PHYSIOLOGY. For the article "Nitrite reductase activity of myoglobin regulates respiration and cellular viability in myocardial ischemia-reperfusion injury," by Ulrike B. Hendgen-Cotta, Marc W. Merx, Sruti Shiva, Joel Schmitz, Stefanie Becher, Johann P. Klare, Heinz-Jürgen Steinhoff, Axel Goedecke, Jürgen Schrader, Mark T. Gladwin, Malte Kelm, and Tienush Rassaf, which appeared in issue 29, July 22, 2008, of *Proc Natl Acad Sci USA* (105:10256–10261; first published July 16, 2008; 10.1073/pnas.0801336105), the authors note two printer's errors. In the last line of the Abstract, "myoglobin knockout mice" appeared inadvertently and should be deleted. In addition, in Fig. 1*A*, the red time course did not print. The corrected figure and its legend appear below.

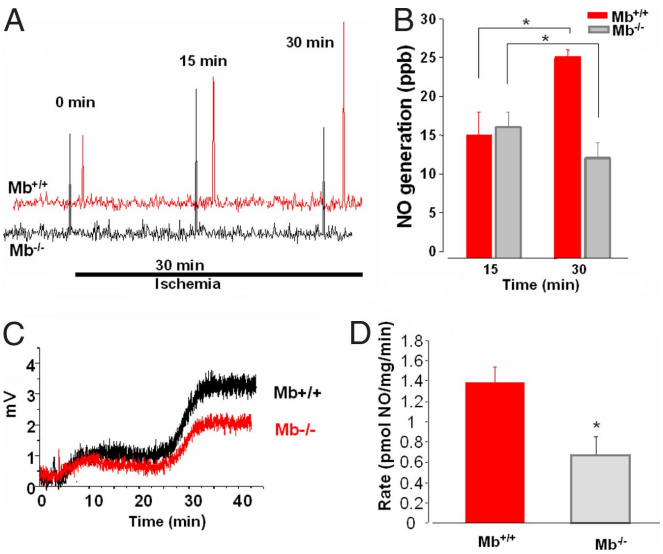

Fig. 1. NO• production from the *ex vivo* retrograde perfused heart during ischemia by using the NO• collection heart chamber. (*A*) Representative time courses of NO• production from nitrite during 30-min ischemia in myoglobin wild-type and knockout hearts measured by chemiluminescence every 15 min. (*B*) NO• generation in myoglobin$^{+/+}$ and myoglobin$^{-/-}$ hearts after 15 and 30 min of ischemia. Application of nitrite (100 μM) showed a time-dependent increase of NO• production in myoglobin$^{+/+}$ hearts from 15 ± 3 ppb to 25 ± 1 ppb ($P < 0.05$; $n = 3$), whereas in myoglobin$^{-/-}$ hearts, a decrease from 16 ± 2 ppb to 12 ± 2 ppb was observed ($P < 0.05$; $n = 3$). (*C*) Representative traces of NO• generation by heart homogenates from myoglobin$^{+/+}$ and myoglobin$^{-/-}$ mice treated with nitrite (1 mM). (*D*) Quantitation of nitrite generation from several curves similar to *C* ($P < 0.05$; $n = 4$). *, $P < 0.05$.

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GENETICS. For the article "Formation of native prions from minimal components *in vitro*," by Nathan R. Deleault, Brent T. Harris, Judy R. Rees, and Surachai Supattapone, which appeared in issue 23, June 5, 2007, of *Proc Natl Acad Sci USA* (104:9741–9746; first published May 29, 2007; 10.1073/pnas.0702662104), the authors note that on page 9743, right column, in the third full paragraph, line 15, it is stated that there are $\approx 1.4 \times 10^7$ *in vitro*-propagated PrP^{Sc} monomers and $\approx 4.3 \times 10^5$ PrP27-30 monomers per LD_{50} unit. However, the values were miscalculated. The correct values are $\approx 1.4 \times 10^8$ *in vitro*-propagated PrP^{Sc} monomers and $\approx 4.3 \times 10^6$ PrP27-30 monomers per LD_{50} unit. These errors do not affect the conclusions of the article.

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Formation of native prions from minimal components *in vitro*

Nathan R. Deleault*, Brent T. Harris†, Judy R. Rees‡, and Surachai Supattapone*§¶

Departments of *Biochemistry, †Pathology, ‡Community and Family Medicine (Biostatistics and Epidemiology), and §Medicine, Dartmouth Medical School, Hanover, NH 03755

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The conformational change of a host protein, PrP^C, into a disease-associated isoform, PrP^{Sc}, appears to play a critical role in the pathogenesis of prion diseases such as Creutzfeldt–Jakob disease and scrapie. However, the fundamental mechanism by which infectious prions are produced in neurons remains unknown. To investigate the mechanism of prion formation biochemically, we conducted a series of experiments using the protein misfolding cyclic amplification (PMCA) technique with a preparation containing only native PrP^C and copurified lipid molecules. These experiments showed that successful PMCA propagation of PrP^{Sc} molecules in a purified system requires accessory polyanion molecules. In addition, we found that PrP^{Sc} molecules could be formed *de novo* from these defined components in the absence of preexisting prions. Inoculation of samples containing either prion-seeded or spontaneously generated PrP^{Sc} molecules into hamsters caused scrapie, which was transmissible on second passage. These results show that prions able to infect wild-type hamsters can be formed from a minimal set of components including native PrP^C molecules, copurified lipid molecules, and a synthetic polyanion.

polyanion | PrP | purified | spontaneous | *de novo*

A critical event in the pathogenesis of prion disease appears to be misfolding of the host-encoded prion protein (PrP^C) into a pathogenic isoform (PrP^{Sc}). According to the "protein-only" hypothesis, infectious mammalian prions may be comprised exclusively of PrP^{Sc} molecules (1). Work using refolded, recombinant proteins in both mammalian and fungal systems has confirmed the remarkable concept that pure proteins can harbor infectious properties (2–4). However, the mechanism by which wild type PrP^C molecules are converted into infectious PrP^{Sc} molecules is currently unknown.

Studies using model systems have also suggested that host-encoded factors other than PrP^C may be required to propagate prions *in vitro* and *in vivo* (5–10). Furthermore, the restricted range of neuronal and nonneuronal cell types that are susceptible to infection by prions also suggests the existence of prion propagation cofactors (11–13). Although no specific cofactors have been identified to date, several studies have shown that various polyanionic compounds, such as host-encoded RNA and proteoglycan molecules, appear to stimulate prion-seeded conversion of PrP^C into PrP^{Sc} molecules *in vitro* (10, 14–17).

A powerful approach to identify the requirements for prion formation is the use of *in vitro* PrP^{Sc} conversion systems, such as the cell-free conversion assay (18–20) and the protein misfolding cyclic amplification (PMCA) technique (21–24). Recently, Castilla *et al.* (22) serially propagated PrP^{Sc} molecules and infectious prions *in vitro* by subjecting brain homogenates to PMCA. Building on this advance, we investigated whether purified PrP^C molecules could function as a substrate for the propagation of PrP^{Sc} molecules and infectious prions *in vitro*. To perform these studies, it was necessary to develop a protocol to purify PrP^C from native brain tissue because recombinant PrP produced in *Escherichia coli* is an inefficient substrate for PMCA reactions, even when reconstituted with crude brain homogenates (25). During the course of these studies, we identified conditions under which purified substrates could propagate infectious prions *in vitro* and also unexpectedly discovered that infectious prions could be generated spontaneously from purified, noninfectious components.

Results

Seeded PrP^{Sc} Propagation with Purified Substrates. We previously reported that partially purified hamster PrP^C molecules efficiently convert into a protease-resistant conformation upon incubation with PrP^{Sc} seed and synthetic polyanions *in vitro* (10). To obtain a more homogeneous preparation of hamster PrP^C substrate for the current studies, we developed a modified purification protocol using a combination of detergent solubilization, Protein A agarose, PrP immunoaffinity, and cation exchange chromatographic steps. This protocol yielded a preparation containing 30- to 33-kDa proteins, as determined by SDS/PAGE (Fig. 1, lane 3). We analyzed the chemical composition of the purified preparation using a variety of sensitive techniques and detected only hamster PrP^C plus equimolar quantities of 20-carbon fatty acids [described in supporting information (SI) *Materials and Methods*].

To test the ability of purified PrP^C to propagate prions *in vitro*, we used the serial dilution/propagation protocol described by Castilla *et al.* (22) (Fig. 2*A*). We first tested the ability of several purified substrate mixtures to facilitate the PMCA propagation of PrP^{Sc} molecules in reactions originally seeded with Sc237 PrP27-30 molecules. The results showed that a substrate mixture containing both purified PrP^C plus synthetic poly(A) RNA molecules successfully propagated PrP^{Sc} molecules for 16 consecutive rounds of PMCA (Fig. 2*B Bottom*), whereas poly(A) RNA or PrP^C molecules alone failed to propagate PrP^{Sc} (Fig. 2*B Top* and *Middle*). These results indicate that the propagation of Sc237 PrP^{Sc} *in vitro* requires both PMCA sonication and an accessory polyanion. No propagation was obtained without sonication (SI Fig. 5).

To determine whether the propagation of other prion strains might also require an accessory polyanion, we performed similar serial PMCA-propagation experiments using 139H PrP27-30 molecules as the initial seed. Again, PrP^C alone failed to propagate PrP^{Sc}, whereas PrP^C plus poly(A) RNA successfully propagated PrP^{Sc} during all 16 days tested (Fig. 2*C*). These results show that *in vitro* propagation of 139H PrP^{Sc} also depends on the presence of an accessory polyanion, and therefore the phenomenon of polyanion

Author contributions: N.R.D. and S.S. designed research; N.R.D., B.T.H., and S.S. performed research; N.R.D., B.T.H., J.R.R., and S.S. analyzed data; and N.R.D., B.T.H., J.R.R., and S.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: PMCA, protein misfolding cyclic amplification; sCJD, sporadic Creutzfeldt–Jakob disease.

See Commentary on page 9551.

¶To whom correspondence should be addressed at: Department of Biochemistry, 7200 Vail Building, Dartmouth Medical School, Hanover, NH 03755. E-mail: supattapone@dartmouth.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0702662104/DC1.

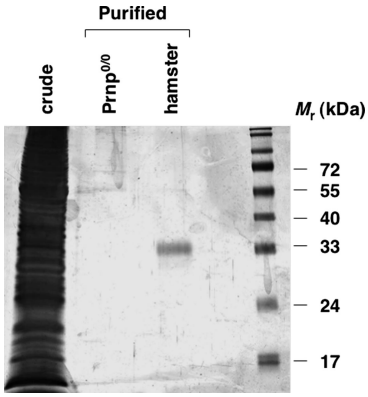

Fig. 1. Silver stain analysis of purified PrP^C substrate. Twelve percent SDS/PAGE showing (from left to right): crude, detergent-solubilized brain supernatant; preparation mock-purified from $Prnp^{0/0}$ mouse brains; preparation purified from normal hamster brains; and molecular weight markers.

dependence is not limited to the propagation of one specific prion strain.

We tested other charged polymeric compounds for their ability to support purified PrP^{Sc} propagation and found that only single-stranded polyanions sufficed for this process (SI Fig. 6). To determine whether accessory polyanions are also required for the maintenance of purified PrP^{Sc} propagation, we used nuclease-treated, PMCA-generated PrP^{Sc} molecules to seed subsequent propagation reactions containing either purified PrP^C substrate alone or PrP^C plus poly(A) RNA. These experiments confirmed that polyanions are required to maintain PMCA propagation of PrP^{Sc} molecules (SI Fig. 7).

We next measured the sensitivity of PMCA using purified substrates. The results of this experiment showed that the minimum dilution of the PMCA product from a typical PrP^{Sc} propagation experiment required to seed PrP^{Sc} formation in the next round is between 1:5,000 and 1:10,000, and the minimum dilution of the PMCA product required to seed PrP^{Sc} formation after three successive propagation rounds is $\approx 10^{-11}$ (equivalent to 1 fl of undiluted PMCA product) (SI Fig. 8). The seven orders of magnitude increase in sensitivity gained by performing three rounds of PMCA propagation resembles the previously reported increased sensitivity of multiple-round PMCA reactions using brain homogenate substrate (24, 26). We estimate that the concentration of PrP^{Sc} molecules present in a sample of undiluted PMCA product to be ≈400 ng/ml as determined by comparison with reference amounts of recombinant PrP. Using this value, we calculate that the minimum number of PrP^{Sc} molecules required to seed PrP^{Sc} formation in a three-round PMCA propagation experiment is ≈7 monomers (no. of monomers = reaction volume 100 μl × limiting dilution $10^{-11}\times$ estimated PrP^{Sc} concentration 400 ng/ml × monomeric PrP molecular weight 35,000 ÷ Avogadro's number). Interestingly, this calculated value is close to the measured minimum size of brain-derived infectious scrapie particles (2–6 PrP^{Sc} monomers) (27–29) as well as the minimum number of PrP^{Sc} monomers (equal to 26) required to initiate serial PMCA propagation reactions in crude homogenates (26). It should be noted that approximately half of the PrP^C preparations generated were less sensitive, i.e., required seed more concentrated than a 10^{-11} dilution of PMCA product to initiate a three-round PMCA propagation experiment; such preparations were not used for subsequent experiments.

Spontaneous Formation of PrP^{Sc} Molecules. During the studies described above, we performed a serial PMCA-propagation experiment using PrP^C plus poly(A) RNA substrate that was not originally seeded with infectious prions. To our surprise, we observed that

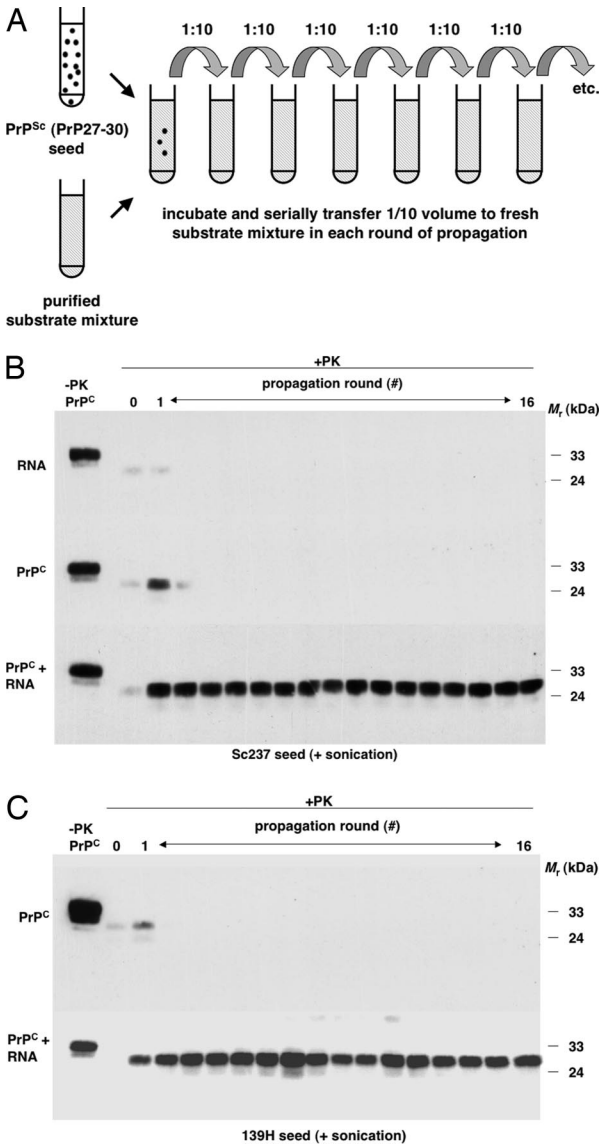

Fig. 2. Seeded PrP^{Sc} propagation by using purified substrates. (*A*) Schematic diagram of serial dilution and propagation paradigm used in subsequent experiments, adapted from Castilla *et al.* (22). (*B* and *C*) Western blots showing samples subjected to 16 rounds of PMCA, serial dilution, and propagation as depicted in *A*. (*B*) Samples originally seeded with Sc237 PrP27-30 were propagated in substrate containing either poly(A) RNA alone (top gel), purified PrP^C alone (middle gel), or PrP^C plus poly(A) RNA (bottom gel). (*C*) Samples originally seeded with 139H PrP27-30 were propagated in substrate containing either purified PrP^C alone (top gel), or poly(A) PrP^C plus poly(A) RNA (bottom gel). In all gels, a sample containing PrP^C not subjected to proteinase K digestion is shown in the first lane as a reference for comparison of electrophoretic mobility (PrP^C-PK). All other samples were subjected to limited proteolysis with 50 μg/ml proteinase K (+PK).

PrP^{Sc} molecules spontaneously appeared during the "mock" propagation of these unseeded substrates (Fig. 3*A*). The spontaneously appearing (*de novo*) PrP^{Sc} molecules migrated on SDS/PAGE with an apparent molecular mass of ≈28 kDa after proteinase K treatment, similar to the apparent molecular mass of brain-derived Sc237 PrP27-30. Once formed, *de novo* PrP^{Sc} molecules serially propagated the formation of PrP^{Sc} molecules for the remainder of the experiment.

We next conducted a control experiment to investigate the possibility that environmental contamination during sonication or sample processing could have been responsible for the apparently

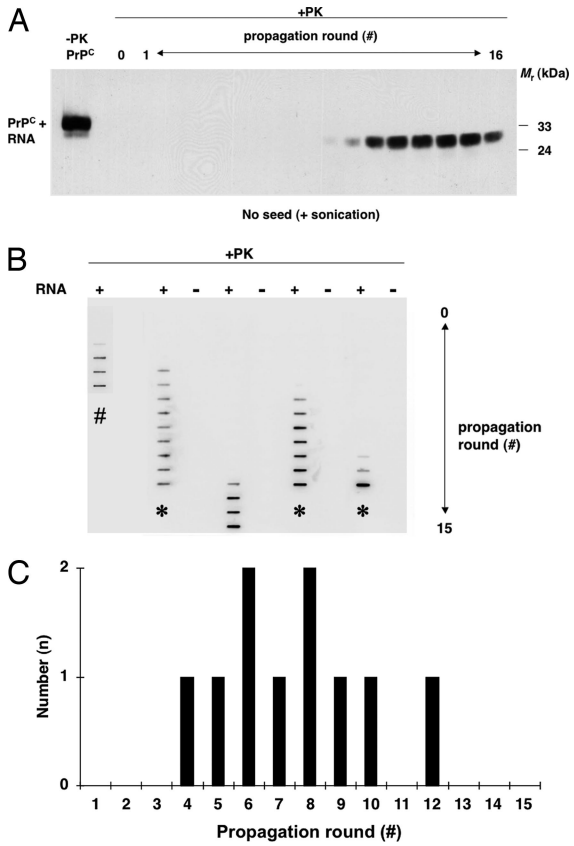

Fig. 3. Formation of PrP^{Sc} molecules *de novo* during serial PMCA propagation of unseeded purified substrates. (*A*) Western blot showing unseeded samples containing PrP^{C} plus poly(A) RNA subjected to 16 rounds of PMCA, serial dilution, and propagation. A sample containing PrP^{C} not subjected to proteinase K digestion is shown in the first lane as a reference for comparison of electrophoretic mobility (PrP^{C}-PK). All other samples were subjected to limited proteolysis with 50 μg/ml proteinase K (+PK). (*B*) Representative slot blot showing the formation of protease-resistant PrP molecules *de novo* in multiple propagation experiments. Unseeded samples were propagated for 15 rounds in either the presence or absence of poly(A) RNA, as indicated by plus (+) and minus (−) symbols, respectively. In experiments designated by asterisks, propagation was not carried beyond the 12th round because a preliminary assay performed at that stage already detected PrP^{Sc} in several preceding rounds. The experiment designated by the # symbol was performed entirely in a prion-free laboratory. (*C*) Histogram showing the temporal distribution of the first detectable PrP^{Sc} signals during serial propagation of unseeded PrP^{C} plus poly(A) RNA substrate mixtures in 10 separate propagation experiments, 7 of which were carried out simultaneously in the same sonicator.

spontaneous production of PrP^{Sc} molecules described above. Specifically, we purified PrP^{C} substrate and performed an unseeded propagation experiment entirely in a different laboratory that never been exposed to prions, using only uncontaminated reagents and equipment including: commercially purchased frozen brains obtained from hamsters raised in an off-site commercial facility, new chemical buffers, a new sonicator directly shipped from the manufacturer, a new tube rack, and new sample tubes taken from a previously unopened container. Daily volume transfers were performed on an open bench within this prion-free environment by using aerosol barrier pipette tips and disposable gloves. Under these conditions, we again observed the spontaneous formation of PrP^{Sc}, indicating that PrP^{Sc} molecules can form under prion-free conditions (Fig. 3*B*, experiment designated with # symbol).

We next performed a series of unseeded propagation experiments to characterize the polyanion dependence and kinetics of spontaneous PrP^{Sc} formation. The results show that, whereas unseeded PMCA reactions containing both PrP^{C} and poly(A) RNA could produce PrP^{Sc} molecules spontaneously, reactions containing PrP^{C} substrate alone failed to produce *de novo* PrP^{Sc} molecules. (Fig. 3*B*). Furthermore, the spontaneous generation of PrP^{Sc} in experiments containing PrP^{C} plus poly(A) RNA appeared to be stochastic; PrP^{Sc} first became detectable during different propagation rounds in different experiments (Fig. 3 *B* and *C*). The stochastic nature of spontaneous PrP^{Sc} formation indicates that this process: (*i*) occurs relatively infrequently (< 1 conversion event per 6×10^{11} input PrP^{C} molecules per PMCA round) and (*ii*) is unlikely to be caused by the amplification of preexisting PrP^{Sc} molecules.

We also compared the detergent solubility and glycosylation patterns of *de novo* PrP^{Sc} molecules with brain-derived PrP27-30 and prion-seeded, *in vitro*-generated PrP^{Sc} molecules. The results of these biochemical studies showed that, like both native and seeded *in vitro*-generated PrP^{Sc} molecules, *de novo* PrP^{Sc} molecules are relatively detergent-insoluble (SI Fig. 9) and contain *N*-linked glycans (SI Fig. 10).

***In Vitro*-Generated, Purified PrP^{Sc} Molecules Are Infectious.** To test whether *in vitro*-generated, purified PrP^{Sc} molecules are infectious, we inoculated wild-type hamsters intracerebrally with various samples derived from our serial PMCA-propagation experiments. The results of these bioassays show that *in vitro*-propagated, serially diluted PrP^{Sc} molecules originally seeded with Sc237 or 139H prions as well as *de novo* PrP^{Sc} molecules formed and propagated without seeds (including a sample prepared in a prion-free environment by using new equipment), all caused scrapie in inoculated hamsters (Table 1). Neuropathological studies revealed typical spongiform degeneration, astrogliosis, and PrP deposition (Fig. 4), accompanied by the accumulation of PrP^{Sc} (SI Fig. 11) in the brains of hamsters inoculated with either seeded or unseeded PrP^{Sc} molecules.

End-point titration data indicate that, during the course of the Sc237-seeded propagation experiment, the level of prion infectivity associated with the final, day-16 PMCA product ($\approx 5 \times 10^4$ LD_{50} per ml) was ≈4-fold lower than the level of infectivity associated with the input Sc237 PrP27-30 seed ($\approx 2 \times 10^5$ LD_{50} per ml) (Table 1). Therefore, during the course of the 16-round, serial dilution experiment, the level of prion infectivity was relatively maintained, whereas the initial PrP^{Sc} seed was diluted $>10^{15}$-fold. In contrast, Sc237-seeded samples propagated in either PrP^{C} or poly(A) RNA substrate alone contained no prion infectivity detectable by bioassay (Table 1). Based on comparisons with known quantities of recombinant PrP on semiquantitative slot-blot assays, we estimate that the concentration of PrP^{Sc} molecules is ≈400 ng/ml in the PMCA product and ≈40 ng/ml in the PrP27-30 seed. Therefore, we calculate that there are $\approx 1.4 \times 10^7$ *in vitro*-propagated PrP^{Sc} monomers (based on molecular mass of 35,000 Da) and $\approx 4.3 \times 10^5$ PrP27-30 monomers (based on molecular mass of 28,000 Da) per LD_{50} unit. It should be noted that there is currently no agreed definition of "PrP^{Sc}," and therefore our method for measuring protease-resistant PrP^{Sc} molecules should be considered operational.

The infectious titer of the inoculum containing *de novo* PrP^{Sc} molecules derived from serial PMCA propagation of PrP^{C} plus poly(A) RNA substrate mixture for 16 rounds was $\approx 5 \times 10^3$ LD_{50} per ml. In contrast, no prion infectivity could be detected in the PrP^{C} plus poly(A) RNA substrate mixture not subjected to PMCA (Table 1). Taken together, these results confirm that prion infectivity was generated *de novo* during the course of the serial PMCA propagation experiment, i.e., in the absence of infectious seed material.

To test whether scrapie caused by *in vitro*-generated PrP^{Sc} molecules is transmissible, we serially passaged brain homogenates prepared from animals originally inoculated with samples containing Sc237-seeded, 139H-seeded, or *de novo* PrP^{Sc} molecules. The results confirm that scrapie caused by any of the three original

Table 1. Transmission of brain-derived and *in vitro*-generated prions to normal Syrian hamsters.

Seed (strain)	Inoculum	Dilution	n/n_0	IP, days*
Sc237	Input PrP27–30	10^0	8/8	87 ± 3
		10^{-1}	4/4	92 ± 2
		10^{-2}	4/4	125 ± 44
		10^{-3}	4/4	116 ± 29
		10^{-4}	2/4	186 ± 94
		10^{-5}	1/3	308
	Seeded in vitro propagation with PrP^C alone	10^0	0/5	>370
		10^{-1}	0/8	>370
	Seeded in vitro propagation with RNA alone	10^0	0/6	>370
		10^{-1}	0/7	>370
	Seeded in vitro propagation with PrP^C + RNA	10^0	8/8	169 ± 31
		10^{-1}	8/8	173 ± 17
		10^{-2}	6/7	185 ± 15
		10^{-3}	3/3	192 ± 22
		10^{-4}	0/4	>370
		10^{-5}	0/4	>370
	Serial passage #1 (in vitro propagation with PrP^C + RNA)	10^{-1}	8/8	94 ± 12
	Serial passage #2 (in vitro propagation with PrP^C + RNA)	10^{-1}	8/8	89 ± 14
139H	Input PrP27–30	10^0	8/8	97 ± 17
		10^{-1}	8/8	113 ± 30
	Seeded in vitro propagation with PrP^C + RNA	10^0	8/8	141 ± 19
		10^{-1}	6/7	181 ± 45
	Serial passage #1 (in vitro propagation with PrP^C + RNA)	10^{-1}	8/8	88 ± 16
	Serial passage #2 (in vitro propagation with PrP^C + RNA)	10^{-1}	8/8	89 ± 16
No seed	Unseeded PrP^C + RNA substrate mixture not subjected to PMCA	10^{-1}	0/6	>360
	Repeat unseeded PrP^C + RNA substrate mixture not subjected to PMCA (completely prion-free environment†)	10^{-1}	0/4	>400
	Unseeded in vitro propagation with PrP^C + RNA	10^0	7/8	134 ± 22
		10^{-1}	7/8	173 ± 16
		10^{-2}	4/5	172 + 9
		10^{-3}	1/4	281
		10^{-4}	1/4	179
		10^{-5}	0/4	>370
	Repeat unseeded in vitro propagation with PrP^C + RNA (completely in prion-free environment†)	10^{-1}	8/8	168 ± 34
	Serial passage #1 (in vitro propagation with PrP^C + RNA)	10^{-1}	8/8	85 ± 10
	Serial passage #2 (in vitro propagation with PrP^C + RNA)	10^{-1}	4/4	87 ± 6

*Mean incubation period (IP) of scrapie sick animals ± SE. All experiments with live animals remaining are ongoing at >370 days.
†These samples were generated in a prion-free laboratory by using new equipment.

inocula could be efficiently transmitted to normal hamsters upon serial passage (Table 1). The brains of terminally ill animals that received the serial passage inocula displayed accumulation of PrP^{Sc} (SI Fig. 11*A*) and severe spongiform degeneration (data not shown).

Strain Properties of *in Vitro*-Generated Prions. Originally, we intended to analyze whether inocula derived from Sc237 and 139H could maintain strain differences upon *in vitro* propagation with purified substrates. However, we were unable to perform this analysis because the incubation time, biochemical, and neuropathological phenotypes of hamsters inoculated with PrP27-30 molecules derived from these two strains showed no statistically significant differences (Table 1; SI Fig. 12*A*, Fig. 4*C*, and SI Fig. 11*B Upper*). The reason for this apparent strain convergence is currently unknown but may be related to the digestion of strain-specific, protease-sensitive $sPrP^{Sc}$ conformers during the PrP27-30 purification procedure (30, 31) or the removal of the N-terminal octarepeat region from protease-resistant $rPrP^{Sc}$ molecules.

We next sought to compare the strain characteristics of animals inoculated with brain-derived PrP27-30 molecules to those of animals inoculated with *in vitro*-generated PrP^{Sc} molecules. Interestingly, animals inoculated with samples containing PrP^{Sc} molecules generated by *in vitro* propagation of Sc237 prions exhibited an altered relationship between incubation time and infectious titer compared with animals inoculated with samples containing the brain-derived Sc237 molecules used as input seed for the propagation experiment (Table 1). At infectious titers between 10^3 and 10^5 LD_{50} units/ml, animals inoculated with Sc237-seeded, *in vitro*-generated PrP^{Sc} molecules had incubation times ≈50 days longer that animals inoculated with brain-derived Sc237 molecules. Animals inoculated with high concentrations of 139H-seeded *in vitro*-generated or *de novo* PrP^{Sc} molecules also displayed long scrapie incubation times (Table 1). All *in vitro*-generated PrP^{Sc} inocula displayed shortened incubation times upon second passage (Table 1).

Clinically, hamsters inoculated with Sc237-seeded, 139H-seeded, or *de novo* PrP^{Sc} molecules were indistinguishable from each other. All three groups of animals showed typical signs of scrapie in the terminal phase, including ataxia, trembling, circling, broad gait, and inability to maintain or regain upright posture. However, nearly all of the animals in these three groups also displayed atypical clinical signs during the early symptomatic phase, namely hyperactivity and the propensity to climb and hang vertically on cage cover bars. In contrast, these clinical signs were seldom observed in animals inoculated with brain-derived PrP27-30. Upon serial passage of Sc237-seeded, 139H-seeded, and unseeded *in vitro*-generated PrP^{Sc} inocula, scrapie incubation times were uniformly shortened to ≈90 days (Table 1), and inoculated animals did not exhibit vertical climbing activity.

We also examined the neuropathology of hamsters inoculated with Sc237-seeded, 139H-seeded, and two independently generated samples of unseeded PrP^{Sc} molecules (SI Fig. 12*A*). The severity of vacuolation produced by one of the unseeded inocula (prepared

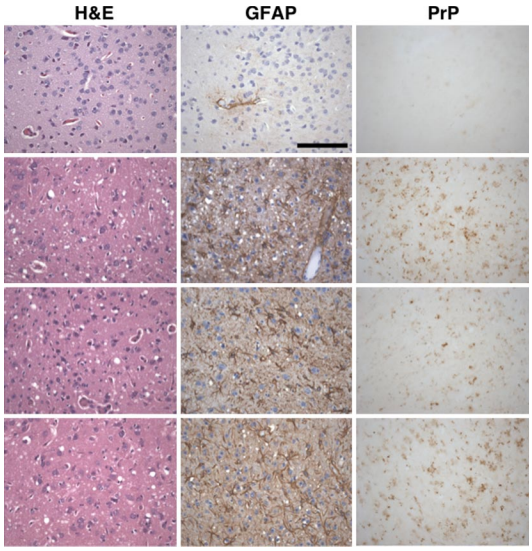

Fig. 4. Representative histological fields of the CA2 hippocampus region in control animals and animals inoculated with *in vitro*-generated PrP^{Sc} molecules. Rows from top to bottom: (top row) uninoculated, normal 190 day old hamster; (second row) terminally ill hamster inoculated with Sc237-seeded, serially propagated PrP^{Sc} molecules; (third row) terminally ill hamster inoculated with 139H-seeded, serially propagated PrP^{Sc} molecules; (bottom row) terminally ill hamster inoculated with spontaneously generated, serially propagated PrP^{Sc} molecules. Hematoxylin and eosin (H&E) staining, as well as glial fibrillary acidic protein (GFAP) and PrP immunohistochemical staining results are shown for each group. (Scale bar, 100 μm.)

completely in a prion-free environment and indicated by #) was significantly lower ($P < 0.05$) than the 139H-seeded inoculum in five of five brain regions, the Sc237-seeded inoculum in four of five brain regions, and the other unseeded inoculum (indicated by filled triangles) in one of five brain regions (cerebellum). Furthermore, the regional pattern of vacuolation produced by this inoculum differed from the patterns produced by other *in vitro*-generated PrP^{Sc} inocula in that relatively little vacuolation was observed in the frontal cortex and hippocampus, compared with other brain regions (SI Fig. 12*A*). The severity of vacuolation produced by the other unseeded inoculum (indicated by filled triangles) more closely resembled those caused by the seeded inocula (SI Fig. 12*A*). There were no statistically significant differences in the PrP deposition scores measured by immunohistochemistry between any of the groups inoculated with seeded or unseeded *in vitro*-generated PrP^{Sc} molecules (SI Fig. 12*B*). No neuropathological abnormalities were observed in control animals inoculated with (*i*) an unseeded PrP^{C} + poly(A) RNA mixture not subjected to PMCA, (*ii*) a seeded sample serially propagated by using PrP^{C} substrate alone, and (*iii*) a seeded sample serially propagated by using poly(A) RNA alone. (SI Fig. 13 and Fig. 4 *B* and *C*). These negative diagnostic results confirm that the PrP^{C} and poly(A) RNA substrates are not themselves contaminated with prions or any other neurotropic infectious agents.

Biochemically, the guanidine denaturation profiles of protease-resistant PrP^{Sc} molecules in the brains of hamsters inoculated with seeded and unseeded *in vitro*-generated PrP^{Sc} molecules as well as brain derived PrP27-30 molecules were nearly indistinguishable (SI Fig. 11*B*).

Discussion

Accessory Polyanions Facilitate Prion Formation *in Vitro*. Our observation that efficient PMCA propagation of infectious prions *in vitro* requires an accessory polyanion raises the possibility that endogenous polyanionic cofactors may participate in prion propagation *in vivo*, either as catalysts or as scaffolds complexed to PrP^{Sc} molecules. Further studies are required to distinguish between these possibilities and to identify the specific endogenous polyanions that facilitate prion propagation *in vivo*. Several classes of negatively charged macromolecules could potentially serve as cofactors for prion propagation, including: nucleic acids (17, 32–38), glycosaminoglycans (14–16), phospholipid-rich membranes (39–42), and chaperone proteins (43). Interestingly, it has been proposed that polyanions could play a broad role in protein folding and misfolding, and the ability of polyanions to facilitate prion conversion may represent a specific example of that general concept (44). It has also been proposed that binding of specific RNA molecules to PrP^{Sc} could determine strain properties (45). We observed that two independently generated samples of *de novo* PrP^{Sc} molecules prepared by using purified PrP^{C} plus synthetic poly(A) RNA substrates produced moderately different regional vacuolation profiles in hamsters. This result indicates that, although polyanions might constrain the folding potential of PrP molecules, purified prions formed spontaneously in the presence of poly(A) RNA can display at least some degree of conformational heterogeneity. More work is required to determine whether polyanions can influence prion strain properties.

It has been previously reported that refolding pure, recombinant PrP into amyloid fibers in the absence of polyanions could produce synthetic mammalian prions (2). Several possible explanations could account for the discrepancy between the results of that study and our demonstration that polyanions are necessary for prion formation *in vitro*. (*i*) Recombinant PrP amyloid fibrils may interact with endogenous polyanions *in situ* after inoculation. An observation consistent with this possible explanation is that synthetic prions formed from recombinant PrP molecules display unique biochemical and neuropathological strain properties upon initial inoculation into transgenic mice expressing truncated PrP^{C} molecules but subsequently cause typical scrapie (resembling infection with the murine RML prion strain) upon serial passage into wild-type mice (2, 46). (*ii*) Polyanions may not be absolutely required to form an infectious prion, but may increase the efficiency of prion conversion. High concentrations of recombinant PrP were used to produce disease with very long incubation times in Tg mice overexpressing PrP, and therefore it is possible that the specific infectivity of PrP amyloid formed without polyanions may be substantially lower than the specific infectivity of PrP^{Sc} molecules formed in the presence of polyanions. The *in vitro*-prions generated in our experiments also produced longer initial incubation times than brain-derived prions; one possible explanation for this effect is that poly(A) RNA may be an imperfect cofactor for forming hamster prions. (*iii*) Differences in specific experimental conditions (e.g., PrP preparation method, buffer composition, reaction pH, or the presence of denaturants) could account for the apparent difference in polyanion requirement between the two systems. More work is required to determine the reason that polyanions are required to produce infections prions in PMCA experiments but not in the *in vitro* folding experiments reported by Legname *et al.* (2).

Spontaneous Generation of Infectious Prions: a Model of Sporadic Prion Disease. The mechanism by which PrP^{Sc} molecules and infectious prions originate in sporadic Creutzfeldt–Jakob disease (sCJD) is not known, and no experimental model of this disease has previously been described. PrP^{Sc} molecules invariably accumulate in the brains of patients with sCJD, and the disease can be experimentally transmitted to normal primates (47). Several hypotheses have been proposed to explain the etiology of sCJD, including: stochastic formation of PrP^{Sc} molecules (48), somatic mutation of PrP sequence in individual brain cells (49), and age-dependent decline in PrP^{Sc} clearance mechanisms (50). Our results suggest that interactions between PrP^{C} molecules and endogenous polyanions may contribute to the relatively infrequent process of prion formation in patients with sCJD.

Several lines of evidence indicate that prions form spontaneously

at low frequency in unseeded experiments, rather than by amplification of preexisting prions. (*i*) Most importantly, *de novo* PrP^{Sc} molecules were generated in a completely prion-free laboratory, by using only new or prion-free equipment and source materials. (*ii*) The appearance of *de novo* PrP^{Sc} molecules appears to be stochastic, whereas one might expect that contaminating PrP^{Sc} molecules would be amplified in a more stereotypic manner during the course serial propagation experiments. (*iii*) *De novo* PrP^{Sc} molecules generated in a prion-free environment were infectious, whereas various negative control samples were not. Interestingly, this sample produced a unique regional profile characterized by relatively mild vacuolation in the frontal cortex and hippocampus, compared with other samples containing either PrP27-30 or *in vitro*-generated PrP^{Sc} molecules.

Although less likely, it is also possible that normal hamsters have low levels of endogenous PrP^{Sc} molecules in their brains. Previous experiments have shown that PrP^{Sc} molecules can persist chronically in animals without causing disease (51–53). In this scenario, the rate of endogenous PrP^{Sc} production in normal animals might be balanced by a putative clearance mechanism, preventing accumulation.

Limiting the Possible Composition of Infectious Prions. The results presented in this article indicate that a purified PrP^{C} preparation plus an accessory polyanion can serve as substrates for *in vitro* prion propagation. Therefore, we can logically limit the possible composition of the scrapie agent to, at most, these defined components. More work is required to determine whether the accessory polyanion component functions as an unbound catalyst or is physically complexed to PrP^{Sc} within infectious prions. We detected approximately equimolar levels of several unsaturated 20-carbon fatty acids, including several isomers of arachidonic acid, in our purified PrP^{C} preparations. The only fatty acid previously identified as a component of the PrP GPI anchor is stearic acid, a saturated 18-carbon compound (54), and therefore it is likely that the copurified fatty acids are not covalently bound to PrP^{C}. Phospholipids have been shown to influence the folding of recombinant PrP molecules (40–42), and it will be interesting in future studies to determine whether lipids might also regulate the formation of PrP^{Sc} molecules in PMCA reactions.

Materials and Methods

PrP^{C} and PrP27-30 molecules were purified by using modified versions of previously published protocols (55). PMCA and serial dilution/propagation experiments were performed as described (22) by using purified substrates instead of brain homogenates. Standard bioassay and neuropathology techniques were used to measure prion infectivity and strain properties (56). PrP^{Sc} stability was assayed as described (46). Detailed descriptions for each of these techniques, statistical methods, and chemical analyses are provided in *SI Materials and Methods*.

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