains were quite distinct. RML gave an incubation time of ~140 days upon initial transmission from CD-1 mice to Tg(MH2M)92/FVB mice, whereas Me7 exhibited a greatly extended incubation period (262 days) upon initial transmission from C57BL mice to Tg(MH2M)92/FVB mice (Fig. 1 and 2). Upon second passage in Tg(MH2M)92/FVB mice, MH2M(Me7) prions yielded a much shorter incubation time (~90 days) whereas MH2M(RML) prions gave an incubation time of 124 days (Fig. 1 and 2). Evidence for the distinct properties of the Me7 and RML strains was extended when these strains were passaged from Tg(MH2M)92/FVB mice into Syrian hamsters (Fig. 3). These findings emphasize the importance of using animals expressing PrP molecules with different sequences to study the biogenesis of prion strains.

PrP sequence variations change the properties of the Me7 strain. The Me7 strain was isolated by passage in C57BL mice from a pool of scrapie-infected sheep brains (26–28). The Me7 strain was cloned repeatedly by limiting dilution and was thought to be one of the most stable of all of the strains that had been studied. Thus, it was unexpected when we produced the SHa(Me7) strain of prions exhibiting short incubation times in hamsters. Earlier studies had shown that Me7 prions from C57BL mice passaged into Syrian hamsters yielded a strain Me7-H with very prolonged incubation periods (44), yet a single passage of Me7 through Tg(MH2M)92/FVB mice profoundly changed the properties of Me7 on subsequent passage in Syrian hamsters. Furthermore, it was reported that Me7 could be transmitted to hamsters and then subsequently back to mice without alteration of their respective strain-specific characteristics upon further passage in mice (44). We have reproduced this finding (67a). Hence, the dramatic difference between Me7-H and SHa(Me7) is caused by differences in the passage history with respect to PrP and presumably arises because the primary structure of MH2M PrP enforces specific conformations of PrP^{Sc} which differ from those adopted by mouse Me7 and/or hamster Me7-H prions.

Since SHa(Me7) and Me7-H prions appear to be equally sensitive to protease digestion (Fig. 7), and alternative methods for distinguishing prion strain conformers are not yet available, we are unable to directly demonstrate a difference in conformation between SHa(Me7) and Me7-H at this time. However, we have performed a similar study using the DY strain, and the results show that the conformation of the prion protein does not change upon passage from Syrian hamsters to Tg(MH2M) mice (70a).

Modification of prion strain propagation by interactions with different PrPs. In earlier studies, expression of SHaPrP at high levels in Tg(SHaPrP) mice was accompanied by a lengthening of incubation period upon inoculation with Mo prions (64) and inhibited conversion of $MoPrP^{C}$ in scrapie-infected mouse neuroblastoma (ScN2a) cells (68). Inactivation of the endogenous MoPrP allele in Tg mice led to a reduction of the incubation period when prions encoded by several different PrP sequences were inoculated (14, 61, 79). In the present

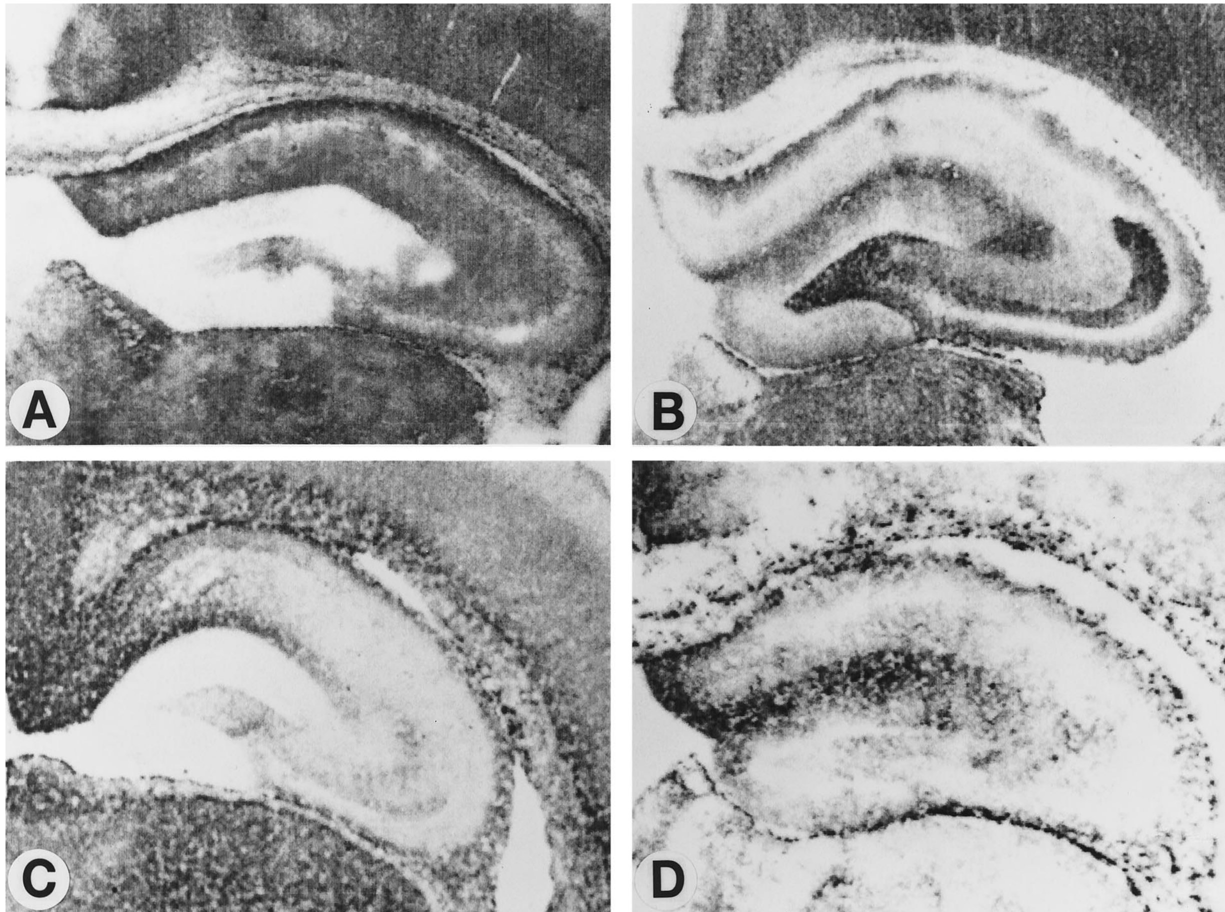

FIG. 5. Histoblot comparison of hamster prion strains in the hippocampus from Syrian hamsters infected with SHa(Me7) (A), Me7-H (B), Sc237 (C), and SHa(RML) (D).

study, the effect of overexpression of MH2M was found to vary when different strains were compared. A marked difference in incubation period was observed upon transmission of MH2M(Me7) prions into Tg(MH2M) mice expressing different levels of chimeric PrP. Short incubation periods were observed when MH2M(Me7) prions were inoculated into either Tg(MH2M)92/FVB or Tg(MH2M)229/FVB mice, but much longer incubation times were observed following inoculation of Tg(MH2M)455/FVB mice, which express relatively low levels (one- to twofold) of chimeric PrP (Table 1; Fig. 1).

The modulation of prion strain propagation by interactions between wt MoPrP and chimeric MH2M PrP is reminiscent of the dissimilar behaviors of strains in $Prnp^{a/a}$, $Prnp^{b/b}$, and $Prnp^{a/b}$ mice. For example, Me7 and RML are propagated readily in $Prnp^{a/a}$ mice and less well in $Prnp^{b/b}$ and $Prnp^{a/b}$ mice, while 22A and 87V strains are best propagated in $Prnp^{b/b}$ mice and replicate only poorly in $Prnp^{a/a}$ and $Prnp^{a/b}$ mice (17). The 87V strain in $Prnp^{a/b}$ mice is particularly notable because the presence of PrP-A seems to inhibit the conversion of PrP-B into PrP^{Sc}. These findings are in accord with the data reported here which demonstrate the variability that differences in the amino acid sequence of two competing PrP molecules have on the propagation of prion strains.

Interactions of PrP isoforms with protein X. Our data are consistent with the notion that the primary structure of PrP may influence the tertiary structure of PrP^{Sc} by determining which conformational states can be tolerated by the polypeptide chain. In our experiments where we observe an inhibitory effect of MoPrP expression upon prion strain propagation in Tg(MH2MPrP) mice, we reason that expression of MH2MPrP inhibits the formation of $MoPrP^{Sc}$ in animals inoculated with Me7, but does so to a lesser extent in Tg(MH2M) mice inoculated with RML (Fig. 1 and 2). Therefore, it seems that strains may differ with respect to the severity of the effect of competition between PrP isoforms of different amino acid sequences. Notably, elimination of competing MoPrP by crossing of the transgene array into a $Prnp^{0/0}$ background reduced the incubation period when the animals were inoculated with either MH2M(Me7) or (MH2M)RML prions, even in lines which express very high levels of MH2MPrP (Fig. 2).

We have proposed that the site of conversion would be a complex of PrP^{Sc}, PrP^{C}, and a putative cellular factor, protein X (79). Viewed in this light, it seems reasonable that the inhibitory effect of MoPrP expression upon MH2M(Me7) prion propagation occurs because MoPrP and MH2M PrP differ enough in sequence that binding of $MoPrP^{C}$ to $MH2MPrP^{Sc}$ creates a nonproductive complex because it is energetically unfavorable for $MoPrP^{Sc}$ to adopt the conformation represented by MH2M(Me7) prions. The increased activation energy barrier for conversion of $MoPrP^{C}$ to $MoPrP^{Sc}$ would slow the conversion process, thereby limiting the available supply of protein X and resulting in an extended incubation period.

Dependence of scrapie prion strain properties upon passage history. In reviewing the published history of many established scrapie strains, we were impressed by the fact that many of the

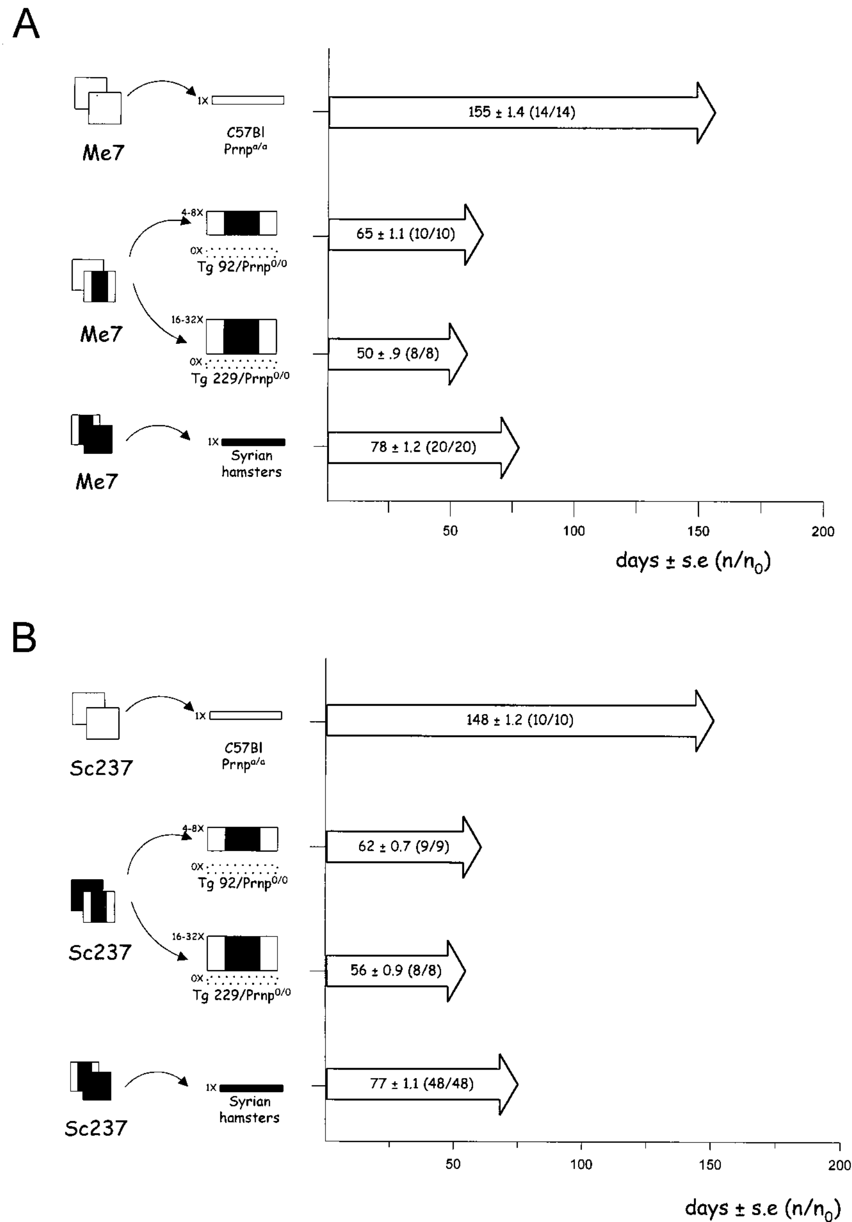

FIG. 6. Similar behaviors of Me7 and Sc237 passaged in mice, hamsters, and Tg(MH2M) mice encoding different levels of PrP. The schematic is as described in the legend to Fig. 1. (A) MH2M(Me7) and SHa(Me7) prions were prepared as depicted in Fig. 1 and 3 and inoculated into Tg(MH2M)92 and Tg(MH2M)229 mice lacking a functional $Prnp^{a/a}$ allele and Syrian hamsters, respectively. For comparison, Me7 prions serially passaged in $Prnp^{a/a}$ mice were inoculated into non-Tg $Prnp^{a/a}$ mice. (B) Mo(Sc237) prions obtained by passaging Sc237 prions to Tg(MH2M)92 mice and subsequently to non-Tg $Prnp^{a/a}$ mice, as well as Tg(MH2M)92-passaged Sc237 prions, were transmitted into non-Tg $Prnp^{a/a}$ mice and Tg(MH2M)92 and Tg(MH2M)229 mice lacking a functional $Prnp^{a/a}$ allele, respectively. For comparison, we inoculated Syrian hamsters with Sc237 prions following two passages in Tg(MH2M) and a single passage in Syrian hamsters. Results obtained were identical to those obtained with Sc237 which had been serially passaged in Syrian hamsters (70).

well-characterized prion strains appear to be derived from a pool of scrapie-infected Cheviot sheep brains. Subsequent passaging of Cheviot sheep scrapie brain homogenates into goats at Compton led to the "drowsy" goat source, from which the Chandler mouse scrapie isolate was derived (20); and from this strain, through a series of passages performed over a number of years, several of the commonly used Mo prion strains were derived, including 139A of mice, which seems to be similar to RML, as well as SHa strains such as 139H and 263K (Table 2). The 263K strain was derived from the same source as Sc237 (43–45, 49). Passage of the Cheviot sheep source directly into $Prnp^{b/b}$ mice and subsequent cloning by limiting dilution led to the 22A strain (27). Passaging of the "drowsy" goat isolate to $Prnp^{a/a}$ mice led to the 79A strain, and passage of the "drowsy" isolate to $Prnp^{b/b}$ animals resulted in the 87V strain (11, 12). Although we are limited to a historical viewpoint in analyzing the origins of many of the distinct scrapie isolates described above, we find it intriguing that in almost every instance where distinct prion strains have been identified, the two strains being compared differ with respect to their passage history.

Since all of the above-mentioned strains may be considered to be derived from a single source, strains that can be traced to

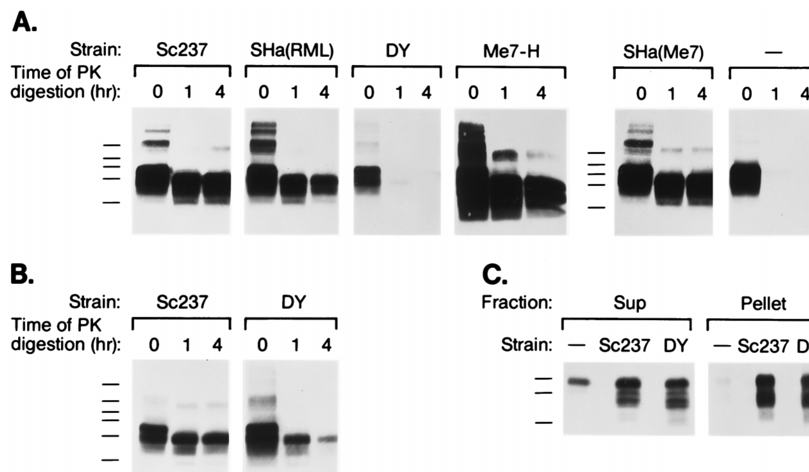

FIG. 7. Comparison of protease sensitivity and 27-30 products of different prion strains. (A) Brain homogenates (1 mg of total protein ml^{-1}) of Syrian hamsters infected with several SHa prion strains were treated with 100 μg of proteinase (PK) ml^{-1} for 0, 1, or 4 h and analyzed by Western blotting using anti-PrP monoclonal antibody 3F4, which recognizes SHaPrP (42). The inocula used were Sc237, SHa(RML), DY, Me7-H, and SHa(Me7). —, uninoculated control. (B) Brain microsomal membrane fractions (1 mg of total protein ml^{-1}) of Syrian hamsters infected with Sc237 or DY were treated with 100 μg of proteinase K ml^{-1} for 0, 1, or 4 h, and analyzed by Western blotting using anti-PrP monoclonal antibody 3F4. (C) Brains from normal or ill hamsters infected either with Sc237 or the DY inoculum were solubilized in cold buffer A (1% Triton X-100 and 1% sodium deoxycholate in phosphate-buffered saline), incubated on ice, and then centrifuged at 15,000 × g for 20 min at 4°C. The supernatant was removed, and then equal percentages of the supernatant (Sup) and pellet (Pellet) fractions were analyzed by Western blotting using anti-PrP antibody 3F4. Molecular size markers are indicated at the left and represent, in descending order, 84, 53, 35, 29, and 21 kDa (A and B) or 35, 29, and 21 kDa (C).

a completely different natural source are of particular biological interest. Passage of the spleen of a scrapie-infected Suffolk sheep into Moredun ($Prnp^{a/a}$) mice and subsequent cloning by limiting dilution led eventually to isolation of the Me7 strain (Table 2) (27, 28, 86). Serial transmission of Me7 to Syrian hamsters led to the stable isolate Me7-H (44). In addition, transmission of bovine spongiform encephalopathy from infected cattle to $Prnp^{b/b}$ mice led to the 301V mouse strain (31), transmission from scrapie-infected cattle to Syrian hamsters produced the MT-C5 hamster isolate (34), and the DY hamster prion strain was obtained by passage of transmissible mink encephalopathy to hamsters (6, 7, 48).

Some investigators have argued the relative levels of di-, mono-, and unglycosylated PrP^{Sc} are strain specific (25). Such an approach has been applied to brain extracts from British patients who have recently died from what is thought to be a new variant of CJD (84). Unlike the case for most sporadic and iatrogenic CJD cases, Western blots showed a preponderance of the diglycosylated PrP 27-30 (25). While our own studies have not provided evidence in support of the notion that Asn-linked glycosylation of PrP^{Sc} is strain specific (Fig. 7), in accord with several other studies (9, 19, 72, 81), it seems possible that under some circumstances, a particular prion strain might be monitored by the relative concentrations of specific PrP^{Sc} glycoforms (25a). In addition, it seems reasonable to suppose that differences in Asn-linked glycosylation of PrP^{Sc} may become evident as a consequence of changes in the conformation of PrP^{Sc}.

Estimating the limits of prion diversity. If prion strains within a single PrP genetic background are found to appear coincident in their properties, despite having been derived from completely different primary sources and being geographically isolated from one another, prion diversity is likely to be limited (66). The apparent identity of Sc237 and Me7 when passaged between identical hosts is made more striking in view of the fact that Me7 appears to be derived from an entirely different primary source, a Suffolk sheep spleen, than are Sc237 and RML, whose ancestries can both be traced to the Compton "drowsy" goat source, which in turn was derived by transmission from scrapie-infected Cheviot sheep (55). Our study clearly shows that despite their common origins, RML and Sc237 are distinct. Their passage histories differ, however, in that Sc237 was obtained from the Chandler mouse isolate, which interpolated an additional series of passages in rats (22, 23, 43). In contrast, we were able to obtain two completely different SHa strains from the same, cloned, primary source, Me7, by including one additional passage in Tg(MH2M) mice. These strains differ in their properties just as much as either of them differ from two isolates, Sc237 and SHa(RML), which were themselves derived from a distinct ancestral source. Thus, the primary structure of PrP encoding prions during the passage history, rather than the original source of inoculum, determines strain characteristics in any particular host.

Differences in prion strain characteristics can be directly traced back to changes in PrP sequence during serial transmission, suggesting that strain characteristics are maintained and propagated through protein-protein interactions between PrP isoforms. This is entirely consistent with a model in which prion strain diversity is contained entirely within PrP. The spectrum of possible PrP conformations must be constrained by the sequence of PrP, and it seems likely that only a limited number of distinct conformations for a PrP^{Sc} of any particular sequence is possible. Intriguingly, results of ionizing radiation inactivation experiments argue that the minimal infectious unit may be a dimer of PrP^{Sc} (3), raising the possibility that one or two distinct PrP^{Sc} conformers can form multiple dimeric forms by variations in the quaternary packing of a very restricted number of monomeric subunits, thereby significantly increasing the diversity obtainable.

New approaches arising from these studies. The construction of Tg(MH2M) mice has opened a new approach to the study of prion strains. These mice have allowed us to use Mo prion strains, which have been previously passaged in a genetic background in which they are stable for many generations, to form distinct isolates in Syrian hamsters, after minimal passaging. By using these isolates, it should be possible to prepare

prion strains, such as Me7, 22A, or RML, that have been passaged in Syrian hamsters.

Physical characterization of highly purified preparations of distinct SHa prion strains may elucidate structural details of the mechanism of prion diversity. Syrian hamsters are superior to mice for many studies of scrapie, but the number of strains available for study in hamsters previously has been relatively small. Syrian hamsters have numerous advantages over mice, including larger brains, higher yields of PrP^{Sc} in large-scale purification, and incubation times that are often relatively short (62, 63). As our studies of prion strains have progressed, it seems increasingly more important to obtain protein structure data on at least two clearly distinct prion strains. To achieve this, these strains must have relatively short incubation times in host animals in which they may be passaged in a stable fashion, yet they must be unambiguously distinct. In addition, structure determination may require that they be readily purified in large quantities. From the data presented here, we believe that at least two and perhaps three distinct strains are now available for this purpose.

The results of our studies provide new approaches to the study of prions in both cultured cells and cell-free systems. First, it should be possible to study the biogenesis of PrP^{Sc} in scrapie-infected murine neuroblastoma cells infected with two different strains, Me7 and RML (9, 75, 76). Our previous studies have used RML exclusively (16), but it is likely that Me7 will be equally infectious for these cells. Second, cell-free systems designed to generate scrapie infectivity (40, 47, 65) might best be studied by using multiple SHa prion strains such as SHa(Me7) and SHa(RML) to generate $SHaPrP^{Sc}$ molecules that could be used in conjunction with the chimeric substrate $MH2MPrP^{C}$. Such a chimeric substrate offers the advantage that bioassays of chimeric prion infectivity in mice should not measure SHa prions (70). Since distinct strains exhibit profound differences in incubation times depending on the sequences of the PrP molecules with which the prions interact (Fig. 1 and 2), the availability of different strains may greatly facilitate such investigations.

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