

# Transgenic Studies Implicate Interactions between Homologous PrP Isoforms in Scrapie Prion Replication

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## Summary

**Transgenic (Tg) mice expressing both Syrian hamster (Ha) and mouse (Mo) prion protein (PrP) genes were used to probe the mechanism of scrapie prion replication. Four Tg lines expressing HaPrP exhibited distinct incubation times ranging from 48 to 277 days, which correlated inversely with HaPrP mRNA and HaPrP<sup>C</sup>. Bioassays of Tg brain extracts showed that the prion inoculum dictates which prions are synthesized de novo. Tg mice inoculated with Ha prions had ~10<sup>9</sup> ID<sub>50</sub> units of Ha prions per gram of brain and <10 units of Mo prions. Conversely, Tg mice inoculated with Mo prions synthesized Mo prions but not Ha prions. Similarly, Tg mice inoculated with Ha prions exhibited neuropathologic changes characteristic of hamsters with scrapie, while Mo prions produced changes similar to those in non-Tg mice. Our results argue that species specificity of scrapie prions resides in the PrP sequence and prion synthesis is initiated by a species-specific interaction between PrP<sup>Sc</sup> in the inoculum and homologous PrP<sup>C</sup>.**

## Introduction

Studies with transgenic (Tg) mice expressing a Syrian hamster prion protein (PrP) gene have shown that virtually all facets of experimental scrapie may be modified by the PrP transgene (Scott et al., 1989). Susceptibility to prions, neuropathology, incubation time, and species tropism of the prions were all found to be influenced by the transgene.

Although progress has been made in deciphering the structure of the infectious prion particle (Gabizon and Prusiner, 1990), little information has been available about the molecular mechanisms that feature in the replication of prion infectivity. A substantial body of evidence has accumulated over the past decade that argues that the scrapie isoform of the prion protein (PrP<sup>Sc</sup>) is a necessary and major component of the infectious scrapie agent (Gabizon and Prusiner, 1990). Although a few investigators have reported the apparent absence of PrP<sup>Sc</sup> in frac-

tions with low or intermediate levels of scrapie infectivity (Aiken et al., 1989; Czub et al., 1988; Manuelidis et al., 1987; Sklaviadis et al., 1989), these reports generally suffer from methodological problems attendant with detecting low levels of PrP<sup>Sc</sup>. For example, multiple published studies have reported that PrP<sup>Sc</sup> could not be found in scrapie-infected neuroblastoma cells (Race et al., 1988; Caughey et al., 1989), but the inability to detect PrP<sup>Sc</sup> has recently been attributed to technical difficulties (Caughey et al., 1990).

While several aspects of scrapie and other prion diseases resemble those of viral illnesses, other facets of these disorders argue that they are distinct entities. Similarities include transmission of scrapie by inoculation, de novo synthesis of prions in the infected host, clinical dysfunction and pathologic changes in the diseased host, and different isolates or "strains" of prions. Features distinguishing prions from viruses include lack of an immune response during scrapie (see Barry and Prusiner, 1987), inability to inactivate prion infectivity by procedures modifying nucleic acids (Prusiner, 1989), an abnormal isoform of the cellular protein, i.e., PrP<sup>Sc</sup>, contained by fractions highly enriched for scrapie infectivity (Gabizon and Prusiner, 1990), genetic linkage between the cellular PrP gene and scrapie incubation times in mice (Carlson et al., 1986) as well as between PrP mutations and Gerstmann-Sträussler-Scheinker syndrome in humans (Hsiao et al., 1989), and the barrier for transmission between species residing in cellular PrP gene translated sequence (Scott et al., 1989). The similarities between prion and viral illnesses have caused some investigators to ignore other features that distinguish these two entities and contend that scrapie must be caused by a virus-like particle (Braig and Diringer, 1985; Murdoch et al., 1990).

Attempts to identify a scrapie-specific nucleic acid by molecular cloning (Duguid et al., 1988; Weitgreffe et al., 1985) or by physical techniques (Meyer et al., 1990) have been unsuccessful to date. Numerous attempts to demonstrate the reduction of infectivity by procedures that selectively modify or hydrolyze nucleic acids have been without success (Bellinger-Kawahara et al., 1987a, 1987b; Gabizon et al., 1987). In contrast, substantial reductions in scrapie infectivity are regularly observed after procedures that denature or hydrolyze proteins are applied (Prusiner, 1982; McKinley et al., 1983).

Tg mice harboring foreign PrP genes have given the first opportunity to program the synthesis of infectious prions (Scott et al., 1989). The studies reported here coupled with a recent study showing that the acquisition of PrP protease resistance is a posttranslational event (Borchelt et al., 1990) provide some insight into the molecular mechanisms operating in the replication of scrapie prions. Our results demonstrate an inverse relationship between the level of PrP expression and the length of scrapie incubation period. Although the Tg mice were capable of producing both hamster (Ha) and mouse (Mo) prions, they were found to produce exclusively one or the other depending

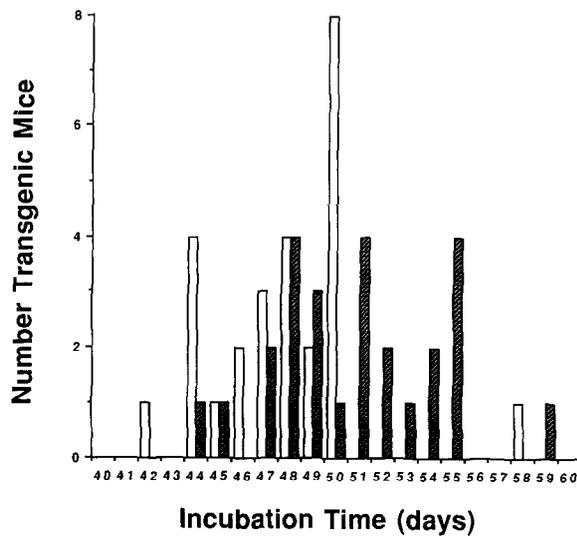


Figure 1. Tg 7 Mice Expressing HaPrP Genes Have the Shortest Scrapie Incubation Times after Inoculation with Ha Prions Recorded to Date

Tg 7 mice were inoculated intracerebrally with  $\sim 10^7$  ID<sub>50</sub> units of Ha prions. Incubation times from inoculation to onset of clinical illness (open bars) and to death (hatched bars) are plotted as a histogram.

on the inoculum. In attempting to reconcile all of the currently available experimental data on the molecular structure of prions, we propose a model for scrapie prion replication in which existing PrP<sup>Sc</sup> molecules serve as a template for the posttranslational conversion of PrP<sup>C</sup> or a precursor into similar PrP<sup>Sc</sup> molecules. In the apparent absence of a scrapie-specific nucleic acid, such a mechanism, albeit rather unorthodox, is consistent with most aspects of scrapie that remain unresolved.

## Results

Mice of Tg line 7 harbor >60 copies of the HaPrP transgene and have the shortest scrapie incubation times ever recorded. The Tg 7 mice inoculated with  $\sim 10^7$  ID<sub>50</sub> units of Ha prions had a mean incubation time of  $48 \pm 1.0$  days for the interval from inoculation to illness and  $51 \pm 0.8$  days for the interval from inoculation to death (Figure 1). This is a nonstochastic process with all of the 26 inoculated animals developing clinical signs of scrapie in a normal distribution followed by death over a mean period of 2.9 days.

### Four Lines of Tg Mice Expressing HaPrP mRNA

Four lines of Tg mice expressing HaPrP<sup>C</sup> were constructed and propagated. Southern blot analysis of the four lines suggests that the transgenes are integrated at one chromosomal site in a tandem array as has been reported for many Tg mice harboring other foreign genes (Scott et al., 1989). Northern blots showed that Tg 69 mice with two to four copies of the transgene expressed the lowest levels of HaPrP mRNA, while Tg 71 with a similar number of transgenes expressed slightly higher levels of HaPrP mRNA. Tg 81 mice with 30–50 copies of the trans-

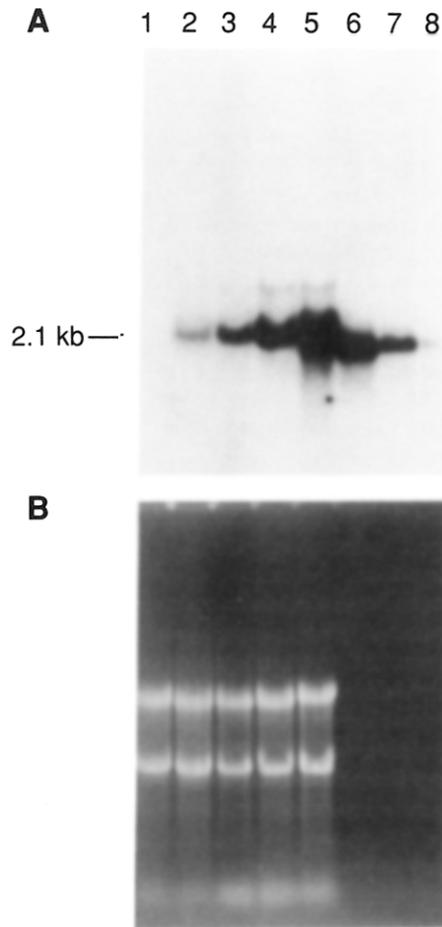


Figure 2. Syrian Hamster PrP mRNA Steady-State Levels in the Brains of Four Tg (HaPrP) Mo Lines

(A) Northern transfer analysis probed with <sup>32</sup>P-labeled AccI–EcoRI fragment excised from the 3' untranslated region of the HaPrP cDNA clone S<sub>11</sub>.

(B) Visualization of total RNA in formaldehyde–agarose gels (0.8%) in which ethidium bromide was added directly to the samples prior to heat denaturation and electrophoresis.

Lanes 1–5 each contain 10 µg of total brain RNA. Lane 1, non-Tg 69; lane 2, Tg 69; lane 3, Tg 71; lane 4, Tg 81; lane 5, Tg 7. Lanes 6–8 contain synthetic RNA transcribed from a pSP64 subclone containing a HaPrP cDNA insert. Lane 6, 20 pg; lane 7, 5 pg; lane 8, 1 pg.

gene expressed substantially higher levels of HaPrP mRNA. The hierarchy of HaPrP mRNA levels for the four Tg lines is illustrated in Figure 2.

The Tg 20 line was produced in the same series of microinjections into (C57BL/6 × LT/Sv)F2 fertilized eggs that yielded Tg 7. Neither HaPrP<sup>C</sup> nor HaPrP mRNA was detectable in Tg 20 mice. Digestion with XbaI yielded an aberrant 3 kb hybridizing fragment rather than the 3.8 kb fragment seen in Tg 7 and in hamsters. Whether a rearrangement or deletion within the HaPrP insert in Tg 20 is responsible for the lack of expression is unknown.

### The Spectrum of Incubation Times

A range of scrapie incubation times varying from  $277 \pm 6.7$  to  $48 \pm 1.0$  days was recorded for the four Tg (HaPrP) Mo lines expressing HaPrP mRNA inoculated with Ha

Table 1. Scrapie Incubation Times in Tg Mice Expressing HaPrP after Inoculation with Either Mo or Ha Prions

Species	Line	Ha Prions <sup>a</sup>			Mo Prions <sup>b</sup>		
		n <sup>c</sup>	Illness (Days ± SE)	Death (Days ± SE)	n <sup>c</sup>	Illness (Days ± SE)	Death (Days ± SE)
Mo	Non 69 <sup>d</sup>	0/20	>370	—	17/17	143 ± 2.9	154 ± 3.6
	Non 71	0/18	>270	—	14/14	148 ± 4.8	162 ± 5.7
	Non 81	0/24	>460	—	21/21	128 ± 1.7	142 ± 2.6
Tg Mo	Tg 20	0/6	>305	—	21/21	134 ± 3.1	154 ± 2.7
	Tg 69	18/18	277 ± 6.7	293 ± 8.1	17/17	166 ± 4.7	173 ± 5.5
	Tg 71	15/17 <sup>e</sup>	176 ± 2.7	182 ± 2.7	19/19	165 ± 3.4	179 ± 2.3
	Tg 81	24/24	75 ± 1.1	75 ± 1.1	20/20	194 ± 3.5	200 ± 3.2
	Tg 7	26/26	48 ± 1.0	51 ± 0.8	25/25	173 ± 4.8	180 ± 4.8
Syrian Ha	LVG:Lak	32/32	89 ± 0.9	101 ± 1.4	0/32	>360	—

<sup>a</sup> Animals were inoculated intracerebrally with 30 µl containing ~10<sup>7</sup> ID<sub>50</sub> units of Ha prions in crude extracts prepared from scrapie-infected Ha brains.

<sup>b</sup> Animals were inoculated intracerebrally with 30 µl containing ~10<sup>6</sup> ID<sub>50</sub> units of Mo prions in crude extracts prepared from scrapie-infected Mo brains.

<sup>c</sup> Number of animals developing clinical signs of scrapie divided by the total number of animals inoculated. Mice dying atypically were virtually always <5% of the total number of animals inoculated and they were therefore excluded (Prusiner, 1987).

<sup>d</sup> Non-Tg mice are littermates of the Tg animals.

<sup>e</sup> Two mice are alive and well at 250 days after inoculation with Ha prions. Whether these mice were mistyped and they are not transgenic remains to be determined.

prions (Table 1). Tg 69 mice with the lowest steady-state levels of HaPrP mRNA had the longest incubation times, while Tg 7 mice with the highest levels of HaPrP mRNA had the shortest incubation times. The Tg 71 and 81 mice had intermediate levels of HaPrP mRNA and displayed incubation times of intermediate length. The Tg 20 mice that failed to express HaPrP mRNA have scrapie incubation times after inoculation with Ha prions exceeding 300 days. These observations demonstrate an inverse relationship between the level of transgene HaPrP mRNA and the length of the incubation time after inoculation with Ha prions.

While non-Tg mice developed scrapie between 128 and 148 days after inoculation with Mo prions, Tg 69 and Tg 71 mice exhibited incubation times of 166 ± 4.7 and 165 ±

3.4 days, respectively. Even greater prolongation of the incubation times after Mo prion inoculation was seen with Tg 81 and Tg 7 mice, where periods of 194 ± 3.5 and 173 ± 4.8 were observed, respectively. Tg 20 mice developed scrapie 134 ± 3.1 days after inoculation with Mo prions consistent with their failure to express the HaPrP transgene. These observations argue that expression of the HaPrP transgene impedes Mo prion synthesis.

#### Species-Specific Inocula Direct Synthesis of Ha or Mo Prions

Inoculation of Tg mice expressing HaPrP genes with Ha prions resulted in the formation of Ha prions as determined by bioassay in hamsters. As shown in Table 2, inoculation of either Tg 71 or 81 mice with Ha prions pro-

Table 2. Production of Ha or Mo Prions in the Brains of Tg Mice

Tg Mice				Bioassays of Prions		
Inoculum <sup>a</sup>	Tg Line	n <sup>b</sup>	Incubation Time <sup>c</sup> (Days ± SE)	Species	n <sup>d</sup>	Log Titer <sup>e</sup> (ID <sub>50</sub> /ml ± SE)
Ha	71	3	163 ± 1.5	Mo	0/30	<1
Ha	71	4	161 ± 1.3	Ha	39/39	8.0 ± 0.20
Ha	81	6	75 ± 0.9	Mo	0/40	<1
Ha	81	6	75 ± 0.9	Ha	47/47	7.5 ± 0.20
Mo	71	3	158 ± 1.3	Mo	14/14	5.5 ± 0.41
Mo	71	3	155 ± 1.6	Ha	0/16	<1
Mo	81	3	173 ± 3.2	Mo	30/30	5.2 ± 0.34
Mo	81	3	173 ± 3.2	Ha	0/24	<1

<sup>a</sup> Ha inoculum was a 10% (w/v) homogenate of scrapie-infected Syrian hamster brain, designated Sc237, diluted 10-fold. Mo inoculum was a 10% (w/v) homogenate of scrapie-infected Swiss CD-1 mouse brain, designated RML. See Experimental Procedures for passage history.

<sup>b</sup> Number of Tg mice on which individual bioassays of their brains were performed.

<sup>c</sup> Mean incubation times for the Tg mice on which bioassays were performed.

<sup>d</sup> Number of mice or hamsters developing scrapie divided by the total number of all animals used for the bioassays. Typically between five and ten animals were used for each bioassay.

<sup>e</sup> Titers were calculated from published curves relating the dose of prions inoculated to the resulting incubation time, as described in Experimental Procedures. Titers are given in ID<sub>50</sub> U/ml of 10% (w/v) brain homogenate.

Table 3. Hamster Bioassay of Prions in Brains of Tg Mice Expressing HaPrP

Inocula	Mouse	Log Titer (ID <sub>50</sub> U/ml ± SE)
Ha brain homogenate	Tg 81 <sup>a</sup>	7.5 ± 0.17
	Tg 81	8.1 ± 0.12
	Tg 81	7.6 ± 0.16
	Tg 71	8.4 ± 0.21
	Non-Tg 71	<1
Purified Ha prion rods	Tg 81 <sup>b</sup>	7.3 ± 0.19
	Tg 81	7.9 ± 0.17
	Tg 81	7.4 ± 0.20
	Non-Tg 81	3.2 ± 0.19
	Non-Tg 81	<1
Ha prion DLPCs <sup>c</sup>	Tg 71 <sup>d</sup>	7.9 ± 0.07
	Tg 71	7.9 ± 0.08
	Tg 71	8.0 ± 0.07
	Non-Tg 71	<1
	Non-Tg 71	<1

<sup>a</sup> Mice sacrificed ~75 days after inoculation except for Tg 71 and non-Tg 71 mice sacrificed at ~160 days.

<sup>b</sup> Mice sacrificed ~75 days after inoculation.

<sup>c</sup> Detergent-lipid-protein complexes.

<sup>d</sup> Mice sacrificed ~160 days after inoculation.

duced high levels of Ha prions, but virtually no Mo prions were detected by bioassay. Conversely, inoculation with Mo prions generated substantial levels of Mo prions, but virtually no Ha prions were found. These findings argue that the origin of the prion inoculum determines whether Ha or Mo prions are produced by Tg (HaPrP) mice capable of supporting the replication of either prion.

Our results also show that the scrapie prion titers in the brains of Tg mice were independent of the length of the incubation time. For example, Tg 71 and Tg 81 mice exhibited Ha prion titers of ~10<sup>8</sup> ID<sub>50</sub>/g of brain, yet the incubation time after inoculation with Ha prions for the Tg 71 mice was 161 ± 1.3 days compared with incubation times of 75 ± 0.9 days for Tg 81.

Development of scrapie in non-Tg mice after inoculation with Ha prions is a stochastic process presumably due to the relative incompatibility between HaPrP<sup>Sc</sup> and

MoPrP<sup>C</sup> (Scott et al., 1989). Crossing the species barrier is a slow and inefficient process (Pattison, 1965) where only a few non-Tg mice inoculated with Ha prions eventually develop scrapie after greatly extended incubation periods (Scott et al., 1989). Other studies argue that these mice synthesize de novo Mo but not Ha prions (Scott et al., 1989; Pattison, 1965; Pattison and Jones, 1968; Bockman et al., 1987). Non-Tg mice inoculated with Ha prions sacrificed at the same time that their Tg littermates developed scrapie were found to have low or undetectable levels of Ha prions as determined by bioassay (Table 3). From earlier studies, non-Tg mice inoculated with Ha prions might be expected to have a residual titer as high as ~10<sup>6</sup> ID<sub>50</sub> U/g of brain (Oesch et al., 1985), yet the highest titer of Ha prions found by bioassays in hamsters was ~10<sup>3</sup> ID<sub>50</sub> U/g of brain; generally the titer was <10 ID<sub>50</sub> U/g of brain in these non-Tg control mice regardless of the Ha prion inoculum used. These experimental measurements demonstrate conclusively that infectious Ha prions are produced de novo in the brains of Tg (HaPrP) mice inoculated with Ha prions but not in the brains of non-Tg littermate controls.

Additional control experiments are shown in Table 4 where the serial passage of Tg 71 and 81 brain extracts from mice inoculated with Ha prions into Tg 71 and 81 mice, respectively, produced incubation periods similar to those observed for Ha prions inoculated into these mice (Table 1). More importantly, two Tg 71 Mo brain extracts inoculated into Tg 81 mice produced clinical signs of scrapie in ~75 days (Table 4). These results are consistent with bioassays in Syrian hamsters where brain extracts from clinically ill Tg 71 and Tg 81 mice inoculated with Ha prions produced disease in hamsters in ~75 days (Scott et al., 1989). Dilution of the extract ~10<sup>5</sup>-fold increased the incubation time for Tg 81 mice from 80 ± 2.6 to 119 ± 2.5 days, showing that the length of the incubation time in Tg (HaPrP) mice is dependent on the dose of Ha prions inoculated. These observations argue that use of Syrian hamsters for bioassay of Ha prions produced in the brains of Tg (HaPrP) mice is a reasonable procedure, although comparisons of endpoint titrations performed in hamsters and Tg mice will be of interest.

Table 4. Serial Passage Prions from the Brains of Tg (HaPrP) Mice Inoculated with Ha Prions

Tg Donor	Recipient	Log Dilt	n <sup>a</sup>	Incubation Times	
				Illness (Days ± SE)	Death (Days ± SE)
Tg 81 Mo	Tg 81 Mo	1	23/23	80 ± 2.6	81 ± 3.4
		5	16/16	119 ± 2.5	125 ± 2.9
	Syrian Ha Swiss Mo	1	47/47	75 ± 1.5	91 ± 2.4
		1	0/40	>270	
Tg 71 Mo	Tg 71 Mo	1	25/25	178 ± 3.2	184 ± 4.2
		1	15/15	75 ± 2.1	78 ± 1.6
	Syrian Ha Swiss Mo	1	24/24	72 ± 1.7	89 ± 1.7
		1	0/30	>230	

<sup>a</sup> Number of animals developing clinical signs of scrapie divided by the total number of animals inoculated. Animals dying atypically were virtually always <5% of the total number of animals inoculated and they were therefore excluded (Prusiner, 1987).

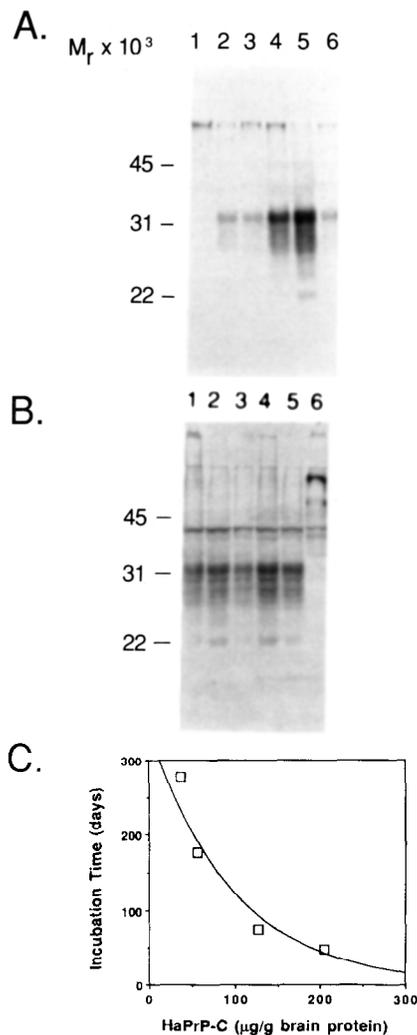


Figure 3. Hamster PrP<sup>C</sup> Steady-State Levels in Four Tg Lines of Mice  
Western immunoblots of brain extracts from non-Tg mice, lane 1; Tg 69 mice, lane 2; Tg 71 mice, lane 3; Tg 81 mice, lane 4; Tg 7 mice, lane 5; Syrian hamster, lane 6.  
(A) Hamster PrP-specific MAb 13A5 was used for immunoblotting.  
(B) Mouse PrP-specific polyclonal antiserum, designated H5A, was raised in Syrian hamsters and used for immunoblotting.  
(C) Plot of scrapie incubation times as a function of the HaPrP<sup>C</sup> steady-state levels in the four Tg (HaPrP) Mo lines. An exponential curve was fitted to the data points obtained from Tables 1 and 5.

### Synthesis of HaPrP<sup>C</sup> and HaPrP<sup>Sc</sup> in Tg Mice

Western immunoblots show a correlation between the translation product HaPrP<sup>C</sup> (Figure 3A) and HaPrP mRNA levels (Figure 2) in the four Tg lines. While the levels of HaPrP<sup>C</sup> increased as the steady-state levels of HaPrP mRNA rose in the four lines, the level of MoPrP<sup>C</sup> was unaltered (Figure 3B).

Steady-state levels of HaPrP<sup>C</sup> in the brains of four Tg (HaPrP) Mo lines were determined by an ELISA as listed in Table 5. The levels of HaPrP<sup>C</sup> as determined by ELISA are in reasonable agreement with those estimated by Western immunoblotting. For example, the levels of HaPrP<sup>C</sup> in Tg 69, 71, and Syrian hamsters appear similar by Western blotting (Figure 3A) and give values of 38, 56, and 52 μg HaPrP/g protein by ELISA, respectively. The scrapie incubation times after inoculation with Ha prions (Table 1) are plotted as a function of the HaPrP<sup>C</sup> concentration in the brains of the four Tg (HaPrP) lines (Figure 3C). The data were fitted to an exponential function with a correlation coefficient of ~0.95.

Since the titers of Ha prions in the brains of Tg 71 and Tg 81 mice at the time of illness were similar, we surmised that it was likely that the levels of HaPrP<sup>Sc</sup> would also be similar. Indeed, the levels of HaPrP<sup>Sc</sup> in all four lines of Tg (HaPrP) mice were similar in their brains at the time of illness (Table 5). These findings are in accord with earlier observations showing that the scrapie prion titer at the time of clinical illness is independent of the incubation time (Kimberlin and Walker, 1977, 1978; Prusiner, 1987), whereas the concentration of PrP<sup>Sc</sup> is directly proportional to the prion titer (McKinley et al., 1983). As shown in Figure 4A and Table 5, HaPrP<sup>Sc</sup> levels in the brains of Tg mice with clinical signs of scrapie ranged between 25% and 50% of those found in Syrian hamster brains. Differences in HaPrP<sup>Sc</sup> levels of 2- to 4-fold would not be expected to be reflected as measureable changes in prion titers (Tables 2 and 3).

While Tg mice expressing the HaPrP gene were found to produce HaPrP<sup>Sc</sup> after inoculation with Ha prions, low levels of protease-resistant HaPrP were detected on Western blots in brain extracts from scrapie-infected Tg mice inoculated with Mo prions (Scott et al., 1989). The virtual absence of Ha prion infectivity found in these Tg mouse brains (Table 2) is at odds with earlier Western blot results

Table 5. PrP<sup>C</sup> and PrP<sup>Sc</sup> Levels in Four Lines of Tg Mice as Determined by ELISA

Animal <sup>a</sup>	Ha Prion-Infected		Mo Prion-Infected	
	HaPrP <sup>C</sup> (μg PrP/g Protein)	HaPrP <sup>Sc</sup> (μg PrP/g Protein)	HaPrP <sup>C</sup> (μg PrP/g Protein)	HaPrP <sup>Sc</sup> (μg PrP/g Protein)
Non-Tg	4 ± 1.2 (3) <sup>b</sup>	1 ± 0.5 (3)	2 ± 1.1 (4)	2 ± 1 (4)
Tg 69	38 ± 6.0 (3)	20 ± 6.0 (3)	16 ± 4.2 (3)	3 ± 1 (3)
Tg 71	56 ± 6.8 (4)	17 ± 7.3 (4)	51 ± 3.4 (3)	1 ± 1 (3)
Tg 81	128 ± 11.8 (3)	22 ± 9.8 (3)	122 ± 12.4 (3)	3 ± 1 (3)
Tg 7	205 ± 41.2 (3)	30 ± 4.0 (3)	195 ± 35.0 (2)	3 ± 1 (2)
Syrian Ha	52 ± 8.0 (3)	72 ± 6.5 (3)	ND <sup>c</sup>	ND

<sup>a</sup> All animals sacrificed upon developing signs of clinical scrapie except for non-Tg mice sacrificed at ~160 days. PrP was determined as described in Experimental Procedures.

<sup>b</sup> Number of animals, from which measurements were made, is given in parentheses.

<sup>c</sup> Not determined.

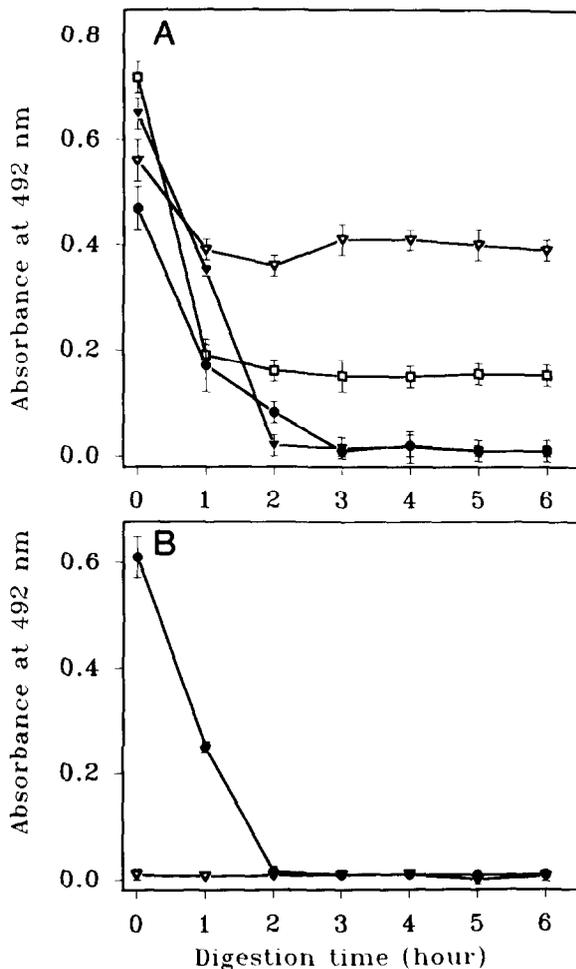


Figure 4. Determinations of HaPrP<sup>C</sup> and HaPrP<sup>Sc</sup> by Limited Proteinase K Digestion  
Brain homogenates (2 mg/ml) were digested at 37°C with proteinase K (67 µg/ml) in the presence of 0.2% Sarkosyl. Aliquots were withdrawn at given times, and phenylmethylsulfonyl fluoride (final concentration 10 mM) was added to stop the reaction. After mixing with equal volume of 2 M guanidine thiocyanate, the mixture was applied to a nitrocellulose membrane. The ELISA was done as described in Experimental Procedures. Each data point is the average of triplicate determinations. Vertical error bars represent standard deviations of the mean. (A) Solid circle, brain homogenate of normal hamster; open triangle, brain homogenate of Sc237-infected hamster; solid triangle, brain homogenate of normal Tg 81 mouse; open square, brain homogenate of Sc237-infected Tg 81 mouse. (B) Solid circle, brain homogenate of RML-infected Tg 81 mouse; open triangle, brain homogenate of RML-infected non-Tg mouse.

(Scott et al., 1989). To investigate this apparent inconsistency, HaPrP<sup>Sc</sup> in the brains of the four Tg Mo lines inoculated with Mo prions was measured by ELISA. No HaPrP<sup>Sc</sup> above background levels was detected by ELISA in Tg (HaPrP) mice inoculated with Mo prions, while it was readily detected if the Tg (HaPrP) mice were inoculated with Ha prions (Figure 4B and Table 5).

The origin of the protease-resistant HaPrP found on Western blots of brain extracts from Tg (HaPrP) mice inoc-

ulated with Mo prions is unknown (Scott et al., 1989). Of note, HaPrP deposition in amyloid plaques in the brains of Tg 81 mice inoculated with mouse prions was observed, as described below. This finding raises the possibility that the protease-resistant HaPrP detected by Western blotting comes at least in part from the entrapment of HaPrP in these deposits. These observations on protease-resistant forms of PrP emphasize the need for scrapie-specific probes and correlative bioassays especially for studies with Tg mice.

### Neuropathology

Consistent with our observations that species-specific inocula dictate whether Ha or Mo prions are produced in Tg (HaPrP) mice (Table 2), we found that the inocula also determine the distribution of spongiform change as well as the formation of PrP amyloid plaques (Table 6). Tg (HaPrP) mice inoculated with Ha prions developed an intense spongiform change confined to gray matter of the hippocampus, thalamus, cerebral cortex, and brainstem, sparing the white matter (Figure 5). Numerous PrP amyloid plaques reactive with the HaPrP-specific monoclonal antibody (MAb) 13A5 (Barry and Prusiner, 1986) were found in the subcallosal and periventricular regions (Figure 6). Interestingly, the mean size of the PrP amyloid plaques was proportional to the level of PrP transgene expression (Table 6; Figure 6). The distribution of spongiform changes and the PrP amyloid plaques resembled those found in Syrian hamsters inoculated with Ha prions (DeArmond et al., 1987a).

Astrocytic gliosis was a prominent feature of the brains of all Tg (HaPrP) mice developing clinical signs of scrapie. Neither the distribution nor extent of gliosis was altered by the scrapie inoculum (Table 6).

Tg 69, 71, and 81 mice inoculated with Mo prions had widespread spongiform change in both the gray and white matter, but the degree of vacuolation was less than that found when the Tg (HaPrP) mice were inoculated with Ha prions (Table 6; Figure 5). Only a few PrP amyloid plaques were found in these Tg (HaPrP) mice inoculated with Mo prions (Figure 6). These plaques reacted with Ha-specific MAbs (Barry and Prusiner, 1986). In non-Tg mice (*Prn<sup>0</sup>*) inoculated with Mo prions, spongiform change in the gray and white matter is commonly seen. HaPrP reactive plaques were not seen in non-Tg mice developing scrapie after inoculation with Mo prions.

### Discussion

Germane to deciphering the molecular mechanism of scrapie prion replication is the species barrier, which results in greatly prolonged incubation periods when prions from one species are inoculated into another (Pattison, 1965; Pattison and Jones, 1968). Recent investigations suggest that the species barrier resides in differences in the coding sequence of the PrP gene (Scott et al., 1989). Equally important are "strains" or isolates of scrapie agent that breed true (Dickinson et al., 1968; Dickinson and Outram, 1979). The results from early studies

Table 6. Species-Specific Prion Inocula Determine the Distribution of Spongiform Change and Deposition of PrP Amyloid Plaques in Tg Mice

Animal	Ha Prions					Mo Prions			
	n <sup>c</sup>	Spongiform Change <sup>a</sup>		PrP Plaques <sup>b</sup>		n <sup>c</sup>	Spongiform Change <sup>a</sup>		PrP Plaques <sup>b</sup>
		Gray	White	Frequency	Diameter <sup>d</sup>		Gray	White	
Non-Tg		ND <sup>e</sup>		ND		10	+	+	-
Tg 69	6	+ <sup>f</sup>	-	Numerous	6.5 ± 3.1 (389)	2	+	+	-
Tg 71	5	+	-	Numerous	8.1 ± 3.6 (345)	2	+	+	-
Tg 81	7	+	-	Numerous	8.3 ± 3.0 (439)	3	+	+	Few
Tg 7	2	ND		Numerous	14.0 ± 8.3 (19)		ND		ND
Syrian Ha	3	+	-	Numerous	5.7 ± 2.7 (247)		ND		ND

<sup>a</sup> Spongiform change evaluated in hippocampus, thalamus, cerebral cortex, and brainstem for gray matter, and the deep cerebellum for white matter.

<sup>b</sup> Plaques in the subcallosal region were stained with HaPrP MAb 13A5, anti-PrP rabbit antiserum R073, and trichrome stain.

<sup>c</sup> Number of brains examined.

<sup>d</sup> Mean diameter of PrP plaques given in microns ± SE with the number of observations in parentheses.

<sup>e</sup> Not determined.

<sup>f</sup> Plus sign = present; minus sign = not found.

were confusing since the two hosts C57BL/6 (*Prn<sup>a</sup>*) and VM (*Prn<sup>b</sup>*) mice have PrP genes that encode prion proteins differing at codons 108 and 189 (Westaway et al., 1987). Recently, inocula prepared from *Prn<sup>a</sup>* and *Prn<sup>b</sup>* mice were found to have different properties when analyzed in *Prn<sup>a</sup>*, *Prn<sup>b</sup>*, and (*Prn<sup>a</sup>* × *Prn<sup>b</sup>*)F1 mice (Carlson et al., 1989). If the genetic origin of the inoculum was the same with respect to the PrP genotype (*Prn-p*) as the inoculated host, the shortest incubation times were observed; altering this relationship lengthened the incubation time. Some isolates recovered from mice and hamsters have been found that breed true independent of the PrP gene sequence (Dickinson et al., 1968; Bruce and Dickinson, 1987; Kimberlin et al., 1989), indicating that inoculated prions impart to those synthesized de novo some structural features that govern their biological properties.

#### Levels of Transgene Expression Modulate Incubation Times

Our results demonstrate an inverse relationship between the steady-state level of HaPrP gene expression and the length of the scrapie incubation time after inoculation of Tg (HaPrP) mice with Ha prions (Table 1; Figure 3C). Tg mice with the highest steady-state levels of HaPrP mRNA and HaPrP<sup>C</sup> had the shortest scrapie incubation times and vice versa.

While our results argue for the importance of PrP mRNA and PrP<sup>C</sup> levels in modulating scrapie incubation times, these are clearly not the only factors capable of influencing the interval between inoculation and onset of clinical signs of neurologic dysfunction. For example, in mice inoculated with Mo prions, a gene controlling the incubation time, designated *Prn-i*, was found to be linked genetically with the PrP gene (Carlson et al., 1986). This linkage has been widely confirmed (Hunter et al., 1987; Carlson et al., 1988; Race et al., 1990). Short and long incubation time alleles of *Prn-i* are not associated with alterations in the steady-state levels of PrP mRNA or PrP<sup>C</sup>, but rather with mutations in the open reading frame (ORF) (Westaway et

al., 1987). However, other observations suggest that *Prn-i* and the MoPrP gene, *Prn-p*, are not necessarily congruent (Carlson et al., 1988).

The HaPrP transgene was also found to regulate the length of incubation time when Mo prions were inoculated. Low copy numbers of the transgene and low levels of HaPrP mRNA expression were associated with a modest lengthening of the incubation time, while high copy numbers and greater levels of HaPrP mRNA were correlated with substantial increases in incubation times (Table 1). Although HaPrP transgene expression led to a prolongation of scrapie incubation times after inoculation with Mo prions, it did not appear to alter MoPrP<sup>C</sup> levels (Figure 3B). Conclusive data addressing this issue must await the production of MoPrP-specific MAbs that can be used in an ELISA of the type reported here (Figure 4; Table 5). All four lines of Tg (HaPrP) mice had MoPrP<sup>C</sup> levels similar to those found in non-Tg mice (Figure 3B). If *Prn-i* and *Prn-p* prove to be congruent, then a plausible explanation for our observations is that HaPrP<sup>C</sup> binds to MoPrP<sup>C</sup> or MoPrP<sup>Sc</sup>, preventing the production of more MoPrP<sup>Sc</sup>. Alternatively, HaPrP<sup>C</sup> might modulate *Prn-i*.

The 42 kb cosmid insert used in our studies (Scott et al., 1989; Basler et al., 1986) forces us to consider the possibility that a non-PrP transgene product is the real arbiter of the Ha scrapie phenotype. Such a non-PrP transgene product might be encoded either by a gene within the intron or a flanking sequence. In one scenario, this hypothetical product would be expected to interact with PrP<sup>Sc</sup> or some as yet unidentified molecule in the scrapie prion inoculum in determining the length of the scrapie incubation times, the de novo synthesis of species-specific prions, and species-specific neuropathologic changes. Alternatively, if the hypothetical non-PrP transgene product is a necessary component of the inoculum, then it would have to purify with PrP<sup>Sc</sup> on MAb immunoaffinity columns, be susceptible to neutralization by polyclonal antibodies raised against PrP 27-30 purified by SDS-PAGE, and initiate polymerization of PrP into amyloid

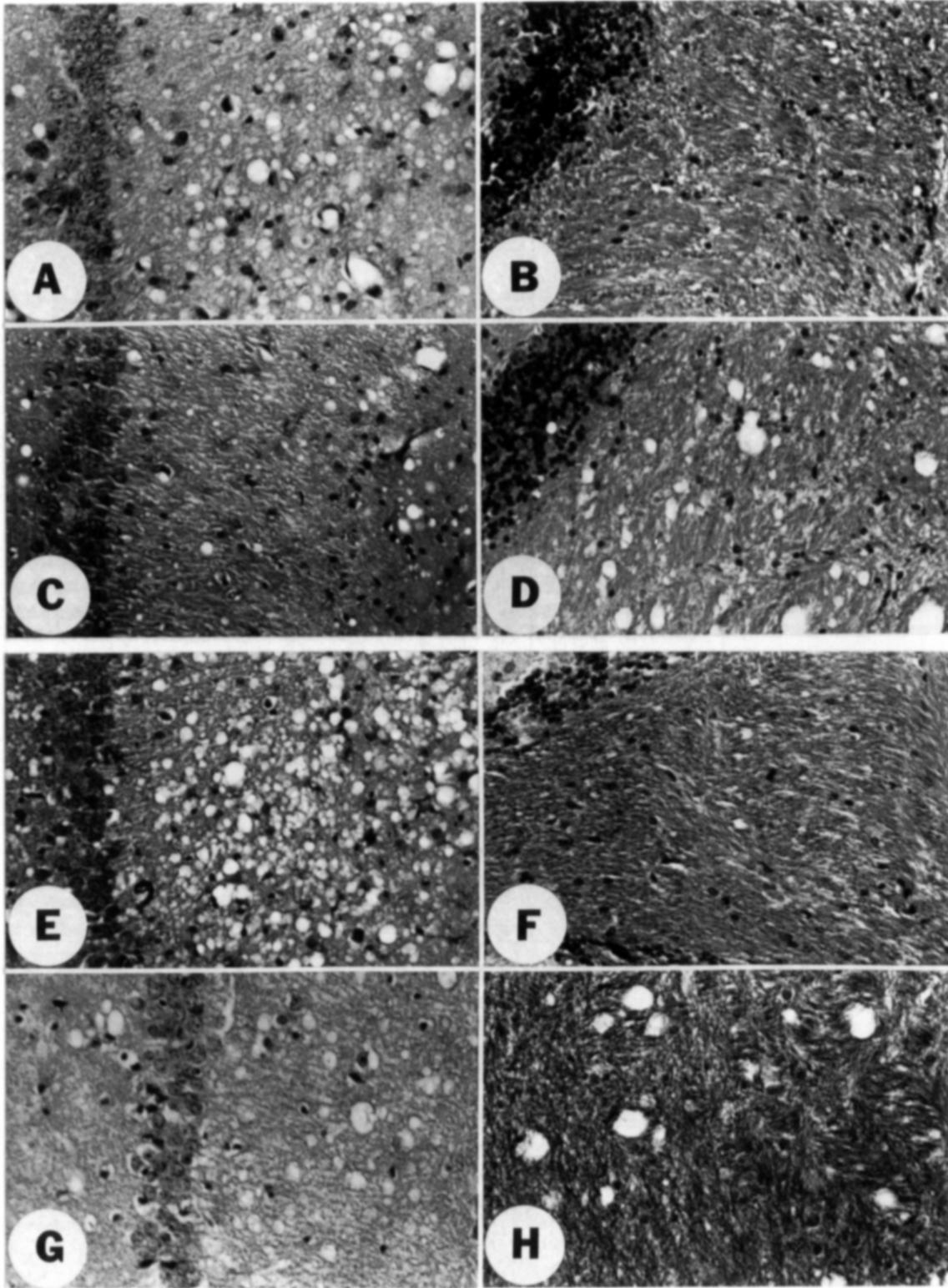


Figure 5. Distribution of Spongiform Degeneration in Two Tg Lines Inoculated with Ha or Mo Prions

The photomicrographs in (A), (C), (E), and (G) are of the hippocampal gray matter (left column), and the photomicrographs in (B), (D), (F), and (H) are of the cerebellar white matter (right column). (A and B) Tg 69 mice inoculated with Ha prions. (C and D) Tg 69 mice inoculated with Mo prions. (E and F) Tg 71 mice inoculated with Ha prions. (G and H) Tg 71 inoculated with Mo prions. Bar is 50  $\mu$ m.

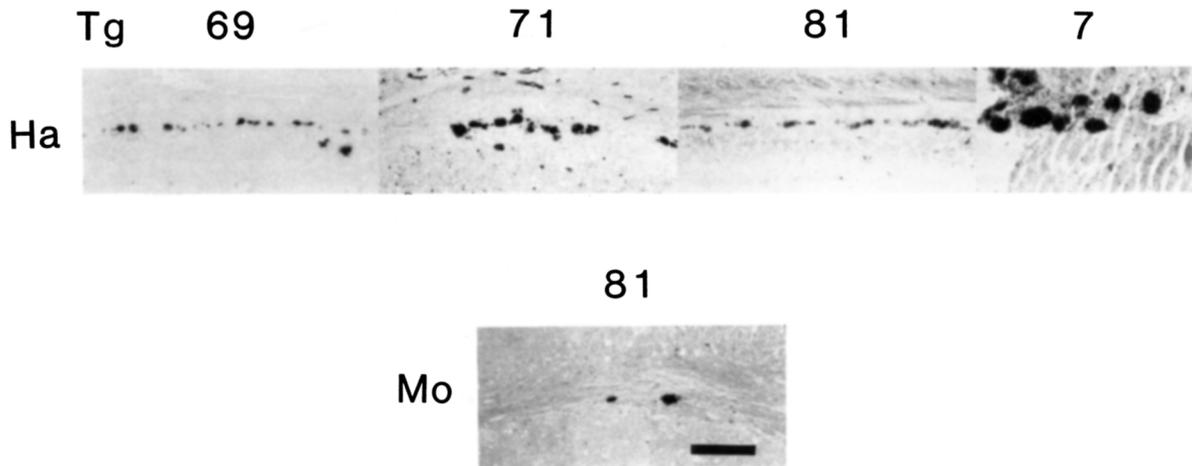


Figure 6. HaPrP Plaques in the Brains of Tg Mice Inoculated with Ha or Mo Prions

The plaques were immunostained with a HaPrP-specific MAb (13A5). In each case, plaques in the subcallosal region are illustrated. The Tg lines are designated by numbers above each micrograph. At the left side of each row, the inoculum is designated: Ha for hamster prions, and Mo for mouse prions. The bar is 50  $\mu$ m.

filaments that are deposited within PrP-immunoreactive plaques.

#### Do PrP Heterodimers Feature in the Synthesis of Infectious Prions?

That Tg (HaPrP) mice synthesize *de novo* the prion homologous to that in the inoculum may give some insight into the molecular mechanism of prion replication. In other words, if Tg (HaPrP) mice are inoculated with Mo prions, then they produce only Mo prions and those inoculated with Ha prions synthesize only Ha prions as determined by bioassays in hamsters and mice. This phenomenon was recorded in multiple mice from both the Tg 71 and 81 lines with different incubation times and steady-state levels of HaPrP<sup>C</sup> expression, attesting to the generality of our observations. Also noteworthy are the results of earlier studies showing that mixtures of Ha and Mo prions inoculated into Syrian hamsters and Swiss CD-1 mice produced scrapie incubation times similar to those observed when Ha and Mo prions were inoculated separately (Scott et al., 1989). It will be of interest to evaluate the effects of mixed inocula as well as combinations of inocula administered at various times on the production of Ha and Mo prions in Tg (HaPrP) mice.

The synthesis of Ha prions in Tg mice must involve one or possibly more translation products of the HaPrP cosmid insert since this is the only additional DNA that Tg (HaPrP) mice harbor compared to their littermate non-Tg controls. Our results argue that it is likely that HaPrP<sup>Sc</sup> in the prion inoculum interacts with the homologous transgene product HaPrP<sup>C</sup> or a precursor thereof in the *de novo* synthesis of more Ha prions. The minimum size of the putative PrP<sup>Sc</sup>-PrP<sup>C</sup> complex is a heterodimer. Interestingly, ionizing radiation studies suggest that the biologically active, i.e., infectious, particle has a molecular weight of 55,000. A particle of this size might be a homodimer composed of two PrP<sup>Sc</sup> molecules (Bellinger-

Kawahara et al., 1986). Alternatively, the hypothetical PrP<sup>Sc</sup>-PrP<sup>C</sup> complex could also be a multimer of PrP<sup>Sc</sup> and PrP<sup>C</sup> and even involve other cellular components (Oesch et al., 1990).

Whether this conversion of PrP<sup>C</sup> or a precursor into PrP<sup>Sc</sup> involves the addition or deletion of a chemical group, a tightly bound ligand, or only a conformational change remains to be established. To date, there is evidence for neither a chemical modification nor a ligand that is unique to the PrP<sup>Sc</sup> isoform. These observations raise the possibility that the difference between PrP<sup>C</sup> and PrP<sup>Sc</sup> is only conformational.

The PrP<sup>Sc</sup> molecule of the putative heterodimer might act as template for the conversion of the PrP<sup>C</sup> into a second PrP<sup>Sc</sup> molecule. Propagation of conformational changes in oligomeric enzyme complexes by constrained interactions among protomers (Monod et al., 1965; Fox et al., 1986) constitutes a modest precedent for the model proposed here. Noteworthy are many other examples of protein-catalyzed folding of protein molecules involving disulfide isomerases (Creighton et al., 1980), *cis trans* prolyl isomerases (Lang et al., 1987; Evans et al., 1987) as well as heat shock proteins (Ostermann et al., 1989), and a variety of other molecules that act as "chaperones" (Hemmingsen et al., 1988). In an ATP-dependent process, hsp60 has been shown to facilitate the folding of cytosolic proteins into protease-resistant molecules after they are imported into mitochondria (Ostermann et al., 1989); perhaps these observations provide a precedent for the acquisition of protease resistance by PrP<sup>Sc</sup>.

If prion synthesis proceeds through a replication intermediate such as a heterodimer of PrP<sup>C</sup> and PrP<sup>Sc</sup>, then it might also explain "strains" or isolates of scrapie prions that breed true. Such a model requires that PrP<sup>Sc</sup> molecules assume different stable conformations that could act as templates for the posttranslational conversion of PrP<sup>C</sup> into new PrP<sup>Sc</sup> molecules.

Cell-free translation of PrP RNA has shown that the same polypeptide chain can assume two different topologies corresponding to secretory and transmembrane forms (Hay et al., 1987a, 1987b). Recent studies identified a region of PrP on the N-terminal side of the membrane-spanning segment designated stop transfer effector (or STE) that regulates topology of PrP depending on which cell-free translational system is used (Yost et al., 1990; Lopez et al., 1990). Although both PrP molecules are protease sensitive and thus similar to PrP<sup>C</sup>, these findings establish that PrP can exist in multiple conformational states.

A heterodimer model is also compatible with the view that Gerstmann-Sträussler-Scheinker syndrome is both a genetic and infectious disease (Hsiao et al., 1989). Mutant PrP<sup>C</sup> molecules might spontaneously fold into the appropriate conformation for PrP<sup>Sc</sup> at some relatively low but finite frequency. If infectious prions are found to be devoid of foreign nucleic acid, then these PrP<sup>Sc</sup> molecules alone or complexed with some other cellular element should be sufficient to initiate the disease.

#### **Genetic Origin of Inoculum Determines Scrapie Neuropathology**

Our results in Tg mice inoculated with either Ha or Mo prions argue that the amino acid sequence of PrP<sup>Sc</sup> molecules is an important determinant of the scrapie phenotype with respect to both the clinical course of the disease and the neuropathologic features characterized by spongiform change and PrP amyloid plaque deposition. We found that the genetic origin of the inoculum in two different Tg Mo lines determined the distribution of vacuolar changes (Table 6; Figure 5). The most parsimonious interpretation of our observations is that PrP<sup>Sc</sup> in the inoculum dictates the *de novo* synthesis of prions, which in turn specifies the neuropathology. That PrP<sup>Sc</sup> in the prion inoculum determines the neuropathology gains further support by our findings that PrP amyloid plaques were numerous in the brains of Tg mice inoculated with Ha prions but infrequent in those injected with Mo prions (Table 6; Figure 6). These amyloid plaques contain filaments composed at least in part of PrP (DeArmond et al., 1985).

Whether our studies have identified the molecules responsible for neuropathologic findings in earlier studies is uncertain. The influence of mouse genotype and scrapie agent isolate on the distribution and extent of vacuolation has been frequently recorded (Fraser et al., 1989). The molecular mechanism responsible for numerous amyloid plaques in the brains of VM (*Prn<sup>b</sup>*) mice inoculated with the 87V isolate of Mo prions remains to be explained (Bruce et al., 1976; Bruce and Dickinson, 1985), but the amyloid plaques are known to contain PrP (Roberts et al., 1986; McBride et al., 1988).

#### **Toward Resolving Some Issues in Prion Biology**

Although our results can be explained by a "scrapie virus" model, there is no experimental evidence to support the existence of a scrapie-specific polynucleotide. In fact, there is a wealth of data that militates against the exis-

tence of this putative scrapie-specific nucleic acid (Prusiner, 1989) and marshalling arguments in favor of a "scrapie virus" becomes increasingly difficult as PrP and scrapie become more intertwined. For example, if we argue that the "scrapie virus" contains a distinct "genome" then to explain our current results we have to suppose that species specificity for scrapie resides both in host-encoded PrP molecules (Scott et al., 1989) and the putative genome. One possibility is that PrP<sup>C</sup> is a cellular receptor for the putative virus. To explain the copurification of PrP<sup>Sc</sup> and infectious particles (Gabizon and Prusiner, 1990) as well as the neutralization of scrapie infectivity with anti-PrP antiserum (Gabizon et al., 1988), PrP<sup>Sc</sup> would have to be either an integral component of the "virus" or at least a tightly bound form of the receptor. The inherited prion diseases such as Gerstmann-Sträussler syndrome, in which point mutations or insertions within the PrP ORF are clearly implicated (Hsiao et al., 1989), might be reconciled with the "virus susceptibility" model by arguing that mutations within the PrP ORF predispose cells to infection. Such a model requires either a "virus" that is ubiquitous in the environment (Weissmann, 1989) or one that is induced by the mutant PrP gene product, for example, an endogenous provirus. Although each of these arguments is plausible by itself, the "scrapie virus" model becomes less tenable when all the information on the molecular properties of prions is considered (Prusiner, 1989).

Our observations argue that the steady-state level of PrP<sup>C</sup> directly modulates the rate of prion biosynthesis. We assume that as the rate of prion production increases, the incubation time decreases since neurologic dysfunction becomes evident when prion accumulation reaches some critical level. Yet, we have no cogent explanation for differences in prion titers found in the brains of Tg 71 and 81 mice at the time of overt disease inoculated with Ha prions compared with those receiving Mo prions (Table 2). Tg (HaPrP) mice inoculated with Ha prions accumulate  $\sim 10^9$  ID<sub>50</sub> units of Ha prions per gram of brain in clinically ill animals as judged by Ha bioassay, while Tg (HaPrP) mice inoculated with Mo prions generate  $\sim 10^6$  ID<sub>50</sub> units of Mo prions. Similar levels of Ha and Mo prions were found in the brains of clinically ill hamsters and mice, respectively, as determined by bioassays (Eklund et al., 1967; Marsh and Kimberlin, 1975; Prusiner et al., 1977, 1982b). Our results argue that the level of a particular prion at the time of clinical illness is a function of the genetic origin of that prion, i.e., the PrP ORF sequence, and not the genetic background, excluding PrP genes, of the host. However, the rate of prion synthesis as measured by the length of the incubation time appears to be a function of the levels of PrP mRNA and PrP<sup>C</sup> (Tables 1 and 5; Figures 2 and 3). From a pragmatic view, these observations, especially those with the Tg 7 mice, suggest that it may be possible to construct Tg mice in the future with incubation times of <10 days. The availability of such animals would transform scrapie research.

The behavior of Tg mice expressing HaPrP genes strengthens the assertion that PrP<sup>Sc</sup> is a necessary and major component of the infectious scrapie prion particle. Compelling evidence argues for the formation of a com-

plex composed of PrP<sup>Sc</sup> and either PrP<sup>C</sup> or a precursor during the replication of scrapie prion infectivity. We are aware of no experimental results that militate against this hypothesis. Creating chimeric Ha-Mo PrP transgenes may allow physical mapping of the domains of the PrP molecule that mediate the interaction between PrP isoforms.

#### Experimental Procedures

##### Sources of Scrapie Prions

A Syrian hamster-adapted isolate of scrapie prions was provided by Dr. Richard Marsh (Marsh and Kimberlin, 1975). This isolate, designated Sc237, was passaged repeatedly in golden Syrian hamsters (LVG:Lak) purchased from Charles River Laboratories (Wilmington, MA). The properties of the Sc237 isolate are similar to those reported for an isolate designated 263K (Kimberlin and Walker, 1977). The Chandler isolate from Swiss mice (Chandler, 1961) was provided by Dr. William Hadlow and was passaged in Swiss mice from a closed colony at the Rocky Mountain Laboratory or in Swiss CD-1 mice obtained from Charles River Laboratories. This murine isolate was designated RML.

##### Determination of Scrapie Incubation Periods

Mice were inoculated intracerebrally with 30  $\mu$ l of prions using a 27-gauge disposable hypodermic needle inserted into the right parietal lobe; hamsters received 50  $\mu$ l intracerebrally. Criteria for diagnosis of scrapie in mice and hamsters have been described (Prusiner et al., 1982b; Carlson et al., 1986). After inoculation, the mice and hamsters were examined for neurologic dysfunction every 3 days. Once clinical signs of scrapie were detected, the animals were examined daily. When some animals whose death was clearly imminent were identified, their brains were taken for histologic examination and confirmation of the diagnosis of scrapie.

##### Bioassays of Ha and Mo Prions

Titers of Ha and Mo prions in brain extracts were determined by incubation time measurements according to the protocols described above. The inoculated dose was calculated from equations derived from published curves relating scrapie incubation times to the dose of prions (Prusiner et al., 1982b; Butler et al., 1988).

##### Construction of Tg Mice

Tg mice were produced by microinjection of DNA constructs into the pronuclei of one-cell mouse eggs, as previously described (Scott et al., 1989). Briefly, the eggs were obtained from superovulated (C57BL/6  $\times$  SJL)F1/J hybrid females mated to (C57BL/6  $\times$  SJL)F1/J males, both of which were purchased from Jackson Laboratories (Bar Harbor, ME) for the Tg 69, Tg 71, and Tg 81 lines constructed at the University of California, San Francisco. (C57BL/6  $\times$  LT/Sv)F1/J mice were used to produce the Tg 7 and Tg 20 mice by a similar protocol at the Jackson Laboratory. The morning following fertilization, all eggs were flushed from the oviducts and placed into the culture medium. Cumulus cells were removed from the eggs with hyaluronidase (1000 U/ml) and subsequently transferred through four drops of medium to wash the eggs free of enzyme. Following the DNA microinjections, the eggs were incubated overnight and transferred to the oviducts of pseudopregnant CD1 recipient mice. Weanling animals were screened for the presence of the HaPrP gene. Tg 69, Tg 71, and Tg 81 founder animals were then mated to (C57BL/6  $\times$  SJL)F1/J mice to yield the first generation of Tg animals. First-generation transgenic animals were again mated to (C57BL/6  $\times$  SJL)F1/J mice to provide second-generation Tg and non-Tg animals that were incorporated into the experimental design. The Tg 7 and Tg 20 founders were mated with C57BL/6 mice in all subsequent crosses.

Tail tissue from newly weaned pups was digested in 0.7 ml of 50 mM Tris-HCl (pH 8.0), 100 mM EDTA, 0.15% SDS buffer containing 35  $\mu$ l of a 10 mg/ml solution of proteinase K. Following incubation overnight, the DNA was extracted first with buffered saturated phenol and second with phenol-CHCl<sub>3</sub> (1:1). Nucleic acid was collected by precipitation with 2 vol of ethanol at room temperature and resuspended in 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. Aliquots of tail DNAs were screened

by slot-blot hybridization using the radiolabeled hamster ORF probe and stringent hybridization conditions as described (Scott et al., 1989).

##### Northern Blots

Total RNA was prepared by a single-step method using acid guanidinium-thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and then lithium chloride precipitation (Puissant and Houdebine, 1989). Frozen brain tissue (~0.5 g) was homogenized at room temperature with 5 ml of a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. Sequentially, 0.5 ml of 2 M sodium acetate (pH 4.0), 5 ml of water-saturated phenol, and 1 ml of chloroform-isoamyl alcohol (49:1) were added to the homogenates and mixed after each addition. The suspension was vortexed for 10–15 s and then put on ice for 15 min. The samples were centrifuged at 10,000  $\times$  g for 20 min at 4°C. The RNA aqueous phase was transferred to a clean tube and precipitated with 5 ml of isopropanol for 2 hr at –20°C. The RNA was pelleted at 10,000  $\times$  g for 10 min at 4°C. The pellet was resuspended in 1 ml of 4 M lithium chloride with vigorous vortexing and then centrifuged at 3000  $\times$  g for 10 min at 4°C. This pellet was dissolved in 1 ml of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, extracted with 1 ml of chloroform-isoamyl alcohol (49:1), and centrifuged at 3000  $\times$  g for 10 min. The aqueous phase was then precipitated with 1 ml of isopropanol in the presence of 0.2 M sodium acetate (pH 5.0) at –20°C overnight. The RNA pellet was resuspended in diethylpyrocarbonate (DEP)-treated water (500  $\mu$ l), and the concentration of RNA was determined by A<sub>260</sub> spectrophotometric analysis. RNA was ethanol precipitated, dried, and resuspended in sample buffer containing 2  $\mu$ l of sterile water, 5  $\mu$ l of deionized formamide, 2  $\mu$ l of formaldehyde, 1  $\mu$ l of 10 $\times$  running buffer, and 1  $\mu$ l of 400  $\mu$ g ml<sup>-1</sup> ethidium bromide (Rosen and Villa-Komaroff, 1990). The samples were mixed thoroughly and heated for 10 min at 65°C. The samples were placed on ice and briefly spun to collect any condensate. The agarose gel (0.8%) containing 6.6% (w/v) formaldehyde was electrophoresed at 3 v/cm in recirculating 1 $\times$  running buffer (20 mM MOPS, 1 mM EDTA, 1 mM sodium acetate [pH 7.0], 6.6% formaldehyde). After electrophoresis the gel was soaked in several changes of DEP-treated water for 30 min, 0.05 N NaOH for 20 min, and then 20 $\times$  SSC for 45 min. The RNA was transferred to nitrocellulose overnight in 10 $\times$  SSC and the blot washed for 15 min in 3 $\times$  SSC and baked for 2 hr at 80°C. The blotted RNA was visualized by UV light to confirm uniform transfer. An AccI-EcoRI probe fragment was excised from the 3' untranslated region of PrP cDNA clone S<sub>11</sub> (Basler et al., 1986) and was random primed radiolabeled with DNA polymerase I Klenow fragment as described (Scott et al., 1989). Hybridization at 42°C for 40 hr was as described previously (Westaway et al., 1987), except the composition of the hybridization buffer was 3 $\times$  SSC, 50 mM HEPES (pH 7.4), 50% formamide, 2.5 $\times$  Denhardt's solution, 100  $\mu$ g/ml heparin (Singh and Jones, 1984), and 0.5% SDS. The final wash was performed in 0.1% SDS, 0.1 $\times$  SSC for 20 min at 68°C.

##### Generation of Synthetic HaPrP RNA

The "pSP PrP 5' minus" is a subclone in which a putative cloning artifact at the 5' end of the Ha cDNA clone S<sub>11</sub> (Basler et al., 1986) was deleted. The S<sub>11</sub> cDNA fragment flanked by EcoRI linkers was cloned into the EcoRI site of pSP64 (Krieg and Melton, 1984; Hay et al., 1987b). This plasmid was digested with SacI and SacII, and the resultant termini were blunt ended and religated to create a deletion running from the SacI site of the pSP64 polylinker to a SacII site within the 5' untranslated region of the cDNA insert. CsCl gradient-purified plasmid was linearized with EcoRI, proteinase K digested, phenol extracted, and ethanol precipitated and transcribed *in vitro* with SP6 RNA polymerase (Promega Corporation, Madison, WI) according to the manufacturer's recommendations. Following RQ1 DNAase treatment (Promega Corporation), the resultant RNA was extracted and precipitated and its concentration determined by absorbance at 260 nm. The RNA was serially diluted in DEP-treated water prior to use.

##### ELISA and Western Blots

The quantitation of PrP from brain homogenates was determined by a modified ELISA (Ijsselmuiden et al., 1987, 1989; Uhl and Newton, 1988). Hamster brains were prepared as 10% homogenates in 0.25 M sucrose. The homogenate was resuspended in 1 M guanidinium thiocyanate in phosphate-buffered saline (PBS)-Tween (0.15 M NaCl, 10

mM sodium phosphate [pH 7.4] with 0.05% Tween 20) to give a final concentration of 2 mg/ml. Samples (5  $\mu$ l) were applied to nitrocellulose membrane (Schleicher and Schuell, Keene, NH) via a manifold filtration unit (Schleicher and Schuell) and the membrane left at room temperature to dry for 30 min. After rinsing with PBS, the nitrocellulose membrane was sequentially treated as follows: incubation at 37°C for 1 hr with 5% nonfat dry milk in PBS; incubation at room temperature for 30 min with avidin (25  $\mu$ g/ml) (Boehringer Mannheim, Indianapolis, IN) in PBS-Tween to block endogenous biotin activity (Wood and Warnke, 1981); incubation with biotin (2  $\mu$ g/ml) (Boehringer Mannheim) in PBS-Tween twice for 15 min; incubation at 4°C overnight with biotinylated MAb 13A5, which recognizes HaPrP but not MoPrP (Barry and Prusiner, 1986); washing with PBS-Tween three times for 10 min; incubation with peroxidase-conjugated streptavidin (Pierce Chemical Co., Rockford, IL) for 90 min at room temperature; washing with PBS-Tween three times for 10 min, and then cutting each dot out with a puncher and placing it into the well of 96-well plate; color development in the dark for 30 min by adding 100  $\mu$ l of 0.05% O-phenylenediamine and 0.015% H<sub>2</sub>O<sub>2</sub> in citrate-phosphate buffer (pH 5.0) to each well (Ambler and Peters, 1984). To stop the reaction, 100  $\mu$ l of 2.5 M sulfuric acid was added, and the resulting solution was transferred to an empty 96-well plate for absorbance measurements at 492 nm with a Titertek Multiskan II spectrophotometer (Flow Laboratories, Rockville, MD).

All measurements were made in triplicate. The SDS-PAGE purified HaPrP 27-30 was prepared as previously described (Endo et al., 1989) and its concentration determined by the amino acid analysis (Stahl et al., 1987). Known amounts of PrP 27-30 (from 4 ng to 0.06 ng) were used to construct a standard curve for correlating optical densities of protein samples to their PrP contents (Leinikki et al., 1978). To determine PrP<sup>Sc</sup>, limited proteinase K digestion was carried out in the brain homogenates to remove PrP<sup>C</sup> immunoreactivities prior to the ELISA (Prusiner et al., 1982a). PrP<sup>C</sup> content was calculated by subtracting the PrP<sup>Sc</sup> from the total PrP (PrP<sup>C</sup> and PrP<sup>Sc</sup>) measured in the absence of proteinase K digestion.

Western immunoblots were performed as described (Towbin et al., 1979; Barry et al., 1985; Oesch et al., 1985; Barry and Prusiner, 1986) with 75  $\mu$ g of protein in brain extracts for each SDS-PAGE lane. The extracts from brains of Tg mice were prepared as follows: brains were disrupted in a Dounce homogenizer in 9 vol of cold lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% NP40, 0.5% deoxycholate in 10 mM Tris-HCl [pH 7.4]). Insoluble debris was removed by centrifugation at 500  $\times$  g for 5 min. The supernatants were aliquoted and stored at -70°C.

Mouse-specific PrP antibodies were raised in Syrian hamsters immunized weekly with 5  $\times$  10<sup>7</sup> MoCHO cells that express recombinant, membrane-bound MoPrP<sup>C</sup> (M. Rogers, D. Serban, D. Lowenstein, D. Bredesen, and S. B. Prusiner, unpublished data). The antiserum induced after four injections, designated H5A, was found to react with MoPrP but not HaPrP.

#### Neuropathologic Studies

Brain tissue was immersion fixed in 10% buffered formalin after sacrifice of the animal. Alternatively, animals were anesthetized with pentobarbital, and the brain was perfusion fixed through the cardiac route with either 10% buffered formalin or periodate-lysine-0.5% paraformaldehyde fixative (DeArmond et al., 1987b). In all cases, the brain tissue was embedded in paraffin, and 8  $\mu$ m thick histological sections were prepared.

Tissue sections mounted on polylysine-coated slides were deparaffinized in xylene followed by two washes in 100% ethanol. Endogenous peroxidase activity was blocked with methanol-hydrogen peroxide (2 ml of 30% H<sub>2</sub>O<sub>2</sub> in 200 ml of methanol) followed by two rinses in PBS. The sections were then treated with proteinase K (5  $\mu$ g/ml) for 10 min at room temperature followed by three rinses with PBS. Each section was treated with 3 M guanidinium thiocyanate for 10 min because this denaturation step was found to greatly enhance immunoreactivity of proteinase K-resistant prion proteins on Western transblots (Serban et al., 1990; Taraboulos et al., 1990). This step significantly increased the intensity of immunoperoxidase staining of nonamyloid PrP in the gray and white matter. It was followed by three rinses with PBS for a total of 30 min. Nonspecific protein binding was blocked with 10% normal goat serum in calcium- and magnesium-free PBS (CMF-PBS) (10 min, room temperature).

The polyclonal PrP antiserum, R073, was diluted 1:100 and the glial fibrillary acidic protein antiserum was diluted 1:250 in CMF-PBS. Tissue sections were incubated with the primary antisera overnight at 4°C. Following two rinses with PBS, the sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA), 1:250 dilution, for 30 min at room temperature. After two rinses with PBS, the sections were incubated with an avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min at room temperature. Two rinses with PBS were then followed by a 5 min reaction with diaminobenzidine solution (5 mg of diaminobenzidine tetrahydrochloride, 68 mg of imidazole, and 7 mg of NaN<sub>3</sub> in 10 ml of CMS-PBS), to which 100  $\mu$ g of 0.0015% H<sub>2</sub>O<sub>2</sub> was added.

The avidin-biotin-peroxidase immunohistochemical procedure for MAbs differed from the above procedure for rabbit antisera in several respects. Ten percent normal horse serum was used to block non-specific protein binding. The primary antibody (MAb 13A5) was diluted 1:1 for overnight incubation at 4°C, and the biotinylated antiserum was horse anti-mouse IgG.

Serial sections were stained by the Masson trichrome method to reveal potential amyloid plaques and spongiform degeneration.

The smallest diameter of all amyloid plaques greater than 3  $\mu$ m in the subcallosal region was measured after tissue sections were stained with the 13A5 MAb. The Bioquant System IV software (R&M Biometrics, Inc., Nashville, TN) was used to compile the measurements and for statistical analysis.

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