DISSERTATION

IN VITRO AMPLIFICATION AND ENHANCED TRANS-SPECIES TRANSMISSION OF CHRONIC WASTING DISEASE PRIONS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY TIMOTHY DANIEL KURT ENTITLED "*IN VITRO* AMPLIFICATION AND ENHANCED TRANS-SPECIES TRANSMISSION OF CHRONIC WASTING DISEASE PRIONS" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

IN VITRO AMPLIFICATION AND ENHANCED TRANS-SPECIES TRANSMISSION OF CHRONIC WASTING DISEASE PRIONS

Chronic wasting disease (CWD) is a prion disease of deer, elk and moose that is spreading rapidly in North America. Like all prion diseases, CWD is associated with conversion of a normal, host-encoded protein, PrP^{C}, to a protease-resistant conformer, PrP^{RES} (also called PrP^{CWD}). That a protein could replicate without nucleic acids constitutes one of the most intriguing, and controversial, premises in modern biology. Little is known about the mechanisms of prion conversion, or how it could occur with the efficiency necessary for the rapid spread of CWD among cervids in nature.

In this dissertation, I demonstrate that it is possible to induce the conversion of PrP^{C} to PrP^{CWD} *in vitro* via two protocols: non-denaturing amplification and serial protein misfolding cyclic amplification (sPMCA). Serial PMCA using brain substrate from transgenic mice that express cervid PrP^{C} [Tg(CerPrP)1536 mice] produced PrP^{CWD} amplification of $>6.5 \times 10^{9}$-fold after six rounds and significant increases in the sensitivity of PrP^{CWD} detection. Efficient *in vitro* amplification of PrP^{CWD} is a significant

step toward potential ante-mortem detection of PrP^{CWD} in the body fluids of CWD-infected animals.

Whether CWD presents a threat to non-cervid species is not yet known. To predict non-cervid susceptibility to CWD, I used sPMCA to amplify PrP^{CWD} in normal brain substrates from several non-cervid species, a method I refer to as trans-species sPMCA. I show that brain homogenates from several CWD-susceptible species, such as ferrets and hamsters, support amplification of PrP^{CWD} by sPMCA, whereas brain homogenates from CWD-resistant species, such as laboratory mice and transgenic mice expressing human PrP^{C} [Tg(HuPrP) mice], do not. I also investigated whether several species that share the environment with cervids would support amplification of PrP^{CWD} by sPMCA. Three North American rodent species (including voles and field mice) supported PrP^{CWD} amplification, whereas several other species (including prairie dogs and coyotes) did not. Analysis of PrP sequences suggests that ability to support amplification of PrP^{CWD} in trans-species PMCA correlates with the presence of asparagine at position 170 of the substrate species PrP. Thus PMCA may offer insights into species barriers to transmission of CWD.

To investigate whether PMCA can be used to estimate susceptibility to CWD infection I inoculated CWD from deer into prairie voles (*Microtus ochrogaster*) and found that prairie voles are somewhat susceptible to CWD from mule-deer. To determine if cross-species amplified PrP^{CWD} was infectious I inoculated products generated by trans-species PMCA into prairie voles. Inoculation of prairie voles with CWD prions amplified by trans-species PMCA resulted in more rapid and consistent disease onset.

Furthermore, immunohistochemical analysis revealed an altered pattern of CWD prion deposition in infected voles in comparison to infected Tg(CerPrP)1536 mice suggesting a new CWD strain has been created. These results indicate that CWD prion amplification assays such as PMCA can be used to increase PrP^{CWD} detection sensitivity, predict species susceptibility to CWD, accelerate adaptation to prion disease in non-cervid species, and create new strains of CWD.

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DEDICATION

I would like to dedicate this work to my wife Liz, who has learned almost as much about prion diseases as I have. Thank you for your enthusiasm and support.

I would also like to dedicate this work to my parents, Richard and Lesley, who encouraged me to think creatively from an early age. Your expectations and example always inspired me to select challenging goals, and yet my accomplishments were never necessary for your love.

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Introduction

The prion diseases

The prion diseases are a unique group of neurodegenerative diseases characterized by progressive dementia and the presence of vacuoles, or spongiform encephalopathy, in the central nervous system. Also known as transmissible spongiform encephalopathies (TSEs), prion diseases are 100% fatal, and have been recognized clinically in humans (e.g. Creutzfeldt-Jakob Disease) since the early 1900s [for review see (Pearce, 2004)], and in animals (e.g. sheep scrapie) for possibly 200 years (Comber, 1772). In 1937, scrapie was detected in approximately 1,500 sheep that had a received a formalin-treated louping-ill vaccine administered two years prior (Gordon, 1946), and it became clear that the scrapie was both transmissible and resistant to formalin inactivation. The scrapie agent was thought to be a "slow-virus" due to the long incubation periods associated with infection (Sigurdsson, 1954), however the disease did not induce inflammatory or humoral immune responses (Porter, Porter, and Cox, 1973) and was also resistant to ionizing and ultraviolet radiation (Alper et al., 1967; Alper, Haig, and Clarke, 1978). Sheep scrapie was eventually transmitted to mice (Chandler and Fisher, 1963) and thus developed into a laboratory model for prion disease. In the late 1960s, kuru, a prion disease of the Fore people of New Guinea, was transmitted to chimpanzees (Gajdusek,

Gibbs, and Alpers, 1966; Gajdusek, Gibbs, and Alpers, 1967) and the similarities between scrapie, kuru and Creutzfeldt-Jakob Disease (CJD) of humans were identified and described (Beck et al., 1966; Gibbs et al., 1968).

In the 1980s and 1990s, an epidemic of neurological disease and spongiform encephalopathy among cattle (bovine spongiform encephalopathy, or BSE) in Great Britain affected over 180,000 animals (Anderson et al., 1996) and became the largest outbreak of prion disease recorded (Wilesmith et al., 1992; Wilesmith et al., 1988). The BSE epidemic is thought to have been caused by the presence of prions in cattle feed, and the recycling of infected carcasses into feed via meat-and-bone-meal supplements (Walker et al., 1991; Wilesmith, Ryan, and Hueston, 1992). The failure to remove BSE-infected cattle from the industrial food chain led to exposure of both humans and animals to BSE-contaminated meat products, and resulted in the transmission of BSE to several non-bovid species (Kirkwood et al., 1993; Kirkwood et al., 1990; Wyatt et al., 1991). Whether BSE originated with the tissues of a spontaneously TSE-infected bovid, or of a scrapie-infected sheep, is still uncertain. Eventually, a form of human Creutzfeldt-Jakob Disease characterized by a new pathological profile (therefore variant, or vCJD) and early age of onset was recognized in humans (Will et al., 1996), and was shown to have resulted from ingestion of BSE-contaminated meat-products (Bruce et al., 1997), despite initial assurances that this was unlikely to happen (Taylor, 1989). By 2006, over 180 people had been infected with vCJD (Collee, Bradley, and Liberski, 2006) and the realization that vCJD could be spread by blood transfusion (Wilson and Ricketts, 2006; Zou, Fang, and Schonberger, 2008) has led to restrictions on blood donation in many countries (Dietz et al., 2007).

Prions and the "protein-only" hypothesis

Transmissible spongiform encephalopathies (TSEs), such as Chronic Wasting Disease (CWD) of cervids, bovine spongiform encephalopathy (BSE) of cattle, scrapie in sheep (SS) and Creutzfeldt-Jakob Disease (CJD) of humans are infectious as well as spontaneous or heritable neurodegenerative diseases. Alper (Alper et al., 1967; Alper, Haig, and Clarke, 1978) originally demonstrated that the infectious agent associated with scrapie was resistant to treatments that inactivated bacteria or viruses, and the term "prion," or *pro*teinaceous *in*fectious particle, later came to denote this infectious agent which apparently lacked nucleic acids (Prusiner, 1982). The identification of the prion protein, which could be purified from diseased brain homogenates and segregated with infectivity, enabled more detailed studies of the molecular and genetic aspects of TSEs (Bolton, McKinley, and Prusiner, 1982; Bolton, Meyer, and Prusiner, 1985).

The normal prion protein (PrP^{C}) is a highly-conserved, host-encoded, protease-sensitive protein that becomes partially protease-resistant (PrP^{RES}) with conversion of α-helical regions to β-sheet conformation during TSE infection (Bolton, McKinley, and Prusiner, 1982; Bolton, Meyer, and Prusiner, 1985; Pan et al., 1993; Sigurdson et al., 2002; Sigurdson et al., 1999; Spraker et al., 2002). Weissman and colleagues originally demonstrated that PrP^{C}-deficient mice were resistant to TSE infection (Bueler et al., 1993; Sailer et al., 1994) and thereby established that PrP^{C} expression is required for susceptibility to prion disease. Concurrently, others observed that heritable TSEs were associated with specific mutations in the *Prnp* gene that encodes PrP^{C} and that result in the spontaneous conversion of PrP^{C} to PrP^{RES} (Goldfarb et al., 1992; Medori et al., 1992).

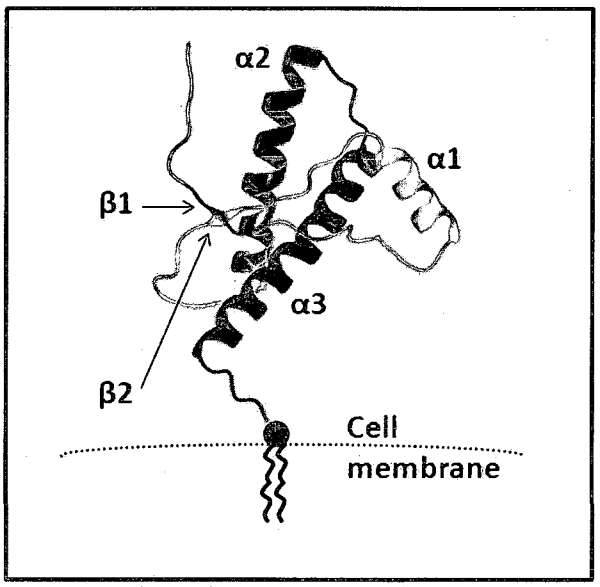
While production of infectious, synthetic prions has not yet been successful, a seminal study by Supattapone and colleagues (Deleault et al., 2007) demonstrated that infectious prions could be created *de novo* from purified mammalian PrP^{C}, and thus provided crucial evidence to support the theory that PrP^{RES} is the infectious agent associated with prion diseases. Collectively, these results have led to widespread acceptance of the "protein-only" prion hypothesis.

PrP structure and physiological function

The normal, protease-sensitive prion protein (PrP^{C}) is a highly conserved, glycosylphosphatidyl-inositol (GPI) -linked protein composed of ~210 amino acids, after removal of a 24-residue C-terminal GPI signal sequence and a 22-residue N-terminal ER targeting sequence (Cobb and Surewicz, 2009). Like most GPI-linked proteins, PrP^{C} is found primarily among lipid rafts in the outer surface of cell membranes (Cobb and Surewicz, 2009). Gene sequencing and NMR studies of recombinant PrP have demonstrated that the primary and tertiary structures of PrP are highly conserved among mammals (Gossert et al., 2005; Lysek et al., 2005; Prusiner, 1998). Five glycine-rich octapeptide repeats comprised of PHGGGWGQ are located within the relatively disordered N-terminal region of PrP, are important for binding putative ligands [reviewed in (Lee et al., 2003)] and may predispose PrP to aggregation (Aguzzi, Baumann, and Bremer, 2008; Goldfarb et al., 1991; Prusiner, 1998). The C-terminal globular domain (residues 120-230) contains a two stranded β-sheet (β1: residues 128-131 and β2: residues 161-164) and three α-helices (Helix 1: residues 143-153, Helix 2: residues 172-

191, Helix 3: residues 199-227). Two α-helices are connected by a disulfide bridge between Cys179 and Cys214 (Prusiner, 1998).

The L1 loop, or "rigid loop," comprises amino acids 166-175 and is located between α-helix 2 and β-strand 2 (Gorfe and Caflisch, 2007; Gossert et al., 2005). NMR studies have demonstrated that the stability of the L1 loop may be associated with susceptibility to different TSEs (Gorfe and Caflisch, 2007; Gossert et al., 2005; Sigurdson et al., 2008b).



Elk PrP Ribbon Diagram Legend. The three α-helices are labeled α1-3, and β-sheet regions are labeled β1 (red) and β2 (yellow). The c-terminal GPI-anchor is solid black. Much of the disordered n-terminal region (red) is not shown because accurate structure for this region cannot be predicted. Model created with PyMOL using PDB code 1XYW (Gossert et al., 2005).

At this time, high resolution structural data for PrP^{RES} are not available due to its insolubility and propensity to aggregate. PrP^{C} and PrP^{RES} can be glycosylated at amino acid positions 181 or 197, resulting in characteristic western blot bands that represent the di-, mono- and unglycosylated PrP forms (Bolton, Meyer, and Prusiner, 1985). After partial protease digestion, PrP^{RES} bands gain electrophoretic mobility consistent with the loss of 60-70 amino acids from the N-terminus of the protein (Bolton, Meyer, and Prusiner, 1985).

The exact physiological function of PrP^{C} is still controversial; PrP^{C}–deficient animals develop normally and exhibit few abnormalities as adults (Bueler et al., 1992). PrP^{C} may bind divalent cations, oligonucleotides and proteoglycans, and may play an important role in cell signaling and cellular responses to reactive oxygen species (Aguzzi, Baumann, and Bremer, 2008; Geoghegan et al., 2007; Gomes, Cordeiro, and Silva, 2008; Isaacs, Jackson, and Altmann, 2006; Snow and Wight, 1989). Many cell types, including neurons, glial cells, dendritic cells, and B cells express PrP^{C} [reviewed in (Isaacs, Jackson, and Altmann, 2006)]. The mechanisms by which PrP^{C}-to-PrP^{RES} formation and aggregation might lead to vacuole formation and neuronal cell death are still unclear.

Chronic Wasting Disease: a prion disease of cervids

Chronic wasting disease (CWD) of cervids (deer, elk and moose) was first identified in the Rocky Mountain region and has since spread to at least fifteen states, two Canadian provinces and one Asian country (Williams, 2005; Williams and Young, 1980).

Clinical symptoms of CWD may include ataxia, hind-limb paresis, and generalized wasting (Sigurdson and Miller, 2003; Williams, 2005; Williams and Young, 1980). Post-mortem analysis usually reveals the presence of CWD prions (PrP^{CWD}) in lymphoid tissues (Fox et al., 2006), as well as spongiform degeneration, reactive astrocytic gliosis, and perivascular and plaque-like PrP^{CWD} deposits in the nervous system (Sigurdson, 2008; Williams, 2005). Areas particularly affected during CWD infection include the obex of the medulla and the hippocampus. Other confirmatory tests for CWD include western blot detection of PrP^{CWD} after digestion with proteinase K to eliminate background PrP^{C}.

The facile transmission and rapid spread of CWD in nature is different from other prion diseases (Miller and Williams, 2003; Miller et al., 2004) and may be due to several factors, including high cervid population densities in North America (Miller and Wild, 2004), the presence of infectious prions in saliva and excreta of infected animals (Haley et al., 2009; Mathiason et al., 2006; Safar et al., 2008), and contamination of the environment with stable, infectious prions (Miller et al., 2004). Higher CWD prevalence has been observed among deer with the S96G and F225S PrP polymorphisms and elk with the V132M PrP polymorphism, although all cervid PrP genotypes are susceptible to CWD. While the known natural host-range of CWD is limited to cervids, some non-cervid species, such as ferrets and hamsters, have been infected experimentally (Bartz et al., 1998; Harrington et al., 2008; Raymond et al., 2007; Sigurdson et al., 2008a). Whether non-cervids outside the laboratory may be infected with CWD is not known. It is thus conceivable that non-cervid CWD reservoirs or vectors exist in nature. Investigations into the *in vivo* susceptibility of many species would be impractical, and

therefore an *in vitro* test to predict species-susceptibility to CWD would be of great value.

The transmission barrier and CWD

Several species, such as laboratory strains of mice (*Mus* species) and transgenic mice expressing human PrP^C [Tg(HuPrP) mice], are considered resistant to CWD (Browning et al., 2004; Kong et al., 2005; Raymond et al., 2007; Tamguney et al., 2006). This resistance is frequently referred to generally as a species or transmission barrier. Transmission barriers are characterized by long incubation periods upon initial passage in the new host. However, these incubation periods will often decrease and become more consistent when tissues from the few infected, or subclinically infected, animals in the initial passage group are used as inocula for serial sub-passage experiments. The process by which infection rates increase and incubation periods shorten and become more consistent is referred to as adaptation, and suggests that the degree of compatibility between PrP^C and PrP^{RES} determines the efficiency of inter-species transmission. Therefore transmission barriers are thought to be mediated primarily by differences between species in PrP^C sequence, although PrP^C concentration, the infectious PrP^{RES} conformation, nucleic acids and other still undefined cofactors may also play a role (Bartz et al., 1994; Harrington et al., 2008; Kong et al., 2005; Piening et al., 2006; Raymond et al., 2000). Transmission barriers can be abrogated by expression of foreign genes associated with TSE susceptibility in the recipient species. For instance, unlike wild-type *Mus* mice, transgenic *Mus* mice that express cervid PrP^C are highly susceptible

to CWD (Browning et al., 2004; Kong et al., 2005; Prusiner et al., 1990; Raymond et al., 2007; Windl et al., 2005). Furthermore, susceptibility to certain TSE strains may be associated with specific polymorphisms in the recipient, e.g. humans that express PrP 129M/M, but not 129V/M or 129V/V, are susceptible to variant CJD (Agrimi et al., 2008; Collinge et al., 1996). Even species with identical PrP^{C} primary structures may be variably susceptible to the same prion strain, indicating that genetic background also plays a role in determining TSE susceptibility (Lloyd et al., 2001; Nonno et al., 2006). Some features of prion strains, e.g. PrP^{RES} western blot properties and histologic lesion profiles, may be altered upon trans-species transmission (Collinge et al., 1996). In addition, TSE strains may gain expanded host-range after trans-species transmission, a phenomenon that could escalate the spread of CWD in nature, and possibly place humans and livestock at risk. Therefore identification of CWD-susceptible non-cervid species, and alterations in CWD strain properties after trans-species transmission, are important aspects of CWD biology.

In vitro prion amplification

The advent of cell-free conversion (Kocisko et al., 1994) and amplification (Lucassen, Nishina, and Supattapone, 2003; Supattapone, 2004) assays has offered the potential to study PrP^{C}-to-PrP^{RES} conversion *in vitro*. These studies have shown that cofactors, such as mammalian RNA and proteoglycans, may be necessary for efficient prion replication (Deleault, Lucassen, and Supattapone, 2003; Saborio et al., 1999; Wong et al., 2001). *In vitro*, these amplification factors are usually provided by 10% (w/v)

brain homogenate substrates, although there is some evidence that use of recombinant protein substrates for amplification may be possible (Atarashi et al., 2007; Deleault et al., 2005).

The development of protein misfolding cyclic amplification (PMCA) by Soto et al. (Saborio, Permanne, and Soto, 2001) increased the power and efficiency of *in vitro* PrP^{C}-to-PrP^{RES} amplification. In PMCA, normal brain homogenates (NBH) supply PrP^{C}, which upon addition of an infectious seed (e.g. CWD-infected deer brain homogenate), is converted into the protease-resistant isoform, PrP^{RES}. Intermittent sonication breaks the PrP^{RES} aggregates and releases newly formed PrP^{RES} seeds, which then convert additional PrP^{C} (Saa, Castilla, and Soto, 2004; Saborio et al., 1999; Soto et al., 2005).

PMCA has been used to amplify prions indefinitely to generate new infectious prions (Castilla et al., 2005), and to detect prions in blood (Castilla, Saa, and Soto, 2005; Saa, Castilla, and Soto, 2006; Thorne and Terry, 2008) and urine (Haley et al., 2009). When PrP^{C} and PrP^{RES} from the same species are used, *in vitro* amplification preserves the biochemical characteristics, infectivity and species barriers of the seed PrP^{RES} (Bossers et al., 1997; Castilla et al., 2008; Castilla et al., 2005; Kocisko et al., 1995; Lucassen, Nishina, and Supattapone, 2003). Thus, the combination of PrP^{C} and PrP^{RES} from different species (trans-species PMCA) offers the potential to assess species barriers to prion disease *in vitro*, in a more time- and cost-effective manner than conventional *in vivo* studies. The studies contained in this dissertation are based on the hypothesis that PrP^{CWD} could convert PrP^{C} from CWD-susceptible species to PrP^{RES} *in vitro*, and that

this new PrP^{RES} generated by the sPMCA protocol indicates the generation, *in vitro*, of new infectious prions.

This thesis therefore seeks to address several unanswered questions in CWD research, including whether CWD may be transmissible to non-cervid species, how PrP^{C} is converted to PrP^{CWD}, and what cofactors might be involved in this process. I hope to shed light on the range of species known to be at risk of CWD-infection in nature through a novel procedure termed protein misfolding cyclic amplification (PMCA) that simulates in 1-2 days the process of normal to abnormal pathogenic prion conversion, a process requiring years *in vivo*. Thereby, these studies could both enhance current knowledge of CWD infection and pathogenesis and pre-empt prolonged and costly *in vivo* studies. This work demonstrates the influence of PrP primary structure on *in vitro* prion conversion, thus further clarifying one important factor that contributes to CWD and prion species barriers. In addition, I identify three North American non-cervid species that may be susceptible to CWD, and show how CWD might appear in one of these species, the prairie vole (*Microtus ochrogaster*). In these ways, this work advances contemporary knowledge regarding CWD and prion disease research.

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CHAPTER 1*:

Efficient *in vitro* amplification of Chronic Wasting Disease PrP^{RES}

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ABSTRACT

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 I report use of both non-denaturing amplification and protein-misfolding cyclic amplification (PMCA) to amplify PrP^{CWD} *in vitro*. Normal brain from deer, transgenic mice [Tg(CerPrP)1536] expressing cervid PrP^{C}, and ferrets supported amplification. PMCA using Tg(CerPrP)1536 normal brain as PrP^{C} substrate produced amplification of >6.5 x 10^{9}-fold after six rounds. Highly efficient *in vitro* amplification of PrP^{CWD} is a significant step toward detection of PrP^{CWD} in body fluids or excreta of CWD-susceptible species.

BACKGROUND

Chronic wasting disease (CWD) of cervids is a transmissible spongiform encephalopathy (TSE), akin to sheep scrapie (SS) and bovine spongiform encephalopathy (BSE). TSE pathogenesis is associated with refolding of the normal prion protein, PrP^{C}, into a partially protease-resistant isomer termed PrP^{RES} (Bolton, McKinley, and Prusiner, 1982; Pan et al., 1993; Prusiner, 1998). A remarkable feature of CWD among prion diseases is its horizontal transmission in nature (Miller and Williams, 2003), suggesting that PrP^{C} conversion is highly efficient in this TSE, and perhaps associated with the presence of infectious prions in body fluids of deer (Mathiason et al., 2006). CWD is also transmissible to and pathogenic in ferrets (Bartz et al., 1998; Sigurdson et al., 2008) and transgenic mice expressing normal cervid PrP^{C} (Browning et al., 2004; Kong et al., 2005; Meade-White et al., 2007).

Raymond and colleagues (Raymond et al., 2000) first demonstrated the conversion of cervid PrP^{C} to PrP^{RES} *in vitro*. Non-denaturing amplification without use of radiolabeling (Deleault, Lucassen, and Supattapone, 2003; Lucassen, Nishina, and Supattapone, 2003), further contributed to understanding the mechanisms of PrP^{C} to PrP^{RES} conversion due to its directness and technical simplicity. Soto, Castilla and colleagues (Castilla et al., 2005; Castilla, Saa, and Soto, 2005; Saborio, Permanne, and Soto, 2001) greatly extended the process and power of *in vitro* PrP^{RES} amplification in developing protein-misfolding cyclic amplification (PMCA). In PMCA, normal brain homogenates (NBH) supply PrP^{C}, which upon addition of infected brain homogenate (seed) is re-folded 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 (Saa, Castilla, and Soto, 2004; Saborio et al., 1999; Soto et al., 2005).

To begin to address the mechanisms of PrP^{C} to PrP^{RES} conversion in CWD and to enhance sensitivity of CWD prion (PrP^{CWD}) detection in deer, I developed two *in vitro* amplification assays: non-denaturing amplification patterned after Lucassen et al. (Lucassen, Nishina, and Supattapone, 2003) and serial PMCA (Fig.1.1) after Soto et al. (Saa, Castilla, and Soto, 2004; Saborio, Permanne, and Soto, 2001). Here I report amplification using CWD-negative brain homogenates from whitetailed deer (*Odocoileus virginianus*), cervid PrP transgenic mice [Tg(CerPrP)1536] (Browning et al., 2004), and ferrets (*Mustela putorius furo)*, a species shown to be susceptible to CWD infection *in vivo* (Bartz et al., 1998; Sigurdson et al., 2008). Furthermore, I amplified deer-origin PrP^{CWD} and ferret-adapted PrP^{CWD} (derived from CWD-infected ferrets) in homologous and heterologous seed-substrate combinations. Amplification was inhibited by nucleases that degrade single-stranded RNA, as shown by Supattapone and colleagues (Deleault, Lucassen, and Supattapone, 2003), and by phospholipase-C, an enzyme that cleaves glycosylphosphatidyl-inositol (GPI)-anchors.

MATERIALS and METHODS

Preparation of tissue homogenates. Whole brains were removed rapidly after sacrifice from CWD-free animals and immediately frozen in liquid nitrogen. For PMCA experiments, animals were perfused at-mortem with PBS containing 5mM EDTA. Normal brain homogenate (NBH) was prepared by homogenization of brains with glass dounce (Kontes) or glass-beads [FastPrep™ (Qbiogene, Irvine, CA) set at 6.5 for 45 s] in

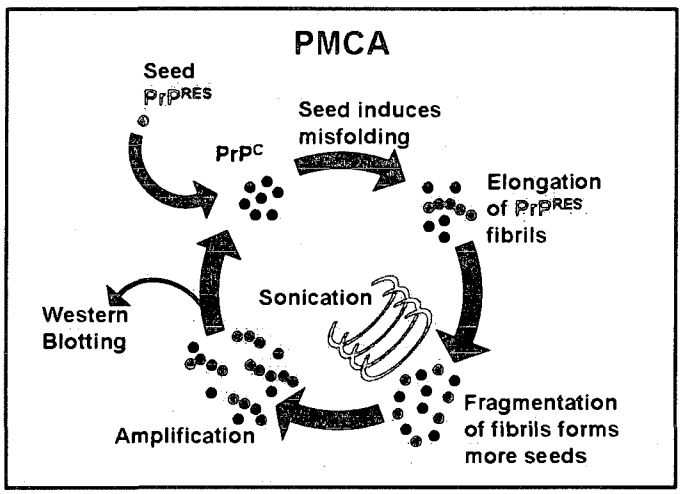

Fig. 1.1. Protein Misfolding Cyclic Amplification (PMCA). For PMCA experiments, CWD-infected brain homogenate containing seed PrP^{RES} was diluted into normal brain homogenate NBH containing PrP^{C}. Samples were incubated at in a cup-horn water bath sonicator at 37°C with intermittent bursts of sonication designed to fragment PrP^{RES} fibrils/aggregates and increase conversion. Newly formed PrP^{RES} was detected by western blot.

9 volumes of cold phosphate-buffered saline (PBS) for non-denaturing experiments, or PBS with Triton X-100, 5mM EDTA, 150mM NaCl and 0.05% saponin (Sigma) plus CompleteMini protease inhibitors (Roche) to a final concentration of 10% (w/v) for PMCA experiments. NBHs were centrifuged at 200g for 30s (non-denaturing experiments) or 2000g for 1 min (PMCA experiments), and the supernatant removed and frozen at -70°C for use. Deer-origin CWD (D10, provided by Dr. Michael Miller, Colorado Division of Wildlife, Fort Collins, CO) consisted of brain from an experimentally infected mule-deer and was prepared as 10% (w/v) homogenate. Ferret-adapted CWD (CSU-1) was comprised of the pooled brains of three experimentally infected ferrets prepared as 20% (w/v) homogenate. These ferrets exhibited typical CWD

symptoms and Ire euthanized 4.5-5 months post-inoculation (Sigurdson et al., 2008). Ferret-adapted CWD isolated 524 and 536 consisted of brain homogenate from ferrets infected with CSU-1.

***In vitro* PrP^{CWD} amplification.** For non-denaturing experiments, PrP^{CWD} was diluted in 50µl PBS plus 1% Triton X-100. All 50µl were then mixed with 50µl of NBH. Dilutions of PrP^{CWD} equivalent to x-fold higher concentrations than input were frozen, not amplified, for use in quantification (for example, see Figs. 1.1, 1.2). 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 and hamster tissues) proteinase K (PK, Invitrogen), and 40 µl of each sample was boiled with 15 µl lithium dodecyl sulfate (LDS, Invitrogen). For PMCA (Fig. 1.1), D10 was diluted to a final concentration of 1:1000 in NBH, with serial 1:3 dilutions in NBH to a final dilution of 1:6561000. Sixty microliters (60µl) of each dilution was incubated at 37°C in a Misonix (Farmingdale, NY) sonicator 3000 containing 160 mL water,
programmed for 96 cycles of 40 second pulse (at power level 7) + 30 minute incubation. After 48 h sonication/incubation (one round), 8.25 µl of each sample was brought to 0.875% SDS and digested with 150 µg/mL PK at 37°C for 20 min and 45°C for 10 min. All samples (final volume 15 µl) were then boiled with 5 µl LDS.

Electrophoresis and immunoblotting. Non-denaturing experiments: samples Ire loaded into 4-12% gels (Invitrogen), transferred to PVDF membranes (Millipore) using BioRad equipment and the membranes blocked using 6% milk powder (Carnation) for >1 h. Membranes were then incubated for 1 h in 2 µg/ml Bar224 mAb (a generous gift from

Dr. Jacques Grassi, CEA/Saclay, France) diluted 1:10000 in the blocking solution described. Membranes were rinsed in tris-buffered saline plus 0.2% Tween-20 and incubated for 45 min in HRP-labeled goat anti-mouse IgG secondary Ab (Jackson Labs) diluted 1:20000 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. PMCA experiments: samples (processed as described above) were loaded into 12% gels; transfer and blocking steps were identical to those described above. Membranes were incubated with Bar224 conjugated directly to HRP diluted 1:20000 in blocking solution for >2 h, then washed in dH_2O plus 0.2% Tween-20 for 30x5 min before applying ECL-plus™. Membranes from PMCA experiments were analyzed using a digital Gel-Doc™ system (Fujifilm) with automated detection of saturation limits; bands Ire quantified using ImageGauge™ (Fujifilm).

Inhibition experiments. To investigate the role of nucleic acids in amplification of PrP^{CWD}, I used Benzonase® (Sigma), DNase (Sigma), RNase H (Invitrogen), RNase V1 (Ambion), and RNases A (Invitrogen) and S7 (Sigma). I also used phosphoinositol phospholipase-C (Invitrogen) to investigate the role of the GPI-anchor in PrP^{C}-to-PrP^{CWD} conversion.

RESULTS

Brain substrates from deer and ferrets support non-cyclic amplification of PrP^{CWD}.

To determine whether deer brain homogenates could support PrP^{CWD} amplification, I mixed 10% (w/v) NBH from uninfected whitetailed deer with an equal volume of diluted brain homogenate from a CWD-infected mule-deer (D10) shown to be infectious to both Tg(CerPrP)1536 mice and whitetailed deer *in vivo* (Browning et al., 2004; Mathiason et al., 2006). This non-denaturing protocol consistently produced ~3-fold PrP^{CWD} amplification by 12 h (Fig. 1.2).

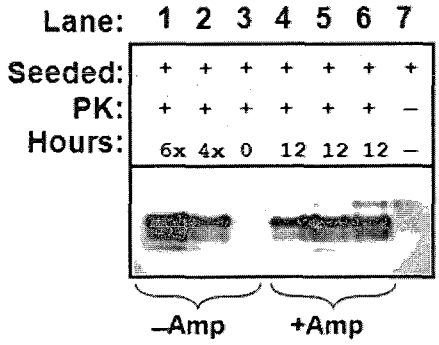

Fig. 1.2. Normal deer brain homogenate supports about 4-fold amplification of PrP^{CWD}. Lanes 1-3: Dilutions of D10 equivalent to 6-times the input (Lane 1, 1:333 dilution), 4-times the input (Lane 2, 1:500 dilution), and the input dilution (Lane 3, 1:2000 dilution) without amplification. Lanes 4-6: the input dilution (1:2000) of D10 after 12 hrs of amplification in three different cerebral cortex homogenates. Lane 7: PrP not digested with PK. "Seeded": samples mixed with D10 (+) or not (–). "PK": samples digested with PK (+) or not (–).

As part of ongoing CWD species barrier studies, our lab has developed ferrets as an alternate animal model of CWD infection (Sigurdson et al., 2008), extending the work of Bartz et al. (Bartz et al., 1998) who first reported ferret susceptibility to CWD. To expand this work to *in vitro* amplification, I spiked CWD-infected ferret brain into NBH made from uninfected normal ferret brain, and at specified times during amplification

samples were removed and frozen for subsequent analysis by western blotting. This resulted in consistent ~4 to 10-fold PrP^{CWD} amplification over a 12 h period (Fig. 1.3A, 1.3B, 1.3C). This result was notable in achieving higher amplification efficiency than obtained with deer NBH and in demonstrating amplification of PrP^{CWD} in a

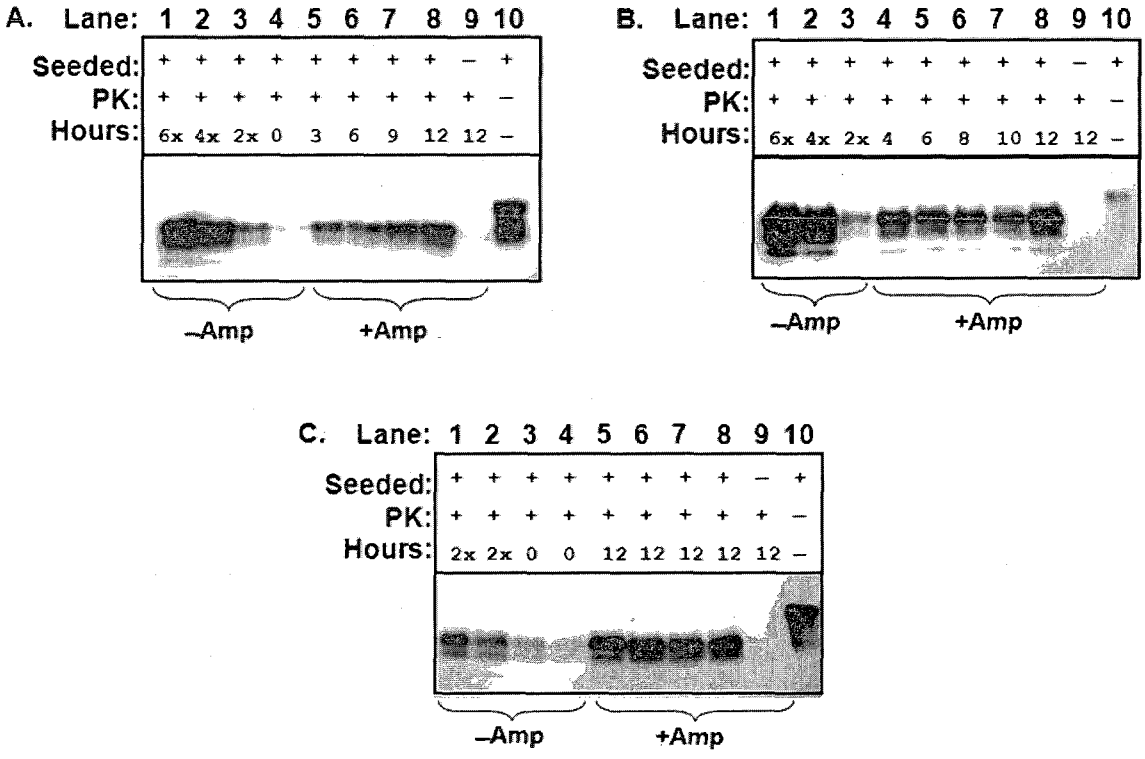

Fig. 1.3. Ferret NBH supports amplification of ferret-adapted CWD.
A: Amplification of a 1:200 dilution of PrP^{CWD} from CSU-1, a ferret-adapted CWD strain, in ferret NBH. B: Amplification of a 1:200 dilution of PrP^{CWD} from 536, a ferret infected with CSU-1, in ferret NBH. C: Amplification of a 1:400 dilution of PrP^{CWD} from 524, a ferret infected with CSU-1, in ferret NBH. "Hours": Each sample was amplified for the indicated number of hours. "6x, 4x, 2x": dilutions of PrP^{CWD} equivalent to x-fold more material than input. "Seeded": samples mixed with PrP^{CWD} (+) or not (–). "PK": samples digested with PK (+) or not (–).

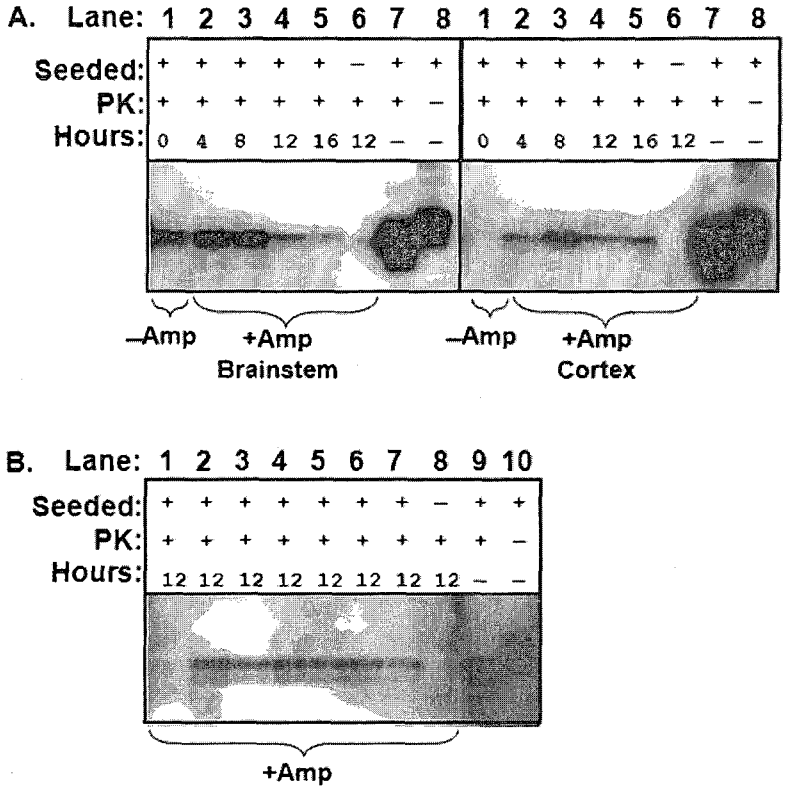

Fig. 1.4. Homogenates from anatomically diverse brain regions support amplification. A: Amplification of a 1:2000 dilution of D10 in deer brainstem (left panel) or cerebral cortex (right panel) homogenate at different timepoints. Lane 7: positive control D10 after PK digestion, Lane 8: D10 not digested with PK. B: Amplification of a 1:200 dilution of CSU-1 in ferret brain. For (B), PrP^{CWD} was diluted into $PrP^{0/0}$ mouse NBH (Lane 1), ferret hindbrain (Lane 2), ferret cerebral cortex (Lane 3), ferret midbrain (Lane 4), ferret cerebral cortex (Lane 5), and ferret whole brain (Lane 6 and7); Lane 8: ferret whole brain not seeded with PrP^{CWD}. Lane 9: positive control CSU-01 after PK digestion, Lane 10: CSU-01 not digested with PK.

CWD-susceptible, non-cervid species. In control experiments, NBH from mice which do not express PrP^{C} ($PrP^{0/0}$ mice) (Bueler et al., 1992) did not support amplification (Fig. 1.4B).

Normal brain homogenates from anatomically diverse brain regions support amplification. Due to the difficulty in preparing whole brain homogenates from large animals (e.g. deer), and to investigate anatomic specificity in supporting amplification, I prepared NBH from various regions of deer and ferret brain for use in amplification. I found that deer brain cortex (Figs. 1.4A right panel and 1.2A) supported higher efficiency amplification than brainstem (Fig 1.4A left panel). Anatomically diverse regions of ferret brain supported amplification to an approximately equivalent degree (Fig. 1.4B).

Non-cyclic amplification of PrP^{CWD} is inhibited by nucleases that degrade single-stranded RNA. Supattapone and colleagues have shown that nucleic acids derived from mammalian tissues are essential for efficient amplification of scrapie PrP^{RES} in hamster normal brain homogenates (Deleault, Lucassen, and Supattapone, 2003). To investigate the role of nucleic acids in amplification of PrP^{CWD}, I obtained a nonspecific nuclease (Benzonase®), a deoxyribonuclease (DNase), a ribonuclease that degrades only RNA contained in RNA-DNA duplexes (RNase H), a ribonuclease that degrades only double-stranded RNA (RNase V1), and ribonucleases that degrade only single-stranded RNA (RNase A, S7) (Table 1.1). Amplification in deer NBH did not appear to be inhibited by any of the nucleases (Fig. 1.5), possibly because amplification efficiency in normal deer substrates was frequently low, thereby inhibitory effects were less noticeable.

Table 1.1. Nucleases used for degradation of RNA and DNA.

Nuclease	Target
Benzonase®	RNA and DNA, all forms
DNase	Single and double-stranded DNA
RNase H	RNA-DNA hybrids
RNase V1	Double-stranded RNA
RNase A	Single-stranded RNA
S7 (micrococcal nuclease)	Single-stranded RNA

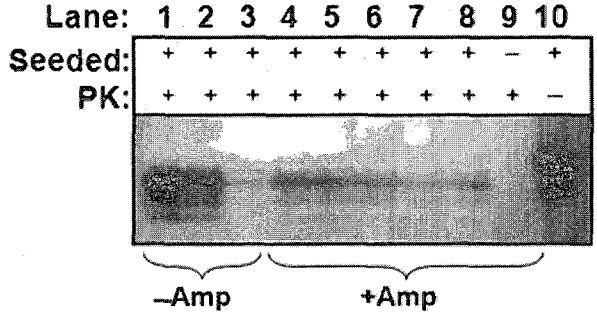

Fig. 1.5. Effects of nuclease treatment on amplification in deer NBH. Lanes 1, 2: Dilutions of PrP^{CWD} equivalent to 6-fold and 4-fold more material than input (a 1:2000 dilution of D10, Lane 3) without amplification. D10 was diluted 1:2000 into NBH treated with 80 U RNase inhibitor (Lane 4), 100 U DNase (Lane 5), 25 U RNase H (Lane 6), 30 U RNase V1 (Lane 7) or 30 μg RNase A (Lane 8) and amplified for 12 hours. Differences in band densities were within the normal range when compared to other experiments.

To further investigate the role of nucleic acids in amplification, I subjected ferret samples to nuclease treatment. Ferret samples incubated with nucleases that degrade single-stranded RNA, such as Benzonase® (Fig. 1.6A), micrococcal nuclease S7 (Fig.

1.6B), or RNase A (Fig. 1.6D) did not amplify ferret-adapted PrP^{CWD} as efficiently as untreated samples. In contrast, incubating samples with RNase V1 (Fig.1.6C), which degrades double-stranded RNA, had no effect on amplification. Likewise, incubating samples with RNase H (Fig.1.6C), which degrades RNA contained in RNA-DNA duplexes, had no effect on amplification.

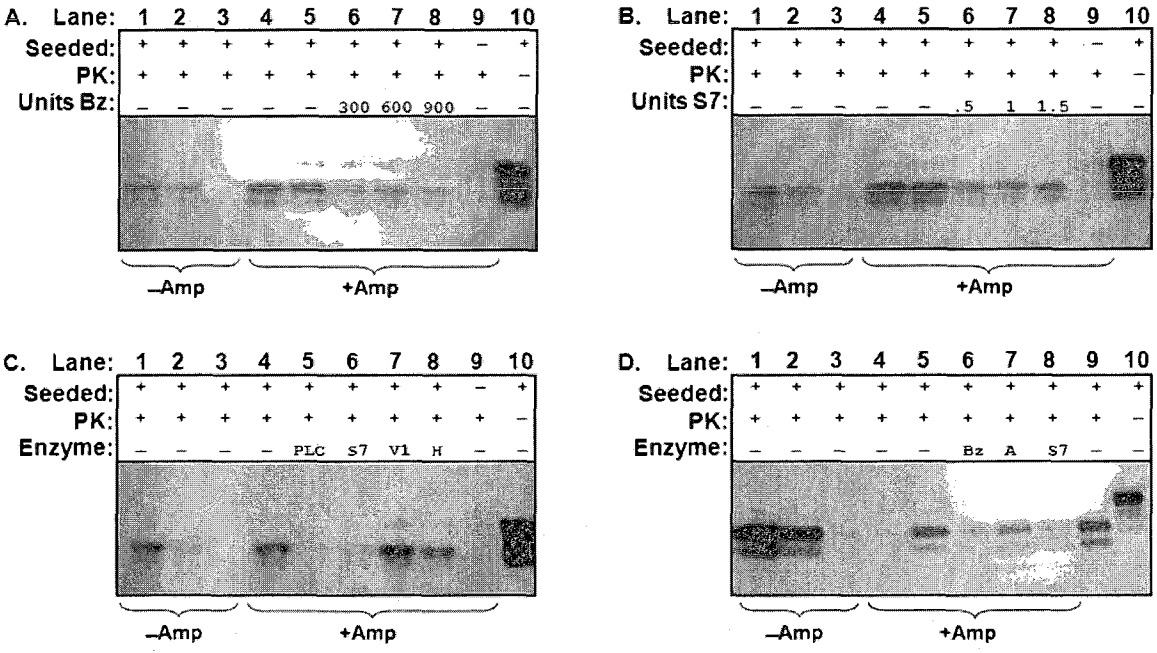

Fig. 1.6. Amplification in ferret NBH is inhibited by degradation of single-stranded RNA. A, B, C: Lanes 1, 2, 3: Dilutions of PrP^{CWD} equivalent to 4-fold and 2-fold more material than input (Lane 3, a 1:400 dilution of CSU-1) without amplification. A, B: NBH incubated with the units of Benzonase® ("Bz") or S7 indicated. C: NBH incubated with 1 U phospholipase C (lane 5), 1 U S7 (lane 6), .5 U RNase V1 (lane 7), or 25 U RNase H (lane 8). D: Lanes 1, 2, 3: Dilutions of PrP^{CWD} equivalent to 4-fold and 2-fold more material than input (Lanes 3 and 4: brain from CWD-infected ferret 524 diluted 1:200 in $PrP^{0/0}$ NBH) without amplification. NBH was incubated with 30 U Benzonase® (lane 6), 30 µg RNase A (lane 7), or 30 U S7 (lane 8). Lane 9, A-C: NBH not seeded with PrP^{CWD}, after PK digestion. Lane 10: NBH not subjected to PK digestion.

Control experiments confirmed nuclease activity (not shown) and that these enzymes did not increase the proteinase K sensitivity of PrP^{RES} (not shown). These results indicate that single-stranded RNA molecules are required for robust non-denaturing amplification of PrP^{CWD}.

Non-denaturing amplification of PrP^{CWD} is inhibited by phosphatidylinositol phospholipase-C (PI-PLC) treatment. To investigate whether PrP must be tethered to lipid membranes for PrP^{C}-to-PrP^{CWD} conversion, I treated samples with phosphatidylinositol phospholipase-C (PI-PLC), an enzyme that cleaves phosphatidylinositol, thus releasing GPI-anchored proteins into solution and leaving diacylglycerol in the lipid membrane.

PI-PLC had an inhibitory effect on amplification in ferret NBH (Fig. 1.6C), a result which suggests that insertion of PrP in lipid membranes is important for PrP^{C}-to-PrP^{CWD} conversion and is consistent with other *in-vitro* conversion studies (Caughey and Raymond, 1991). However, cleavage of phosphatidylinositol or GPI-anchors associated with other proteins could also have affected our results. It is not known whether other GPI-anchored proteins besides PrP play a role in amplification. Control experiments indicated that treatment with PI-PLC did not increase the sensitivity of PrP^{CWD} to protease digestion (not shown).

Deer and ferret NBH support trans-species amplification of PrP^{CWD}. In attempts to investigate whether non-denaturing amplification would reflect species barriers to CWD, I seeded normal brain homogenates from deer, ferrets and hamsters with PrP^{CWD} from

D10 or CWD-infected ferrets. Deer PrP^{CWD} amplified in NBH from both deer and ferret NBH, just as both these species are susceptible to CWD (1.7A), but not in NBH from Syrian hamsters, a species which may be somewhat resistant to CWD (Bartz et al., 1998; Raymond et al., 2007). Ferret PrP^{CWD} amplified in deer and ferret NBH, as might be expected given that this TSE was derived from cervids, and ferrets are susceptible to CWD. Ferret PrP^{CWD} also amplified slightly in hamster NBH (Fig. 1.7B), which is consistent with the studies of Bartz et al. (Bartz et al., 1998), which demonstrated that hamsters may be more susceptible to ferret-adapted CWD. Both PrP^{CWD} strains amplified more efficiently in homologous, rather than heterologous, species combinations of PrP^{CWD} and NBH.

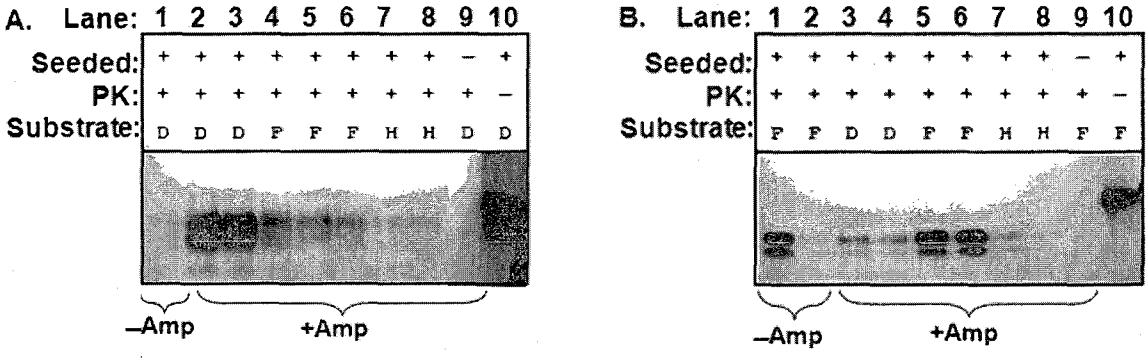

Fig. 1.7. Non-denaturing trans-species amplification of PrP^{CWD}. A: A 1:2000 dilution of D10 was amplified in NBH from whitetailed deer (D), ferret (F) or hamster (H). Lane 1: an equivalent dilution of D10 not amplified. B: A 1:2000 dilution of PrP^{CWD} from CWD-infected ferret 524 was amplified in NBH from whitetailed deer (D), ferret (F) or hamster (H). Lane 1: 2-fold more material than input (Lane 2) without amplification.

Cyclic amplification of PrP^{CWD} in deer and Tg(CerPrP)1536 mouse brain. To increase the amount of PrP^{CWD} generated by *in vitro* amplification, I next applied the protein misfolding cyclic amplification (PMCA) protocol to amplify PrP^{CWD} from mule-deer. Whitetailed deer NBH was spiked with CWD+ deer brain homogenate D10, the same infectious PrP^{CWD} source that was used in the non-denaturing experiments described above. After incubation with intermittent sonication (see Fig. 1.8), western blotting detection of PrP^{CWD} revealed a final amplification yield of ~6 to 27-fold, as calculated by band intensity relative to non-amplified, starting dilutions (Fig. 1.8). 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.

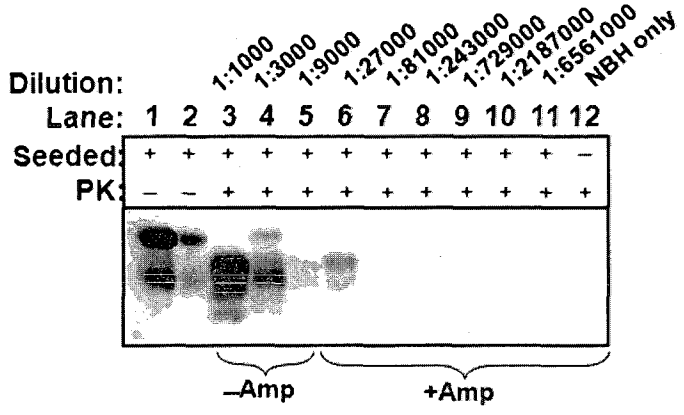

Fig. 1.8. PMCA applied to amplify PrP^{CWD} in deer NBH. Lanes 1 and 2: undigested NBH diluted 1:250 and 1:500, respectively, after sonication/incubation, Lanes 3-5: Serial 1:3 dilutions of D10 in NBH, starting at 1:1,000 relative to whole brain, not amplified, Lanes 6-11: Continuing serial 1:3 dilutions of D10 in NBH from 1:27,000-1:6,561,000, after 48h of PMCA, Lane 12: unseeded NBH subjected to PMCA followed by digestion with proteinase K.

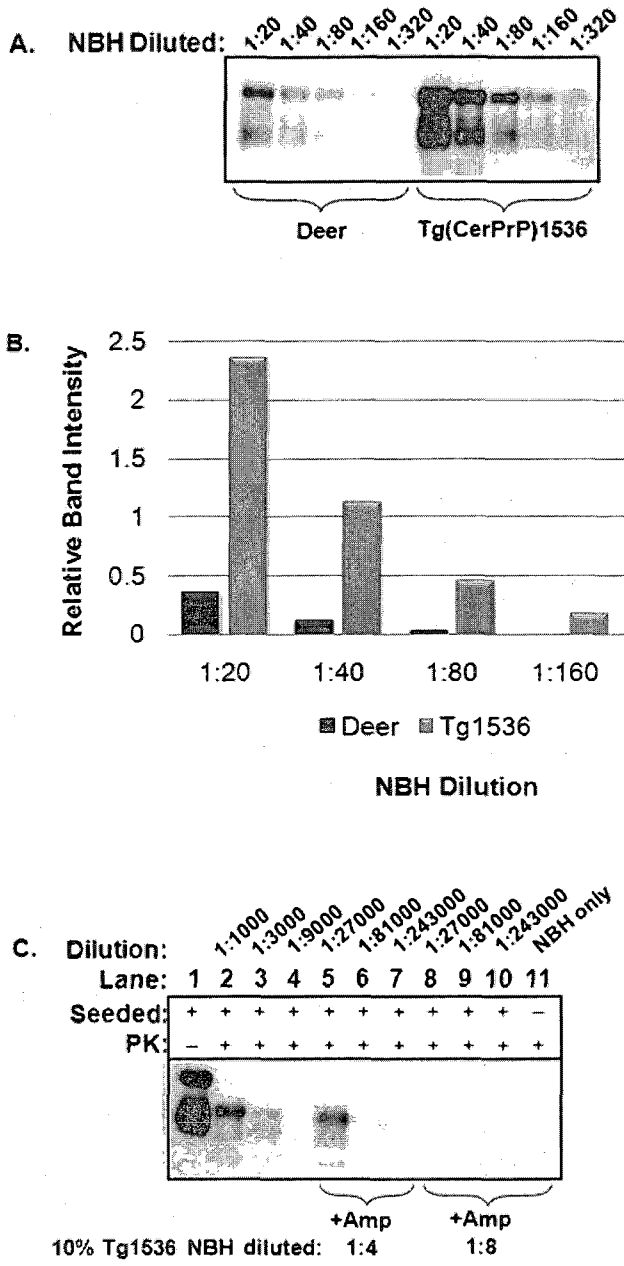

Fig. 1.9. Semi-quantitative estimation of PrP^C in brains of deer vs. Tg(CerPrP)1536 mice and effect of PrP^C concentration on PMCA. A: Serial 1:2 dilutions of NBH were analyzed by western blot. Lanes 1-5 are deer NBH; Lanes 6-10 are Tg(CerPrP)1536 NBH. B: western blot bands, of which size and density correspond to amount of PrP^C, were quantified and plotted for comparison. C: Tg(CerPrP)1536 NBH was diluted 1:4 (Lanes 5-7, 11) or 1:8 (Lanes 8-10) in $PrP^{0/0}$ for PMCA. Lane 1: undigested NBH diluted 1:250, Lanes 2-4: Serial 1:3 dilutions of D10 in NBH, starting at 1:1,000 relative to whole brain, not amplified, Lanes 5-7 and 8-10: D10 diluted 1:27,000; 1:81,000; and 1:243,000 after PMCA, Lane 11: unseeded NBH subjected to PMCA followed by digestion with proteinase K.

To improve upon these results, I prepared NBH from pooled whole brains of Tg(CerPrP)1536 mice (Browning et al., 2004), animals in which the amino acid sequence of PrP^C is identical to that of whitetailed deer. Semi-quantitative estimation of PrP^C levels in Tg(CerPrP)1536 mouse brain vs. deer brain homogenates by western blot analysis revealed ~5 to 8-fold greater PrP^C expression in the brain tissue of these mice (Fig. 1.9A, B). When Tg(CerPrP)1536 NBH was diluted 1:4 into $PrP^{0/0}$ brain (Bueler et al., 1992), to approximate the PrP^C concentration of deer, amplification yield (Fig. 1.9C) was very similar to that obtained using deer NBH (Fig. 1.8). As might be anticipated, when Tg(CerPrP)1536 mouse NBH was diluted 1:8 into $PrP^{0/0}$ brain, no amplification was detected (Fig. 1.9C).

Following these results, I substituted Tg(CerPrP)1536 mouse NBH for deer NBH entirely, and PMCA yield increased ~20 times, to over 200-fold per round (Fig. 1.10A, first panel). 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 6 rounds (Fig. 1.10B, last panel). Theoretically, serial PMCA attaining ~200-fold increases at each round would result in $\sim 6.4 \times 10^{13}$-fold total increase after 6 rounds. To maintain characteristic western blot PrP^{CWD} signals I diluted samples less than 200-fold at each round, resulting in a slightly lower final yield. More importantly, Tg(CerPrP)1536 NBH served as a very efficient substrate for PrP^{CWD} amplification relative to deer NBH, likely reflecting the greater expression of PrP^C relative to deer.

Previously, Raymond and colleagues (Raymond et al., 2000) observed highly efficient conversion of whitetailed deer PrP^C using mule-deer derived PrP^{CWD} as a spike source in a cell-free conversion system employing radiolabeled substrate PrP^C. Therefore

I examined further the premise that in PMCA the more efficient amplification in Tg(CerPrP)1536 vs. deer NBH was due principally to the substrate PrP^C concentration vs. other factors such as higher levels of conversion-enhancing co-factors [e.g. single-stranded RNA (Deleault, Lucassen, and Supattapone, 2003) or heparan sulfate

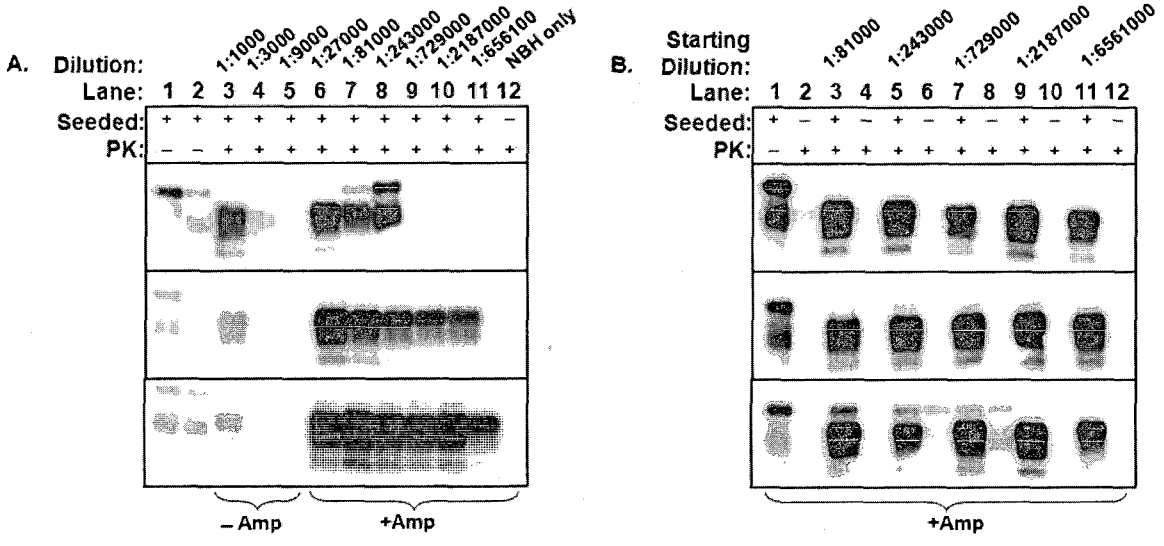

Fig. 1.10. Amplification of PrP^{CWD} in Tg(CerPrP)1536 by PMCA. *For rounds 1-3* (A): Lanes 1 and 2: undigested NBH diluted 1:250 and 1:500, respectively, after sonication/incubation, Lanes 3-5: Serial 1:3 dilutions of D10 in NBH, starting at 1:1,000 relative to whole brain, not amplified, Lanes 6-11: Continuing serial 1:3 dilutions of D10 in NBH from 1:27,000-1:6,561,000, after 48h of PMCA, Lane 12: unseeded NBH subjected to PMCA. For rounds 2 and 3, samples from Lanes 6-11 from the preceding round were diluted 1:10 into fresh NBH and subjected to another 48h PMCA. *For rounds 4-6* (B): Lane 1: undigested NBH diluted 1:250 after sonication/incubation, Lanes 2-11: For round 4, material from lanes 7-11 of round 3 was diluted 1:100 into fresh NBH and subjected to PMCA; these samples Ire then diluted 1:100 into fresh NBH for rounds 5 and 6.

proteoglycan (Wong et al., 2001)] in mouse brain. I added deer whole brain RNA, heparan sulfate proteoglycan, and increasing concentrations of $PrP^{0/0}$ mouse NBH to the deer NBH; these did not improve amplification yield (not shown). I then evaluated

addition of deer NBH to Tg(CerPrP)1536 NBH; this did not inhibit amplification in comparison to an equivalent addition of $PrP^{0/0}$ mouse brain, making the presence of inhibitory factors in deer brain less likely (Fig. 1.11).

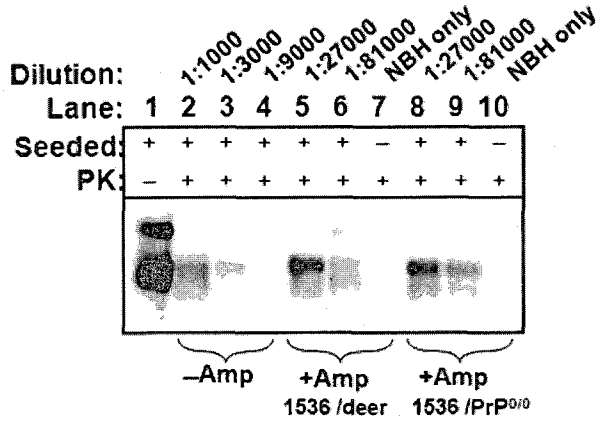

Fig. 1.11. Deer brain homogenate does not inhibit PMCA in Tg(CerPrP)1536 NBH. Tg(CerPrP)1536 NBH was diluted 50:50 with either deer (Lanes 5-7, 11) or $PrP^{0/0}$ mouse (Lanes 8-10) NBH for PMCA. Lane 1: undigested NBH diluted 1:250, Lanes 2-4: Serial 1:3 dilutions of D10 in NBH, starting at 1:1,000 relative to whole brain, not amplified, Lanes 5-7 and 8-10: D10 diluted 1:27,000; 1:81,000; and 1:243,000 after PMCA, Lanes 7 and 10: unseeded NBH subjected to PMCA followed by digestion with proteinase K.

DISCUSSION

In this study I demonstrate the amplification of PrP^{CWD} in normal brain homogenates (NBH) from deer and ferrets. The unexpected, low amplification efficiency using deer NBH is unlikely to be due to a species barrier between mule deer (our PrP^{CWD} source) and whitetailed 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 (Nishina et al., 2006; Priola and Lawson, 2001). The presence of serine vs. glycine at PrP^{C} position 96 in whitetailed deer has been associated with greater resistance to CWD in whitetailed deer and transgenic mice (Meade-White et al., 2007; O'Rourke et al., 2004), however, all whitetailed deer used for NBH in these studies were of the more susceptible 96G/G or 96G/S PrP genotypes. Finally, deer NBH did not inhibit amplification using Tg(CerPrP)1536 NBH. Thus efficient *in vitro* amplification of PrP^{CWD} by PMCA appears to be a reflection of the higher concentration of PrP^{C} in Tg(CerPrP)1536 mouse vs. deer brain.

DeLeault et al. (Deleault, Lucassen, and Supattapone, 2003) originally demonstrated that single-stranded RNA molecules are essential for *in vitro* scrapie prion amplification. Here I replicate and confirm those studies using PrP^{CWD} and ferret NBH. The role of RNA in PrP^{C}-to-PrP^{RES} conversion is unclear. Several studies have demonstrated that RNA, and other polyanionic molecules such as heparan sulfate, can induce oligomerization of PrP *in vitro* and/or may act as a scaffold that facilitates PrP^{C}-to-PrP^{RES} interaction and conversion (Deleault et al., 2005; Deleault, Lucassen, and Supattapone, 2003; Geoghegan et al., 2007; Gomes, Cordeiro, and Silva, 2008; Gomes et al., 2008; Liu et al., 2007; Vasan, Mong, and Grossman, 2006). It is also possible that an as-yet-unidentified RNA oligomer specifically induces PrP^{C}-to-PrP^{RES} conversion. This RNA species could be the hypothetical molecular chaperone referred to as "protein X" (Kaneko et al., 1997; Telling et al., 1995). To isolate a specific RNA oligomer involved in conversion, one could add cell fractions to purified PrP^{C} and PrP^{RES}, and then attempt the amplification procedure. Interestingly, in my experiments addition of RNA or heparan sulfate proteoglycan did not enhance amplification results, however small

changes in efficiency may not have been apparent due to the large quantities of putative cofactors present in crude brain homogenates.

Studies by Caughey et al. (Caughey et al., 1990) have indicated that PI-PLC has little effect on PrP^{RES} compared to PrP^{C}. Here I demonstrate that incubation of ferret NBH with PI-PLC inhibits amplification. Our results suggest that PrP^{C} must be inserted into lipid membranes for efficient non-denaturing amplification, a result that is consistent with the studies of Birkmann et al. (Birkmann and Riesner, 2008). However, I cannot exclude the possibility that these results could be due to the action of PI-PLC on phosphatidylinositol or other GPI-anchored proteins. Ultimately, this finding contributes to our understanding of PrP^{C}–to-PrP^{RES} conversion in cell-free systems.

Our results with trans-species amplification using ferret and deer tissues indicate that PrP^{CWD} can be amplified in non-homologous substrate NBH. Both of these species are susceptible to CWD. Whether ferret-adapted CWD is pathogenic to cervids is not known, however most other TSEs are pathogenic in the original host even after adaptation to a new species (Race et al., 2002; Race et al., 2001). That Syrian hamster NBH did not support amplification of PrP^{CWD} from deer is consistent with the observation by Bartz et al. that this species is less susceptible to deer CWD than ferret-adapted CWD (Bartz et al., 1998). It is also possible that the non-denaturing amplification assay is not powerful enough to induce conversion of hamster PrP^{C} by deer PrP^{CWD}. In future experiments I will investigate trans-species amplification using serial PMCA.

At minimum, our studies indicate that NBH from ferrets and Tg1536 mice can support amplification of PrP^{CWD}, and that amplification efficiency is correlated with PrP^{C}

concentration. Studies utilizing PMCA may yield further insight into whether other non-cervid species may support *in vitro* PrP^{CWD} amplification and whether these results may forecast *in vivo* CWD susceptibility. I also demonstrate highly efficient amplification of PrP^{CWD} using brain substrate from Tg(CerPrP)1536 mice. The magnitude of PrP^{CWD} conversion obtained with serial PMCA may make possible the *in vitro* detection of PrP^{CWD} in body fluids and excreta of infected animals.

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CHAPTER 2*:

Trans-species amplification of PrP^{CWD} and correlation with rigid loop 170N

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ABSTRACT

Chronic wasting disease (CWD) is an efficiently transmitted spongiform encephalopathy of cervids. Whether CWD represents a threat to non-cervid species remains speculative. Here I show that brain homogenates from several CWD-susceptible non-cervid species, such as ferrets and hamsters, support amplification of PrP^{CWD} by serial protein misfolding cyclic amplification (sPMCA), whereas brain homogenates from CWD-resistant species, such as laboratory mice and transgenic mice expressing human PrP^{C} [Tg(HuPrP) mice], do not. I also investigated whether several North American species that share the environment with cervids would support amplification of PrP^{CWD} by sPMCA. Three native rodent species, including voles and field mice, supported PrP^{CWD} amplification, whereas other species (e.g. prairie dog, coyote) did not. Analysis of PrP sequences suggests that ability to support amplification of PrP^{CWD} in trans-species sPMCA is correlated with the presence of asparagine at position 170 of the substrate species PrP. Serial PMCA may offer insights into species barriers to transmission of CWD.

BACKGROUND

Chronic wasting disease (CWD) of deer, elk and moose is a prion disease first identified in the Rocky Mountain region and now recognized in 15 states, Canada, and one Asian country (Williams, 2005; Williams and Young, 1980). Like other transmissible spongiform encephalopathies (TSEs) such as ovine scrapie, bovine spongiform encephalopathy (BSE) and human Creutzfeldt-Jakob Disease (CJD), CWD is caused by the conversion of normal, protease-sensitive PrP^{C} protein to a misfolded, protease-resistant conformation (PrP^{RES}) which accumulates in the central nervous and lymphoid systems and leads to wasting and spongiform encephalopathy (Sigurdson et al., 2002; Sigurdson et al., 1999; Spraker et al., 2002).

The facile spread of CWD is different from most TSEs and may reflect the transmission of infectious prions from the saliva and excreta of infected cervids (Mathiason et al., 2006; Safar et al., 2008). While the known natural host range for CWD is limited to cervids, some non-cervid species, e.g. ferrets and hamsters, can be infected experimentally (Bartz et al., 1998; Harrington et al., 2008; Raymond et al., 2007; Sigurdson et al., 2008a). Trans-species transmission of prion diseases is infrequent due to the species barrier phenomenon, which may be mediated by differences in PrP^{C} sequence, prion strain, and other still unknown factors (Bartz et al., 1994; Harrington et al., 2008; Kong et al., 2005; Piening et al., 2006; Raymond et al., 2000). Because *in vivo* susceptibility studies in candidate outbred species are protracted and costly, a comprehensive analysis of CWD species barriers by direct *in vivo* exposure has yet to emerge. Thus, the existence of non-cervid reservoirs for CWD in the wild remains conceivable.

The advent of protein misfolding cyclic amplification (PMCA) for *in vitro* prion amplification (Saborio, Permanne, and Soto, 2001) offers the potential to assess the CWD species barrier by evaluating the permissiveness of a given species brain substrate to support PrP^{C}-to-PrP^{RES} conversion (Jones et al., 2007; Saa, Castilla, and Soto, 2006; Soto et al., 2005). When PrP^{C} and PrP^{RES} from the same species are used, *in vitro* amplification preserves the biochemical characteristics, infectivity and species barriers of the seed PrP^{RES} (Bossers et al., 1997; Castilla et al., 2008; Castilla et al., 2005; Kocisko et al., 1995; Lucassen, Nishina, and Supattapone, 2003). Several researchers have demonstrated efficient amplification of CWD PrP^{RES} (PrP^{CWD}) by serial protein misfolding cyclic amplification (sPMCA) using transgenic mice [Tg(CerPrP) mice] over-expressing cervid PrP^{C} as brain substrate (Green et al., 2008; Kurt et al., 2007; Meyerett et al., 2008).

The plausibility of trans-species sPMCA is supported by cell-free conversion studies which have shown that some conversion may occur when combining PrP^{C} and PrP^{RES} from different species (Kocisko et al., 1994; Piening et al., 2006; Priola, Chabry, and Chan, 2001; Raymond et al., 2000). Here I apply sPMCA to demonstrate that brain homogenates from species shown to be susceptible to CWD infection *in vivo* also support amplification of CWD prions *in vitro* (e.g. ferrets and hamsters) whereas relatively resistant species [e.g. laboratory mice (*Mus* spp.)] (Browning et al., 2004; Raymond et al., 2007; Sigurdson et al., 2006; Williams and Young, 1980) do not. I extended this approach to include species abundant in North America likely to be exposed to CWD in the wild and that therefore have potential to serve as reservoirs or laboratory models for CWD. Interestingly, I found that all tested species that expressed asparagine at PrP

position 170 supported trans-species amplification of PrP^{CWD}. All but one species that expressed serine at PrP position 170 failed to support trans-species amplification of PrP^{CWD}.

MATERIALS AND METHODS

Transgenic mice encoding cervid PrP. Transgenic mice expressing cervid PrP were generated in the Telling lab (Browning et al., 2004) and have been used previously in sPMCA (Green et al., 2008; Kurt et al., 2007; Meyerett et al., 2008).

Production of transgenic mice encoding human PrP. Transgenic mice expressing human PrP encoding either methionine (M) or valine (V) at codon 129, referred to as HuPrP-M129 and HuPrP-V129 respectively, were generated in the Telling lab. In all cases, Tg mice were maintained on a FVB/$Prnp^{0/0}$ background. Tg(HuPrP-M129)$6816^{+/-}$ mice and Tg(HuPrP-V129)$7823^{+/-}$ mice were used in these studies.

Sources and preparation of brain homogenates. Donor animals were euthanized according to ACUC approved protocols and immediately perfused (except prairie dogs), to remove as much blood as possible, with phosphate buffered saline (PBS) plus 5 mM EDTA. At least two individuals (in most cases three) of each species, and one coyote, were used. Deer brain was provided by David Osborn (Warnell School of Forestry, University of Georgia). Tg(CerPrP)$1536^{+/-}$ mice (Browning et al., 2004) were housed at CSU. Ferrets were obtained from Marshall Farms Inc. BALB/c mice were provided by James Perry and Anne Avery at CSU. Prairie deer mice and white-footed mice (*Peromyscus maniculatus bairdii* and *P. leucopus*, respectively) were obtained from the University of South Carolina Genetic Stock Center and were housed at CSU. Prairie

voles *(Microtus ochrogaster)* were obtained from Thomas Curtis (University of Oklahoma) and were housed at CSU. Coyote brain (*Canis latrans*) was obtained from the National Wildlife Research Center Utah Field Station with the help of John Shivik and Stacey Brummer. Mink brain was a gift from Jason Bartz (Creighton University). Syrian Golden hamsters were obtained from Harlan Labs. Armenian and Chinese hamsters were a gift from Greg Raymond, Richard Race, Brent Race and Byron Caughey at the Laboratory of Persistent Viral Diseases, Rocky Mountain Veterinary Branch, NIAID, NIH. Cat brain was harvested from animals that were involved in other studies being conducted in the CSU pathogen-free facility and were made available by Sue VandeWoude. Prairie dogs were obtained from the Black-Footed Ferret Conservation Center, US Fish and Wildlife Service, thanks to Paul Marinari. Macaque monkey brain was obtained from Tulane Primate Research Center. Transgenic mice expressing human prion protein, referred to as Tg(HuPrP)6816$^{+/-}$ and Tg(HuPrP)7823$^{+/-}$ were housed at the University of Kentucky/Telling lab.

Preparation of brain homogenates. Normal-brain homogenates (NBH) were prepared as previously described (Kurt, Perrott et al. 2007).

CWD-positive brain homogenates were prepared as follows: (1) D10 was prepared from a CWD-infected mule deer (generously provided by Michael Miller, Colorado Division of Wildlife), and (2) 104 was prepared from a whitetailed deer experimentally infected with CWD source LAO1 (Colorado Division of Wildlife) and housed at CSU (Mathiason et al., 2006; Safar et al., 2008). The CWD-positive brain homogenates were prepared at a final concentration of 20% (w/v) and were not subjected to centrifugation.

Serial PMCA procedure. To eliminate possible contamination, NBH was thawed on ice and loaded into 96 well plates (TempPlate III, USA Scientific) in a laboratory that had never been used for prion research. The plate was then transported to the prion research laboratory where CWD-positive brain homogenate was diluted into the NBH to comprise a total volume of 50 µl (unseeded, NBH-only controls also comprised 50 µl). Non-amplified dilutions (–PMCA in figures) Ire frozen at -70°C for the duration of the experiment for comparison with amplified (+PMCA) samples. The PrP^{CWD} concentrations of –PMCA samples were equivalent to corresponding +PMCA samples *after* sPMCA. The plate was placed in a Misonix 3000 sonicator containing 200 mL distilled water, leaving 2-3 mm between the horn and the plate bottom. The plate was subjected to 40 s bursts at power level 7 followed by 30 min incubations at 37° C for 48 hr (this comprising 1 round of PMCA), and the samples were diluted 1:2 into fresh NBH for each new round (control dilutions were diluted identically at the start of the experiment and were then frozen, not amplified). These settings yielded the most efficient amplification of PrP^{CWD} in our experiments.

Electrophoresis and Istern blotting. After sPMCA, samples were digested with proteinase K (PK) (Invitrogen) before transfer and blotting. Due to innate differences in protease sensitivity of PrP^{C} between each substrate species, equivalent amounts of parallel non-seeded samples were used as a guide to assure complete PrP^{C} digestion. Samples using Tg(CerPrP)$1536^{+/-}$ mouse, prairie dog, mink, coyote and cat NBH as substrate were brought to a final SDS concentration of 0.25% prior to digestion with 100 µg/mL PK for 30 min at 37° C folloId by 10 min at 45° C. Ferret, Tg(HuPrP)$6816^{+/-}$ and Tg(HuPrP)$7823^{+/-}$ substrates were digested similarly but without incubation at 45° C.

Deer, hamster, vole, *Peromyscus* and BALB/c mouse substrate samples were digested with 100 μg/mL PK, and macaque with 50 μg/mL PK, for 30 min at 37^0 C. All samples (including unseeded, NBH-only controls) had a final volume of 10 μl after addition of PK.

Electrophoresis and transfer to PVDF membranes were performed as previously described (Kurt, Perrott et al. 2007). I screened each species to find optimal antibodies based on PrP^C detection (Table 2.1). Antibodies used for figures are as follows: For

Table 2.1. Antibodies used for the detection of PrP. Each species was screened to find antibodies for western blot detection of PrP. These antibodies were used in attempt to detect newly formed PrP^{RES} in trans-species sPMCA experiments.

Species	Antibodies that detect PrP
Deer and Tg(CerPrP)1536 mice	Bar224, 12F10, L42, SAF53, SAF54, SAF70, SAF83, SAF84, 8G8, 12B2, R30, R505
Ferret, mink	Bar224, 12F10, L42
Hamster	Bar224, SAF83, 3F4, 3O8
Prairie vole	Bar224, SAF83, SAF84
Peromyscus mice	SAF83
Mus mouse	SAF83, 6H4
Tg(HuPrP) mice	12F10, L42, 6H4
Cat	Bar224, L42, 3F4, 3O8, 7D9
Coyote	Bar224, 12F10, L42, SAF84, 7D9
Prairie dog	Bar224
Macaque	Bar224, 12F10, L42, 3F4, 3O8, 7D9

detection of PrP in Tg(CerPrP)$1536^{+/-}$, cat, coyote, ferret, prairie dog, mink, vole and deer samples, membranes were incubated in Bar224 mAb (a gift from Jacques Grassi, CEA,

Saclay, France) conjugated directly to horse radish peroxidase (HRP) for at least 1 h. For detection of hamster, *Peromyscus* and BALB/c *Mus* samples, membranes were incubated in mAb SAF83 (Cayman Chemical) for at least 1 h, washed several times, then incubated in HRP-labeled goat anti-mouse IgG secondary Ab (Jackson Labs) diluted 1:20000. For detection of PrP in Tg(HuPrP)6816$^{+/-}$ and Tg(HuPrP)7823$^{+/-}$ mouse samples, membranes were incubated in mAb 12F10 (Cayman Chemical) for at least 1 h, washed several times, then incubated in HRP-labeled goat anti-mouse IgG secondary Ab diluted 1:20000. Macaque samples were incubated in mAb 7D9 (Abcam) for at least 1 h, washed several times, then incubated in HRP-labeled goat anti-mouse IgG secondary Ab diluted 1:20000. All membranes were washed several times in dH_20 containing 0.2% Tween-20 before application of ECL-plus™ chemiluminescent reagents (Amersham). Data were generated using a digital Fuji-Doc™ gel documentation system (Fuji) with automated detection of saturation limits, and densitometric analyses were performed with ImageGauge™ quantification software. Successful amplification was indicated by increases in PrP^{CWD} relative to –PMCA samples or by the presence, after sPMCA, of protease-resistant bands in samples containing otherwise undetectable concentrations of PrP^{CWD}, concurrent with the absence of PrP^{CWD} in non-seeded, NBH-only control samples. I analyzed samples after 3 or 4 rounds of sPMCA and performed up to eight rounds of sPMCA in attempts to obtain conversion in those species that did not support PrP^{CWD} amplification. Data shown are representative of multiple experiments using 2-3 individuals of each species and one coyote.

PCR and *PRNP* sequencing. DNA was extracted from NBH of coyote, *Peromyscus* mice and prairie voles by addition of 500 µl chloroform-phenol isoamyl-alcohol (IAA) to 1 ml NBH. The samples were then inverted 10 times and centrifuged at 12,000x g for 5 minutes at room temperature before the aqueous layer was removed and combined with IAA and these steps repeated as described. The aqueous layer was then removed and mixed with 0.1 volumes of 3M sodium acetate, followed by 2.5 volumes of 100% ethanol. Samples were stored at -20^0 C for 48 h before centrifugation at 14,000x g for 30 min at 4^0 C, at which time the fluid was decanted and the pellet allowed to dry. The DNA was re-suspended in 50 µl of 1x TE buffer. For Polymerase Chain Reaction (PCR), the following forward and reverse primers were used for each species: (1) *Microtus ochrogaster*: GTGGAACAAGCCCAGTAAGCCAAA and ATGGTGATGTTGACGCAATCGTGC, ATGGACTGATGTGGGTCTCTGCAA and CGTGCACGAAGTTGTTCTGGTTGT, (2) *Peromyscus bairdii*: ACTCTTTGTGGCTACGTGGACTGA and ACGGGCCGATAGTACACTTGGTTA, (3) *P. leucopus*: ACTCTTTGTGGCTACGTGGACTGA and TGACTGTGTGCTGCTTGATGGTGA, (4) *Canis latrans*: GGTGAAAAGCCACATAGGCGGC and CAGCGAGATGAGGAGGATCACGG. PCR products Ire excised from the gel using Invitrogen PureLink Quick Gel Extraction Kit and were cloned using the TOPO TA Cloning Kit (K4500-01, Invitrogen). At least two clones of from each species were sequenced (Macromolecular Resources, Colorado State University) (GenBank accession nos. **FJ232956**, **FJ232957**, **FJ232958** and **FJ232959**) and then used to deduce amino acid sequence.

RESULTS

Species susceptible to CWD infection *in vivo*:

Deer, Tg(CerPrP) mouse, and ferret brain homogenates support PrP^{CWD} amplification. To determine whether *in vitro* PrP^{CWD} amplification is demonstrable in a susceptible species, I first performed sPMCA using normal-brain homogenates (NBH) from whitetailed deer (*Odocoileus virginianus*), a natural host for CWD. For these experiments, CWD-positive deer brain 104 was diluted 1:10 into NBH from whitetailed deer and subjected to sPMCA with 1:2 dilutions into fresh NBH at each successive round for a total of 4 rounds. I previously reported (Kurt et al., 2007), and here confirm, that deer brain homogenates support ~5 fold increases in PrP^{RES} in sPMCA (Fig. 2.1A, left panel), thus NBH from the native CWD-susceptible species will support PrP^{C}-to-PrP^{RES} conversion *in vitro*. I extended this work using NBH from Tg(CerPrP)$1536^{+/-}$ mice, which express cervid PrP^{C} at ~4-fold the concentration of that in deer brain. Using Tg(CerPrP)$1536^{+/-}$ NBH and the CWD-positive deer brain D10, PrP^{CWD} amplification was at least 100 to 250-fold per round of PMCA (Kurt et al., 2007) and amplification was consistently achieved with starting dilutions up to 1:16000, whereas the equivalent un-amplified dilutions (–PMCA samples) were not detectable by western blot (Fig. 2.1A, right panel).

To initiate trans-species sPMCA studies, I first used NBH from ferrets *(Mustela putorius furo),* a species that is susceptible to CWD (Bartz et al., 1998; Sigurdson et al., 2008a), as a PrP^{C} conversion substrate. Ferret NBH supported amplification at starting dilutions of up to 1:16000 of D10 (Fig. 2.1B).

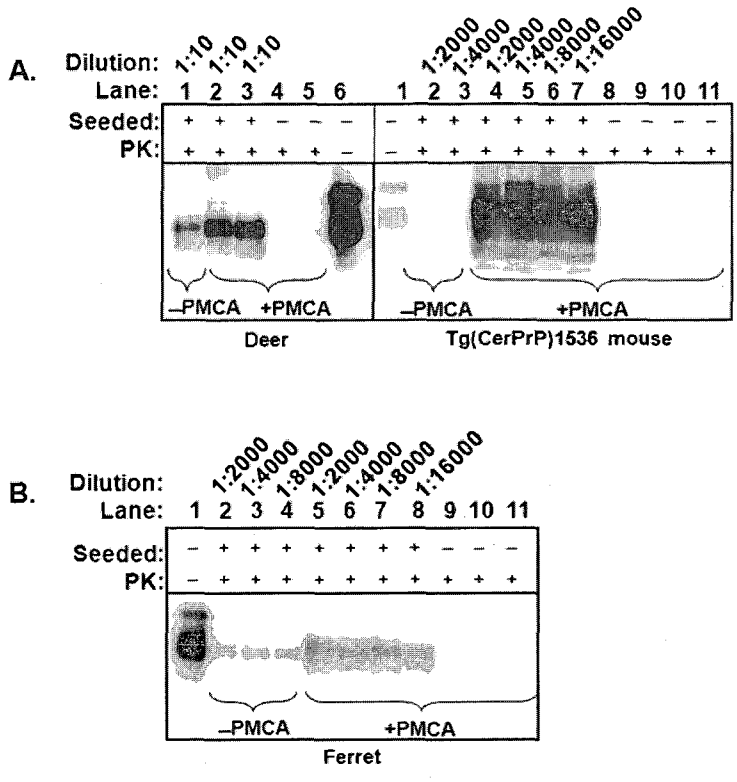

Fig. 2.1. NBH from deer, Tg(CerPrP)1536 mouse and ferret support amplification of PrP^{CWD}. A: left panel: Amplification of PrP^{CWD} in whitetailed deer NBH initiated by diluting CWD-positive brain deer brain 104 1:10 into the NBH. Serial PMCA was performed with 1:2 dilutions into fresh NBH at each subsequent round for a total of 4 rounds. Two replicates each of PrP^{CWD}-seeded (lanes 2-3) and unseeded (lanes 4-5) samples are shown. Lane 1: A dilution (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples (labeled +PMCA) after sPMCA. Lane 6: Deer NBH only, showing PrP^{C} not digested with PK. **A**: right panel: Amplification in Tg1536 NBH was initiated by 1:2000-1:16000 dilutions CWD-positive brain D10, followed by 1:2 dilutions into fresh NBH at each subsequent round for a total of 4 rounds. Lane 1: Tg1536 NBH only, showing PrP^{C} not digested with PK. Lanes 2-3: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples (labeled +PMCA) after sPMCA. **B:** Amplification in ferret NBH was accomplished by 1:2000-1:16000 dilutions of D10 and 4 rounds of sPMCA. Lane 1: Ferret NBH only, showing PrP^{C} not digested with PK. Lanes 2-3: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples (labeled +PMCA) after sPMCA. "Seeded": samples seeded (+) or not seeded (-) with CWD-positive brain homogenate, "PK": samples digested (+) or not (-) with proteinase K, "PMCA": samples subjected (+) or not (-) to the sPMCA protocol described.

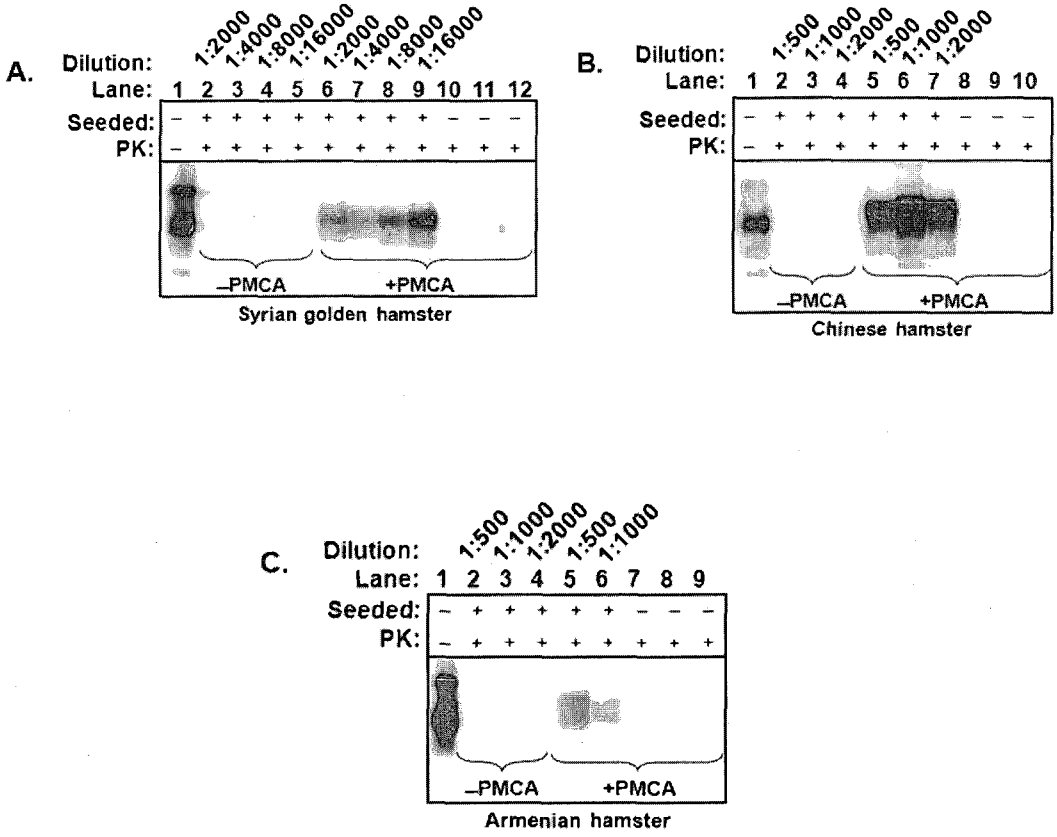

Fig. 2.2. NBH from Syrian golden, Chinese and Armenian hamsters support amplification of PrP^{CWD}. **A:** Amplification of PrP^{CWD} in Syrian golden hamster NBH was initiated by diluting CWD-positive brain D10 1:2000-1:16000 into the NBH. Serial PMCA for all hamsters was performed with 1:2 dilutions into fresh NBH at each subsequent round for a total of 4 rounds. Lanes 2-5: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. **B:** Amplification of PrP^{CWD} in Chinese hamster NBH was initiated by diluting CWD-positive brain D10 1:500-1:2000 into the NBH. Lanes 2-4: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. **C:** Amplification of PrP^{CWD} in Armenian hamster NBH was initiated by diluting CWD-positive brain D10 1:500-1:1000 into the NBH. Lanes 2-4: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. A, B and C, Lane 1: Hamster NBH only, showing PrP^{C} not digested with PK. "Seeded": samples seeded (+) or not seeded (-) with CWD-positive brain homogenate, "PK": samples digested (+) or not (-) with proteinase K, "PMCA": samples subjected (+) or not (-) to the sPMCA protocol described.

In control experiments, D10 added to PrP-null mouse ($PrP^{0/0}$) brain homogenate did not amplify, indicating that the majority of PrP^{C} which is converted in sPMCA comes from the NBH vs. the PrP^{CWD} seed material (not shown).

Species relatively less-susceptible to CWD infection *in vivo*:

Hamster brain homogenates have varying ability to support PrP^{CWD} amplification. Raymond *et al* (Raymond et al., 2007) have demonstrated that Syrian golden (*Mesocricetus auratus*), Chinese (*Cricetulus griseus*) and Armenian (*Cricetulus migratorius*) hamsters are variably susceptible to intracerebral inoculation of CWD. I have recently confirmed the *in vivo* susceptibility of Syrian golden hamsters to CWD (100% infected after inoculation with D10, Hoover lab, unpublished). To investigate differences in the ability of hamster species to support CWD amplification *in vitro*, I harvested NBH from Armenian, Chinese and Syrian golden hamsters for sPMCA. I found that in three experiments, Syrian golden hamster NBH supported amplification of 1:8000-1:16000 dilutions of PrP^{CWD} (Fig. 2.2A). Chinese hamster NBH consistently supported amplification of up to 1:2000 dilutions of mule-deer PrP^{CWD} (Fig. 2.2B) and Armenian hamsters supported amplification of up to ~1:1000 dilutions of D10 (Fig. 2.2C). NBH from Djungarian and Turkish hamster also supported amplification of PrP^{CWD} (Fig. 2.3). It is not known whether Turkish hamsters are susceptible to CWD *in vivo*.

Semi-quantitative estimation of PrP^{C} levels in NBHs from these hamsters revealed that PrP^{C} expression was relatively similar among the species (Fig. 2.4), and not predictive of ability to support sPMCA.

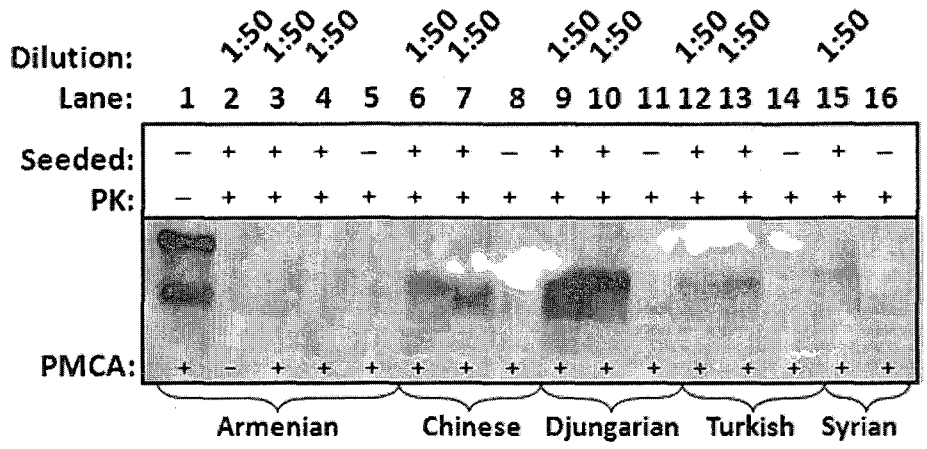

Fig. 2.3. NBH from Djungarian and Turkish hamsters also support amplification of PrP^{CWD}. Amplification of PrP^{CWD} in hamster NBH was initiated by diluting CWD-positive brain D10 1:50 into the NBH. Serial PMCA for all hamsters was performed with 1:2 dilutions into fresh NBH at each subsequent round for a total of 3 rounds. Lane 1: Hamster NBH only, showing PrP^{C} not digested with PK. Lane 2: Dilution (labeled PMCA –) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Each species NBH is denoted at bottom. "Seeded": samples seeded (+) or not seeded (-) with CWD-positive brain homogenate, "PK": samples digested (+) or not (-) with proteinase K, "PMCA": samples subjected (+) or not (-) to the sPMCA protocol described.

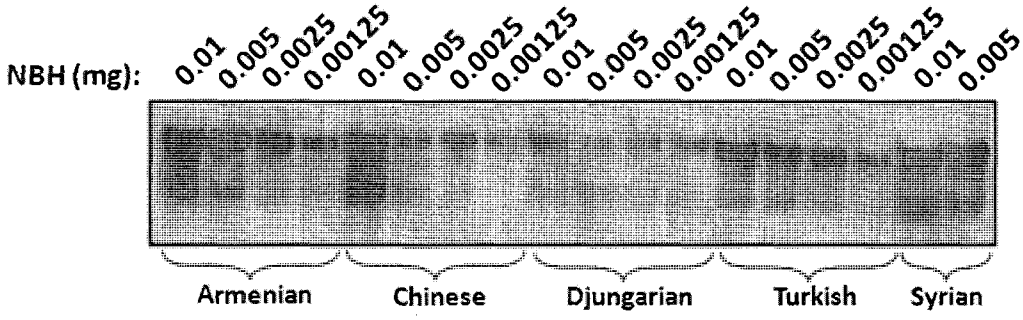

Fig. 2.4. Semi-quantitative estimation of PrP^{C} concentration in NBH from various hamster species. NBH from the hamster species used in sPMCA were analyzed by densitometry. Milligrams (mg) of NBH are indicated at top, species are denoted at bottom.

Mink brain homogenates did not support PrP^{CWD} amplification. American mink *(Mustela vison)* are closely related to ferrets and differ from the latter by very few residues in PrP amino acid sequence (Bartz et al., 1994), however, recent studies suggest that a relatively strong species barrier exists restricting CWD transmission to mink by even the intracranial route (Harrington et al., 2008). In our experiments mink NBH did not support amplification of PrP^{CWD} even when a high concentration (a 1:10 dilution) of D10 seed was used in order to provide as much seed material as possible (Fig. 2.5). Higher concentrations of D10 were not feasible due to the difficulty in distinguishing potentially new PrP^{RES} from input seed. In these experiments western blot PrP^{CWD} signals degraded with successive rounds of sPMCA (Fig. 2.5).

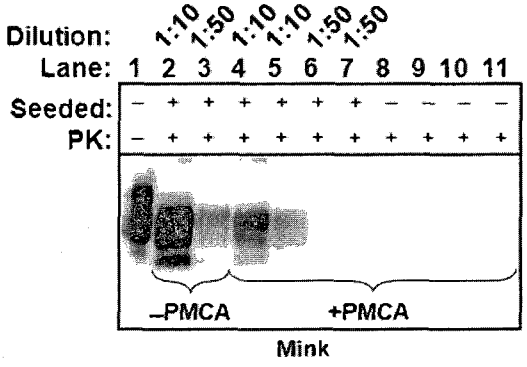

Fig. 2.5. Mink NBH does not support amplification of PrP^{CWD}. For attempts to amplify PrP^{CWD} in mink, CWD-positive brain was diluted 1:10 (lanes 4-5) or 1:50 (lanes 6-7) into the NBH and subjected to 3 rounds of sPMCA with 1:2 dilutions into fresh NBH at each subsequent round (final concentrations Ire 1:40 or 1:200, respectively). Lanes 2-3: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Mink NBH only, showing PrP^{C} not digested with PK.

Semi-quantitative estimation of PrP^C levels in ferret vs. mink revealed that PrP^C expression was relatively similar in these species (Fig. 2.6). Thus it is unlikely that the failure of mink NBH to support amplification of PrP^{CWD} is due only to PrP^C concentration in this species.

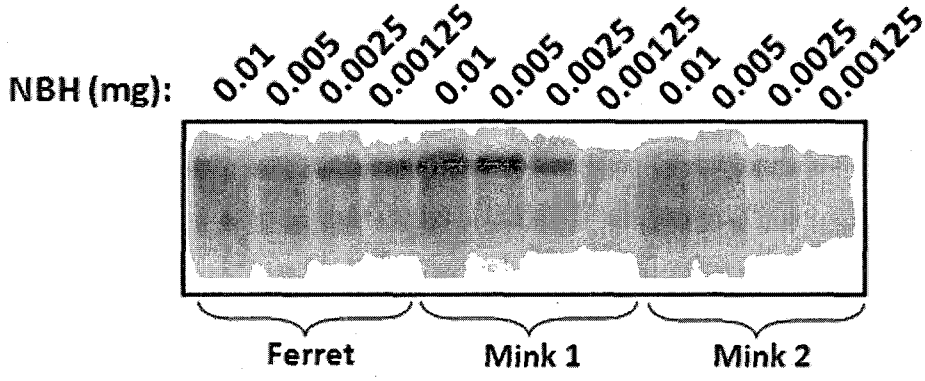

Fig. 2.6. Semi-quantitative estimation of PrP^C concentration in NBH from ferret and mink. NBH from one ferret and two mink used in sPMCA were analyzed by densitometry. Milligrams (mg) of NBH are indicated at top and species are denoted at bottom.

Species relatively resistant to CWD infection *in vivo*:

BALB/c mouse brain failed to support PrP^{CWD} amplification. Common laboratory mouse (*Mus*) strains are considered resistant to CWD (Browning et al., 2004; Raymond et al., 2007; Sigurdson et al., 2006). Therefore I evaluated NBH from BALB/c mice (selected as a common laboratory mouse strain expressing wild-type *Mus* PrP^C) as a potential negative control in trans-species sPMCA. BALB/c NBH did not

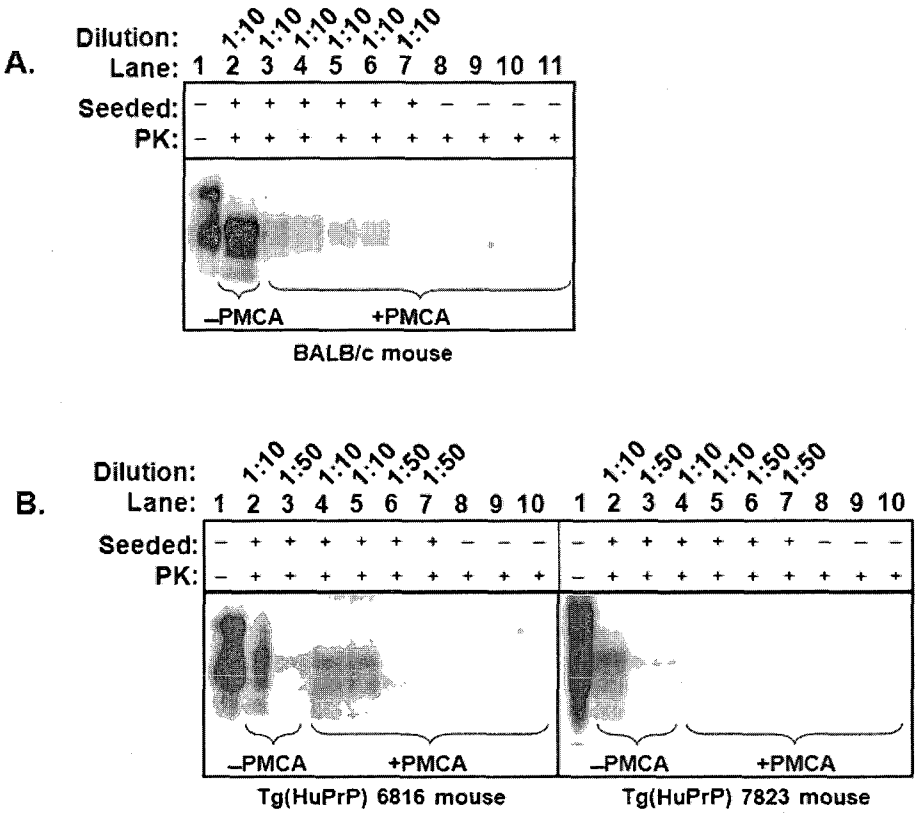

Fig. 2.7. NBH from BALB/c,Tg(HuPrP-M129), and Tg(HuPrP-V129) mice does not support amplification of PrP^{CWD}. A: For attempts to amplify PrP^{CWD} in BALB/c mice, CWD-positive brain was diluted 1:10 (4 replicates, lanes 3-6) into the NBH and subjected to 4 rounds of sPMCA. Lane 1: BALB/c mouse NBH only, showing PrP^{C} not digested with PK. Lane 2: A dilution (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. **B:** For amplification of PrP^{CWD} in Tg mice expressing human PrP, CWD-positive brain was diluted 1:10 (2 replicates, lanes 4-5) or 1:50 (2 replicates, lanes 6-7) into each NBH and subjected to 3 rounds of sPMCA. Lanes 2-3: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Tg(HuPrP) mouse NBH only, showing PrP^{C} not digested with PK.

support amplification of PrP^{CWD}, even when a 1:10 starting dilution of D10 seed was used (Fig. 2.7A). As with other NBH that did not support amplification, western blot PrP^{CWD} signals degraded with each successive round.

Human PrP transgenic mouse brain failed to support PrP^{CWD} amplification *in vitro*. I attempted to amplify PrP^{CWD} using NBH from two strains of transgenic mice hemizygous for transgenes expressing human PrP. Tg mice expressing human PrP encoding either M or V at codon 129 were generated in the Telling lab by microinjection of fertilized embryos from FVB/$Prnp^{0/0}$ mice. The resulting founders were mated to FVB/$Prnp^{0/0}$ mice to produce lines that were hemizygous for the transgene array. Expression of $HuPrP^{C}$ in the CNS of mice was examined by western blotting with mAb 6H4. Mice from the Tg(HuPrP-M129)$6816^{+/-}$ and Tg(HuPrP-V129)$7823^{+/-}$ lines expressed HuPrP in the CNS at approximately 16- and 5-fold the level of PrP in the brains of wild type mice. Neither strain NBH supported amplification of 1:10 or 1:50 starting dilutions of D10 (Fig. 2.7B).

Species for which CWD susceptibility is unknown:

Prairie dog brain homogenates failed to support PrP^{CWD} amplification. Prairie dogs *(Cynomys ludovicianus)* are ground-dwelling rodents prevalent in the Western United States, including within CWD-enzootic areas, and would likely be exposed indirectly to CWD. Prairie dog brain did not support PrP^{C}-to-PrP^{RES} conversion when seeded with 1:10 or 1:50 dilutions CWD-positive brain (Fig. 2.8A).

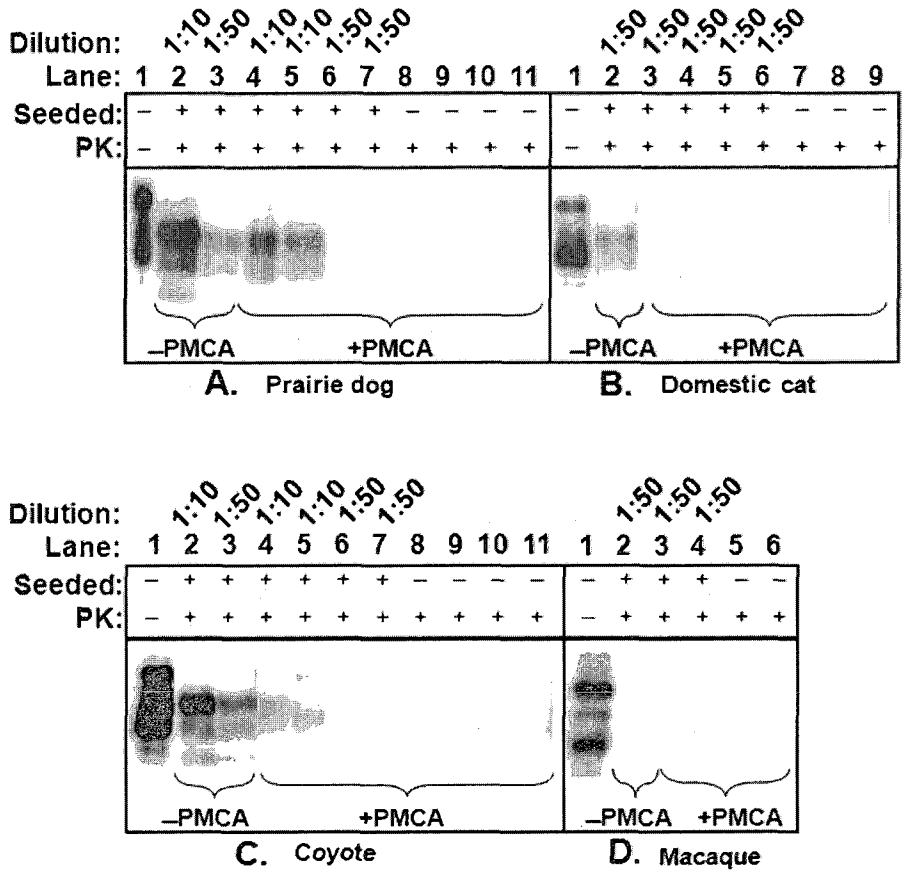

Fig. 2.8. Prairie dog, domestic cat, wild coyote and macaque monkey NBH do not support amplification of PrP^{CWD}. **A:** For attempts to amplify PrP^{CWD} in prairie dogs, CWD-positive brain was diluted 1:10 (2 replicates, lanes 4-5) or 1:50 (2 replicates, lanes 6-7) into the NBH and subjected to 4 rounds of sPMCA. Lanes 2-3: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Prairie dog NBH only, showing PrP^{C} not digested with PK. **B:** For the cat experiment shown (representative of 3 cats), CWD-positive brain was diluted 1:50 into the NBH and subjected to 8 rounds of sPMCA (4 replicates, lanes 4-7). Lane 2: A dilution (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Cat NBH only, showing PrP^{C} not digested with PK. **C:** For attempts to amplify PrP^{CWD} in coyote, CWD-positive brain was diluted 1:10 (2 replicates, lanes 4-5) and 1:50 (2 replicates, lanes 6-7) and subjected to 4 rounds of sPMCA. Lanes 2-3: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Coyote NBH only, showing PrP^{C} not digested with PK. **D:** For attempts to amplify PrP^{CWD} in macaque NBH, CWD-positive brain was diluted 1:50 and subjected to 4 rounds of sPMCA (2 replicates, lanes 3-4). Lane 2: A dilution (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: Macaque NBH only, showing PrP^{C} not digested with PK.

Domestic cat and coyote brain homogenates did not support PrP^{CWD} amplification. Canid and felid carnivore species may be directly exposed to CWD by predation and scavenging. Domestic cats (*Felis catus*), in addition to exotic felids, are susceptible to BSE, leading to cases of feline spongiform encephalopathy in several European countries (Pearson et al., 1992; Sigurdson and Miller, 2003; Wells and McGill, 1992). However, their susceptibility to CWD has not been determined. Interestingly, cat NBH did not support PrP^{CWD} amplification (Fig. 2.8B).

I harvested NBH from one coyote (*Canis latrans*) and performed trans-species sPMCA. Coyote NBH also did not support amplification of PrP^{CWD} (Fig. 2.8C). I subjected NBH that did not support sPMCA, such as from cat and coyote, to up to 8 rounds of PMCA and no change in results was produced (see cat, Fig. 2.8B).

Rhesus macaque brain homogenates did not support PrP^{CWD} amplification. At least one species of non-human primate is susceptible to CWD (Marsh et al., 2005). I obtained NBH from three rhesus macaques (*Macaca mulatta*) for trans-species sPMCA. Macaque NBH did not support support amplification of PrP^{CWD} (Fig. 2.8D).

Prairie vole and field mouse brain homogenates support PrP^{CWD} amplification. In our search for non-cervid species susceptible to CWD I examined PrP^{C}-to-PrP^{RES} conversion using NBH from several North American rodents. These studies were prompted in part by the work of Chandler *et al* (Chandler, 1971; Chandler and Turfrey, 1972) and Nonno *et al* (Nonno et al., 2006) demonstrating that field voles (*Microtus agrestis*) and bank voles (*Myodes glareolus*) are susceptible to scrapie. I therefore

assessed the prairie vole (*Microtus ochrogaster*), a common North American species, for its capacity to amplify PrP^{CWD} in trans-species sPMCA. In three experiments, I found that prairie vole NBH consistently supported amplification of PrP^{CWD} at starting dilutions of up to 1:50 to 1:100 D10 brain within four rounds of sPMCA (Fig. 2.9A). These results were similar to those obtained using ferret, Chinese hamster and Tg1536 mouse NBH,

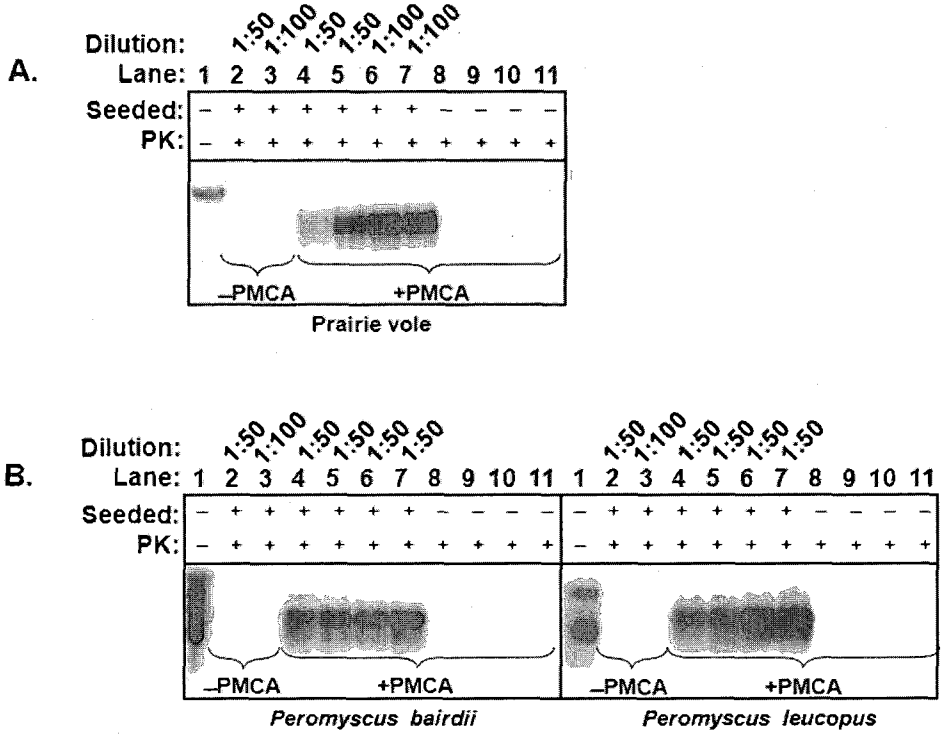

Fig. 2.9. NBH from prairie vole and *Peromyscus* mice support amplification of PrP^{CWD}. **A:** For amplification of PrP^{CWD} in prairie voles, CWD-positive brain was diluted 1:50 (2 replicates, lanes 4-5) or 1:100 (2 replicates, lanes 6-7) into the NBH and subjected to 3 rounds of sPMCA. Lanes 2-3: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: PrP^{C} not digested with PK. **B**: For amplification of PrP^{CWD} in *Peromyscus* mice, CWD-positive brain was diluted 1:50 (4 replicates, lanes 4-7) into the NBH and subjected to 3 rounds of sPMCA. Lanes 2-3: Dilutions (labeled –PMCA) frozen at -70°C for the duration of the experiment and equivalent to the amplified samples after sPMCA. Lane 1: PrP^{C} not digested with PK.

and suggested to us that prairie voles may be susceptible to CWD, a hypothesis I am currently testing in ongoing infectivity studies. Concurrently, I became aware of the work of Johnson, Heisey and colleagues who have reported ongoing *in vivo* studies indicating that other North American vole species are susceptible to CWD (C. Johnson, D. Heisey and colleagues, personal communication).

I next examined two species of common North American field mice, *Peromyscus leucopus* and *Peromyscus maniculatus bairdii,* by trans-species sPMCA. *P.m. bairdii* in particular has a geographic range that overlaps that of CWD-positive cervids (Baker, 1983). Both species of *Peromyscus* mice consistently supported amplification of dilutions of D10 up to 1:100 (Fig. 2.9B). Studies examining the *in vivo* susceptibility of *Peromyscus* mice to CWD are also in progress. Again separately, Heisey, Johnson and colleagues have gathered data that suggest both of these *Peromyscus* species are susceptible to CWD (C. Johnson, D. Heisey and colleagues, personal communication).

Cyclic amplification of PrP^{CWD} correlated with position 170 of the substrate species PrP.

Host PrP primary structure is associated with susceptibility to particular TSEs (Jewell et al., 2005; Laplanche et al., 1993; O'Rourke et al., 2004; Westaway et al., 1994). I compared the PrP primary structures of the animals I used for sPMCA (cloning of prairie dog *Prnp* has not yet been successful) in an effort to find amino acids that might help explain why NBH from certain species support sPMCA while others do not. Of particular interest was the loop between the β2 strand and α2 helix (PrP residues 166-175), which is more stable in elk than mice (Gorfe and Caflisch, 2007; Gossert et al.,

2005) and may contribute to the transmission barrier between these species (Sigurdson et al., 2008b). Of the species I studied, all of those that express asparagine at PrP position 170 (i.e. Tg(CerPrP)1536 mouse, *Peromyscus* mouse, prairie vole, Syrian, Chinese and Armenian hamster) supported amplification of PrP^{CWD} by sPMCA (Fig. 2.10). In contrast, only one (ferret) of the eight species (ferret, *Mus* mouse, two strains of Tg(HuPrP) mouse, cat, coyote, mink, macaque) that express serine at position 170 supported amplification of PrP^{CWD} (Fig. 2.10), indicating that 170N facilitates PrP^{CWD} amplification. I did not find correlations at any other PrP amino acid positions (Fig.2.11) and ability to support sPMCA did not correlate with degree of sequence similarity (Fig.2.12).

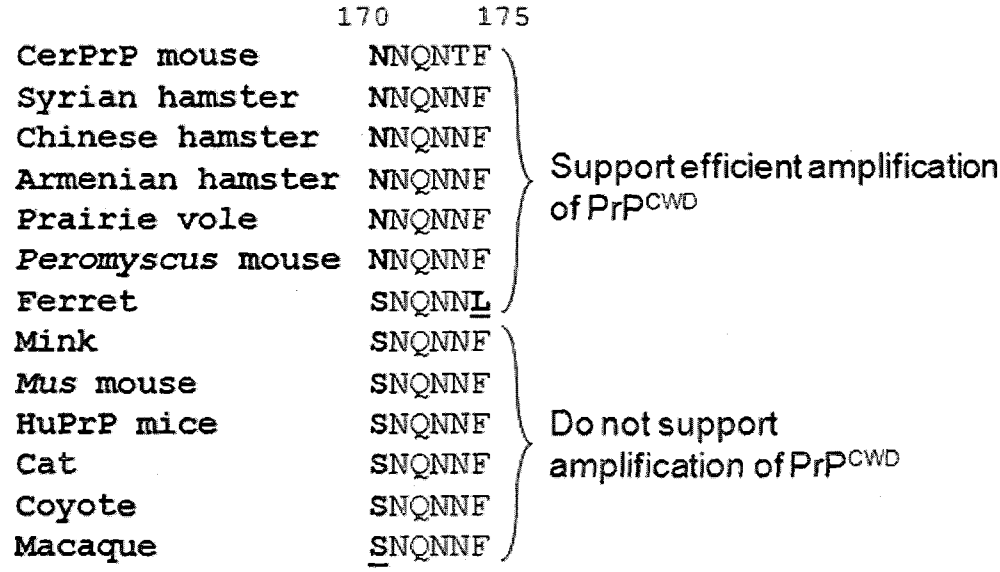

Fig. 2.10. Trans-species amplification of PrP^{CWD} correlated with position 170 of the substrate species PrP. PrP sequence 170-175 (part of the β2-α2 loop) of the species used for NBH in trans-species sPMCA. All the species tested expressed asparagine (N) or serine (S) at position 170. The ferret sequence encodes leucine (L) at position 175.

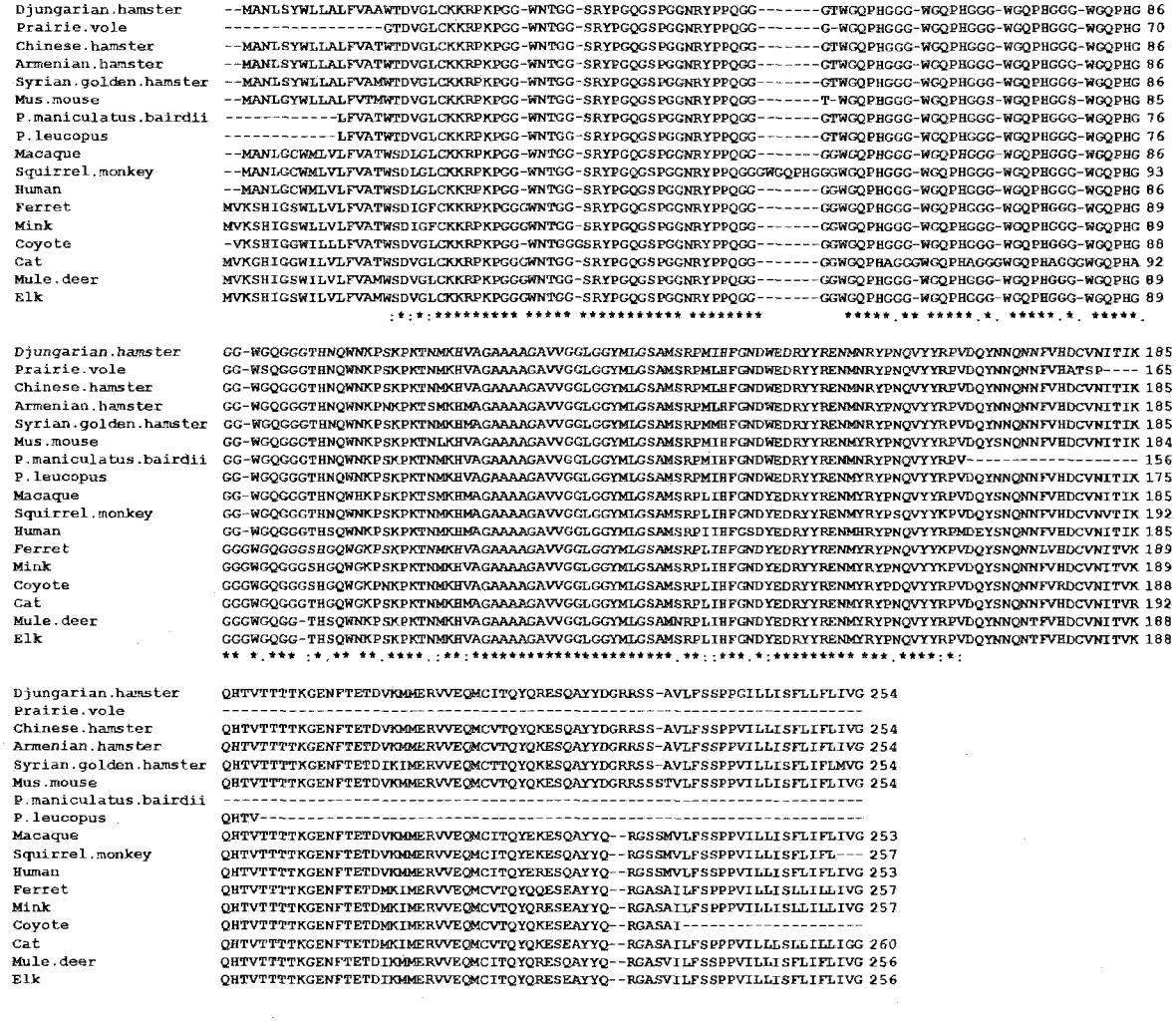

Fig. 2.11. Full PrP sequence alignment for all species. The PrP amino acid sequences for all species used as NBH in sPMCA are shown, as well as two CWD-susceptible species not used in sPMCA [elk and squirrel monkey (Marsh et al., 2005)]. Only partial sequence data are available for some species (prairie vole, *Peromyscus* mice, coyote). (*) all amino acid residues in that column are identical, (:) conserved substitutions are present, (.) semi-conserved substitutions are present, (-) residue gap or data absent.

	Similarity Score
Mule.deer > Elk	99
Mule.deer > Cat	91
Mule.deer > Coyote	90
Mule.deer > Syrian.golden.hamster	87
Mule.deer > Chinese.hamster	87
Mule.deer > Armenian.hamster	86
Mule.deer > Djungarian.hamster	87
Mule.deer > P.maniculatus.bairdii	92
Mule.deer > P.leucopus	92
Mule.deer > Ferret	91
Mule.deer > Mink	92
Mule.deer > Mus.mouse	86
Mule.deer > Human	89
Mule.deer > Macaque	89
Mule.deer > Squirrel.monkey	87
Mule.deer > Prairie.vole	89

Fig. 2.12. Similarities among PrP amino acid sequences for all species. The PrP amino acid sequences for all species (plus elk and squirrel monkey) were compared using ClustalW2. Similarity scores were calculated based on whether amino acids in the same position are identical, absent, and whether substitutions are conserved or semi-conserved.

DISCUSSION

Here I demonstrate that CWD prions can be amplified in normal-brain homogenates (NBH) from several outbred species whose habitat overlaps with that of free-ranging cervids. My findings complement and extend recent studies that suggest sPMCA results may correlate with susceptibility to prion diseases (Castilla et al., 2008; Green et al., 2008). I have begun *in vivo* analyses to assess whether prairie voles (*Microtus* spp.) or field mice (*Peromyscus* spp.) are susceptible to CWD infection and whether species adaptation occurs via trans-species sPMCA. The recent findings of Green *et al* and Castilla *et al* provide support for the plausibility of the latter (Castilla et al., 2008; Green et al., 2008). Moreover, reports from several investigators provide

evidence that PrP^{RES} generated by sPMCA is infectious (Castilla et al., 2008; Castilla et al., 2005; Deleault et al., 2007; Green et al., 2008).

In the present study, sPMCA experiments were considered positive if they resulted in the generation of new protease-resistant material in PrP^{CWD}-seeded (+PMCA) samples but not in non-PrP^{CWD}-seeded (–PMCA) samples. Accurate calculation of comparative amplification efficiency using non-cervid NBH can be complicated by differences in the antibody affinity and PK sensitivity of cervid (our PrP^{CWD} source) vs. non-cervid prion proteins. To avoid this potential pitfall I attempted to estimate relative amplification efficiency by finding the dilutions of input PrP^{CWD} which could consistently be amplified within 3-4 rounds of sPMCA. I performed up to eight rounds of sPMCA, used different CWD-positive seed sources (D10 and 104), and used several detection antibodies in attempts to obtain conversion in species that did not support PrP^{CWD} amplification, but this did not result in PrP^{C} conversion in those species. I performed confirmatory western blots to ensure that our findings could be replicated and interpreted consistently. These assays increased our confidence that all PrP^{RES} generated in our experiments was protease-resistant and that all NBH PrP^{C} was digested and did not contribute to signal as background.

The detectable signal of input PrP^{CWD} (–PMCA) samples in species that did not support sPMCA is due to the high concentration of starting material used in attempt to initiate sPMCA in these species. For example, I used a 1:10 input dilution of PrP^{CWD} in attempts to obtain conversion in BALB/c mice and HuPrP mice (Fig. 2.4). The final dilution of this sample after 4 rounds of PMCA was 1:80 (Round 1 = 1:10, Round 2 = 1:20, Round 3 = 1:40, Round 4 = 1:80), which is detectable by western blot without

PMCA (see –PMCA samples). The samples subjected to PMCA (+PMCA) sometimes exhibit a weaker western blot signal than predicted based on the –PMCA samples, which is indicative of a failure to support amplification. The deterioration of signal is most likely caused by the increased exposure of PrP^{CWD} to proteinase K (PK) following physical separation of PrP^{CWD} aggregates (Piening et al., 2005). Proteolysis during PMCA is unlikely given that protease inhibitors were added to all homogenates. I have observed deterioration of PrP^{RES} signals when using NBH from species known to be TSE-resistant, such as transgenic *Prnp*$^{0/0}$ mice (not shown). The variability in input PrP^{CWD} signals between blots is explained by the different concentrations of PK used for each species (due to innate differences in PrP^{C} sensitivity and the need to completely digest NBH PrP^{C}) and the different antibodies used for western blotting (e.g. deer and BALB/c mouse samples were both digested with 100 µg/mL PK, but different antibodies were used for detection).

Our ongoing *in vivo* studies should yield data to aid in correlating amplification efficiency *in vitro* with susceptibility *in vivo*, an obviously critical issue. Thus far I have several non-cervid species (transgenic CerPrP mice, ferrets, hamsters, wild-type *Mus* mice, transgenic HuPrP mice, *Peromyscus* mice) in which the *in vivo* and *in vitro* results have generally correlated. However, *in vivo* and *in vitro* species barriers to conversion may be affected by prion strain, infectious titer of the inoculum, the genetic background of the recipient species, and PrP^{C} expression levels. One of our PrP^{CWD} sources (D10) amplified in NBH from several hamster species, which is consistent with the higher attack rates of this inoculum in Syrian golden hamsters (100%, Hoover lab, unpublished) than reported with other CWD inocula (Raymond et al., 2007). Corresponding *in vivo*

studies in many non-cervid species (e.g. felids, canids) would require lengthy observation periods before definitive conclusions regarding susceptibility could made.

PrP^C concentration has also been shown to play a role in determining the ability to support amplification of PrP^{CWD} (Kurt et al., 2007). I estimated the PrP^C concentration of brain homogenates from several species by western blot and did not find differences that could explain our results. Some of the species I used, e.g. the transgenic HuPrP mice, express high levels of PrP^C and still did not support amplification.

PrP sequence also influences the efficiency of *in vitro* prion conversion and species barriers to disease transmission (Bossers et al., 1997; Nonno et al., 2006; Piening et al., 2006; Priola, Chabry, and Chan, 2001). I sequenced the PrP gene from each of the species examined (cloning of prairie dog *Prnp* has not yet been successful) for which published data were not available, deduced the primary structures, and compared these with published sequences for the other species. The amino acids present at position 222 (225 in deer) and 92 (96 in deer), both of which are associated with susceptibility to CWD in cervids (Jewell et al., 2005; O'Rourke et al., 1999; O'Rourke et al., 2004), did not correlate with ability or inability to support amplification.

I then examined the L1 loop (residues 166-175), which is more stable in elk than mice primarily due to the presence of asparagine rather than serine at position 170 (Gorfe and Caflisch, 2007; Gossert et al., 2005). Of the animals I used, all of those that express asparagine at PrP position 170 (i.e. Tg(CerPrP)1536 mouse, *Peromyscus* mouse, prairie vole, Syrian, Chinese and Armenian hamsters) supported amplification of PrP^{CWD} by sPMCA. In contrast, only one (ferret) of the eight animals (laboratory mouse, both Tg(HuPrP) mice, cat, coyote, ferret, mink, macaque) that express serine at position 170

supported amplification of PrP^{CWD}. The mobility of the loop is influenced by other residues such as Phe175 (Gorfe and Caflisch, 2007), and the unique presence of leucine, rather than phenylalanine (and the corresponding phenyl ring), at position 175 of ferret PrP may allow greater stability in the L1 loop. This might explain the greater PrP^{C}-to-PrP^{CWD} conversion observed when I used ferret NBH, despite the presence of Ser170. Asparagine and serine are both hydrophilic amino acids, and position 170 would be solvent-exposed (Fig. 2.8) according to the NMR structure published by Wuthrich and colleagues (Gossert et al., 2005). Likewise, leucine, present in ferret at position 175, and phenylalanine, present in all other species at position 175, are both hydrophobic. The loop region, including residues 170 and sheep polymorphism 171, is thought play a role in determining PrP conversion/susceptibility in several species (Bossers et al., 1997; Christen et al., 2008; Gorfe and Caflisch, 2007; Piening et al., 2006; Sigurdson et al., 2008b; Westaway et al., 1994). I did not find correlations with any other PrP amino acid positions, including 155 (Piening et al., 2006; Priola, Chabry, and Chan, 2001), nor with the degree of sequence similarity in comparison to deer PrP. Similarity scores were generated using ClustalW2 and reflect whether amino acids in the same position are identical, absent, and whether substitutions are conserved or semi-conserved. Although only partial sequences were obtained for several species, the majority of the missing residues lie within the N-terminal and C-terminal signal sequences which are removed during processing *in vivo*.

Certain other species which are susceptible to CWD, such elk (*Cervus elaphus*) and squirrel monkey (*Saimiri sciureus*), also express asparagine at PrP position 170. It is likely that NBH from these species would support amplification of PrP^{CWD} by sPMCA.

In contrast, sheep and cattle express serine at 170, and in preliminary studies (not shown) did not support sPMCA. To further investigate the role of position 170 and the rest of the L1 loop in determining ability to support PrP^{C}-to-PrP^{CWD} conversion, one could generate recombinant PrP with various amino acid substitutions in the L1 region. *Mus* mice engineered to express 170N and 174T are susceptible to CWD, suggesting that these residues control susceptibility (Sigurdson et al., 2008b). It would be interesting to generate *Mus* mice expressing 175L, like ferret, for sPMCA and *in vivo* susceptibility studies. More detailed NMR studies regarding the effects of 175L on loop stability might yield additional information on how the L1 region affects PrP^{C}-to-PrP^{CWD} conversion.

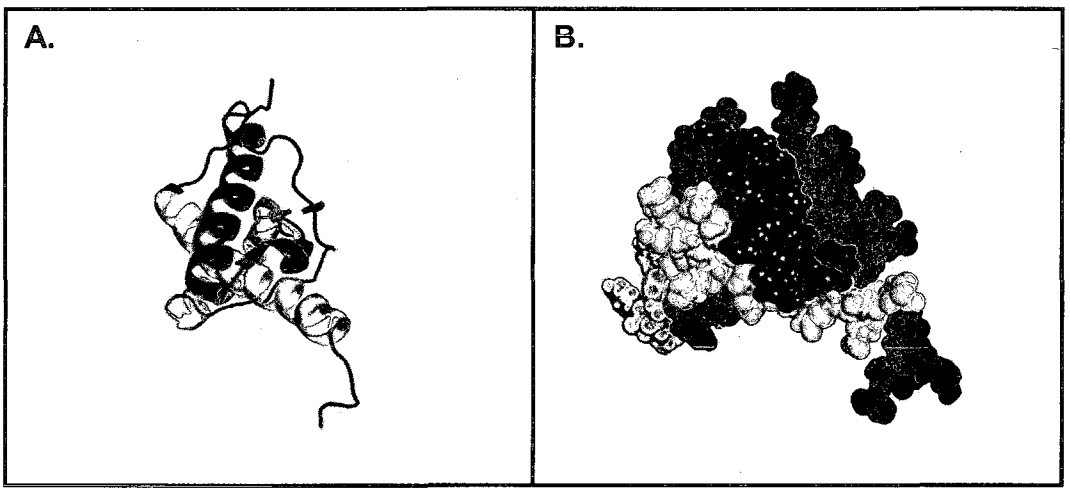

Fig. 2.8. Ribbon and space-filling diagrams of the globular domain of elk PrP^{C}. A: Ribbon model of the elk PrP^{C} structure illustrating the position of the L1 loop, residues 166-175 (red). **B:** Space-filling model of elk PrP^{C} illustrates the position of residue 170 (red). Carbohydrates not shown. Cyan residues: helix one, pink residues: helix two, yellow residues: helix 3, blue residues: β-strands and unstructured regions. Models created with PyMOL using PDB code 1XYW (Gossert et al., 2005).

In our experiments, mink brain homogenates did not support sPMCA even though this species exhibits some susceptibility to CWD *in vivo* (Harrington et al., 2008). This clearly supports the premise that other factors in the brain and periphery *besides* PrP sequence (Deleault et al., 2005; Deleault, Lucassen, and Supattapone, 2003; Sigurdson et al., 2002; Sigurdson et al., 1999) affect susceptibility *in vivo*. This premise is supported by studies that show host genetic background affects TSE susceptibility (Lloyd et al., 2001) and demonstrates that extrapolations from negative *in vitro* conversion results should be made with caution.

This study demonstrates successful trans-species sPMCA using a diverse range of animal species. Many of these findings corroborate data from other studies. For instance, Bartz *et al* (Bartz et al., 1998) and Sigurdson *et al* (Sigurdson et al., 2008a) demonstrated that ferrets are susceptible to CWD *in vivo*. I found that ferret NBH supported amplification of high dilutions of input PrP^{CWD}. Raymond *et al* (Raymond et al., 2007) demonstrated that hamsters are variably susceptible to CWD *in vivo*. I found that the capacity of hamster NBH to amplify PrP^{CWD} depended on the species: Syrian golden and Chinese hamster NBH supported robust amplification of PrP^{CWD}, and Armenian hamster NBH supported minimal amplification. As might have been anticipated, wild-type laboratory mouse *(Mus)* NBH did not support sPMCA, just as these animals fail to support CWD amplification *in vivo* (Browning et al., 2004; Raymond et al., 2007; Sigurdson et al., 2006; Williams and Young, 1980). Finally, I was not able to amplify PrP^{CWD} using NBH from HuPrP transgenic mice, a result which is consistent with findings from the *in vivo* studies of Kong *et al* (Kong et al., 2005) and Tamguney *et al* (Tamguney et al., 2006) using transgenic mice expressing human PrP^{C}.

I am mindful that susceptibility to prion disease is affected by dose, route of inoculation, host lifespan and other factors, and therefore is difficult to quantify. Although our sPMCA results (with the exception of mink) were consistent with *in vivo* studies demonstrating susceptibility or resistance to CWD in the same species, *in vivo* studies are still the gold standard for determining TSE susceptibility. Thus I have initiated *in vivo* studies in a subset of species that support sPMCA as described here, in an effort to explore whether sPMCA can actually be used to predict *in vivo* susceptibility.

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CHAPTER 3

Trans-species Amplification and Enhanced Transmission of CWD Prions

ABSTRACT

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy of cervids now present in much of the US and Canada. Whether non-cervid species could be occasionally infected or serve as CWD reservoirs or vectors is not known. I previously demonstrated *in vitro* amplification of CWD prions in normal brain homogenates from various species including prairie voles (*Microtus ochrogaster*), an indigenous North American rodent. Here I show that prairie voles are somewhat susceptible to CWD from mule-deer. Inoculation of prairie voles with CWD prions amplified in prairie vole brain homogenate by serial protein misfolding cyclic amplification (sPMCA) resulted in shorter and more consistent incubation periods. Immunohistochemical analysis revealed an altered pattern of CWD prion deposition in infected voles in comparison to infected transgenic (CerPrP) mice. These results suggest that sPMCA accelerates and enhances CWD transmission to non-cervid species.

BACKGROUND

Chronic Wasting Disease (CWD) is a fatal transmissible spongiform encephalopathy (TSE) of cervids, including deer, elk and moose. CWD is the only TSE found in free-ranging wildlife; its rapid spread in nature may reflect the potential transmission of prions from saliva and excreta of infected cervids (Haley et al., 2009; Mathiason et al., 2006; Miller and Williams, 2003; Safar et al., 2008) and/or the persistence of infectious prions in the environment (Johnson et al., 2007; Miller et al., 2004; Seidel et al., 2007). While the known natural host range for CWD is limited to cervids, recent studies have expanded the number of non-cervid species, e.g. ferrets, mink, and hamsters, that can be infected experimentally (Bartz et al., 1998; Harrington et al., 2008; Raymond et al., 2007; Sigurdson et al., 2008). It is not known whether non-cervid species may contribute to the spread of CWD as reservoirs or vectors. Inter-species transmission of prion diseases (e.g. from deer to non-cervids) is frequently less efficient than intra-species transmission, a TSE transmission phenomenon commonly referred to as the species barrier. Species barriers may be mediated by differences in PrP^C sequence, strain conformation (Raymond et al., 2007), dose, route of inoculation, and other still unknown factors (Bartz et al., 1994; Harrington et al., 2008; Kong et al., 2005; Piening et al., 2006; Raymond et al., 2000).

I recently demonstrated (Kurt et al., 2009) that PrP^C from several non-cervid species, e.g. prairie voles (*Microtus ochrogaster*) and prairie deer mice (*Peromyscus maniculatus bairdii*), can be converted to PrP^{CWD} *in vitro* by serial Protein Misfolding Cyclic Amplification (sPMCA). To determine whether these species might be

susceptible to CWD *in vivo*, and whether sPMCA affects inter-species TSE transmission, I compared prairie voles inoculated with PrP^{CWD} generated in sPMCA experiments with prairie voles inoculated with PrP^{CWD} from CWD-infected mule-deer.

MATERIALS AND METHODS

Transgenic mice encoding cervid PrP. Transgenic mice expressing cervid PrP were generated in the Telling lab (Browning et al., 2004) and have been used previously in sPMCA and CWD studies (Green et al., 2008; Kurt et al., 2007; Kurt et al., 2009; Meyerett et al., 2008).

Sources and preparation of brain homogenates for sPMCA. Donor animals were housed and euthanized according to ACUC approved protocols. $Tg(CerPrP)1536^{+/-}$ mice (Browning et al., 2004) were housed at Colorado State University (CSU). Prairie voles *(Microtus ochrogaster)* were obtained from Thomas Curtis (University of Oklahoma) and were housed at CSU. Prairie deer mice (*Peromyscus maniculatus bairdii*) were obtained from the University of South Carolina Genetic Stock Center and were housed at CSU. Normal brain homogenates (NBH) were prepared as previously described (Kurt, Perrott et al. 2007). CWD-positive brain homogenate from deer D10 (D10) was prepared from a CWD-infected mule deer (generously provided by Michael Miller, Colorado Division of Wildlife). D10 has been used previously in several CWD bioassay and sPMCA studies (Browning et al., 2004; Kurt et al., 2007; Mathiason et al., 2006; Meyerett et al., 2008).

Serial (s)PMCA procedure. To eliminate possible contamination, NBH was thawed on ice and loaded into 96 well plates (TempPlate III, USA Scientific) in a laboratory that was never used for prion research. The plate was then transported to the prion research laboratory where CWD-positive brain homogenate was diluted into the NBH to comprise a total volume of 50 µl (unseeded, NBH-only controls also comprised 50 µl). Non-amplified dilutions (–PMCA in figures) were frozen at -70°C for the duration of the experiment and used for western blot comparison with amplified (+PMCA) samples. The plate was placed in a Misonix 3000 sonicator containing 200 mL distilled water leaving 2-3 mm between the horn and the plate bottom, and subjected to 40 s bursts at power level 7 followed by 30 min incubations at 37° C for 48 hr (this comprising 1 round of PMCA), and the samples were diluted 1:2 into fresh NBH for each new round. Control dilutions were diluted identically at the start of the experiment and then frozen, not amplified. These settings yielded the most efficient amplification of PrP^{CWD} in our experiments.

Inocula. For intracerebral (IC) inoculations, 10% (w/v) brain homogenate samples containing 1% Triton X-100 Ire diluted 1:10 in sterile saline containing Pen/Strep (100 U/mL) to reduce detergent concentration to 0.1%. Thus animals were inoculated intracerebrally with 25 µl of 1% (w/v) brain homogenate using a Kendall 3/10 mL insulin syringe inserted into the left parietal lobe. Prior to inoculation, animals were sedated using ketamine/xylazine anesthesia according to approved ACUC protocols. Upon termination, the brain hemisphere contralateral (right) to the inoculation site was removed and fixed for histology, and the other hemisphere (left) frozen for western blot analysis. Male and female animals in all studies were inoculated between one and five months old.

Animals Ire monitored weekly for the appearance of neurological dysfunction (ataxia, hind-limb paresis, inactivity or hyperactivity), and daily upon detection of any of these symptoms.

Histology and Immunohistochemistry. Upon removal, hemi-brains were fixed in 10% formalin for at least 48 hrs. Each was then cut into six coronal sections of equal width, treated with 88% formic acid for 1 hr, rinsed continuously with water for at least 2 hrs and embedded in paraffin. Paraffin-embedded sections (6 μm) were mounted onto positively charged glass slides by the Colorado State Veterinary Diagnostic Laboratory. Slides were then heated at 65^0 C for 45 min, deparaffinized, and rehydrated through graded ethanol. Antigen retrieval was performed using a Retriever™ system and DAKO Target Retrieval Solution (DAKO, Hamburg, Germany). Tissues were blocked in 3% methanol for 1 hr followed by TNB buffer (Perkin-Elmer) for 1 hr, and stained using Tyramide Signal Amplification (Perkin-Elmer). Tg1536 mouse tissues were stained for PrP using HRP-conjugated monoclonal antibody Bar224 at a 1:500 dilution, and vole tissues were stained using SAF83 (Cayman Chemical) at a 1:500 dilution followed by HRP-conjugated goat anti-mouse secondary antibody diluted 1:500. PrP^{CWD} was visualized by incubation in AEC+ (DAKO) for 30 min with hematoxylin counterstain, and images were then captured using an Olympus Vanox-S microscope (Olympus) with DP70 digital camera.

Electrophoresis and western blotting. Electrophoresis and western blotting of sPMCA samples was performed as previously described (Kurt et al., 2009). Briefly, for *in vivo* studies, brains were removed and one hemisphere homogenized (10% w/v) in PBS

containing 1% Triton X-100 before centrifugation at 2000 x g for 1 min and removal of the supernatant for analysis by western blotting. For western blotting, samples using Tg(CerPrP)1536$^{+/-}$ mouse brain homogenate were brought to a final SDS concentration of 0.25% prior to digestion with 100 µg/mL proteinase K (PK) for 30 min at 37° C followed by 10 min at 45° C. Vole brain samples were digested with 100 µg/mL PK for 30 min at 37^{0} C. Samples had a final volume of 10 µl after addition of PK, and were boiled in 0.33% NLS buffer (Invitrogen) for 3 minutes after PK digestion.

Electrophoresis and transfer to PVDF membranes were performed as previously described (Kurt et al., 2007; Kurt et al., 2009). After transfer, membranes were incubated in blocking solution (0.5% nonfat dry milk in PBS with .05% Tween-20) and antibodies using the Millipore Snap-ID system. All membranes were incubated in Bar224 mAb (a gift from Jacques Grassi, CEA, Saclay, France) conjugated directly to horse radish peroxidase (HRP). All membranes were washed using dH20 containing 0.2% Tween-20 before application of ECL-plus™ chemiluminescent reagents (Amersham). Data were generated using a digital Fuji-Doc™ gel documentation system (Fuji) with automated detection of saturation limits, and densitometric analyses were performed using ImageGauge™ quantification software.

RESULTS

A species barrier limits transmission of CWD from mule-deer to prairie voles. To investigate whether prairie voles (*Microtus ochrogaster*) might be susceptible to CWD, I inoculated animals (in cohorts of 9) with 25 µl of 1% (w/v) CWD-infected mule-deer (D10) brain homogenate by the intracerebral (IC) route. The D10 brain homogenate has been used in several previous bioassay and sPMCA studies (Browning et al., 2004; Kurt et al., 2007; Kurt et al., 2009; Meyerett et al., 2008). Negative control animals were inoculated IC with brain homogenate from a CWD-negative deer. None of the negative control animals developed symptoms of neurological disease within 550 days post-inoculation (dpi). One of the 9 voles inoculated with D10 brain homogenate developed clinical symptoms of neurological disease, including hyperactivity, ataxia, and hind-limb paresis, and was euthanized at 463 dpi. Immunohistochemistry (IHC) revealed PrP^{CWD} deposition throughout the brain of this animal, with greatest accumulation in the thalamus (Fig. 3.1A). Some cell-associated PrP^{CWD} was observed in the hippocampus. Large plaques were not observed. Spongiform degeneration, with vacuoles ~5-20 µm in diameter, appeared to generally colocalize with PrP^{CWD} deposition. Purkinje cell loss was noted in sections of cerebellar cortex (Fig. 3.1A). These IHC data contrasted with those from Tg(CerPrP)1536 mice (see Fig. 3.5B, middle row), in which PrP^{CWD} is found primarily as plaques in the hippocampus. PrP^{CWD} was easily detected in brain homogenate from this animal by western blot (Fig. 3.1B)

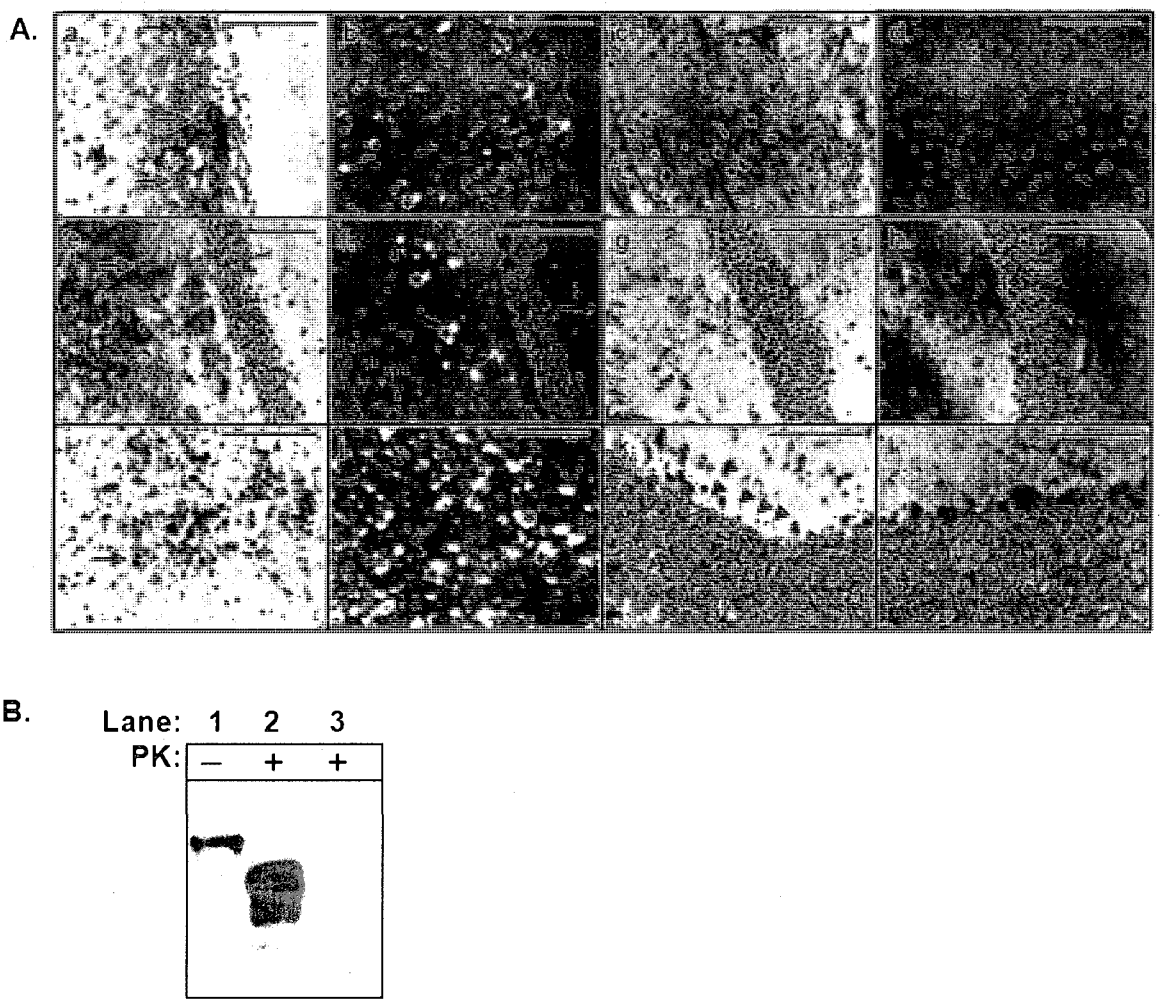

Fig. 3.1. Voles inoculated with CWD from mule deer accumulate PrP^{RES} in the CNS. A: Sections of brain tissue from a vole inoculated with brain homogenate from a CWD-positive deer and euthanized at 463 dpi (a, b, e, f, i, j, k), and a vole inoculated with brain homogenate from a CWD-negative deer (c, d, g, h, l), Ire subjected to immunohistochemistry (a, c, e, g, i) or hematoxylin/eosin (b, d, f, h, j, k, l) stain. Brain sections shown correspond to thalamus (a-d), hippocampus (e-h), unidentified brain region (i, j), and cerebellum (k, l). Arrows indicate PrP^{RES}; asterisks (*) indicate vacuoles; arrowheads (panel k only) indicate loss of Purkinje cells. Scale bars = 100 μm. B: Western blot of brain homogenate from a vole inoculated with brain homogenate from a CWD-positive deer and euthanized at 463 dpi (lanes 1, 2) and a vole inoculated with brain homogenate from a CWD-negative deer (lane 3). Samples were either treated with proteinase K ("PK+"), or not ("PK—").

sPMCA abrogates the species barrier and enhances CWD infection in prairie voles. I previously demonstrated that normal brain homogenates (NBH) from prairie voles support amplification of mule-deer CWD prions by sPMCA [see Chapter 2, (Kurt et al., 2009)]. To investigate whether sPMCA can be used to generated infectious prions *in vitro*, and whether sPMCA alters CWD strain properties, I inoculated prairie voles with PrP^{RES} generated by amplification of D10 in vole NBH by sPMCA. This resulted in new PrP^{RES} material I refer to as vole PMCA-PrP^{RES} (VPMCA-PrP^{RES}). Briefly, to generate VPMCA-PrP^{RES}, D10 was diluted 1:200 into vole NBH and amplified for 9 rounds of sPMCA with 1:2 dilutions into fresh NBH at each round. The final concentration of D10 in VPMCA-PrP^{RES} was 1:51200 after 9 rounds of sPMCA and 1:512000 after the material was diluted 1:10 for IC inoculation. I used densitometric analysis to estimate the total PrP^{RES} concentration of VPMCA-PrP^{RES} relative to D10, and found that it was equivalent to ~1:600 dilution of D10 (Fig. 3.2).

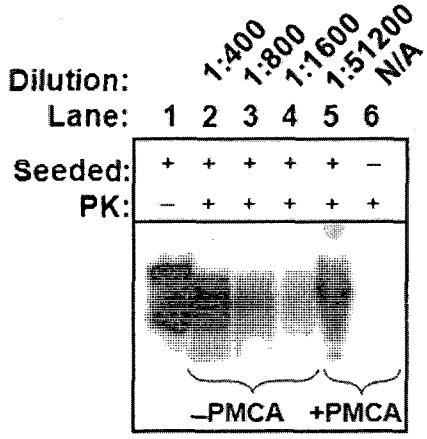

Fig. 3.2. Amplification of mule-deer CWD prions in prairie vole NBH. To generate vole PrP^{RES} by sPMCA (VPMCA-PrP^{RES}), I diluted D10 1:50 into vole NBH and amplified it for nine rounds of sPMCA. The final dilution of D10 in the vole NBH after nine PMCA rounds was 1:51200, and densitometric analysis revealed the PMCA product to be equivalent to a 1:600 dilution of D10.

VPMCA-PrP^{RES} would have been equivalent to a 1:6000 dilution of D10 after 1:10 dilution for IC inoculation of voles.

All nine voles inoculated with VPMCA-PrP^{RES} developed symptoms of neurological disease including rapid movement, ataxia and frequent rolling into dorsal recumbency with difficulty regaining normal, sternal posture (Fig. 3.3).

Fig. 3.3. Clinical TSE in prairie voles inoculated with VPMCA-PrP^{RES}. Among neurological signs such as ataxia and hind-limb paresis, prairie voles frequently rolled into dorsal recumbency (animal in upper left) and had difficulty regaining normal, sternal posture. Animal in lower right is in normal orientation.

Upon euthanasia, the presence of PrP^{CWD} in voles was confirmed by western blotting (Fig. 3.4A) and IHC (Fig. 3.4B). PrP^{CWD} was evident throughout the brain in voles inoculated with VPMCA-PrP^{RES}, with greatest accumulation in the thalamus. Prion deposits were primarily diffuse and granular, and vacuoles were ~5-20 μm in diameter. In contrast, Tg1536 mice had less PrP^{CWD} accumulation in the thalamus and greater

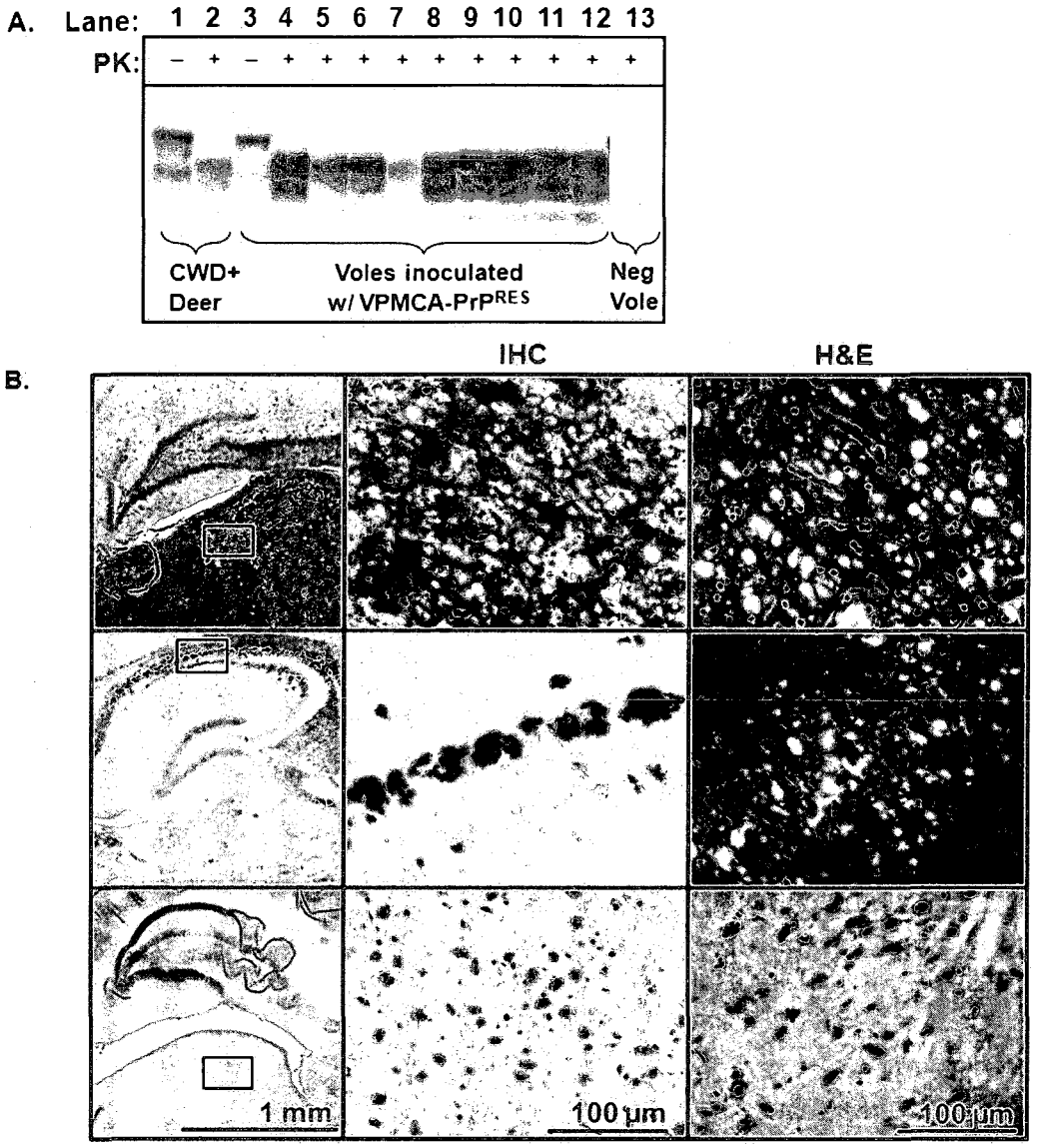

Fig. 3.4. Confirmation of vole CWD infection by western blot and immunohistochemistry (IHC). A: 10 ul of 10% brain homogenates were analyzed by western blot. Voles inoculated with VPMCA-PrP^{RES} are indicated. CWD-positive deer brain (lanes 1-2) is provided for reference. "Neg vole" consisted of brain material from a vole inoculated with brain homogenate from a CWD-negative deer. **B:** Top row: brain sections from a sick vole inoculated with VPMCA-PrP^{RES}, middle row: brain sections from a sick Tg(CerPrP)1536 mouse inoculated with D10, bottom row: brain sections from a vole inoculated with brain homogenate from a CWD-negative deer. Hippocampus and thalamus can be seen in column at left. Inset denotes area of detail in middle column. Brain sections in left and middle columns developed by immunohistochemistry. Brain sections in right column stained with hematoxylin/eosin. Scale bars valid for each column.

staining in the hippocampus, including large plaque-like deposits up to ~50 μm in diameter. Negative control animals were inoculated with unseeded NBH that had been subjected to an equivalent number of sPMCA rounds, or D10 diluted 1:512000 or with brain from a CWD-negative deer. None of the animals in these groups demonstrated clinical signs of prion disease within 550 days, or the presence PrP^{RES} in brain tissue by post-mortem analysis (Fig. 3.4). All voles inoculated with VPMCA-PrP^{RES} were euthanized by 239 ± 17 dpi (Fig. 3.5). In contrast, the nine Tg1356 mice inoculated IC with a 1:500 dilution of D10 were euthanized by 259 ± 20 dpi (Fig. 3.5). These differences in incubation period were statistically significant (t-test, $p < 0.05$).

DISCUSSION

In this study I demonstrate that CWD prions (PrP^{CWD}) generated *in vitro* by sPMCA are infectious. All nine voles inoculated IC with a 1:512000 dilution of D10, after sPMCA, developed clinical signs of CWD and were positive for PrP^{CWD} by western blot and IHC by 239 ± 17 dpi, whereas voles inoculated with a 1:512000 dilution of D10 not subjected to sPMCA developed neither clinical signs of CWD nor detectable levels of PrP^{CWD} in the CNS. I also demonstrate that *in vitro* amplification of PrP^{CWD} in non-cervid, i.e. prairie vole, brain homogenates enhances transmission of CWD to that species, given that prairie voles inoculated with VPMCA-PrP^{RES} developed CWD more rapidly than Tg(CerPrP)1536 mice (239 ± 17 dpi vs. 259 ± 20 dpi, respectively, $p < 0.05$). This was true despite the lower PrP^{RES} titer of the VPMCA-PrP^{RES} inoculum, determined using densitometry analysis to be equivalent to a 1:6000 dilution of D10,

whereas Tg(CerPrP)1536 mice received a 1:500 dilution of D10. Thus, voles received 1000-fold less D10 than the Tg(CerPrP)1536 mice, and 10-fold less total PrP^{RES}, yet still succumbed to disease more rapidly than the Tg(CerPrP)1536 mice. It is important to note that PrP^{C} levels in the CNS of Tg(CerPrP)1536 mice are 4-5 times higher than deer (Kurt et al., 2007) and wild-type FVB mice (Browning et al., 2004) and that these animals are considered highly susceptible to CWD (Browning et al., 2004; Haley et al., 2009; Meyerett et al., 2008).

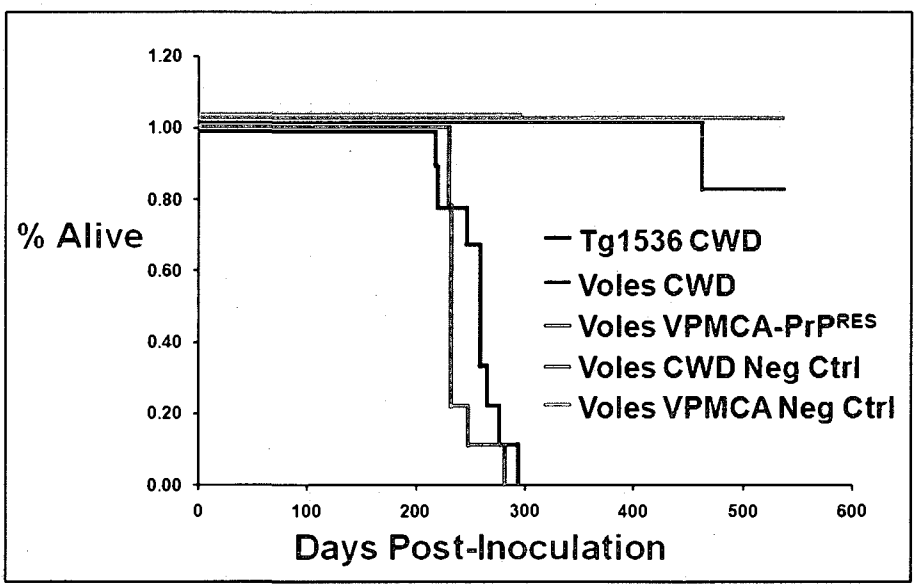

Fig. 3.5. Prairie voles inoculated IC with VPMCA-PrP^{RES} develop neurological signs more rapidly than Tg(CerPrP)1536 mice inoculated with D10. Nine voles inoculated with VPMCA-PrP^{RES} (red) equivalent to a 1:6000 dilution of D10 were euthanized by 239 ± 17 dpi. In contrast, the nine Tg(CerPrP)1536 mice inoculated with a 1:500 dilution of D10 (blue) were euthanized at 259 ± 20 dpi. One vole inoculated with a 1:500 dilution of D10 (black) was euthanized at 463 dpi, the rest are alive at 550 dpi. Negative controls (green) are still alive at 550 dpi. All animals were confirmed positive for PrP^{RES} by western blot and immunohistochemistry. Animals that were euthanized due to non-prion related disease were not included the graphs, and were confirmed negative for PrP^{RES} by standard immuno-assays.

The pattern of PrP^{CWD} deposition in the CNS of CWD-infected prairie voles was distinct from that in deer and CerPrP mice (Browning et al., 2004; Fox et al., 2006; Sigurdson, 2008; Spraker et al., 2002; Williams and Young, 1980). It is unlikely this represented the original D10 inoculum given that animals were injected in the left parietal lobe and the right brain hemisphere was used for IHC analysis. Furthermore, PrP^{CWD} accumulation and spongiform degeneration were observed in regions of the brain distant from the inoculation site, indicating replication and spread of prions. PrP^{CWD} accumulation in prairie vole brains was highest in the thalamus, with less accumulation in the hippocampus, cerebral cortex, and granular layer of the cerebellar cortex. Deposits were generally granular and diffuse, and no large plaques were observed. Purkinje cell loss was noted in the cerebellar cortex, although PrP^{CWD} deposition in the Purkinje layer was not evident. In contrast, CWD-infected Tg(CerPrP)1536 mice displayed high levels of PrP^{CWD} in the hippocampus, with less accumulation in the thalamus and cerebellum. PrP^{CWD} deposits in the Tg1536 mice typically occurred as large, patchy plaques. No loss of Purkinje cells was noted in CWD-infected Tg(CerPrP)1536 mice, however this may be due to the lack of PrP^{C} expression by Purkinje cells in this transgenic mouse strain (Fischer et al., 1996; Rossi et al., 2001). The differences in neuropathological features between CWD-infected voles and Tg(CerPrP)1536 mice are consistent with the alterations in strain properties sometimes observed upon trans-species passage of CWD and other TSEs (Raymond et al., 2007; Tamguney et al., 2009; Yokoyama et al., 2009). Control experiments will be performed to confirm that these differences in pathology are due to disease and not due to differences in PrP^{C} expression patterns between Tg1536 mice and voles.

The unique pathologic features of infected voles also provide some insights into prion disease mechanisms. Purkinje cells were lost, yet no PrP^{RES} accumulation was observed in the Purkinje cell layer, suggesting that the neurodegeneration occurred due to altered or dysfunctional cell-signaling and not cellular PrP^{RES} accumulation. Each Purkinje cell is thought to receive excitatory input from the parallel fibers of over 150,000 glutaminergic granule cells via AMPA receptors (Barbour, 1993). Furthermore, Purkinje cells are very susceptible to excitotoxic degeneration via AMPA receptor stimulation and this is thought to play a role in prion-related neurodegeneration (Brorson et al., 1995; Scallet and Ye, 1997). In my studies, diseased voles displayed significant PrP^{RES} accumulation in the granule cell layer of the cerebellum but not in the Purkinje or molecular layers, suggesting that Purkinje cell death is due altered signals resulting in excitotoxic degeneration. Thus, this work suggests that cell death due to PrP^{RES} accumulation may occur by an indirect mechanism.

Whether passage of CWD in prairies voles has resulted in a new CWD strain is not yet known and will require serial sub-passage in voles, and possibly back-passage in Tg(CerPrP)1536 mice. The pathologic appearance of CWD in prairie voles is unique but shares some features with fatal familial insomnia, e.g. thalamic neuron degeneration (Manetto et al., 1992; Parchi et al., 1995), as well as kuru and some types of CJD, e.g. Purkinje cell loss, granule cell loss and PrP^{RES} accumulation in the thalamus (Armstrong et al., 2009; McLean, 2008; Watanabe and Duchen, 1993). Interestingly, certain strains of PrP-deficient mice (Ngsk, Zürich II and Rcm0) exhibit marked Purkinje cell death, although this phenotype appears to be due to over-expression of the PrP-like Doppel

protein vs. the absence of PrP^C expression in these cells (Anderson et al., 2004; Rossi et al., 2001; Sakaguchi et al., 1996; Silverman et al., 2000).

Castilla et al. (Castilla et al., 2008) recently demonstrated that mouse prions could be amplified in normal brain homogenate (NBH) substrate from hamsters, and vice versa, and that this was followed by enhanced transmission to the species used for substrate. Similarly, Green et al. (Green et al., 2008) demonstrated amplification of mouse scrapie in NBH from Tg(CerPrP)1536 mice followed by transmission of the amplified scrapie prions to Tg(CerPrP)1536 mice. Our study confirms these findings using CWD prions and prairie voles, an out-bred species with geographic range that encompasses CWD-endemic areas (Baker, 1983; Wilson and Reeder, 2005). It is not yet known whether prairie voles or other indigenous rodent species may be occasionally infected with CWD or serve as reservoirs or vectors for CWD infection in cervids.

I previously demonstrated that NBH from other non-cervid species (e.g. *Peromyscus* spp. mice) support PrP^C amplification (Kurt et al., 2009), and investigations into CWD-susceptibility in these species are ongoing. I am also interested in whether sPMCA enhances transmission by the oral route in prairie voles and other non-cervid species, which could provide a mechanism by which studies that require serial sub-passages can be hastened.

In this study I have shown that an indigenous North American rodent, the prairie vole, is somewhat susceptible to CWD. Amplification of CWD prions by sPMCA enhanced the transmission of CWD to prairie voles and accelerated adaptation, indicated by a more rapid and consistent incubation period. These studies usually require serial

sub-passage and may be time-consuming and expensive. The sPMCA procedure may accelerate trans-species transmission studies and thus offer unique insight into transmission barriers and species susceptibility.

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Conclusion

In the preceding chapters I demonstrated the successful *in vitro* amplification of Chronic Wasting Disease prions (PrP^{CWD}) with and without sonication. I obtained ~4-fold increases when PrP^{CWD} from mule-deer was amplified in normal brain homogenate (NBH) from whitetailed deer without sonication. However, I obtained ≥ 200-fold increases when PrP^{CWD} was amplified in Tg(CerPrP)1536 NBH by serial protein misfolding cyclic amplification (sPMCA). Efficient amplification was associated with the higher PrP^{C} concentration of Tg(CerPrP)1536 vs. deer NBH. Ultimately, the increased PrP^{CWD} detection sensitivity obtained with PMCA may allow *in vitro* detection of CWD prions in biological fluids from infected animals.

I have also demonstrated that NBH from several CWD-susceptible non-cervid species, including hamsters and ferrets, support amplification of PrP^{CWD} by sPMCA. In contrast, NBH from several CWD-resistant species, including *Mus* mice and transgenic (HuPrP) mice, did not support PrP^{CWD} amplification. These results correlated well with *in vivo* susceptibility or resistance in these species. Some species (e.g. mink) may be somewhat susceptible to CWD but not support amplification, thus extrapolations from unsuccessful sPMCA experiments must be made with caution. For now, bioassays remain the gold standard in determining susceptibility to CWD *in vivo*. I expanded the

number of species used in sPMCA to several present in CWD-endemic areas and thus potentially exposed to CWD, including coyotes, prairie voles, *Peromyscus* spp. mice, and prairie dogs. Many of these species had not been used in sPMCA before. I found that NBH from coyotes, prairie dogs, cats, and macaques did not support amplification. Interestingly, NBH from prairie voles and *Peromyscus* spp. mice supported robust amplification of PrP^{CWD}. It is not yet known whether these species may be occasionally infected in nature.

To investigate whether PrP^{C} sequence differences are associated with the ability to support PrP^{CWD} amplification, I sequenced the *Prnp* gene from all of the species used in sPMCA for which this information was not already published. I then deduced and compared the PrP primary structures. Ability to support amplification correlated strongly with the presence of asparagine (Asn), but not serine (Ser), at position 170 of substrate PrP. PrP position 170 is located in the L1 loop (residues 166-175), between the β2 strand and α2 helix, a region that is more stable in elk than mice (Gorfe and Caflisch, 2007; Gossert et al., 2005) and may contribute to the CWD transmission barrier between these species (Sigurdson et al., 2008). Of the species I studied, all that express asparagine at PrP position 170 (i.e. Tg(CerPrP)1536 mouse, *Peromyscus* mouse, prairie vole, Syrian, Chinese and Armenian hamster) supported amplification of PrP^{CWD} by sPMCA. In contrast, only one (ferret) of the eight species (ferret, *Mus* mouse, two strains of Tg(HuPrP) mouse, cat, coyote, mink, macaque) that express serine at position 170 supported amplification of PrP^{CWD}, indicating that 170Asn facilitates PrP^{CWD} amplification. I did not find correlations at any other PrP amino acid positions.

In all of our sPMCA experiments, I found that newly-formed PrP^{CWD} resembled the seed material (mule-deer brain D10) in western blot migration patterns. This suggests that prion strain may have an important role in determining certain physico-chemical properties of prions created *in vitro*, such as proteinase K cleavage sites and which PrP^{RES} glycoforms are dominant. Alternatively, these properties may be determined less by the PrP^{RES} seed than the presence of compatible PrP^{C} species in the NBH.

Finally, I demonstrated that PrP^{CWD} generated *in vitro* is infectious and that sPMCA enhances trans-species transmission. Voles inoculated intracerebrally (IC) with a 1:512000 dilution of D10, after sPMCA, developed clinical signs of CWD and were positive for PrP^{CWD} by western blot and IHC by 239 ± 17 dpi, whereas voles inoculated with a 1:512000 dilution of D10 not subjected to sPMCA, or unseeded NBH subjected to sPMCA, developed neither clinical signs of CWD nor detectable levels of PrP^{CWD} in the CNS. Interestingly, prairie voles inoculated with PrP^{CWD} generated by sPMCA developed CWD more rapidly than Tg1356 mice (239 ± 17 dpi vs. 259 ± 20 dpi, respectively), even though the prairie vole inoculum had a lower PrP^{RES} titer (equivalent to a 1:6000 dilution of D10 vs. 1:500 for Tg1536 mice). Thus, voles received 10-fold less total PrP^{RES}, yet succumbed to disease more rapidly than the Tg1536 mice. These results suggest that sPMCA accelerated CWD adaptation to prairie voles and enhanced transmission to this non-cervid species.

The pattern of PrP^{CWD} deposition in the CNS of CWD-infected prairie voles was distinct from that seen in deer and transgenic (CerPrP) mouse models of CWD (Browning et al., 2004; Fox et al., 2006; Sigurdson, 2008; Spraker et al., 2002; Williams

and Young, 1980). PrP^{CWD} accumulation in prairie vole brains was highest in the thalamus, with less accumulation in other brain areas such as the hippocampus and cerebral cortex. Deposits were generally granular and diffuse, and large plaques were absent. Thalamic neuron degeneration was evident and Purkinje cell loss was noted in the cerebellar cortex, although no PrP^{CWD} deposition in the Purkinje layer was noted. In contrast, CWD-infected Tg(CerPrP)1536 mice displayed more PrP^{CWD} accumulation in the hippocampus and less accumulation in the thalamus. PrP^{CWD} deposits in Tg1536 mice typically occurred as large patchy plaques. The differences in neuropathological features between CWD-infected voles and Tg1536 mice are consistent with alterations in prion strain properties sometimes observed upon trans-species transmission of TSEs (Raymond et al., 2007; Tamguney et al., 2009; Yokoyama et al., 2009). The changes in PrP^{CWD} accumulation pattern, in addition to the more rapid and consistent incubation periods in voles, are suggestive of a new CWD strain. Serial sub-passage in voles will be necessary to confirm these results. It is possible that prairie vole-CWD also has a altered host range in relation to mule-deer CWD. Whether sPMCA enhances transmission by the oral route in prairie voles and other non-cervid species remains unknown but under investigation.

It would be advantageous to investigate the ability of non-central nervous system (CNS) tissues and cell types to support amplification by sPMCA. Non-CNS tissue substrates could constitute a more physiologically-relevant model of peripheral prion replication, and thus shed light on susceptibility to CWD infection by natural routes of exposure. Recent advances using recombinant PrP^{C} as a substrate for sPMCA (Atarashi et al., 2007) also warrant investigation. It is not known whether recombinant cervid PrP^{C}

would support amplification of PrP^{CWD}, but this methodology may offer high PrP^{CWD} detection sensitivity and consistency without the need for crude-brain homogenates.

In conclusion, I have shown that brain homogenates from both cervids and non-cervids can support sPMCA, that amplification is correlated with 170Asn in the substrate PrP^{C}, and that sPMCA accelerates adaptation of CWD to an indigenous North American rodent, the prairie vole. The use of an *in vitro* assay to model prion autocatalytic replication and susceptibility to TSEs is a novel and thought-provoking alternative to otherwise lengthy and expensive *in vivo* studies. It will be interesting to see what the future holds for this methodology and the field of CWD research at large.

Fin

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