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Highly Infectious Prions Generated by a Single Round of Microplate-Based Protein Misfolding Cyclic Amplification

Mohammed Moudjou, Pierre Sibille, Guillaume Fichet, Fabienne Reine, Jérôme Chapuis, Laetitia Herzog, Emilie Jaumain, Florent Laferrière, Charles-Adrien Richard, Hubert Laude, Olivier Andréoletti, Human Rezaei, Vincent Béringue

INRA (Institut National de la Recherche Agronomique), UIR892, Virologie Immunologie Moléculaires, Jouy-en-Josas, France; Franklab, Montigny-le-Bretonneux, France; UMR INRA ENVT 1225, Interactions Hôtes Agents Pathogènes, École Nationale Vétérinaire de Toulouse, Toulouse, France.

M.M. and P.S. contributed equally to this work.

ABSTRACT Measurements of the presence of prions in biological tissues or fluids rely more and more on cell-free assays. Although protein misfolding cyclic amplification (PMCA) has emerged as a valuable, sensitive tool, it is currently hampered by its lack of robustness and rapidity for high-throughput purposes. Here, we made a number of improvements making it possible to amplify the maximum levels of scrapie prions in a single 48-h round and in a microplate format. The amplification rates and the infectious titer of the PMCA-formed prions appeared similar to those derived from the in vivo laboratory bioassays. This enhanced technique also amplified efficiently prions from different species, including those responsible for human variant Creutzfeldt-Jakob disease. This new format should help in developing ultrasensitive, high-throughput prion assays for cognitive, diagnostic, and therapeutic applications.

IMPORTANCE The method developed here allows large-scale, fast, and reliable cell-free amplification of subinfectious levels of prions from different species. The sensitivity and rapidity achieved approach or equal those of other recently developed prion-seeded conversion assays. Our simplified assay may be amenable to high-throughput, automated purposes and serve in a complementary manner with other recently developed assays for urgently needed antemortem diagnostic tests, by using bodily fluids containing small amounts of prion infectivity. Such a combination of assays is of paramount importance to reduce the transmission risk in the human population and to identify asymptomatic carriers of variant Creutzfeldt-Jakob disease.

Prion diseases are infectious neurodegenerative disorders affecting a broad range of mammalian species, including Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and chronic wasting disease in cervids (1). The causal proteinaceous agent, termed a prion, propagates by converting the α-helix-rich host-encoded prion protein PrP\textsuperscript{C} into a misfolded, β-sheet-enriched conformer designated PrP\textsuperscript{Sc}. The abnormal form PrP\textsuperscript{Sc} is believed to replicate by recruiting and converting PrP\textsuperscript{C} into higher-order aggregates, through a so-called seeded polymerization process (2, 3). Fragmentation of PrP\textsuperscript{Sc} assembles is thought to generate new PrP\textsuperscript{Sc} seeds to sustain the conversion (4). Distinct strains of prions are recognized phenotypically, based on different incubation times, neuropathological features, and PrP\textsuperscript{Sc} biochemical properties in experimentally infected rodents. Strain biological properties are believed to be encoded in different, specific PrP\textsuperscript{Sc} conformers (5–8). Several lines of evidence support PrP\textsuperscript{Sc} as the main molecular determinant of prion replication and infectivity (2, 3). Among them is the possibility to generate prion infectivity by PrP\textsuperscript{Sc}-templated conversion of PrP\textsuperscript{C} under cell-free conditions using protein misfolding cyclic amplification (PMCA) assays. This technique has emerged in the last decade as a very efficient procedure to amplify prions in a test tube (9). PMCA exploits the ability of PrP\textsuperscript{Sc} to template the conversion of PrP\textsuperscript{C} by repetitive cycles of incubation and sonication, leading to the amplification of minute amounts of PrP\textsuperscript{Sc}. The sensitivity achieved allows detection of PrP\textsuperscript{Sc} present at low levels in biological tissue or fluid samples, including blood, urine, feces, or cerebrospinal fluid (10–14). The mechanisms by which such efficient in vitro amplification is achieved are essentially unknown. By analogy with the nucleation/polymerization process, incubation of PrP\textsuperscript{Sc} seeds with PrP\textsuperscript{C}-containing substrate is thought to favor PrP\textsuperscript{Sc} conversion and growth of PrP\textsuperscript{Sc} aggregates. Sonication is thought to fragment the polymers, thus providing new seeds for conversion. The PMCA-generated products are infectious and share (in general) similar biochemical and structural properties and biological strain properties with the prion strain seed that serves for amplification (15–19). There are, however, some clear discrepancies in the amounts of prion infectivity generated among the studies (16, 18–20). Significant variations in the laboratory-specific methodologies employed to amplify PrP\textsuperscript{Sc} could explain the discrepancies observed. Importantly, the PMCA reaction mixtures employed were not always supplemented with beads, which were shown to significantly improve the level and the reproduc-
ibility of the amplification (21, 22), by putatively favoring the fragmentation of the generated polymers. The infectious titer of the amplicon was also frequently measured after several rounds of amplification. Repetitive, long-term incubations and sonications may alter infectivity. Besides, the higher number of rounds increases the probability of generating false-positive amplifications (23).

In this study, we first report significant simplification and improvements of the PMCA technique, leading to high throughput and highly efficient amplification of PrPSc from several prion strains from different species in a single 48-h round. We then demonstrate by endpoint dilution quantitation in reporter mice that this method restores an infectivity titer comparable to that of in vivo brain-derived prions, whatever the input dilution seeding the PMCA reaction.

RESULTS

Endpoint titration of 127S prions using mb-PMCA. The experimental conditions leading to miniaturized bead-PMCA (mb-PMCA) were established with brain material from transgenic tg338 mice overexpressing ovine PrPC. These mice were infected or not with the 127S scrapie strain, a prototypal “fast” strain killing the mice within 2 months (24, 25). The procedure was performed by seeding tg338 mouse brain lysate containing ovine PrPC- substrate with serial 10-fold dilutions of 127S-infected brain homogenate and running 96 sonications/cycles for a round of 48 h. The amplicons were treated with proteinase K (PK) before detection of PK-resistant PrPSc (PrPres) signals by dot blotting and Western blotting. Without amplification, PrPres is detected until the brain homogenate is diluted 103-fold (Fig. 1a).
Maximal Prion Amplification in a Single Round of PMCA

FIG 2  mb-PMCA of 127S prions by using different concentrations of PrP<sup>Sc</sup> substrate. Brain homogenate from tg338 mice infected with 127S prions was serially diluted in substrate containing either pure tg338 mouse brain homogenate (BH) or tg338 BH mixed with PrP<sup>0/0</sup> BH or PMCA buffer, as indicated. The mixture was then subjected to a single round of mb-PMCA. All samples were digested with PK before Western blot analysis. Undigested normal brain homogenate (PrP<sup>Sc</sup>) and PK-digested, infected brain homogenate (PrP<sup>0/0</sup>) are provided as electrophoretic references. Molecular mass markers (kDa) are indicated on the left.

Proving by 10<sup>7</sup>-fold the sensitivity of the technique. “Standard” PMCA protocols (26) were initially followed using individual PCR tubes, brain from perfused tg338 mice to prepare the substrate, phosphate-buffered saline (PBS)–Triton as PMCA buffer, no beads added, and a final reaction volume of 100 μl. In our hands, this often resulted in variations in the amplification efficiency and difficulties in completely digesting PrP<sup><sub><sup></sub></sup></sup> with PK, even in the unseeded control samples (data not shown). When PBS was replaced with Tris in the PMCA buffer, better PrP<sup><sub><sup></sub></sup></sub> amplification and reduced PrPC undigested background were obtained. Under these conditions, PrP<sup><sub><sup></sub></sup></sub> was detected in PMCA reaction mixtures seeded with 10<sup>-4</sup>-diluted 127S brain homogenate (Fig. 1b). Addition of 3 ceramic beads during the reaction markedly increased the sensitivity and the reproducibility of the amplification; PrP<sup><sub><sup></sub></sup></sub> was detected routinely from reaction mixtures seeded with 10<sup>-5</sup>-diluted inoculum (Fig. 1b). Addition of one Teflon bead was also beneficial, as previously described (21). Use of strips of 8 PCR tubes instead of individual tubes placed in rigorously defined positions in the cup horn led to systematic and robust amplification of PrP<sup><sub><sup></sub></sup></sub> in 10<sup>-7</sup>-diluted inoculum (Fig. 1b). Encouraged by these observations, we shifted to 96-well PCR microplates. The final PMCA volume was reduced to 36 μl. Using this new experimental design (referred to as mb-PMCA), highly efficient and reproducible detection of PrP<sup><sub><sup></sub></sup></sub> was achieved in reaction mixtures seeded with 10<sup>-7</sup>-diluted brain material (Fig. 1c). PrP<sup><sub><sup></sub></sup></sub> was occasionally detected from amplification of 10<sup>-12</sup> dilutions (see, for example, Fig. 2). Reactions seeded with higher dilutions (up to 10<sup>-13</sup>) did not show any positive PrP<sup><sub><sup></sub></sup></sub> signal (Fig. 1c). Specificity was further assessed by submitting the mb-PMCA samples seeded with 10<sup>-13</sup>-diluted material to a second round with fresh substrate. No PrP<sup><sub><sup></sub></sup></sub> could be detected (data not shown). Two to six unseeded control samples per plate were systematically included in each mb-PMCA. No PrP<sup><sub><sup></sub></sup></sub> was detected in these control samples in a total of more than 220 mb-PMCA reactions (i.e., the equivalent of 21,000 individual tubes) (for example, Fig. 1c), thus highlighting the high specificity of the reaction. Taken together, these findings suggest that the input seed limiting dilution of 127S prions should be established at 10<sup>-11</sup> to 10<sup>-12</sup> and can be amplified by mb-PMCA in one 48-h round of 96 cycles of sonication/incubation.

PrP<sup><sub><sup></sub></sup></sub> and other brain factors are limiting mb-PMCA efficacy.

We next examined whether the high sensitivity of the mb-PMCA procedure was linked to the amount of PrP<sup><sub><sup></sub></sup></sub> present in the substrate lysate, as tg338 mouse brain overexpresses approximately 8-fold PrP<sup><sub><sup></sub></sup></sub> compared to sheep brain (27). Performing mb-PMCA with serial 10-fold dilutions of 127S input seeds mixed with substrate containing 100%, 50%, or 25% tg338 brain lysate (the latter dilutions were performed in 10% PrP-knockout brain lysates made in PMCA buffer) led to a 2-log<sub>10</sub> reduction of the efficacy of the amplification (Fig. 2). In marked contrast, diluting tg338 brain lysate by 1:2 in PMCA buffer reduced by 6 log<sub>10</sub> the efficacy (Fig. 2), suggesting that PrP<sup><sub><sup></sub></sup></sub> was not the sole limiting factor in PMCA conversion efficacy.

We estimated the PrP<sup><sub><sup></sub></sup></sub> conversion yield during the 48-h mb-PMCA procedure. 127S PrP<sup><sub><sup></sub></sup></sub>, but not PrP<sup><sub><sup></sub></sup></sub>, resists thermolysin digestion (28). Measuring the ratio of thermolysin-resistant PrP<sup><sub><sub></sub></sub></sup> to total PrP would thus provide information on the percentage of mb-PMCA-converted PrP<sup><sub><sub></sub></sub></sup>. Samples from two independent mb-PMCA protocols were serially diluted and typed phenotypically (26). Previously, all the prions tested have been serially passaged and typed phenotypically on the ad hoc transgenic mice. As summarized in Table 1, mouse prions 139A, 22L, RML, and Chandler were efficiently amplified using transgenic tg20 mouse brain (29) as the substrate for mouse PrP<sup><sub><sub></sub></sub></sup>. PrP<sup><sub><sub></sub></sub></sup> was routinely detected from input seeds diluted up to 10<sup>-10</sup>-fold (n ≈ 6 experiments). ME7 prion amplification was 1,000 times less efficient. The PrP<sup><sub><sub></sub></sub></sup> glycoprofile of the amplified products resembled that of the inoculum, with a prominent monoglycosylated PrP form typical of these mouse prions (Fig. 4 and data not shown). PrP<sup><sub><sub></sub></sub></sup> from 263K, Sc237, and HY hamster prions was amplified from 10<sup>-7</sup>-diluted input seeds in one round using hamster PrP transgenic mouse brain (tg7 line [25]) as the substrate (n ≈ 10 experiments). The DY hamster strain was less efficiently amplified (10<sup>-3</sup>). The PrP<sup><sub><sub></sub></sub></sup> electrophoretic and glycoform profiles of the nonamplified and mb-PMCA-amplified prions were similar (Fig. 4 and data not shown). Finally, we assessed the efficacy of mb-PMCA for human variant CJD
minute amounts of PrPSc from different prion strains in a single round. These experiments allowed us to determine that the lowest dose resulting in positive transmission (as based on the appearance of clinical signs and detection of PrPSc in brain) was observed at the 10−7 dilution of 127S brain inoculum (Table 2; Fig. 5). mb-PMCA products were generated from reaction mixtures seeded with this limiting dilution or with 100-fold-more-diluted or -more-concentrated seeds. The amplification generated with the 10−7 seed was 10-fold diluted up to a 10−7 dilution for complete endpoint titration while the amplicons obtained with the 10−5 and 10−7 seeds were diluted 101-, 103-, 105-, and 107-fold. Two unseeded samples run in parallel were 10-fold diluted. All the dilutions were prepared separately and immediately inoculated into recipient tg338 mice by the intracerebral route. The results are summarized in Table 2. At the time of writing, more than 400 days after the experimental infection, mice inoculated with unseeded controls were still alive and healthy. Whatever the initial dilution of the 127S seeds that served for mb-PMCA, an attack rate of 100% was observed in tg338 mice inoculated with all the amplicons diluted up to 105- to 109-fold. At the 10−7 dilution, 1/5 (10−7 and 10−9 seed) or 2/5 (10−5 seed) tg338 mice were still infected, an attack rate reminiscent of that observed for nonamplified 127S-infected brain. In other words, one round of PMCA was sufficient to regenerate infectivity to levels identical to those reached in the brains of intracerebrally inoculated tg338 mice at the terminal stage of disease. For the fully titrated amplicon generated with the 10−9 input seed, the relationship between prion concentration and mouse incubation period appeared superimposable on that observed with prions derived from terminally sick mouse brains (Fig. 5), suggesting similar multiplication rates between cell-free and brain-derived prions upon injection in the tg338 mouse brain.

The clinical signs of tg338 mice inoculated with serially diluted amplicons were identical to those of mice infected intracerebrally with 127S prions, including notably hyperexcitability, waddling, and rolling gait. Brain and spleen samples from these animals accumulated PrPSc with a glycosylation and tissue-specific (28) mobility profile similar to that found in 127S-infected brain and spleen, respectively (Fig. 6a and b and data not shown). The regional distribution of PrPSc in the brains of tg338 mice infected with the amplicons resembled that observed with 127S inoculated intracerebrally at equivalent dilutions (24) (Fig. 6c and data not shown). The PrPSc staining was pronounced in the septum, cor-

Table 1: Endpoint titration of mouse, hamster, and human prions by a single round of mb-PMCA

| Species | Prion strain | PrPSc substrate (mouse line) | Limiting dilution of brain material |
|---------|--------------|------------------------------|-----------------------------------|
| Mouse   | 139A         | Mouse (tg20)                 | 10−8                              |
|         | RML          |                              | 10−9                              |
|         | Chandler     |                              | 10−7                              |
|         | 22L          |                              | 10−9                              |
|         | BSE          |                              | 10−10                             |
|         | ME7          |                              | 10−6                              |
|         | Sc237        | Hamster (tg7)                | 10−7                              |
|         | 263K         |                              | 10−7                              |
|         | HY           |                              | 10−7                              |
|         | DY           |                              | 10−5                              |
| Human   | vCJD         | Human M129 (tg650)           | 10−8                              |

mb-PMCA generates highly infectious prions. We finally sought to determine whether highly efficient mb-PMCA of PrPSc was associated with similarly efficient amplification of infectivity. This was done with 127S prions using the tg338 mouse bioassay.

According to endpoint titration by the intracerebral (IC) route, the infectious titer of 127S prions in tg338 mouse brain is 10^{9.2} lethal dose 50 (LD_{50}) per gram (25). These experiments allowed us to determine that the lowest dose resulting in positive transmission (as based on the appearance of clinical signs and detection of PrPSc in brain) was observed at the 10−7 dilution of 127S brain inoculum (Table 2; Fig. 5). mb-PMCA products were generated from reaction mixtures seeded with this limiting dilution or with 100-fold-more-diluted or -more-concentrated seeds. The amplification generated with the 10−7 seed was 10-fold diluted up to a 10−7 dilution for complete endpoint titration while the amplicons obtained with the 10−5 and 10−7 seeds were diluted 101-, 103-, 105-, and 107-fold. Two unseeded samples run in parallel were 10-fold diluted. All the dilutions were prepared separately and immediately inoculated into recipient tg338 mice by the intracerebral route. The results are summarized in Table 2. At the time of writing, more than 400 days after the experimental infection, mice inoculated with unseeded controls were still alive and healthy. Whatever the initial dilution of the 127S seeds that served for mb-PMCA, an attack rate of 100% was observed in tg338 mice inoculated with all the amplicons diluted up to 105- to 109-fold. At the 10−7 dilution, 1/5 (10−7 and 10−9 seed) or 2/5 (10−5 seed) tg338 mice were still infected, an attack rate reminiscent of that observed for nonamplified 127S-infected brain. In other words, one round of PMCA was sufficient to regenerate infectivity to levels identical to those reached in the brains of intracerebrally inoculated tg338 mice at the terminal stage of disease. For the fully titrated amplicon generated with the 10−9 input seed, the relationship between prion concentration and mouse incubation period appeared superimposable on that observed with prions derived from terminally sick mouse brains (Fig. 5), suggesting similar multiplication rates between cell-free and brain-derived prions upon injection in the tg338 mouse brain.

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pus callosum, habenula, hypothalamus, lateral and posterior hypothalamic area, and brain stem.

Together, our data led to the conclusion that the mb-PMCA amplicons are strongly related, if not identical, to 127S prions phenotypically and that one round of mb-PMCA restores infectivity levels similar to that of 127S original brain material.

DISCUSSION

The sensitivity of PMCA in amplifying prions has long been recognized. By using 96-well microplates, beads, and a reduced reaction volume, we now show for the first time specific and highly sensitive prion amplification in a single round, thus greatly limiting the probability of false-positive samples. We also provide evidence that the PMCA-amplified products can be highly infectious, exhibiting a titer similar to that found in the parental brain material.

The PMCA method is constantly under continuous adaptation and improvements, due to inconsistent robustness and reproducibility, thus compromising its use for large-scale purposes. Two important technical limitations were circumvented in our study:

TABLE 2 Incubation times of tg338 mice inoculated with serial 10-fold dilutions of brain-derived or mb-PMCA-generated 127S prions

| Dilution | Incubation time in days ± SEM (n/ni) |
|----------|------------------------------------|
|          | PMCA generated                     |
|          | 10^-5 seed | 10^-7 seed | 10^-9 seed | Brain derived |
| 10^-1    | 63 ± 2 (5/5) | 60 ± 1 (4/4) | 59 ± 1 (5/5) | 64 ± 1 (5/5) |
| 10^-3    | 74 ± 1 (5/5) | 72 ± 2 (5/5) | 74 ± 3 (5/5) | 79 ± 2 (5/5) |
| 10^-4    | ND         | ND          | 82 ± 2 (5/5) | 85 ± 1 (5/5) |
| 10^-5    | 91 ± 2 (5/5) | 98 ± 3 (5/5) | 111 ± 11 (5/5) | 99 ± 2 (5/5) |
| 10^-6    | ND         | ND          | 117 ± 11 (5/5) | 119 ± 7 (4/5) |
| 10^-7    | 134; 147 (2/5) | 124 (1/5) | 139 (1/5) | 157 (1/5) |
| Unseeded no. 1 | >400             |              |            |              |
| Unseeded no. 2 | >400             |              |            |              |

a n/ni, number of affected/number of inoculated tg338 mice; ND, not done.
The sensitivity reached by the mb-PMCA assay exceeded that of 127S prions to levels detectable by conventional Western blotting. This effect appears optimal in a reduced reaction volume, suggesting a link with the distribution of sonicator, allowing more than 110 samples to be run at the same time (see Fig. S1 in the supplemental material). The observation that ceramic beads greatly improved the efficiency of PMCA is congruent with previous observations made with Teflon beads (21, 22). Beads are believed to promote efficient fragmentation of PrPSc polymers, thus increasing the number of seeds available for conversion (21). The observation that the number and the size of beads added to the conversion appeared less important than their chemical composition (our unpublished data and reference 21) might suggest a more complex physical involvement of the beads during the incubation/sonication cycles and by decreasing the volume necessary to the reaction with the PCR microplate format. This led to the use of the maximum surface offered by the sonicator, allowing more than 110 samples to be run at the same time (see Fig. S1 in the supplemental material). The observation that ceramic beads greatly improved the efficiency of PMCA is congruent with previous observations made with Teflon beads (21, 22). Beads are believed to promote efficient fragmentation of PrPSc polymers, thus increasing the number of seeds available for conversion (21). The observation that the number and the size of beads added to the conversion appeared less important than their chemical composition (our unpublished data and reference 21) might suggest a more complex physical involvement of the beads in the PMCA reaction. This effect appears optimal in a reduced reaction volume, suggesting a link with the distribution of sonication energy.

Using the so-called mb-PMCA protocol, a single 48-h round was sufficient to achieve amplification of the highest dilution of 127S prions to levels detectable by conventional Western blotting. The sensitivity reached by the mb-PMCA assay exceeded that of the tg338 bioassay by 10^5 to 10^3-fold. This difference is consistent with other studies (17, 33). A significant part of the inoculated or generated prions might be degraded in animal bioassays (36, 37), a phenomenon that would be limited in cell-free assays. Remarkably, the technical improvements employed were beneficial to other prion strains, such as mouse, hamster, and human vCJD prions, without, at variance with previous studies (35, 38, 39), an apparent need to adapt the conditions to each prion strain. Variation in the power amplitude of the sonicator (10 to 80%) was without notable influence on the sensitivity achieved with 127S, 263K, Chandler, and vCJD prions (data not shown), suggesting that these samples received optimal sonication energy in the mb-PMCA format. However, some prion sources, notably those responsible for certain CJD subtypes, fairly resist mb-PMCA (unpublished observations), suggesting that certain cell-free prion polymerizations would necessitate cofactors or different experimental conditions. The maximum dilution of prion brain inoculum detected by mb-PMCA varied with certain strain types (Table 1). This may reflect differences in infectious titers among strains. Comparatively, ME7 and DY are at least 100-fold less infectious than RML/Chandler and HY prions in tga20 mice (40) and hamsters (41), respectively. The sensitivity of detection achieved with vCJD prions would be compatible with that necessary for reliable detection of this agent in blood (42), an important public health concern given the current uncertainties about the number of individuals incubating the disease (43–45).

We confirm here that PMCA sensitivity is dependent on the concentration of PrPC in the PMCA substrate (46–48). However, despite its remarkable sensitivity, the PrPSc conversion yield achieved by the mb-PMCA assay was approximately 20%. This low conversion yield might suggest that all brain PrPSc species are not convertible, a hypothesis consistent with the deposition of PrPSc in (strain-dependent) specific brain areas in infected animals (49–51). Alternatively but not exclusively, other brain factors might be a limiting factor. Supporting this hypothesis, a 2-fold dilution of PMCA substrate in PMCA buffer instead of PrP0/0 brain lysate dramatically decreased the mb-PMCA efficiency. RNAs or poly(A) and lipids have been shown to be instrumental in efficient PMCA conversion (52–54). The high-throughput screening capacities of mb-PMCA will be helpful to investigate this further and to search for potent cellular factors involved in the PrPSc conversion on a large scale.

Our 20% conversion yield sharply contrasted with the 100% conversion yield previously reported by using beads in the PMCA reaction (21). These differences might be due to different prion sources/PrP substrate combinations and/or to the quantification methods used. Here, we quantified the densitometric ratio of PrPSc amplicons generated with input seeds diluted 100-fold more...
than the 127S prion limiting dilution in the tg338 bioassay, thus excluding any contribution of the parental seed to the infectivity measured. This tight relationship between prion replication dynamics in cell-free and animal assays contrasts with previously published results. Shikiya and Bartz (18) found, as we did, that their PMCA protocol generated high-titer prions; however, the rate of prion amplification was significantly altered. Notably, they titrated PMCA-generated HY prions after the 10th round of amplification, which may have altered the amplicon infectious properties. Other contradictory studies, which were also based on multiround PMCA, divided the infectivity titer by the amount of PrPSc generated by the PMCA reaction, so as to calculate PrPSc specific infectivity (16, 19). A number of pieces of experimental evidence suggest a quantitative disconnection between infectivity and PrPSc in the brains of prion-infected animals (59). Specifically for 127S and the extensively used Sc237 hamster prions, a subpopulation of PrPSc assemblies would support most of the infectivity, making a significant proportion of PrPSc assemblies relatively innocuous (25, 60). We may have regenerated high-titer 127S prions by a single round of mb-PMCA because this “most infectious” PrPSc subpopulation was preferentially amplified. Accordingly, we demonstrated with another “fast” ovine prion strain, exhibiting a behavior similar to that of 127S in tg338 mice (25), that the subset of PrPSc assemblies that carried the major part of prion infectivity also exhibited by far the highest templating activity by mb-PMCA (61).

In summary, the mb-PMCA assay allows large-scale, fast, and reliable cell-free amplification of subinfectious levels of prions from different species. The sensitivity and rapidity achieved approach or equal those of other prion-seeded conversion assays (62), such as the quaking-induced conversion (QuIC) assay (63). At variance with the latter (62, 64), mb-PMCA can regenerate large amounts of prion infectivity. Such a simplified assay may be amenable to high-throughput, automated purposes and serve, in a complementary manner with QuIC-like (62) or solid-phase binding (42) assays, for urgently needed preclinical diagnostic tests, by using bodily fluids containing small amounts of prion infectivity.

MATERIALS AND METHODS

Ethics statement. All animal experiments have been performed in strict accordance with EU directive 2010/2063 and were approved by the local ethics committees of the authors’ institutions (Comethea; permit number 12/034).

Transgenic mice and prion strains. The ovine (tg338 line; Val136-Arg154-Gln171 allele), human (tg650 line; Met129 allele), hamster (tg7 line), and mouse (tga20) PrP transgenic lines have been described previously (24, 25, 29, 30, 43, 65). These lines are homozygous and overexpress
about 8-, 6-, 4-, and 10-fold the heterologous PrPC level on a mouse PrP-null background, respectively.

The 127S scrapie prion strain has been obtained through serial transmission and subsequent biological cloning by limiting dilutions of PG127 field scrapie isolate totg338 mice (24, 27). The 127S infectious titer is 10^2.5 50% lethal doses (LD_{50})/g of tg338 brain (25). Pools of 127S-infected tg338 mice brains were prepared as 20% (wt/vol) homogenate in 5% glucose by use of a tissue homogenizer (Precellys 24 Ribolyzer; Ozyme; Bertin Technologies, France). The homogenate was diluted half to 10% in PMCA buffer (see below) to obtain the 10^-2 dilution of the inoculum and stored at −80°C. All subsequent dilutions refer to this 10% homogenate starting material.

Mouse prion strains 139A, 22L, RML, Chandler, ME7, and mouse-adapted bovine spongiform encephalopathy (BSE); hamster prion strains 263K, Sc237, HY, and DY; and human VVJD prions have been serially passaged on tga20, tg7 (25), and tg650 mice (30), respectively.

PMCA. Mouse brain lysate from uninfected tg338 mice was used as the substrate for 127S scrapie prions. Mouse brain lysates from tga20 mice, tg7 mice, and tg650 mice were used as the substrates for mouse, hamster, and human prions, respectively. Two- to 12-month-old mice were euthanized. The survival time was defined as the number of days from inoculation to euthanasia. Their brains and spleens were removed for analysis. Each sample was supplemented with SDS (0.3 to 0.6% final concentration) at 37°C for 1 h. The brain tissue was left at 4°C for 30 min and briefly clarified by centrifugation at 1,000 × g for 2 min at 4°C. The resulting supernatant, corresponding to the PrPC substrate lysate, was briefly clarified by centrifugation at 1,000 × g for 2 min at 4°C. The supernatant, corresponding to the PrPC substrate lysate, was collected, aliquoted, and stored at −80°C. Protein misfolding cyclic amplification (PMCA) performed with either young (6- to 10-week) or old (1-year) mouse brain homogenates yielded the same results. Moreover, brain perfusion prior to collection and homogenization appeared not to be of any influence on the performance of the mb-PMCA (data not shown).

PMCA was performed in a final volume of either 100 μl or 36 μl of lysate per well, in either single PCR tubes (conventional method); in 2-, 4-, or 8-PCR-tube strips; or with a 96-well PCR microplate (Axygen, Union City, CA, USA). Each tube or well was first filled with ceramic beads (3 beads of 1.23 mm in diameter; Matrixx, France). Two ceramic beads of 2.4 mm or one Tollen bead of 2.38 mm in diameter (Marbet et Lemarié, Pantin, France) was also efficient. A 4-μl aliquot of the analyte inoculum (10^-2 dilution) was suspended in 36 μl of healthy tg338 brain lysate to obtain the 10^-3 dilution. Then, a series of 10-fold dilutions was made by adding 4 μl from the previous inoculum dilution to the next 36-μl containing tube or well. Individual tubes, tube strips, or microplates were placed on a Plexiglas rack designated for the cup horn of the S300 or Q700 sonicator (Misonix, Farmingdale, NY, USA, or Delta Labo, Colombelles, France) and subjected to 96 cycles of 30 s of sonication at 220- to 240-W power (level 6 to 7 for the S300 or 30% amplitude of the Q700 sonicator) followed by 29 min 30 s of incubation at 37°C. The cup horn was filled with 300 ml of water (or 4 M guanidium hydrochloride solution) circulating with rubber tubing in a water bath maintained at a temperature of 35 to 36°C. When needed, subsequent rounds of PMCA were realized using a 1/10 dilution of the products of the previous PMCA round as the template. At the end of the PMCA, the tubes or microplates were removed and aliquots from each sample were taken to be analyzed for their PrPsc content.

Protease digestion of PMCA products. To analyze the production of proteinase K (PK)-resistant PrPsc species during PMCA, 10 to 18 μl of each sample was supplemented with SDS (0.3 to 0.6% final concentration) and treated with PK (115 μg/ml final concentration) at 37°C for 1 h. The PK digestion was stopped by adding an equal volume of 2X Laemmli denaturation sample buffer and heating at 100°C for 5 min. The samples were then stored at −20°C until dot blotting and Western blotting.

To analyze the levels of thermolysin-resistant species, PMCA products were first diluted 1/5 in TNT buffer (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100) to decrease the EDTA concentration which prevents thermolysin digestion. The samples were then treated with 200 μg/ml of thermolysin (Sigma, St. Louis, MO, USA) at 37°C for 1 h before denaturation as described above.

Dot blotting analysis of PrPsc. Given the significant number of samples generated with the PMCA procedure, a quick dot blot analysis was set up and turned out to be practical and efficient in getting an overview of the PMCA results. Ten to 20 μl of each PK-digested PMCA sample was removed and transferred into a 96-well microplate containing 50 μl of 1% SDS and 20% glycerol. The samples were then transferred onto a nitrocellulose membrane placed on the dot blot apparatus (Whatman, France) connected to a vacuum system. The membrane was removed and rinsed twice with Tris-buffered saline (TBS)−0.1% Tween 20 before incubation with the primary antibody (biotinylated Sh3a1 [66 anti-PrP monoclonal antibody]) for 15 to 30 min at room temperature. After 3 washes of 5 min each, the membrane was incubated with streptavidin-coupled horseradish peroxidase for 15 to 30 min at room temperature and processed for detection with the enhanced chemiluminescence (ECL) reagent (GE Healthcare, Saclay, France).

SDS-PAGE and Western blotting. PMCA samples were run on either 4 to 12% or 12% Bis-Tris NuPAGE precast gels (Invitrogen, Cergy-Pontoise, France) or on Criterion XT 12% Bis-Tris precast gels (Bio-Rad, Hercules, CA, USA), electrotransferred onto nitrocellulose membranes with the semidy electrotransfer system (Bio-Rad), and probed with biotinylated Sh3a1 anti-PrP monoclonal antibody. Secondary antibody incubation and ECL detection were performed as described above. When necessary, the PrPsc content of PMCA samples was determined with GeneTools software after acquisition of the signals with a GeneGnome digital imager (Syngene, Frederick, MD, USA).

Endpoint titration of PMCA products in tg338 mice. A strict protocol based on the use of disposable equipment and preparation of all inocula in a class II microbiological cabinet was followed to avoid any cross-contamination. Serial 10-fold dilutions of PMCA products were prepared in sterile 5% glucose containing 5% bovine serum albumin, and 20 μl of each dilution was immediately inoculated into individually identified 6- to 10-week-old tg338 recipient mice (n = 5 mice per dilution) by the intracerebral route. The inoculated animals were observed daily for the appearance of prion disease. Animals at the terminal stage of disease were euthanized. The survival time was defined as the number of days from inoculation to euthanasia. Their brains and spleens were removed for PrPsc analysis by Western blotting and histoblotting as previously described (24, 25). For the histoblotting procedure, brains were rapidly removed from euthanized mice and frozen on dry ice. Cryosections were cut at 8 to 10 mm, transferred onto Superfrost slides, and kept at −20°C until use. Histoblot analyses were performed on 3 brains per dilution per ampiclon, using the 12F10 anti-PrP antibody (67).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00829-13/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.

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