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Deer Prion Proteins Modulate the Emergence and Adaptation of Chronic Wasting Disease Strains

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ABSTRACT

Transmission of chronic wasting disease (CWD) between cervids is influenced by the primary structure of the host cellular prion protein (PrP^C). In white-tailed deer, *PRNP* alleles encode the polymorphisms Q95 G96 (wild type [wt]), Q95 S96 (referred to as the S96 allele), and H95 G96 (referred to as the H95 allele), which differentially impact CWD progression. We hypothesize that the transmission of CWD prions between deer expressing different allotypes of PrP^C modifies the contagious agent affecting disease spread. To evaluate the transmission properties of CWD prions derived experimentally from deer of four *PRNP* genotypes (wt/wt, S96/wt, H95/wt, or H95/S96), transgenic (tg) mice expressing the wt allele (tg33) or S96 allele (tg60) were challenged with these prion agents. Passage of deer CWD prions into tg33 mice resulted in 100% attack rates, with the CWD H95/S96 prions having significantly longer incubation periods. The disease signs and neuropathological and protease-resistant prion protein (PrP-res) profiles in infected tg33 mice were similar between groups, indicating that a prion strain (Wisc-1) common to all CWD inocula was amplified. In contrast, tg60 mice developed prion disease only when inoculated with the H95/wt and H95/S96 CWD allotypes. Serial passage in tg60 mice resulted in adaptation of a novel CWD strain ($H95^+$) with distinct biological properties. Transmission of first-passage tg60CWD-$H95^+$ isolates into tg33 mice, however, elicited two prion disease presentations consistent with a mixture of strains associated with different PrP-res glycotypes. Our data indicate that H95-*PRNP* heterozygous deer accumulated two CWD strains whose emergence was dictated by the PrP^C primary structure of the recipient host. These findings suggest that CWD transmission between cervids expressing distinct PrP^C molecules results in the generation of novel CWD strains.

IMPORTANCE

CWD prions are contagious among wild and captive cervids in North America and in South Korea. We present data linking the amino acid variant Q95H in white-tailed deer cellular prion protein (PrP^C) to the emergence of a novel CWD strain ($H95^+$). We show that, upon infection, deer expressing H95-PrP^C molecules accumulated a mixture of CWD strains that selectively propagated depending on the *PRNP* genotype of the host in which they were passaged. Our study also demonstrates that mice expressing the deer S96-*PRNP* allele, previously shown to be resistant to various cervid prions, are susceptible to $H95^+$ CWD prions. The potential for the generation of novel strains raises the possibility of an expanded host range for CWD.

Chronic wasting disease (CWD) is an emerging prion disease or transmissible spongiform encephalopathy (TSE) of cervids, affecting free-ranging white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), elk (*Cervus elaphus canadensis*), and moose (*Alces americanus*) (1, 2). CWD occurs in captive herds of these species in North America and in red deer (*Cervus elaphus*) and sika deer (*Cervus nippon*) in South Korea (1, 3, 4). Reindeer (*Rangifer tarandus*), also known as caribou, are susceptible to experimental infection (5).

TSEs are slowly progressive, fatal neurodegenerative disorders for which no effective treatment or vaccine is available. Neuropathological changes include prion protein deposits, spongiform degeneration, neuronal loss, and astrogliosis. These hallmarks are diagnostic for CWD in cervids, scrapie in sheep and goats, bovine spongiform encephalopathy (BSE), as well as kuru, iatrogenic Creutzfeldt-Jakob disease (iCJD), and variant Creutzfeldt-Jakob disease (vCJD) in humans (6–10).

The pathogenesis of TSEs is associated with misfolded prion protein (PrP^{Sc}; or PrP^{CWD} for cervid infections), whose ability to propagate, persist, and trigger neuropathology requires the expression of host *PRNP*-encoded cellular prion protein (PrP^C). Prion propagation involves the posttranslational misfolding of normal cellular PrP molecules into pathognomonic, transmissible, generally protease-resistant prion protein (PrP-res) conformers that progressively accumulate in brain and other tissues (11–14). The primary structure of PrP^C influences host susceptibility to infection, its disease progression, and its neuropathological and

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biochemical profiles (15–23). Knockout (*Prnp*) mice are refractory to experimental infection with mouse-adapted scrapie (24).

The difficulty of prion transmission from one species to another is defined as the species barrier, and that between individuals of the same species with different *PRNP* genotypes is defined as the transmission barrier and is influenced by the primary structure of the recipient's PrP^C (15, 17, 19, 20, 25, 26). This barrier does not necessarily render the host refractory to infection and is impacted by the invading prion strain (20, 21, 26, 27). Prions can exhibit strain diversity. Strains are distinguished on the basis of their host range, clinical presentation, disease progression, and neuropathological and PrP biochemical profiles (28–31). The propagation of prion strains is dependent on both the *PRNP* genotype of the recipient and the properties of the invading agent (27, 32). For example, sheep expressing the V136-R154-Q171 PrP^C (GenBank accession number AJ567988) are most susceptible to classical scrapie, while sheep with distinct *PRNP* genotypes have reduced susceptibility (33–35). The strain of the agent also plays a role, as sheep expressing A136-R154-R171 PrP^C (GenBank accession number AJ567985) or A136-H154-Q171 PrP^C (GenBank accession number AJ567983) are susceptible to atypical scrapie, although they are relatively resistant to classical scrapie (31, 36). Similarly, the PrP^C primary structure and the invading agent modulate human susceptibility to prion infection; polymorphisms at codon 129 affect susceptibility to vCJD, kuru, and iCJD (20, 21, 26, 27, 37–40), while the G127V mutation renders carriers resistant against kuru (41).

In regions of North America where CWD is enzootic, transmission occurs between cervids expressing heterologous PrP^C molecules (PrP^C allotypes [18]). Analysis of *PRNP* allelic frequencies in wild and captive white-tailed deer identified two PrP^C polymorphisms, Q95H and G96S, that impact susceptibility to CWD (42–44) (GenBank accession numbers AF156185, AF156184, and AY275711). Homozygous wild-type (wt; Q95 G96) deer are most susceptible to CWD and have relatively short incubation periods. In contrast, deer heterozygous for the S96/wt, H95/wt, and H95/S96 alleles had extended incubation periods, suggesting that S96-PrP^C and H95-PrP^C impact CWD prion propagation (45). Miller et al. (46) reported similar observations in experimentally challenged S96/wt and S96/S96 deer when the incubation periods for those deer were compared to those for wt/wt white-tailed deer and mule deer. To further explore the diversity of CWD strains and the consequences of propagation in deer expressing different PrP^C primary structures, brain homogenates from CWD-infected white-tailed deer of different *PRNP* genotypes (wt/wt, S96/wt, H95/wt, or H95/S96 [45]) were inoculated into transgenic (tg) mice expressing the deer wt or S96 allele (47, 48). Our data show that CWD prions passaged in deer expressing H95-PrP^C have altered transmission properties. The H95/wt and H95/S96 CWD allotypes efficiently triggered prion disease in tg mice with S96-*PRNP* genotypes, leading to the identification and adaptation of a novel CWD strain. Transmission of first-passage tg60CWD-$H95^+$ prions into tg33 mice resulted in two distinct prion disease phenotypes which resembled those observed after primary passage of H95-PrP heterozygous deer CWD in both tg lines.

MATERIALS AND METHODS

Deer CWD inocula. Four CWD agents consisting of 10% or 1% (wt/vol) brain homogenates (Bh) in phosphate-buffered saline were used for transmission studies (45). The inocula were designated on the basis of their specific *PRNP* genotypes. CWD brain homogenates were obtained from orally infected white-tailed deer expressing different PrP^C molecules: homozygous Q95 G96 (wt/wt), heterozygous Q95 S96/wt (S96/wt), heterozygous H95 G96/wt (H95/wt), and H95 G96/Q95 S96 (H95/S96) (45). Brain homogenate from an uninfected white-tailed deer was used as a negative control. Frozen sagittal brain halves were homogenized (blended) to 20% (wt/vol) in cold phosphate buffer (1.3 M NaCl, 70 mM $Na_2HPO_4{\cdot}2H_2O$, 30 mM $NaH_2PO_4{\cdot}2H_2O$, pH 7.4), aliquoted, and stored at −80°C. Subsequently, aliquots were homogenized in a 50-ml syringe by passage through needles of different sizes (18 gauge to 21 gauge).

Transmission studies in transgenic mice. Animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Health Sciences Animal Care and Use Committee of the University of Alberta Animal Care and Use Committee. Bioassays were performed with transgenic mouse lines expressing the deer wt allele ($tg33^{+/+}$ and $tg33^{+/-}$ mice) or the S96-*PRNP* allele (tg60 mice, which express 30% less PrP^C than $tg33^{+/+}$ mice). (47, 48). Weanling pups were inoculated intracerebrally with 30 μl of deer CWD brain homogenates. Animals were monitored for the appearance of clinical signs and disease progression. Individual incubation periods are expressed as the number of days postinoculation (dpi) and were calculated from the time that the mice were inoculated until the time that clinical disease was established. The distribution of incubation periods between groups of tg33 mice was compared using the Kruskal-Wallis test with Dunn's multiple-comparison posttest ($P < 0.05$). Survival times postinoculation between tg60 mice inoculated with the H95/wt deer CWD allotype and the H95/S96 deer CWD allotype were compared using the Mann-Whitney test ($P < 0.05$). The statistical analysis of transmission experiments was performed with GraphPad Prism (version 5.04) software.

Isolates derived from tg60 mice infected with the H95/wt or H95/S96 deer CWD allotype (tg60CWD-H95/wt and tg60CWD-H95/S96 isolates, respectively) were used for syngeneic and allogeneic passages. One tg60CWD-H95/wt isolate (10%, wt/vol) was transmitted in tg33 and tg60 mice. A tg60CWD-H95/S96 isolate (10%, 1%, 0.0001%, wt/vol) was also passaged into both tg lines.

Histopathological analysis. Brain tissues from at least 5 (range, 5 to 11) tg mice per inoculum group were formalin fixed and paraffin embedded for histopathological analysis. Sagittal brain sections were obtained from 2 to 4 mice in each group of animals receiving each inoculum, and coronal brain sections were obtained from 3 to 7 mice in each group. Six consecutive slides of both sagittal and coronal brain sections (4 coronal sections from each brain) were examined as follows: 2 slides (4 to 6 μm thick) were stained with hematoxylin and eosin (H&E) to evaluate the sections for spongiform degeneration, and the other 4 slides were immunostained for PrP^{CWD} deposition and glial fibrillary acidic protein (GFAP)-positive astroglia. The sagittal paramedian brain sections were 0.36 to 0.60 mm lateral from the brain midline. All immunostaining experiments included CWD-positive tissue and negative mock-infected control sections. Differences in PrP^{CWD} deposition patterns could exist in areas of the central nervous system that were not examined. For the purpose of comparison, identification of the structures in H&E-stained slides was performed according to the mouse brain atlas (49).

Lesion profile analysis was performed using coronal brain sections as described previously (50). The lesion profile scores for the first passage in tg33 mice were obtained from 3 to 7 mice per inoculum group. For the CWD-affected tg60 mice, lesion profiles were obtained by scoring 4 mice per inoculum group. The density of spongiform lesions in nine gray matter areas from the brains of prion disease-affected mice were scored by three independent observers in a blind manner. The scores are reported as the mean ± standard deviation.

PrP^{CWD} deposits were visualized by immunostaining using anti-PrP monoclonal antibody BAR224 or 8G8 (0.2 μg/ml diluted 1:2,000 or 1:100, respectively; Bertin Pharma, formerly Spi-Bio). Briefly, brain slides were pretreated with high-pressure autoclaving (2.1×10^5 Pa) for 30 min in

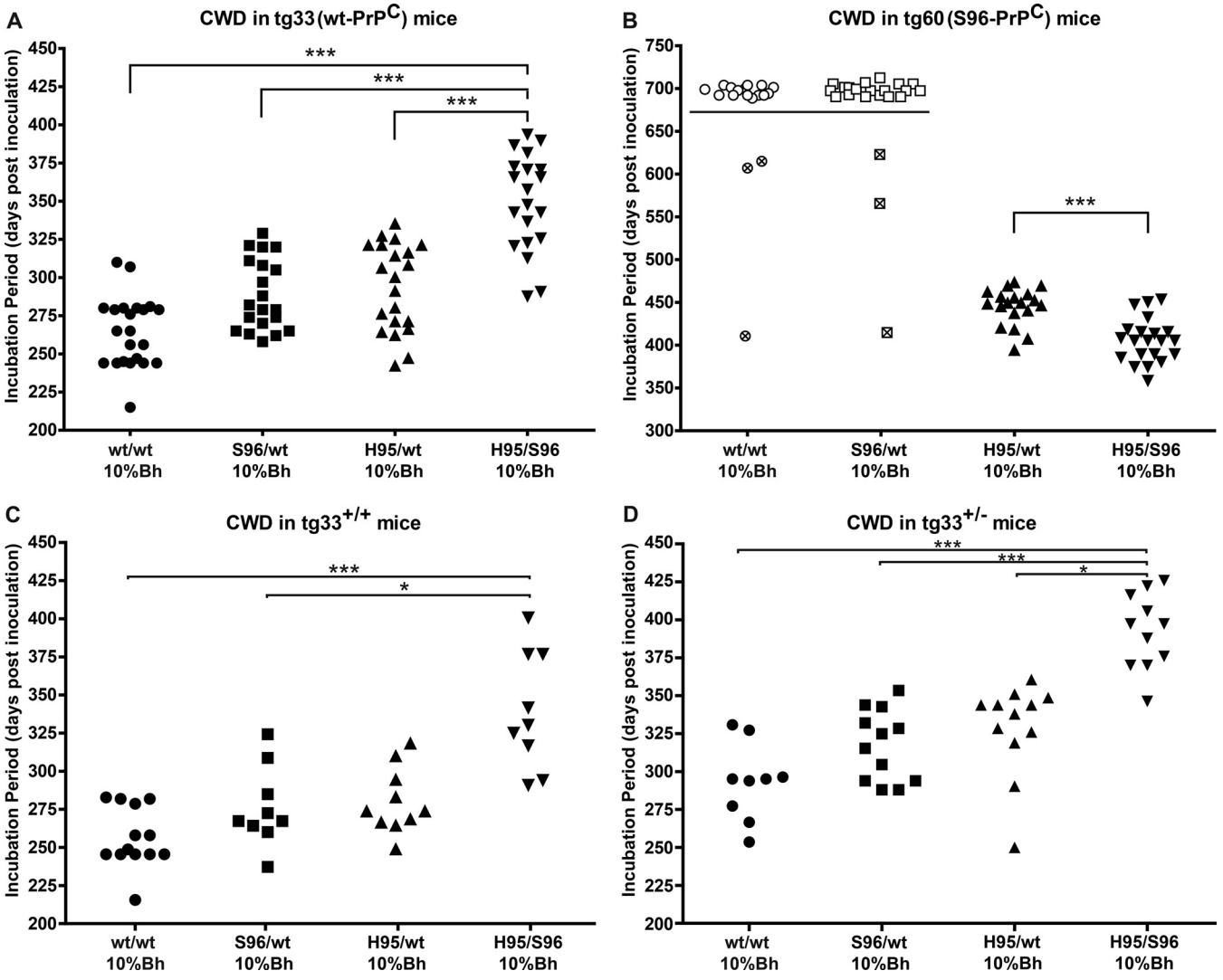

FIG 1 Transmission of CWD allotypes into transgenic mice expressing deer wt or S96-PrP^C. (A) Susceptibility of tg33 (wt-PrP^C) mice to infection with 10% (wt/vol) Bh from deer with CWD. (B) S96-PrP^C (tg60) mice developed clinical prion disease only when inoculated with CWD prions derived from deer expressing H95-PrP^C. Mice inoculated with wt/wt (open circles) or S96/wt (open squares) CWD prions did not show clinical signs. Symbols with crosses represent animals euthanized due to intercurrent disease. (C and D) Comparison of incubation periods in tg33$^{+/+}$ and tg33$^{+/-}$ mice. ***, significant differences between groups (the Kruskal-Wallis test with Dunn's multiple-comparison posttest, $P < 0.05$). The Mann-Whitney test ($P < 0.05$) was used to compare the distribution of incubation periods in tg60 mice.

citric acid (10 mM), pH 6.0, at 121°C, followed by treatment with 98% formic acid for 30 min and 4 M guanidine thiocyanate for 2 h at room temperature. Astrogliosis was evaluated by immunostaining of glial fibrillary acidic protein using an anti-GFAP antibody (0.5 mg/ml diluted 1:1,000; BD Biosciences) after hydrated autoclaving for epitope exposure. Immunohistochemical detection was achieved with biotinylated secondary antibodies according to the manufacturer's instructions (ARK animal research kit; Dako). Tissue sections were scanned with a NanoZoomer 2.0RS scanner (Hamamatsu Photonics) and analyzed using NanoZoomer digital pathology software (Hamamatsu Photonics).

Immunoblot analysis. Brain tissues from tg mice were homogenized to 10% (wt/vol) in sterile water using disposable syringes and needles of decreasing diameters (18 gauge to 21 gauge), aliquoted, and stored at −80°C. The brain homogenate protein content was determined using a micro-bicinchoninic acid assay kit (Life Technologies). For the proteinase digestion reactions, 50 to 70 μg total protein (final sample protein concentration, 1 to 1.4 mg/ml) was treated with 150 μg/ml of proteinase K (Life Technologies) for 45 min at 37°C. Reactions were terminated by boiling the samples in 2.5× Laemmli buffer (150 mM Tris-HCl, pH 6.8, 0.5% bromophenol blue, 25% glycerol, 5% [wt/vol] SDS, 12.5% β-mercaptoethanol) at 95°C for 10 min. Samples (10 to 15 μg) were resolved on 12% NuPAGE bis-Tris gels (Life Technologies) and transferred onto polyvinylidene difluoride Immobilon-P membranes (Millipore). The membranes were blocked for 1 h at room temperature with 5% (wt/vol) nonfat dry milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20 (TBST). Detection was performed using primary monoclonal antibody BAR224 (0.2 μg/ml diluted 1:10,000 in 5% [wt/vol] nonfat dry milk in TBST; Bertin Pharma) or 8G8 (0.2 μg/ml diluted 1:2,000; Bertin Pharma), secondary horseradish peroxidase-conjugated goat anti-mouse IgG antibody, and chemiluminescent substrate (diluted 1:10,000; Life Technologies). Images were acquired on X-ray film (Super Rx; Fujifilm). PrP-res glycoform ratios were determined using three animals per inoculum group; samples were resolved by Western blotting and detected with X-ray film. Quantification of PrP-res ratios was performed using ImageJ soft-

TABLE 1 Prion disease in tg-deer-*PRNP* mice inoculated with white-tailed deer and tg mouse-passaged CWD prions

Inoculum	tg-deer -*PRNP* mouse line	Bh dose (%)	No. of mice positive[a]/total no. of mice tested	Incubation period range (dpi)	PrP-res type	PrP^{CWD} distribution pattern
Deer CWD wt/wt	tg33	10	22/22	215–310	High MM[b]	Widespread
		1	8/8	256–345	High MM	Widespread
	tg60	10	0/18	>700	Negative	Not determined
		1	0/10	>600	Negative	Not determined
Deer CWD S96/wt	tg33	10	20/20	258–329	High MM	Widespread
		1	8/8	225–357	High MM	Widespread
	tg60	10	0/21	>700	Negative	Not determined
		1	0/10	>600	Negative	Not determined
Deer CWD H95/wt	tg33	10	21/21	242–335	High MM	Widespread
		1	10/10	316–356	High MM	Widespread
	tg60	10	19/19	394–473	Low MM	Localized
		1	10/10	465–608	Low MM	Localized
Deer CWD H95/S96	tg33	10	20/20	288–394	High MM	Widespread
		1	9/9	323–433	High MM	Widespread
	tg60	10	21/21	359–454	Low MM	Localized
		1	16/16	359–554	Low MM	Localized
tg60CWD-H95/wt	tg33	10	15/15	340–383	High MM	Widespread
	tg60	10	16/16	310–380	Low MM	Localized
tg60CWD-H95/S96	tg33	10	7/7	373–409	High or low MM	Localized or widespread
	tg33	1	8/8	397–448	High or low MM	Localized or widespread
	tg33	0.0001	8/8	329–490	High or low MM	Localized or widespread
	tg60	10	18/18	331–369	Low MM	Localized
Uninfected deer wt/wt	tg33	10	0/6	>560	Negative	Negative
	tg60	10	0/5	>560	Negative	Negative

[a] Clinically positive animals.
[b] MM, molecular mass.

ware (NIH). Independent triplicate measurements from each sample group were averaged, and the values were compared using GraphPad Prism (version 5.04) software.

RESULTS

Transmission of experimental CWD into tg-deer-*PRNP* mice. To evaluate the transmission properties of CWD prions derived from experimentally infected white-tailed deer of different *PRNP* genotypes (45), tg33 mice (expressing deer wt-PrP^C) or tg60 mice (expressing deer S96-PrP^C) (47, 48) were inoculated intracerebrally with deer CWD brain homogenates of 10% or 1% (wt/vol). All CWD inocula (wt/wt, S96/wt, H95/wt, H95/S96) resulted in clinical prion disease in mice expressing deer wt-PrP^C ($tg33^{+/+}$ and $tg33^{+/-}$ mice) (Fig. 1 and Table 1). Mice presented with similar disease signs, including hyperactivity, kyphosis, ataxia, and myoclonus. Clinical signs variably progressed into a general weakening, at which time the animals were euthanized. tg33 mice inoculated with the H95/S96 CWD agent had significantly longer incubation periods than mice receiving the other CWD inocula (the Kruskal-Wallis test with Dunn's multiple-comparison posttest $P < 0.05$) (Fig. 1A, C, and D). No significant differences in the incubation periods were observed between tg33 mice inoculated with the S96/wt, H95/wt, or wt/wt CWD inoculum (the Kruskal-Wallis test with Dunn's multiple-comparison posttest, $P > 0.05$) (Fig. 1).

In contrast to the susceptibility of the tg33 mouse line, mice expressing deer S96-PrP^C (tg60) developed clinical disease only when inoculated with CWD agents derived from deer expressing the H95-*PRNP* allele (Fig. 1B). Mice inoculated with the H95/S96 and H95/wt CWD allotypes were clinically positive for prion disease between 359 and 473 dpi. Affected mice became lethargic with myoclonus, kyphosis, labored breathing, and ataxic gait characterized by limb weakness. Incubation periods were significantly different between tg60 mice inoculated with the H95/S96 CWD agent and tg60 mice challenged with the H95/wt CWD agent (Mann-Whitney test, $P < 0.05$). S96-PrP^C mice inoculated with the wt/wt and S96/wt CWD agents did not develop prion disease at >700 days postinoculation.

Neuropathology of tg-deer-*PRNP* mice infected with CWD agents. To define the neuropathological hallmarks and assess differences between groups of mice inoculated with the different CWD inocula, sagittal and coronal brain sections were examined histologically for spongiform changes and immunohistochemically for PrP^{CWD} aggregates and GFAP-positive astroglia (Fig. 2).

CWD-infected tg33 (wt-PrP^C) mice presented with extensive pathology in various brain regions and were characterized by neuronal loss, spongiform change, the widespread accumulation of PrP^{CWD} aggregates, and astrogliosis (Fig. 2A to H). The distribution of pathological changes in the brain (i.e., PrP^{CWD} distribution) was similar between mice inoculated with the four CWD inocula (Fig. 3A to G). The average spongiform change scores of various brain structures were similar among the infected tg33 mice (Fig. 2Q). In general, the lesions (vacuolation and PrP^{CWD} accumulation) observed in the forebrain and cerebellum agree with previous results obtained with this transgenic mouse line after infection with CWD prions from other sources (47). Additionally, the granular layer of the cerebellum had areas of neuronal loss, where dense PrP^{CWD} aggregates surrounded by GFAP-positive astrocytes were revealed in consecutive tissue sections (Fig. 2F to G). The spongiform changes and cell death in the cerebellum of tg33 mice were less conspicuous in the molecular layer and more

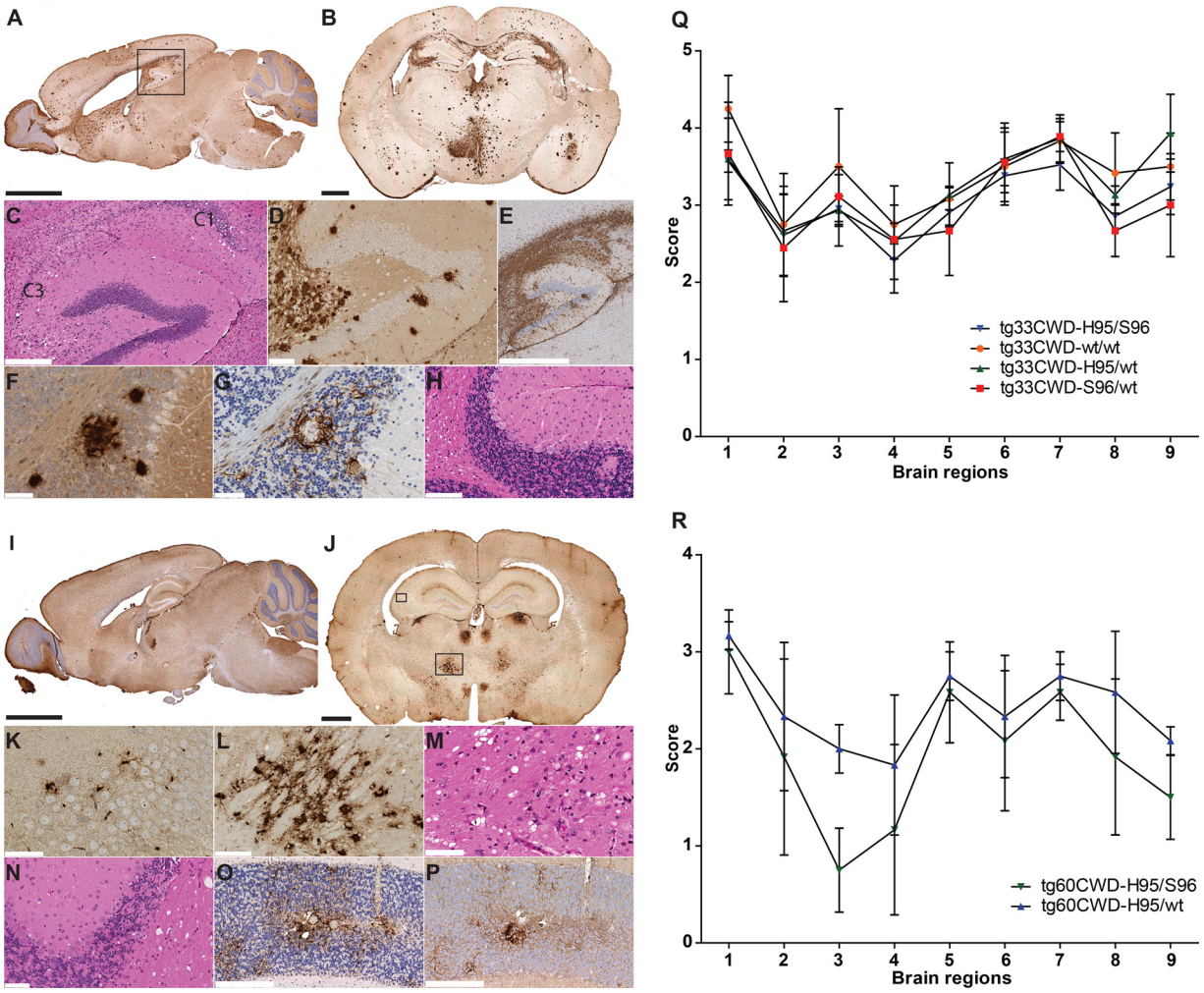

FIG 2 Neuropathology of tg-deer-*PRNP* mice following the first passage of white-tailed deer CWD allotypes. (A and B) Accumulation of wt-PrP^{CWD} aggregates in tg33 mice. The regional distribution of wt-PrP^{CWD} aggregates was similar in mice receiving different CWD inocula (Fig. 3A to G). (C to E) Hippocampal degeneration (box in panel A) was characterized by spongiform change and a loss of pyramidal neurons of the Ammon's horn (C1 and C3), accompanied by the extensive accumulation of PrP^{CWD} aggregates and abundant astrocytosis (GFAP). (F and G) Cerebellum pathology involved the loss of granular neurons and the presence of prion protein deposits flanked by astrocytes (as seen in sequential tissue sections). (H) Vacuolation was observed in Purkinje neurons and cerebellar white matter. (I and J) Detection of S96-PrP^{CWD} aggregates in tg60 mice infected with the H95/wt and H95/S96 CWD allotypes. The distribution of PrP^{CWD} aggregates was similar between animals receiving the $H95^+$ deer CWD agent (Fig. 3J to K). (K) S96-PrP^{CWD} aggregates in the hippocampus were noticeable at a higher magnification of the small box in panel J. (L and M) Abnormal prion protein deposits and spongiosis in thalamic nuclei shown by a higher magnification of the large box in panel J. (N to P) Cerebellar pathology included white matter vacuolation (N) and astrocytosis (O) that colocalized with diffuse and punctate protein aggregates (P). (Q) Lesion profile of tg33 mice infected with deer CWD allotypes. (R) Lesion profile of tg60 mice infected with the $H95^+$ deer CWD agent. Brain regions are as follows: 1, medulla; 2, cerebellum; 3, superior colliculus; 4, hypothalamus; 5, thalamus; 6, hippocampus; 7, septum; 8, posterior cortex; 9, anterior cortex. Bars, 2.5 mm (A and I), 1 mm (B and J), 850 μm (E), 300 μm (C, O and P), 125 μm (D, L and H), and 60 μm (F, G, K, M, and N). PrP^{CWD} detection was achieved with anti-PrP monoclonal antibody BAR224. (A) Brain section from a tg33 mouse infected with S96/wt CWD prions at 270 dpi; (B) brain section from a tg33 mouse infected with H95/S96 CWD prions at 387 dpi, (I) brain section from a tg60 mouse infected with H95/S96 CWD prions at 375 dpi; (J) brain section from a tg60 mouse infected with H95/S96 CWD prions at 414 dpi.

abundant in the white matter and the Purkinje cell layers (Fig. 2H). Infection of tg33 mice resulted in more prominent PrP^{CWD} accumulation in the corpus callosum (Fig. 2A and B) than that described in other studies (47).

The susceptibility of S96-PrP^C (tg60) mice to CWD agent infection was strongly influenced by the invading CWD allotype. All tg60 mice exposed to the H95/wt or H95/S96 CWD agent developed clinical prion disease with similar neuropathologies (Fig. 2I to P and R and 3J to K). The distribution and severity of the neuropathological changes observed in diseased tg60 mice infected with $H95^+$ deer CWD allotypes followed a consistent lesion pattern (Fig. 2I to P and R and 3J to K). Spongiform degeneration and abnormal S96-PrP^{CWD} aggregates were localized in the caudoputamen and the corpus callosum and extended down the septum to the diagonal band nucleus (Fig. 3K). Both vacuolation and PrP^{CWD} deposition were of milder intensity in the cerebral cortex and hippocampus than in the other brain areas; however, immunohistochemical staining revealed the presence of small, punctate S96-PrP^{CWD} aggregates at higher magnification (Fig. 2K). Pathological changes were more severe in various regions of the thalamus, including the medial-dorsal, ventral-medial, and ventral anterior-lateral thalamic nuclei (Fig. 2I to J, L, and M and 3J) and

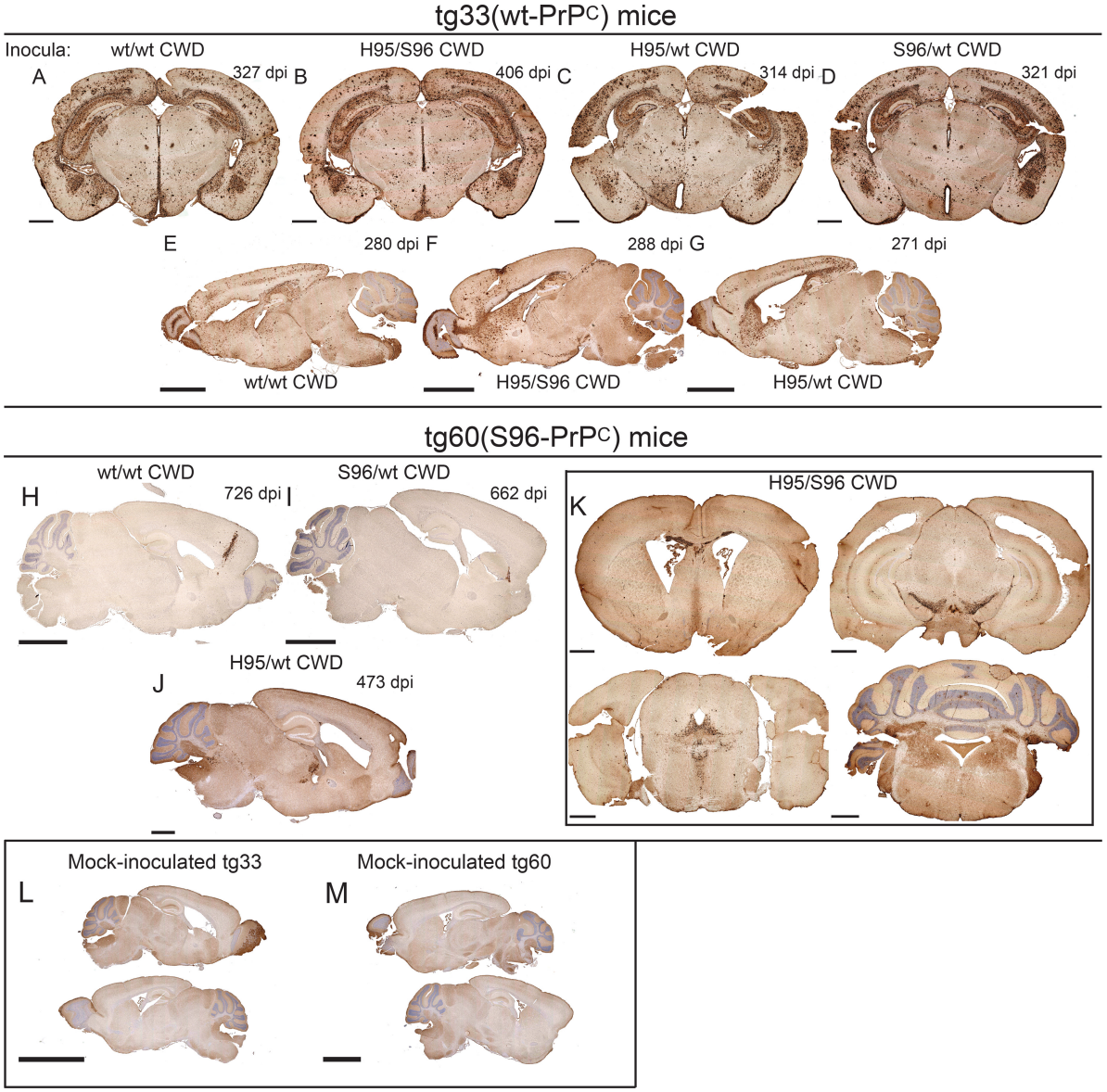

FIG 3 Distribution of PrP^{CWD} aggregates in the brains of tg-deer-*PRNP* mice inoculated with different white-tailed deer CWD allotypes. (A to G) PrP^{CWD} aggregates in the brains of tg33 mice inoculated with different white-tailed deer CWD allotypes. (H and I) Abnormal PrP aggregates were detected after >700 dpi in the brains of tg60 mice without clinical signs inoculated with the wt/wt or S96/wt CWD allotypes. (J and K) Only tg60 mice inoculated with $H95^+$ CWD allotypes had clinical signs and were consistently positive for PrP^{CWD} aggregates. (K) Coronal brain sections from a clinically ill tg60 mouse infected with $H95^+$ CWD prions (414 dpi). (L and M) Brain sections of mock-infected tg33 and tg60 mice. Bars, 1 mm (A to D, J, and K), 2.5 mm (E to I and M), and 5 mm (L). Tissue sections were stained with anti-PrP monoclonal antibody BAR224.

also involved the zona incerta, cerebral peduncle, and subthalamic and hypothalamic nuclei (Fig. 2I to J and 3J). In the midbrain, lesions were localized in the substantia nigra adjacent to the ventral tegmental area and extended to periaqueductal gray and adjacent structures, including the raphe nucleus, mesencephalic reticular formation, and superior cerebellar peduncle (Fig. 2I and 3K). Pathology was also observed in the hindbrain and affected various regions, including the median raphe nucleus and pontine reticular nucleus (Fig. 3K). In the cerebellum, the spongiform change was the most prominent in the white matter; however, small vacuoles were also observed in the molecular, Purkinje, and granular layers, with the granular layer showing loss of granular neurons (Fig. 2N). PrP^{CWD} staining revealed either diffuse deposits (lightly stained) or larger confluent aggregates in the cerebellar nuclei and the granular layer (Fig. 2I and P and 3K).

A few tg60 (S96-PrP^C) mice that did not have clinical signs and that were challenged with the wt/wt or S96/wt CWD agents (3/28 and 3/31 mice, respectively) had detectable prion aggregates at >700 days postinoculation (Fig. 3H to I), highlighting the low efficacy of these CWD agents for establishing infection in this transgenic line. The accumulation of PrP^{CWD} aggregates in these particular mice did not follow the PrP^{CWD} distribution patterns described in the other mice.

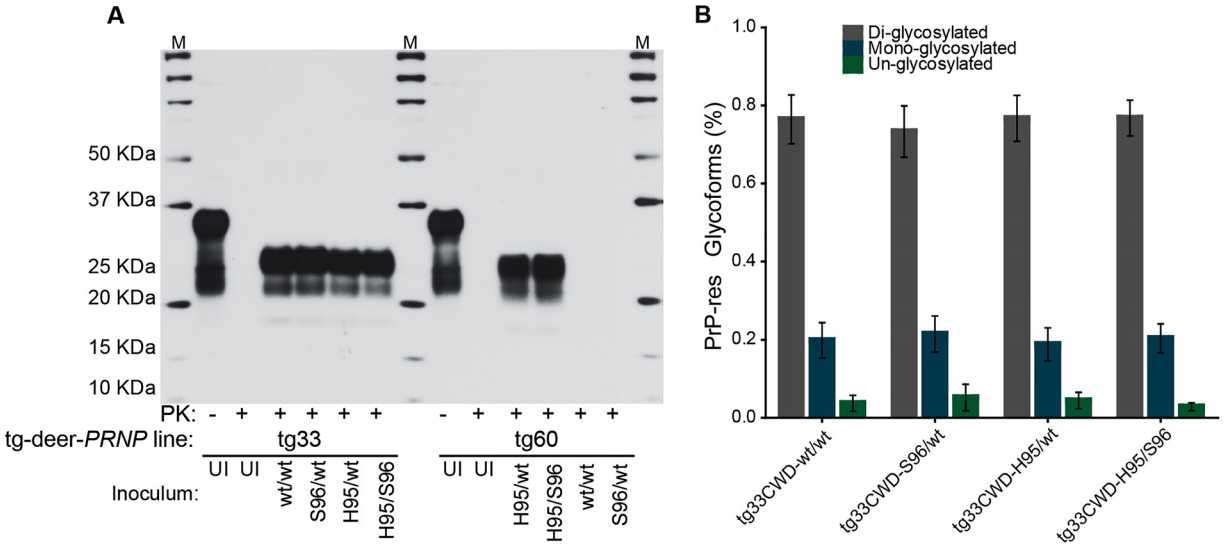

FIG 4 Disease-associated PrP-res in tg-deer-*PRNP* mice inoculated with different CWD allotypes. (A) PrP-res from brains of prion-affected tg33 (wt-PrP^C) and tg60 (S96-PrP^C) mice. Brain homogenates were digested with proteinase K (PK) and analyzed by SDS-PAGE and Western blotting. Lanes M, molecular size markers. PrP-res from tg33 mice had similar molecular masses after enzymatic cleavage (A) and equivalent glycoform ratios (B). S96-PrP-res has a lower molecular mass and was detectable only in brain homogenates derived from tg60 mice infected with H95$^+$ CWD allotypes. UI, homogenates from tg mice inoculated with uninfected deer brain homogenate. PrP-res detection was achieved with anti-PrP monoclonal antibody BAR224.

PrP-res glycotypes in transgenic mice expressing deer PrP^C. Distinct PrP-res isoforms have been associated with different prion strains (28, 51, 52). PrP-res can vary in their molecular masses, glycoform ratios, and other biochemical properties related to the structural stability of the abnormal PrP conformers (53). These properties have been interpreted to be conformational differences in the structures of the misfolded PrP molecules that carry the information that defines different prion strains (28, 51–53). To compare the PrP-res in mice infected with the different CWD inocula, brain homogenates were digested with proteinase K and analyzed by Western blotting using anti-PrP monoclonal antibodies 8G8 (which recognizes deer PrP amino acid residues 98 to 113) or Bar224 (which epitope comprises deer PrP residues 144 to 154). All clinically affected tg33 mice were PrP-res positive; no differences with respect to molecular masses and glycoform patterns were observed (Fig. 4). Although the electrophoretic profile of PrP-res from clinically affected tg60 mice was similar between mice inoculated with the H95/wt or H95/S96 CWD allotypes, this PrP-res type was distinct from that observed in tg33 mice. The gel migration of the proteinase K cleavage products indicated that S96-PrP-res has a lower molecular mass than wt-PrP-res (Fig. 4 and 5). PrP-res was not detected at 700 dpi in brain homogenates from tg60 mice inoculated with the wt/wt or S96/wt CWD allotypes.

Serial transmission of passage 1 tg60CWD-H95$^+$ isolates into tg-deer-*PRNP* mice. To evaluate the transmission properties of tg60 (S96-PrP^C) mouse-passaged CWD prions, we inoculated these isolates into both the tg33 and tg60 mouse lines. Serial transmission of first-passage tg60CWD-H95$^+$ isolates back into tg60 mice (syngeneic passage) resulted in a reduction of the incubation periods (Fig. 5A and Table 1). The disease signs, biochemical PrP-res glycotype, and neuropathology resembled those after first passage (Fig. 5B to D).

Passage of tg60CWD-H95$^+$ isolates into tg33 mice (allogeneic passage) resulted in two different prion disease presentations. After exposure to 10% (wt/vol) tg60CWD-H95/wt brain homogenate, the mice had extended incubation periods compared to those of tg33 mice infected with the H95/wt deer CWD allotype (Fig. 6A and Table 1). Disease signs and pathological hallmarks were similar to those described during the first passage of deer CWD prions in tg33 mice, characterized by hyperactivity, a widespread distribution of aggregates in the brain, and high-molecular-mass PrP-res (Fig. 6B and C). Transmission of the tg60CWD-H95/S96 isolate into tg33 mice resulted in divergent prion disease phenotypes. Inoculation of 10% brain homogenates resulted in extended incubation periods, with some mice developing disease signs and pathology characteristic of tg33 mice infected with the deer CWD agents, while others developed disease signs and neuropathology that resembled the disease phenotype described for tg60 mice (Fig. 6). Evaluation of proteinase K-resistant PrP in brain homogenates from affected mice revealed PrP-res glycotypes of distinct molecular masses (Fig. 6B). Passage of 1% and 0.0001% (wt/vol) brain homogenates resulted in further extension of the incubation period and increased the abundance of mice presenting with lethargy (like tg60 mice), accompanied by the accumulation of low-molecular-mass PrP-res and localized deposition of PrP aggregates in brain (Fig. 6).

DISCUSSION

To explore the transmission properties of CWD prions derived from white-tailed deer of four different *PRNP* genotypes (45), we inoculated transgenic mice expressing deer prion proteins associated with susceptibility (tg33 mice expressing deer wt-PrP^C) or resistance (tg60 mice expressing deer S96-PrP^C) to CWD prions (47, 48). Transmission of the deer H95/wt and H95/S96 CWD allotypes resulted in the emergence of a distinct CWD strain (H95$^+$). This novel prion agent was identified when brain homogenates from deer containing H95-PrP molecules were transmitted into tg60 mice. Passage of these deer brain homogenates into tg33 mice, however, resulted in a prion disease phenotype indistin-

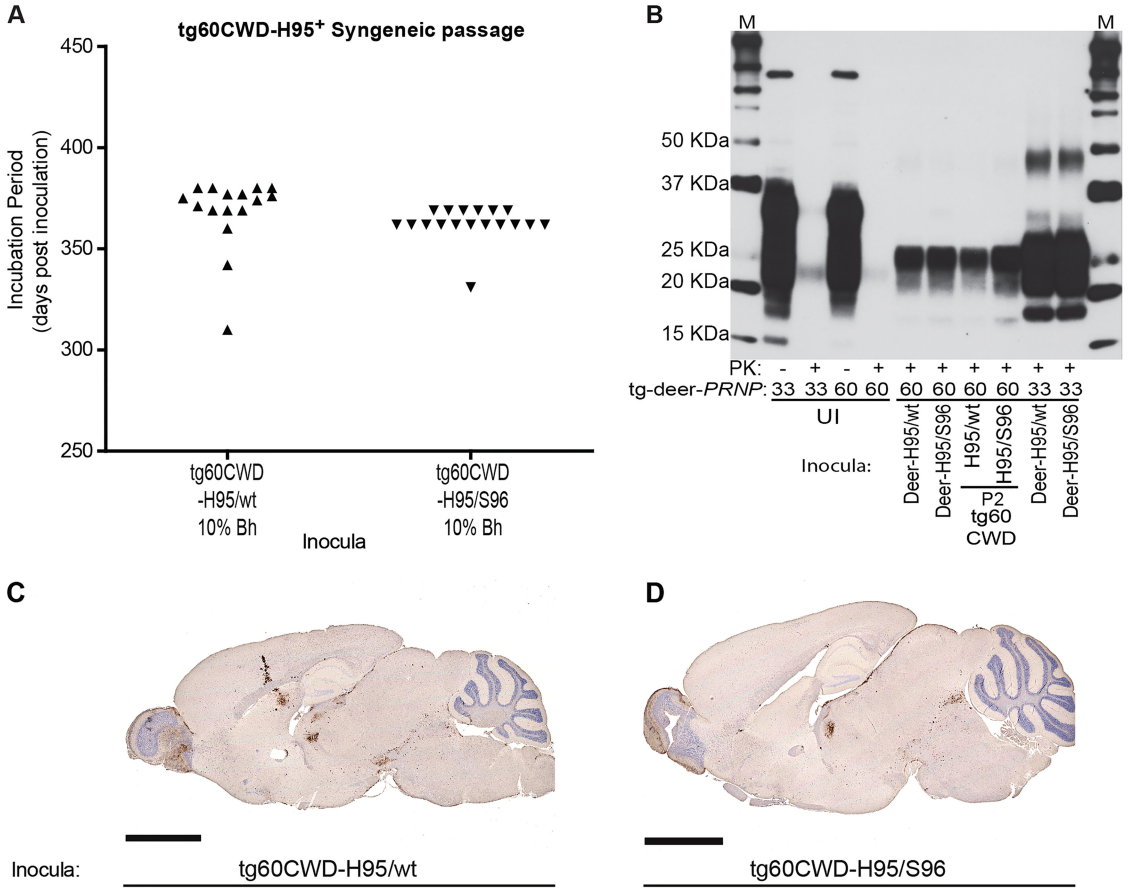

FIG 5 Serial passage of tg60 (S96-PrP^{C}) mouse-passaged CWD prions. (A) Syngeneic transmission of tg60CWD-H95^{+} isolates into tg60 mice led to reduction in the incubation period following intracerebral inoculation of 10% (wt/vol) brain homogenates. (B) S96-PrP-res properties were maintained following secondary passage in tg60 mice. S96-PrP-res has a lower molecular mass than wt-PrP-res derived from tg33 mice. Lanes M, molecular size markers. (C and D) Distribution of S96-PrP^{CWD} aggregates in the brains of tg60 mice infected with tg60CWD-H95^{+} prions. Immunohistochemical comparison revealed a similar distribution of abnormal PrP^{CWD} aggregates, as observed in tg60 mice from the first passage of H95^{+} deer CWD agent (Fig. 2I to J and 3J and K). Detection of abnormal PrP (B to D) was performed with antibody BAR224. Bars, 2.5 mm.

guishable from that observed following infection with the wt/wt or S96/wt CWD agents. The ability of H95^{+} CWD agent to cause clinical prion disease in tg60 mice, which have been shown to be resistant to other CWD isolates, indicates that a new strain has emerged (45, 47, 48). Our data show that the passage of CWD (wt/wt pool) through deer with the H95/wt and H95/S96 allotypes resulted in a mixture of at least two CWD strains, distinguishable on the basis of the tg-deer-*PRNP* genotype in which they were propagated.

Upon first passage into tg33 mice, all deer CWD agents resulted in similar disease signs, PrP-res glycotypes, and neuropathological features, suggesting that expression of wt-PrP^{C} favored the propagation of a CWD strain (prion conformer) common to all inocula. We refer to this agent as "Wisc-1." Our results suggest that Wisc-1 is similar to strain CWD-1 described by Angers et al. (54). The white-tailed deer sample analyzed in the study of Angers et al. (54) was a wt/wt CWD isolate from Wisconsin. Whether Wisc-1 and CWD-1 are identical is difficult to ascertain, as the white-tailed deer agents were passaged in different transgenic mice. The H95^{+} CWD strain differs from the Wisc-1, CWD-1, and CWD-2 strains (54, 55).

We found that inoculation of the H95/S96 CWD agent into tg33 mice resulted in incubation periods significantly different from those obtained by inoculation of CWD prions of the other allotypes. The absence of wt-PrP^{CWD} in this inoculum and, thus, the lack of homologous prion conversion likely contributed to the prolonged incubation period. The presence of more than one prion conformer within this inoculum may result in competition between agents, leading to propagation interference and extension of the incubation periods (56–59).

Incubation periods were not significantly different between tg33 mice infected with the wt/wt, H95/wt or S96/wt CWD agents. Additionally, all tg33 mice presented the same prion disease phenotype irrespective of the CWD inoculum that they received. One possible interpretation for the phenotypic similarities observed between tg33 mice is that the Wisc-1 conformers have an adaptive advantage in hosts (either in deer or in tg mice) expressing wt-PrP^{C}. The differences in incubation periods between the H95/wt CWD allotype- and H95/S96 CWD allotype-infected tg33 mice suggest that the PrP^{C} sequence in these deer impacted the proportion of accumulated CWD strains. It has previously been demonstrated in hamster coinfection experiments that the ratio of the strains in a prion mixture influences the emergence of the fastest-replicating or dominant strain (56, 57, 59).

The differential susceptibility to prion infection is modulated by PrP^{C} amino acid sequence variability and the invading prion

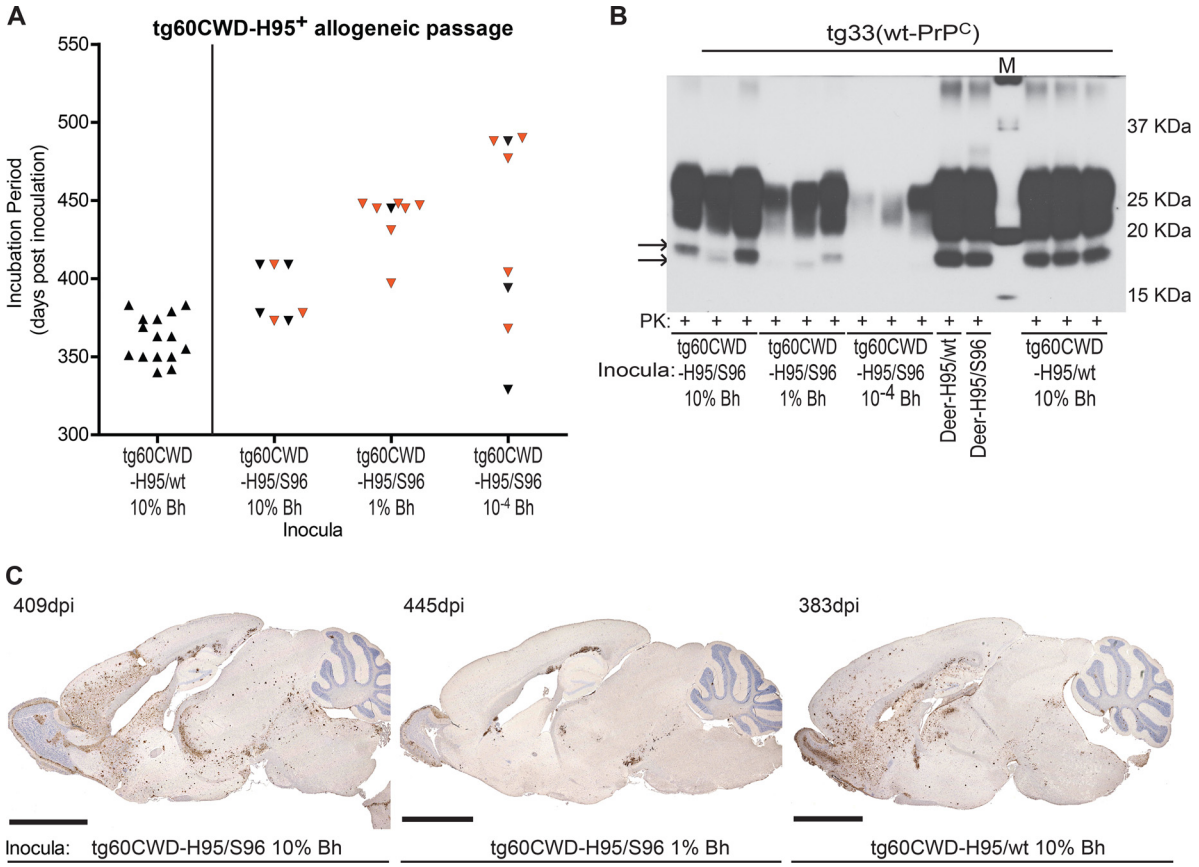

FIG 6 Allogeneic transmission of tg60 (S96-PrP^C) mouse-passaged CWD prions into tg33 mice. (A) Incubation periods of tg33 mice upon challenge with passage 1 tg60CWD-H95$^+$ isolates. Passage of tg60CWD-H95/S96 brain homogenates gave rise to different clinical presentations (hyperactivity versus lethargy) resembling the disease phenotypes described for both tg-deer-*PRNP* lines during the first passage of deer CWD prions. Black symbols, tg33 animals with hyperactive disease presentation, high-molecular-mass PrP-res, and a widespread distribution of brain PrP^{CWD} aggregates; orange symbols, tg33 mice with a lethargic presentation, low-molecular-mass PrP-res, and a localized distribution of PrP^{CWD} aggregates. (B) PrP-res glycotypes in brains of tg33 mice inoculated with different tg60CWD-H95$^+$ isolates. Infected tg33 mice accumulated different proteinase K-resistant PrP types resembling those observed after the first passage of deer CWD prions. Lane M, molecular size markers. (C) Divergent histological phenotypes in tg33 mice infected with tg60CWD-H95/S96 or tg60CWD-H95/wt brain homogenates. Bars, 2.5 mm. Detection of abnormal PrP was performed with anti-PrP monoclonal BAR224.

strain (15, 17, 20, 21, 25, 26, 60). Both natural and experimental infections support the association of S96-PrP^C with reduced susceptibility and the slower progression of CWD (3, 42–48, 55). tg60 (S96-PrP^C) mice were previously shown to be resistant to CWD isolates from different cervid species (47, 48). In our study, tg60 mice inoculated with the wt/wt or S96/wt CWD agents did not present with clinical disease after >700 dpi; however, mice receiving the H95/wt and H95/S96 CWD allotypes developed disease signs and presented consistent neuropathology and PrP-res glycotypes. A second passage of the tg60CWD-H95$^+$ isolates into tg60 mice resulted in a reduction of the incubation periods and similar phenotypic characteristics.

Allogeneic transmission of the first-passage tg60CWD-H95$^+$ isolates into tg33 mice resulted in the development of prion disease with two distinct phenotypes resembling those caused by the Wisc-1 and H95$^+$ prion strains. While some animals presented with hyperactivity and displayed a widespread accumulation of disease-associated PrP in the brain as well as high-molecular-mass PrP-res, others were lethargic with localized PrP^{CWD} deposits and a distinct PrP-res glycotype. Transmission of a diluted tg60CWD-H95$^+$ inoculum resulted in more mice presenting the tg60-like phenotype. This suggests that the tg60 donor mouse, which preferentially amplified the H95$^+$ strain, contained a persistent Wisc-1 fraction that was amplified upon passage at a high dose (10% Bh) in tg33 mice. Transmission of lower doses of the inoculum likely altered the proportion of the two prion conformers, favoring the propagation of the H95$^+$ strain. A similar outcome was observed when dilutions of the transmissible mink encephalopathy agent were passaged in hamsters, resulting in the isolation of the Hyper and Drowsy strains (56). Transmission of tg60CWD-H95$^+$ isolates into tg33 mice indicates that individual tg60 mice accumulated mixtures of CWD agents. Although prion transmission experiments in tg mice do not always recapitulate what is observed in the wild (i.e., tg60 mice are resistant to a number of different CWD strains, whereas 96S homozygous deer are naturally infected), natural scrapie and CWD isolates have been shown to contain strain mixtures that can be differentiated by serial passage in mouse models or by histopathological and biochemical analyses (54, 61–63).

Deer with S96-*PRNP* alleles can be infected with the CWD agent but have extended preclinical periods, suggesting that they could be infectious over longer periods of time than wt

homozygous deer (45, 46, 48). Additionally, in areas where CWD is endemic, white-tailed deer with S96-*PRNP* alleles likely have a fitness advantage over deer with the more susceptible genotypes, and as a result, the resistance allele may become more abundant in the population (64). An increase in the S96-*PRNP* allele frequency could also affect the potential for the selection of CWD strains able to infect deer with resistant genotypes. Likewise, other *PRNP* alleles associated with extension of the CWD preclinical phase, such as H95-*PRNP*, could also be subjected to a disease-driven increase in white-tailed deer populations. Our transmission data show that deer expressing H95-PrP accumulate a CWD strain capable of infecting deer with S96-*PRNP* genotypes, unlike other CWD agents. An increase in the frequency of H95-*PRNP* would also increase the likelihood of the emergence of $H95^+$ CWD prions. Our data suggest that white-tailed deer expressing different PrP^C allotypes can accumulate and transmit CWD strain mixtures.

CWD epizootics involve multiple factors, including the contagious nature of the agent, host-pathogen interactions, agent strains, and cervid population genetics. Our data indicate that CWD strain emergence is modulated by amino acid polymorphisms in the cervid PrP. CWD transmission between hosts with different *PRNP* genotypes (65) has the potential to generate and select novel prion conformations. Deer expressing H95-PrP^C accumulate CWD prions with different transmission properties, as exemplified by its ability to infect resistant S96-*PRNP* mice. Finally, our study highlights the importance of characterizing the diversity of CWD strains and their potential for interspecies transmission, as various mammalian species are susceptible to experimental CWD infection (66–69). Although several lines of evidence suggest that humans are resistant to CWD prions (70–73), not all CWD strains have been tested for their zoonotic potential. Our results demonstrating that $H95^+$ deer CWD prions have transmission properties different from those of CWD prions composed of wt-PrP or S96-PrP suggest the need for evaluation of the transmissibility of CWD allotypes.

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