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# The emergence of classical BSE from atypical/Nor98 scrapie

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**Atypical/Nor98 scrapie (AS) is a prion disease of small ruminants. Currently there are no efficient measures to control this form of prion disease, and, importantly, the zoonotic potential and the risk that AS might represent for other farmed animal species remains largely unknown. In this study, we investigated the capacity of AS to propagate in bovine PrP transgenic mice. Unexpectedly, the transmission of AS isolates originating from 5 different European countries to bovine PrP mice resulted in the propagation of the classical BSE (c-BSE) agent. Detection of prion seeding activity in vitro by protein misfolding cyclic amplification (PMCA) demonstrated that low levels of the c-BSE agent were present in the original AS isolates. C-BSE prion seeding activity was also detected in brain tissue of ovine PrP mice inoculated with limiting dilutions (endpoint titration) of ovine AS isolates. These results are consistent with the emergence and replication of c-BSE prions during the in vivo propagation of AS isolates in the natural host. These data also indicate that c-BSE prions, a known zoonotic agent in humans, can emerge as a dominant prion strain during passage of AS between different species. These findings provide an unprecedented insight into the evolution of mammalian prion strain properties triggered by intra- and interspecies passage. From a public health perspective, the presence of c-BSE in AS isolates suggest that cattle exposure to small ruminant tissues and products could lead to new occurrences of c-BSE.**

prion | atypical scrapie | c-BSE

**T**ransmissible spongiform encephalopathies (TSEs), or prion diseases, are fatal neurodegenerative disorders that affect a large spectrum of mammalian species. These conditions include scrapie in small ruminants, classical bovine spongiform encephalopathy (c-BSE) in cattle, and sporadic Creutzfeldt-Jakob disease (sCJD) or variant CJD (vCJD) in humans.

The fundamental event in prion propagation is the conversion of the normal cellular prion protein (PrP<sup>C</sup>) into an abnormal disease-associated isoform (PrP<sup>Sc</sup>) in tissues of infected individuals. PrP<sup>C</sup> is completely degraded by digestion with proteinase K (PK), whereas PrP<sup>Sc</sup> is N-terminally truncated, resulting in a PK-resistant core termed PrP<sup>res</sup> (1). According to the prion concept, PrP<sup>Sc</sup> is the principal, if not sole, component of the transmissible prion agent (2), and PrP<sup>res</sup> is a disease marker for prion diseases (1, 3). Particular biochemical properties of PrP<sup>Sc</sup>, such as detergent solubility, PK resistance, and electromobility evidenced by Western blot can be used to distinguish between different prion agents or strains (4, 5).

Intraspecies transmission of prion disease between individuals is typically quite efficient. In contrast, interspecies transmission of prions can be unpredictable, with apparent failure of disease transmission on many occasions. In other cases, clinical prion disease may not be evident but, rather, there is the presence of subclinical infection (6). When interspecies prion transmission does occur, the propagating agent can remain identical to the

original prion strain or can display different biological properties compared to the original inoculum (7, 8). This complex set of outcomes for interspecies prion challenge are collectively referred to as the transmission barrier phenomenon.

After identification of the gene encoding PrP, it was soon discovered that differences in amino acid sequence between host PrP<sup>C</sup> and donor PrP<sup>Sc</sup> constitutes the principal determinant of the transmission barrier. For example, the resistance of wild-type mice to clinical prion disease induced by hamster scrapie is abrogated by transgenic expression of hamster PrP<sup>C</sup> in mice (9, 10). As a consequence, mice genetically engineered to express particular species forms of PrP sequence, in the absence of endogenous mouse PrP, have emerged as relevant models to experimentally characterize the outcome of prion strain transmission between species (11). It is also now well established that strain properties have a significant impact on the ability of prions to cross the species barrier. For instance, human vCJD can be transmitted readily to conventional mice, but it is extremely difficult for sCJD to propagate in the same mouse lines (12, 13). Furthermore, the amino acid sequence of PrP<sup>Sc</sup> influences the efficacy of interspecies

## Significance

**The origin of transmissible BSE in cattle remains unestablished. Sheep scrapie is a potential source of this known zoonotic. Here we investigated the capacity of sheep scrapie to propagate in bovine PrP transgenic mice. Unexpectedly, transmission of atypical but not classical scrapie in bovine PrP mice resulted in propagation of classical BSE prions. Detection of prion seeding activity by in vitro protein misfolding cyclic amplification demonstrated BSE prions in the original atypical scrapie isolates. BSE prion seeding activity was also detected in ovine PrP mice inoculated with limiting dilutions of atypical scrapie. Our data demonstrate that classical BSE prions can emerge during intra- and interspecies passage of atypical scrapie and provide an unprecedented insight into the evolution of mammalian prions.**

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The authors declare no competing interest.

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prion transmissions, since studies in human PrP transgenic mouse models indicate that the human species barrier is more permeable to sheep-passaged BSE compared to its cattle counterpart (14).

From a public health perspective, the transmission barrier phenomenon and its capacity to limit the interspecies propagation of prion disease has long been considered as an effective protection of humans against animal TSEs (15). However, in 1996, the new human prion disease vCJD was observed in people in the United Kingdom. Multiple lines of evidence indicated that vCJD was the likely consequence of dietary exposure of humans to the agent responsible for c-BSE in cattle, an epizootic prion disease that has spread in bovine hosts through the recycling of prion-contaminated animal carcasses in the animal food chain (16). Since the emergence of vCJD, considerable efforts have been deployed to characterize not only the zoonotic potential of animal prions but also their capacity to propagate in farmed animal species.

Atypical/Nor98 scrapie (AS) probably represents the largest geographically spread known animal prion disease. Since its original discovery in 1998 in Norway, AS has been identified in most EU member states, in Asia, and in North and South America (17). AS has also been detected in Australia and New Zealand, 2 countries that were believed to be free of animal TSEs (18, 19). Retrospective studies carried out in archived animal tissues identified an AS case in a sheep that died in 1972 in the United Kingdom, demonstrating that the disease has been present in small ruminant populations for many decades (20).

Bioassay in ovine PrP transgenic mice provided evidence that AS comprised a single prion strain (21–23). The AS prion strain was associated with a multiband PrP<sup>res</sup> signature that contrasted with those normally observed in small ruminant TSE cases (24). Since there is no statistical difference in the apparent prevalence of atypical scrapie between sheep flocks in general and those flocks where a positive case had been identified, atypical scrapie is considered by many as a noncontagious prion disease that arises sporadically in sheep and goats (25). However, atypical scrapie can be experimentally transmitted via the oral route in small ruminants, resulting in a similar clinicopathological phenotype to that observed in natural

cases (26). Consequently, the origin of atypical scrapie (spontaneous disorder versus acquired disease) remains an open question.

In this study, we used mice transgenic for bovine PrP (tgBov mouse line) to characterize the capacity of sheep AS isolates to cross the bovine transmission barrier. Unexpectedly, the TSE agent that propagated in tgBov mice was indistinguishable from the prion strain that was responsible for the c-BSE epizootic in cattle. In addition, our sensitive detection of c-BSE by in vitro PMCA methodology indicated that this bovine prion strain was present as a minor prion strain in the original sheep AS isolates, and that AS prion strain replication in an ovine PrP host was accompanied by the generation of c-BSE prions. Collectively, these data provide compelling evidence for the emergence and the propagation of zoonotic mammalian prions during intra- and interhost transmission of the AS prion strain.

## Results

A panel of 8 atypical scrapie (AS) cases collected from sheep and goats in 5 different European countries was obtained (Table 1). All of the AS isolates displayed a multiband abnormal PrP (PrP<sup>res</sup>) Western blot profile that was considered to be specific for small ruminant AS (SI Appendix, Fig. S1). This panel of AS isolates was transmitted to the VRQ ovine PrP transgenic mice (tg338). The transmission properties such as incubation period (SI Appendix, Table S1), vacuolar lesion profile (Fig. 1A), and PrP<sup>res</sup> Western blot profile in the brain (SI Appendix, Fig. S1) of the propagated AS isolates observed after 2 or 3 iterative passages in tg338 were similar and were the same as previously reported for AS passage in tg338 mice (21–23).

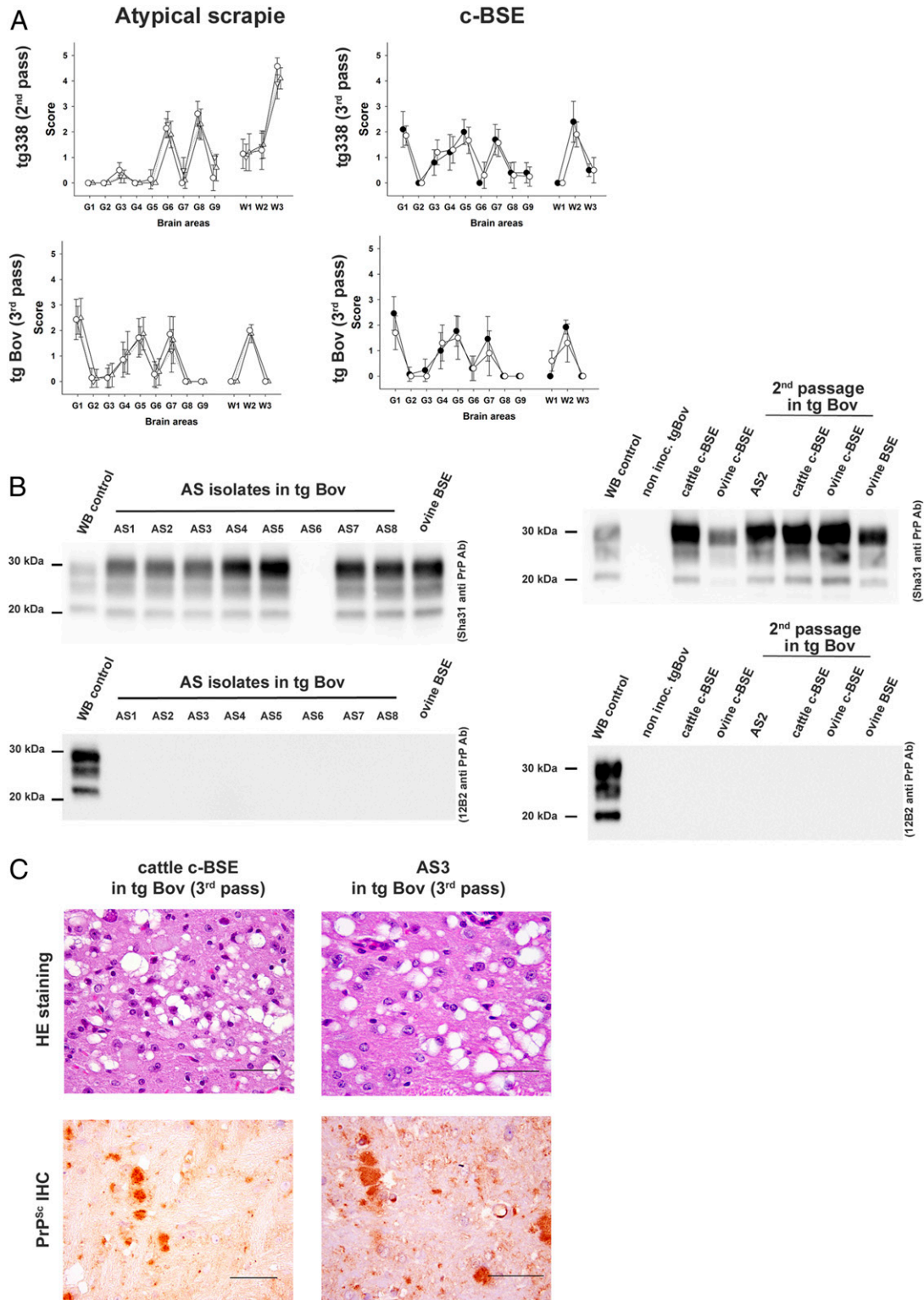
The panel of 8 AS isolates was individually serially transmitted (2 or 3 iterative passages) in bovine PrP transgenic mice (tgBov; Table 1). On first passage, signs of clinical prion disease were observed in a low proportion of inoculated tgBov mice. PrP<sup>res</sup> was detected by Western blot in the brains of clinically affected mice and in some mice that displayed no apparent clinical signs of prion disease when euthanized at the end of their life expectancy. No PrP<sup>res</sup> accumulation was observed in tgBov mice after inoculation with several of the AS isolates (AS 2, AS 3, AS 5, AS 6, and AS 7), as was the case with classical scrapie PS42 (Table 1).

**Table 1. Inoculation of atypical scrapie isolates in bovine PrP (tgBov) expressing mice**

Identifiant	Isolates		TgBov					
			First passage		Second passage		Third passage	
			Positive mice	Incubation, mean ± SD	Positive mice	Incubation, mean ± SD	Positive mice	Incubation, mean ± SD
AS 1	Fr	ARQ*/ARQ	1/6	533	3/5	317 ± 63	7/7	235 ± 16
AS 2	Sp	ARR/ARQ	0/6	>650	7/9	354 ± 26	5/5	273 ± 5
AS 3	Sp	ARQ/ARH	0/6	>650	2/11	504, 525 <sup>‡</sup>	12/12	269 ± 13
AS 4	Nor	ARQ*/ARQ*	3/4	395 ± 44	6/6	230 ± 17	6/6	271 ± 18
AS 5	Sp	ARQ/ARQ	0/6	>650	0/4	>650	NA	—
AS 6	Sp	ARQ/ARH	0/6	>650	1/4	>650 <sup>‡</sup>	NA	—
AS 7 <sup>†</sup>	It	ARQ/AHQ	0/6	>650	1/7	424	6/6	286 ± 14
AS 8	Po	ARQ/ARQ	1/5	439	5/5	297 ± 14	6/6	250 ± 4
PS42	Fr	VRQ/VRQ	0/6	>650	0/6	>650	0/6	>650
Ovine c-BSE	Fr	ARQ/ARQ	6/6	254 ± 19	6/6	234 ± 12	6/6	232 ± 6
Cattle c-BSE	Fr	—	6/6	295 ± 12	6/6	265 ± 35	6/6	243 ± 7

Transgenic mice that express the bovine PrP (tgBov) were inoculated intracerebrally (6 to 12 mice, 20 µL per mouse) with 8 sheep or goat (<sup>†</sup>) atypical scrapie (AS) isolates originating from 5 different countries, France (Fr), Spain (Sp), Norway (Nor), Italy (It) or Portugal (Po). The AS affected animals displayed a different PRNP genotype at codons 136, 154, and 171. Some also displayed a F/L dimorphism at codon 141 (\*). Cattle classical BSE (c-BSE), ovine c-BSE (first passage of cattle c-BSE in an ARQ/ARQ sheep by the intracerebral route), and classical scrapie (PS42) isolates were inoculated in the same mouse model. After first and second passages, clinically affected or asymptomatic mice that had lived for more than 500 d post inoculation were pooled and used for subsequent passage in the same line. Mice were considered positive when abnormal PrP deposition was detected in the brain. Incubation periods (in days) are shown as mean ± SD except when less than 50% of the mice were found to be positive. In that case, the incubation periods of the positive mice are individually presented. NA, not available. Cattle c-BSE transmission in tgBov data from ref. 51. PS42 transmission data from ref. 15.

<sup>‡</sup>Abnormal PrP positive and found dead animals (without symptoms).



**Fig. 1.** Brain lesion profile and PrP<sup>res</sup> Western blot profiles in tgBov and tg338 mice inoculated with atypical/Nor98 scrapie (AS) or c-BSE. Groups of mice ( $n \geq 6$ ) that express either ovine VRQ PrP (tg338 mice) or bovine PrP (tgBov mice) were intracerebrally challenged with atypical scrapie isolates (AS) or an ovine classical BSE isolate (c-BSE). (A) After 2 or 3 iterative passages in each mouse line, a standard lesion profile was established by scoring the vacuolar changes observed in predefined brain areas. In c-BSE graphs, ●, ovine c-BSE; ○, cattle c-BSE. In AS graphs, ○, AS 1; △, AS 2; ▽, AS 3. (B) The accumulation of PK-resistant PrP (PrP<sup>res</sup>) in the brain of tgBov mice was established by Western blot using anti-PrP monoclonal antibodies Sha31 (epitope 145-YEDRYRE-152) and/or 12B2 (epitope 89-WGQG-93). The same Western blot PrP<sup>res</sup> control (classical scrapie isolate) was used on all of the gels labeled as WB control. Cattle c-BSE and an ovine c-BSE (original isolate and isolate passaged in tgBov) were included as controls. (C) Vacuolar lesions (thalamus level, conventional histology; hematoxylin-eosin) and abnormal PrP deposition (mesencephalon: tegmentum, immunohistochemistry using 6H4 anti-PrP antibody, epitope 147-DYEDRYRE-155) in tgBov mice inoculated with AS3 and cattle BSE. (Scale bars: vacuolar lesions, 25  $\mu$ m; abnormal PrP deposition, 50  $\mu$ m.)

Second passage of the AS isolates in tgBov mice was performed using either first-passage PrP<sup>res</sup>-positive brains or pooled PrP<sup>res</sup>-negative brains as inoculum. During this process, 7 out of the 8 AS isolates caused the occurrence of clinical prion disease in a proportion of animals in each group inoculated. On third passage (available for AS 1, 2, 3, 4, and 7), 100% attack rates and mean incubation periods that ranged between 235 and 286 dpi were recorded (Table 1).

At each passage stage, a 3-band PrP<sup>res</sup> Western blot profile characterized by a prominent di-glycosylated PrP band was observed in the brains of the clinically positive tgBov mice (Fig. 1B). Strikingly, the lesion profile (Fig. 1A), the PrP<sup>res</sup> Western blot profile (Fig. 1B), and the histopathological lesions (Fig. 1C) in the brains of AS inoculated tgBov mice were identical to those observed for transmission of c-BSE (sheep or cattle origin) to tgBov mice. Importantly, the inoculation of one classical scrapie isolate (PS42) to the same mouse models resulted in the occurrence of a 100% attack rate for prion disease with a short incubation period in tg338 mice (SI Appendix, Table S1), but no clinical disease or PrP<sup>res</sup> accumulation in the brains of tgBov (Table 1).

In order to further characterize the nature of the TSE agent that propagated in tgBov mice inoculated with the AS isolates, prions obtained after second passage (isolate AS 2) or third passage (isolate AS 3) in this mouse line were transmitted (2 iterative passages) to VRQ (tg338) and ARQ (tgARQ) ovine PrP transgenic mice (Table 2). The incubation periods (Table 2 and SI Appendix, Table S1), the lesion profile (Fig. 2A), and the PrP<sup>res</sup> Western blot profile in the brain (Fig. 2B) of tg338 mice inoculated with tgBov-adapted AS isolates clearly differed from those observed in the same mouse line inoculated with the original AS isolates.

No PrP<sup>res</sup> deposition could be detected in the spleen of tg338 mice inoculated with the original AS isolates (Fig. 3A). Conversely, transmission in tg338 of tgBov-adapted AS isolates was associated with a PrP<sup>res</sup> accumulation in the spleen, as was transmission of ovine and cattle c-BSE (Fig. 3A). PrP<sup>res</sup> WB profile in the spleen of tg338 mice that were inoculated with c-BSE and AS isolates passaged in tgBov were identical (Fig. 3B).

Transmission of tgBov-adapted AS isolates in both tg338 and tgARQ mice resulted in prion incubation periods (Table 2), brain vacuolar lesion profiles (Fig. 2A), PrP<sup>res</sup> Western blot profile patterns (Fig. 2B), and PrP<sup>res</sup> distribution patterns in the brain (Fig. 3A) that were similar to c-BSE passaged in tg338 and tgARQ mice. Collectively, these results demonstrate beyond reasonable doubt that our transmission of AS in a bovine PrP host resulted in the propagation of the c-BSE agent. Since the bioassays reported here were performed in 3 independent institutes (located in France and Spain) that used inoculum prepared by 5 distinct

laboratories, we exclude the possibility of a cross-contamination of the original AS isolates by the c-BSE agent.

Two hypotheses could explain the emergence of the c-BSE agent in tgBov mice after their inoculation with AS isolates. First, c-BSE prions could be present at a low level in the original AS isolates. The high sensitivity of tgBov mice for detection of the c-BSE agent could allow this potentially low level of bovine prions to be identified during passage of the original AS isolates in the bovine PrP host. Alternatively, the occurrence of c-BSE in AS-inoculated tgBov mice could result from a mutation of AS strain properties triggered by passage across the bovine transmission barrier for this particular ovine prion strain.

In order to explore the origin of the c-BSE agent observed in tgBov mice inoculated with AS, we employed *in vitro* protein misfolding cyclic amplification (PMCA), a methodology that mimics prion replication *in vitro*, but in an accelerated form, allowing amplification of minute amounts of PrP<sup>Sc</sup> and prion infectivity (27). In PMCA, a PrP<sup>C</sup>-containing substrate is combined with a seed that contains PrP<sup>Sc</sup>. Following repeated cycles of incubation and sonication, the amount of PrP<sup>Sc</sup> increases.

PMCA has been previously reported to amplify the c-BSE agent with great efficacy using either tgARQ or tgBov mouse brain homogenate as substrate (28). Using this protocol, 2 (tgARQ substrate) or 3 (tgBov substrate) amplification rounds were sufficient to reach the detection limit for c-BSE prion seeding activity (SI Appendix, Fig. S2). The level of prion infectivity and prion seeding activity of a reference sheep-passaged c-BSE isolate were endpoint-titrated by both bioassays in tgBov mice and PMCA, respectively (SI Appendix, Table S2). The infectious prion titer of the sheep-passaged c-BSE isolate was  $\sim 10^{7.2}$  LD<sub>50</sub>/g IC in tgBov mice. The prion seeding titer (SA<sub>50</sub>) was estimated to be  $\sim 10^{11.1}$  SA<sub>50</sub>/g using tgARQ mouse tissue as substrate and  $10^{11.05}$  SA<sub>50</sub>/g using tgBov mouse tissue as substrate. Considering the fact that mice were inoculated using 20  $\mu$ L of sample and the PMCA reactions were seeded using 5  $\mu$ L of the same sample, the PMCA can be considered to be about 1,500 fold more sensitive than the bioassay in tgBov. This also means that 1 c-BSE LD<sub>50</sub> in tgBov mice corresponds to  $\sim 1,500$  SA<sub>50</sub> assessed by PMCA.

In addition to its high sensitivity, *in vitro* PMCA can reproduce, at least partly, the transmission barrier phenomenon observed during the *in vivo* prion bioassay (29). Therefore, amplification of prion seeding activity in AS isolates by PMCA using tgBov mouse tissue as substrate offered an opportunity to characterize the potential impact of the bovine transmission barrier on AS strain properties.

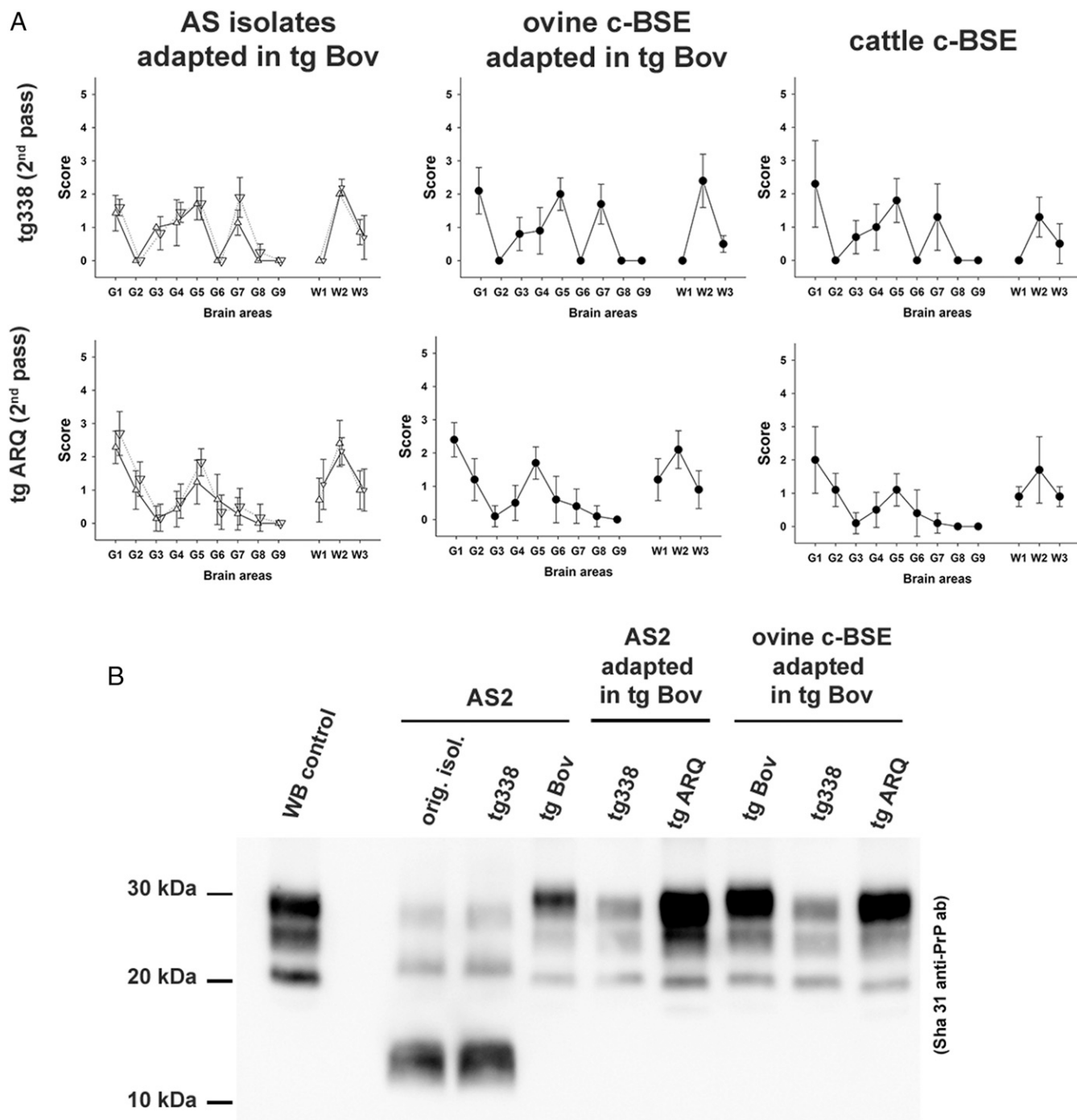
The AS isolates that were originally transmitted to tgBov mice (except AS8) and 18 additional AS isolates (originating from Norway, France, and Portugal) were subjected to PMCA (Table 3). Each AS isolate was used to seed reactions containing either

**Table 2. Inoculation of atypical scrapie adapted in tgBov and c-BSE isolates in ovine PrP expressing mouse models (tg338 and tgARQ)**

Identifiant	Origin	Tg338				TgARQ			
		First passage		Second passage		First passage		Second passage	
		Positive mice	Incubation, mean $\pm$ SD	Positive mice	Incubation, mean $\pm$ SD	Positive mice	Incubation, mean $\pm$ SD	Positive mice	Incubation, mean $\pm$ SD
AS 2	Second pass in TgBov	6/6*	>650	6/6	617 $\pm$ 75	6/6	350 $\pm$ 9	6/6	260 $\pm$ 3
AS 3	Third pass in TgBov	6/6*	>650	6/6	672 $\pm$ 83	6/6	354 $\pm$ 21	6/6	257 $\pm$ 2
Ovine c-BSE	Second pass in TgBov	6/6*	>750	6/6	653 $\pm$ 32	6/6	270 $\pm$ 12	6/6	259 $\pm$ 4
Cattle c-BSE	Cattle	6/6*	>700	6/6	682 $\pm$ 52	6/6	321 $\pm$ 16	6/6	263 $\pm$ 7

Transgenic mice that express the VRQ (tg338) or ARQ (tgARQ) variants of ovine PrP were inoculated intracerebrally (6 mice, 20  $\mu$ L per mouse) with atypical scrapie isolates or ovine c-BSE isolate that had previously been adapted in tgBov (2 iterative passages). Cattle BSE was also included as control. After first passage, clinically affected or asymptomatic mice that had lived for more than 500 d post inoculation were pooled and used for second passage in the same line. Mice were considered positive when abnormal PrP deposition was detected in the brain. Incubation periods (in days) are shown as mean  $\pm$  SD.

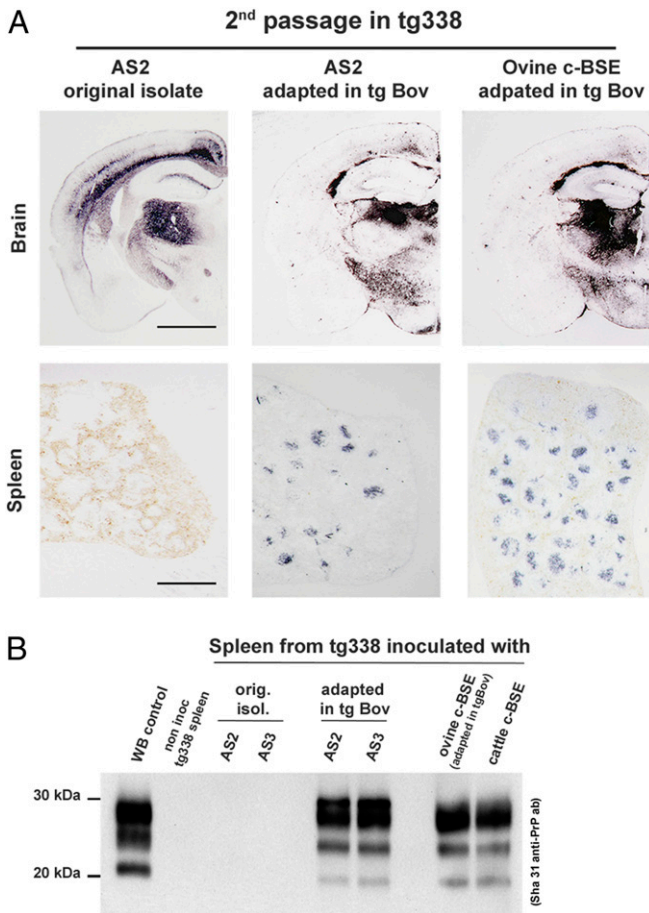
\*Abnormal PrP positive and found dead animals (without symptoms).



**Fig. 2.** Brain lesion profile and PrP<sup>res</sup> Western blot in the brain of tgARQ and tg338 mice inoculated with AS scrapie adapted in tgBov. Groups of mice ( $n \geq 6$ ) that express ovine VRQ PrP (tg338 mice) or ovine ARQ PrP (tgARQ mice) were intracerebrally challenged with atypical scrapie isolates (AS) or an ovine c-BSE isolate that had previously been adapted (2 iterative passages) in tgBov mice. (A) After 2 iterative passages in each mouse line, a standard lesion profile was established by scoring the vacuolar changes observed in predefined brain areas. In AS graphs,  $\Delta$ , AS 2;  $\nabla$ , AS 3. (B) The Western blot profile of PK-resistant PrP (PrP<sup>res</sup>) in the original AS isolates and in the brain of inoculated mice was established by Western blot using anti PrP monoclonal antibody Sha31 (epitope 145-YEDRYRE-152). The same Western blot PrP<sup>res</sup> control (classical scrapie isolate) was used on all of the gels labeled as WB control.

bovine PrP or ovine ARQ PrP substrate (10 to 18 replicates per substrate). After amplification, PrP<sup>res</sup> was detected by Western blot in a low proportion of the reactions seeded, with 19 out of the 25 AS isolates for tgBov and tgARQ combined (Table 3). In most instances, a similar proportion of PrP<sup>res</sup>-positive PMCA reactions were observed when either bovine PrP or ovine ARQ PrP was used as substrate. However, in some cases ( $n = 3$ ), a low

number of PrP<sup>res</sup>-positive reactions were observed when bovine PrP was used as substrate (in the case of AS 10) or when ovine ARQ PrP was used as substrate (in the cases of AS 9 and AS 25). Whatever combination of AS isolate and substrate PrP was used, the PrP<sup>res</sup> Western blot profile in PMCA-positive reaction products and its reactivity with 12B2 antibody were indistinguishable from those observed for PMCA reaction products seeded with



**Fig. 3.** Brain and spleen PrP<sup>res</sup> accumulation in tg338 mice inoculated with AS scrapie adapted in tgBov. Groups of mice ( $n \geq 6$ ) that express ovine VRQ PrP (tg338 mice) were intracerebrally challenged with atypical scrapie isolates (AS) and AS that had previously been adapted (2 iterative passages) in tgBov mice. In parallel, cattle c-BSE isolate and ovine BSE isolate (adapted in tgBov) were transmitted (2 iterative passages) in tg338 mice. (A) In tg338 mice (second passage), the PrP<sup>res</sup> distribution pattern in the brain (thalamic coronal section) and in the spleen was established by paraffin-embedded tissue blot using anti-PrP monoclonal antibody Sha31 (epitope 145-YEDRYRE-152). (Scale bars: brain, 160  $\mu$ m; spleen, 100  $\mu$ m.) (B) The Western blot profile of PK-resistant PrP (PrP<sup>res</sup>) in the spleen of tg338 mice (second passage) inoculated with AS isolates and AS isolates passaged in tgBov and c-BSE (cattle and ovine origin) was established using anti-PrP monoclonal antibody Sha31.

authentic ovine c-BSE prions (Fig. 4). No PrP<sup>res</sup> was observed in PMCA reactions that were unseeded ( $n = 120$ ) or in those reactions seeded ( $n = 60$ ) with prion-free sheep brain homogenate (representative samples shown in Fig. 4). It should be noted that the PrP amino sequence was 100% homologous between certain AS isolates (AS 5, AS 26) and the ovine PrP substrate (tgARQ) used in PMCA reactions. Therefore, in vitro amplification of c-BSE prions in PMCA reactions seeded with these AS isolates using ovine ARQ PrP as substrate cannot be a consequence of mutation of prion strain properties triggered by a transmission barrier.

Taken together, the tgBov mouse bioassay and PMCA results strongly support the view that a low level of c-BSE prions was initially present in at least 21 out of the 26 AS isolates tested.

To further clarify the origin of the c-BSE agent detected in AS isolates, 2 of these isolates (AS 25 and AS 26) were endpoint-titrated in tg338 mice (1/10 dilution series, 6 or 7 tg338 mice inoculated per dilution). For both isolates, the last positive transmissions were observed in mice that received a  $10^{-6}$  log<sub>10</sub> dilution of the original 10% w/vol brain material (*SI Appendix, Table S3*). The

brains of these endpoint-titration tg338 mice were subsequently subjected to PMCA. Irrespective of the substrate used for PMCA, either bovine or ovine ARQ PrP, PrP<sup>res</sup> was observed in a similar proportion of the PMCA reactions seeded with either the original AS isolates or AS isolates passaged in tg338 mice (Table 4). The PrP<sup>res</sup> Western blot profile observed in all of the PMCA-positive reactions was identical to that seen in reactions seeded with authentic c-BSE prions (Fig. 5). PMCA reactions seeded with brain homogenate prepared from age-matched noninoculated tg338 mice remained PrP<sup>res</sup>-negative (Table 4 and Fig. 5).

Considering the level of c-BSE seeding activity originally present in isolates AS 25 and AS 26 (less than 100 SA<sub>50</sub>/mL; Table 4), there is an extremely low level of probability that one of the 6 tg338 mice inoculated with 20  $\mu$ L of a  $10^{-6}$  diluted AS isolate ( $<2 \cdot 10^{-6}$  SA<sub>50</sub> per dose of inoculum) could be exposed to 1 infectious dose of c-BSE agent (1 c-BSE LD<sub>50</sub> is  $\sim 1,500$  SA<sub>50</sub>). Consequently, the presence of c-BSE prion seeding activity in the brains of tg338 mice inoculated with a  $10^{-6}$  log<sub>10</sub> dilution of original AS isolate implies that a low titer of c-BSE prions was generated during the propagation of ovine AS prions in a host that expressed ovine PrP, namely tg338 mice.

## Discussion

The mechanism(s) that lead to an alteration in the phenotype of prion strains as these transmissible entities undergo transmission between different host species remain uncertain. This is despite the identification that differences in amino acid sequence between host PrP<sup>C</sup> and donor PrP<sup>Sc</sup>, together with prion strain identity, are principal determinants of the transmission barrier (9, 10). Based on the concept that conformation of PrP<sup>Sc</sup> molecules/aggregates encode prion strain information (2, 4, 5, 30, 31), at least 2 non-exclusive hypotheses, “deformed templating” (32, 33) and the “conformational selection model” (32–35), have been proposed to explain the mutation of prion strains.

The deformed templating hypothesis postulates that a prion strain replicates as a clone of PrP<sup>Sc</sup> molecules/aggregates. When confronted by a transmission barrier that does not allow clonal prion replication, the propagation process is modified so that “altered” PrP<sup>Sc</sup> structural variants are generated in an attempt to convert the new host PrP<sup>C</sup>. While the majority of these presumably fail to replicate efficiently in the new host, variants eventually emerge that are successful and adapt to the new PrP environment through multiple trial-and-error replication events. In this deformed templating model, confrontation of the transmission barrier serves as the triggering event that initiates the generation of new prion variant(s) and as a filter for their selection (35).

The conformational selection model proposes that a prion strain naturally propagates in its host as an ensemble of PrP<sup>Sc</sup> conformers dominated by a stable energetically favorable conformation responsible for the observed prion strain phenotype. Furthermore, this model predicts that the number of stable PrP<sup>Sc</sup> conformers is limited for each PrP amino acid sequence, which would explain the existence of a finite number of stable prion strains that can propagate in a given species. It is further proposed that, during transmission of a prion strain to a new host, one of the less dominant PrP<sup>Sc</sup> conformers of those present in the ensemble is selected with a resultant change, or mutation, in the properties of the newly propagating prion strain. In the conformational selection model, the transmission barrier acts simply as a selective filter for new prion variants, and ease of permeation of the barrier results from the extent of overlap of PrP<sup>Sc</sup> conformers that exist between the interacting species (32, 33).

Our data reported here showed that c-BSE prions are present as a minor variant in natural isolates of ovine AS. In addition, transmission of ovine AS to bovine PrP mice demonstrated that c-BSE can emerge during these transmissions as the dominant prion strain. These results provide a cogent argument in favor of

**Table 3. Protein Misfolding Cyclic Amplification seeding activity in atypical scrapie isolates**

Identifiant	Isolates		PMCA positive reactions	
	Origin	Genotype	TgBov substrate	TgARQ substrate
AS 1	Fr	ARQ*/ARQ	3/12	5/12
AS 2	Sp	ARR/ARQ	2/12	3/12
AS 3	Sp	ARQ/ARH	3/12	4/18
AS 4	No	ARQ*/AFRQ	12/12	9/12
AS 5	Sp	ARQ/ARQ	4/12	3/12
AS 6	Sp	ARQ/ARH	5/12	1/12
AS 7	It	ARQ/AHQ	1/12	2/12
AS 8	Po	ARQ/ARQ	ND	ND
AS 9	Nor	ARR/ARQ	0/12	2/10
AS 10	—	ARQ*/AHQ	1/12	0/10
AS 11	—	AHQ/ARQ	3/12	7/12
AS 12	—	ARR/ARQ	0/12	0/12
AS 13	—	ARR/AHQ	0/12	0/12
AS 14	—	ARQ/AHQ	1/12	1/12
AS 15	—	ARR/ARR	3/12	1/12
AS 16	—	ARR/AHQ	1/12	3/10
AS 17	—	ARQ*/AHQ	1/12	1/10
AS 18	—	ARQ*/AHQ	0/12	0/10
AS 19	Po	ARR/ARR	0/12	0/12
AS 20	—	ARR/AHQ	3/12	1/12
AS 21	—	ARR/ARR	0/12	0/10
AS 22	—	ARQ*/AHQ	0/12	0/12
AS 23	—	ARQ*/ARQ	1/12	1/12
AS 24	—	ARQ*/ARQ	2/12	4/12
AS 25	Fr	AHQ/AHQ	0/12	1/12
AS 26	—	ARQ/ARQ	1/12	1/12
TSE free sheep	—	ARQ/ARQ	0/12	0/12
Unseeded	—	—	0/120	0/120

Twenty-six AS scrapie cases (1/50 diluted 10% brain homogenates) originating from 5 different countries (France [Fr], Spain [Sp], Italy [It], Portugal [Po] and Norway [Nor]) were used to seed PMCA reactions (5 µL of seed per reaction). The AS affected animals displayed different *Prnp* genotypes at codons 136, 154, and 171. Two different PMCA substrates were used. The first one was prepared using brains from transgenic mice overexpressing the ARQ variant of the sheep prion protein (tgARQ). The second was prepared using brains from transgenic mice overexpressing the bovine prion protein (tgBov). For each isolate and substrate, 10 to 18 individual replicates were tested. Reactions were subjected to 3 amplification rounds. After each round, reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round. PMCA reaction products (third amplification round) were analyzed by Western blot for the presence of PrP<sup>res</sup>. The number of PrP<sup>res</sup> Western blot positive reactions/total number of reactions are reported. Unseeded reactions and reactions seeded with brain homogenate prepared from a TSE-free sheep were included as specificity controls. ND, not done.

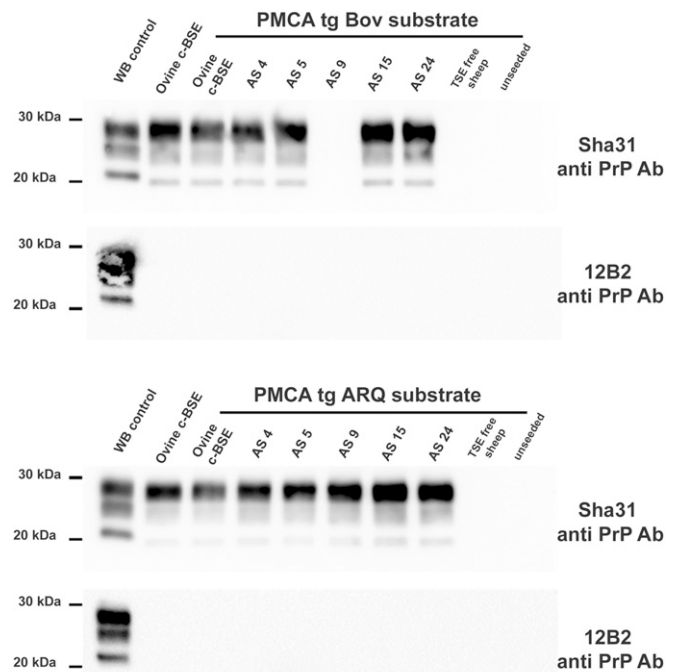
\*F/L dimorphism displayed at codon 141.

the conformational selection model as the mechanism for prion strain mutation during interspecies prion transmission. This would be expected to occur by selection of a preexisting PrP<sup>Sc</sup> variant in AS isolates, one best suited to the new replicative environment. Within this conceptual framework, the occurrence of prion strain mutation is dependent upon the particular repertoire of PrP<sup>Sc</sup> variants associated with distinct prion strains. This notion is supported by our observation that c-BSE prions emerged during serial transmission of ovine AS in tgBov mice but not from serial passage of classical scrapie in the same mouse line [Table 1 and Cassard et al. (15)].

The diversity of prion strains that exist in small ruminants remains undefined, although it is established that at least 5 different natural ovine prion strains exist, including AS (6, 36–39). According to the conformational selection model, each of these

different ovine prion strains is associated with a unique and stable PrP<sup>Sc</sup> conformer and a distinct set of minor variants. The tgBov mouse line has previously been reported to support the propagation of a variety of natural ovine prions, of which several displayed significantly shorter incubation periods than c-BSE (15). Strikingly, in our experiments, the diversity of prion variants in the AS isolates (7 different cases) revealed by the serial passage in tgBov was restricted to the c-BSE agent (Table 1). This consistent emergence of a single prion strain argues against the view that AS prion replication in sheep can randomly generate all of the existing stable PrP<sup>Sc</sup> variants associated with a particular ovine PrP<sup>C</sup> amino acid sequence. Instead, our data support the view that individual prion strains are associated with a restricted repertoire of stable PrP<sup>Sc</sup> variants in a given host. Whether AS is unique in its ability to generate c-BSE prion particles during its replication process remains to be established.

Classical BSE was first recognized in 1984 and 1985 as a novel prion disease affecting cattle in the United Kingdom (40). Epidemiological data clearly established that the number of cases of c-BSE was amplified by the recycling of infected animal carcasses into cattle feed in the form of meat and bone meal (MBM) (41). Since bovine prion disease had not been recognized in cattle prior to the c-BSE epizootic and the disease is apparently non-contagious between cattle, several hypotheses were proposed to explain its emergence. These range from the spontaneous occurrence



**Fig. 4.** PrP<sup>res</sup> detection in PMCA reactions seeded with atypical/Nor98 scrapie isolates. Protein misfolding cyclic amplification (PMCA) reactions were seeded with atypical/Nor98 scrapie (AS) isolates (1/50 diluted 10% brain homogenate) that had been identified in 5 European countries (Table 3). PMCA reactions seeded with brain homogenate from a TSE-free sheep (originating from New Zealand) and unseeded PMCA reactions were included as specificity controls. PMCA substrate consisted of brain homogenate from either bovine PrP (tgBov) or ovine PrP (tgARQ) mice. PMCA reactions were subjected to 3 (tgARQ substrate) or 4 (tgBov substrate) amplification rounds, each comprising 96 cycles (10 s sonication, 14 min and 50 s incubation at 39.5 °C) in a Qsonica700 device. The PMCA reactions were analyzed by Western blot for the presence of abnormal PK-resistant PrP (PrP<sup>res</sup>) using anti-PrP monoclonal antibodies Sha31 (epitope 145-YEDRYRE-152) and/or 12B2 (epitope 89-WGQGG-93). Each Western blot included a classical scrapie isolate (labeled as WB control) and an ovine c-BSE isolate as controls.



**Table 4. PMCA seeding activity in atypical scrapie passed in tg338**

Case	PMCA seeds		PrP <sup>res</sup> positive PMCA reactions		Seeding activity, SA <sub>50</sub> /mL	
	Origin		TgBov substrate	TgARQ substrate	TgBov substrate	TgARQ substrate
AS 25	Sheep		0/12	1/12	0 (0–10 <sup>1.86</sup> )*	10 <sup>1.40</sup>
	First passage in tg338 (neat)	Mouse 1	2/12	1/12	10 <sup>1.72</sup>	10 <sup>1.40</sup>
	Second passage in tg338 (neat)	Mouse 1	2/12	1/12	10 <sup>1.72</sup>	10 <sup>1.40</sup>
	End-point titration in tg338 (10 <sup>-6</sup> dilution)	Mouse 1	2/12	3/12	10 <sup>1.72</sup>	10 <sup>1.92</sup>
		Mouse 2	1/12	3/12	10 <sup>1.40</sup>	10 <sup>1.92</sup>
	Mouse 3	2/12	2/12	10 <sup>1.72</sup>	10 <sup>1.72</sup>	
AS 26	Sheep		1/12	1/12	10 <sup>1.40</sup>	10 <sup>1.40</sup>
	First passage in tg338 (neat)	Mouse 1	2/12	0/12	10 <sup>1.72</sup>	0 (0–10 <sup>1.86</sup> ) *
	Second passage in tg338 (neat)	Mouse 1	2/12	0/12	10 <sup>1.72</sup>	0 (0–10 <sup>1.86</sup> ) *
	End-point titration in tg338 (10 <sup>-6</sup> dilution)	Mouse 1	2/12	1/12	10 <sup>1.72</sup>	10 <sup>1.40</sup>
		Mouse 2	1/12	1/12	10 <sup>1.40</sup>	10 <sup>1.40</sup>
		Mouse 1	0/12	0/12	—	—
	Noninoculated tg338	Mouse 2	0/12	0/12	—	—
		Mouse 2	0/12	0/12	—	—
		Mouse 3	0/12	0/12	—	—

Two sheep atypical scrapie (AS) isolates were selected. The 10% w/vol brain homogenates were inoculated into tg338 mice (2 iterative passages). Groups of 6 tg338 mice were inoculated intracerebrally with 20 µL of serial 10-fold dilutions of the same homogenates. Transmission was observed in 3 (AS 25) and 2 (AS 26) mice inoculated with 10<sup>-6</sup> brain homogenate. No transmission was observed at lower dilutions. PMCA reactions (12 replicates) were seeded with 1/50 diluted brain homogenate (10% w/vol) from 1) the original sheep, 2) the second passage tg338 mice (pool of brains), and 3) individual brain from positive tg338 in the endpoint titration experiment. Brain homogenates (10% w/vol) from age-matched, noninoculated tg338 mice were also used as seeds (1/50 diluted). Two different PMCA substrates were used. The first one was prepared using brains from transgenic mice overexpressing the ARQ variant of the sheep prion protein. The second was prepared using brains from transgenic mice overexpressing the bovine prion protein (tgBov). Reactions were subjected to up to 4 amplification rounds. After each round, reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round. PMCA reaction products were analyzed by Western blot for the presence of PrP<sup>res</sup>. The number of PrP<sup>res</sup> Western blot-positive reactions/total number of reactions are reported. Seeding activity titers were estimated using the Spearman–Kärber’s limiting dilution titration method (most likely value) or, when no positive reaction was observed, by the Poisson’s probabilistic model. Titers are given as the number of PMCA SA<sub>50</sub> per milliliter of 10% brain homogenate. \*Most likely value and IC 95% as described by Brown et al. (66).

of c-BSE in cattle to the passage and adaptation of a prion originating from another species (42, 43). Our studies here that show the presence of c-BSE prions in AS isolates, combined with the demonstrated presence of AS in the United Kingdom long before the appearance of the c-BSE epizootic in cattle, suggests that the recycling of AS cases in MBM might be a source of bovine prion disease (20). In addition to its potential role in the initial emergence of c-BSE in cattle, the presence of c-BSE prions in natural cases of AS has current and direct implications for both the continued risk of this ovine prion disease to other farmed animals and for human exposure risks. The distribution of AS cases are widespread across the world (17–19). A recent retrospective analysis of surveillance data collected over a period exceeding 10 y in the European Union (EU) concluded that the prevalence of detected AS cases has remained relatively stable in the different member states, with between 2 and 6 positive cases per 10,000 tested animals per year. This implies that a substantial number of AS-infected animals could enter either the animal or human food chain each year (44, 45), and each case represents a potential source of exposure to the c-BSE agent for farmed animals (MBM derived from rendered small ruminants) and human consumers (consumption of healthy slaughtered animals), respectively. The epidemiological features of AS within the EU is likely to reflect the situation of the disease in other countries that breed and maintain small ruminants.

In Europe, the c-BSE crisis and the emergence of vCJD resulted in the implementation of a strong and coherent policy (EU regulation 999/2001) aimed at control and eradication of this animal prion disease. The total feed ban on the use of MBM in animal feed and the systematic retrieval from the food chain of ruminant tissues that have the potential to contain high levels of prion infectivity, so-called Specified Risk Material (SRM) measures, were instrumental for control of c-BSE in cattle and prevention of dietary human exposure to these bovine prions (46, 47). As a side effect, these measures also strongly limited the exposure of farmed animals and human consumers to the other TSE agents circulating in farmed animal species, including AS.

With the decline of the c-BSE epizootic in cattle and the combined increase in pressure from industry, EU authorities have begun to consider discontinuing certain TSE control measures. The abrogation of the SRM measures for small ruminants and the partial reauthorization of the use of processed animal protein, formerly known as MBM, in animal feed are part of the EU authorities’ agenda. Our observation of the presence of the c-BSE agent in AS-infected small ruminants suggests that modification of the TSE control measures could result in an increased risk of exposure to c-BSE prions for both animals and humans. Whether or not this exposure will result in further c-BSE transmission in cattle and/or humans remains an open and important question.

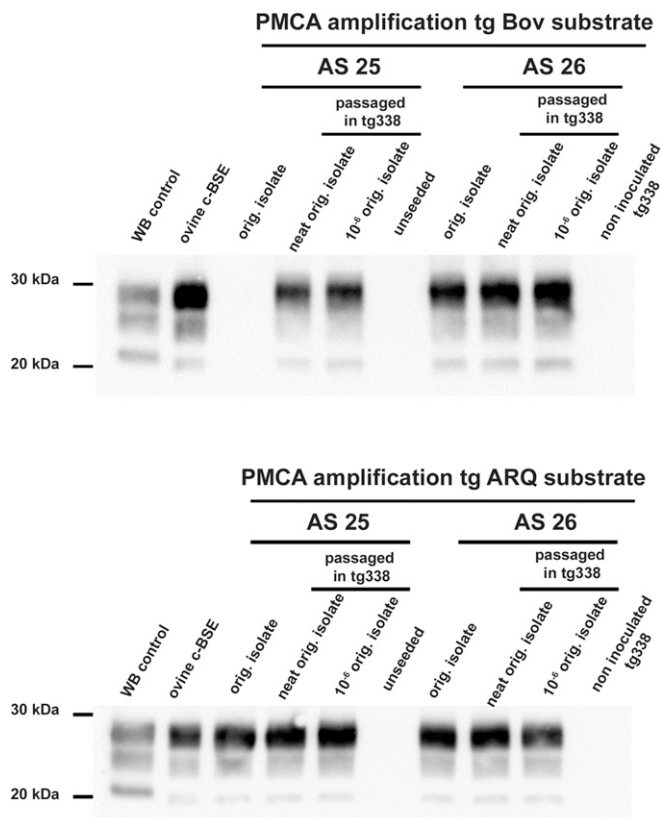
**Methods**

**Ethics Statement.** All animal experiments were performed in compliance with institutional and French national guidelines and in accordance with the European Directives 86/609/EEC and 2010/63/EU. In France, the animal experiments that are part of this study (national registration 01734.01) were approved by the local ENVT ethics committee. Experiments developed in CISA-INIA (Madrid, Spain) were approved by the Committee on the Ethics of Animal Experiments of the Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria and the General Directorate of the Madrid Community Government (permit numbers: CEEA 2009/004 and PROEX 228–16). Mouse inoculations were performed under anesthesia (isoflurane).

Experiments developed in IRTA-CReSA (Barcelona, Catalonia) involving animals were approved by the animal experimentation ethics committee of the Autonomous University of Barcelona (reference number 585–3487) in agreement with Article 28, sections (a), (b), (c), and (d) of the “Real Decreto 214/1997 de 30 de Julio,” European Directive 86/609/CEE, and the European Council Guidelines included in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

Mice that displayed clinical signs were anesthetized with isoflurane before sacrifice using CO<sub>2</sub> inhalation.

**Atypical/Nor98 Scrapie Cases and Control Sheep.** Natural atypical scrapie (AS) cases identified through active or passive surveillance programs were selected according to their geographical origin (France, Spain, Italy, Norway, and Portugal) and PRNP genotypes (SI Appendix, Table S1 and Table 1). These cases



**Fig. 5.** PMCA seeding activity detection in 2 bioassay endpoint-titrated atypical/Nor98 isolates. Two atypical/Nor98 isolates, AS 25 and AS 26 (Table 3), were endpoint-titrated in tg338 mice (1/10 dilution series, 6 tg338 mice per dilution). For both isolates, the last positive transmissions were observed in mice that received a  $10^{-6}$  dilution of the original 10% w/vol brain material (SI Appendix, Table S3). The original AS isolates and the brains of clinically affected mice inoculated with neat and  $10^{-6}$  diluted isolates were used to seed PMCA reactions that either used tgARQ or tgBov as substrate. PMCA reactions seeded with age-matched inoculated tg338 mice and unseeded reactions were included as specificity controls. Reactions were subjected to 3 (tgARQ substrate) or 4 (tgBov substrate) amplification rounds, each comprising 96 cycles (10 s sonication, 14 min and 50 s incubation at 39.5 °C) in a Qsonica700 device. The PMCA reactions were analyzed by Western blot for the presence of abnormal PK-resistant PrP ( $\text{PrP}^{\text{res}}$ ) using the Sha31 (epitope 145-YEDRYRE-152) and/or the 12B2 (89-WGQGG-93) anti-PrP antibodies. Each Western blot included a classical scrapie isolate (labeled as WB control) and an ovine c-BSE isolate as controls.

have been originally classified as AS by TSE national reference laboratories in each country. All of the cases corresponded to sheep except the AS 7 case (goat).

In all cases, PrP genotype was checked by sequencing exon 3 of the *PRNP* gene as previously described (48). The polymorphisms at codons 136 (A/V), 154 (H/R), and 171 (R/Q/H), which have been demonstrated to strongly influence the susceptibility to TSE in sheep, are indicated (49). Additionally, the presence of a phenylalanine at codon 141 (F/L), which has been shown to impact on the susceptibility to atypical/Nor98 scrapie, are also indicated (Tables 1 and 4) (23, 48). Brain material collected in TSE-free Poll-Dorset sheep (APHA) was used as control (50).

**c-BSE Isolates.** Cattle and ovine classical BSE (c-BSE) isolates were used as control. The cattle c-BSE isolate was a natural case originating from France. This isolate was used in previous studies aimed at the characterization of c-BSE strain properties through transmission to mice overexpressing the PrP sequence of various host species (51). The ovine c-BSE isolate was obtained by the intracerebral inoculation of the same cattle c-BSE isolate in ARQ/ARQ TSE-free sheep (first passage) as described in Andréoletti et al., 2004 (50).

**Mouse Bioassays.** Bioassays were carried out using mice expressing bovine PrP (tgBov/tg110) (52, 53) and/or mice expressing ovine ARQ (tgARQ) (54) or VRQ (tg338) PrP (55).

Groups of 6- to 10-wk-old female mice ( $n \geq 6$ ) were anesthetized and inoculated with 20  $\mu\text{L}$  of a 10% tissue homogenate in the right parietal lobe using a 25-gauge disposable hypodermic needle. Mice were observed daily, and their neurological status was assessed weekly. When clinically progressive TSE disease was evident, the animals were euthanized and their brains harvested. Half of the brain was fixed by immersion in 10% formol saline and the other half was frozen at  $-20^\circ\text{C}$ . Tissues from animals found dead were frozen (no formalin fixation). In animals where no clinical signs were observed, mice were killed at the end of their natural lifespan (650 to 750 d). In those cases, incubation periods reported in Tables 1 and 2 as  $>650$  dpi corresponded to the survival time observed in at least 3 out of the 6 mice.

**PMCA Amplification.** Brains from tgBov, tgARQ, and tg338 mice were used to prepare the PMCA substrates. PMCA was performed as previously described (28, 56). Briefly, PMCA reactions (50  $\mu\text{L}$  final volume) were seeded with 5  $\mu\text{L}$  of sample to be tested. PMCA reactions were then subjected to 3 amplification rounds, each comprising 96 cycles (10 s sonication, 14 min and 50 s incubation at 39.5 °C) in a Qsonica700 device. After each round, reaction products (1 volume) were mixed with fresh substrate (9 volumes) to seed the following round. The PMCA reaction products were analyzed by Western blot for the presence of PK-resistant PrP. Each WB line was loaded with the equivalent of 20  $\mu\text{L}$  of PK-digested PMCA product. Each PMCA run included a reference ovine BSE sample (10% brain homogenate) as a control for the amplification efficiency. Unseeded controls (2 unseeded controls for 8 seeded reactions) were also included in each run.

**Western Blot Detection of Abnormal PrP.** PK-resistant abnormal PrP ( $\text{PrP}^{\text{res}}$ ) extraction and Western blot were performed as previously described (57). Immunodetection was performed using 2 different PrP-specific monoclonal antibodies, Sha31 (1  $\mu\text{g}/\text{mL}$ ) (58) and 12B2 (4  $\mu\text{g}/\text{mL}$ ) (59), which recognize the amino acid sequences YEDRYRE (145–152) and WGQGG (89–93), respectively (60).

**Paraffin-Embedded Tissue Blot.** Paraffin-embedded brain tissue from inoculated mice was analyzed as previously described (61–63).

**Lesion Profiling and Abnormal PrP Immunohistochemistry.** Vacuolar brain lesion profiles were established following the method described by Fraser et al. (64). In situ  $\text{PrP}^{\text{Sc}}$  immune labeling was performed as previously described (63) using 6H4 anti-PrP antibody (epitope:  $_{147}\text{DYEDRYRE}_{155}$  of the bovine PrP concentration 3  $\mu\text{g}/\text{mL}$ ).

**Infectious and Seeding Activity Titer Estimates.** A series of 1/10 dilutions of a reference 10% w/vol brainstem from an ovine-BSE (ARQ/ARQ) isolate and 2 AS isolates (AS 25 and AS 26) were prepared. Successive 1/10 dilutions of brain homogenate were inoculated intracerebrally (20  $\mu\text{L}$ ) into tgBov or tg338 mice ( $n = 6$  per inoculum). Dilutions of the same c-BSE isolate were used to seed PMCA reactions that used brain tissue from either bovine PrP (tgBov mice) or ovine ARQ PrP (tgARQ mice) as substrate. Twelve individual replicates of each sample dilution were tested. Reactions were then subjected to 3 amplification rounds. PMCA reaction products (third amplification round) were analyzed by Western blot for the presence of  $\text{PrP}^{\text{res}}$ . The titer of prion seeding activity was estimated by the Spearman–Kärber’s method (65).

For AS and AS passed in tg338 isolates (10% brain homogenate), 1/50 diluted material was used to seed 12 individual reactions (tgBov or tgARQ substrates). After 3 amplification rounds, the number of  $\text{PrP}^{\text{res}}$  Western blot-positive reactions per total number of reactions was established. These ratios were used to estimate seeding activity titers (SA/ $\mu\text{L}$  of 10% brain homogenate) by the limiting dilution titration method (application of Poisson’s probabilistic model) described by Brown et al. (66) or by Spearman–Kärber’s method. According to Fisher et al. (67) and as previously used for prion infectivity comparisons (68), 1 SA<sub>50</sub> was considered to be equivalent to 0.693 SA.

**Data Availability.** All data are available in the manuscript and SI Appendix.

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