A Copine family member, Cpne8, is a candidate quantitative trait gene for prion disease incubation time in mouse

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Abstract Prion disease incubation time in mice is determined by many factors including genetic background. The prion gene itself plays a major role in incubation time; however, other genes are also known to be important. Whilst quantitative trait loci (QTL) studies have identified multiple loci across the genome, these regions are often large, and with the exception of Hectd2 on Mmu19, no quantitative trait genes or nucleotides for prion disease incubation time have been demonstrated. In this study, we use the Northport heterogeneous stock of mice to reduce the size of a previously identified QTL on Mmu15 from approximately 25 to 1.2 cM. We further characterised the genes in this region and identify Cpne8, a member of the copine family, as the most promising candidate gene. We also show that Cpne8 mRNA is upregulated at the terminal stage of disease, supporting a role in prion disease. Applying these techniques to other loci will facilitate the identification of key pathways in prion disease pathogenesis.

Keywords Prion · Incubation time · Mouse · Mmu15 · Cpne8

Introduction

Transmissible spongiform encephalopathies or prion diseases are progressive neurodegenerative disorders for which there is no effective treatment and are invariably fatal. They occur in several mammalian species including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease in humans. Although there is variation between disease types, they are characterised by a clinically silent long incubation period and share the neuropathological features of neuronal loss, spongiform change (vacuolation) and deposition of an abnormal form of the prion protein. Prion diseases are naturally and experimentally transmissible, including to laboratory mice.

Experimental transmission of prions to different strains of inbred mice shows highly reproducible variation in incubation time, reflecting a strong genetic influence [1–4]. The similarities between the mouse and human genome, and conservation of these diseases across mammalian species, suggest that the identification of quantitative trait genes in mice will not only highlight common disease pathways and new therapeutic targets but will also identify susceptibility genes in human. The main genetic determinant of incubation time and susceptibility in both mouse and humans is variation in the prion gene (Prnp) itself. In mice, Prnpa (Leu-108, Thr-189) and Prnpb (Phe-108, Val-189) mice have short and long incubation times, respectively [4, 5]. However, prion transmissions to different Prnpa allele mice show significant variation in incubation time, suggesting that other genes are also important [3, 4, 6]. Quantitative trait studies using a variety of mouse crosses and prion strains have successfully identified several loci across the genome [7–11]; however, the underlying quantitative trait genes (QTG) for these regions have not yet been identified. In these crosses, no quantitative trait locus (QTL) for prion disease incubation time was detected on Mmu19; however, using a heterogenous stock of mice, we previously identified...
Hectd2 as a QTG [12]. In a C57BL/FaDk × RIII/FaDk backcross inoculated intracerebrally with BSE prions, Manolakou et al. [10] identified a QTL of significant linkage on Mmu15. The two-way cross resulted in low-resolution mapping such that the interval spans approximately 25 cM and is therefore too large for individual candidate gene analysis. Several strategies are available for fine mapping including the use of heterogeneous stocks (HS) of mice [13–15]. The Northport HS was produced from semi-randomly mating eight different parental strains (A/J, AKR/J, BALB/cJ, CBA/J, C3H/HeJ, C57BL/6J, DBA/2J and LP/J) over multiple generations [16]. This breeding scheme results in offspring with chromosomes that contain multiple recombinations, modelling an outbred population with the advantage that all the parental alleles are known. In combination with the development of appropriate multipoint linkage programmes, HS populations have successfully been used to obtain mapping resolution of 1–2 cM [16–19]. In this study, we use the Northport HS to refine the region of linkage on Mmu15 and identify Cprn8 as a potential quantitative trait gene for prion disease incubation time.

Materials and methods

Mice and prion infection

RIIIS/J mice were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA), and all other inbred lines were obtained from Harlan, UK. Twenty-eight pairs of Northport HS mice were obtained from R. Hitzemann (Portland, Oregon, USA) at generation 35. The offspring were mated semi-randomly, avoiding shared grandparents, to obtain 49 mating pairs. Approximately 1,000 offspring (generation 37) were used for inoculation. Mice were anaesthetised with isofluorane/O₂ and inoculated intracerebrally into the right parietal lobe with 30 μl Chandler/RML prions as previously described [7]. Incubation time was calculated retrospectively after a definite diagnosis of prion disease had been made and defined as the number of days from inoculation to the onset of clinical signs [20]. All procedures were conducted in accordance with institutional and UK regulations on animal welfare.

Genotyping

DNA was extracted from 0.5 cm tail biopsies using a Promega DNA extraction kit and resuspended in 50 μl TE (10 mM Tris–HCL, 1 mM EDTA, pH 7.5). A 1:10 dilution of this stock was used as template for subsequent PCRs. Microsatellites were selected from the UCSC Mouse Genome Informatics web site (www.informatics.jax.org) and checked that they were polymorphic within the parental strains of the HS. Twenty microsatellite markers from Mmu15, (D15Mit234-D15Mit43) (Electronic supplementary material, Table 1), with an intermarker distance of ~1–3 cM, were genotyped in approximately 400 animals which represent the extreme 20% of both sides of the incubation time distribution. Fluorescently labelled and standard oligonucleotides were synthesised by Sigma-Genosys. PCR reactions were all carried out in 5 μl on 96-well plates using MegaMix Blue (Microzone Ltd.) according to the manufacturer’s instructions using 5 pmol of each primer. PCR conditions were determined empirically, but in general, cycling conditions using a PTC-225 (MJ Research) thermal cycler were as follows: 94°C for 10 min; 94°C 30 s, 55°C 30 s, 72°C 30 s for 35 cycles; 72°C for 5 min. Products of appropriate size and fluorescence were pooled before further processing. Reactions were ethanol-precipitated, washed in 70% ethanol and resuspended in a total of 10 μl including 5.8 μl MegaBACE loading solution (GE Healthcare) and 0.2 μl MegaBACE ET400-R size standard (GE Healthcare). Dilution (1/10) in MegaBACE loading solution was used for analysis. Fragments were heat-denatured at 94°C for 2 min before loading onto a MegaBACE1000 capillary sequencer (GE Healthcare). Samples were injected at 3 kV for 45 s and run at 10 kV for 60 min. Fragment sizes were analysed using Genetic Profiler v1.1 (GE Healthcare). Multipoint linkage analysis was carried out using HAPPY (http://www.well.ox.ac.uk/happy).

Primers and probes for genotyping SNPs (Electronic supplementary material, Table 4) were designed using criteria defined by Applied Biosystems and PrimerExpress software (Applied Biosystems). MGB probes labelled with either Vic or Fam were purchased from Applied Biosystems, and primers for amplification were obtained from Sigma-Genosys. All reactions were carried out in 5 μl (5 pmol of each primer and 1 pmol of each probe) using the Allelic Discrimination function on a 7500 Fast real-time PCR system (Applied Biosystems) using QuantiTect probe PCR kit (Qiagen). Conditions were 95°C for 5 min; 95°C 15 s, 60°C 60 s for 40 cycles.

Sequencing

Genomic DNA for the parental strains were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Genomic sequence and intron/exon structure for each gene was established using the UCSC (http://genome.ucsc.edu/) and ENSEMBLE (http://www.ensembl.org/index.html) genome browsers. PCR products were designed to cover the open reading frame, 5′ and 3′ untranslated region, intron–
exon boundaries and potential promoter sequences as defined by the literature for each gene or as predicted by PROSCAN. Primers were designed using Primer 3 (http://frodo.wi.mit.edu/). PCR products were generated in 25 µl as above with 10 pmol of each primer. Forty cycles were carried out as above except for the annealing and extension steps of 60°C 45 s, 72°C 60 s. PCR products were cleaned using Microclean (Microzone Ltd.) according to the manufacturer’s instructions and resuspended in H2O. PCR product (100–200 ng) was added to a 15-µl sequencing reaction including 5 pmol of either the forward or reverse primer, 1 µl BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems) and 5 µl Better buffer (Microzone Ltd.). Cycling conditions were 95°C 30 s, 50°C 15 s, 60°C 120 s for 30 cycles. Reactions were ethanol-precipitated as above and resuspended in 10 µl MegaBACE loading solution. Samples were injected at 3 kV for 40 s and run at 9 kV for 100 min on a MegaBACE1000 capillary sequencer.

RNA extraction and quantitative RT-PCR

RNA was extracted from whole brains from either uninfected or RML terminally sick mice. Tissue was homogenised using a Ribolyser according to the manufacturer’s instructions. RNA was prepared using either RNaseasy Maxi (Qiagen) kit or TRIreagent (Ambion) according to the manufacturer’s instructions. Samples were treated with DNaseI (Qiagen) and purified further using RNaseasy Mini (Qiagen) columns according to the manufacturer’s instructions. Four micrograms total RNA was reversed-transcribed for each sample to ensure no genomic DNA contamination. Cpone8 quantitative PCR was carried out on a 7500 Fast real-time PCR system (Applied Biosystems) in a total volume of 15 µl including 1 µl cDNA (200–300 ng) and RoxMegamixGold (Microzone Ltd.) according to the manufacturer’s instructions. Primers and probe (3 pmol) were designed using PrimerExpress software (Applied Biosystems) and supplied by Sigma Genosys. Endogenous controls (GAPDH and β-actin) were VIC-labelled and supplied as a kit by Applied Biosystems. Primers and probe (supplied by Applied Biosystems) for an additional endogenous control, Thy-1, were designed and used as above. Thy-F-5'-CGGCCAGGTACACCCCTCCTTA-3', R-5'-GTGTGAGGGACATCACGACTGG-3' and probe (3 pmol) 5'-Fam-CTGCAGAGGAAATATGTGACCAACGC-Tamra-3' were designed using PrimerExpress software (Applied Biosystems) and supplied by Sigma Genosys. Endogenous controls (GAPDH and β-actin) were VIC-labelled and supplied as a kit by Applied Biosystems. Primers and probe (supplied by Applied Biosystems) for an additional endogenous control, Thy-1, were designed and used as above. Thy-F-5'-CAGGCCACCTTTGGGATACC-3'; Thy-R-5'-TGGAACTATATCCCGACCAACCT-3'; probe 5'-VIC-ACGTACCGCTCCGCGTACC-Tamra-3'. Endogenous controls were duplexed with the Cpone8 reaction according to the manufacturer’s instructions (GAPDH and β-actin) or for Thy-1 using the same primer and probe concentrations as for Cpone8. All reactions were carried out in triplicate using the following cycling conditions: 95°C 5 min; 95°C 15 s, 60°C 60 s for 40 cycles.

Results

A previous study identified a 25-cM region on Mmu15 linked to BSE prion incubation time in mice [10]. To reduce this interval, we used the Northport HS of mice. The original cross used C57BL/FaDk and RIII/FaDk mice which are not present in the Northport HS; however, we reasoned that the C57BL/6J alleles present in the HS mice would be a reasonable substitute for the C57BL/ FaDk allele. Primary passage of BSE prions across the species barrier from cattle to mice results in incubation times in excess of 400 days, which can be very variable and frequently results in a less than 100% attack rate. To avoid these issues, we used the Chandler/RML mouse adapted scrapie prion strain which was derived originally from goat scrapie but has been passaged multiple times in mice [21] and no longer presents a species barrier to mice. Although it is possible that the Mmu15 QTL is specific to the BSE strain of prions and might not be relevant in mouse scrapie, our ultimate goal is to identify candidate genes that play a role in the fundamental processes of prion disease and are therefore likely to be independent of prion strain.

Approximately 1,000 HS mice at generation 37 were inoculated intracerebrally with Chandler/RML prions. Incubation times (in days) were determined (n=1,052) and were used as the quantitative trait. The incubation times conformed to a normal distribution (Anderson-Darling normality test) with a mean of 147±15 (SD) with a range of 103–229 days, thus confirming that both “long” and “short” incubation time alleles segregate in the population. To reduce the amount of genotyping, we analysed the mice from the extreme 20% of both ends of the incubation time distribution (approximately n=400) as this contains most of the power available in the cross. Twenty microsatellite markers from Mmu15 (D15Mit234–D15Mit43) at approximately 1–3 cM intermarker distances were genotyped in the HS mice (Electronic supplementary material, Table 1). Multipoint linkage analysis was carried out using the HAPPY programme (http://www.well.ox.ac.uk/happy) [17] which identified a peak of linkage at the interval D15Mit241–D15Mit262 (~logP=4.52) and the adjacent interval D15Mit262–D15Mit34 (~logP=4.48; Fig. 1a and Electronic supplementary material, Table 1). Significant linkage was taken as ~logP>3 as defined by a permutation test (n=1,000) carried out by HAPPY. This QTL explains
7.3% of the observed variance which is similar to that reported for BSE prions. Based on information from the UCSC Genome Browser NCBI build 37 (http://genome.ucsc.edu), the region from D15Mit241–D15Mit34 is 1.2 cM (3.6 Mb), which represents a considerable reduction from the original locus and is small enough for a candidate gene approach to be feasible.

Current database interrogation (NCBI build 37) estimates that the D15Mit241–D15Mit34 interval contains 39 known transcripts. D15Mit241–D15Mit262 contains only one Genbank transcript (AK020896), which contains three exons with the potential to encode a protein of 70 residues and is of unknown function. D15Mit241 maps within the intron 2 of this transcript. Sequencing AK020896 in the HS parental strains identified two polymorphisms in exons 1 and 2, respectively. These are likely to represent SNPs in the 5'UTR and map proximal to D15Mit241. We did not exclude AK020896 on this basis; however, we extended our analysis to include the D15Mit262–D15Mit34 interval (3.47 Mb). This contains 38 transcripts of which we sequenced 29 in the HS parental strains (see Electronic supplementary material, Table 2 for a list of sequenced genes). These 29 genes include all genes in the region predicted to have a role in the central nervous system. Excluded genes included those with known non-CNS tissue-specific functions. Sequencing was not exhaustive, and we focused primarily on the cDNA transcript (ORF, 5' and 3'UTRs), intron–exon boundaries and potential promoters as defined by the literature for each gene or PROSCAN (http://www-bimas.cit.nih.gov/molbio/proscan). Most genes proved not to be very polymorphic in the HS parental strains, with only 88 polymorphisms being identified across the whole region. Most genes contained one or two polymorphisms, but the greatest number were seen primarily in Rabl2a (33) and Cpne8 (38). All variants were assessed using a function of HAPPY that uses progenitor strain information to identify quantitative trait nucleotides by reconstructing ancestral haplotypes [22]. The most significant candidates from this analysis were: Rabl2a (−logP=6.02), Syt10 (−logP=5.4) and Cpne8 (−logP=5.1). A graphic display from HAPPY is shown in Fig. 1b, and details of all identified individual SNPs are shown in Electronic supplementary material, Table 3. Highly significant SNPs are boxed to illustrate the associated gene

Table 1 Most significant strain distribution patterns

| Strain distribution pattern | Genes   | −logP | Comment                  |
|----------------------------|---------|-------|--------------------------|
| D15Mit241–D15Mit34         |         |       |                          |
| (A, C3H, C57, CBA) (AKR, BALB, DBA, LP) | Rabl2a  | 6.02  | Exon 7 S276G And 1 3'UTR |
| (A, AKR,C3H, C57, CBA, LP) (BALB, DBA) | Syt10   | 5.40  | Exon 1 G46G              |
| (A, C3H, CBA, LP) (AKR, BALB, C57, DBA) | Cpne8   | 5.11  | 6 Intronic and 3 3'UTR  |

Fig. 1 HAPPY multipoint linkage analysis for D15Mit234–D15Mit43. Results are displayed on the y-axis as −log of the P value with cM along Mmu15 on the x-axis (a) and Mb along Mmu15 on the x-axis for b. a Log probability plot (additive model). The peak of linkage is seen for the interval D15Mit241–D15Mit262 (−logP=4.52) and the adjacent interval D15Mit262–D15Mit34 (−logP=4.48). These data are generated by analysis of HS mice (n=400) from both extremes of the incubation time distribution. For details of intervals, see Electronic supplementary material, Table 1. b HAPPY analysis (additive merged model) for all polymorphisms detected in the interval D15Mit37–D15Mit34. SNPs are derived from sequencing the HS parental lines and data are analysed according to the methods of Yalcin et al [22]. Details for individual SNPs are given in Electronic supplementary material, Table 3. Highly significant SNPs are boxed to illustrate the associated gene.
one SNP reaches the level of significance, and this is a
synonymous change in exon 1 (G46G). For Cpne8, nine
significant SNPs were detected, six of which were intronic
and three were seen in the 3’UTR.

To verify these data, we genotyped representative
significant SNPs from Rabl2a, Syt10 and Cpne8 in the
HS animals and analysed the data by ANOVA (Table 2
and Electronic supplementary material, Table 5). Surprisingly,
the Rabl2a SNP was not significant (P=0.84), but SNPs for
Syt10 (P=0.0019) and Cpne8 (P=0.0002) were both highly
significant. The original linkage data for Mmu15 came from
a C57BLxRIII cross which suggest that if we are detecting
an effect from the same gene in both crosses, then the
functional polymorphism should be present in both crosses.
We therefore sequenced the candidate SNPs in RIIIS/J and
compared this to the C57BL/6J alleles. For the single Syt10
SNP (Gly46Gly), C57BL/6J and RIIIS/J share the same
allele, which suggests that this is unlikely to be the QTN.
For the nine significant Cpne8 variants (six intronic, three
in the 3’UTR), C57BL/6 and RIIIS have different alleles,
which is consistent with Cpne8 being the most promising
candidate gene in this region. No clear function can be
assigned to the Cpne8 SNPs; however, it is possible that
because our sequencing was not exhaustive, we have not
seen the functional SNPs but that they share the same strain
distribution pattern. Using RT-PCR, we examined the
Cpne8 transcript across different mouse strains for splice
variants, but at the level of detection by ethidium bromide-
stained agarose gel, we could not detect any alternative
splicing events (data not shown).

As an additional screening tool, we also looked at the
expression of mRNA by real-time RT-PCR in the HS
parental lines for Cpne8 (Fig. 2a). RNA was extracted from
whole brains of 6- to 8-week-old male mice (except LP).
Samples were analysed by real-time RT-PCR and normal-
ised using the geometric mean of the quantity obtained
from three endogenous controls (GAPDH, β-actin and Thy-
l). To look for genotype-related differential expression, the
strains were grouped according to the most significant
strain distribution pattern seen in Cpne8 as represented by
the 3’UTR SNP (CPNE3UB T/C) where T = A, C3H, CBA
and C = AKR, BALB, C57, DBA. No significant difference
in expression was seen between the two genotypes (Fig. 2b).
We also compared the level of Cpne8 expression between
C57BL/6 (CPNE3UB C) and RIIIS (CPNE3UB T),
and no significant difference was detected (Fig. 2c).
In addition, we compared Cpne8 mRNA expression with
published incubation times for the inbred lines, and no
correlation was observed (R²=0.28) [3, 4, 23].

To see if Cpne8 mRNA expression is associated with
prion infection, we compared Cpne8 levels in the brains of
uninfected and RML prion infected mice at the terminal
stage of disease (Fig. 2c). A significant increase (∼2.5-fold)
was seen in disease in both C57BL/6 (P=2.7×10⁻⁵) and
RIIIS strains (P=7.8×10⁻⁶). No significant difference was
seen between RML-infected C57BL/6 and RIIIS strains.

**Discussion**

Whilst multiple QTL for prion disease incubation time in
mice have been described, with the exception of Hectd2, no
candidate genes for these loci have been identified [7–12].
Using a heterogenous stock of mice, we have successfully reduced the size of the Mmu15 QTL from approximately 25 to 1.2 cM. Sequence analysis and subsequent testing of the SNPs identified many genes, with SNPs reaching the threshold of significance (−logP>3); however, Syt10 and Cpne8 were identified as the most promising candidate genes. In studies of this nature, it is not always possible to eliminate the possibility that genes excluded by us may still be contributing to the QTL. Further, our sequencing of the region was not exhaustive, and it is possible that the most significant strain distribution pattern could be seen in other genes or intergenic regions.

Although SNPs in both Syt10 and Cpne8 gave highly significant results, we discounted Syt10 because these were not polymorphic between C57BL/6 and RIIIS strains. This was based on the assumption that the quantitative trait gene and nucleotides are the same in the original C57BL × RIII backcross and the HS. Although a reasonable assumption, it is possible that this may not be the case because we used C57BL/6J mice, and the original cross used C57BL mice which may harbour differences between them. In addition, the original QTL was identified in mice inoculated with BSE prions, whereas we have used mouse-adapted scrapie prions which are known to be distinct prion strains.

Based on the assumptions detailed above, Cpne8 is the most promising candidate gene in the region. Highly significant SNPs were found in the 3′UTR. These SNPs were not associated with an alteration in mRNA level; however, it is possible that they influence mRNA structure and translation efficiency. Although we sequenced the putative promoter, 5′UTR, ORF and 3′UTR of Cpne8, it is possible that we may not have seen the functional SNPs if these are contained within introns or more distant regulatory regions.

Differences in mRNA expression levels were not associated with genotype or incubation time differences; however, we did see a ×2.5-fold increase in the disease state independent of mouse strain, suggesting that Cpne8 may be involved in the disease process.

Little is known about the function of Cpne8; however, it is a member of the copine family of proteins which are Ca2+-dependent phospholipid binding proteins thought to be involved in membrane trafficking [24]. PrP is a glycosyl-phosphatidylinositol anchored protein located primarily on the plasma membrane. During its synthesis, it is trafficked through the endoplasmic reticulum and Golgi towards the plasma membrane. During its synthesis, it is trafficked through the endoplasmic reticulum and Golgi towards the plasma membrane where it is associated with lipid rafts. PrP is thought to be constantly recycled from the plasma membrane through clathrin-mediated endocytosis (for review, see [25]). The cellular location of PrP conversion to the abnormal form, PrPSc, and the importance of these sites in prion pathogenesis are yet to be fully described. However, pathogenic mutations of PrP, associated with familial prion disease, have shown abnormal cellular localisations [26–28]. This suggests that the correct trafficking of PrP is critical and proteins such as CPNE8 may be important in regulating this process. Although Syt10 is not a good candidate gene for the C57BL × RIIIS cross, it is highly significant in the HS, and interestingly, Syt10 is a member of the synaptotagmin family which are also thought to be Ca2+-dependent phospholipid binding proteins [29].

In conclusion, we believe that Cpne8 is the most promising candidate gene for the Mmu15 QTL. This is the first example of a prion disease incubation time QTL being reduced in size by fine mapping and for a candidate gene to be identified. Similar approaches applied to other QTL across the genome should reveal other candidate genes that will elucidate the key pathways involved in prion disease pathogenesis.

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