Crossing the Species Barrier by PrP^{Sc} Replication In Vitro Generates Unique Infectious Prions

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SUMMARY

Prions are unconventional infectious agents composed exclusively of misfolded prion protein (PrP^{Sc}), which transmits the disease by propagating its abnormal conformation to the cellular prion protein (PrP^{C}). A key characteristic of prions is their species barrier, by which prions from one species can only infect a limited number of other species. Here, we report the generation of infectious prions by interspecies transmission of PrP^{Sc} misfolding by in vitro PMCA amplification. Hamster PrP^{C} misfolded by mixing with mouse PrP^{Sc} generated unique prions that were infectious to wild-type hamsters, and similar results were obtained in the opposite direction. Successive rounds of PMCA amplification result in adaptation of the in vitro-produced prions, in a process reminiscent of strain stabilization observed upon serial passage in vivo. Our results indicate that PMCA is a valuable tool for the investigation of cross-species transmission and suggest that species barrier and strain generation are determined by the propagation of PrP misfolding.

INTRODUCTION

Prion diseases also known as transmissible spongiform encephalopathies (TSEs) are infectious neurodegenerative diseases affecting the brain of humans and several species of mammals (Collinge, 2001). Creutzfeldt-Jakob disease (CJD) is the most common TSE in humans, and scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease (CWD) in cervids are the most prevalent prion diseases in animals. Unlike conventional infectious microorganisms, the TSE agent appears to be devoid of genetic material and instead composed exclusively by a misfolded form of the prion protein (PrP^{Sc}) (Prusiner, 1998). PrP^{Sc} has the unprecedented ability to replicate in the body by inducing the misfolding of the cellular form of the prion protein (PrP^{C}).

One of the characteristics of the agent responsible for prion diseases is its ability to infect some species and not others (Hill and Collinge, 2004; Moore et al., 2005). This phenomenon is known as species barrier. Even between close species, the species barrier is manifested as an incomplete attack rate and a prolongation of the time it takes for animals to develop the clinical disease when injected with another species' infectious material (Hill and Collinge, 2004). Primary interspecies transmission is usually not very efficient, and it takes a long time for the prion replication process to reach the point at which full-blown clinical disease appears. After sequential passages, the PrP^{Sc} in the new host adapts, resulting in a shortage of the incubation period and stabilization of the new strain (Hill and Collinge, 2004).

Compelling evidence indicates that the species barrier is largely controlled by the sequence of PrP (Moore et al., 2005). Unfortunately, we cannot predict the degree of a species barrier simply by comparing the prion proteins from two species. The barrier has to be measured by experimental studies in animals. These studies are long and costly, and in the case of the human species barrier, the studies have to be done with experimental models, the validity of which is not absolutely guarantied. Evaluation of the species barrier is of tremendous medical importance for risk assessment and to implement regulatory measures to avoid spreading of diseases (Moore et al., 2005). At this time, the epidemiological evidence suggests that among animal TSEs only cattle BSE has been transmitted to humans, generating a variant form of CJD (vCJD) (Will et al., 1996). It is unlikely that sheep scrapie is a concern for humans, because the disease has been described for centuries and no increased prevalence of human prion diseases has been found in scrapie-endemic areas (Caramelli et al., 2006; Hunter, 1998). However, the appearance of "atypical" strains of scrapie, as well as the known transmission of BSE to sheep, has generated new concerns of human infections with sheep-derived material (Buschmann and Groschup, 2005; Hunter, 2003). Similarly, the possibility that some of the newly identified animal prion diseases, such as CWD, could be transmitted to humans cannot be ruled out at the present time (Williams, 2005; Xie et al., 2005).

Recently, we reported the generation of infectious prions in vitro by amplification of PrP^{Sc} misfolding in the test tube (Castilla et al., 2005). For these experiments, we used a technology termed PMCA (protein misfolding cyclic amplification) that mimics in vitro some of the fundamental steps involved in PrP^{Sc} replication in vivo at an accelerated rate (Saborio et al., 2001). During PMCA, small quantities of PrP^{Sc} are mixed with excess of PrP^{C}, and through a cyclical process involving incubation and sonication, prion propagation occurs in an autocatalytic way. With this procedure, prions can replicate indefinitely in the test tube and, after successive rounds of dilutions followed by PMCA amplification, PrP^{Sc} used to begin the reaction can be eliminated, and only in vitro-generated misfolded protein remains in the sample (Castilla et al., 2005). Inoculation of PMCA-generated prions into wild-type animals resulted in a disease with the same clinical, neuropathological, and biochemical features as the disease produced by brain-derived infectious material (Castilla et al., 2005). The conclusion drawn from these findings is that all of the information required to propagate the infectious properties is enciphered in the structure of PrP^{Sc}. This is further supported by recent studies from Supattapone and coworkers in which infectious prions were generated in vitro by PMCA with purified PrP^{C} and PrP^{Sc} with the sole addition of synthetic polyanions (Deleault et al., 2007).

The goal of this study was to attempt crossing the species barrier in vitro to generate unique infectious prions in a cell-free system. For these studies, we used mice and hamsters, two experimental rodent systems widely employed in TSE studies and for which several prion strains are available (Bruce, 2003; Kimberlin and Walker, 1988). The PrP sequence shows nine differences between these two animal species (Figure 1A). Infectivity studies have shown that there is a large barrier for prion transmission between these species (Kimberlin et al., 1989; Kimberlin and Walker, 1988; Race et al., 2002). Our findings show that incubation of PrP^{C} from one of the species with PrP^{Sc} from the other resulted in new PrP^{Sc} that was infectious to wild-type animals. Interestingly, a detailed examination of the infectious, neuropathological, and biochemical features of the disease that was produced revealed characteristics that were different from other known prion strains. These results indicate that the prions generated in vitro by crossing of the mouse-hamster barrier represent new strains. Strikingly, studies of the infectious characteristics of these newly generated prions after different rounds of PMCA showed that the procedure not only enabled crossing of the species barrier but also resulted in stabilization of the new strain in vitro by successive rounds of amplification. Our findings show that prions can be propagated in vitro across the species barrier, leading to the generation and adaptation of unique prion strains.

RESULTS

Crossing the Mouse-Hamster Species Barrier to Generate New Hamster Prions

To assess whether prions can be generated in vitro across the species barrier, we used hamsters and mice, two widely studied rodent experimental models of TSEs (Bruce, 2003; Kimberlin and Walker, 1988; Morales et al., 2007). A PMCA experiment done with our standard conditions for amplification of mouse RML prions showed no detectable formation of PrP^{Sc} when hamster PrP^{C} was used as a substrate (Figure 1A). Conversely, a robust PrP^{Sc} generation was observed with mouse PrP^{C} substrate. For this experiment, we mixed a 1000-fold dilution of RML PrP^{Sc}

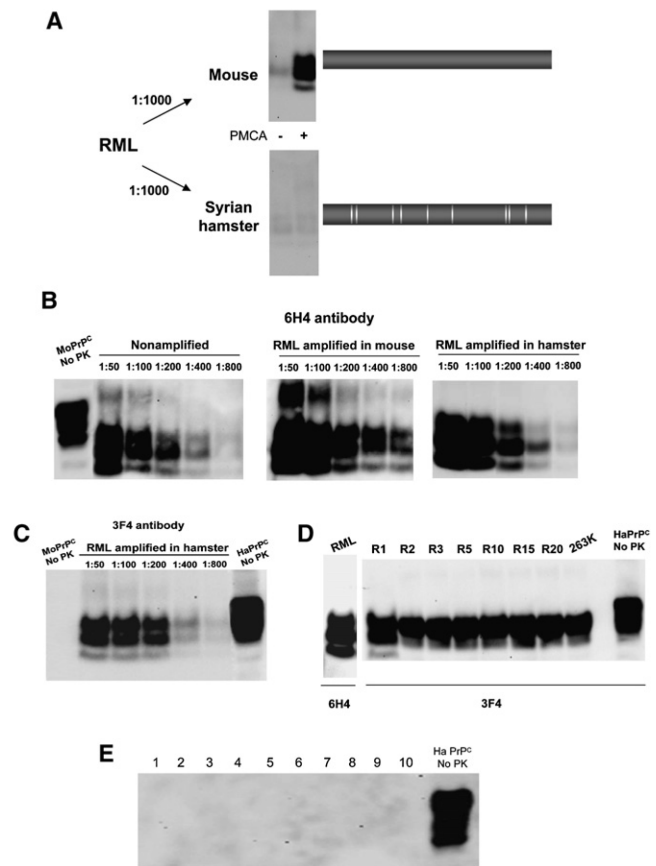


Figure 1. In Vitro Conversion of Hamster PrP^{C} Induced by Mouse RML PrP^{Sc}

(A) RML brain homogenate was diluted 1000-fold into either mouse or hamster normal brain homogenate and subjected to 96 PMCA cycles. The blot shows the results with and without PMCA in each species. At the right side, we show a scheme of PrP indicating the position in which there are amino acid differences between mice and hamsters.

(B) To attempt forcing conversion, we incubated larger quantities (dilutions 1:50 through 1:800) of RML PrP^{Sc} with mouse (central panel) or hamster (right panel) PrP^{C}. All samples (except for the control samples in the left panel labeled "nonamplified") were subjected to 96 PMCA cycles, and PrP^{Sc} signal was detected after PK digestion by western blot with the 6H4 antibody.

(C) The same samples as those in the right panel of (B) were developed with the 3F4 antibody.

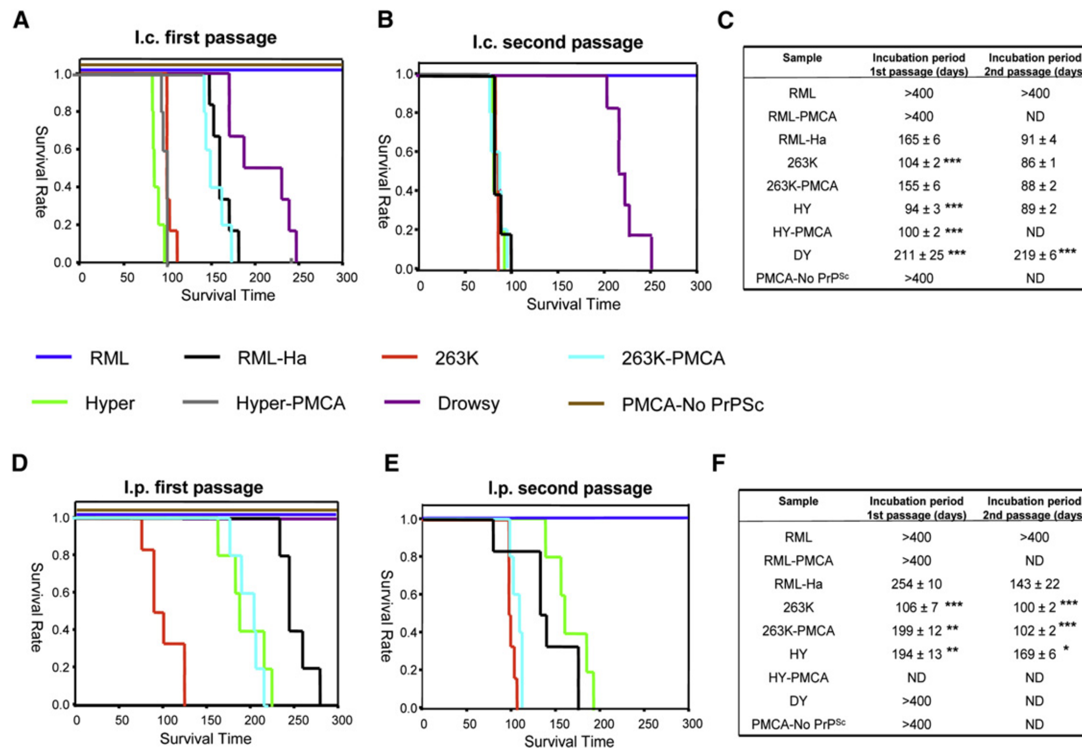
(D) The newly generated RML-Ha PrP^{Sc} was serially passed in hamster brain homogenate by a series of 1:10 dilution followed by 48 PMCA cycles. "R" indicates the number of rounds of PMCA; i.e., R5 represent the samples after five serial rounds of PMCA.

(E) For the assessment of spontaneous generation of PrP^{Sc} by PMCA, samples from brain of ten different hamsters were subjected to the same process of serial PMCA as in (D). PrP^{Sc} formation was analyzed by western blot after PK treatment in each PMCA round. The figure shows the results obtained after 20 rounds of PMCA. In the experiments shown in this figure, all samples were treated with PK, except when indicated.

into 10% brain homogenates of healthy hamsters and mice, respectively. We reasoned that if in vivo it takes longer for prions to replicate across species barriers, then in PMCA we should also encounter more difficulties to convert PrP^C when using PrP^{Sc} from a different species. To attempt forcing the in vitro conversion, we added a higher proportion of PrP^{Sc}-containing mouse brain homogenate into the hamster substrate. A range of dilutions from 50- to 800-fold were tested, but the problem with these experiments is that the large concentration of RML PrP^{Sc} used as inoculum makes it difficult to estimate convincingly whether new PrP^{Sc} generation was obtained (Figure 1B). Fortunately, the 3F4 monoclonal antibody can recognize hamster but not mouse PrP (Lund et al., 2007). Using this antibody for western blot, we could clearly observe that protease-resistant hamster PrP^{Sc} was being produced when the reaction was done with low dilutions (from 1:50 to 1:200) of mouse RML PrP^{Sc} (Figure 1C). When the amplification was attempted with 800-fold diluted PrP^{Sc}-containing mouse brain homogenate, only a very faint signal was observed, confirming the results obtained in Figure 1A and the idea that the combination of PrP^C and PrP^{Sc} from different species impairs PMCA efficiency.

Newly generated hamster PrP^{Sc} starting from RML prions was propagated many times in vitro by serial PMCA in order to remove by dilution the initial amount of mouse scrapie brain material added to begin prion replication (Figure 1D). As described before, using this procedure, we can completely remove all molecules of brain-derived PrP^{Sc} from the sample (Castilla et al., 2005). Hamster PrP^{Sc} of RML origin efficiently propagates in vitro at the expense of hamster PrP^C. Interestingly, in the first PMCA round, the glycoform distribution pattern of the in vitro-generated hamster PrP^{Sc} was comparable to the RML profile showing the three glycoform bands (Figure 1D). After further PMCA rounds, this pattern changed to become undistinguishable from PrP^{Sc} associated to the typical hamster strains, such as 263K (Figure 1D) or Hyper (HY), in which the diglycosylated band is highly predominant. This result suggests that the characteristics of the newly generated PrP^{Sc} are being adapted to the new species during successive PMCA cycling, reminiscent of the adaptation process occurring in vivo upon serial passage of the infectious material. After 20 serial rounds of PMCA, representing a dilution equivalent to 10^{-22} with respect to the brain (since the first round contains a 100-fold dilution of the material), our estimation is that no molecules of mouse brain PrP^{Sc} should be present in the sample. This in vitro-generated material was termed RML-Ha PrP^{Sc} to emphasize the RML origin of this new hamster misfolded prion protein. To make sure that newly formed PrP^{Sc} was indeed coming from conversion of hamster PrP^C induced by mouse PrP^{Sc} and not just spontaneous "de novo" formation of PrP^{Sc} in hamsters (Deleault et al., 2007), we did a large experiment to analyze in detail the possibility of spontaneous generation of PrP^{Sc} and infectivity under our experimental conditions. Samples of healthy brain homogenate from ten different hamsters were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples (Figure 1E).

Inoculation of wild-type hamsters with RML-Ha PrP^{Sc} (produced after a 10^{-22} dilution of RML scrapie brain homogenate) produced disease in 100% of the animals by both intracerebral (i.c.) and intraperitoneal (i.p.) routes (Figure 2). The disease exhibits the clinical characteristics typical of hamster scrapie, including hyperactivity, motor impairment, head wobbling, muscle weakness, and weight loss. The incubation time in the first passage was 165 $\pm$ 6 days by i.c. inoculation (Figures 2A and 2C). This is longer than the incubation time obtained with hamster scrapie strains, such as 263K and HY, in which a similar quantity of PrP^{Sc} produces disease at around 100 days by this route (Figures 2A and 2C). However, in agreement with our previously reported data (Castilla et al., 2005), when hamster 263K prions were replicated in vitro by PMCA, the newly generated PrP^{Sc} produced disease with a delay similar to that observed with the RML-Ha material (Figures 2A and 2C). The delay in our previous study was eliminated upon a second passage in vivo, in which the new infectious material was stabilized to acquire properties undistinguishable from in vivo-derived 263K (Figures 2B and 2C). Interestingly, in the HY hamster prion strain, PMCA-generated material did not show any statistically significant difference compared to in vivo-produced prions (Figures 2A and 2C). These results suggest that in vitro replication of prions by PMCA maintains the strain characteristics, at least in respect to the incubation periods. To assess the stability of RML-Ha and estimate the stabilized incubation period, we performed a second passage. As shown in Figure 2B, the incubation time of RML-Ha prions was decreased to around 90 days, which is very similar to that obtained with 263K and HY but different from the Drowsy (DY) strain. These results suggest that RML-Ha prions behave similarly to the 263K strain; both in vitro-generated prions show a delay in the first passage that gets corrected upon a second in vivo passage. This feature is not displayed by other hamster prion strains, such as HY, or other species of prions (see below for the results in mice), where PMCA-generated prions exhibited the same incubation period in the first passage as in vivo-produced infectious material. As expected, hamsters inoculated with RML prions did not develop disease during the time of the experiment (>400 days). Animals inoculated with hamster brain homogenate subjected to 20 rounds of PMCA in the absence of PrP^{Sc} (control for the de novo generation of PrP^{Sc}) did not develop disease more than 400 days after inoculation (Figures 2A and 2C). Intraperitoneal inoculations of the infectious material showed a clear difference between the three prion strains used as reference, with 263K being the fastest and DY not producing disease by this route (Figure 2D). The incubation period produced by i.p. inoculation of RML-Ha prions was longer than that of the 263K and HY strains, with an average of 254 days in the first passage. This is also longer than 263K prions amplified in vitro by PMCA, which produced disease after 199 days postinoculation in the first passage (Figures 2D and 2F). A second in vivo passage again stabilized PMCA-generated 263K prions to produce disease at a time indistinguishable from that of brain-derived 263K infectious material. The second passage of RML-Ha prions showed that the stabilized incubation period for the i.p. route was on average around 140 days, which is significantly higher than 263K or 263K-PMCA material but shorter than HY prions (Figures 2E and 2F). The differences remained stable in a third passage (data not shown). These results indicate that in some aspects, RML-Ha prions are similar to the agent in the 263K strain but in other features are intermediate between 263K



C

Sample	Incubation period 1st passage (days)	Incubation period 2nd passage (days)
RML	>400	>400
RML-PMCA	>400	ND
RML-Ha	165 ± 6	91 ± 4
263K	104 ± 2 ***	86 ± 1
263K-PMCA	155 ± 6	88 ± 2
HY	94 ± 3 ***	89 ± 2
HY-PMCA	100 ± 2 ***	ND
DY	211 ± 25 ***	219 ± 6 ***
PMCA-No PrP^{Sc}	>400	ND

F

Sample	Incubation period 1st passage (days)	Incubation period 2nd passage (days)
RML	>400	>400
RML-PMCA	>400	ND
RML-Ha	254 ± 10	143 ± 22
263K	106 ± 7 ***	100 ± 2 ***
263K-PMCA	199 ± 12 **	102 ± 2 ***
HY	194 ± 13 **	169 ± 6 *
HY-PMCA	ND	ND
DY	>400	ND
PMCA-No PrP^{Sc}	>400	ND

Figure 2. Infectivity of Newly Generated RML-Ha PrP^{Sc} after Crossing the Species Barrier

RML-Ha PrP^{Sc} samples amplified by 20 serial PMCA rounds were inoculated i.c. or i.p. into six wild-type hamsters. For controls, we inoculated similar quantities of PrP^{Sc} from RML or three distinct hamster strains (263K, Hyper, and Drowsy). We also show the data obtained by inoculation of in vitro-generated prions through 20 serial rounds of PMCA by incubation of 263K (263K-PMCA) or Hyper (HY-PMCA) PrP^{Sc} with healthy hamster brain homogenate and RML replicated at expenses of mouse PrP^C (RML-PMCA). The figure also show the results obtained by inoculation of the material produced after 20 rounds of PMCA with unseeded normal hamster brain homogenate (PMCA-No PrP^{Sc}). (A) and (D) show the survival curves obtained after i.c. and i.p. inoculation, respectively, of the in vitro-generated RML-Ha after 20 rounds of PMCA. (B) and (E) show the survival curves of the second passage (i.e., animals were inoculated with material obtained from the brain of sick animals in the experiments depicted in [A] and [D]) after i.c and i.p inoculation, respectively. (C) and (F) show the average incubation periods of the experiments done by i.c. and i.p. inoculation of various samples. The values correspond to the average ± standard error. The data was analyzed by ANOVA and the Dunnett multiple comparison post-test. Each set of data was compared to the results obtained with the RML-Ha strain, and significant differences are highlighted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$). ND, not done.

and HY prions, providing a first indication that the material obtained by crossing of the mouse-hamster species barrier represents a unique hamster prion strain.

To further assess the characteristics of the disease produced by in vitro-generated RML-Ha prions, we studied in detail the neuropathological and biochemical features of the brain damage. Histopathological studies showed that animals inoculated with RML-Ha prions exhibit the typical brain lesions of scrapie, including spongiform degeneration, astroglyosis, and PrP^{Sc} deposition (Figures 3A–3C). Quantitative studies of the vacuolation profile in different brain areas showed that RML-Ha-infected hamsters showed the largest extent of spongiosis in medulla and cerebellum and less damage in hippocampus, cortex, and colliculum (Figure 3D). This pattern of brain damage was similar to that observed in 263K-inoculated animals and statistically different from that obtained in hamsters injected with HY and DY (Figure 3D). However, the extent of both astroglyosis (Figure 3B) and PrP^{Sc} accumulation (Figure 3C) in the medulla of RML-Ha-infected animals was lower than that in 263K-sick animals and similar to that observed in HY-injected hamsters (Figures 3B and 3C). These data suggest again that the RML-Ha prions are a unique strain with properties intermediate between the previously known 263K and HY hamster strains.

Comparative studies of the biochemical characteristics of PrP^{Sc} obtained from the brain of sick animals after inoculation with RML-Ha, 263K, HY, and DY were done by analysis of the electrophoretical pattern of the protein, its susceptibility to proteolytic degradation, and its resistance to denaturation. For comparison of the protease resistance profile, similar quantities of PrP^{Sc} from the new RML-Ha prions and PrP^{Sc} obtained from the brain of sick hamsters inoculated with the prion strains 263K, HY, and DY were treated for 60 min with various concentrations of proteinase K (PK) (Figure 4A). RML-Ha PrP^{Sc} was highly resistant to large PK concentrations. The misfolded protein associated to the newly generated strain was more resistant than HY or DY and similarly (but still significantly more) susceptible to PK digestion than 263K PrP^{Sc} (Figure 4A). The PK concentration in which 50% of the protein was degraded (PK50) was highest for PrP^{Sc} associated to RML-Ha, followed by 263K, HY, DY, and RML (Table S1 available online).

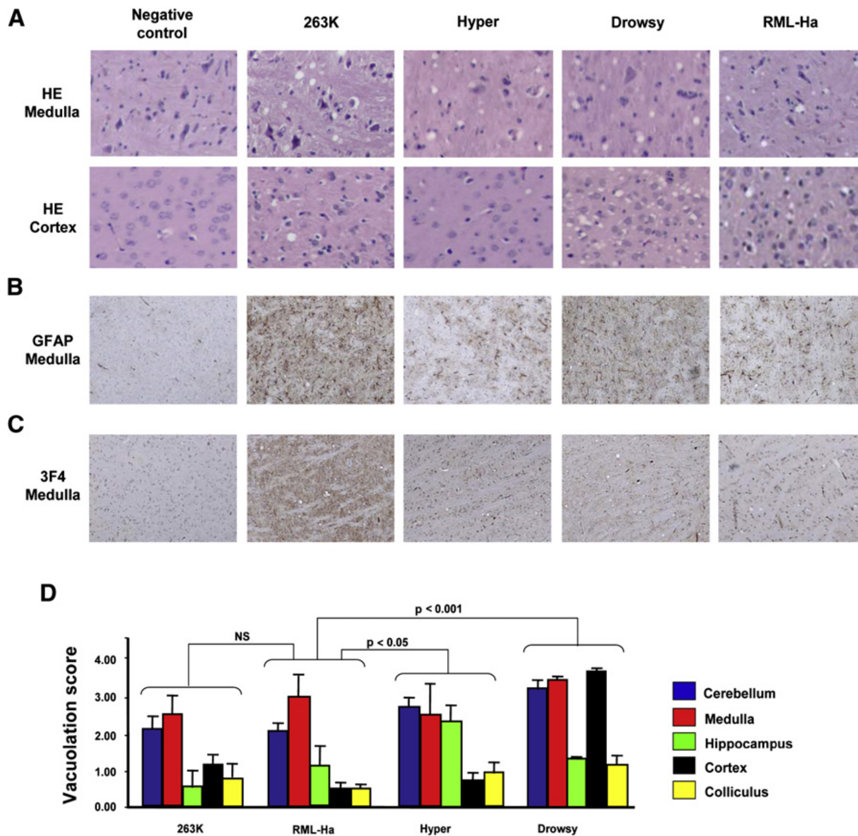


Figure 3. Histopathological Features of the Disease Induced by Inoculation of Hamsters with PMCA-Generated RML-Ha PrP^{Sc}

Brain from sick animals in which disease was produced by inoculation with the in vitro-generated RML-Ha PrP^{Sc} (first passage) or the known hamster strains 263K, Hyper, and Drowsy were analyzed by histological studies. As a control, we used the brain of a hamster inoculated with PBS and sacrificed without disease at 350 days after inoculation.

(A) Spongiform degeneration was evaluated after hematoxilin-eosin staining of medulla and occipital cortex sections and visualized by microscopy at a 40× magnification.

(B) Reactive astroglyosis was evaluated by histological staining with glial fibrillary acidic protein antibody.

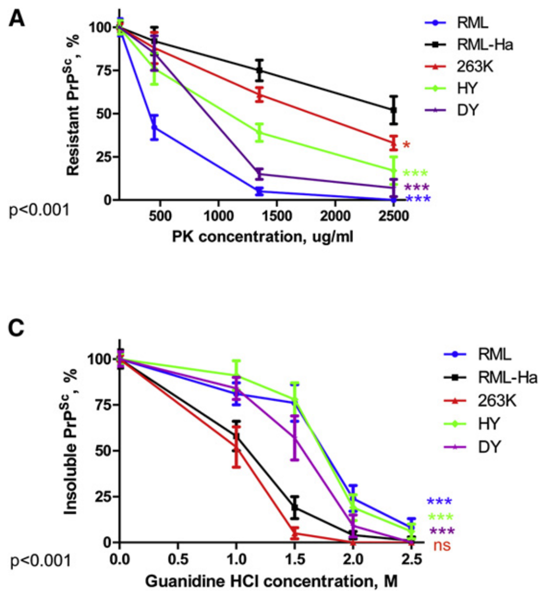
(C) PrP accumulation in these animals was evaluated by staining of the tissue with the 3F4 anti-PrP monoclonal antibody.

(D) The vacuolation profile in each brain area was estimated with a semiquantitative scale, as described in the Experimental Procedures. The brain areas used were the following: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). We also included in the analysis brain sections from animals inoculated with the other hamster prion strains. The values represent the average ± standard error of the extent of vacuolation from the five animals analyzed in each set. Statistical analysis by two-way ANOVA with brain regions and prion origin as the variables indicated that differences were highly significant ($p < 0.001$). To assess the significance of the differences between each known prion strain and RML-Ha, we used the Dunnett multiple comparison post-test, and the p values for each combination are shown.

Another characteristic we studied was the electrophoretic mobility and glycosylation pattern of PrP^{Sc} associated to distinct strains. The predominant glycoform for the hamster strains (including the newly generated RML-Ha) is the diglycosylated band, whereas mouse RML PrP^{Sc} shows a more even distribution of the three bands with the main one being the monoglycosylated form. To assess the size of the protein after PK cleavage, we performed endoglycosidase treatment to remove the glycosylated chains (Figure 4B). Whereas PrP^{Sc} associated to the DY strain has a higher electrophoretical mobility, no significant differences were observed among the other proteins. Another biochemical property of misfolded PrP often used to differentiate prion strains is its resistance to chemical denaturation (Safar et al., 1998). Clear differences were observed in the guanidine concentrations required to denature PrP^{Sc} associated to different strains (Figure 4C). The concentration of the chaotropic agent needed to denature 50% of PrP^{Sc} RML-Ha was 1.11 M, substantially different from the 1.69, 1.56, and 1.72 M required for the proteins associated to HY, DY, and RML, respectively (Table S1).

Crossing the Hamster-Mouse Species Barrier to Generate and Stabilize New Mouse Prions

To study the barrier between these rodent species in the opposite direction, we mixed 263K hamster prions with mouse healthy brain homogenate. As before, when a standard PMCA assay was done by dilution of 263K brain homogenate 1000-fold into mouse healthy brain material, we did not see detectable generation of mouse PrP^{Sc} (data not shown). However, when a higher quantity of hamster PrP^{Sc} was added, we were able to generate new mouse PrP^{Sc} (termed 263K-Mo) that could be propagated by serial rounds of PMCA to reach a dilution of the hamster brain homogenate equivalent to 10^{-17} (Figure 5A). Since there are not available antibodies capable of recognizing mouse PrP but not hamster PrP, we could not compare the electrophoretical pattern of PrP^{Sc} generated in the first rounds of PMCA with the profile of PrP^{Sc} typically observed in mouse and hamster strains. However, the western blot pattern of 263K-Mo after 15 rounds of PMCA (when no more molecules of 263K PrP^{Sc} are present) is similar to the one observed for RML and other ovine-derived mouse strains, despite a slightly faster migration (Figure S1A) that will be investigated in more detail later. To assess whether newly generated PrP^{Sc} was indeed coming from conversion of mouse PrP^{C} induced by 263K hamster PrP^{Sc} and not just spontaneous "de novo" formation of PrP^{Sc} in mice, we did an experiment to analyze the possibility of spontaneous generation of PrP^{Sc} and infectivity under our experimental conditions. Samples of healthy brain homogenate from ten different mice were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples (Figure S1B).



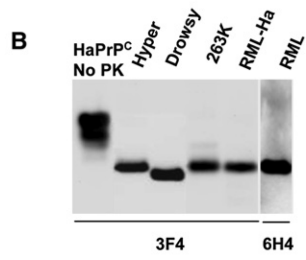


Figure 4. Biochemical Characterization of RML-Ha PrP^{Sc}

Samples from brains of animals inoculated with RML-Ha PrP^{Sc} (first passage in vivo) were used to study the PK resistance profile (A), the relative mobility after deglycosylation and PK treatment (B), and the susceptibility to guanidine denaturation (C). For controls, we used samples from RML or three distinct hamster strains (263K, Hyper, and Drowsy). The results in (A) and (C) correspond to the quantitative evaluation of western blots by densitometric analysis from three independent animals. The data represent the average ± standard error. The data were analyzed by ANOVA and the Dunnett multiple comparison post-test. Each set of data was compared to the results obtained with the RML-Ha strain, and significant differences are highlighted with asterisks (* = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$).

To assess whether mouse PrP^{Sc} generated in vitro from hamster 263K is infectious to wild-type mice and to determine whether the infectious properties are being adapted upon serial PMCA passages, we inoculated several rounds of in vitro-generated material into mice (Figure 5A). Despite the fact that the same amount of PrP^{Sc} was inoculated (as determined by western blot), striking differences in the infectious properties were seen among in vitro-generated prions in distinct rounds of PMCA (Figure 5B). Only two of the six mice inoculated with material produced in the first round of PMCA showed disease symptoms, which appear at a very long time after inoculation (around 500 days) (Figures 5B and 5C). A complete attack rate was observed when animals were inoculated with material produced after three serial rounds of PMCA. However, the incubation period was long (around 310 days on average), and there was a large dispersion among animals (Figures 5B and 5C). The incubation period became stable, short (around 165 days), and there was little dispersion after the six serial rounds of PMCA. These findings indicate that upon successive rounds of PMCA, the newly generated prion, after crossing the species barrier, is becoming adapted and stabilized to the new host, a process very similar to what is seen after several passages in vivo. The large dispersion of incubation times observed in the third round of PMCA suggests that more than one strain has been generated upon crossing of the species barrier and that successive in vitro amplification leads to the selection and cloning of the most efficient of these strains. The incubation time for 263K-Mo after 15 rounds of PMCA (equivalent to a 10^{-17} dilution of the 263K inoculum) was around 165 days, similar to the one produced by scrapie-adapted mouse strains, such as RML, but different from that of the bovine strain 301C (Figure 5D). In vitro replication of the mouse strains RML and 301C at expense of mouse PrP^{C} produced PrP^{Sc} with identical properties as the brain-derived material, reflected as an indistinguishable incubation period (Figure 5D). As expected, mice inoculated with hamster 263K prions did not develop disease during the time of the experiment (>500 days). No disease was also observed in animals inoculated with mouse brain homogenate subjected to 20 rounds of PMCA in the absence of PrP^{Sc}, which corresponds to the control experiment for the de novo generation of PrP^{Sc} (Figure 5D).

To analyze whether the newly generated 263K-Mo infectious material corresponded to a new strain of mouse prions, we studied the histopathological and biochemical features of the brain damage. Animals affected with the disease produced by inoculation of 263K-Mo showed extensive vacuolation in the medulla and hypocampus and moderate but clearly detectable damage in the cerebellum (Figures 6A and 6D). The pattern of spongiform degeneration does not correspond with any of the previously known mouse strains studied and indeed is statistically significantly different to the vacuolation profile produced by RML and 301C prions (Figure 6D). Differences were also detected in the extent of brain inflammation produced by 263K-Mo, since the degree of astroglyosis was less prominent than the one observed in animals inoculated with RML or 301C prions (Figure 6B). The profile of PrP^{Sc} accumulation consisted mostly of diffuse deposition and was not clearly different from the one observed in the other strains (Figure 6C). Then we studied the biochemical characteristics of PrP^{Sc} obtained from the brain of animals infected with 263K-Mo. Electrophoretical migration was assessed after PK digestion and endoglycosidase treatment to remove glycosylation chains. The PK-resistant core of PrP^{Sc} migrated slightly faster than RML but slightly slower than 301C, with an estimated molecular weight of 20 KDa (Figures 7A and 7B). These results indicate that the cleavage site after PK digestion is different from all of the currently known mouse strains. This is important because it is thought that differences in the PK cleavage site reflect disparities in the folding or aggregation of the protein (Chen et al., 2000; Collinge et al., 1996). To further search for biochemical differences, we subjected the protein to proteolytic degradation by using various concentrations of PK. 263K-Mo PrP^{Sc} was much more resistant to PK than to RML (Figure 7C), with a PK_{50} (the PK concentration needed to

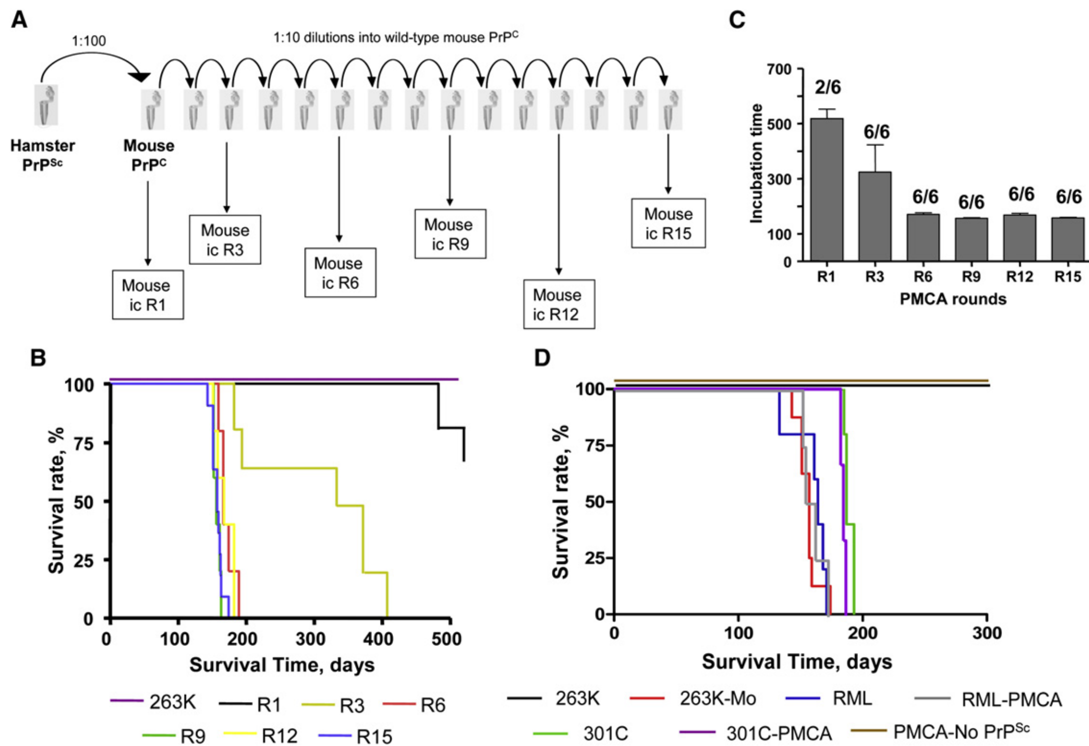


Figure 5. In Vitro Conversion of Mouse PrP^C Induced by Hamster 263K PrP^{Sc} Generates Infectious Prions

(A) Schematic representation of the dilutions done and the PMCA rounds used for our in vivo infectivity experiments.

(B) Survival curve observed after inoculation of six wild-type mice with the material generated after several rounds of PMCA. "R" indicates the number of rounds of PMCA. As a control, the animals were inoculated with 263K hamster prions.

(C) Average and standard error of the incubation times and attack rates observed after inoculation of wild-type mice with the material produced after different rounds of PMCA.

(D) Comparison of survival curves for the stabilized 263K-Mo infectious material (after 15 rounds of PMCA) with those obtained with RML and 301C, two mouse strains of different origin. We also show the data obtained by inoculation of in vitro-generated prions through 20 serial rounds of PMCA by incubation of RML (RML-PMCA) or 301C (301C-PMCA) PrP^{Sc} with healthy mouse brain homogenate. The figure also show the results obtained by inoculation of the material produced after 20 rounds of PMCA with unseeded normal mouse brain homogenate (PMCA-No PrP^{Sc}), which correspond to the control for de novo generation of prions. For all of these experiments, the material was inoculated i.c. as described in the Experimental Procedures.

degrade half of the protein) of 1450 μg/ml (Figure 7D), much larger than the values obtained for RML (240 ug/ml) and 301C (430 ug/ml) (Table S2). Interestingly, the high resistance of PrP^{Sc} is typical of the hamster prions (Table S1), and indeed, 263K, the parental strain of the newly generated mouse prions, has a PK_{50} of around 1700 μg/ml.

DISCUSSION

The phenomenon of the species barrier, by which the agent coming from one species can infect only a limited number of other species, is a typical feature of prion diseases. The molecular basis of this process is not well-understood, but it is thought to be controlled by the structure and folding of the prion protein (Moore et al., 2005; Vanik et al., 2004). As with the related phenomenon of prion strains, it is difficult to imagine how an infectious agent lacking genetic material and composed by a single protein can encode the structural diversity and specificity required to control strains variability and species selectivity (Soto and Castilla, 2004).

In addition to the intriguing molecular mechanism behind the species barrier, understanding this phenomenon has profound implications for public health. Indeed, one of the scariest medical problems of the last decades has been the emergence of a new and fatal human prion disease (variant CJD) originated by cross-species transmission of BSE from cattle (Will et al., 1996). BSE has not only been transmitted to humans. The extensive use of cow-derived material for feeding other animals led to the generation of new diseases in exotic felines, nonhuman primates, and domestic cats (Doherr, 2003). Worrisomely, the transmission of BSE into these different species could create new prion strains with unique biological and biochemical characteristics and thus a potentially new hazard for human health. More frightening is perhaps the possibility that BSE has been passed into sheep and goats. Studies have already shown that this transmission is possible and actually relatively easy (Foster et al., 1993). The disease produced is clinically similar to scrapie, but since it comes from BSE it has the potential to be infectious to humans. Another concern is CWD, a disorder affecting farm and wild species of cervids (Sigurdson and Aguzzi, 2006; Williams, 2005). The

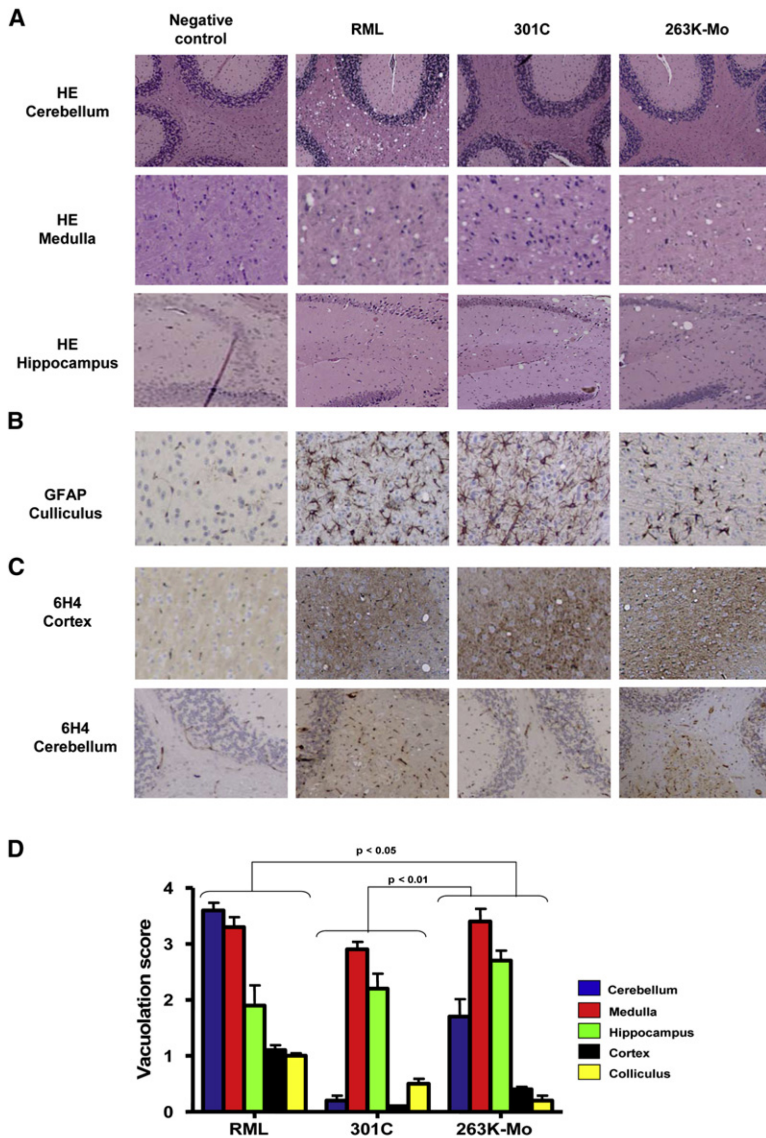


Figure 6. Histopathological Features of the Disease Induced by Inoculation of Mice with PMCA-Generated 263K-Mo PrP^{Sc}

Brains from sick mice in which disease was produced by inoculation with the newly generated 263K-Mo prions after 15 rounds of PMCA (first passage) or the known mouse strains RML and 301C were analyzed by histological studies. As a control, we used brain of a mouse inoculated with PBS and sacrificed without disease at 350 days after inoculation.

(A) Spongiform degeneration was evaluated after hematoxilin-eosin (HE) staining of three different brain areas (cerebellum, medulla, and hippocampus) and was visualized at a 40× magnification.

(B) Reactive astrogliosis was evaluated in the inferior culliculus by staining with glial fibrillary acidic protein antibody.

(C) PrP accumulation in these animals was evaluated in the occipital cortex and cerebellum by staining of the tissue with the 6H4 antibody.

(D) The vacuolation profile in each brain area was estimated with a semiquantitative scale, as described in the Experimental Procedures. The brain areas used were the following: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). We also included in the analysis brain sections from animals inoculated with RML and 301C. The values represent the average ± standard error of the extent of vacuolation from the five animals analyzed in each set. Statistical analysis by two-way ANOVA with brain regions and prion origin as the variables indicated that differences were highly significant ($p < 0.001$). To assess the significance of the differences between each known prion strain and 263K-Mo, we used the Dunnett multiple comparison post-test, and the p values for each combination are shown.

origin of CWD and its potential to transmit to humans are currently unknown. This is worrisome, considering that CWD has became endemic in some parts of the USA and that the number of cases continues to increase (Williams, 2005). CWD transmissibility studies have been performed in many species in order to predict how this disease could be spread by the consumption of CWD meat (Sigurdson and Aguzzi, 2006). Transmission of CWD to humans cannot be ruled out at present, and a similar infective episode to BSE involving CWD could result in catastrophic consequences.

The exciting scientific problem coupled with the relevant public-health issue prompted us to develop strategies to reproduce the species-barrier phenomenon in the test tube. We reported previously the generation of infectious prions in vitro by cyclic replication of the protein misfolding process featuring the pathogenesis of prion diseases (Castilla et al., 2005). These results were reproduced and extended by other groups to better dissect the elements required for prion replication (Deleault et al., 2007; Weber et al., 2007). The PMCA technology has been adapted to replicate prions from various species (Deleault et al., 2005; Jones et al., 2007; Kurt et al., 2007; Murayama et al., 2007; Sarafoff et al., 2005; Soto et al., 2005) and even to use bacterially produced recombinant PrP as substrate (Atarashi et al., 2007). The conclusion drawn from these studies together with the findings reported in this manuscript is that propagation of the PrP^{Sc} misfolding results in formation of infectious material, which maintains the strains and species-barrier properties of the original prions. Qualitatively similar conclusions have been obtained for yeast prions, which are a group of “infectious proteins” that behave as a non-Mendelian genetic element and transmit biological information in the absence of nucleic acid (Wickner et al., 1995). Recent studies showed that bacterially produced N-terminal fragments of the yeast prions Sup35p and Ure2p when transformed into amyloid fibrils were able to propagate the prion phenotype to yeast cells (Brachmann et al., 2005; King and Diaz-Avalos, 2004; Tanaka et al., 2004). Infection of yeast with different conformers led to generation of distinct prion strains in vivo (Brachmann et al., 2005; Tanaka et al., 2004). Remarkably, yeast prions also show the species-barrier phenomenon, and recent data indicate that strain conformation is the critical determinant of cross-species prion transmission (Tanaka et al., 2005).

In the current study, we demonstrate the generation of new infectious prions across the species barrier. For this purpose, we

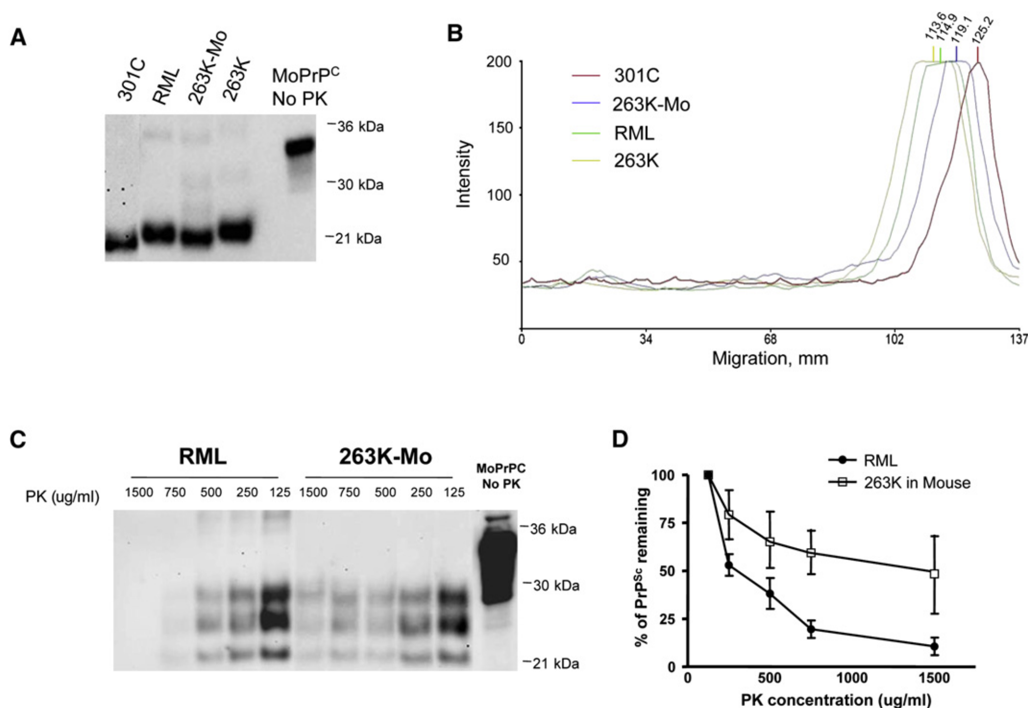


Figure 7. Biochemical Characteristics of 263K-Mo PrP^{Sc}

(A) Samples from brains of mice inoculated with 263K-Mo, RML, or 301C were used to study the electrophoretical migration after deglycosylation and PK treatment.

(B) For assessment of the electrophoretical differences among distinct strains, the blot in (A) was scanned and analyzed by software included in the UVP image analysis system to locate the exact position of the bands.

(C) The PK resistance profile of 263K-Mo PrP^{Sc} was studied and compared with RML.

(D) The results of the experiment shown in (B) were quantitated by densitometric analysis. The data in the figure represent the average ± standard error from three independent animals. The differences were statistically significant as evaluated by one-way ANOVA ($p < 0.01$).

mixed PrP^{Sc} from one species with PrP^{C} from a different animal species and subjected the mixture to serial rounds of PMCA to generate, propagate, and stabilize new prion strains. Hamster PrP^{Sc} generated from mouse RML prions was infectious to wild-type hamsters. Detailed analysis of the disease characteristics and comparison with the illness produced by several known hamster prion strains indicate that the in vitro-generated infectious material across the species barrier corresponds to a new prion strain in hamsters (termed RML-Ha). The main differences of the RML-Ha were on the incubation times after i.p. inoculation, the extremely high resistance to PK degradation, and the pattern of brain damage (Table S1). Similarly, PrP^{Sc} generated by conversion of mouse PrP^{C} with hamster PrP^{Sc} from the 263K strain was shown to be infectious to wild-type mice, with an incubation period comparable to that obtained after inoculation with some of the mouse-adapted scrapie strains, such as RML. Again, the disease produced by the new prions (termed 263K-Mo) was clearly distinguishable from the one produced by some of the currently known mouse prion strains. The major differences were seen in the electrophoretical migration, extremely high resistance to proteolytic degradation, and pattern of brain spongiform degeneration (Table S2). To rule out that newly generated PrP^{Sc} in these experiments was coming from "de novo" spontaneous conversion of PrP^{C} into PrP^{Sc} during PMCA, we used samples of healthy brain homogenate from ten different mice and hamsters that were subjected to serial rounds of PMCA amplification in the absence of PrP^{Sc} seed. After up to 20 serial rounds of PMCA, we did not observe de novo formation of PrP^{Sc} in any of the samples. This material was inoculated into wild-type animals, and no disease was observed more than 400 days after inoculation. These results strongly indicate that the generation of PrP^{Sc} reported in the present study was due to interspecies prion conversion. Nevertheless, we would like to highlight that recently we have been able to generate in vitro PrP^{Sc} de novo without addition of PrP^{Sc} seed (data not shown). However, to reach this aim, the PMCA conditions need to be modified. The modifications include changes on the PMCA parameters (length of incubation and potency of sonication), preincubation, or pretreatment of the normal brain homogenate to induce/stabilize PrP misfolding prior to PMCA. These findings suggest that de novo formation of PrP^{Sc} can be experimentally distinguished from replication of preformed PrP^{Sc}, indicating that the biochemical, conformational, or stability properties of the PrP structures involved in both processes are probably different. Standard PMCA conditions, as those used in the current study, do not result in spontaneous PrP^{Sc} formation.

Interestingly, in our serial PMCA amplifications of RML PrP^{Sc} into hamster PrP^{C}, we observed a progressive change on the western blot profile of the newly generated RML-Ha PrP^{Sc}. Indeed, in the first round of PMCA, the glycoform distribution

pattern was reminiscent of RML and later switched to a profile typical of the hamster strains, characterized by the predomination of the diglycosylated form (Figure 1D). Our interpretation of this result was that consecutive rounds of PMCA may enable the new prion strain to adapt and stabilize. To further study this possibility in our experiments in which mouse prions were generated from 263K hamster prions, we inoculated the material generated after various rounds of PMCA. Strikingly, similar amounts of PrP^{Sc} generated after one and three rounds of PMCA produced disease with incomplete attack rates and/or very long incubation periods (Figures 5B and 5C). Incubation time stabilized after six rounds of serial PMCA, suggesting that at this point the new strain is fully adapted. These findings suggest that PMCA is not only able to reproduce the interspecies transmission of prions but is also able to mimic the strain adaptation process observed in vivo. In vivo adaptation and stabilization of prions generated after crossing the species barrier takes at least four consecutive passages, which requires several years of work (Race et al., 2001, 2002). Conversely, strain adaptation by PMCA takes only 2 or 3 weeks. Importantly, the kinetics of adaptation in vitro and in vivo, as well as the characteristics of the stabilized material, are very similar. Indeed, it has been reported that three serial passages of 263K in mice produce disease in all animals, with an incubation time of around 300 days (Race et al., 2002). This result is very similar to the data obtained with the material generated in vitro after three successive rounds on PMCA replication (Figures 5B and 5C). Moreover, less than three in vivo passages produced an incomplete attack rate, and more than three passages are needed to obtain a stable and low incubation period (Race et al., 2002), which is in the same range of our 263K-Mo infectious material. Finally, similar to our in vitro data, the in vivo cross-species transmission between hamsters and mice also led to the generation of unique prion strains (Race et al., 2001, 2002). Although we are tempted to speculate that each PMCA round has the same effect on strain adaptation as did each in vivo passage, more experiments with other species combinations are needed to reach this conclusion.

In summary, our results show that all elements controlling interspecies transmission of prions are contained in a cell-free system and that new prion strains can be generated, adapted, and stabilized upon crossing the species barrier in vitro by PMCA. These findings provide additional support for the prion hypothesis, suggesting that species-barrier transmission and strain generation are determined by the propagation of PrP misfolding. Furthermore, the data demonstrate that PMCA is a valuable tool for the investigation of the strength of the barrier between diverse species, its molecular determinants, and the expected features of the new infectious material produced. Finally, our findings suggest that the universe of possible prions is not restricted to those currently known but that likely many unique infectious foldings of the prion protein may be produced and that one of the sources for this is cross-species transmission.

EXPERIMENTAL PROCEDURES

Preparation of Tissue Homogenates

Healthy and sick animals were perfused with phosphate-buffered saline (PBS) plus 5 mM ethylenediaminetetraacetic acid (EDTA) before the tissue was harvested. Ten percent brain homogenates (w/v) were prepared in conversion buffer (PBS containing 150 mM NaCl, 1.0% Triton X-100, and the complete cocktail of protease inhibitors from Boehringer Mannheim, Mannheim, Germany). The samples were clarified by a brief, low-speed centrifugation (1500 rpm for 30 s) with an Eppendorf centrifuge (Hamburg, Germany), model 5414.

Serial Replication of Prions In Vitro by PMCA

Aliquots of 10% brain homogenate from clinically sick mice infected with RML or 301C and hamsters infected with 263K, HY, or DY prions were diluted into 10% hamster or mouse healthy brain homogenate. Samples were loaded onto 0.2 ml PCR tubes and positioned on an adaptor placed on the plate holder of a microsonicator (Misonix Model 3000, Farmingdale, NY). Each PMCA cycle consisted of 30 min incubation at 37°C followed by a 20 s pulse of sonication set at potency of 7. Samples were incubated without being shaken immersed in the water of the sonicator bath. After a round of PMCA cycles, a 10 μl aliquot of the amplified material was diluted into 90 μl of more normal brain homogenate, and a new round of PMCA cycles was performed. This procedure was repeated several times to reach the final dilutions indicated in the text. The detailed protocol for PMCA, including reagents, solutions, and troubleshooting, has been published elsewhere (Castilla et al., 2006; Saa et al., 2005).

Proteinase K Degradation Assay

The standard procedure for digestion of PrP^{Sc} consists of subjecting the samples to incubation in the presence of PK (50 μg/ml) for 60 min at 37°C. The digestion was stopped by addition of electrophoresis sample buffer, and the protease-resistant PrP was revealed by western blotting. So that the profile of PK sensitivity for in vitro- and in vivo-generated PrP^{Sc} could be studied, the samples were incubated for 60 min at 37°C with different concentrations of PK ranging from 0 to 2500 μg/ml. The PK_{50} values represent the concentration of PK needed to digest half of the protein, and these values are estimated on the basis of the densitometric analysis of three replicated western blots.

Guanidine Denaturation Assay

Samples were incubated with different concentrations of guanidine hydrochloride for 2 hr at room temperature with shaking. Thereafter, samples were incubated in the presence of 10% sarkosyl for 30 min at 4°C and centrifuged at 100,000 × g for 1 hr in a Biosafe Optima MAX ultracentrifuge (Beckman Coulter, Fullerton, CA). The pellet of the centrifugation was resuspended in conversion buffer and treated with PK as described above. Equivalent aliquots of pellet were analyzed by western blot. The Gdn_{50} value corresponds to the concentration of guanidine hydrochloride required to denaturate 50% of the protein, and these values were estimated on the basis of the densitometric analysis of three replicated western blots.

Protein Deglycosylation Assay

PrP^{Sc} samples were first digested with PK as describe above. After addition of 10% sarkosyl, samples were centrifuged at 100,000 × g for 1 hr at 4°C, supernatant was discarded, and the pellet resuspended in 100 μl of glycoprotein denaturing buffer (New England Biolabs, Beverly, MA) and incubated for 10 min at 100°C. Thereafter, 26 μl of 50 mM sodium phosphate (pH 7.5) containing 1% nonidet P-40 and 3 μl of peptide N-glycosidase F (New England Biolabs, Beverly, MA) were added. Samples were incubated for 2 hr at 37°C, and the reaction was stopped by the addition of electrophoresis buffer and samples were analyzed by western blot.

Western Blot

Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions, electroblotted into nitrocellulose membrane, and probed with 6H4 (for mouse samples) and 3F4 (for hamster samples) antibodies at a 1:5000 dilution. The immunoreactive bands were visualized by enhanced chemoluminesence assay (Amersham, Piscataway, NJ) with an UVp image analysis system. So that the quantity of PrP^{Sc} in the western blot would be assessed, densitometric analyses were done by triplicate.

PrP^{Sc} Quantification

To inject the same quantity of PrP^{Sc} from each preparation, we compared the samples by western blotting after PK digestion. To obtain a reliable and robust quantification, we ran several different dilutions of the sample in the same gel, to avoid artifacts due to saturation of the signal or to too weak of a signal.

Infectivity Studies

In vivo infectivity studies were done in C57Bl6 female mice or Golden Syrian female hamsters, purchased from Charles river. Animals were 4 to 6 weeks old at the time of inoculation. Anesthesized animals were injected stereotaxically into the right hippocampus with 2 or 4 μl of the mouse or hamster infectious material, respectively. For the i.p. infectivity studies, 100 μl of the sample were injected into the peritoneal cavity. The quantity of infectious material injected corresponds to the plateau portion of the incubation period; therefore, small differences in the amount of infectivity should not change incubation period unless there are strain differences. The onset of clinical disease was measured by scoring of the animals twice a week. For mice, the following scale was used: 1, normal animal; 2, roughcoat on limbs; 3, extensive roughcoat, hunckback, and visible motor abnormalities; 4, urogenital lesions; and 5, terminal stage of the disease in which the animal presented with cachexia and lies in the cage with little movement. For hamsters, the following scoring scale was used: 1, normal animal; 2, mild behavioral abnormalities including hyperactivity and hypersensitivity to noise; 3, moderate behavioral problems including tremor of the head, ataxia, wobbling gait, head bobbing, irritability, and aggressiveness (or lethargy in case of the DY strain); 4, severe behavioral abnormalities including all of the above plus jerks of the head and body and spontaneous backrolls; and 5, terminal stage of the disease in which the animal lies in the cage and is no longer able to stand up. Animals scoring level 4 during two consecutive weeks were considered sick and were sacrificed to avoid excessive pain via exposition to carbonic dioxide. Brains were extracted, the right cerebral hemisphere was frozen and stored at $-70^{\circ}C$ for biochemical examination of PrP^{Sc} with western blots, and the left hemisphere was used for histology analysis.

Histopathological Studies

Brain tissue was fixed in 10% formaldehyde solution, cut in sections, and embedded in paraffin. Serial sections (6 μm thick) from each block were stained with hematoxylin-eosin, or incubated with monoclonal antibodies recognizing PrP or the glial fibrillary acidic protein, via our previously described protocols (Castilla et al., 2005). Samples were visualized with a Zeiss microscope. The vacuolation profile was estimated by consideration of both number and size of spongiform degeneration in five different brain areas: occipital cortex, cerebellum (mostly white matter), medulla (spinal 5 nucleus, interpolar part), inferior colliculum, and hippocampus (CA1 and CA2 regions). Each analyzed brain area was scored from 0 to 4 according to the extent of vacuolation in slides stained with hematoxilin-eosin and visualized at a 40× magnification. Samples were analyzed blindly by two different persons, and the scores represent the average of the two determinations.

Statistical Analysis

The differences in incubation periods, histopathological profile of brain damage, and biochemical characteristics of PrP^{Sc} were analyzed by ANOVA, followed by the Dunnett Multiple Comparison post-test to estimate the significance of the differences between the newly generated strains and each of the other hamster and mouse prion strains studied. For these studies, the data were analyzed with the GraphPad Instat, version 3.05 software.

SUPPLEMENTAL DATA

Supplemental Data include one figure and two tables and can be found with this article online at http://www.cell.com/cgi/content/full/134/5/757/DC1/.

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REFERENCES

Atarashi, R., Moore, R.A., Sim, V.L., Hughson, A.G., Dorward, D.W., Onwubiko, H.A., Priola, S.A., and Caughey, B. (2007). Ultrasensitive detection of scrapie prion protein using seeded conversion of recombinant prion protein. Nat. Methods *4*, 645–650.

Brachmann, A., Baxa, U., and Wickner, R.B. (2005). Prion generation in vitro: amyloid of Ure2p is infectious. EMBO J. *24*, 3082–3092.

Bruce, M.E. (2003). TSE strain variation. Br. Med. Bull. *66*, 99–108.

Buschmann, A., and Groschup, M.H. (2005). TSE eradication in small ruminants–quo vadis? Berl. Munch. Tierarztl. Wochenschr. *118*, 365–371.

Caramelli, M., Ru, G., Acutis, P., and Forloni, G. (2006). Prion diseases: Current understanding of epidemiology and pathogenesis, and therapeutic advances. CNS Drugs *20*, 15–28.

Castilla, J., Saá, P., Hetz, C., and Soto, C. (2005). In vitro generation of infectious scrapie prions. Cell *121*, 195–206.

Castilla, J., Saa, P., Morales, R., Abid, K., Maundrell, K., and Soto, C. (2006). Protein misfolding cyclic amplification for diagnosis and prion propagation studies. Methods Enzymol. *412*, 3–21.

Chen, S.G., Zou, W., Parchi, P., and Gambetti, P. (2000). PrP(Sc) typing by N-terminal sequencing and mass spectrometry. Arch. Virol. Suppl. 209–216.

Collinge, J. (2001). Prion diseases of humans and animals: Their causes and molecular basis. Annu. Rev. Neurosci. *24*, 519–550.

Collinge, J., Sidle, K.C., Meads, J., Ironside, J., and Hill, A.F. (1996). Molecular analysis of prion strain variation and the aetiology of 'new variant' CJD. Nature *383*, 685–690.

Deleault, N.R., Geoghegan, J.C., Nishina, K., Kascsak, R., Williamson, R.A., and Supattapone, S. (2005). Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions. J. Biol. Chem. *280*, 26873–26879.

Deleault, N.R., Harris, B.T., Rees, J.R., and Supattapone, S. (2007). Formation of native prions from minimal components in vitro. Proc. Natl. Acad. Sci. USA *104*, 9741–9746.

Doherr, M.G. (2003). Bovine spongiform encephalopathy (BSE)–infectious, contagious, zoonotic or production disease? Acta Vet. Scand. Suppl. *98*, 33–42.

Foster, J.D., Hope, J., and Fraser, H. (1993). Transmission of bovine spongiform encephalopathy to sheep and goats. Vet. Rec. *133*, 339–341.

Hill, A.F., and Collinge, J. (2004). Prion strains and species barriers. Contrib. Microbiol. *11*, 33–49.

Hunter, N. (2003). Scrapie and experimental BSE in sheep. Br. Med. Bull. *66*, 171–183.

Hunter, N. (1998). Scrapie. Mol. Biotechnol. *9*, 225–234.

Jones, M., Peden, A.H., Prowse, C.V., Groner, A., Manson, J.C., Turner, M.L., Ironside, J.W., MacGregor, I.R., and Head, M.W. (2007). In vitro amplification and detection of variant Creutzfeldt-Jakob disease PrPSc. J. Pathol. *213*, 21–26.

Kimberlin, R.H., and Walker, C.A. (1988). Pathogenesis of experimental scrapie. Ciba Found. Symp. *135*, 37–62.

Kimberlin, R.H., Walker, C.A., and Fraser, H. (1989). The genomic identity of different strains of mouse scrapie is expressed in hamsters and preserved on reisolation in mice. J. Gen. Virol. *70*, 2017–2025.

King, C.Y., and Diaz-Avalos, R. (2004). Protein-only transmission of three yeast prion strains. Nature *428*, 319–323.

Kurt, T.D., Perrott, M.R., Wilusz, C.J., Wilusz, J., Supattapone, S., Telling, G.C., Zabel, M.D., and Hoover, E.A. (2007). Efficient in vitro amplification of chronic wasting disease PrPRES. J. Virol. *81*, 9605–9608.

Lund, C., Olsen, C.M., Tveit, H., and Tranulis, M.A. (2007). Characterization of the prion protein 3F4 epitope and its use as a molecular tag. J. Neurosci. Methods *165*, 183–190.

Moore, R.A., Vorberg, I., and Priola, S.A. (2005). Species barriers in prion diseases–brief review. Arch. Virol. Suppl. 187–202.

Morales, R., Abid, K., and Soto, C. (2007). The prion strain phenomenon: Molecular basis and unprecedented features. Biochim. Biophys. Acta *1772*, 681–691.

Murayama, Y., Yoshioka, M., Yokoyama, T., Iwamaru, Y., Imamura, M., Masujin, K., Yoshiba, S., and Mohri, S. (2007). Efficient in vitro amplification of a mouse-adapted scrapie prion protein. Neurosci. Lett. *413*, 270–273.

Prusiner, S.B. (1998). Prions. Proc. Natl. Acad. Sci. USA *95*, 13363–13383.

Race, R., Raines, A., Raymond, G.J., Caughey, B., and Chesebro, B. (2001). Long-term subclinical carrier state precedes scrapie replication and adaptation in a resistant species: Analogies to bovine spongiform encephalopathy and variant Creutzfeldt-Jakob disease in humans. J. Virol. *75*, 10106–10112.

Race, R., Meade-White, K., Raines, A., Raymond, G.J., Caughey, B., and Chesebro, B. (2002). Subclinical scrapie infection in a resistant species: Persistence, replication, and adaptation of infectivity during four passages. J. Infect. Dis. *186*, S166–S170.

Saa, P., Castilla, J., and Soto, C. (2005). Cyclic amplification of protein misfolding and aggregation. Methods Mol. Biol. *299*, 53–65.

Saborio, G.P., Permanne, B., and Soto, C. (2001). Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. Nature *411*, 810–813.

Safar, J., Wille, H., Itri, V., Groth, D., Serban, H., Torchia, M., Cohen, F.E., and Prusiner, S.B. (1998). Eight prion strains have PrP(Sc) molecules with different conformations. Nat. Med. *4*, 1157–1165.

Sarafoff, N.I., Bieschke, J., Giese, A., Weber, P., Bertsch, U., and Kretzschmar, H.A. (2005). Automated PrPres amplification using indirect sonication. J. Biochem. Biophys. Methods *63*, 213–221.

Sigurdson, C.J., and Aguzzi, A. (2006). Chronic wasting disease. Biochim. Biophys. Acta *1772*, 610–618.

Soto, C., and Castilla, J. (2004). The controversial protein-only hypothesis of prion propagation. Nat. Med. *10*, S63–S67.

Soto, C., Anderes, L., Suardi, S., Cardone, F., Castilla, J., Frossard, M.J., Peano, S., Saá, P., Limido, L., Carbonatto, M., et al. (2005). Pre-symptomatic detection of prions by cyclic amplification of protein misfolding. FEBS Lett. *579*, 638–642.

Tanaka, M., Chien, P., Naber, N., Cooke, R., and Weissman, J.S. (2004). Conformational variations in an infectious protein determine prion strain differences. Nature *428*, 323–328.

Tanaka, M., Chien, P., Yonekura, K., and Weissman, J.S. (2005). Mechanism of cross-species prion transmission: An infectious conformation compatible with two highly divergent yeast prion proteins. Cell *121*, 49–62.

Vanik, D.L., Surewicz, K.A., and Surewicz, W.K. (2004). Molecular basis of barriers for interspecies transmissibility of mammalian prions. Mol. Cell *14*, 139–145.

Weber, P., Giese, A., Piening, N., Mitteregger, G., Thomzig, A., Beekes, M., and Kretzschmar, H.A. (2007). Generation of genuine prion infectivity by serial PMCA. Vet. Microbiol. *123*, 346–357.

Wickner, R.B., Masison, D.C., and Edskes, H.K. (1995). [PSI] and [URE3] as yeast prions. Yeast *11*, 1671–1685.

Will, R.G., Ironside, J.W., Zeidler, M., Cousens, S.N., Estibeiro, K., Alperovitch, A., Poser, S., Pocchiari, M., Hofman, A., and Smith, P.G. (1996). A new variant of Creutzfeldt-Jakob disease in the UK. Lancet *347*, 921–925.

Williams, E.S. (2005). Chronic wasting disease. Vet. Pathol. *42*, 530–549.

Xie, Z., O'Rourke, K.I., Dong, Z., Jenny, A.L., Langenberg, J.A., Belay, E.D., Schonberger, L.B., Petersen, R.B., Zou, W., Kong, Q., et al. (2005). Chronic wasting disease of elk and deer and Creutzfeldt-Jakob disease: Comparative analysis of the scrapie prion protein. J. Biol. Chem. *281*, 4199–4206.