

# Assessment of the PrP<sup>c</sup> Amino-Terminal Domain in Prion Species Barriers

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## ABSTRACT

Chronic wasting disease (CWD) in cervids and bovine spongiform encephalopathy (BSE) in cattle are prion diseases that are caused by the same protein-misfolding mechanism, but they appear to pose different risks to humans. We are interested in understanding the differences between the species barriers of CWD and BSE. We used real-time, quaking-induced conversion (RT-QuIC) to model the central molecular event in prion disease, the templated misfolding of the normal prion protein, PrP<sup>c</sup>, to a pathogenic, amyloid isoform, scrapie prion protein, PrP<sup>Sc</sup>. We examined the role of the PrP<sup>c</sup> amino-terminal domain (N-terminal domain [NTD], amino acids [aa] 23 to 90) in cross-species conversion by comparing the conversion efficiency of various prion seeds in either full-length (aa 23 to 231) or truncated (aa 90 to 231) PrP<sup>c</sup>. We demonstrate that the presence of white-tailed deer and bovine NTDs hindered seeded conversion of PrP<sup>c</sup>, but human and bank vole NTDs did the opposite. Additionally, full-length human and bank vole PrP<sup>c</sup>s were more likely to be converted to amyloid by CWD prions than were their truncated forms. A chimera with replacement of the human NTD by the bovine NTD resembled human PrP<sup>c</sup>. The requirement for an NTD, but not for the specific human sequence, suggests that the NTD interacts with other regions of the human PrP<sup>c</sup> to increase promiscuity. These data contribute to the evidence that, in addition to primary sequence, prion species barriers are controlled by interactions of the substrate NTD with the rest of the substrate PrP<sup>c</sup> molecule.

## IMPORTANCE

We demonstrate that the amino-terminal domain of the normal prion protein, PrP<sup>c</sup>, hinders seeded conversion of bovine and white-tailed deer PrP<sup>c</sup>s to the prion forms, but it facilitates conversion of the human and bank vole PrP<sup>c</sup>s to the prion forms. Additionally, we demonstrate that the amino-terminal domain of human and bank vole PrP<sup>c</sup>s requires interaction with the rest of the molecule to facilitate conversion by CWD prions. These data suggest that interactions of the amino-terminal domain with the rest of the PrP<sup>c</sup> molecule play an important role in the susceptibility of humans to CWD prions.

Chronic wasting disease (CWD), bovine spongiform encephalopathy (BSE; mad cow disease) and Creutzfeldt-Jakob disease (CJD) are prion diseases of cervids, cattle, and humans, respectively. These neurodegenerative diseases are caused by the pathogenic misfolding of the normal prion protein (PrP<sup>c</sup>) to an amyloid conformation (scrapie prion protein, PrP<sup>Sc</sup>), the accumulation of which causes neuronal death (1, 2). CWD was first identified in the western United States in the 1960s in captive mule deer (*Odocoileus hemionus hemionus*) (3) and has since spread into free-ranging and farmed cervid populations in 24 states and in Norway, the Republic of Korea, and two Canadian provinces (4). BSE was identified in the United Kingdom in the 1980s and is hypothesized to have been spread as a result of feeding animal by-products to cattle (5, 6). The BSE epidemic led to the culling of over 4.5 million cattle, and over 200 people died from variant CJD, a form of CJD thought to be acquired from consumption of BSE-tainted beef (7–12). So far, there is no evidence of transmission of CWD to humans, but the BSE epidemic suggests that prions have zoonotic potential.

Efforts to define the host range of both BSE and CWD have included experimental infections of nonhost species. CWD inoculations have resulted in prion propagation in cattle, sheep, hamsters, voles, felines, ferrets, and some nonhuman primates (13–20). BSE inoculations have resulted in prion propagation in deer, sheep, and some nonhuman primates (21–26). Moreover, natural BSE infections have occurred in felines and several species of zoo-

ungulates, in addition to humans (9, 11, 12). Transgenic mice expressing human PrP<sup>c</sup> (TgHu) have been used to model human susceptibility to animal prion diseases. Published reports of CWD inoculation of TgHu mice have reported no prion propagation although studies employing protein misfolding cyclic amplification (PMCA) have yielded more ambiguous results (27). Prion propagation has been reported in approximately 50% of TgHu mice inoculated with BSE (28–35). Despite these reports, prion species barriers remain mostly empirical. While the most important known factor in transspecies transmission is the compatibility of the PrP<sup>c</sup> amino acid sequences, there is evidence that the tertiary and quaternary structures of PrP<sup>Sc</sup> also influence prion transmission (36–39).

Multiple studies have identified specific PrP<sup>c</sup> regions that may control susceptibility or resistance to conversion by nonhomologous prions, but the role of the amino-terminal domain (N-ter-

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terminal domain [NTD], which we define as amino acids [aa] 23 to 90, according to mouse numbering) in species barrier maintenance has not been tested (40, 41). Transgenic mice with an amino-terminal PrP<sup>c</sup> truncation that resulted in expression of only aa 90 to 231 had no overt changes in phenotype compared to that of wild-type mice, while larger truncations caused spontaneous neurodegenerative disease (42). A transgenic mouse expressing chimeric mouse-hamster PrP<sup>c</sup> (aa 90 to 231) had reduced susceptibility to prion infection, which varied with the strain of mouse-adapted scrapie used for infection (43). Mice that expressed mouse aa 90 to 231 propagated prions upon inoculation but had delayed disease onset and lower PrP<sup>Sc</sup> titers (44). Mice expressing mouse aa 88 to 231 had reduced susceptibility to infection compared with mice expressing full-length PrP<sup>c</sup>, but disease onset was accelerated when the inoculum consisted of PrP<sup>Sc</sup> with the same truncation (45, 46). Overall, these results suggest that amino acids 23 to 90 are involved in prion propagation and disease progression.

Evidence from *in vitro* studies indicates that the amino-terminal fragment of the protein (amino acids 23 to 144) will misfold easily without addition of a prion seed and that the human fragment converted faster than either the hamster or mouse fragment (47). Other investigators determined that the aggregation mechanism and resulting aggregate morphology were heavily dependent on physical conditions (pH) and truncation of the protein (aa 90 to 231 versus the full-length protein) (48, 49). These *in vitro* data indicate that the absence of aa 23 to 90 has dramatic effects on the mechanism and on the likelihood of misfolding. We were interested in the region from aa 23 to 90 not only for its effect on amyloidogenicity but also for its role in preventing (or facilitating) templated misfolding between species.

We chose to compare full-length recombinant PrP<sup>c</sup> (rPrP<sup>c</sup>; aa 23 to 231) to truncated rPrP<sup>c</sup> comprised of aa 90 to 231 for several reasons: (i) the PrP fragment of aa 27 to 30, an important molecule identified early in prion disease research, is comprised of amino acids 90 to 231 (50); (ii) amino acids 23 to 90 comprise the N2 cleavage product in the disease state (51); and (iii) the fragment of aa 90 to 231 is commonly used as the substrate for prion detection in real-time, quaking-induced conversion (RT-QuIC) assays (52). Specifically, we hypothesized that the amino terminal domain (amino acids 23 to 90; NTD) decreases an rPrP<sup>c</sup> molecule's propensity to form amyloid and that it plays a role in defining *in vitro* species barriers. We have assessed species barriers using real-time, quaking-induced conversion (i) because it makes possible assessment of conversion efficiency (by detection of amyloid formation in real time) and (ii) because we can study the effects of changes to the PrP<sup>c</sup> primary structure without the complexity of a transgenic mouse or cell culture system.

## MATERIALS AND METHODS

**Recombinant PrP cloning and expression.** The coding regions for the bank vole and bovine prion proteins (*PRNP* genes) were kindly provided by Glenn Telling and Jifeng Bian, and we obtained cDNA for the white-tailed deer (WTD), human, and chimeric *PRNP* genes from Genewiz, Inc. We cloned the sequences for the full-length PrP (amino acids 23 to 231) into the pet100D expression system (Life Technologies). We cloned the sequences for the truncated PrP (amino acids 90 to 231) into a vector kindly provided by Byron Caughey. We transformed and amplified the plasmids in *Escherichia coli* Top 10 cells (Life Technologies) and then stored the plasmids in *E. coli* BL21 Star cells (Life Technologies) in glycerol stocks at  $-80^{\circ}\text{C}$ . To express PrP, we added BL21 cells from the glycerol stock to 5 ml of LB medium and antibiotics (5  $\mu\text{g}/\text{ml}$  ampicillin for full-

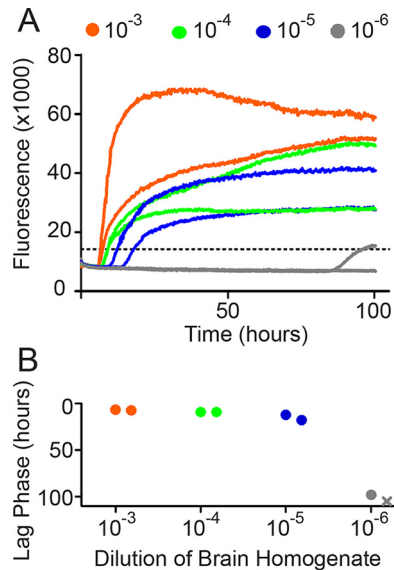
length constructs and 25  $\mu\text{g}/\text{ml}$  kanamycin for truncated constructs) and grew the culture overnight, with shaking at  $37^{\circ}$ . We used the 5-ml culture to inoculate 1 liter of LB medium plus antibiotics as described above. We added auto-induction reagents for a final concentration of 0.5 M  $(\text{NH}_4)_2\text{SO}_4$ , 1 M  $\text{KH}_2\text{PO}_4$ , 1 M  $\text{Na}_2\text{HPO}_4$ , 0.5% glycerol, 0.05% glucose, 0.2%  $\alpha$ -lactose, and 0.001 M  $\text{MgSO}_4$ . We harvested the bacteria when the optical density at 600 nm ( $\text{OD}_{600}$ ) reached approximately 3.0 for full-length constructs and 1.7 for truncated constructs. We either froze the cell pellets at  $-80^{\circ}\text{C}$  for up to 5 days or immediately lysed the cells and harvested inclusion bodies, which we isolated according to the manufacturer's protocol with Bug Buster and Lysonase (EMD-Millipore).

**Recombinant PrP purification.** We solubilized inclusion bodies in 8 M guanidine hydrochloride (GdnHCl) and 100 mM  $\text{Na}_2\text{HPO}_4$  and rotated the solution overnight at room temperature. We bound the denatured rPrP to Superflow nickel resin (Qiagen) and refolded the rPrP<sup>c</sup> at a rate of 0.75 ml/min on the column in buffer with a gradient from 6.0 M GdnHCl, 100 mM  $\text{Na}_2\text{HPO}_4$ , and 10 mM Tris (pH 8.0) to the same buffer with no GdnHCl (wash buffer). We used a gradient from the wash buffer to 1.0 M imidazole, 100 mM  $\text{NaH}_2\text{PO}_4$ , and 10 mM Tris (pH 5.5) with a flow rate of 2.0 ml/min to elute full-length PrP. For truncated PrP, the final gradient ended with 0.5 M imidazole. We filtered and dialyzed the eluted rPrP at a concentration of  $\sim 0.4$  mg/ml in two changes of 4.0 liters of 20 mM  $\text{NaH}_2\text{PO}_4$  (pH 5.5). After dialysis, we filtered and stored the rPrP at  $4^{\circ}\text{C}$ , its concentration having been determined by the  $A_{280}$  and Beer's law. We confirmed the purity of the rPrP samples by gel electrophoresis (12% bis-Tris gels with  $1\times$  morpholinepropanesulfonic acid [MOPS; Bio-Rad], 190 V, 55 min) and staining with Coomassie brilliant blue (Bio-Rad).

**Preparation of prion seed material.** We homogenized brain tissue from deer, cattle, or humans at 10% weight/volume in  $1\times$  phosphate-buffered saline (PBS) using a FastPrep bead beater (MB Biomedicals). Single-use aliquots of 10% homogenate were stored at  $-80^{\circ}\text{C}$ . Both prion-positive and prion-negative brain materials were prepared. Prion-positive brain material was used for the experiments, while prion-negative brain was used for the unseeded controls. The BSE sample was classical BSE (cBSE), and the sporadic CJD (sCJD) sample was MM1 (type 1).

**Real-time, quaking-induced conversion (RT-QuIC) for full-length rPrP.** We combined 0.1 mg/ml rPrP with a premixed reaction solution that contained a final concentration of 400 mM NaCl, 1 mM EDTA, and 20 mM  $\text{NaH}_2\text{PO}_4$ . Then, we added freshly made thioflavin T (ThT; final concentration, 10  $\mu\text{M}$ ) for a final volume of 98  $\mu\text{l}$  per well in a 96-well plate. We thawed aliquots of 10% brain homogenate and serially diluted the samples into  $1\times$  PBS plus 0.1% SDS. We added 2  $\mu\text{l}$  of diluted brain seed to 98  $\mu\text{l}$  of protein substrate mix in the well of an optical-bottom, 96-well plate. An RT-QuIC experiment consisted of 400 cycles (100 h) of shaking and incubation at  $45^{\circ}\text{C}$ ; specifically, plates were shaken for 1 min (700 rpm, double orbital) followed by 1 min of rest. Fluorescence (450-nm excitation and 480-nm emission; 20 flashes/well) was recorded every 15 min using a gain setting of 1700 in a FLUOstar microplate reader (BMG Labtech). To compare full-length PrP directly to truncated PrP with the same molarity (0.6 nM), we increased the concentration of full-length rPrP to 0.14 mg/ml and that of the truncated rPrP to 0.10 mg/ml (see Fig. 2). These conditions were optimized for use with full-length rPrP. For the experiments shown in Fig. 4, conditions were as follows: 0.6 nM rPrP, 320 mM NaCl, and 0.1% SDS at  $42^{\circ}\text{C}$ .

**RT-QuIC for truncated PrP.** We combined 0.07 mg/ml rPrP with a premixed reaction solution that contained a final concentration of 320 mM NaCl, 1 mM EDTA, and 20 mM  $\text{NaH}_2\text{PO}_4$ . We added freshly made thioflavin T (ThT; final concentration, 10  $\mu\text{M}$ ) for a final volume of 98  $\mu\text{l}$  per well in a 96-well plate. We thawed and serially diluted aliquots of 10% brain homogenate into  $1\times$  PBS plus 0.05% SDS. We added 2  $\mu\text{l}$  of diluted brain seed to 98  $\mu\text{l}$  of protein substrate mix in the well of an optical-bottom, 96-well plate. An RT-QuIC experiment consisted of at least 250 cycles (62.5 h) of shaking and incubation at  $42^{\circ}\text{C}$ ; specifically, plates were shaken for 1 min (700 rpm, double orbital), followed by 1 min of rest.



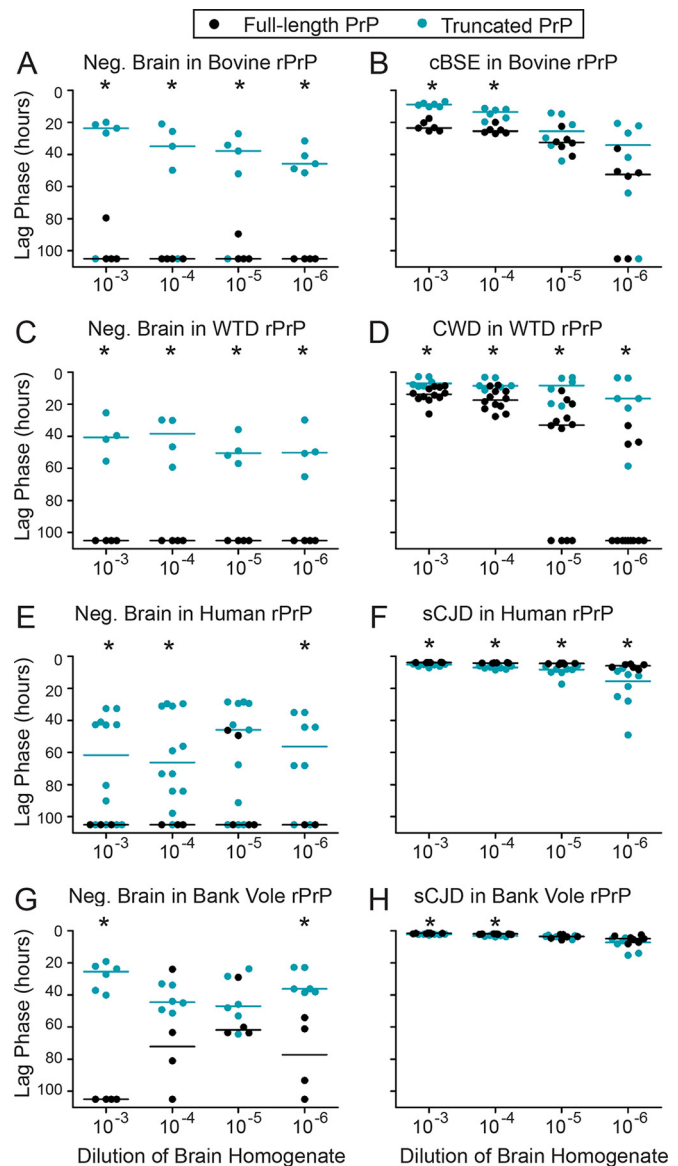
**FIG 1** Data analysis workflow. (A) Raw data are recorded as fluorescence every 15 min. We show two technical replicates for this example. (B) We plot the time required to reach the threshold ( $+5$  SD above the mean background) versus the dilution of 10% brain homogenate added to the reaction mixture. The X indicates an estimated value for a sample that did not cross the threshold during the experiment. We have reversed the  $y$  axis to make it easier to identify the fastest conversions as the highest points.

Fluorescence (450-nm excitation and 480-nm emission; 20 flashes/well) was recorded every 15 min using a gain setting of 1700 in a FLUOstar microplate reader (BMG Labtech). These conditions were optimized for use with truncated rPrP.

**Data analysis and statistics.** The RT-QuIC readout is fluorescence, which was recorded at 15-min intervals (Fig. 1A). To assess the efficiency of reactions, we compared the lag phase for amyloid formation. First, we calculated a fluorescence threshold by averaging the baseline fluorescence for every well in the plate and then adding 5 standard deviations (SD). We used Omega software (BMG Labtech) to determine the lag phase, i.e., the time at which the fluorescence in a given well exceeded the threshold (Fig. 1B). If the fluorescence for a sample never crossed the threshold during the experiment, we estimated a value of 105 h (an example is marked by a gray X in Fig. 1B). Raw lag phase data are displayed as scatterplots with lines to indicate the medians. We analyzed the lag phase data using non-parametric, rank-based tests (Wilcoxon-Mann-Whitney [WMW] and Wilcoxon signed-rank tests). If all values for one sample were equal, WMW tests were invalid, so we used Wilcoxon signed-rank tests instead. We chose these tests for several reasons: (i) our data are often nonnormal (right skewed due to replicate wells that do not cross the threshold in the course of the experiment); (ii) our number of replicates is relatively small (8 to 12); and (iii) we must choose values to substitute for the replicate wells that do not cross the threshold. The choice of a substitute value would affect the mean (and the results of any statistical tests that relies on means, like  $t$  tests), but would not affect rank-based statistical analysis since those values will have the longest lag phase regardless of which value we choose. We chose to represent the median instead of mean for the same reason: the median is not affected by our choice of a value for wells that do not cross the threshold. We described differences as statistically significant at a  $P$  value of  $<0.05$ , which is indicated in the figures.

## RESULTS

**Removal of the amino-terminal domain affects efficiency of seeded conversion and increases spontaneous conversion of rPrP<sup>C</sup>.** To investigate the effect of the amino-terminal domain



**FIG 2** Removal of the amino-terminal domain affects rate of seeded conversion and increases spontaneous conversion of rPrP. Brain homogenate (prion positive or negative) was added to the homologous substrate, in either its full-length or truncated version. Reaction conditions were designed for increased amyloidogenicity and were the same for all experiments shown in this figure (RT-QuIC conditions for full-length PrP are as described in Materials and Methods). Scatterplots represent raw data, and lines indicate the medians. \*,  $P < 0.05$  (WMW test), for the difference between the lag phases for full-length and truncated substrates. (A) Truncated bovine rPrP spontaneously formed amyloid faster than full-length bovine rPrP (in the presence of normal brain homogenate). (B) cBSE seeded truncated bovine rPrP faster than full-length. (C) Truncated white-tailed deer rPrP spontaneously formed amyloid faster than full-length white-tailed deer rPrP (in the presence of normal brain homogenate). (D) CWD seeded truncated white-tailed deer rPrP faster than full-length. (E) Truncated human rPrP spontaneously formed amyloid faster than full-length human rPrP (in the presence of normal brain homogenate). (F) sCJD seeded full-length human rPrP faster than truncated. (G) Truncated bank vole deer rPrP spontaneously formed amyloid faster than full-length bank vole rPrP (in the presence of normal brain homogenate). (H) sCJD seeded full-length bank vole rPrP faster than truncated.

N-Bo-Hu-C Chimera	KKRPKP <b>GGG</b> WNTGGSRYPGQGS <b>PGGN</b> RYPPQGGGGW <b>QPHGGG</b> W <b>QPHGGG</b> W <b>QPHGGG</b> W
Bovine	KKRPKP <b>GGG</b> WNTGGSRYPGQGS <b>PGGN</b> RYPPQGGGGW <b>QPHGGG</b> W <b>QPHGGG</b> W <b>QPHGGG</b> W
N-Hu-Bo-C Chimera	KKRPKP-GGWNTGGSRYPGQGS <b>PGGN</b> RYPPQGGGGW <b>QPHGGG</b> W <b>QPHGGG</b> W <b>QPHGGG</b> W
Bank Vole	KKRPKP-GGWNTGGSRYPGQGS <b>PGGN</b> RYPPQGGGG <b>T</b> W <b>QPHGGG</b> W <b>QPHGGG</b> W <b>QPHGGG</b> W
Human	KKRPKP-GGWNTGGSRYPGQGS <b>PGGN</b> RYPPQGGGGW <b>QPHGGG</b> W <b>QPHGGG</b> W <b>QPHGGG</b> W
	*****
N-Bo-Hu-C Chimera	GQPHGGG <b>WQPHGGG</b> W <b>GG</b> THSQWNKPSKPKTNMKHMAGAAAAGAVVGGGLGGYMLGSA
Bovine	GQPHGGG <b>WQPHGGG</b> W <b>GG</b> THSQWNKPSKPKTNMKHVAGAAAAGAVVGGGLGGYMLGSA
N-Hu-Bo-C Chimera	GQPHGGG <b>WQPHGGG</b> W <b>GG</b> THSQWNKPSKPKTNMKHVAGAAAAGAVVGGGLGGYMLGSA
Bank Vole	GQPHGGG <b>WQ</b> -----GGTHNQWNKPSKPKTNIKHVAGAAAAGAVVGGGLGGYMLGSA
Human	GQPHGGG <b>WQ</b> -----GGTHSQWNKPSKPKTNMKHMAGAAAAGAVVGGGLGGYVLGSA
	*****
N-Bo-Hu-C Chimera	MSRPLI <b>HFG</b> SDYEDRYREN <b>MHRY</b> PNQVY <b>YR</b> PMDEYS <b>NQ</b> NNFVHDCVNITIKQHTVTTTT
Bovine	MSRPLI <b>HFG</b> SDYEDRYREN <b>MHRY</b> PNQVY <b>YR</b> PVDQY <b>S</b> NQNNFVHDCVNITVKEHTVTTTT
N-Hu-Bo-C Chimera	MSRPLI <b>HFG</b> SDYEDRYREN <b>MHRY</b> PNQVY <b>YR</b> PVDQY <b>S</b> NQNNFVHDCVNITVKEHTVTTTT
Bank Vole	MSRPMI <b>HFG</b> NDWEDRYREN <b>MNRY</b> PNQVY <b>YR</b> PVDQY <b>Y</b> NNQNNFVHDCVNITIKQHTVTTTT
Human	MSRPLI <b>HFG</b> SDYEDRYREN <b>MHRY</b> PNQVY <b>YR</b> PMDEYS <b>NQ</b> NNFVHDCVNITIKQHTVTTTT
	****:****.*:*****.*****:*.*:*****:*.*****
N-Bo-Hu-C Chimera	KGENFTETDV <b>KMM</b> ERVVEQMCITQYERESQAYYQ <b>RGS</b>
Bovine	KGENFTETDI <b>KMM</b> ERVVEQMCITQYQRESQAYYQ <b>RGA</b>
N-Hu-Bo-C Chimera	KGENFTETDI <b>KMM</b> ERVVEQMCITQYQRESQAYYQ <b>RGA</b>
Bank Vole	KGENFTETDV <b>KMM</b> ERVVEQMCV <b>TQY</b> QKESQAYY <b>EGRS</b>
Human	KGENFTETDV <b>KMM</b> ERVVEQMCITQYERESQAYYQ <b>RGS</b>
	*****:*****:***:*****:

**FIG 3** Primary sequence alignment. The amino acid sequences of bovine, human, bank vole, and the N-Hu-Bo-C and N-Bo-Hu-C chimeras are aligned. Green letters indicate differences between human and bovine NTD sequences, and red letters indicate differences between bank vole and human PrP<sup>c</sup> NTD sequences. The red bars indicate the junctions where human and bovine NTDs meet in the chimeras.

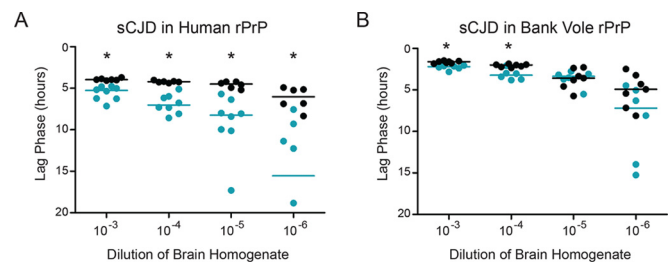
(NTD) on the propensity of rPrP<sup>c</sup> to form amyloid, we added the homologous prion seed (or negative brain material) to each species' rPrP<sup>c</sup>, either truncated or full-length (e.g., CWD to deer PrP). To ensure that any differences in the median amyloid formation lag phase would be due to differences in the rPrP/seed compatibility, not to other reaction conditions, we performed the RT-QuIC reactions with 0.6 nM rPrP, salt, SDS, and EDTA and at the same temperatures (full-length conditions are described in Materials and Methods).

We found for all species that truncated rPrP<sup>c</sup> was more prone to spontaneous conversion (after addition of negative brain material) than the full-length substrate (Fig. 2A, C, E, and G). The addition of cBSE brain to truncated bovine rPrP<sup>c</sup> (aa 90 to 231) resulted in faster amyloid conversion than addition of the same seed to full-length bovine rPrP<sup>c</sup> (Fig. 2B). Likewise, the addition of CWD seed to truncated white-tailed deer rPrP<sup>c</sup> resulted in faster amyloid conversion than addition of the same CWD material to full-length white-tailed deer rPrP<sup>c</sup> (Fig. 2D).

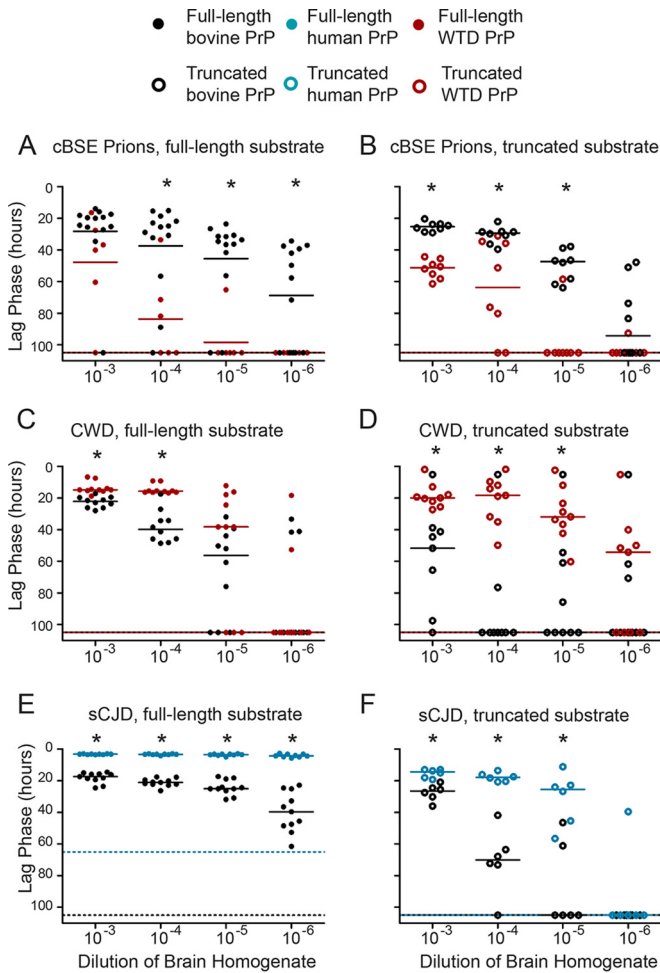
The addition of sporadic CJD (sCJD) MM1 seed to truncated or full-length human rPrP<sup>c</sup> (methionine at position 129) resulted in very fast amyloid conversion. However, conversion of the truncated human rPrP<sup>c</sup> was significantly slower than that of the full-length human rPrP<sup>c</sup> (Fig. 2F). Because the bank vole and human *PRNP* sequences are very similar in the NTD (Fig. 3), we compared truncated to full-length bank vole rPrP<sup>c</sup> as well. As with the human rPrP<sup>c</sup>, both the truncated and full-length bank vole rPrP<sup>c</sup>s formed amyloid very quickly upon addition of sCJD prions (which have been demonstrated to convert bank vole PrP effectively [53, 54]). Also as with the human rPrP<sup>c</sup>, conversion of the full-length bank vole rPrP<sup>c</sup> was statistically faster than that of

truncated rPrP<sup>c</sup> (53, 54) (Fig. 2H). Because the conversions of human and bank vole rPrP<sup>c</sup>s were so fast, the data are also shown on a smaller scale (Fig. 4A and B).

**The amino-terminal domain is not essential for preferential seeding of rPrP species by their native prions.** Once we completed the direct comparisons of truncated rPrP to full-length rPrP, we modified the reaction conditions used for the truncated rPrP to improve specificity. We decreased the salt, SDS, PrP concentration, and temperature to reduce the amyloidogenicity of the



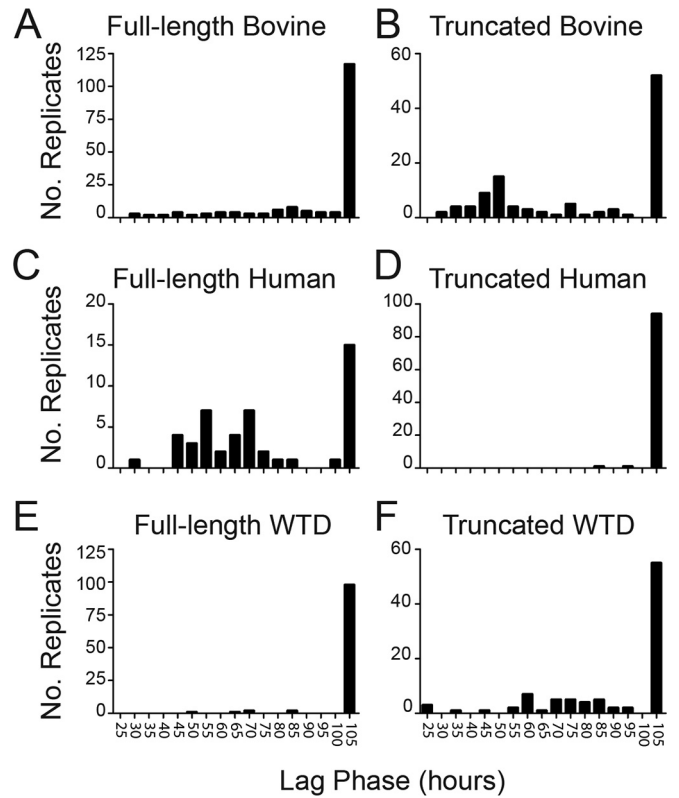
**FIG 4** sCJD converts human and bank vole rPrP<sup>c</sup>s very efficiently. These data are also displayed in Fig. 2, but they are shown here on a smaller y axis, which makes it easier to visualize the differences between the points. As described in the legend of Fig. 2, prion-positive human brain homogenate was added to human rPrP<sup>c</sup> substrate in either its full-length or truncated version (A) and to bank vole rPrP<sup>c</sup> in either its full-length or truncated form (B). Reaction conditions were designed for increased amyloidogenicity and were the same for all experiments shown in this figure (RT-QuIC conditions for full-length PrP are as described in Materials and Methods). Scatterplots represent raw data, and lines indicate the medians. \*,  $P < 0.05$  (WMW test), for the difference between the lag phases for full-length and truncated substrates.



**FIG 5** The amino-terminal domain is not essential for preferential seeding of rPrP species by their native prions. Prion-positive or prion-negative brain homogenate was added to both a homologous rPrP<sup>c</sup> substrate and a nonhost substrate, either truncated or full-length. Full-length rPrP experiments were performed under RT-QuIC conditions for full-length PrP, as described in Materials and Methods. Truncated rPrP experiments were performed under RT-QuIC conditions for truncated PrP, as described in Materials and Methods and unlike the conditions used for the experiments shown in Fig. 2. Scatterplots represent raw data, and lines indicate the medians. Dotted lines indicate the median lag phases for unseeded misfolding for truncated substrates (species are identified by color). \*, *P* < 0.05 (WMW test), for the difference between the lag phases for full-length and truncated substrates. (A) cBSE prions converted full-length bovine rPrP<sup>c</sup> faster than white-tailed deer rPrP<sup>c</sup>. (B) cBSE prions also converted bovine rPrP<sup>c</sup> faster than white-tailed deer rPrP<sup>c</sup> when the substrates were truncated. (C) CWD prions converted white-tailed deer rPrP<sup>c</sup> faster than bovine rPrP<sup>c</sup> when the substrates were full-length. (D) CWD prions converted white-tailed deer rPrP<sup>c</sup> faster than bovine rPrP<sup>c</sup> when the substrates were truncated. (E) sCJD prions converted human rPrP<sup>c</sup> faster than bovine rPrP<sup>c</sup> when the substrates were full-length. (F) sCJD prions converted human rPrP<sup>c</sup> faster than bovine rPrP<sup>c</sup> when the substrates were truncated.

reaction mixture. Experiments shown in subsequent figures were performed under these more specific conditions (details are given in Materials and Methods).

We hypothesized that the NTD may play a role in defining an rPrP<sup>c</sup> molecule as bovine or human or white-tailed deer. For the RT-QuIC assay, we expected a prion seed to cause the most efficient conversion in its host substrate (38), but we hypothesized



**FIG 6** Distribution of unseeded controls. A frequency histogram of the negative controls (negative brain material) is displayed for full-length bovine substrate (A), truncated bovine substrate (B), full-length human substrate (C), truncated human substrate (D), full-length white-tailed deer (WTD) substrate (E), and truncated WTD substrate (F). Data represent the frequency of unseeded controls crossing the threshold for each lag phase.

that this might no longer be the case when the NTD was removed. Therefore, we added each prion of interest (cBSE, CWD, and sCJD) to its homologous substrate and to a nonhomologous substrate. For cBSE, CWD, and sCJD, there was no indication that the NTD is necessary for the prion to preferentially seed its host rPrP<sup>c</sup>. cBSE prions caused amyloid conversion faster in bovine rPrP<sup>c</sup> than in nonhost rPrP<sup>c</sup> (white-tailed deer), whether the rPrP<sup>c</sup> was amino-terminally truncated or full-length (Fig. 5A and B). CWD prions converted white-tailed deer rPrP<sup>c</sup> faster than nonhost rPrP<sup>c</sup> (bovine), whether the rPrP<sup>c</sup> was truncated or full-length (Fig. 5C and D). sCJD prions caused amyloid conversion faster in human rPrP<sup>c</sup> than in the nonhost (bovine) protein, whether or not the rPrP<sup>c</sup> was truncated (Fig. 5E and F). Therefore, the NTD was not essential for prion seeds to most efficiently seed their host rPrP<sup>c</sup>s. The amyloid formation in unseeded controls is indicated by the dotted lines in Fig. 5, and the distribution of these data is displayed in Fig. 6. Additionally, each data set in Fig. 5 was compared to unseeded controls, and the results are shown in Table 1.

**The amino-terminal domain enables CWD conversion of human rPrP<sup>c</sup> *in vitro*.** Above, we showed that homologous prions are better seeds for their native rPrP<sup>c</sup>s, despite truncation (Fig. 5). However, when we assessed the behavior of nonhomologous prions in human rPrP<sup>c</sup>, we observed that the truncated human rPrP<sup>c</sup> is also less permissive to CWD prions than the full-length human rPrP<sup>c</sup> (Fig. 7A).

TABLE 1 Statistical analysis of data in Fig. 5

Prion seed and substrate	Fig. no.	Dilution	P <sup>a</sup>
<b>BSE</b>			
FL bovine	5A	10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	<0.05
		10 <sup>-6</sup>	<0.05
FL WTD		10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	NS
		10 <sup>-6</sup>	NS
Truncated bovine	5B	10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	<0.05
		10 <sup>-6</sup>	NS
Truncated WTD		10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	NS
		10 <sup>-6</sup>	<0.05
<b>CWD</b>			
FL WTD	5C	10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	<0.05
		10 <sup>-6</sup>	NS
FL bovine		10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	<0.05
		10 <sup>-6</sup>	NS
Truncated WTD	5D	10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	<0.05
		10 <sup>-6</sup>	NS
Truncated bovine		10 <sup>-3</sup>	NS
		10 <sup>-4</sup>	NS
		10 <sup>-5</sup>	NS
		10 <sup>-6</sup>	NS
<b>sCJD</b>			
FL human	5E	10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	<0.05
		10 <sup>-6</sup>	<0.05
FL bovine		10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	<0.05
		10 <sup>-6</sup>	<0.05
Truncated human	5F	10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	<0.05
		10 <sup>-5</sup>	<0.05
		10 <sup>-6</sup>	<0.05
Truncated bovine		10 <sup>-3</sup>	<0.05
		10 <sup>-4</sup>	NS
		10 <sup>-5</sup>	NS
		10 <sup>-6</sup>	NS

<sup>a</sup> Difference from the unseeded control value. NS, not significant.

**The amino-terminal domain improves CWD conversion of bank vole rPrP<sup>c</sup> *in vitro*.** Because bank vole and human PrP<sup>c</sup>s have only one amino acid difference in their NTDs (Fig. 3), we hypothesized that the full-length bank vole rPrP<sup>c</sup> might also be more susceptible to conversion by CWD prions than the truncated bank vole rPrP<sup>c</sup>.

We tested the effect of amino-terminal truncation on the promiscuity of the bank vole rPrP<sup>c</sup> (Fig. 7B). Again, CWD caused conversion slightly more efficiently in full-length bank vole PrP<sup>c</sup> than in truncated bank vole PrP<sup>c</sup>.

**The human amino-terminal domain does not increase susceptibility when substituted into another rPrP<sup>c</sup>.** Since the human NTD behaved differently than that of bovine or white-tailed deer in its effect on amyloidogenicity, we hypothesized that the human NTD may confer increased promiscuity to the molecule and that the substitution of the human NTD into bovine rPrP<sup>c</sup> would increase the susceptibility of the chimeric rPrP<sup>c</sup> to CWD prions. To test this, we developed a chimera containing the human NTD and bovine carboxy-terminal domain (N-Hu-Bo-C). cBSE caused PrP<sup>c</sup> conversion with indistinguishable lag phases in the N-Hu-Bo-C chimera and bovine rPrP<sup>c</sup>, which suggests that the NTD substitution does not reduce the homologous seeding of bovine rPrP<sup>c</sup> with cBSE (Fig. 8A). sCJD prions converted the human rPrP<sup>c</sup> faster than the N-Hu-Bo-C chimera, indicating that the human NTD alone was not sufficient to define the rPrP<sup>c</sup> substrate as human (Fig. 8C). Finally, seeding efficiencies with CWD were equivalent in the N-Hu-Bo-C chimera and in bovine rPrP<sup>c</sup>, suggesting that the substitution of the human amino-terminal domain did not increase the propensity of bovine rPrP<sup>c</sup> to be converted by CWD prions (Fig. 8E).

**CWD prions convert human rPrP<sup>c</sup> efficiently, regardless of amino-terminal domain sequence.** Given the above result, we alternatively hypothesized that human rPrP<sup>c</sup> is susceptible to conversion by CWD prions independent of NTD sequence, as long as the NTD is present. We created the N-Bo-Hu-C chimera, which substitutes the bovine NTD for the human NTD, to investigate the importance of the NTD in the CWD species barrier. When we added cBSE prions to the N-Bo-Hu-C chimera, the conversion of the chimera was statistically indistinguishable from that with the full-length human rPrP<sup>c</sup> (Fig. 8B). When we added sCJD prions, the N-Bo-Hu-C chimera likewise was statistically indistinguishable from full-length human rPrP<sup>c</sup> (Fig. 8D). Finally, when we added CWD prions, the chimera was statistically indistinguishable

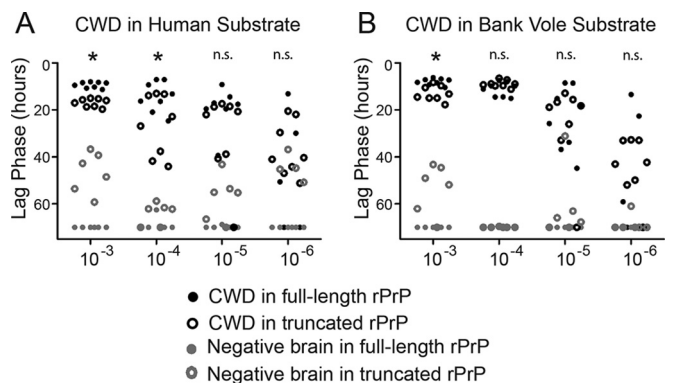
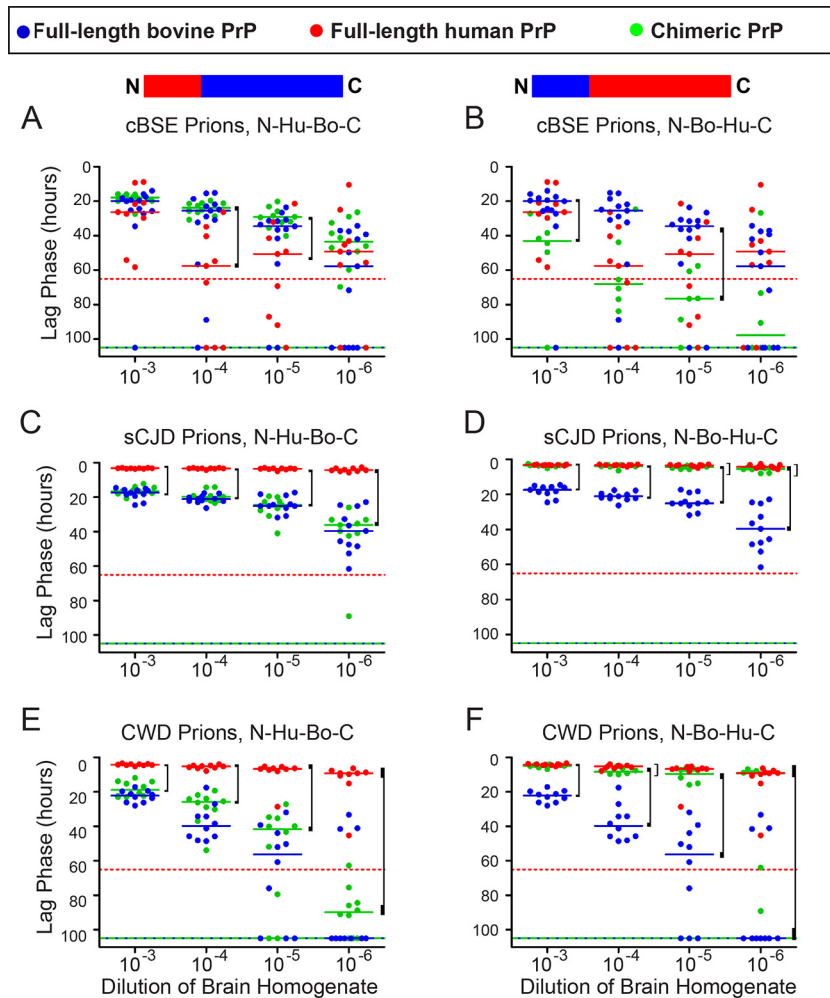


FIG 7 The amino-terminal domain enables CWD conversion of human rPrP<sup>c</sup>. (A) Prion-positive or prion-negative brain homogenate from white-tailed deer was added to truncated or full-length human rPrP<sup>c</sup>. Experiments for both were performed under the same conditions, which are described in Materials and Methods. (B) Prion-positive or prion-negative brain homogenate from white-tailed deer was added to truncated or full-length bank vole rPrP<sup>c</sup>. Scatterplots represent raw data. \*,  $P < 0.05$  (WMW test), for the difference between the CWD susceptibilities of full-length and truncated substrates.



**FIG 8** The human rPrP<sup>c</sup> is susceptible to conversion by CWD prions regardless of its amino-terminal domain sequence. However, the human amino-terminal domain does not confer increased susceptibility to other species' rPrP<sup>c</sup>s. Prion-positive or prion-negative brain homogenate from white-tailed deer (CWD), cattle (cBSE), or humans (sCJD) was added to full-length human, bovine, or chimeric rPrP<sup>c</sup>. These experiments were performed under RT-QuIC conditions for full-length PrP, as described in Materials and Methods. Scatterplots represent raw data, and lines indicate the medians. Dotted lines indicate the median lag phases for unseeded misfolding for truncated substrates (species are identified by color). Brackets indicate a significant difference between either full-length bovine or full-length human and the chimera ( $P < 0.05$ , WMW). (A) cBSE prions convert bovine and N-Hu-Bo-C chimeric rPrP<sup>c</sup>s with the same efficiencies, but human rPrP<sup>c</sup> is faster. (B) cBSE prions convert human and N-Bo-Hu-C chimeric rPrP<sup>c</sup>s with the same efficiencies, while the conversion of bovine rPrP<sup>c</sup> is faster. (C) sCJD prions convert bovine and N-Hu-Bo-C chimeric rPrP<sup>c</sup>s with the same efficiencies, but human rPrP<sup>c</sup> is faster. (D) sCJD prions convert human and N-Bo-Hu-C chimeric rPrP<sup>c</sup>s with the same efficiencies, while the conversion of bovine rPrP<sup>c</sup> is slower. (E) CWD prions convert bovine and N-Hu-Bo-C chimeric rPrP<sup>c</sup>s with the same efficiencies, but conversion of human rPrP<sup>c</sup> is faster. (F) CWD prions convert human and N-Bo-Hu-C chimeric rPrP<sup>c</sup>s with the same efficiencies, while the conversion of bovine rPrP<sup>c</sup> is slower.

able from full-length human rPrP<sup>c</sup> (Fig. 8F). These results suggest that the presence of an amino-terminal domain preserves the susceptibility of full-length human rPrP<sup>c</sup> to CWD prions even if it does not have exactly the same sequence as the human NTD.

## DISCUSSION

Many researchers have investigated the role of the amino-terminal domain (NTD; aa 23 to 90) of PrP<sup>c</sup> in normal phenotype, disease progression, and amyloidogenicity (43–45, 47–49, 55–57). However, the role of the NTD in transspecies transmission of prions has not been reported. Here, we used real-time, quaking-induced conversion (RT-QuIC) to compare the conversion efficiency of white-tailed deer (WTD), bovine, bank vole, or human PrP<sup>c</sup>, with or without the NTD, in the presence of CWD, cBSE, and sCJD or without prions.

We demonstrated that truncated (aa 90 to 231) WTD, bovine, bank vole, and human PrP<sup>c</sup>s spontaneously form amyloid faster than the full-length proteins. This suggests that the NTD reduces the amyloidogenicity of PrP<sup>c</sup>. However, the efficiency of conversion in the presence of a prion seed relies upon the compatibility of the prion seed with the substrate and upon the amyloidogenicity of the PrP<sup>c</sup> substrate. Truncated bovine and WTD rPrP<sup>c</sup>s formed amyloid faster than full-length rPrP<sup>c</sup>s upon addition of a seed, whereas truncated human and bank vole rPrP<sup>c</sup>s converted more slowly than full-length rPrP<sup>c</sup>s upon addition of a seed. This suggested that the NTDs of human and bank vole may increase the compatibility of the seed and substrate in some way. Next, we tested the effect of the NTD on the permissiveness of rPrP<sup>c</sup> to conversion by homologous and heterologous prions. cBSE, CWD, and sCJD converted their native

TABLE 2 Summary of results for NTD of various species' rPrP<sup>c</sup> proteins<sup>a</sup>

Species PrP <sup>c</sup>	NTD role in permissiveness (Fig. 5)	NTD role in rate of misfolding (Fig. 2)	NTD required to recognize host (Fig. 3)	Chimera behaves like (Fig. 8)
Bank vole	↑	↑	No	
Bovine	↔	↓	No	
Human	↑	↑	No	
White-tailed deer	↔	↓	No	
N-Hu-Bo-C				Bovine
N-Bo-Hu-C				Human

<sup>a</sup> Additional details are provided in the indicated figures. Increased permissiveness or misfolding is indicated by an upward arrow. Decreased permissiveness or misfolding is indicated by a downward arrow. No effect is indicated by a double-headed arrow.

substrates more efficiently than nonnative substrates, regardless of truncation. This suggested that a prion seed is able to recognize its host PrP<sup>c</sup> without the NTD and that the NTD does not play an essential role in defining a PrP<sup>c</sup> molecule as one species or another. We observed previously that human rPrP<sup>c</sup> is susceptible to conversion by CWD prions (38). Here, we demonstrated that CWD converted truncated human rPrP<sup>c</sup> less efficiently than it converted full-length human rPrP<sup>c</sup>, suggesting that the susceptibility of human rPrP<sup>c</sup> to CWD prions may involve the NTD. The results are summarized in Table 2.

Chimeric PrP<sup>c</sup> molecules are commonly used in prion research to focus on the region of the protein responsible for resistance to prion conversion (58–62). We were particularly interested in whether the NTD of human PrP<sup>c</sup> could increase susceptibility to transspecies conversion by CWD prions, so we created a chimera with a human NTD substituted into bovine PrP<sup>c</sup> (N-Hu-Bo-C) and a chimera with the bovine NTD substituted into human PrP<sup>c</sup> (N-Bo-Hu-C). We hypothesized that the human NTD would increase promiscuity, so we tested the susceptibility of the N-Hu-Bo-C chimera to seeded conversion. However, N-Hu-Bo-C was not different from bovine rPrP<sup>c</sup>, whether homologous or nonhomologous prions were used as seeds. We concluded that the human NTD alone did not confer additional susceptibility to conversion. Alternatively, we hypothesized that the presence of any NTD to human substrate would have the effect of increasing the susceptibility of the chimera versus that of the truncated rPrP<sup>c</sup>. Indeed, we found that N-Bo-Hu-C had essentially the same permissiveness as full-length human rPrP<sup>c</sup> to conversion by human, cBSE, and CWD prions. The importance of the NTD for human rPrP<sup>c</sup> susceptibility recalls transgenic mouse studies wherein truncation around amino acid 90 results in reduced attack rate and PrP<sup>Sc</sup> accumulation (43, 44). It is possible that the human and bank vole PrP<sup>c</sup>s have a site in their globular domains that interacts with the NTD to facilitate seeded conversion, but not spontaneous conversion.

Together, the above data suggest that the PrP<sup>c</sup> NTD alone does not control species barriers *in vitro* but that interaction of the amino-terminal domain with the rest of the protein may be involved in the susceptibility of human rPrP<sup>c</sup> to *in vitro* conversion by CWD prions. Multiple investigators have studied the role of specific regions of the PrP<sup>c</sup> core and carboxy-terminal domain in prion disease species barriers. Reports have identified the  $\beta$ 2- $\alpha$ 2 loop and amino acids 165 to 175 as regions controlling transspecies transmission of prions, particularly of CWD prions to humans (35, 40). A favored interpretation of these data describes a steric zipper, wherein very few amino acid changes can severely disrupt the tertiary structure. Several polymorphisms between hu-

man and WTD PrP<sup>c</sup>s exist in this region, which may explain the apparent resistance of humans to CWD (39, 40). However, our data indicate that the variation in the  $\beta$ 2- $\alpha$ 2 loop does not prevent *in vitro* conversion, which suggests that factors in addition to the PrP<sup>c</sup>-PrP<sup>Sc</sup> interactions we modeled *in vitro* must influence *in vivo* conversion.

Our empirical observation that unseeded conversion of truncated rPrP<sup>c</sup> was more efficient than that of full-length rPrP<sup>c</sup> made us wonder whether the NTD impeded conversion universally or in a species-specific manner, serving to protect against spontaneous misfolding or against transspecies infection. We hypothesized that the NTD may play a protective role in transspecies prion conversion, perhaps in addition to the  $\beta$ 2- $\alpha$ 2 loop. However, our data suggest that the NTD does not control transspecies prion conversion but that it may contribute to the promiscuity of human and bank vole rPrP<sup>c</sup>. It is possible that the NTD, which is natively unfolded, has some interaction with other regions of the rPrP<sup>c</sup> or with the PrP<sup>Sc</sup> seed. A recent report suggests a structural mechanism by which the NTD may interact with the globular domain (63). These observations suggest that full-length protein should be used in structural studies of human PrP<sup>c</sup> and of the mechanisms of its seeded or spontaneous conversion.

We found that human rPrP<sup>c</sup> can be readily converted to an amyloid state by CWD prions and that the NTD facilitates this conversion. As there is little evidence for the susceptibility of humans to CWD, the biologic significance of our observation remains to be determined. However, the role of the NTD in this *in vitro* phenomenon may be important to the *in vivo* mechanism as well. RT-QuIC, transgenic mouse bioassay, and PMCA measure different outcomes. This report compares the efficiency of initial amyloid formation, while bioassay and PMCA reflect total accumulation of protease-resistant PrP<sup>Sc</sup>, which may explain the difference in the apparent susceptibilities of full-length human rPrP<sup>c</sup> in these models. The molecular underpinnings for species barriers and transspecies prion conversion remain a complex yet important problem in prion biology. We propose that an interaction between the amino-terminal domain and the globular domain facilitates *in vitro* susceptibility of human rPrP<sup>c</sup> to conversion by CWD prions. Such an interaction may be important in assessing the susceptibility of humans to animal prion diseases.

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