Introduction

According to the protein only hypothesis, prions consist mainly, if not solely, of PrPSc, aggregate conformers of cellular prion protein (PrPC). PrPSc acts as a template and triggers the conversion of PrPC molecules in newly formed PrPSc, leading to prion propagation. This model is supported by protein misfolding cyclic amplification (PMCA),1 a technique that mimics in vitro the autocatalytic PrPC to PrPSc conversion, by seeding healthy brain homogenates as a source of PrPC with minute amounts of prions, and fostering the reaction through multiple cycles of sonication and incubation, which ultimately leads to a vast excess of infectious prions.2 Prions exist as strains that differ in their pathological phenotype and faithfully propagate in the same host, being thus associated with PrPSc having the same amino acid sequence; lacking any evidence of genetic information, it has been proposed that strains represent different PrPSc conformers.3-6 Although strain characteristics are usually maintained on serial passages in the same host, seminal studies have shown that prion strains can mutate upon experimental transmission to a different host, or even when serially passaged into the same host.7 More recently, it has been shown that crossing in vitro the species barrier between mice and hamsters by heterologous PMCA led to the generation of new strains,8 thus mimicking in vitro previous observations.9,10

While prion mutation during interspecies transmissions could be explained by the species barrier, which imposes new conformational constraints to PrPSc propagation due to amino acid mismatches with the host PrPC,6,11 it has been less clear how strain mutation occurs during propagation in the same host. Recent advances in this field were made possible by the development of sensitive and strain-selective cell-based assays, such as the cell panel assay,12 allowing to study prion propagation under different selective environments and to differentiate strains based on their cell tropism and sensitivity to various drugs.13 These studies provided strong evidence that prions undergo mutation14 and selective amplification15 when grown in cell culture, showing that prion populations are intrinsically heterogeneous and exhibit features of Darwinian evolution. Interestingly, in several instances mutated drug-resistant populations positively selected in the presence of drug, reverted upon in vivo passage or by removal of the drug.14,16,17 This led the authors to hypothesize that the behavior of prion populations could be explained in the framework of the quasispecies theory,13 similarly to error prone replication organisms such as RNA viral populations.18 In this scenario, a prion strain would be composed by an ensemble of different conformational molecules that are maintained under host selection; when the host selection changes, the ensemble would shift from one to another dominant conformational molecule. Similar conclusions have been drawn in studies dealing with prions in fungi.19

We have previously shown that bank voles are a sensitive and permissive bioassay for many different prion strains,20-23 and that these features are reflected when using vole brain homogenates as substrate for prion amplification.24 Having set up extremely

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sensitive and controlled conditions for in vitro amplification of prions by using bank vole PrP<sup>C</sup> as an ultra-efficient substrate,<sup>24</sup> in this study we aimed at investigating the intrinsic mutational ability of prion strains in serial homologous PMCA reactions, in the absence of strong selective constraints such as PrP sequence mismatches, PrP<sup>C</sup> modifications, RNA-depletion or the addition of drugs. To this aim, we explored the evolutionary potential of 2 prion strains submitted to different passage regimens, i.e., either propagated through large population passages or subjected to repeated bottleneck events.

### Results

In preliminary studies aimed at verify the consistency of PMCA reactions, we found that the position of tubes in the PMCA rack was associated with prion replication kinetics, putatively due to an irregular distribution of ultrasound waves during sonication. By reducing the number of tubes in the PMCA rack (Supplemental Material [text]; Fig. S1) this effect was considerably reduced. Thus, all experiments were done using only 24 tubes placed in the central portion of the rack and changing the position of each tube at every PMCA round.

Two vole prion strains were selected for the experiments: v586, derived in previous studies from vole PMCA-generated PrP<sup>Sc</sup> and then passaged in vivo,<sup>24</sup> characterized by a high replication efficiency (Fig. 1A) and a BSE-like protease-resistant core of PrP<sup>Sc</sup> (PrPres)<sup>24</sup>; and SS21, a vole-adapted scrapie strain, with 100 fold less efficient replication efficiency (Fig. 1A) and a scrapie-like PrPres type.<sup>22,25</sup>

Our aim was to compare the outcome, in terms of PrPres typing, of prion populations replicated for serial 48h PMCA rounds in high or low PrP<sup>Sc</sup>/PrP<sup>C</sup> ratio, in an attempt to mimic large populations passages (high PrP<sup>Sc</sup>/PrP<sup>C</sup>) vs. bottleneck events (low PrP<sup>Sc</sup>/PrP<sup>C</sup>). Unexpectedly, PrP<sup>Sc</sup> was progressively lost in preliminary experiments with v586 continuously propagated at low PrP<sup>Sc</sup>/PrP<sup>C</sup> (i.e., seeded at 10<sup>-7</sup> in serial PMCA), although v586 initially gave high PrP<sup>Sc</sup> yields in similar experiments with v586 continuously propagated at low PrP<sup>Sc</sup>/PrP<sup>C</sup> (i.e., seeded at 10<sup>-7</sup> in serial PMCA). To overcome this effect, bottleneck events were mimicked by alternating 10<sup>-7</sup> and 10<sup>-2</sup> seeded PMCA rounds (Fig. 1B). Five v586 populations were studied with this new set-up, 2 propagated with large population passages (v586/a, v586/b), and 3 subjected to bottleneck events (v586/c, v586/d, v586/e). None of the populations which underwent bottleneck events were lost, although v586/d and v586/e showed a decrease

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**Figure 1.** Experimental design. (A) Serial 10-fold dilutions of v586 and SS21 were subjected to a single round of PMCA, PK digested and analyzed by western blotting with antibody SA F84. The amplification rate was derived from the last positive dilution yielding high PrP<sup>Sc</sup> signal, and was ~10<sup>-7</sup> for v586 and ~10<sup>-5</sup> for SS21. In the first three lanes of each blot were loaded the dilutions 10<sup>-7</sup>, 10<sup>-5</sup>, and 10<sup>-4</sup> of the inoculum not subjected to PMCA. These values were used to set up our experimental design. (B) PMCA experiments using normal brain homogenates (in blue) as substrate were seeded with brain homogenates from terminally affected voles (in red), and serially propagated through successive PMCA rounds by using two parallel experimental regimens: high PrP<sup>Sc</sup>/PrP<sup>C</sup> seeding (upper panel), in which prions were continuously passaged by 1:10 dilution; low PrP<sub>c</sub>/PrP<sub>c</sub> seeding (lower panel) which starts from an highly diluted seed and then is propagated by alternating bottleneck passages and large population passages in order to recover the population size.
in PrP Sc (Fig. 2A), suggesting a decrease in replication rate. After 5 PMCA rounds, i.e., a replication factor of $-10^3$ for the populations propagated at high PrP Sc/PrP C and $-10^7$ for those at low PrP Sc/PrP C, an upward shift of PrP res was observed only in population v586/a, subjected to high PrP Sc/PrP C replication, but not in the three populations which underwent bottleneck events (Fig. 2A). This different PrP res pattern emerged after 2 PMCA rounds and was propagated until the fifth round (Fig. 2A).

In order to confirm that the slight increase in molecular weight of PrP res observed in v586/a was indeed due to a different PK cleavage site of PrP Sc, we used a sensitive discriminative western blot which measures the relative binding of C-terminal and N-terminal anti-PrP mAbs to PrP Sc treated with high concentration of PK. By choosing the N-terminal mAb directed to an epitope near the expected PK cleavage site, the C-terminal/N-terminal ratio reflects the proportion of PrP res molecules having the epitope chosen (N-terminal mAb) compared with the whole population (C-terminal mAb). As v586 is mostly cleaved C-terminally to the epitope of 12B2, it was anticipated that the upward shift of v586/a should have been reflected by a sensible increase in 12B2 binding and by a decrease of the ratio between SAF84 (PrP-core antibody) and 12B2. Indeed, v586/b-to-e populations were similar to the original v586 and showed high SAF84/12B2 ratio, while the higher MW of v586/a (Fig. 2B) was accompanied by a decrease of SAF84/12B2 ratio (Fig. 2C).

A similar experimental design (high PrP Sc/PrP C seeded at $10^{-2}$ and low PrP Sc/PrP C alternatively seeded at $10^{-3}$ and $10^{-2}$) was used to study the in vitro evolution of PrP Sc from SS21. Unexpectedly, 2 out of the 3 populations propagated at low PrP Sc/PrP C were barely detectable after 5 PMCA rounds, i.e., an expected replication factor of $10^{14}$, and eventually became negative in successive rounds (Fig. 3A). After 9 PMCA rounds, only 3 populations were positive by WB, after an overall replication factor of $10^9$ for SS21/a and SS21/b and $10^{24}$ per SS21/c. Again, an upward shift of PrP res emerged from one population, SS21/a, subjected to high PrP Sc/PrP C propagation. The last PMCA round yielding a positive reaction was used to compare the five populations by discriminatory immunoblotting with mAbs SAF84 and SAF32, whose epitope is partially lost in PrP res fragments from vole-adapted scrapie strains. This confirmed the upward shift of SS21/a, with SAF84/SAF32 ratio

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**Figure 2.** Evolution of PrP res in v586. (A) Western blot analysis of PrP res from v586-derived populations, as indicated. PrP res from different PMCA rounds (roman numbers) were analyzed using antibody SAF84, showing an upward shift of v586/a, and poor PrP res yields in odd rounds of v586/c, v586/d and v586/e. (B) Epitope mapping of PrP res from v586-derived populations analyzed in replica blots with antibodies SAF84 or 12B2, in comparison with v586 and a scrapie control. v586/a showed an intermediate PrP res profile between the inoculum (BSE-like PrPres profile) and the control scrapie. (C) Graph depicting the SAF84/12B2 antibody ratio (y axis, log scale) for samples shown in panel B.
greatly decreased in SS21/a compared with all other SS21-derived populations (Fig. 3B and C).

The same PrP\textsuperscript{res} typing approach was applied to follow PrP\textsuperscript{Sc} conformational variations in each of the 9 consecutive PMCA rounds of the mutated SS21/a. This analysis showed that the upward shift of MW and the decrease in SAF84/SAF32 ratio, and so a change in the dominant PrP\textsuperscript{Sc} conformational variant, were evident after 2 rounds, but disappeared at the fourth and sixth ones, before being apparently “fixed” by the ninth round (Fig. 4A). When this PrP\textsuperscript{Sc} variant obtained after 9 PMCA rounds was used as seed in 9 replicate tubes and propagated for 1 additional round, the mutant biochemical signature was lost in 2 out of 9 tubes (Fig. 4B), indicating a stochastic preferential propagation of different PrP\textsuperscript{Sc} conformers, or a dynamic equilibrium among PrP\textsuperscript{Sc} sub-populations with slightly different conformations. In agreement, every attempt to “fix” this conformational variant by end-point dilution failed.

Given the unexpected loss of 2 out of 3 populations propagated at low PrP\textsuperscript{Sc}/PrP\textsuperscript{PC}, we determined the amplification rates of all the SS21-derived populations by seeding serial 10-fold dilutions of their last positive PMCA round (the ninth for SS21/a, SS21/b and SS21/c and the fourth for SS21/d and SS21/e). In agreement with previous findings (Fig. 3), the in vitro amplification of SS21/d and SS21/e was extremely inefficient, as PrP\textsuperscript{res} was barely detectable only from dilution 10\textsuperscript{-2} (data not shown). Among the 3 populations positive after 9 PMCA rounds, SS21/a and SS21/b showed an amplification rate similar or even higher than that of the starting inoculum. In contrast, SS21/c, the most efficient of the 3 population propagated at low PrP\textsuperscript{Sc}/PrP\textsuperscript{PC}, was at least 100 times less efficient than those propagated at high PrP\textsuperscript{Sc}/PrP\textsuperscript{PC} (Fig. 5). Thus, a remarkable loss of fitness was associated with relatively few bottleneck passages in the SS21-derived populations propagated at low PrP\textsuperscript{Sc}/PrP\textsuperscript{PC}.

**Discussion**

It is now accepted that PMCA is able to reproduce several aspects of prion biology, including prion strain mutation and
It is conceivable that the in vitro environment offers less constraint to prion replication than live animals or cells, due to the absence of active clearance and cell division, which are key players of conformers selection in ex vivo models. In agreement, it was shown that PMCA was able to propagate equally well the scrapie strain RML in cell lysates from either, RML-susceptible or RML-resistant cells, thus overcoming cell-specific susceptibility.

It has been proposed that prion populations are composed of a heterogeneous ensemble of conformational variants which behaves as quasispecies, which would imply that mutation is an intrinsic feature of prions. PMCA appeared to us as an appropriate and practical technique to investigate prion mutability in absence of PrP sequence mismatches, PrPSc modifications, RNA-depletion or treatments with drugs. Importantly, PMCA allows prion populations to undergo very large amplification factors in a relatively short time period, so that we were able to follow prion populations for a very high number of replications. Indeed, with v586 we observed an increase up to $10^7$ fold of the original population in 48 h, with a much higher kinetic of accumulation than an extremely rapid in vivo model. Another key feature of PMCA is that it enables to recover the original population size in a given time period when starting from prion dilutions differing as much as 5 orders of magnitude (see Figure 1). We took advantage of this feature to follow prion populations propagated by transferring different population sizes at each PMCA round, mimicking large population vs bottleneck passages, a very useful experimental set up for studying the evolutionary features of quasispecies populations of RNA viruses.

As a read-out for conformational mutations of PrPSc we used a PrPRes typing technique proved to be a reproducible and fast way to reveal even minor changes of PK-cleavage sites of PrPSc. On the other hand, PrPRes typing only looks for prion variants in which the conformational change involves a shift of the protease-resistant core of PrPSc, which are conceivably much less than the existing PrPSc conformational variants. This necessarily led to underestimate the number of existing variants, notwithstanding we observed the emergence of conformational variants in 1 out of 5 populations with both strains used as starting material. These conformational variants were either positively selected as the dominant population, such as in v586/a,
or in dynamic equilibrium with parental variants, as in SS21/a. Interestingly, v586/a and SS21/a mutant populations emerged after a relatively low number of replications, i.e., after 2 large population passages or -100 fold amplification of the starting population. In contrast, no variations were observed in 6/6 populations subjected to bottleneck events, although these last were grown for a much higher replication factor, up to 10^{15} for SS21/c. These results might suggest that PrP<sup>Sc</sup> conformational variants with a greater protease-resistant core were present as minor sub-populations in brain-derived v586 and ss21 strains, and were transferred and positively selected by the in vitro environment after large population passages, but instead diluted away in populations propagated at low PrP<sup>Sc</sup>/PrP<sup>C</sup> ratio. The repeated conformational changes observed in the population SS21/a could thus represent a dynamic equilibrium between two prevalent PrP<sup>Sc</sup> conformations, which could have been positively or negatively selected by uncontrolled variations of the experimental conditions, such as those we have observed while setting up these experiments (Fig. S1). An alternative and less realistic explanation would be that prion replication with PMCA is extremely error prone, so to allow mutation and positive selection of the variant after few cycles of replication.

Although no PrP<sup>Sc</sup> variants were observed in the prion populations subjected to repeated bottleneck events, throughout our experiments we observed a lower than expected amplification rate in prion populations propagated through serial high dilution passages. Indeed, although v586 yielded as much as PrP<sup>Sc</sup> as in the starting material when seeded at 10<sup>-7</sup> dilution, populations serially propagated through 10<sup>-7</sup> passages were eventually lost after a few passages. Even when introducing a large population transfer to allow the recovery of population size between 2 bottleneck passages, 2 out of 3 SS21-derived populations became undetectable after 5 PMCA rounds, as their amplification rate had become extremely low after 2 bottleneck passages. SS21/c, whose population size was kept relatively high until the ninth PMCA round, had a ~100 times lower amplification rate than the original population, supporting the view of a generalized fitness decrease in the populations propagated in the bottleneck setting up these experiments (Fig. S1). An alternative and less realistic explanation would be that prion replication with PMCA is extremely error prone, so to allow mutation and positive selection of the variant after few cycles of replication.

These findings were surprising in light of recent studies showing an increase in fitness of brain-derived prion strains after 6 PMCA rounds, as it would be expected by positive selection of the conformations more fit in the new environment. Why did large population transfers of SS21 allow maintenance of the fitness and accumulation of a conformational variant, while repeated bottleneck events led to a strong decrease of the fitness with no evidence of changes in PrP<sup>Sc</sup> type? Interestingly, a possible explanation comes from the quasispecies theory and from studies with RNA viruses supporting the notion that the starting population size affects the evolutionary outcome of a given population under a new selective environment. Indeed, populations with high mutation rate are composed by a cloud of variants shaped by environmental constraints, so that sampling a low number of replicative units (bottlenecks) to start a new population implies a loss of “genomic” information compared with large population passages, and thus a loss in adaptability.

In conclusion, our experiments have shown the emergence of mutant PrP<sup>Sc</sup> conformational variants during standard in vitro replication by PMCA. In contrast to previous in vitro studies, in which prion mutants emergences had been forced through PrP sequence mismatches or by changes in the replication environment, in our studies prion mutation and selection occurred under constant environmental conditions. Furthermore, we observed a decline of fitness in populations grown through repeated bottlenecks events, which is tentatively explained as random drift of a highly heterogeneous prion population due to repeated founder events. Overall, these findings support the emerging view that mutability is an intrinsic property of prion strains.

**Material and Methods**

**PMCA**

Substrates were prepared using 2–3 mo old bank voles homozygous for methionine at codon 109 (Bv109M). Voles were euthanized using carbon dioxide and then perfused with PBS plus 5 mM EDTA. All the brains dissected were immediately homogenized in Conversion Buffer (PBS 1×, pH 7.4; 0.15 M NaCl; 1% Triton X) with the Roche Complete Protease Inhibitor Cocktail (1 tablet in 50 ml CB) as 10% w/v; the pool obtained was divided into small aliquots and stored at -80 °C. All the procedures for substrate preparation were conducted in a prion free laboratory, and the aliquots needed were taken in the prion lab only immediately before the beginning of each PMCA round.

Seeds were prepared using brain tissue from Bv109M terminally affected with strains SS21 or v586. Tissues were homogenized in PBS (10% w/v) containing Complete Protease Inhibitor Cocktail (Roche) using disposable Teflon pestles directly in 1.5 mL Eppendorf tubes, and stored at -20 °C. This homogenate was serially diluted 10-fold in normal bank vole homogenate to generate a dilution curve from 10<sup>-2</sup> to 10<sup>-8</sup> (where 10<sup>-2</sup> means 1% w/v of infected brain homogenate) using the procedure described in Cosseddu et al., to avoid contamination.

PMCA was performed using the Misonix S3000 sonicator, with procedures specifically designed to avoid cross-contamination of samples as described in Cosseddu et al. Briefly, to avoid cross-contamination within the PMCA machine 0.5 mL screw cap Multiply-Safecup (Sarstedt) sealed with parafilm were used, and we avoided to work simultaneously with the 2 strains either in the sonicator rack or under the biological hood. At the end
of each round, 5 μl of each reaction mix were serially 10-fold diluted in fresh substrate and the needed dilution subjected to the new PMCA round, according to the experimental design depicted in Figure 1. The amplification factor was determined as the reciprocal of the dilution factor that a given population accumulated during PMCA passages.

Western blot analysis
Fifteen μl of seeds or PMCA reactions were added to 15 μl of TRIS-HCl sarcosyl 4% and digested with 100 μg/mL of proteinase K (Sigma-Aldrich) for diagnosis or with 200 μg/mL of proteinase K for epitope mapping. Electrophoresis and western blotting were performed as previously described. 28

The membrane were then analyzed with anti-PrP monoclonal antibody SAF84 (a.a. 167–173; 1.2 μg/ml), and SAF32 (octarepeat; 4.8 μg/ml). Following incubation with horseradish peroxidase-conjugated anti-mouse immunoglobulin (Pierce Biotechnology) at 1:13000, the PrP bands were detected by enhanced chemiluminescent substrate (SuperSignal Femto, Pierce) and VersaDoc imaging system (Bio-Rad). The chemiluminescence signal was quantified by QuantityOne software (Bio-Rad).

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/prion/article/28468/

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