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Polymorphisms at codons 108 and 189 in murine PrP play distinct roles in the control of scrapie incubation time

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Susceptibility to transmissible spongiform encephalopathies (TSEs) is associated strongly with PrP polymorphisms in humans, sheep and rodents. In mice, scrapie incubation time is controlled by polymorphisms at PrP codons 108 (leucine or phenylalanine) and 189 (threonine or valine), but the precise role of each polymorphism in the control of disease is unknown. The L108F and T189V polymorphisms are present in distinct structural regions of PrP and thus provide an excellent model with which to investigate the role of PrP structure and gene variation in TSEs. Two unique lines of transgenic mice, in which 108F and 189V have been targeted separately into the endogenous murine *Prnp*^{a} gene, have been produced. TSE inoculation of inbred lines of mice expressing all allelic combinations at codons 108 and 189 has revealed a complex relationship between PrP allele and incubation time. It has been established that both codons 108 and 189 control TSE incubation time, and that each polymorphism plays a distinct role in the disease process. Comparison of ME7 incubation times in mouse lines that are heterozygous at both codons has also identified a previously unrecognized intramolecular interaction between PrP codons 108 and 189.

INTRODUCTION

The transmissible spongiform encephalopathies (TSEs) are a group of fatal, neurodegenerative diseases that include scrapie in sheep, bovine spongiform encephalopathy in cattle, chronic wasting disease in deer and elk and Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker disease and fatal familial insomnia in man. TSE diseases are thought to be caused by conversion of a normal cellular glycoprotein (PrP^{C}) into an abnormal, disease-associated isoform (PrP^{Sc}) that accumulates in diseased tissue (Prusiner, 1996). This conversion of PrP^{C} to PrP^{Sc} is accompanied by an alteration in conformation from predominantly α-helix to β-sheet structure and development of partial resistance to digestion with proteinase K (PK). It has been proposed that PrP^{Sc} constitutes infectivity (Prusiner, 1982) and acts as a seed or template in the conversion of host PrP^{C} to PrP^{Sc} (Jarrett & Lansbury, 1993; Prusiner, 1996; Weissmann, 1996). Whether PrP^{Sc} is indeed the infectious agent remains to be established, but the conversion of PrP^{C} to PrP^{Sc} is clearly a critical event in the pathogenesis of these diseases.

The host PrP gene (*Prnp*) has a major influence over the outcome of TSE disease. PrP polymorphisms have been shown to alter incubation time and TSE susceptibility in mice (Moore *et al.*, 1998), sheep (Goldmann *et al.*, 1994) and man (Palmer *et al.*, 1991; Goldfarb *et al.*, 1992). Classical genetic analysis of the control of TSE incubation time in mice identified the presence of a single gene (*Sinc*) encoding two alleles (*s*7 and *p*7), which programmed short and long incubation times, respectively, for the ME7 strain of scrapie (Dickinson *et al.*, 1968). Similar differences in incubation time have been found between NZW/LacJ and Iln/J mouse strains when infected with the Chandler isolate, identifying a single locus, *Prni*, that controlled incubation time, which was linked closely to *Prnp* (Carlson *et al.*, 1986). Following isolation and characterization of the gene encoding PrP in mice (*Prnp*) (Basler *et al.*, 1986; Locht *et al.*, 1986), mouse strains carrying *Sinc s7* were shown to have a *Prnp* gene encoding 108L_189T ($Prnp^{a}$) and those carrying *Sinc p7* had a *Prnp* gene encoding 108F_189V ($Prnp^{b}$) (Westaway *et al.*, 1987). However, due to the different genetic backgrounds of these lines of inbred mice, it was difficult to confirm that the *Prnp*, *Sinc* and *Prni* genes were congruent and that the polymorphisms in *Prnp* were responsible for the control of scrapie incubation time in mice.

The introduction of 108F and 189T into the murine $Prnp^{a}$ gene by gene targeting produced a line of transgenic mice (FV/FV) that differed from the 129/Ola parental line by only the targeted polymorphisms. Inoculation of these mice with TSE infectivity produced incubation times similar to those in $Prnp^{b}$ mice, demonstrating that *Sinc*, *Prni* and *Prnp* were indeed the same gene and that the codon 108 and/or 189 polymorphisms were the major factors controlling TSE incubation time in mice (Moore *et al.*, 1998; Barron *et al.*, 2003; Barron & Manson, 2004).

In order to determine the individual involvement of the codon 108 and 189 polymorphisms in disease and the mechanism by which they control TSE incubation time in mice, 108F and 189V have been introduced separately into the murine $Prnp^{a}$ gene by gene targeting. These new alleles are designated $Prnp^{a[108L_189V]}$ ($Prnp^{LV}$) and $Prnp^{a[108F_189T]}$ ($Prnp^{FT}$). Inoculation of mice that were homozygous for $Prnp^{a}$, $Prnp^{b}$, $Prnp^{LV}$ or $Prnp^{FT}$ and their heterozygous crosses with mouse scrapie have demonstrated the involvement of both polymorphisms in the control of incubation time.

METHODS

Production of targeting vectors. An 8524bp 129/Ola *Prnp*^{a} genomic DNA clone spanning PrP exon 3 was isolated from a λ DASH II 129/Ola HM-1 embryonic stem (ES) cell genomic DNA library and cloned into pBluescript II. The wild-type PrP open reading frame (ORF) encoding *Prnp*^{a} (108L_189T) was modified by using *in vitro* mutagenesis to express 108F or 189V alone, rather than both, as described previously (Moore *et al.*, 1998). Vectors p108F and p189V were modified to introduce an HSV-TK module and a *LoxP*-flanked PGK/HPRT minigene downstream of exon 3 for selection (Fig. 1). This resulted in the generation of 15·7 kb constructs p108F_*LoxP*/HPRT/TK and p189V_*LoxP*/HPRT/TK, showing a 6·4 kb region of 5′ homology and a 1·4 kb region of 3′ homology flanking the *LoxP*/HPRT minigene. The *LoxP*-flanked HPRT minigene was derived from pBT*LoxP*_HPRTv2 and consists of a PGK-driven HPRT minigene (Selfridge *et al.*, 1992) in pBTSKII+ flanked by two 38 bp *LoxP* sites, allowing the Cre-mediated excision of the *Lox*P-flanked HPRT minigene.

Generation of ES lines and mice with PrP codon 108 and 189 alterations. For each experiment, 200 μg p108F_*LoxP*/HPRT/TK or p189V_*LoxP*/HPRT/TK was linearized by digestion with *Not*I and electroporated into HM-1 ES cells (50×10^{6} cells, 900 V, 3 μF). All tissue-culture reagents and conditions were as described previously (Thompson *et al.*, 1989; Selfridge *et al.*, 1992; Moore & Melton, 1995). ES cells surviving hypoxanthine/aminopterin/thymidine and ganciclovir drug selection were screened for homologous-recombination events by PCR with primer pair 262W (5′-AGCCTACCCTCTGGTAGATTGTCG-3′) and W6991 (5′-CCCAATTCTCTCCTTCTAAGGGAG-3′) to amplify the HPRT minigene [94 °C (60 s), 66 °C (60 s), 72 °C (95 s); 35 cycles]. Targeted clones amplified a 1·75 kb targeting-specific band and were further screened for PrP codon 108 or 189 alterations by a combination of PCR and restriction digestion. The PrP ORF was amplified with primers A996 (5′-GTGGCTGGGGACAACCCCAT-3′) and B188 (5′-GCCTAGACCACGAGAATGCG-3′) to generate a 624 bp PCR product [94 °C (30 s), 65 °C (30 s), 72 °C (60 s); 35 cycles]. The codon 108 alteration was detected by the loss of an *Mnl*I site following the single nucleotide change (CTC to TTC). The codon 189 alteration was detected by digestion with *Bst*EII as described previously (Moore & Melton, 1995).

Cre deletion of the HPRT minigene. The *LoxP*-flanked PGK/HPRT module was excised from targeted alleles by transient transfection of ES cells with the Cre recombinase expression construct pMCCreN (Gu *et al.*, 1993). Clones were transfected with 150 μg pMCCreN (10×10^{6} cells, 900 V, 3 μF) and selected by 6-thioguanine. Drug-resistant clones were screened for deletion of the *LoxP*-flanked HPRT minigene by PCR with primers W7878 (5′-AGTCAGGGAGGAGTAACACAGAAGG-3′) and W6991 [94 °C (60 s), 66 °C (60 s), 72 °C (95 s); 35 cycles]. This generated a 1·61 kb product from the wild-type allele and a slightly larger band of 1·66 kb from the HPRT-deletant allele. This size difference arises from the retention of a single 38 bp *LoxP* site and a portion of the pBluescript multiple-cloning site. ES cell clones were

examined by Southern analysis for the expected gene structure and the absence of additional recombination events, as described previously (Moore *et al.*, 1995).

Generation of mice. Germ-line chimeras were generated as described previously (Thompson *et al.*, 1989). Briefly, HM-1 ES cells with the desired alterations were introduced into BALB/c 3·5-day-old blastocysts and transferred to pseudopregnant MF1 recipients. Chimeras were crossed directly to 129/Ola stock-generating mice with PrP codon 108 and 189 alterations, which were co-isogenic with the parental strain 129/Ola Hsd (Harlan). Germ-line pups were screened by a combination of PCR and *Mnl*I or *Bst*EII restriction digestion, as described above. 129/Ola littermates heterozygous for the targeted PrP allele that were also wild-type at the deleted HPRT locus in HM-1 cells were crossed to establish the line.

Northern blot analysis. Total RNA was isolated from terminal brains by using RNAzol B, based on the guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski & Sacchi, 1987). Total RNA (50 μg) was separated on a 1·0 % agarose/formaldehyde denaturing gel and transferred to Hybond-N (Amersham Biosciences) by capillary transfer overnight. RNA was fixed to the membrane by baking at 80 °C for 2 h before probing. A 936 bp *Kpn*I–*Eco*RI fragment from *Prnp* exon 3 was used to generate the PrP probe. Membranes were hybridized overnight by using ULTRAhyb (Ambion). As a loading control, membranes were reprobed for 18S rRNA by using a 275 bp PCR-generated murine DNA fragment.

Western blot analysis. Homogenates of frozen brain tissue [10 % (w/v)] were prepared in NP40 buffer [0·5 % (v/v) NP40, 0·5 % (w/v) sodium deoxycholate, 150 mM NaCl, 50 mM Tris/HCl (pH 7·5)]. Clarified homogenates (10 000 ***g*** for 10 min at 10 °C) were incubated with or without PK (20 μg ml^{-1}) for 1 h at 37 °C and the reaction was terminated by the addition of PMSF to 1 mM. Samples were prepared at 10 mg ml^{-1} in SDS-PAGE sample buffer (Novex; Invitrogen), incubated at 90 °C for 20 min and separated on 12 or 4–12 % Novex Tris/glycine acrylamide gels (Invitrogen). Proteins were transferred onto a PVDF membrane by electroblotting and incubated overnight at room temperature with mouse anti-PrP monoclonal antibody 7A12 (Li *et al.*, 2000) at a dilution of 50 ng ml^{-1}, or with rat anti-tubulin monoclonal antibody (Abcam) at 250 ng ml^{-1}. Proteins were visualized with horseradish peroxidase-conjugated anti-mouse or anti-rat secondary antibody diluted to 200 ng ml^{-1} (Jackson ImmunoResearch) and a chemiluminescence detection kit (Roche Diagnostics). Membranes were exposed to X-ray film for periods ranging from 10 s to 10 min.

Preparation of inoculum. Inocula were prepared from the brains of C57BL mice with terminal ME7 scrapie and the brains of VM mice with terminal 301V disease. A 1 % homogenate of each sample was prepared in sterile saline prior to use as an inoculum. All mice were inoculated intracerebrally with 20 μl inoculum under anaesthesia. All experimental protocols were submitted to the Local Ethical Review Committee for approval before mice were inoculated. All

experiments were performed under licence to and in accordance with the UK Home Office Regulations [Animals (Scientific Procedures) Act 1986].

Scoring of clinical TSE disease. The presence of clinical TSE disease was assessed as described previously (Dickinson *et al.*, 1968). Animals were scored for clinical disease without reference to the genotype of the mouse. Genotypes were confirmed for each animal by PCR analysis of tail DNA at the end of the experiment. Incubation times were calculated as the interval between inoculation and cull due to terminal TSE disease. Mice were killed by cervical dislocation at the terminal stage of disease, at termination of the experiment (between 600 and 700 days) or for welfare reasons due to intercurrent illness. Half-brains were fixed in 10 % formal saline for 48 h, followed by decontamination in 98 % formic acid for 1 h. The remaining half-brain was frozen at –70 °C for biochemical analysis. Fixed brain tissue was dehydrated in alcohol and impregnated in wax during a 7 h automated processing cycle. Sections were cut coronally at four levels and mounted on Superfrost slides.

Lesion profiles. Sections were haematoxylin/eosin-stained and scored for vacuolar degeneration on a scale of 0–5 in nine standard grey-matter areas and three standard white-matter areas, as described previously (Fraser & Dickinson, 1967).

Genotyping of mouse-tail DNA. A 2–3 cm portion of tail was removed post-mortem from each mouse. DNA was prepared from a 1 cm piece of tail by digestion overnight at 37 °C in tail lysis buffer [300 mM sodium acetate, 1 % SDS, 10 mM Tris (pH 8), 1 mM EDTA, 200 μg PK ml$^{<MIN>1}$)] and subsequent extraction with an equal volume of phenol/chloroform. DNA was precipitated with 2-propanol, washed with 70 % ethanol and resuspended in 100 μl TE buffer [10 mM Tris, 1 mM EDTA (pH 7·4)]. A 605 bp PCR fragment for the *Prnp* ORF was generated from purified tail DNA with an ORF primer, PrP5′> (5′-GTGGCTGGGGACAACCCCAT-3′), and an exon 3 3′-untranslated region primer, PrP3′< (5′-GCCTAGACCACGAGAATGCG-3′). Cycle conditions were: 35 cycles, 94 °C (30 s), 65 °C (30 s), 72 °C (1 min). The *Prnp*$^{\mathrm{LV}}$ allele was detected by the absence of a *Bst*EII site within this product compared with the wild-type *Prnp*$^{\mathrm{a}}$ allele. The *Prnp*$^{\mathrm{FT}}$ allele was detected by the absence of an *Mnl*I site compared with the wild-type *Prnp*$^{\mathrm{a}}$ allele.

RESULTS

Construction of the mouse lines

108F and 189V were introduced separately into the *Prnp*^{a} allele of 129/Ola-derived HM-1 ES cells by using a Cre–*Lox* gene-targeting strategy (Lakso *et al.*, 1992; Gu *et al.*, 1993). These targeting events produced ES cell clones expressing *Prnp*$^{a[108F_189T]}$ (*Prnp*^{FT}) or *Prnp*$^{a[108L_189V]}$ (*Prnp*^{LV}). Presence of the targeted mutations was verified by the loss of an *Mnl*I restriction site at codon 108 (CTC to TTC alteration) and the loss of a *Bst*EII restriction site at codon 189 (ACC to GTC alteration) (Fig. 1). Southern blot analysis using probes derived from the intron/exon 2 region, from intron 2 and from exon 3 confirmed that no further alterations had occurred in the mouse genome during targeting (data not shown). DNA sequence analysis was carried out to ensure that the *Prnp*^{FT} and *Prnp*^{LV} alleles had undergone no detectable deletions, insertions or other rearrangements during the targeting process. However, the gene-targeting strategy resulted in the retention of a 38 bp *LoxP* site and a small portion of the pBluescript multiple-cloning site (~100 bp) 2 kb downstream of exon 3 of the *Prnp* gene (Fig. 1). The *LoxP* site was distant from the intergenic exons between *Prnp* and the closely located *Prnd* (doppel) and was thus not expected to influence the expression of either gene.

HM-1 ES cell clones bearing the targeted *Prnp*^{FT} or *Prnp*^{LV} alleles were injected into 3·5-day-old BALB/c blastocysts to create chimeric mice (Moore *et al.*, 1995), which were crossed with 129/Ola mice to establish the FT/FT and LV/LV transgenic lines co-isogenic with 129/Ola. The identical genetic background of the lines ensured that any observed alterations in incubation time and disease pathology were a direct result of the targeted mutations and were not due to the effects of genes in mice of other genetic backgrounds.

Expression of PrP in the gene-targeted lines

Expression levels of *Prnp* mRNA and mature PrP^{C} were investigated in FT/FT and LV/LV brain and compared with those in wild-type 129/Ola mice (LT/LT) and the existing gene-targeted transgenic line expressing *Prnp*$^{a[108F_189V]}$ (FV/FV) (Moore *et al.*, 1998). Northern blot (Fig. 2) and Western blot (Fig. 3) analysis of tissues from each line confirmed expression from the *Prnp*^{FT} and *Prnp*^{LV} alleles and indicated that the levels of expression in the three homozygous lines (FV/FV, LV/LV and FT/FT) were indistinguishable from those of wild-type 129/Ola mice. *Prnp* gene expression was therefore not affected by the process of gene targeting, the introduction of a single polymorphism or the presence of the *LoxP* site 3′ of the *Prnp* gene. Thus, any alteration in incubation time and neuropathology observed on inoculation of these animals with TSE infectivity should be attributable to the specific alterations at codons 108 and 189, and not due to any differences in *Prnp* gene-expression levels.

Interactions between codon 108 and 189 polymorphisms and ME7 scrapie

The ME7 strain of mouse scrapie is passaged in mice that are homozygous for the *Prnp*^{a} gene (LT/LT) and produces short incubation times in *Prnp*^{a} (LT/LT) mice and prolonged incubation times in *Prnp*^{b} (FV/FV) mice. Following ME7 challenge, LT/FV mice had incubation

times of 223 days, intermediate between those of the parental lines LT/LT (155 days) and the gene-targeted FV/FV (295 days). This pattern of allelic interaction recapitulates that observed between wild-type *Prnp*^{a}, *Prnp*^{b} and F1 crosses and demonstrates that the gene-targeted FV allele can reproduce the dominance pattern observed in mice with wild-type PrP alleles. Incubation times of 168 and 261 days were obtained in FT/FT and LV/LV lines of mice, respectively (Table 1), which were distinct from those in wild-type LT/LT and gene-targeted FV/FV mice, indicating that neither 108F nor 189T is solely responsible for the increased incubation time in FV/FV mice. The most significant change in incubation time was due to the T to V alteration at codon 189, producing a difference of ~100 days between LT/LT and LV/LV lines. A much smaller difference of 13 days was produced by the L to F polymorphism at codon 108 (FT/FT), demonstrating that both codon 108 and 189 polymorphisms can alter murine scrapie incubation times independently.

In order to assess the full effect of the polymorphisms in both the homozygous and heterozygous situations, LV/LV and FT/FT mice were crossed with wild-type LT/LT mice, gene-targeted FV/FV mice and each other to produce the full repertoire of genetic combinations (Table 1). This breeding strategy produced 10 lines of transgenic mice, each with a different PrP allelic combination. Each line of mice was inoculated intracerebrally with ME7 and the resulting incubation times are shown in Table 1 and Supplementary Figure.

Introducing heterozygosity at codons 108 and 189 revealed that the control of incubation time by these two polymorphisms is not as straightforward as suggested by data from the homozygous lines (Table 1). There is no obvious pattern of allelic combination associated with increasing incubation time (see Supplementary Figure), with the exception that the two shortest incubation times are in mice that are homozygous for 189T (155 and 168 days) and the two longest are in mice that are homozygous for 189V (295 and 317 days), in keeping with identity at codon 189 with the source of the agent (LT/LT). However, homozygosity at codon 189 does not always produce extreme incubation times, as LT/FT and LV/LV mice have intermediate incubation times of 196 and 261 days, respectively (Table 1).

An association between genotype and incubation time can be identified when the 10 lines are examined by the allelic combinations at either codon 108 or codon 189 (Table 1). When lines are sorted into groups by codon 189 genotype (189TT, 189VV or 189FV), the effect of codon 108 on incubation time is the same in each of the three groups: the shortest incubation times occur when the codon at 108 is homozygous for either L or F, although the shorter of the two is always 108LL, matching that of the source of the inoculum (LT/LT). Therefore, at codon 108, heterozygosity always leads to increased incubation times with respect to the combination at codon 189, despite the inoculum matching one allele at codon 108. Indeed, incubation times in excess of those in *Prnp*^{b} (FV/FV) mice were observed in FV/LV mice (Table 1 and Supplementary Figure). However, when sorted by codon 108 genotype (108LL, 108FF or 108LF), a different pattern of incubation time is observed. For each of the three groups, homozygosity for threonine at codon 189 (which matches the source of the inoculum) gives the shortest incubation times and homozygosity for valine (which does not match the inoculum) gives the longest incubation times, whilst the heterozygotes (TV) produce intermediate

incubation times. Hence, at codon 108, ME7 incubation times follow the pattern LL<FF<LF, whilst at codon 189, the pattern of incubation time is TT<TV<VV. These results show that the effect on incubation period depends upon the combination of PrP alleles, particularly the presence of homozygosity or heterozygosity at codons 108 and 189 and on identity with the source of the inoculum at these positions in PrP.

Interactions between codon 108 and 189 polymorphisms and the 301V strain

In contrast to ME7 challenge, LT/LT mice inoculated with the *Prnp*^{b} (FV/FV)-passaged strain 301V have long incubation periods of 240 days. Similarly, FV/FV mice that have long ME7 incubation periods produce short incubation periods of 125 days with 301V. The role of codons 108 and 189 in the control of 301V incubation time was explored by challenge of FT/FT and LV/LV mice. Incubation times of 141 and 202 days were obtained in the LV/LV and FT/FT lines of mice, respectively, compared to 125 days in FV/FV mice and 240 days in LT/LT mice (Table 1). Hence, in agreement with the data obtained from inoculations with ME7, both codons 108 and 189 are responsible for the control of incubation time for the 301V agent strain. Similarly to ME7, the greatest effect was produced by the T to V alteration at codon 189 (~100 days reduction). However the L to F change produced a greater effect on incubation time with 301V (~60 days reduction) than with ME7, again showing that challenge with an agent that shows identity at codon 108 or 189 results in the shortest incubation times (Table 1). The full range of allelic combinations has not been examined with this strain of TSE agent. In addition, no incubation time is available for the LT/FT line. Six LT/FT mice were culled due to welfare reasons between 327 and 377 days and all showed positive TSE pathology on examination of the brain tissue, suggesting an incubation time in excess of that of the LT/LT line (240 days). However, the combinations that were examined have produced patterns of incubation-time alteration identical to those identified with ME7 (Table 1 and Supplementary Figure), with the exception that the F1 cross LT/FV has an incubation time in excess of those of both parental lines. This overdominance is characteristic of 301V and can be observed with other TSE strains. Thus, the pattern of incubation time for 301V appears to be FF<LL<LF at codon 108 and VV<TV<TT at codon 189. This pattern is the same as that observed for ME7, where heterozygosity results in extended incubation times for 108LF, but intermediate incubation times with 189TV. The shortest incubation times for both strains of agent reveal a preference for identity with the genotype of the donor of infectivity (LT/LT for ME7 and FV/FV for 301V).

Effect of polymorphisms on PrP^{Sc} production

Detergent homogenates were prepared from three terminal ME7 brains of each genotype. The homogenates were treated with PK and analysed by immunoblotting to assess the levels of PK-resistant PrP present in each model. Equal levels of PK-resistant PrP were detected between the three mice of each genotype (data not shown). Levels were also similar between the 10 different lines of mice (Fig. 4), suggesting that the codon 108 and 189 polymorphisms have no effect on PrP^{Sc} levels in the brains of mice culled with clinical ME7 infection. This was also true for the lines challenged with 301V (data not shown).

Effect of polymorphisms on vacuolar pathology

The targeting of vacuolar pathology in the brain was similar for all allelic combinations infected with ME7 (Fig. 5a–c). Thus, the codon 108 and 189 polymorphisms in host PrP do not influence the distribution of vacuolar pathology with the ME7 strain of scrapie. However, the polymorphisms appeared to affect the degree of vacuolation. In general, lines containing 189TT (LT/LT, FT/FT and LT/FT) or 189TV with homozygosity at 108 (LT/LV and FT/FV) had short incubation times (<215 days) and gave very similar vacuolation profiles. Lines displaying higher vacuolation scores had longer incubation times and contained 189VV (FV/FV, LV/LV or FV/LV) or were heterozygous at both 108 and 189 (LV/FT). However, the LT/FV heterozygote gave a vacuolation profile more similar to that in LT/LT mice (Fig. 5c). Alternatively, these observations may simply reflect incubation time of disease, with high degrees of vacuolation being associated with extended incubation times.

The effect of the codon 108 and 189 polymorphisms on targeting was more prominent in 301V-infected lines, where the targeting of vacuolation varied between LT/LT and FV/FV mice (Fig. 5d–f). 189VV mice (LV/LV) and 189TV mice homozygous at codon 108 (LV/LT) gave profiles similar to those of FV/FV mice, whilst 189TT mice (FT/FT and LT/FT) gave profiles similar to those of LT/LT mice (Fig. 5d–f). The LT/FV heterozygote produced an intermediate vacuolation profile (Fig. 5f). The degree of vacuolation with 301V therefore does appear to be controlled by the codon 108 and 189 polymorphisms and not by incubation time, as extended incubation times in FT/FT and LT/FT mice resulted in lower vacuolation scores in areas 7–9 compared to FV/FV mice (Fig. 5a–b).

DISCUSSION

Previous experiments have shown that the introduction of 108F_189V into the murine *Prnp*^{a} gene had a major effect on TSE disease, producing incubation times similar to those in *Prnp*^{b} mice for both *Sinc s7*-derived (ME7, 139A, 79A and 301C) and *Sinc p7*-derived (301V and 22A) strains (Moore *et al.*, 1998; Barron *et al.*, 2003; Barron & Manson, 2004). These results showed that amino acids 108 and 189 in murine PrP exert the major control over scrapie incubation time in *Prnp*^{a} and *Prnp*^{b} lines of mice, but could not determine whether either or both of the polymorphisms were responsible for this control. By targeting each polymorphism separately into the murine *Prnp*^{a} gene, we have been able to analyse the specific effects of codon 108 and 189 polymorphisms in co-isogenic mice, both independently and in several different allelic combinations. Inoculation of these lines with two scrapie strains has shown that both codons 108 and 189 in murine PrP are responsible for control of scrapie incubation time in mice, as incubation times in LV/LV and FT/FT mice are distinct from those produced in LT/LT and FV/FV mice. The shortest incubation times were obtained when PrP expressed from both alleles matched the PrP in the inoculum (LT/LT for ME7 and FV/FV for 301V). Correspondingly, long incubation times were obtained for LT/LT mice with 301V and FV/FV mice with ME7. However, from analysis of the other allelic combinations, it was apparent that shortened incubation times were also obtained in lines where codon 189 matched the source of the inoculum and the residues on both alleles at codon 108 were homogeneous. Of these, the shortest incubation times occurred when these residues at codon 108 matched the source of inoculum. In lines heterozygous at both codons 108 and 189 (with LT and FV molecules in one case, and LV and FT molecules expressed in the other), shorter incubation times were observed in the line expressing PrP that matched the source of inoculum (LT/LT for ME7). The importance of homozygosity at codon 108 and identity with the inoculum at codon 189 suggests that these areas of the protein play very different roles in the disease process.

The control of incubation time is thought to be due to PrP sequence identity between the host and the donor of infectivity. However, our results show that short incubation times are obtained in FT/FT mice inoculated with LT/LT-derived ME7 and in LV/LV mice inoculated with FV/FV-derived 301V, despite the sequence incompatibility between donor and host at codon 108. Hence, although codon 108 polymorphisms can affect scrapie incubation time independently, the major control is due to identity with the source of inoculum at codon 189. The inoculation of mice expressing different combinations of *Prnp*^{LT}, *Prnp*^{FV}, *Prnp*^{LV} and *Prnp*^{FT} produced a complex series of incubation times (see Supplementary Figure). It was clear that, in addition to identity with the inoculum at codon 189, homozygosity at codon 108 consistently gave rise to shorter incubation times in both 108L/L- and 108F/F-expressing lines. Heterozygosity at codon 108 always resulted in extended incubation times with respect to the genotype at codon 189. Although the series of allelic crosses produced for these experiments are described as homozygous and heterozygous at codons 108 and 189, expression from both *Prnp* alleles in a heterozygote will result in the presence of two different PrP populations in the cell. It is interactions between these proteins, and not between *Prnp* alleles, that will determine

the incubation time of disease. Hence, the nature or combination of PrP variants in the recipient may be more important than PrP identity with the inoculum, indicating that interactions between amino acids 108 and 189, either on the same or different proteins, is a critical part of disease propagation.

It has been proposed that the production of PrP^{Sc} is a two-step process that involves binding of PrP-res (PK-resistant PrP) to PrP-sen (PK-sensitive PrP), followed by the conversion of PrP-sen to PrP-res (Caughey, 2001). However, it is unknown which process requires PrP amino acid identity. Cell-free conversion experiments have shown that mouse PrP-res (108L_111V) will convert mouse PrP-sen expressing the 3F4 epitope (108M_111M), but not hamster PrP-sen (also possessing the 3F4 epitope), even though both proteins were found to bind to mouse PrP-res (Horiuchi *et al.*, 2000). Deletion mutants lacking residues 34–113 (MoPrPΔ34–113) also bound to mouse PrP-res and converted, albeit with reduced efficiency (Lawson *et al.*, 2001). These results imply that amino acid 108 is not required for either initiation of conversion or binding to PrP^{Sc}, but may be involved in the control of the rate of conversion and final conformation of PrP-res (Lawson *et al.*, 2004). Moreover, MoPrPΔ34–113 was found to bind heterologous PrP^{Sc}, but, unlike the full-length molecule, did not prevent the conversion of homologous PrP^{C}, again suggesting that this region is involved in the control of conversion, but not binding (Lawson *et al.*, 2001). The Δ34–113 truncation of PrP^{C} was also found to affect the solubility of the molecule, as loss of these residues reduced the percentage of PrP-sen that was seen to self-aggregate when incubated under cell-free assay conditions in the absence of PrP-res. This region may therefore be responsible for some degree of multimerization/aggregation of PrP-sen, which may be beneficial for conversion (Lawson *et al.*, 2001).

The individual effects of codon 108 and 189 polymorphisms in PrP have also been modelled *in vitro* by expressing recombinant 108F_189T and 108L_189V PrP in *Escherichia coli* (Brown *et al.*, 2000). These intermediate forms of PrP were found to be less stable than both PrP-A (108L_189T) and PrP-B (108F_189V) and were observed to lose their normal conformation and gain some PK resistance over time. It was proposed that inheritance of either 108F or 189V alone may be a disadvantage in terms of mouse survival, and that mice expressing either of these polymorphisms separately could potentially develop spontaneous disease (Brown *et al.*, 2000). However, the recently reported existence of a $Prnp^{c}$ (108F_189T)-expressing line (Lloyd *et al.*, 2004) and the data presented here for transgenic FT/FT and LV/LV lines show that such mice are viable and do not develop any neurological phenotype during their lifespan. PrP expressed in the brains of transgenic LT/LT and FV/FV mice is PK-sensitive and expressed at levels identical to those in wild-type mice of both genotypes. These results demonstrate clearly that *in vitro* and *in vivo* studies can produce very different outcomes; these may be due to the difference in PrP post-translational modification and/or the physiological environment of the native protein.

From the data presented here, we predict that: (i) polymorphisms at PrP codon 189 control the initial interaction and binding with the agent, as incubation times reflect a preference for sequence identity between the host and inoculum at codon 189; and (ii) polymorphisms at codon 108 control the rate of conversion of PrP^{C} to PrP^{Sc}, where the ability to induce

multimerization of PrP^C and increase the efficiency of conversion is favoured when all PrP expressed in a cell is homogeneous in the N-terminal region. Alternatively, the preference for codon 108 homozygosity may reflect a more complex interaction between host PrP^C and PrP^{Sc} that is less efficient in the presence of heterogeneous protein populations. However, the importance of codon 108 homozygosity in murine scrapie transmission may explain the role of PrP codon 129 in human TSE disease, where the majority of CJD cases occur in individuals who are homozygous for methionine or valine at codon 129 (Palmer *et al.*, 1991; Zeidler *et al.*, 1997; Alperovitch *et al.*, 1999). There is an under-representation of M/V heterozygotes with CJD when compared to the distribution of genotypes in the normal population (Palmer *et al.*, 1991), suggesting that a similar mechanism requiring N-terminal homogeneity controls the efficiency of disease transmission in humans.

The results of transmissions to the 108L/F_189T/V lines of transgenic mice have therefore shown that codon 189 polymorphisms exert the major control over scrapie incubation time, but that the efficiency of disease transmission is increased when codon 108 is homozygous, suggesting that a multimer of PrP^C may be involved in conversion of PrP during TSE disease. Several other studies have suggested the involvement of PrP dimers in disease (Priola *et al.*, 1995; Warwicker, 1997, 2000; Meyer *et al.*, 2000; Jansen *et al.*, 2001; Meier *et al.*, 2003), yet most existing dimer models do not consider dimerization of the N-terminal region of PrP, due to the lack of structural information available for this region. Combined with the *in vivo* data produced in this study, the observations that amino acids 90–121 are retained after PK cleavage of PrP^{Sc} and that deletion of this region affects susceptibility to disease (Fischer *et al.*, 1996; Shmerling *et al.*, 1998; Flechsig *et al.*, 2000; Lawson *et al.*, 2001; Supattapone *et al.*, 2001) prove that this is a structurally important part of the molecule that is involved in the control of disease incubation time.

Analysis of LV/LV and FT/FT transgenic mice has therefore shown that both amino acid polymorphisms at codons 108 and 189 in murine PrP are involved in the control of scrapie incubation time, and that distinct regions of PrP may play different roles in the disease process. Interactions within a single PrP molecule and between molecules must therefore underlie the mechanism by which incubation times are controlled. Such structural interactions may provide the key to replication of TSE infectivity.

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Fig. 1. Gene-targeting strategy. Wild-type PrP clones were derived from a 129/Ola HM-1 ES cell genomic library and amino acids were modified by site-directed mutagenesis. Targeting vectors were constructed by the introduction of a PGK/HPRT cassette (flanked by *LoxP* sites) and an HSV/TK cassette for selection purposes. After homologous recombination in ES cells, the PGK/HPRT cassette was removed with Cre recombinase. Primer pair W262 and W6991 was used to determine successful homologous recombination in targeted ES cells. Primer pair W7878 and W6991 was used to screen for the Cre-mediated deletion of the PGK-HPRT cassette (see Methods). In the presence of the cassette, the PCR fragment is too large to amplify. Successfully removed cassette produces a PCR product larger than the wild-type allele, due to the retention of a single LoxP site and part of the pBluescript multiple-cloning site after Cre recombination. Presence of 108F or 189V polymorphism was confirmed by PCR of the ORF using primers A996 and B188 and digestion of the product with *Mnl*I (108F) or *Bst*EII (189V). B, *Bam*HI; E, *Eco*RI; P, *Pst*I; RV, *Eco*RV; S, *Sal*I.

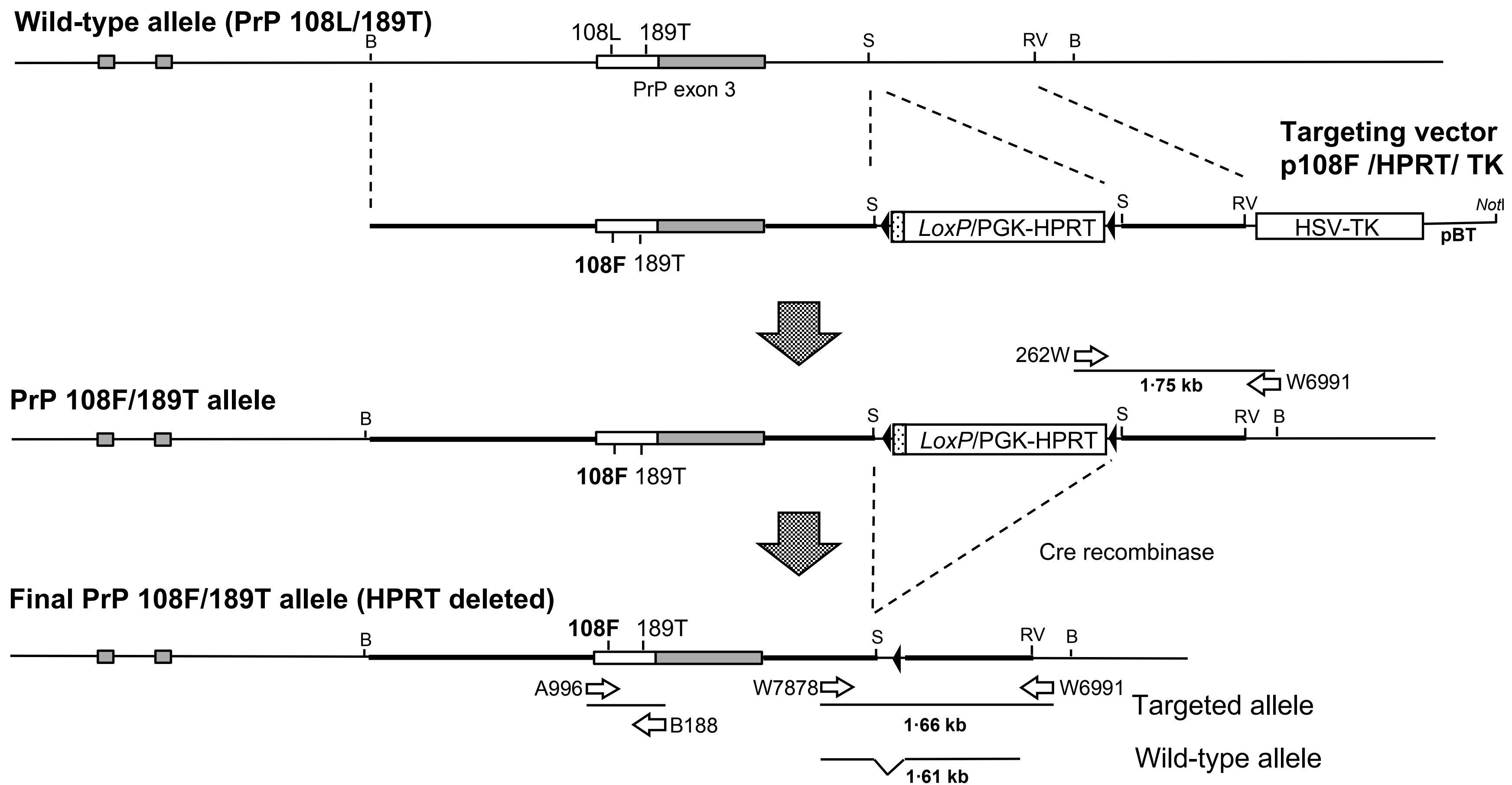


Fig. 2. Northern blot of RNA extracted from mice expressing codon 108 and 189 polymorphisms. Lanes: 1, *Prnp*^{a}; 2, PrP$^{-/-}$; 3, *Prnp*$^{LV/FT}$; 4, *Prnp*$^{FV/FV}$; 5, *Prnp*$^{FV/-}$; 6, *Prnp*$^{LT/LV}$; 7, *Prnp*$^{LV/LV}$; 8, *Prnp*$^{LT/FT}$; 9, *Prnp*$^{FT/FT}$; 10, *Prnp*^{a}. Top panel was probed with a 936 bp *Kpn*I–*Eco*RI fragment from *Prnp* exon 3; bottom panel was probed with a PCR-generated 275 bp DNA fragment corresponding to the 18S rRNA sequence as a loading control.

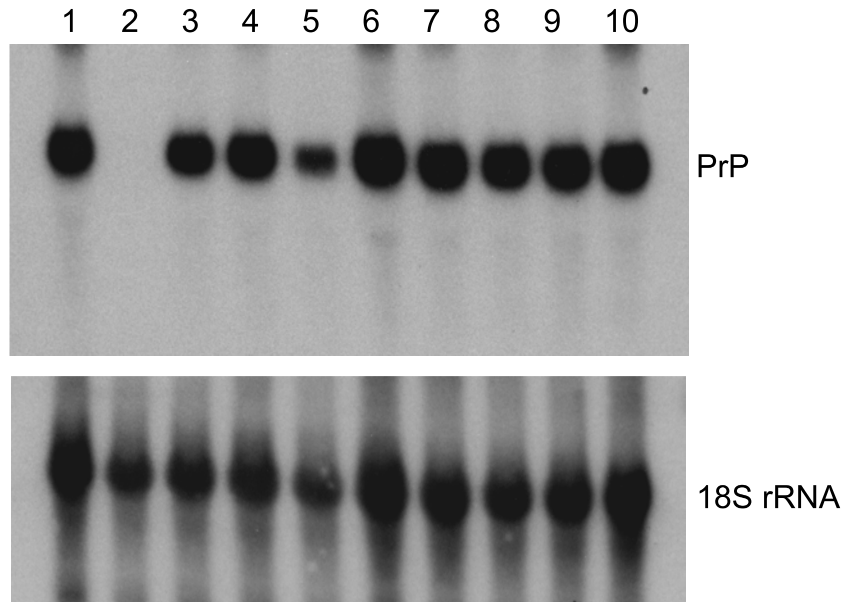


Fig. 3. Western blot of uninfected mice expressing the codon 108 and 189 polymorphisms. Lanes: 1, wild-type 129/Ola mouse (LT/LT); 2, LV/LV mouse; 3, FT/FT mouse; 4, FV/FV mouse. Top panel was probed with anti PrP monoclonal antibody 7A12; bottom panel was probed with anti-tubulin monoclonal antibody as a loading control.

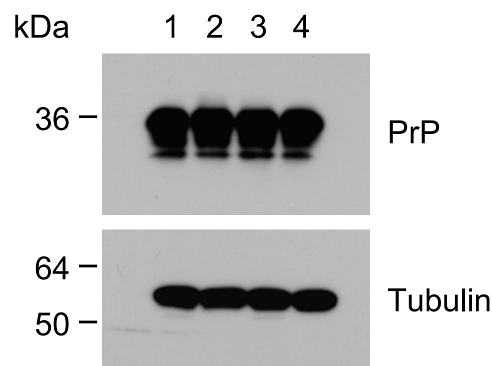


Fig. 4. Western blot analysis of PrP^{Sc} levels in different allelic combinations inoculated with ME7. Lanes 1–4, ME7-infected homozygous lines: 1, LT/LT; 2, FV/FV; 3, LV/LV; 4, FT/FT. Lanes 5–10, ME7-infected heterozygous lines: 5, LT/FV; 6, LV/LT; 7, LV/FV; 8, FT/LT; 9, FT/FV; 10, FT/LV. All samples were treated with PK at 20 μg ml<MIN>1 for 1 h at 37 °C. Blot was probed with anti-PrP monoclonal antibody 7A12. Equivalent levels of PrP^{Sc} are present in all lines of mice.

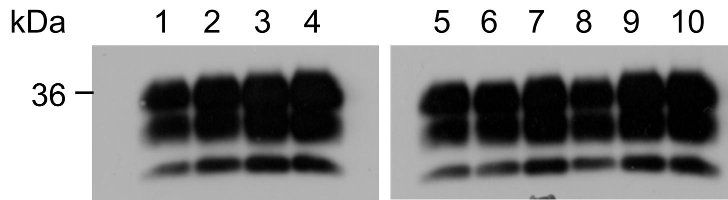


Fig. 5. Lesion profiles of LV/LV and FT/FT transgenic mice infected with ME7 and 301V. The extent of vacuolar change in the brain was assessed semi-quantitatively in nine areas of grey matter and three areas of white matter by lesion profiling, as described previously (Fraser & Dickinson, 1967). Animals were scored on a scale of 0–5 in each specific area; mean scores (calculated from a minimum of six animals) are shown graphically (error bars±SEM). Grey-matter scoring areas: 1, dorsal medulla: 2, cerebellar cortex; 3, superior colliculus; 4, hypothalamus; 5, medial thalamus; 6, hippocampus; 7, septum; 8, cerebral cortex; 9, forebrain cerebral cortex. White-matter scoring areas: 1*, cerebellar white matter; 2*, midbrain white matter; 3*, cerebral peduncle. (a) ME7 in lines with profiles similar to LT/LT mice; (b) ME7 in lines with profiles similar to FV/FV mice; (d) 301V in lines with profiles similar to LT/LT mice; (e) 301V in lines with profiles similar to FV/FV mice; (c, f) LT/LT, FV/FV and LT/FV lines with ME7 and 301V, respectively.

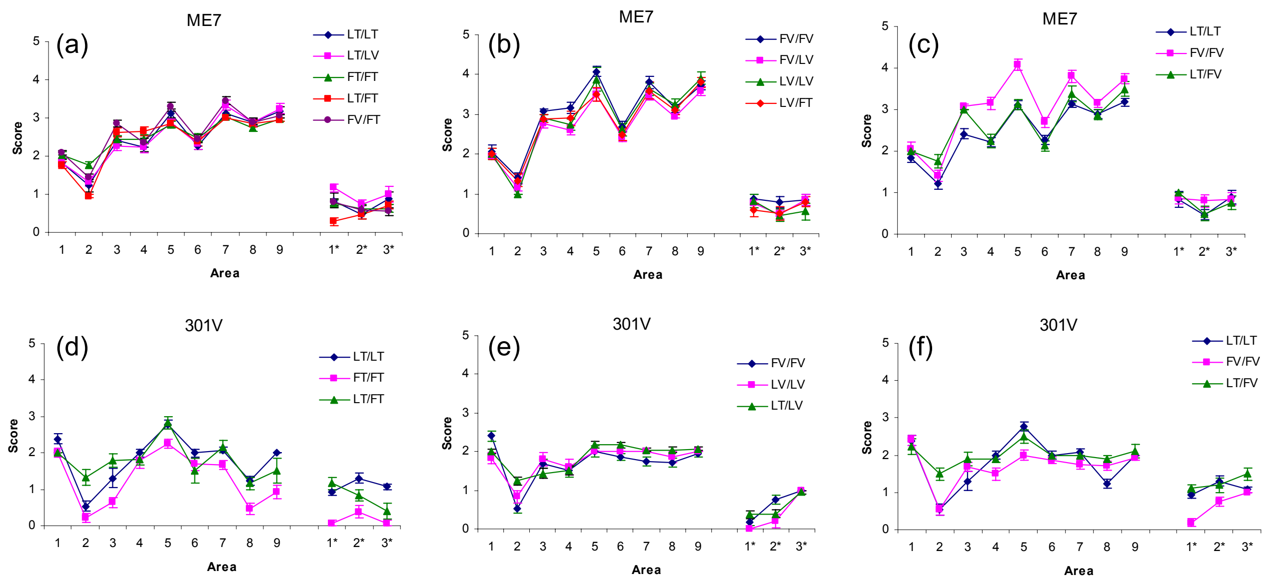


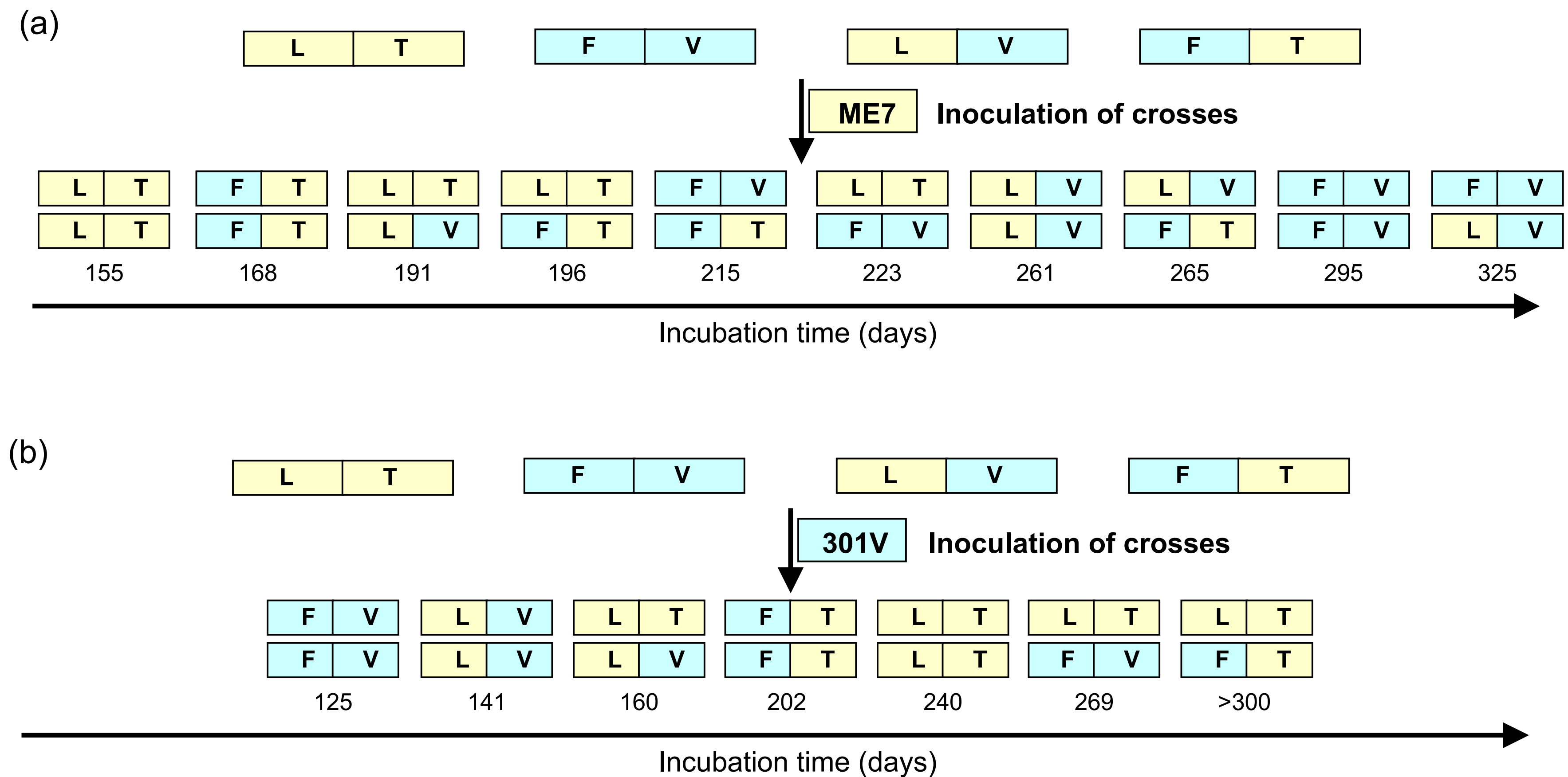
Table 1. Incubation times of ME7 and 301V in mice with different genetic combinations at codons 108 and 189

Mouse line	*Prnp* genotype	ME7 (LT/LT)		301V (FV/FV)	
		Mean incubation time (days) ± SEM	***n******	**Mean incubation time (days) ± SEM**	***n******
LT/LT	$Prnp^{LT}$	155 ± 2	9	240 ± 6	14
FV/FV	$Prnp^{FV}$	295 ± 7	10	125 ± 1	17
LV/LV	$Prnp^{LV}$	261 ± 5	11	141	7
FT/FT	$Prnp^{FT}$	168 ± 1	13	202 ± 1	13
LT/FV	$Prnp^{LT}/Prnp^{FV}$	223 ± 4	6	269 ± 2	10
LT/LV	$Prnp^{LT}/Prnp^{LV}$	191 ± 2	11	160 ± 2	27
LT/FT	$Prnp^{LT}/Prnp^{FT}$	196 ± 2	21	>300†	6†
FV/LV	$Prnp^{FV}/Prnp^{LV}$	325 ± 7	9	NA	NA
FV/FT	$Prnp^{FV}/Prnp^{FT}$	215 ± 4	10	NA	NA
LV/FT	$Prnp^{LV}/Prnp^{FT}$	265 ± 3	12	NA	NA

*No. mice in each group. All groups displayed 100 % susceptibility.

†Six mice culled at 327–377 days with intercurrent disease, all showing positive TSE pathology.

NA, No data.



Supplementary Figure. ME7 and 301V incubation times in mice expressing codon 108 and 189 polymorphisms. Diagrammatic representation of the ME7 and 301V incubation times shown in Table 1 in all allelic combinations of 108/189 transgenic mice. Lines are colour-coded to display homology with (a) ME7 (LT/LT in yellow) and (b) 301V (FV/FV in blue). Progression of incubation time is indicated from left to right and incubation times for each line are shown. This representation of the data aids in the visualization of the importance of codon 189 identity with the agent and codon 108 homozygosity in maintaining shortened incubation times.