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Efficient In Vitro Amplification of Chronic Wasting Disease PrP^{RES} ▽

Timothy D. Kurt,[1] Matthew R. Perrott,[1] Carol J. Wilusz,[1] Jeffrey Wilusz,[1] Surachai Supattapone,[2] Glenn C. Telling,[3] Mark D. Zabel,[1] and Edward A. Hoover[1]*

Department of Microbiology, Immunology, and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colorado[1]; Department of Biochemistry, Dartmouth Medical School, Hanover, New Hampshire[2]; and Department of Molecular Biology and Genetics, University of Kentucky, Lexington, Kentucky[3]

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Chronic wasting disease (CWD) of cervids is associated with conversion of the normal cervid prion protein, PrP^{C}, to a protease-resistant conformer, PrP^{CWD}. Here we report the use of both nondenaturing amplification and protein-misfolding cyclic amplification (PMCA) to amplify PrP^{CWD} in vitro. Normal brains from deer, transgenic mice expressing cervid PrP^{C} [Tg(cerPrP)1536 mice], and ferrets supported amplification. PMCA using normal Tg(cerPrP)1536 brains as the PrP^{C} substrate produced $>6.5 \times 10^{9}$-fold amplification after six rounds. Highly efficient in vitro amplification of PrP^{CWD} is a significant step toward detection of PrP^{CWD} in the body fluids or excreta of CWD-susceptible species.

Chronic wasting disease (CWD) of cervids is a transmissible spongiform encephalopathy (TSE), akin to sheep scrapie and bovine spongiform encephalopathy. TSE pathogenesis is associated with refolding of the normal prion protein, PrP^{C}, into a partially protease resistant isomer termed PrP^{RES} (2, 15, 17). A remarkable feature of CWD among prion diseases is its horizontal transmission in nature (12), suggesting that PrP^{C} conversion is highly efficient in this TSE and perhaps is associated with the presence of infectious prions in the body fluids of deer (10). CWD is also transmissible to and pathogenic in ferrets (1) and transgenic mice expressing normal cervid PrP^{C} (3, 8, 11).

Raymond and colleagues (18) first demonstrated the conversion of cervid PrP^{C} to PrP^{RES} in vitro. Nondenaturing amplification without the use of radiolabeling (7, 9) further contributed to understanding of the mechanisms of PrP^{C}-to-PrP^{RES} conversion due to its directness and technical simplicity. Soto, Castilla, and colleagues (5, 6, 21) greatly extended the process and power of in vitro PrP^{RES} amplification by developing protein-misfolding cyclic amplification (PMCA). In PMCA, normal brain homogenates (NBH) supply PrP^{C}, which, upon addition of an infected brain homogenate, is refolded into the protease-resistant isoform, PrP^{RES}. Breakage of aggregates by use of sonic bursts releases the newly formed PrP^{RES} and extends the enciphering process (19, 20, 22).

To begin to address the mechanisms of PrP^{C}-to-PrP^{RES} conversion in CWD and to enhance the sensitivity of CWD prion (PrP^{CWD}) detection in deer, we developed two in vitro amplification assays: nondenaturing amplification patterned after the technique of Lucassen et al. (9) and serial PMCA modeled after the method of Soto and colleagues (19, 21). Here we report amplification using CWD-negative brain homogenates from white-tailed deer (*Odocoileus virginianus*), cervid PrP transgenic mice [Tg(cerPrP)1536 mice] (3), and ferrets (*Mustela putorius furo*), a species shown to be susceptible to CWD infection in vivo (1).

Preparation of tissue homogenates. Whole brains were removed rapidly after sacrifice from CWD-free animals and were immediately frozen in liquid nitrogen. For PMCA experiments, animals were perfused at death with phosphate-buffered saline (PBS) containing 5 mM EDTA. NBH were prepared by homogenization of brains with a glass Dounce homogenizer (Kontes) or glass beads (FastPrep; set at 6.5 for 45 s; Qbiogene, Irvine, CA) in 9 volumes of cold PBS (nondenaturing experiments) or PBS with Triton X-100, 5 mM EDTA, 150 mM NaCl, and 0.05% saponin (Sigma) plus CompleteMini protease inhibitors (Roche) to a final concentration of 10% (wt/vol) (PMCA experiments). NBH were centrifuged at $200 \times g$ for 30 s (nondenaturing experiments) or $2{,}000 \times g$ for 1 min (PMCA experiments), and the supernatants were removed and frozen at −70°C for use. A CWD-positive sample of deer origin (D10; provided by Michael Miller, Colorado Division of Wildlife, Fort Collins) consisted of a brain from an experimentally infected mule deer and was prepared as a 10% (wt/vol) homogenate. A ferret-adapted CWD-positive sample (CSU-1) was composed of the pooled brains of three experimentally infected ferrets prepared as a 20% (wt/vol) homogenate. These ferrets exhibited typical CWD symptoms and were euthanized 4.5 to 5 months postinoculation (M. R. Perrott et al., unpublished data).

In vitro PrP^{CWD} amplification. For nondenaturing experiments, D10 was diluted 1:200 and CSU-1 was diluted 1:40 in PBS plus 1% Triton X-100 to final dilutions of 1:2,000 and 1:200, respectively, relative to whole brain. For amplification, 50 μl of each was mixed with 50 μl of homologous-species NBH. Dilutions of D10 or CSU-1 equivalent to specific multiples of input were frozen, not amplified, for use in quantification (Fig. 1). Amplified samples were incubated at 37°C with continuous shaking in a Thermomixer R (Invitrogen). Afterwards, all samples were digested for 1 h at 37°C with 50 μg/ml (deer tissues) or 30 μg/ml (ferret tissues) proteinase K (PK;

* Corresponding author. Mailing address: Department of Microbiology, Immunology, and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523. Phone: (970) 491-7587. Fax: (970) 491-0523. E-mail: edward.hoover@colostate.edu.

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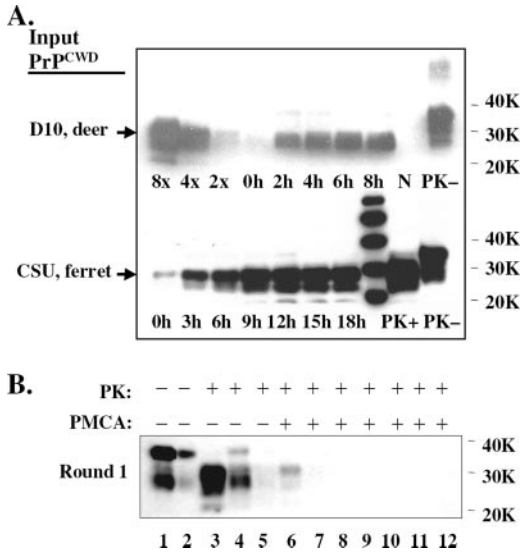

FIG. 1. Deer and ferret NBH support amplification. (A) (Top panel) Deer NBH was used to amplify PrP^{CWD} from an infected mule deer (D10). (Bottom panel) Ferret NBH was used to amplify PrP^{CWD} from CSU-1, a ferret-adapted CWD strain. 8×, 4×, and 2×, dilutions of D10 NBH equivalent to eight-, four-, and twofold more material, respectively, than the input, represented by "0 h"; N, NBH subjected to the amplification protocol and digested with PK; PK+ and PK−, positive brain either digested or not digested with PK, respectively. (B) PMCA applied to amplify PrP^{CWD} in deer NBH. Lanes 1 and 2, undigested NBH diluted 1:250 and 1:500, respectively, after sonication/incubation; lanes 3 to 5, serial 1:3 dilutions of D10 in NBH, starting at 1:1,000 relative to whole brain, not amplified; lanes 6 to 11, continuing serial 1:3 dilutions of D10 in NBH, from 1:27,000 to 1:6,561,000, after 48 h of PMCA; lane 12, NBH subjected to sonication/incubation, followed by digestion with PK.

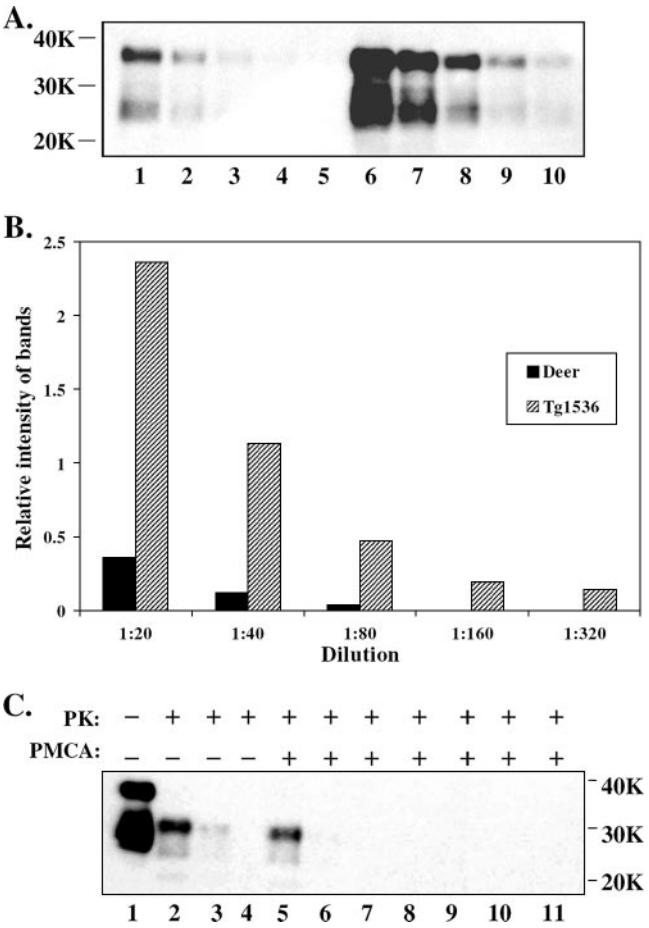

FIG. 2. Semiquantitative estimation of PrP^{C} in brains of deer versus Tg(cerPrP)1536 mice and effect of PrP^{C} concentration on PMCA. (A) Serial 1:2 dilutions of NBH were analyzed by Western blotting. Lanes 1 to 5, deer NBH; lanes 6 to 10, Tg(cerPrP)1536 NBH. Molecular weights, in thousands (K), are indicated on the left. (B) Western blot bands, the size and density of which correspond to the amount of PrP^{C}, were quantified and plotted for comparison. (C) Tg(cerPrP)1536 NBH was diluted 1:4 (lanes 5 to 7 and 11) or 1:8 (lanes 8 to 10) in a $PrP^{0/0}$ brain for PMCA. Lane 1, undigested NBH diluted 1:250; lanes 2 to 4, serial 1:3 dilutions of D10 in NBH, starting at 1:1,000 relative to whole brain, not amplified; lanes 5 to 7 and 8 to 10, D10 diluted 1:27,000, 1:81,000, and 1:243,000, respectively, after PMCA; lane 11, NBH subjected to sonication/incubation, followed by digestion with PK.

Invitrogen), and 40 μl of each sample was boiled with 15 μl lithium dodecyl sulfate (Invitrogen). For PMCA, D10 was diluted to a final concentration of 1:1,000 in NBH, with serial 1:3 dilutions in NBH to a final dilution of 1:6,561,000. Sixty microliters of each dilution was incubated at 37°C in a Misonix (Farmingdale, NY) sonicator 3000 containing 160 ml water, programmed for 96 cycles of a 40-s pulse (at power level 7) plus 30 min of incubation. After 48 h of sonication/incubation (one round), 8.25 μl of each sample was brought to 0.875% sodium dodecyl sulfate and digested with 150 μg/ml PK at 37°C for 20 min and at 45°C for 10 min. All samples (final volume, 15 μl) were then boiled with 5 μl lithium dodecyl sulfate.

Electrophoresis and immunoblotting. For nondenaturing experiments, samples were loaded onto 4 to 12% gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore) using Bio-Rad equipment, and the membranes were blocked using a solution of 6% milk powder (Carnation) for >1 h. Membranes were then incubated for 1 h in 2 μg/ml monoclonal antibody Bar224 (a generous gift from Jacques Grassi, CEA/Saclay, France) diluted 1:10,000 in the blocking solution described above. Membranes were rinsed in Tris-buffered saline plus 0.2% Tween 20 and were incubated for 45 min in horseradish peroxidase-labeled goat anti-mouse immunoglobulin G as a secondary antibody (Jackson Laboratories) diluted 1:20,000 in blocking solution. They were rinsed again before immersion in ECL-plus chemiluminescent reagents (Amersham), exposure to BioMax film (Kodak, Rochester, NY), and development using a Mini Medical/90 film processor (AFP Imaging). Only films clearly below saturation level were used for quantification in Adobe Photoshop. For PMCA experiments, samples (processed as described above) were loaded onto 12% gels; transfer and blocking steps were identical to those described above. Membranes were incubated for >2 h with Bar224 that had been conjugated directly to horseradish peroxidase and diluted 1:20,000 in blocking solution. Membranes were then washed 30 times in distilled H_2O plus 0.2% Tween 20, for 5 min each time, before the application of ECL-plus. Membranes from PMCA experiments were analyzed using a digital Gel-Doc system (Fujifilm) with automated

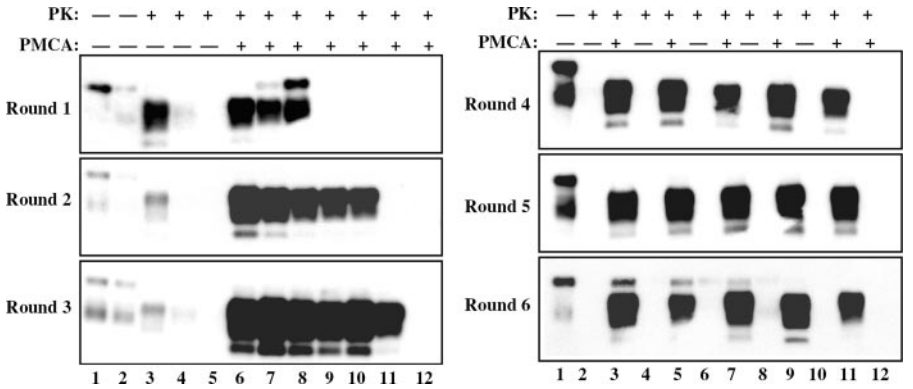

FIG. 3. Amplification of PrP^{CWD} in Tg(cerPrP)1536 mouse NBH by PMCA. (Left panels) Rounds 1 to 3. Lanes 1 and 2, undigested NBH diluted 1:250 and 1:500, respectively, after sonication/incubation; lanes 3 to 5, serial 1:3 dilutions of D10 in NBH, starting at 1:1,000 relative to whole brain, not amplified; lanes 6 to 11, continuing serial 1:3 dilutions of D10 in NBH, from 1:27,000 to 1:6,561,000, after 48 h of PMCA; lane 12, unspiked NBH subjected to sonication/incubation and digestion with PK. For rounds 2 and 3, samples from lanes 6 to 11 from the preceding round were diluted 1:10 into fresh NBH and subjected to another 48 h of PMCA, followed by immunoblotting. (Right panels) Rounds 4 to 6. Lane 1, undigested NBH diluted 1:250 after sonication/incubation; lanes 2 to 11, each sample either amplified by PMCA or not, as indicated; lane 12, NBH subjected to sonication/incubation, followed by digestion with PK. For round 4, material from lanes 7 to 11 from the previous round was diluted 1:100 into fresh Tg(cerPrP)1536 mouse NBH and subjected to PMCA. For rounds 5 to 6, material from lanes 3, 5, 7, 9, and 11 was diluted 1:100 in fresh Tg(cerPrP)1536 mouse NBH and subjected to PMCA.

detection of saturation limits; bands were quantified using ImageGauge (Fujifilm).

Brain substrates from deer and ferrets support noncyclic amplification of PrP^{CWD}. To determine whether deer brain homogenates could support PrP^{CWD} amplification, we mixed 10% (wt/vol) NBH from uninfected white-tailed deer with an equal volume of a diluted brain homogenate from a CWD-infected mule deer (D10) shown to be infectious to both Tg(cerPrP)1536 mice and white-tailed deer in vivo (3, 10). At specified times during amplification, samples were removed and frozen for subsequent analysis by Western blotting. This nondenaturing protocol consistently produced ~3-fold PrP^{CWD} amplification by 8 h (Fig. 1A, top panel). Interestingly, most of the conversion activity occurred within the first 2 to 4 h of incubation.

As part of ongoing CWD species barrier studies, we have developed ferrets as an alternate animal model of CWD infection (C. J. Sigurdson et al., unpublished data; Perrott et al., unpublished), extending the work of Bartz et al. (1), who first reported ferret susceptibility to CWD. To expand this work to in vitro amplification, we spiked CWD-infected ferret brains into NBH made from uninfected normal ferret brain; this resulted in consistent ~5- to 10-fold PrP^{CWD} amplification over a 12-h period (Fig. 1A, bottom panel). This result was notable in achieving higher amplification efficiency than was obtained with deer NBH and in demonstrating amplification of PrP^{CWD} in a CWD-susceptible noncervid species.

Cyclic amplification of PrP^{CWD} in deer and Tg(cerPrP)1536 mouse brains. To increase the amount of PrP^{CWD} generated by in vitro amplification, we next applied the PMCA protocol to amplify PrP^{CWD} from a mule deer. White-tailed deer NBH was spiked with the CWD-positive D10 deer brain homogenate, the same infectious PrP^{CWD} source that was used in the nondenaturing experiments described above. After incubation with intermittent sonication, detection of PrP^{CWD} by Western blotting revealed a final amplification yield of ~6- to 27-fold, as calculated by the band intensity relative to nonamplified, starting dilutions (Fig. 1B). Serial amplification, in which amplified material is diluted into fresh NBH and subjected to additional cycles of PMCA to increase the overall yield, was unsuccessful using deer NBH, presumably due to the sporadic, low amplification yields (i.e., ≤10-fold) of these experiments.

To improve upon these results, we prepared NBH from pooled whole brains of Tg(cerPrP)1536 mice (3), animals in which the amino acid sequence of PrP^{C} is identical to that in white-tailed deer. Semiquantitative estimation of PrP^{C} levels in Tg(cerPrP)1536 mouse brain versus deer brain homogenates by Western blot analysis revealed ~5- to 8-fold greater PrP^{C} expression in the brain tissues of these mice (Fig. 2A and B). When Tg(cerPrP)1536 NBH was diluted 1:4 into $PrP^{0/0}$ brain (4), to approximate the PrP^{C} concentration in deer, the amplification yield (Fig. 2C) was very similar to that obtained using deer NBH (Fig. 1B). As might be anticipated, when Tg(cerPrP)1536 mouse NBH was diluted 1:8 into $PrP^{0/0}$ brain, no amplification was detected (Fig. 2C).

Following these results, we substituted Tg(cerPrP)1536 mouse NBH for deer NBH entirely, and the PMCA yield increased ~20 times, to more than 200-fold per round (Fig. 3, round 1). Moreover, serial PMCA, diluting amplified material into fresh Tg(cerPrP)1536 NBH for each successive round, resulted in a yield of $>6.56 \times 10^{9}$-fold after just six rounds (Fig. 3, round 6). Theoretically, serial PMCA attaining ~200-fold increases at each round would result in a $\sim 6.4 \times 10^{13}$-fold total increase after six rounds. To maintain characteristic Western blot PrP^{CWD} signals, we diluted samples less than 200-fold at each round (Fig. 3), resulting in a slightly lower final yield. More importantly, the Tg(cerPrP)1536 NBH served as a very efficient substrate for PrP^{CWD} amplification relative to deer NBH, likely reflecting the higher expression of PrP^{C} relative to that in deer.

Previously, Raymond and colleagues (18) observed highly efficient conversion of white-tailed deer PrP^{C} by using mule deer-derived PrP^{CWD} as a spike source in a cell-free conversion system employing radiolabeled PrP^{C} as a substrate. Therefore, we examined further the premise that in PMCA, the more

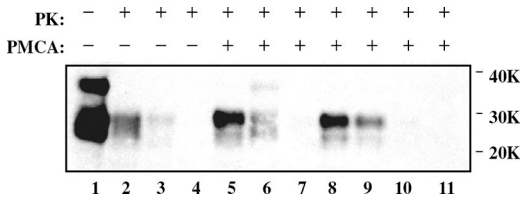

FIG. 4. A deer brain homogenate does not inhibit PMCA in a Tg(cerPrP)1536 NBH. A Tg(cerPrP)1536 NBH was diluted 50:50 with either deer (lanes 5 to 7 and 11) or $PrP^{0/0}$ (lanes 8 to 10) NBH for PMCA. Lane 1, undigested NBH diluted 1:250; lanes 2 to 4, serial 1:3 dilutions of D10 in NBH, starting at 1:1,000 relative to whole brain, not amplified; lanes 5 to 7 and 8 to 10, D10 diluted 1:27,000, 1:81,000, and 1:243,000, respectively, after PMCA; lane 11, NBH subjected to sonication/incubation followed by digestion with PK.

efficient amplification in Tg(cerPrP)1536 mouse versus deer NBH was due principally to the substrate PrP^C concentration as opposed to other factors such as higher levels of conversion-enhancing cofactors (e.g., single-stranded RNA [7] or heparan sulfate proteoglycan [23]) in mouse brains. We added increasing concentrations of $PrP^{0/0}$ mouse NBH to the deer NBH; this did not improve the amplification yield (data not shown). We then evaluated the addition of deer NBH to Tg(cerPrP)1536 NBH; this did not inhibit amplification in comparison to addition of an equivalent amount of a $PrP^{0/0}$ mouse brain, making the presence of inhibitory factors in deer brains less likely (Fig. 4). The low amplification efficiency obtained by using deer NBH is unlikely to be due to a species barrier between mule deer (our PrP^{CWD} source) and white-tailed deer (our NBH source). These two cervid species have identical PrP^C amino acid sequences and brain PrP glycosylation patterns, variables that have been shown to affect in vitro conversion (13, 16). The presence of serine versus glycine at PrP^C position 96 in white-tailed deer has been associated with greater resistance to CWD in white-tailed deer and transgenic mice (11, 14). All white-tailed deer used for NBH in these studies were of the more susceptible 96G PrP genotype. Thus, efficient in vitro amplification of PrP^{CWD} by PMCA appears to reflect the higher concentration of PrP^C in Tg(cerPrP)1536 mouse brains versus deer brains.

In summary, we report efficient amplification of PrP^{CWD} by using brain substrates from Tg(cerPrP)1536 mice. The magnitude of PrP^{CWD} conversion obtained by serial PMCA may make possible in vitro detection of PrP^{CWD} in the body fluids and excreta of infected animals. These studies have been initiated.

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