

Genetic Susceptibility to Prion Diseases in Humans and Mice

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Abstract: Prion diseases are fatal transmissible neurodegenerative disorders of both animals and humans associated with prolonged incubation periods and include scrapie, bovine spongiform encephalopathy (BSE) and Creutzfeldt-Jakob disease (CJD). The arrival of variant CJD (vCJD) and the recognition that it is causally related to BSE, to which there has been widespread dietary exposure, has led to considerable public health concerns. According to the protein-only hypothesis, prions are principally or entirely composed of an abnormal isoform (PrP^{Sc}) of host-encoded cellular prion protein (PrP^C). Human prion diseases have inherited, sporadic and acquired aetiologies. The inherited prion diseases are all associated with coding mutations in the human PrP gene (*PRNP*) and PrP polymorphisms are known to affect susceptibility, incubation time and disease phenotype. Although *PRNP* is the major genetic determinant of prion disease susceptibility, it is becoming clear that other genes play an important role. Genetic studies in humans are limited by the small numbers of affected individuals and therefore to identify these genes several large mouse crosses have been analysed and multiple loci on at least eight different chromosomes now identified. To date, the regions identified are large and the identification of candidate genes remains challenging. However, the development of alternative mouse crosses offers the prospect of fine mapping, which, together with microarray analysis and increased sequence information, now makes identifying these susceptibility genes a realistic goal. Characterisation of these mouse alleles and then their human homologues may allow the identification of at-risk individuals for BSE prion infection, allow better prediction of any vCJD epidemic, and ultimately should identify new proteins and biochemical pathways which will contribute to our understanding of prion pathogenesis and provide new targets for therapeutic intervention.

Key Words: Prion, *PRNP*, mutation, polymorphism, incubation time, quantitative trait locus (QTL), candidate genes.

PRION DISEASES

Prion diseases or transmissible spongiform encephalopathies are fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals. Prion diseases are characterised by their prolonged incubation periods and distinctive neuropathology which includes spongiform change, gliosis, neuronal loss and an accumulation in affected brains of an abnormal isomer (PrP^{Sc}) of the host-encoded cellular prion protein (PrP^C).

HUMAN PRION DISEASES – ROLE OF *PRNP*

The aetiology of human prion disease is unique in that it is manifested in three forms: sporadic, acquired or inherited. Sporadic CJD makes up ~85% of all human prion disease however its cause remains unknown. Acquired prion disease is rare and is transmitted through exposure to infectious material. Iatrogenic transmission has occurred through exposure to contaminated neurosurgical instruments, tissue grafts or human cadaveric pituitary hormones. Other human acquired diseases include kuru, which reached epidemic proportions in the Fore region of Papua New Guinea and was transmitted by the consumption of infected material during cannibalistic mortuary feasts. More recently, variant CJD

(vCJD) has been recognised as an acquired prion disease caused by a BSE-like prion strain.

Inherited prion diseases make up ~15% of all human prion diseases and they include CJD, Gerstmann-Sträussler-Scheinker disease (GSS) and fatal familial insomnia (FFI). They are late onset disorders that exhibit autosomal dominant Mendelian inheritance with linkage to chromosome 20p. Classical genetic studies identified mutations in the prion gene (*PRNP*) and showed that they were causative. Subsequently, over 30 mutations have been described including premature stops, amino acid substitutions and insertions of octapeptide repeats Fig. (1).

The three distinctive aetiologies are unified by the protein-only hypothesis [1] that places an abnormal conformational isoform of the prion protein central to the whole disease process and states that PrP^{Sc} is the principal if not sole component of the infectious agent [2].

In human PrP a common polymorphism occurs at codon 129 which encodes either a valine or methionine residue Fig. (1). Within the United Kingdom Caucasian population, 37% are methionine homozygotes (MM), 12% are valine homozygotes (VV) and 51% are heterozygotes (MV) [3]. This naturally occurring variant is known to have a major impact on both incubation time and disease susceptibility. Studies of the kuru epidemic have shown that incubation times vary from 5 years to > 40 years [4]. All codon 129 genotypes succumb to disease however homozygotes have a shortened incubation time relative to heterozygotes [5]. In inherited prion disease, the age at onset in a family with a

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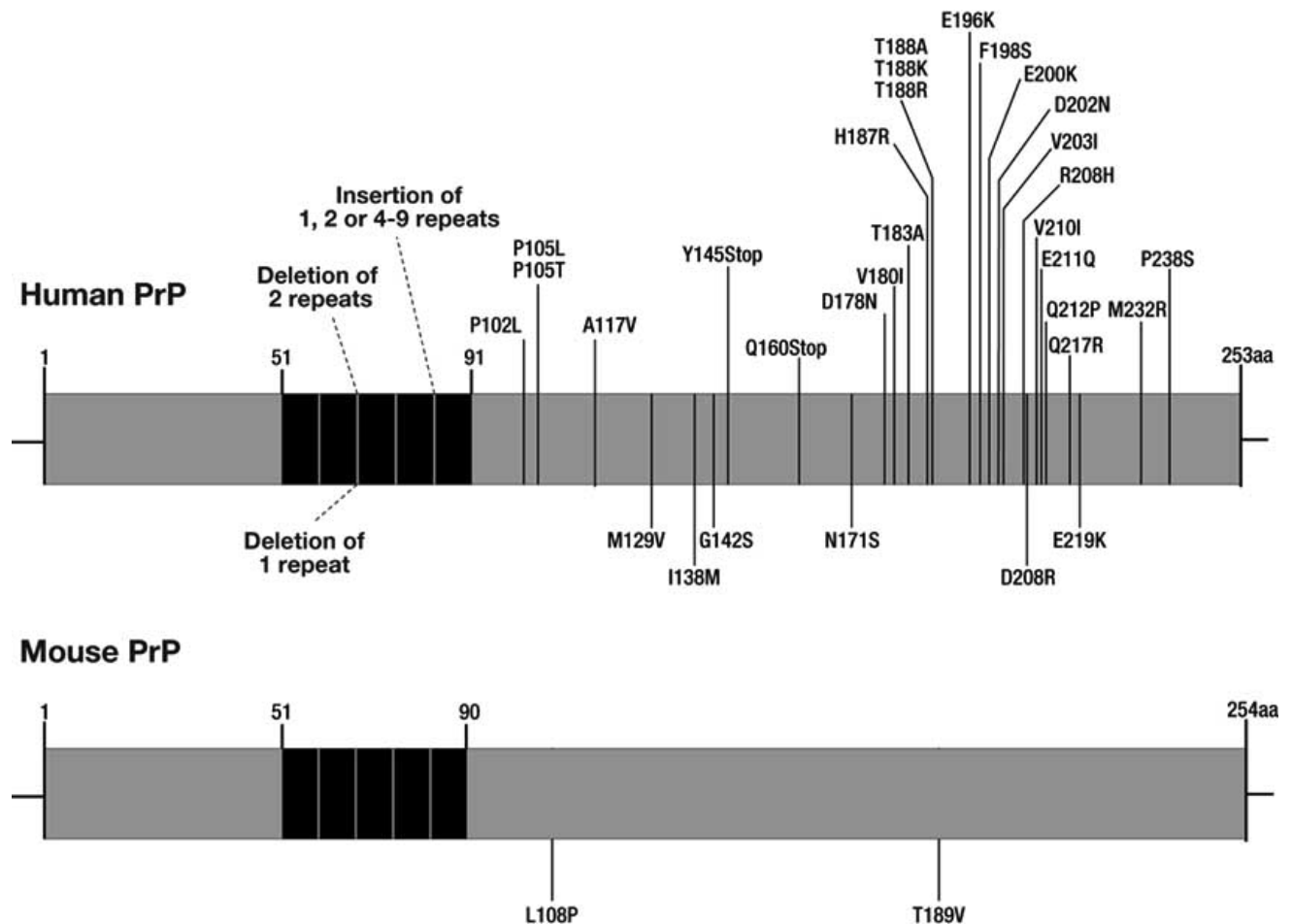


Fig. (1). Schematic representation of coding changes in human and mouse prion proteins. For human PrP, pathogenic mutations and polymorphic variants are shown above and below the protein respectively. For mouse PrP only polymorphisms are shown. For both proteins the five octapeptide repeats are shown as black blocks. Numbers refer to codon numbers (1-253 or 254 amino acids). Mutations are designated by the wild-type amino acid preceding the codon number, followed by the mutant residue. All residues are shown using single letter amino acid abbreviations.

six-octapeptide-repeat insertion is earlier for methionine homozygotes as compared to heterozygotes [6, 7]. Similarly, in sporadic and iatrogenic disease, homozygosity for either methionine or valine at codon 129, predisposes individuals to disease [8, 9]. In addition, mean incubation times for iatrogenic CJD, caused by exposure to infected pituitary hormones, are shorter for homozygotes than for heterozygotes [10]. To date, all patients with vCJD have been methionine homozygotes [11-13]. These data suggest that heterozygosity at codon 129 is protective in delaying the onset of disease but does not provide full resistance. Based on our experience of other prion diseases it seems likely that other genotypes will develop a prion disease following BSE prion exposure with longer incubation times although it is possible these patients may have phenotypes distinct from vCJD [4, 14]. Heterozygosity may be protective by inhibiting homologous protein-protein interactions thereby reducing the rate of PrP^{Sc} accumulation [8]. Codon 129 genotype may also affect the efficiency with which defined human prion strains can propagate [4]. Variation at codon 129 may not be the only

protective human PrP polymorphism as in Japan, heterozygosity at codon 219 (Glu219Lys), confers resistance to sporadic CJD [15].

Kuru imposed strong balancing selection on the Fore population essentially eliminating *PRNP* 129 homozygotes. Elderly survivors of the kuru epidemic, who had multiple exposures at mortuary feasts, are, in marked contrast to younger unexposed Fore, or elderly Europeans, predominantly *PRNP* 129 heterozygotes [16]. Worldwide *PRNP* haplotype diversity and coding allele frequencies suggest that kuru-like epidemics imposed strong balancing selection at this locus during the evolution of modern humans [16]. While it cannot be excluded that *PRNP* heterozygosity is protective against some unknown common disease the known powerful protection against prion disease argues that acquired prion disease epidemics occurred in prehistoric populations either through an endemic animal prion disease able to cross the transmission barrier or through cannibalistic practices [16].

INCUBATION TIME IN MICE – ROLE OF *PRNP*

Prion diseases are characterised by prolonged, clinically silent, incubation periods. In experimental transmissions to mice this is measured in days from the time of prion inoculation to the onset of clinical signs. When the experimental conditions are kept constant, the incubation times are highly reproducible with remarkably small standard deviations. Many factors have been shown to influence incubation time. As one might expect, the dose of infectious material is important as is the route of administration, with the incubation times being shorter when prions are inoculated directly into the brain compared to peripheral inoculation or oral dosing. Primary passage of prions into a new species, for example inoculating BSE prions into mice, is associated with a much more prolonged incubation period than passage within the same species. Second passage, in this example to further mice, is associated with a considerable shortening of incubation period. This prolongation of incubation on primary passage is known as a species or transmission barrier. This may be due to the amino acid sequence differences between the host PrP^C and the incoming PrP^{Sc} in addition to other species specific factors. Prion strain types are also known to contribute to transmission barriers and to affect incubation time [4]. Distinct prion strains are distinguished by their biological properties – producing characteristic incubation periods and patterns of neuropathology in defined inbred lines of mouse [17-20] – or more recently by biochemical differences in PrP^{Sc}. Prion strains differ in conformation [18-21] and glycosylation of PrP^{Sc} [19], allowing rapid molecular strain typing [22]. The level of host PrP^C expression is also known to be an important determinant of incubation time. Knockout mice for *Prnp* (*Prnp*^{0/0}) are unable to replicate PrP^{Sc} and never develop disease while mice with just one copy of *Prnp* (*Prnp*^{+/-}) show an enhanced resistance to scrapie infection relative to mice carrying the usual two copies of *Prnp* [23]. Further work using transgenic mice over-expressing PrP^C has shown an inverse correlation between the level of PrP^C and incubation time [23-25].

With the same experimental procedure and the same inoculum, the major determinant of incubation time is mouse strain. The influence of mouse genes on incubation time was first described by Dickinson and MacKay in 1964 when they identified a mouse strain (VM/Dk) with an unusually long incubation time when challenged with mouse-adapted scrapie [26]. Surveys of incubation periods in a range of inbred lines of mice inoculated with mouse-adapted scrapie show that each mouse strain has a well defined characteristic incubation time for a given prion strain [27-30]. These incubation times fall into two groups where a “short” incubation time is 100-200 days and a “long” incubation time is greater than 255 days [30]. Classical genetic studies using the Me7 mouse scrapie strain defined a single gene, *Sinc*, regulating incubation time with two alleles *s7* and *p7* encoding ‘short’ and ‘long’ incubation times, respectively [27]. Further screens of inbred mice with the Chandler/RML mouse scrapie strain identified an additional *Sinc p7* mouse strain (I/Ln) which led the way to linkage studies in a cross between I/Ln and NZW/LacJ, a *Sinc s7* strain [28, 31]. These studies identified a single locus closely linked to the prion gene (*Prnp*), named *Prni*, also with two alleles, *N* and *I* which define short and long incubation times respectively

and was considered to be the same locus as *Sinc* [31]. Although *Prnp* was an excellent candidate gene for *Sinc/Prni* these crosses were unable to confirm this suspicion.

In 1987, Westaway *et al.*, identified two amino acid polymorphisms in the mouse prion gene Fig. (1) which they suggested would provide the molecular basis for the affect of *Sinc/Prni* alleles on incubation time [29]. *Prnp*^a (108-Leu, 189-Thr) and *Prnp*^b (108-Phe, 189-Val) alleles occurred only in ‘short’ and ‘long’ incubation time mice respectively [29]. Although *Sinc/Prni* and *Prnp* were widely believed to be congruent, various approaches including genetic crosses, the use of congenic and transgenic mice, failed to rule out the possibility that *Sinc/Prni* was a gene closely linked to *Prnp* [32-36]. Final confirmation that the amino acid sequence of PrP plays a key role in determining incubation time came from gene targeting experiments where the *Prnp*^b allele amino acids (108-Phe, 189-Val) were engineered into a *Prnp*^a allele mouse so that a transgenic mouse was produced that had exactly the same genetic makeup as a *Prnp*^a allele mouse except at codons 108 and 189 of PrP and had an incubation time that resembled that of a *Sinc p7* mouse for a mouse passaged BSE strain [37].

Until recently, it was widely believed that mice either had the *Prnp*^a or *Prnp*^b allele. Genotypes have been described for approximately 60 inbred mouse lines and of these only eight have the *Prnp*^b allele [30]. The *b* allele is thought to have only arisen once during the evolution of these mice as all the strains tested are likely to share common ancestors derived from Abbie Lathrop’s farm [30, 38]. However, during a screen of different lines of inbred mice, a new allele was discovered [39]. MAI/Pas mice were derived from wild mice trapped near Illmitz, Austria in 1981 and established as an inbred line by Jean-Louis Guénet at the Pasteur Institute, Paris [38]. *Prnp* sequencing revealed a new allele, termed *Prnp*^c, which appears to be a hybrid of the *a* and *b* alleles, encoding 108-Phe and 189-Thr. The origins of *Prnp*^c are unclear however, a *de novo* mutation may be more likely than a recombination event between an *a* and *b* allele mouse as Southern blot analysis indicates that MAI/Pas mice share polymorphisms 5’ and 3’ of the *PRNP* ORF with *Prnp*^b mice suggesting that the mutation at codon 189 arose on a *b* allele background [39]. The incubation time of MAI/Pas mice with Chandler/RML scrapie (360±11 days) is longer than that described for *b* allele mice (Table 1) however, some of this effect is likely to be the result of other genes, as congenic mice carrying the *Prnp*^c allele on a C57BL6 background (*Prnp*^a) (C57.MAI-prnp) have an incubation time which is intermediate between the two extremes (255±12 days) (Table 1, [39]).

Because of the use of different mouse strains, prion strains and experimental procedures it is often difficult to compare the incubation times listed in various publications. However, it is now apparent that there is no clear cut distinction between the ‘short’ and ‘long’ incubation time mice [39]. Within *Prnp*^a mice there is a very broad range of incubation times with Chandler/RML scrapie prions inoculated intra-cerebrally, ranging from 108±1 days (NZW/OlaHsd) to 221±5 days (PWK/Pas) (Table 1). Published data for *Prnp*^b mice suggests a range from 255±14 days (I/Ln) to 313±3 days (JU/FaCt) and the only *Prnp*^c mice have an

incubation time of 360 ± 11 days (MAI/Pas) [29-31]. The range of incubation times within a given *Prnp* genotype suggest that many genes other than *Prnp* also have a role in determining prion disease incubation time, and it is these genes that are responsible for blurring the edges between the distinctive effects of PrP sequence polymorphisms.

Table 1. Incubation Times Following Intracerebral Inoculation with Chandler/RML Mouse Scrapie

Strain	Incubation time (days) \pm sem
a allele mice – laboratory strains	
NZW/OlaHsd	108 ± 1 (n=38)
SJL/OlaHsd	122 ± 1 (n=37)
FVB/NHsd	131 ± 1 (n=33)
SM/J	133 ± 1 (n=47)
SWR/OlaHsd	135 ± 1 (n=36)
RIIS/J	135 ± 1 (n=34)
C57BL6/JOlaHsd	143 ± 1 (n=42)
a allele mice – trapped wild mice	
PERA/Kamei	151 ± 1 (n=26)
CAST/Ei	188 ± 3 (n=16)
PWK/Pas	221 ± 5 (n=12)
b allele mice	
JU/FaCt	313 ± 3 (n=24)
VM/Dk	300 ± 3
I/Ln	255 ± 14
c allele mice	
MAI/Pas	360 ± 11 (n=20)
C57.MAI-prnp	255 ± 12 (n=9)

All incubation time data are taken from the following sources:

Lloyd *et al. Proc. Natl. Acad. Sci. USA* **2001**, *98*: 6279-6283; Lloyd *et al. Mamm. Genome* **2004**, *15*: 383-389; Carlson *et al. Cell* **1986**, *46*: 503-511.

INCUBATION TIME IN MICE – IDENTIFICATION OF OTHER GENES

From early experiments to identify *Sinc/Prni* and looking at the variation in incubation time within a given *Prnp* genotype, it is evident that genes other than *Prnp* influence incubation time [29-31, 33]. The first additional locus to be implicated is located within the D subregion of the major histocompatibility complex (*H-2*) on mouse chromosome 17 and is termed *Pid-1* (prion incubation determinant-1) [28]. In this study, the *H-2* complex was specifically targeted because the lymphoid system is known to have a role in the peripheral early replication of prions and was studied by recording incubation times in mice congenic for different *H-2* alleles [28]. The advent of high-throughput genotyping technologies has greatly enhanced our ability to map new loci and current strategies allow researchers to carry out whole genome screens without any prior knowledge of the loci under investigation. To date, five studies have successfully identified multiple loci on eight mouse chromosomes [40-43].

Prion disease incubation time, measured in days, is a continuous or quantitative trait therefore it can be used as a phenotype in linkage studies. The general approach for mapping prion disease quantitative trait loci (QTL) has been to select two strains of mice with significantly different incubation times but with the same *Prnp* allele (*a*), and to use these strains as the basis for generating either a backcross or an F2-intercross. The backcross has the advantage of simplifying the analysis by only having two possible genotypes at each locus rather than three but has the disadvantage that only dominant alleles can be identified and only one rather than two meiotic events can be studied for each animal. All the animals in the cross were challenged with prions and their incubation times recorded. In all the crosses described, the distribution of incubation times in the cross was broad and approximated to a normal distribution suggesting that multiple genes are involved and that they co-segregate independently in the cross. For the genome wide screen, polymorphic markers were chosen with an inter-marker distance of 10-20cM to give coverage of every chromosome. Two different approaches were used for genotyping: either every animal in the cross was genotyped [41-44] or to save time and money, the extreme ~20% of each end of the incubation time distribution was genotyped [40], as it is estimated that most of the statistical power may be derived from the tails of the distribution [45]. Linkage analysis provides a statistical measurement of the correlation between genotype and phenotype. In this type of study, significant linkage is expressed as a lod score > 4.3 for an F2 intercross or > 3.3 for a backcross with a genome-screen P-value of < 0.05 . [46].

The advantage of using mouse F2 or backcrosses for QTL studies is that very large numbers of progeny can be used and the analysis is simplified by having only two different alleles segregating. However, two parental strains of mice are unlikely to contain all the possible alleles that may modulate the phenotype. This makes the analysis simpler but means that limiting the search to two strains of mice also limits the number of QTL that can be identified. In the QTL studies of prion disease incubation time different groups have adopted different strategies which makes the data hard to compare but maximises the number of detectable loci. Four different combinations have been used: CAST x NZW (F2 intercross) [41, 44]; CAST x SJL (F2 intercross) [40], C57 x RIII (reciprocal backcross and F2 intercross) [42, 43]. Another potential variant is the use of different prion strains. Part of the definition of a prion strain is that it produces a characteristic incubation time in a given strain of mice therefore it is possible that different QTL will be affected by different prion strains. Incubation time is also particularly affected by the presence or absence of a species or transmission barrier therefore this provides yet another source of QTL variation. The difficulty with this strategy is that inoculating mice across a species barrier produces very long and highly variable incubation times and not all animals show signs of clinical disease. This degree of intra-strain variation makes detecting inter-strain variation much more challenging and may only be possible for a few loci which have a major effect. All the QTL studies published to date have used an intra-cerebral route of inoculation, however peripheral or oral challenge may involve additional QTL

especially with regards to peripheral replication and pathogenesis, and may also be more relevant for modelling QTL that are important in acquired human prion diseases. Not enough data exists on peripheral challenges to mice, especially *via* the oral route, therefore it remains a possibility that too much intra-strain variation will exist to make these studies feasible.

The data from the five published studies are summarised in Table 2. Twenty-one loci have been mapped which cover eight different mouse chromosomes. The data are difficult to compare, however, it is possible that some of these loci are represented in more than one study. To compare mouse strain specific QTL it is possible to compare the Chandler/RML inoculated crosses. Both these studies identified multiple loci on chromosome 11. Not only do the peaks of linkage map very close together (*D11Mit36* 43.7cM and *D11Mit219* 43 cM) but the confidence intervals span 10-20cM suggesting that these loci overlap. *D11Mit36* is also seen, at a suggestive level of linkage, in another CAST x NZW F2 cross, challenged with a different strain of prion. In this cross, the inoculum is mouse passaged BSE, which allows a direct comparison with the Chandler/RML challenged CAST x NZW cross because the only difference is prion strain without the presence of a species barrier. The identification of this locus in three studies reinforces the statistical significance of the data and because all three studies used CAST as the 'long' incubation time parental strain, it is possible that the strong effect of this locus is derived from the CAST strain and may be independent of prion strain. It should be noted that in the CAST x NZW Chandler/RML study, the lod scores are very high and retain a significant level over most of Mmu11. It is therefore likely that multiple loci are present on Mmu11 and it is possible that different yet closely linked genes are responsible for the QTL detected in each of the crosses. In these types of crosses and genome scans it is the effect of the region that is detected and not the effect of individual genes. Therefore, it is possible that multiple genes in the same region may be affecting the phenotype especially as the clustering of genes with related functions is a common occurrence in the mammalian genome. Indeed, other groups have demonstrated this with diverse phenotypes such as high-temperature-growth in yeast and seizure susceptibility in mice [47, 48]. The ability to detect a QTL in a given cross will depend not only on the QTL's contribution to the total variance but also whether the genes in the cluster act in *trans* or in *cis*. Genes acting in *trans* may cancel each other out but genes that act in *cis*, even if their individual effects are small, may amplify the effect over the region so that together they make a significant enough contribution to the total variance that the locus becomes detectable in the genome screen.

Loci on chromosome 2 have also been identified in more than one study. The loci identified in both CAST x NZW F2 intercrosses may represent the same underlying genes as the regions show considerable overlap [41, 44]. These were identified using different prion strains suggesting that these loci may act independently of prion strain. An interesting observation at this locus is that the mean incubation time for Chandler/RML inoculated F2 mice, homozygous for the CAST allele, is significantly shorter (152 ± 24 days) than the mean incubation time for F2 mice homozygous for the NZW

allele (165 ± 27 days, $P < 0.005$). This is the opposite effect to that observed in the parental lines. This highlights the fact that a given mouse strain will harbour a mixture of alleles, some that tend to increase the incubation time and others that have the opposite effect and the parental incubation time reflects the combined interactions of multiple alleles.

Table 2. Summary of Published QTL for Mouse Prion Disease Incubation Time

Cross	Inoculum	Closest Marker and map position (cM)	Lod Score
CAST x NZW F2 intercross ^a (n=1009)	Chandler/RML mouse scrapie	<i>D2Mit107</i> (61.2)	8.15
		<i>D2Mit194</i> (66.7)	4.51
		<i>D2Mit266</i> (98.4)	4.60
		<i>D11Mit36</i> (43.7)	56.41
		<i>D11Mit179</i> (49.2)	53.91
		<i>D12Mit97</i> (42.6)	5.86
		<i>D12Mit28</i> (47.0)	7.55
	<i>D12Mit141</i> (51.4)	5.68	
CAST x SJL F2 intercross ^b (n=153)	Chandler/RML mouse scrapie	<i>D9Mit91</i> (17)	5.70
		<i>D11Mit260</i> (34.3)	5.44
		<i>D11Mit219</i> (43)	5.66
		<i>D11Mit213</i> (55)	4.88
CAST x NZW F2 intercross ^c (n=124)	Mouse passaged BSE	<i>D2Mit304</i> (59.0)	5.57
		<i>D2Mit106</i> (61.2)	5.91
		<i>D2Mit194</i> (66.7)	5.90
		<i>D11Mit36</i> (43.7)	3.96
RIII x C57 F1 x C57 backcross ^d (n=515)	BSE	<i>D2Mit61</i> (34)	5.8
		<i>D8Mit266</i> (43)	5.2
		<i>D4Mit27</i> (42.5)	4.5
		<i>D15Mit159</i> (49.6)	3.8
F1 x RIII backcross ^d (n=512)	BSE		
RIII x C57 F2 intercross ^e (n=282)	Me7 scrapie	<i>D5Mit95</i> (68.0)	4.7*

^aLloyd *et al. Proc. Natl. Acad. Sci. USA* **2001**, 98: 6279-6283.

^bStephenson *et al. Genomics* **2000**, 69: 47-53.

^cLloyd *et al. Neurogenetics* **2002**, 4: 77-81.

^dManolakou *et al. Proc. Natl. Acad. Sci. USA* **2001**, 98: 7402-7407.

^eMoreno *et al. Genetics* **2003**, 165: 2085-2091.

* Females only.

The peak of linkage at this locus corresponds to the location of the prion gene itself. *Prnp* is an excellent candidate for this QTL, however, no amino acid differences exist between CAST and NZW and no significance differences were detected in PrP expression levels [44]. NZW and CAST

mice are only distantly related, [38] therefore multiple polymorphisms are expected between the two strains at this and other loci. However, differentiating between neutral and functionally significant polymorphisms is a major challenge.

Further evidence to support a role for the *Prnp* locus in prion disease susceptibility comes from human association studies. McCormack and colleagues identified three polymorphisms in the promoter and a regulatory region of intron 1 and showed that these are more common in sporadic CJD patients than controls, however, the sample numbers were very small and a high level of statistical significance was not established [49]. Mead *et al.* carried out a more detailed study which identified polymorphisms over a region spanning > 35Kb, and included *PRNP* [50]. The codon 129 methionine/valine polymorphism is known to be a strong susceptibility factor for prion disease [5-9, 11, 12] and a 10Kb region of complete linkage disequilibrium has been described around the *PRNP* ORF [50], therefore both studies needed to take this strong effect into account. By analysing 186 sporadic CJD patients and 86 vCJD patients, Mead *et al.* showed that a polymorphism ~23Kb upstream from *PRNP* was, independently of codon 129, associated with the risk of developing sCJD ($P=0.003$) but not vCJD [50]. This variation may reflect the different aetiologies associated with sporadic and variant CJD or it may reflect the reduced statistical power in a smaller population.

All the other loci identified (Table 2) appear only in one cross therefore it is likely that they are either mouse strain, prion strain or species barrier specific. The inbred lines studied to date represent only a small proportion of the allelic variation that exists in the mouse genome. Therefore the study of other strains of mice, and different crosses, may reveal additional QTL.

FINE MAPPING QTL

The identification of QTL that modulate prion disease incubation time has been very successful, however, all these loci span very large regions (~20cM) of their respective chromosomes and contain too many genes for candidate gene analysis. The classical approach to fine mapping is to generate congenic lines of mice where a locus from one strain of mouse is transferred onto the genetic background of another strain by repeated backcrossing until the genetic background is considered to be >99.9% homozygous. This can be achieved by 10 generations of backcrossing or by the faster method of speed-congenics which is a marker-assisted breeding scheme that selects the most homozygous animals for producing the next generation [51, 52]. Once the congenic line has been established it must then be phenotyped to see if the transferred allele affects the phenotype. Because a single locus only contributes a small amount to the total variance there is a risk that after several years of mouse breeding no detectable phenotypic difference will be present in the congenic line. However, if a phenotype difference can be detected congenic mice can be very useful. We and others are pursuing this strategy but it remains to be seen if it will bear fruit. Congenic mice generated in this way may be very useful to prove the existence of a QTL but may still have regions that are too large for a candidate gene approach. If this is the case, a panel of sub-congenics may be generated

by further backcrossing thereby narrowing the region to a manageable size (1-2cM). Unfortunately, this is a very laborious and time consuming method especially when measuring a phenotype such as prion disease incubation time which itself is lethal and may have an incubation time of over a year.

Several alternative genetic strategies have been suggested including the use of advanced intercrossed lines and outbred stocks [53]. The aim of both strategies is to fine map by increasing the number of recombinations available for analysis in a single mouse. Both crosses rely on multiple generations however the advanced intercross relies on only two different parental lines while outbred stocks are more diverse. In our laboratory we are using a heterogeneous stock (HS) of mice for fine mapping [54]. The HS mice are generated from eight parental strains which are bred semi-randomly over multiple generations (30-60). The chromosomes of the HS mice therefore resemble a fine-mosaic of the parental haplotypes and have successfully provided mapping resolution of less than 1cM [55]. To take advantage of the high density of recombination break points genotyping markers need to be positioned approximately every 1cM. Genotyping at this density makes the cost of a whole genome screen unfeasible; therefore we are only screening regions that have been identified previously in other crosses. To date, this method has successfully mapped QTL to 2cM regions on chromosome 11 and 15 (unpublished data). These regions are now small enough to consider a candidate gene approach.

DIFFERENTIAL GENE EXPRESSION

Alternative approaches to identifying quantitative trait genes include differential gene expression. This can be done on a genome scale by the use of microarrays or on an individual gene basis by quantitative RT-PCR. Microarrays may contain up to 39,000 (Affymetrix mouse arrays) transcripts therefore they provide a very powerful tool for identifying functional strain differences. Many differentially expressed genes would be expected between two un-related mouse strains therefore a critical part of this analysis is to map the transcripts back to loci previously identified in genetic crosses or to identify the pathways in which they act which may have upstream or downstream components mapping to the previously identified QTL. This may prove a highly fruitful approach but one potential pitfall is the lack of knowledge about which stage during disease pathogenesis a given gene may be differentially expressed. We may be very fortunate that the differential expression is detectable in normal, un-inoculated mice but it is perhaps more likely that differential expression may only occur in response to some part of the disease process. To address these questions it may be necessary to examine a variety of tissues (e.g. whole brain, defined brain regions, spleen) at different time point during disease progression. Gene expression studies throughout prion disease pathogenesis may also reveal which genes and pathways are switched on and off at various time points. Techniques such as differential display reverse-transcriptase and subtractive cDNA cloning have already identified differentially expressed genes such as erythroid differentiation-related factor (EDRF) [56], glial fibrillary acid protein, metallothionein II and B chain alpha-crystallin [57], α -2 microglobulin [58], cathepsin S, C1q γ -chain of complement

apolipoprotein D and two previously unknown genes named scrapie-responsive gene (ScRG) 1 and 2 [59]. It is hoped that the increased throughput and quantitation offered by microarray technology will provide further insights into this important area.

CANDIDATE GENE IDENTIFICATION

The quality of the sequence databases now available makes positional gene cloning more a case of positional gene selection. The difficulty of candidate gene selection for prion disease incubation time is the lack of sound biological information about the possible biochemical pathways that may be involved. Candidate genes worthy of investigation would be expected to be expressed in the central nervous system (or spleen and other lymphoreticular tissue) and functionally involved in processes such as apoptosis, protein folding, protein degradation and clearance, protein trafficking, synaptic function, signal transduction or in direct interactions with PrP^C. As more insights into prion disease pathogenesis are gained so the list of potential functional candidates will expand.

Initial evaluation of candidate genes will involve detailed sequence comparisons between key strains of mice as any detectable genetic difference must by definition arise due to polymorphisms between the strains. The ideal scenario would be to have full genomic sequence spanning the regions of interest from all key strains so that haplotype blocks can be established thereby narrowing the regions of the search to the polymorphic blocks. Low resolution maps currently exist for some strains [60] but these are not yet comprehensive enough to be very useful as they often mask smaller blocks of haplotype difference that may be of critical importance. Until genomic sequence is available for more strains individual researchers will still need to laboriously characterise their own regions of interest. To reduce the size of the task efforts will concentrate on regions that are known to be functionally important such as open reading frames, splice sites, promoters and other known regulatory regions.

The identification of polymorphisms in important regulatory sites will not be sufficient to establish the role of a given gene in prion disease. The major challenge for this field as it moves from locus to gene to nucleotide will be the need to establish a causal relationship between genotype and phenotype. *In-vitro* studies may help to establish that a polymorphism affects function but a phenotype such as incubation time can only be characterised properly in an animal model. A combination of knockouts, complementation studies and transgenics will undoubtedly be the way forward [61, 62].

GENETIC BACKGROUND AND PRION STRAIN SELECTION

Many different prion strains have been described and these are distinguished on the basis of their characteristic incubation time and neuropathology when passaged in mice [17]. These distinctive phenotypes are preserved on multiple passage in the same host. For many years the existence of multiple strains was considered to be a stumbling block for the protein-only hypothesis [1] which proposes that an abnormal isoform (PrP^{Sc}) of a normal cellular protein (PrP^C)

is the major, if not sole, component of the infectious unit [2]. However, it is now clear that strain properties are associated with differences in the conformation [18-21], metal occupancy [63] and glycosylation [19] of PrP^{Sc}. PrP^{Sc} types may be distinguished by differing molecular mass of fragments following partial proteinase K digestion and by differing ratios of di-, mono- and un-glycosylated PrP^{Sc} as visualised on western blots. Using this technique, four principal PrP^{Sc} types have been described in association with distinct phenotypes of CJD [19, 22]. Classical CJD, both sporadic and iatrogenic, is associated with PrP^{Sc} types 1-3. Variant CJD (vCJD) is unique amongst the human PrP^{Sc} types as it is the only strain that shows a di-glycosylated band dominant pattern (designated type 4) on a western blot [19, 22]. This characteristic glycoform ratio is also seen in BSE and provided the first strong experimental evidence that vCJD was causally linked to BSE prion exposure [19], a conclusion supported by subsequent biological strain typing experiments [14, 64]. The characteristic BSE molecular signature is seen in all examined United Kingdom infected cow brains and suggests that BSE is caused by a single strain of agent [65].

All vCJD cases seen to date have a similar clinical and neuropathological phenotype and have occurred in individuals that are homozygous for methionine at *PRNP* codon 129 and have type 4 PrP^{Sc} in the brain. However, new data from transgenic models suggest that in some cases BSE prion infection may induce two distinct phenotypes: the first being the classical vCJD phenotype with type 4 PrP^{Sc} and prominent florid plaques in the brain and the second with type 2 PrP^{Sc} and a neuropathological profile consistent with that seen in sporadic CJD [66]. The transgenic animals in which these experiments were carried out were made on a mixed genetic background suggesting that genetic modifier loci may be involved in determining why the phenotype splits. Further supporting evidence comes from parallel studies in inbred lines of mice. Following primary passage of BSE in C57BL/6 and FVB mice, the characteristic di-glycosylated band dominant PrP^{Sc} pattern is seen on western blots, however, in SJL and RIIS lines, a mono-glycosylated pattern is seen [66]. All the mice used were *Prnp^a* and were inoculated with the same inoculum of BSE which suggests that the strain selection is determined by host genetic factors. These findings have serious implication as they suggest that some populations of both humans and other species with diverse genetic backgrounds, and exposed to BSE, may develop prion diseases that are not recognisable as being caused by BSE by any current methodologies. Recent reports from France and Italy have now identified alternative strains of BSE in cattle [67, 68] further supporting these data. Genetic mapping studies similar to those undertaken for incubation time QTL are underway to identify the genes that regulate this strain selection.

HUMAN STUDIES

The ultimate goal of all the studies described above is to identify prion disease susceptibility genes in mice that will also be relevant for human disease. Mice provide an excellent model for these studies because they are able to recapitulate the major characteristics of the disease phenotype while also enabling geneticists to work with the very large numbers of

individuals and “families” that are required to reach statistical significance. The similarities between the mouse and human genomes holds real hope that genes identified in mice will be similarly involved in humans.

Looking for susceptibility genes in human populations is always challenging and using affected families or sibling pairs is often the technique of choice. Alternatively, population wide association studies can be done where allele frequencies are statistically compared between an affected and control population. However, association studies are currently unsuitable for genome wide screens and rely on testing polymorphisms in known candidate genes. This may be very useful in some cases however it requires prior knowledge and assumptions regarding disease pathogenesis which may be inaccurate and by definition will not identify any new or unexpected susceptibility genes. Most association studies rely on genotyping polymorphisms that are considered to be functional, however the presence of linkage disequilibrium allows a more indirect approach. In this approach, the polymorphisms tested may not themselves be functionally important but are in linkage disequilibrium with the causative variant, therefore they act as markers for the real association. The discovery of blocks of linkage disequilibrium throughout the human genome suggest that whole genome screens may eventually be possible (For review see [69]). The expanding SNP (Single Nucleotide Polymorphism) database, the reduction in genotyping costs and the genome wide haplotype maps developed by the International HapMap Project bring these studies a step closer (For review see [70]). Whether the association study uses a direct or an indirect approach, it is particularly important that the control and test populations are well matched especially in terms of genetic origin. They also require large numbers to reach significance levels that stand up to statistical corrections made for multiple testing (For review of association study design see [71]). For human prion diseases these criteria are particularly challenging as the numbers involved are small. Several reports of association studies have been published but with mixed results.

In addition to the associations of the *PRNP* locus with sporadic CJD detailed above [49, 50] there have also been studies looking at polymorphisms in other candidate genes. An interesting candidate gene came to light with the identification of a mouse PrP homologue, designated *Doppel* (*Dpl*). *Dpl* maps within 16Kb of *Prnp*, encodes a protein of 179 residues and demonstrates amino acid sequence homology with the C-terminal of PrP (19% identity, 50% similarity) [72]. Overexpression of *Dpl* in the brain of *Prnp*^{0/0} mice [72, 73] and the purkinje cells of transgenic mice [74] mice results in a cerebellar ataxia associated with Purkinje cell degeneration. However, these results reflect ectopic expression in transgenic mice and do not represent the normal pattern of *Dpl* expression which is high in the adult mouse testis but undetectable in the brain [72, 75]. Even though *Dpl* has been shown to be implicated in male fertility [76] and has no apparent role in mouse models of prion disease [77, 78], considerable interest still remains in the role of *Dpl*.

The human homologue of *Dpl* (*DPL*) is encoded by the gene *PRND* and maps within 25Kb of *PRNP*. Several polymorphisms have been identified within *PRND*, including

a common coding polymorphism (M174T), which have been used in association studies [79, 80]. Of the four studies reported, two report a significant association between the codon 174 polymorphism and sporadic CJD [81, 82], and the two other studies report no strong association [79, 80]. These results highlight the difficulties of replicating the results of association studies carried out using small numbers of samples.

A second *PRNP* homologue, designated *PRNT* (30% amino acid identity, 44% similarity) has also been identified downstream of *PRNP* and *PRND* [75]. *PRNT* expression is limited to the testis [75] and to our knowledge has not been investigated with respect to prion diseases.

Additional candidate genes investigated include the microtubule-associated-protein *Tau* [83] and the major histocompatibility gene complex (MHC, called HLA in humans) [84-86]. *Tau* mutations are associated with familial frontotemporal dementia on chromosome 17 [87] however, polymorphisms in the *Tau* gene have also been associated with other neurodegenerative disorders including progressive supranuclear paralysis [88, 89], corticobasal degeneration [90] and Parkinson's disease [91]. Polymorphism in *Tau* were studied in 29 CJD patients from Catalonia (Spain) but no association was found. This is perhaps not surprising with such a small sample size which was also a mixture of sporadic (confirmed and probable), and inherited prion disease [83].

Unlike sporadic CJD, vCJD shows a prominent accumulation of PrP^{Sc} in the lymphoreticular system. This may be as a result of the route of infection, prion strain specific effects or host genetic factors. The major histocompatibility complex is well known to be associated with susceptibility to other infectious diseases and has also been implicated in prion incubation time in mouse studies [28]. A study by Jackson *et al.* typed 50 vCJD and 26 sporadic CJD patients and identified a significantly reduced frequency (P=0.001) of the class II antigen HLA-DQ7 in vCJD patients (12%) relative to controls (36%). Sporadic CJD frequencies (46%) were not significantly different to controls [84]. However another two studies failed to replicate this finding. Laplanche *et al.* looked at only 6 French vCJD cases [86] and Pepys and colleagues analysed 76 vCJD samples [85]. All of these studies are by necessity small and therefore it is difficult to assess whether or not any of these genes are truly associated with prion disease susceptibility. It may be possible to carry out large enough studies with sporadic CJD patients, however while the numbers of affected vCJD individuals remains small there is no real prospect of significantly increasing the confidence one can ascribe to these data. Supporting evidence for these loci may be provided by some of the mouse genetic studies, however the only real test for validating these data may be the demonstration of functional significance in cellular and animal models.

CONCLUSIONS

It is well established that the prion protein, in both its normal and abnormal forms, is central to the aetiology and pathology of prion diseases and that mutations and polymorphisms in the coding sequence can have profound effects on disease susceptibility. However, the prion protein cannot

act alone and it is clear that other proteins collectively play a major role in the disease process. It is the polymorphisms in these genes that account for the significant variation in disease susceptibility that is observed in the population. The identification of these genes and their different alleles may allow the identification of at-risk individuals, provide better parameters for human epidemic modelling and ultimately identify new proteins and biochemical pathways that will contribute to our understanding of prion pathogenesis and provide new targets for therapeutic intervention.

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ABBREVIATIONS

BSE	=	Bovine Spogiform Encephalopathy
CJD	=	Creutzfeldt-Jakob disease
vCJD	=	Variante Creutzfeldt-Jakob disease
GSS	=	Gerstmann-Straussler-Scheinker disease
FFI	=	Fatal familial insomnia
PRNP	=	Human prion gene
Prnp	=	Mouse prion gene
PrP ^c	=	Cellular prion protein
PrP ^{Sc}	=	Scrapie isoform of prion protein
QTL	=	Quantitative trait loci
ORF	=	Open reading frame
RT-PCR	=	Reverse transcriptase polymerase chain reaction
HS	=	Heterogeneous stock
SNP	=	Single nucleotide polymorphism
MHC	=	Major histocompatibility gene complex
HLA	=	Human leukocyte antigen

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