Delimiting the Location of the Scrapie Prion Incubation Time Gene on Chromosome 2 of the Mouse

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ABSTRACT

Scrapie is a transmissible neurodegenerative disease caused by unusual pathogens called prions. The interval between inoculation and illness for experimental mouse scrapie is dramatically influenced by an incubation time gene (*Prn-i*) that is linked to *Prn-p*, the structural gene for prion protein (PrP). Although prion proteins from mouse strains with short and long scrapie incubation times differ by two amino acids, mice with discordant disease phenotype and *Prn-p* genotype occur in segregating crosses, suggesting recombination between *Prn-p* and a distinct incubation time locus. In addition, expression of $Prn\text{-}p^b$ transgenes from long incubation time mice shortened, rather than prolonged, incubation time. In this study, mice carrying chromosomes with meiotic crossovers near *Prn-p* were analyzed for scrapie incubation time phenotype. The results indicated that *Prn-i* (should it exist) must lie within an interval 0.67 cM proximal and 0.22 cM distal to *Prn-p*. The results also suggest that the cumulative effects of other genes, rather than meiotic recombination, were responsible for the putative recombinants of earlier studies. However, the effect of $Prn\text{-}p^b$ transgene expression in abbreviating scrapie incubation time was mitigated when the transgenes were transferred to mice with an endogenous long incubation time allele. Thus, $Prn\text{-}p^b$ transgenes and *Prn-i* may modulate scrapie pathogenesis by different mechanisms.

SCRAPIE is a naturally occurring progressive degeneration of the central nervous system in sheep that is transmitted by a novel class of infectious pathogens called prions (PRUSINER 1982). A cardinal feature of scrapie is its long incubation period, the interval between inoculation of infectious particles and onset of clinical illness. Early studies on natural and experimental scrapie established the importance of host genotype, as well as prion properties, in determining incubation time (PARRY 1983; DICKINSON and FRASER 1979). DICKINSON and MACKAY (1964) identified a strain of mouse, VM/Dk, which when inoculated with the ME7 scrapie isolate had an incubation period of 280 days compared with incubation periods between 140 and 180 days in eight other strains. This major difference in incubation period was due to the predominant influence of a single autosomal gene, designated *Sinc* (DICKINSON, MEIKLE and FRASER 1968). Progress in understanding the genetic control of susceptibility to scrapie was greatly accelerated by molecular dissection of the disease process.

In addition to the long incubation time of scrapie and its ability to cause devastating degeneration of the CNS in the absence of an inflammatory response, several unusual properties distinguish prions from conventional infectious agents (PRUSINER 1991). An abnormal isoform of a host-encoded cellular protein is a crucial, and possibly the only, functional component of the scrapie prion. This protein, designated PrP^{Sc}, accumulates in the brain during the course of disease and differs from its benign counterpart, PrP^{C}, by post-translational modifications that have yet to be defined (BORCHELT *et al.* 1990). The first evidence that the prion protein (PrP) gene might modulate susceptibility to prion disease came from genetic linkage studies in mice (CARLSON *et al.* 1986). Scrapie incubation times in I/LnJ mice were extraordinarily long, ranging from 200 to over 400 days, compared with incubation times of less than 160 days in most other mouse strains. Backcross and restriction fragment length polymorphism analyses with I/LnJ mice and NZW/LacJ mice, a strain showing a short incubation time (120 days), demonstrated that incubation period was controlled by a single dominant gene (designated *Prn-i*) linked to the PrP locus (*Prn-p*). Subsequently, the scrapie incubation time gene *Sinc*, described more than 25 years ago (DICKINSON, MEIKLE and FRASER 1968), also was found to be linked to *Prn-p* (HUNTER *et al.* 1987), suggesting that *Sinc* and *Prn-i* may be synonymous.

Evidence that *Prn-p* itself controls scrapie incubation time was provided by the sequence of genomic clones encompassing the entire open reading frames of the NZW/LacJ ($Prn\text{-}p^a$) and I/LnJ ($Prn\text{-}p^b$) alleles

(WESTAWAY *et al.* 1987). These two alleles encode distinct proteins, PrP-A and PrP-B, which differ at residues 108 (Leu/Phe) and 189 (Thr/Val). The *Prn-p*^{b} allele has been found only in inbred strains of mice that have long scrapie incubation periods, suggesting congruence of the scrapie incubation time gene and *Prn-p* (WESTAWAY *et al.* 1987; CARLSON *et al.* 1988).

However, all seven strains of mice that share the *b* allele of *Prn-p* probably descended from the I/LnJ progenitor stock, and both recombination suppression and segregation distortion were observed in crosses involving C57BL/6J and I/LnJ (CARLSON *et al.* 1988). These phenomena would favor co-inheritance of *Prn-p*^{b} and linked genes. Mice with discordant incubation time phenotype and *Prn-p* genotype were observed at comparable frequencies among offspring of four independent test crosses, suggestive of meiotic recombination between *Prn-p* and a linked scrapie incubation time locus. One of 66 offspring of the (NZW × I/Ln)F_1 × NZW backcross first used to demonstrate linkage of *Prn-p* to the scrapie incubation time gene (CARLSON *et al.* 1986) showed a recombinant phenotype for a presumptive distance of 1.5 ± 1.5 cM separating the two loci, similar to a distance of 2.3 ± 2.2 cM obtained from 44 mice in a similar backcross (RACE *et al.* 1990). Putative meiotic recombinants were also observed among (NZW × I/Ln)F_2 mice (4/90 or 4.8 ± 1.3 cM) (CARLSON *et al.* 1988) and in a (P/J (*Prn-p*^{b}) × NZW)F_1 × NZW backcross (2/34 or 5.9 ± 4.0 cM) (RACE *et al.* 1990). Because the lethal nature of the scrapie bioassay precluded progeny testing, it was not possible to determine whether phenotypically discordant mice detected in earlier experiments corresponded to true recombinants between *Prn-i* and *Prn-p*.

Results from transgenic (Tg) mice also indicated that expression of PrP-B proteins alone is not sufficient to program extended incubation times (WESTAWAY *et al.* 1991). Tg mice that expressed *Prn-p*^{b} transgenes not only failed to reproduce the long incubation time phenotype of normal mice expressing *Prn-p*^{b}, but also had shorter incubation times than nontransgenic *Prn-p*^{a} mice; one interpretation of this result is that the scrapie incubation time locus was not included within the cosmid insert used to construct the transgenic animals.

Further attempts to resolve the issue of the congruency of *Prn-i* and *Prn-p* are reported here.

MATERIALS AND METHODS

Mice: Segregating crosses and congeneic strains of mice were produced at the McLaughlin Research Institute (MRI), and the tail tip (~1 cm) was removed from each individual as a source of DNA when indicated. Tg (*Prn-p*^{b}) 15 and Tg (HaPrP) 7 mice, produced by microinjection of (C57BL/6J × LT/Sv)F_2 fertilized eggs as previously described (WESTAWAY *et al.* 1991; PRUSINER *et al.* 1990), also were maintained at MRI. Individual animals were numbered by ear punches, and shipped to the University of California, San Francisco, for inoculation with scrapie prions and diagnosis of neurologic dysfunction.

Scrapie prions: The Chandler murine scrapie isolate in its fourth passage in Swiss mice was kindly provided by WILLIAM HADLOW (Rocky Mountain Laboratory, Hamilton, Montana). This isolate was passed in NZW/LacJ mice, and a 10% homogenate in 0.8 M sucrose was prepared from the brains of clinically ill animals. Homogenates were stored frozen (−70°) and diluted immediately prior to use.

Scrapie incubation time: All mice were inoculated intracerebrally with 30 μl of a 10-fold dilution of 10% homogenate using a 26-gauge hypodermic needle. Mice were given a full neurological examination twice per week and were observed daily once neurologic signs were apparent. The clinical signs of disease produced by the Chandler isolate in long and short incubation time mice have been described previously (CARLSON *et al.* 1989). The *Prn-p* genotypes of the mice were not known by the animal care specialists responsible for scrapie diagnosis.

Hybridization probes: JANE PARNES (Stanford University) provided a mouse genomic *B2m* clone from which 600-bp *Sac*I-*Kpn*I fragment was isolated for use as a probe (PARNES and SEIDMAN 1982). A 2-kb *Bam*HI insert containing mouse *Il-1a* cDNA was isolated from the pIL-1 1301 plasmid donated by PETER LOMEDICO (Hoffmann-LaRoche Inc.) (LOMEDICO *et al.* 1984). Rat *Hdc* cDNA was isolated as a 2.3-kb insert from a plasmid provided by DAVID JOSEPH (University of North Carolina) (JOSEPH *et al.* 1990), and a 2.6-kb genomic fragment of mouse *Cenpb* was isolated using *Sac*II and *Eco*RI from a plasmid donated by KEVIN SULLIVAN (Scripps Clinic) (SULLIVAN and GLASS 1991). TOM WILKIE (California Institute of Technology) provided a 575-bp fragment amplified using the polymerase chain reaction from his *Spr8*-containing plasmid 738-70 (T. WILKIE, N. A. JENKINS and N. COPELAND, unpublished). A 1.7-kb mouse genomic fragment flanking an ecotropic virus integration site was isolated from a plasmid donated by RICK BEDIGIAN (The Jackson Laboratory) and used to identify *Evi-4* (H. G. BEDIGIAN, unpublished). A 2-kb insert from a hamster PrP cDNA clone was used to identify *Prn-p* as previously described (OESCH *et al.* 1985). An *Scg-1* mouse cDNA was excised with *Xho*I as a 2.3-kb insert from a plasmid given us by MAJAMBU MBIKAY (Institut de recherches clinique de Montréal) (LINARD *et al.* 1990), and NANCY JENKINS and HEE-SUP SHIN provided a plasmid with a 7.6-kb genomic mouse *D2Hss1* *Bam*HI insert (H.-S. SHIN, unpublished). URBAN DEUTSCH provided a 313-bp *Pax-1* fragment in pSPT19, released by cutting with *Eco*RI and *Hind*III (BALLING, DEUTSCH and GRUSS 1988).

Probes were labeled with [α^{32}P]dCTP using random oligonucleotides as primers and the Klenow fragment of DNA polymerase I as described previously (CARLSON *et al.* 1988).

Restriction Fragment Length Polymorphism (RFLP) analysis: Genomic DNA was prepared as described previously (CARLSON *et al.* 1986), digested with restriction endonuclease according to the manufacturer's specifications, and electrophoresed on appropriate percentage agarose gels. Denaturation and alkaline transfer of the DNA was by overnight blotting with 0.4 N NaOH. Prehybridization, hybridization and washing was as previously described (CARLSON *et al.* 1988).

Restriction endonuclease site polymorphisms distinguishing alleles of *B2m*, *Il-1a* and *Prn-p* have been previously described (CARLSON *et al.* 1988), as have polymorphisms distinguish the *un* allele from wild type at the *Pax-1* locus (BALLING, DEUTSCH and GRUSS 1988). B6 (2.9 kb) and I/

LnJ (6.6 kb) *Pax-1* alleles were distinguished by *Bam*HI digestion. *Bam*HI digestion also was used to distinguish the B6 (3.4 kb) and I/Ln (1.7 kb) alleles of *Spr8*, while *Msp*I was used to distinguish *Spr8* in B10.UW (4.1 kb) from I/LnJ (1.4 kb). *Msp*I differentiated alleles at *Cenpb* (B6, 860 bp; I/Ln, 990 bp; B10.UW, 1.9 kb), *D2Hss1* (B6, 13.7 kb; I/Ln 13.0 and 2.0 kb; B10.UW, 17 kb), *Evi-4* (B6, 1.1 kb; I/Ln, 4.3, 5.4 and 8.8 kb; B10.UW, 1.7, 2.1 and 3.8 kb) and the B10.UW (1.9 and 0.93 kb) and I/LnJ (1.0 kb) alleles at *Scg-1*. *Pvu*II was used to distinguish B6 (3.4 kb) from I/LnJ (2.2 kb) at *Scg-1*. *Pst*I revealed B6 (5.9 kb) and I/LnJ (4.0 kb) alleles at *Hdc* for typing the congeneic lines. Only distinguishing fragment sizes are listed.

Statistical analysis: Scrapie incubation time data were analyzed by standard statistical tests for normalcy of distribution, for the presence of "outliers" using the "box and whiskers" test, and Student's *t* test for the significance of difference between genotypes using the Statworks (Data Metrics, Inc.) and Statview (Abacus Concepts) statistical packages for the Macintosh. Recombination frequencies and standard errors were determined as described in GREEN *et al.* (1981).

RESULTS

Scrapie incubation times in mice with chromosomes resulting from recombination near *Prn-p* and a genetic map of the *Prn-p* region: These experiments were designed to obtain a population of mice that theoretically would be enriched for recombinants between *Prn-p* and *Prn-i*. Previous mapping studies suggested that *Prn-p* was located in the vicinity of the wellhaarig (*we*) mutation and proximal to undulated (*un*) on chromosome 2 (CARLSON *et al.* 1988; SIRACUSA *et al.* 1990). Wellhaarig homozygotes have a wavy first coat, most strongly evident between 10 and 21 days of age (HERTWIG 1942). Undulated homozygotes have a shortened and kinky tail, with the caudal vertebrae reduced in size but not in number (WRIGHT 1947); a point mutation in the paired-rule homeobox gene *Pax-1* is responsible for the defect (BALLING, DEUTSCH and GRUSS 1988). A cross was established to determine the location of *Prn-p* relative to *we* and *un*; if *Prn-p* were located between these two markers, visual screening for *we–un* recombinant mice would allow ready identification of animals with chromosomes resulting from recombination near *Prn-p*.

(B6.I- *Il-1a*^{d} *Prn-p*^{b} N6F2 × B10.UW/Sn) F_1 mice were backcrossed to B10.UW/Sn. B6.I- *Il-1a*^{d} *Prn-p*^{b}/Co is a congeneic strain produced by backcrossing the *Prn-p*^{b} allele from I/LnJ onto C57BL/6J; following the sixth backcross (N6) the mice were intercrossed (F_2) to obtain *Prn-p*^{b} homozygotes. In addition to *Prn-p*, the interleukin-1α (*Il-1a*) allele is derived from the I/LnJ donor; the β2 microglobulin *b* allele (*B2m*^{b}) is from the C57BL/6J background. B6.I-*Il-1a*^{d} *Prn-p*^{b}/Co mice are wild type at the *we* and *un* loci and have a long scrapie incubation time (379 ± 8 days, $n = 10$). B10.UW/Sn mice are homozygous for *we* and *un*, exhibiting the wavy coat and kinky tail phenotypes, and carry the *a* allele of *B2m* and the *a* allele of *Prn-p*; B10.UW mice have a short scrapie incubation time (131 ± 2 days, $n = 4$). To determine the location of *Prn-p* relative to *we* and *un*, 153 offspring of the backcross were typed by analysis of RFLPs for *B2m*, *Il-1a* and *Prn-p* (CARLSON *et al.* 1988), in addition to assessing inheritance of the wavy coat and kinky tail phenotypes. The results, shown in Table 1, indicated that the most likely gene order is *B2m–Il-1a–we–Prn-p–un*; for simplicity, alleles from the B6.I congeneic strain are designated "I," while those from B10.UW are designated "B." Note that there was a deficiency of *un* homozygotes, possibly reflecting decreased viability or segregation distortion.

TABLE 1

***Prn-p* is located between *we* and *un*: parental origin of alleles transmitted by (B6.I/Co × B10.UW/Sn)F_1 mice**

B2m	*Il-1a*	*we*	*Prn-p*	*un*	*n*
I[a]	I	+	I	+	90
B[a]	B	*we*	B	*un*	45
B	B	*we*	B |[b]	+	6
I	I	+	I |	*un*	3
B	B	*we* |	I	+	3
I	I	+ |	B	*un*	2
B	B |	+	I	+	1
I	I |	*we*	B	*un*	0
B |	I	+	I	+	3
I |	B	*we*	B	*un*	0
				Total	153

[a] "I" indicates allele derived from the B6.I-*Il-1a*^{d} *Prn-p*^{b}/Co parent, while "B" indicates allele derived from the B10.UW (*Prn-p*^{a}) parent.

[b] | indicates crossover site.

A total of 451 backcross offspring, including the 153 mice described above, were observed for separation of the *we/we* and *un/un* phenotypes, and 24 recombinants were identified and typed for *Prn-p*. Six were recombinants in the *we–Prn-p* interval (1.3 ± 0.5 cM), while 18 were *Prn-p–un* recombinants (4.0 ± 0.9 cM). Because these genetic distances are similar to the presumptive intervals between *Prn-i* and *Prn-p* obtained in other crosses (1.5–5.9 cM), at least some of the mice carrying *we–un* recombinant chromosomes would be expected to be *Prn-i–Prn-p* recombinants if the scrapie incubation time locus is distinct from the prion protein gene.

The *we–un* recombinant mice were mated with RIIIS/J mice and their offspring inoculated with scrapie prions. RIIIS/J mice have the *c* haplotype of *Prn-p* which can be distinguished from the *a* and *b* haplotypes by restriction site polymorphisms flanking the open reading frame; codons 108 and 189 of *Prn-p*^{c} are identical to those of *Prn-p*^{a}, and RIIIS/J mice have a short scrapie incubation time (136 ± 3 days) (CARLSON *et al.* 1988). All inoculated offspring were typed for *B2m* (RIIIS/J has the *a* allele), *Prn-p* and *Pax-1* (*un*). Twenty of the 24 *we–un* recombinant mice produced offspring, and mean (± SE) incubation times for

TABLE 2

Concordance of *Prn-p* genotype and scrapie incubation time in mice with chromosomes in which *Prn-p* and flanking markers recombined

Recombinant chromosome							No.[a]	No. of offspring with recombinant chromosome	Incubation time (±SE)
B2m	*we*		*Prn-p*			*un*[b]			
a	*we*		*a*	×[c]	+		9	31	143 ± 2
b	+		*b*	×	*un*		7	19	236 ± 5
b	+	×	*a*		*un*		1	5	146 ± 3
a	*we*	×	*b*		+		3	10	237 ± 3

[a] The number of recombinant chromosomes recovered from 451 (B6.I/Co × B10.UW/Sn)F_1 × B10.UW/Sn backcross mice that were transmitted. An additional four recombinants were identified but failed to breed, died or were not mated. See text and Table 3 for details.

[b] *un* genotype of recombinant offspring determined using *Pax-1* RFLP (BALLING, DEUTSCH and GRUSS 1988).

[c] "×" indicates crossover site.

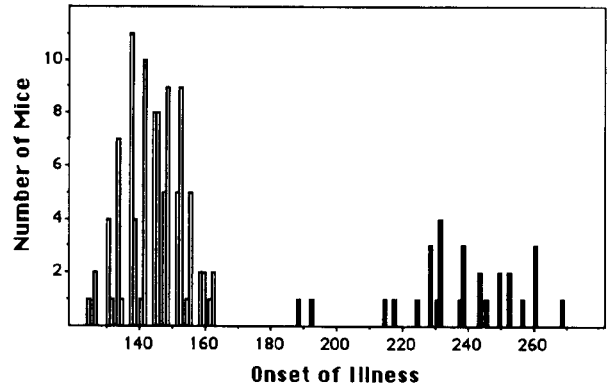

FIGURE 1.—Concordance of *Prn-p* genotype and incubation time phenotype in offspring of mice carrying recombinant chromosomes with crossover sites in the vicinity of *Prn-p*. Results from 128 mice, including 63 that inherited nonrecombinant chromosomes, are shown. Open bars indicate *Prn-p*^{a}/*Prn-p*^{c} mice and filled bars indicate *Prn-p*^{b}/*Prn-p*^{c} mice. The two *Prn-p*^{b}/*Prn-p*^{c} mice with incubation times of 188 and 193 days were offspring of a single recombinant mouse; two littermates that inherited the nonrecombinant *Prn-p*^{a} chromosome both had 155-day incubation periods.

offspring that inherited recombinant chromosomes are shown in Table 2. Scrapie incubation time was concordant with *Prn-p* genotype for both *Prn-p–un* and *we–Prn-p* recombinants. There were no individual mice whose incubation time and *Prn-p* genotype were discordant, including offspring that inherited nonrecombinant chromosomes; times for onset of illness for all 128 progeny of the 20 *we–un* recombinants are illustrated in Figure 1. Clearly, a distinct incubation time locus, if it exists, does not lie proximal to *we* nor distal to *un*.

To delimit further the location of *Prn-i*, DNA samples from the 24 *we–un* recombinant mice were typed for additional loci mapping in the vicinity of *Prn-p*. Probes for *Il-1a* (LOMEDICO *et al.* 1984), centromere protein b (*Cenpb*) (SULLIVAN and GLASS 1991), a receptor for G proteins (*Spr8*) (T. WILKIE, N. A. JENKINS and N. COPELAND, unpublished information), ecotropic virus integration site-4 (*Evi-4*) (H. G.BEDIGIAN, unpublished information), an anonymous DNA marker, D2Hss1 (H.-S. SHIN, unpublished information) and secretogranin-1 (*Scg-1*) (LINARD *et al.* 1990) were used. Table 3 summarizes these results. There was no discordance between *Il-1a* and *we* among the *we–un* recombinants, which was not surprising given the single recombinant (Table 1) which suggested that *Il-1a* lies proximal to *we*. No recombinants between *Evi-4* or *Scg-1* and *Prn-p* were observed. Although no *Prn-p–Scg-1* recombinants were observed in this study, results from an interspecific backcross positioned *Scg-1* distal to *Prn-p* (1 recombinant among 132 offspring) (JENKINS *et al.* 1991). In an even larger backcross involving *we* and *un*, D. ROOPENIAN and colleagues (personal communication) also failed to observe *Prn-p–Evi-4* recombinants, indicating that the genetic distance between these two loci is very small.

A single recombinant indicated that *D2Hss1* lies distal to *Prn-p* between *Scg-1* and *un*. A linkage map for this region of chromosome 2 is presented in Figure 2; genetic distances were calculated assuming that there were no double recombinants within the *we–un* interval among the backcross offspring. In addition to indicating a probable gene order of *Il-1a–we–Cenpb–Spr8–Evi-4/Prn-p–Scg-1–D2Hss1–Pax-1*, these results delimit the chromosomal region that must contain the prion incubation time locus. Offspring of three *Spr8–Prn-p* recombinants and of the single *Prn-p–D2Hss1* recombinant had incubation times concordant with their *Prn-p* genotype (Table 3). Therefore, *Prn-i* cannot lie proximal to *Spr8*, which is located 0.67 ± 0.38 cM proximal to *Prn-p* nor distal to *D2Hss1* which is only 0.22 ± 0.22 cM from *Prn-p* in this cross.

Scrapie incubation times in congeneic mouse strains: Development of congeneic mouse strains also selects for recombination near the target locus (SNELL 1948). The isolation of *Prn-p*^{b} on the C57BL/6J (B6) genetic background began by crossing B6 and I/LnJ and backcrossing the F_1 hybrids to B6. Offspring were typed for *Prn-p* and *Prn-p*^{a}/*Prn-p*^{b} heterozygous individuals were again backcrossed to B6. Typing and backcrossing to B6 was repeated for a minimum of 10 generations, when heterozygotes were intercrossed, selected for donor-type homozygosity, and maintained as inbred lines. Backcross offspring were also typed for *B2m* and *Il-1a*, and three distinct *Prn-p*^{b} congeneic lines were produced–one with the differential chromosomal segment including the *B2m*^{a}, *Il-1a*^{d} and *Prn-p*^{b} alleles from I/LnJ, one with the I/Ln *Il-1a*^{d} and *Prn-p*^{b} alleles but the *B6 B2m*^{b} allele, and the third

TABLE 3

Crossover sites in 24 *we–un* recombinants from 451 (B6.I × B10.UW)F_1 × B10.UW backcross mice and scrapie incubation times of their offspring

Il-1a *we*	*Cenpb*	*Spr8*	*Evi-4* *Prn-p*	*Scg-1*	*D2Hss1*	*un*	No.	Incubation time ± SE (number of recombinant offspring)[a]
B	B	B	B	B	B	I	9[b]	143 ± 2 (29)
I	I	I	I	I	I	B	8[c]	236 ± 5 (19)
B	B	B	B	B	I	I	1	133, 152 (2)
I	I	I	I	I	B	B	0	
B	B	B	I	I	I	I	3[d]	237 ± 3 (10)
I	I	I	B	B	B	B	0	
B	B	I	I	I	I	I	1	No offspring
I	I	B	B	B	B	B	1	No offspring
B	I	I	I	B	I	I	0	
I	B	B	B	B	B	B	1	146 ± 3 (5)

[a] An additional 63 offspring inherited the nonrecombinant chromosome. These mice are included in Figure 1.
[b] Only 8 of the 9 recombinants produced offspring.
[c] Only 7 of the 8 recombinants produced offspring.
[d] All 3 recombinants transmitted the recombinant chromosome to their offspring.

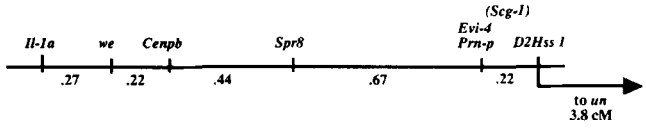

FIGURE 2.—Linkage map of the *Prn-p* region of mouse chromosome 2. Map distances in the *we* to *un* interval were determined by typing 24 *we–un* recombinant mice identified among 451 backcross offspring (Table 3). The *Il-1a–we* interval was calculated from results of 153 backcross offspring (Table 1). No recombinants between *Scg-1* and *Prn-p* were observed in this cross, but results from an earlier study positioned this locus distal to *Prn-p*; *Scg-1* is enclosed in parentheses to indicate this fact.

that was *Prn-p^b* but carried the *B6 B2m^b* and *Il-1a^b* alleles. A fourth line congeneic for I/Ln-derived *B2m^a* that carried the B6 Il-1a^b and *Prn-p^a* alleles was also produced. Mice from each line were inoculated with scrapie prions and were also typed for histidine decarboxylase (*Hdc*), *Il-1a*, *Cenpb*, *Spr8*, *Evi-4*, *Scg-1*, *D2Hss1* and *Pax-1*. Typing and prion incubation time results are presented in Table 4. As do the "recombinant capture" experiments, these results indicate that if a distinct incubation time locus exists, it cannot lie proximal to *Spr8*. The distal markers *Scg-1* and *D2Hss1* were derived from the donor I/LnJ strain in each line, but all three *Prn-p^b* congeneic strains carried the B6 allele at *Pax-1*.

Endogenous long incubation time allele mitigates the abbreviating effect of *Prn-p^b* transgene expression. The results from "recombinant capture" and congeneic strains are compatible with the view that *Prn-p*-linked differences in scrapie incubation time result from either or both of the two amino acid differences between the PrP-A and PrP-B proteins, and suggest that other factors may account for the discordant mice observed in previous studies. If this were the case, however, *Prn-p^b* transgenes might be expected to prolong prion incubation times of *Prn-p^a* mice. In fact, incubation times in Tg (*Prn-p^b*) mice were shorter than those of non-Tg animals (WESTAWAY *et al.* 1991). The cos6.I/LnJ-4 insert used to construct *Prn-p^b* transgenic mice encompasses ~6 kb of 5′- and >15.5 kb of 3′-flanking sequences and contains both untranslated exons located ~11.5 kb upstream from the open reading frame-encoding third exon. Hence *Prn-p^b* expression from the transgene is under the control of its own promoter; all five transgenic lines expressed *Prn-p^b* mRNA and had total PrP^C levels greater than those of non-Tg mice (WESTAWAY *et al.* 1991). Prion incubation times in five independent lines constructed with the *Prn-p^b*-cosmid insert had incubation times ranging from 75 ± 2 days (Tg 94, >50 *Prn-p^b* copies in the transgene array) to 104 ± 2 days (Tg 15, 3 transgene copies); non-Tg mice had incubation times of approximately 140 days. Overexpression of PrP^C ranged from two- to fourfold for low transgene copy number lines to eightfold for lines with high transgene copy number as assessed through an enzyme-linked immunosorbent assay of nitrocellulose-bound brain protein (WESTAWAY *et al.* 1991).

The influence of an endogenous long scrapie incubation time allele in combination with *Prn-p^b* transgenes was tested by inoculating offspring of B6.I-*Prn-p^b*/Co crossed with Tg (*Prn-p^b*) 15 hemizygous mice. These mice were all *Prn-p^a*/*Prn-p^b* and either hemizygous or negative for the 3-copy *Prn-p^b* transgene array. Incubation times in these mice were compared with those of transgene positive and negative *Prn-p^a* homozygous mice as presented in Table 5.

Tg (*Prn-p^b*) mice with an "authentic," endogenous *Prn-p^b* allele had longer scrapie incubation times than mice with the transgene array expressed on a *Prn-p^a* homozygous background (166 ± 2 *vs.* 104 ± 2 days).

TABLE 4

Scrapie incubation time in B6.I congeneic mouse strains

Strain	*B2m*	*Hdc*	*Il-1a*	*Cenpb*	*Spr8*	*Evi-4 Prn-p*	*Scg-1*	*D2Hss1*	*Pax-1*	Scrapie incubation time ± SE
C57BL/6J	B[a]	B	B	B	B	B	B	B	B	143 ± 4
I/LnJ	I	I	I	I	I	I	I	I	I	314 ± 14
B6.I-*Prn-p*^{b}/Co	B	B	B	B	B	I	I	I	B	360 ± 16
B6.I-*Il-1a*^{d} *Prn-p*^{b}/Co	B	B	I	I	I	I	I	I	B	379 ± 8
B6.I-*B2m*^{a} *Prn-p*^{b}/Co	I	I	I	I	I	I	I	I	B	366 ± 10
B6.I-*B2m*^{a}/Co	I	I	B	B	B	B	B	B	B	144 ± 5

[a] "B" indicates allele derived from C57BL/6J, while "I" indicates allele derived from I/LnJ.

This result indicates that the endogenous *Prn-p*^{b} allele, or its linked *Prn-i* locus, functions differently from *Prn-p*^{b} transgenes. It is important to stress that *Prn-p*^{b} copy number cannot account for this result. Although the mice with 4 copies of *Prn-p*^{b} and 1 copy of *Prn-p*^{a} have longer incubation times than mice with 3 copies of *Prn-p*^{b} and 2 copies of *Prn-p*^{a}, additional copies of *Prn-p*^{b} as transgenes shortens, rather than prolongs incubation time. The abbreviating effect of *Prn-p*^{b} transgenes, however, is still evident in mice carrying an "authentic" *Prn-p*^{b} allele; the 166 ± 2-day incubation times of transgene positive *Prn-p*^{a}/*Prn-p*^{b} mice were shorter than those of transgene negative animals (255 ± 11 days).

Endogenous *Prn-p*^{b} expression had no effect on hamster (Ha) prion incubation time in Tg mice expressing a Ha PrP transgene (Table 5). Results from studies with Tg (HaPrP) mice implicate interactions between homologous PrP isoforms in prion replication (PRUSINER *et al.* 1990); Tg (HaPrP) mice inoculated with Ha prions produce only hamster infectivity, while those inoculated with mouse scrapie agent produce only mouse prions. The neutrality of *Prn-p*^{b} in Ha prion inoculated Tg (HaPrP) mice implies that the mouse long incubation time allele does not function simply by protecting the host against prion-mediated neurological damage. It is more likely that differences in incubation time between *Prn-p*^{a} and *Prn-p*^{b} mice reflect events involving prion replication and interaction between PrP^C and PrP^{Sc}.

DISCUSSION

The strongest evidence for the existence of a distinct, *Prn-p*-linked prion incubation time locus was the occurrence of mice with discordant *Prn-p* genotype and incubation time phenotype among the offspring of several independent test crosses (CARLSON *et al.* 1986, 1988; RACE *et al.* 1990). Our failure to capture true-breeding recombinants between *Prn-i* and *Prn-p* cannot be taken as proof that the two genes are congruent, but the results argue strongly that the discordant mice in earlier studies did not result from meiotic recombination between the two loci.

The "recombinant capture" studies were performed to identify and propagate recombinant chromosomes in the interval defined by the *we* and *un* markers. The results indicate that a putative incubation time locus must lie within the *Spr8–D2Hss1* interval which is 0.89 ± 0.41 cM. Although the cross used in attempting to capture recombinants involved different strains of mice than those which produced putative *Prn-i–Prn-p* recombinants, it is unlikely that recombination suppression was responsible for lack of mice with discordant *Prn-p* genotype and incubation time phenotype. Strong recombination suppression in the *B2m–Prn-p* interval was observed in the B6 × I/LnJ crosses used in the production of *Prn-p* congeneic mice; in these crosses, results from 280 mice yielded distances of *B2m*–1 cM–*Il-1a*–0.4 cM–*Prn-p* (CARLSON *et al.* 1988). In other crosses in the same study, the *B2m–Prn-p* interval was 6.25 ± 2.7 cM (B6 × MA/MyJ, 80 mice) and the *Il-1a–Prn-p* interval was 4.0 ± 1.5 cM (NZW/LacJ × I/LnJ, 102 mice), similar to the 5.9 ± 1.9 cM *B2m–Prn-p* and 3.9 ± 1.6 cM *Il-1a–Prn-p* intervals reported here. Although it is well known that recombination frequencies often differ between different mouse strain combinations, there is no suggestion of a deficiency of recombinants in the *we–un* interval in our recombinant capture studies. In addition, RFLP typing of four putative recombinant (NZW/LacJ × I/LnJ)F2 mice for loci flanking *Prn-p* did not reveal any consistent pattern indicative of recombination (CARLSON *et al.* 1988). The lack of recombinants between *Il-1a* and *Prn-p* among the four discordant mice, along with the fact that incubation times of 6 mice that were *Il-1a–Prn-p* recombinants were concordant with *Prn-p* genotype, suggested that the incubation time locus was not likely to lie proximal to *Prn-p*. Two of the four discordant mice were recombinants between *Pax-1* and agouti; results described in this paper clearly indicate *Prn-i* cannot lie distal to *D2Hss1*.

If meiotic recombination does not account for the repeated observation of discordant mice among offspring of crosses between mouse strains with short and long scrapie incubation times, what is responsible?

TABLE 5

Endogenous *Prn-p*^{b} expression mitigates the abbreviating effect of *Prn-p*^{b} transgene on mouse scrapie incubation time but has no effect on hamster prion incubation time in Tg (HaPrP) mice

Mice	Transgene	*Prn-p* genotype	Scrapie inoculum	Onset of illness (*n*)	Death (*n*[a])
Tg 15	*Prn-p*^{b}	*a/a*	Mouse	104 ± 2 (33)	124 ± 4 (10)
Non-Tg[b]	None	*a/a*	Mouse	134 ± 3 (21)	154 ± 2 (21)
(B6.I × Tg 15)F_1	*Prn-p*^{b}	*a/b*	Mouse	166 ± 2 (11)	174 ± 3 (9)
(B6.I × Tg 15)F_1	None	*a/b*	Mouse	255 ± 7 (11)	287 ± 5 (11)
Tg 7	HaPrP	*a/a*	Hamster	47 ± 1 (12)	48 ± 2 (12)
Tg 7	HaPrP	*a/b*	Hamster	47 ± 2 (6)	50 ± 1 (6)

[a] Fewer individuals for times of death than for onset of illness indicated that mice were killed for analysis.
[b] Non-Tg mice include Tg (HaPrP) 20 mice which contain a rearranged transgene that is not expressed. Tg 20 mice were produced by inoculation of (B6 × LT/Sv)F_1 eggs as were Tg 15 and Tg 7.

Mice which exhibited short incubation times, in spite of their *Prn-p*^{a}/*Prn-p*^{b} genotype, could represent "mutation" in the prion isolate to a short incubation time variant. Although there is no physical evidence in favor of a nucleic acid genome in the scrapie prion, true-breeding prion isolates, which can be propagated in the same inbred host strain, define agent-based information which is independent of the amino acid sequence of PrP (BRUCE and DICKINSON 1987). However, selection for prion "mutants" cannot explain discordant mice which have incubation times too long for their *Prn-p*^{a} genotype. A more probable interpretation invokes genes unlinked to *Prn-p* affecting incubation time.

Non-*Prn-p* genes may account for putative *Prn-i*–*Prn-p* recombinants: The influence of minor genes on scrapie incubation time has been observed in several studies (BRUCE and DICKINSON 1985; CARLSON *et al.* 1986, 1988). For example, parental *Prn-p*^{a} NZW/LacJ mice had significantly shorter incubation times than *Prn-p*^{a} homozygous backcross offspring (CARLSON *et al.* 1986). Similarly, genes unlinked to *Prn-p* determine the relatively long (~170 days) incubation periods of MA/MyJ and CAST/Ei mice (CARLSON *et al.* 1988). A major histocompatibility complex (*H-2D*)-linked locus influence on incubation time for mouse-adapted Creutzfeldt-Jakob disease prions has been reported (KINGSBURY *et al.* 1983), but no *H-2* effect was noted in similar studies (MOHRI and TATEISHI 1989; CARLSON *et al.* 1986). Simultaneous inheritance of several "minor" loci, each with a tendency to shorten incubation period, might result in a "short" phenotypic variant mouse with a *Prn-p*^{a}/*Prn-p*^{b} genotype; a similar argument can be invoked for long incubation period mice that are *Prn-p*^{a} homozygous. Supporting the postulate that the cumulative effects of genes unlinked to *Prn-p* are responsible for the apparent recombinants is the fact that no deviant mice were observed among 128 offspring of *we–un* recombinant mice inoculated with scrapie prions. C57BL/6 (B6) and C57BL/10 (B10) mice, the backgrounds of the congeneic strains used in the cross, are closely related and reported to differ for only 6 of approximately 235 loci that have been typed in both strains (RODERICK and GUIDI 1989). I/LnJ and NZW/LacJ were independently derived strains; typing results for both strains are only available for 54 loci, but 12 of these differ between the strains. Failure to observe phenotypically deviant mice in the "recombinant capture" experiments might reflect the similarity of B6 and B10, while the deviants in other crosses may reflect convergence of non-*Prn-p* genes at a reproducible frequency.

Non-PRNP genes also may modulate prion disease phenotype in humans. For example, a 30-year span in age of onset has been seen in affected individuals within a single Gerstmann-Sträussler-Scheinker syndrome (GSS) family with codon 102 leucine mutant PRNP (HSIAO *et al.* 1989). Because there were no differences in PrP primary structure between affected members of the family, the disparity in age of onset must be due to other factors. A similar variation in the time of onset of spontaneous disease (from 125 to >300 days) has been seen in hemizygous Tg (GSS) mice derived from a single founder (HSIAO *et al.* 1990); the founding parents of this line were both (C57BL/6J × SJL)F_1 and numerous alleles, in addition to the transgene, are segregating.

PrP transgenes and *Prn-i* modulate incubation times by different mechanisms: It is clear that expression of PrP-B proteins does not necessarily produce long scrapie incubation times. Tg mice that overexpress *Prn-p*^{b} have shorter, rather than longer, incubation times than non-Tg *Prn-p*^{a} mice. Similarly, *Prn-p*^{a}/*Prn-p*^{b} mice with short incubation times have occurred at a low frequency in several independent segregating crosses. Although our results indicate that meiotic recombination is unlikely to account for these discordant mice, they cannot be taken as proof that control of scrapie incubation time reflects the differences at codons 108 and 189 between the two alleles of *Prn-p*. The finding that an "authentic" *Prn-p*^{b} allele

or its linked incubation time locus mitigates the abbreviating effect of *Prn-p*^{b} transgene expression could suggest that the information responsible for control of scrapie incubation time was not included within the cosmid insert used to construct the Tg animals.

However, all available evidence indicates that the cosmid insert used to construct Tg (*Prn-p*^{b}) mice is a faithful representation of the *Prn-p*^{b} allele of I/LnJ mice (D. WESTAWAY, C. M. COOPER and S. B. PRUSINER, unpublished results). It is worth noting that expression of the *Prn-p*^{b} transgene appears to be position-independent in that five Tg lines, each with a different chromosomal insertion site, all expressed transgene-encoded mRNA in rough proportion to the number of copies in the transgene array (WESTAWAY *et al.* 1991). Expression of endogenous *Prn-p* might be influenced by its chromosomal environment; loss of this positional constraint might lead to aberrant, as well as excessive, expression of the transgene. Endogenous *Prn-p*^{b} expression does not abolish the effect of the transgenes; *Prn-p*^{a}/*Prn-p*^{b} Tg (*Prn-p*^{b}) mice had much shorter incubation times than non-Tg *Prn-p* heterozygous mice.

In mice transgenic for a Syrian hamster PrP gene (HaPrP), the incubation time for hamster prions is inversely correlated with the amount of HaPrP^C expressed as assessed with a monoclonal antibody that does not cross react with mouse PrP (PRUSINER *et al.* 1990). Although antibodies discriminating the PrP-A and PrP-B allotypes are not available, a similar trend was noted in Tg (*Prn-p*^{b}) mice; lines Tg 93H, Tg 94 and Tg 117 with higher transgene copy number expressed greater amounts of PrP mRNA and PrP^C and had shorter incubation times than low copy number lines Tg 93L and Tg 15 (WESTAWAY *et al.* 1991). These studies suggest that the supply of PrP^C for conversion to the malignant PrP^{Sc} isoform exerts a strong influence on scrapie incubation time. However, it is unlikely that quantitative variation in the amount of PrP^C expressed accounts for the dramatic difference in incubation times between mice carrying the *a* or *b* alleles of *Prn-p*. The amounts of PrP^C in the brains of *Prn-p*^{a} and *Prn-p*^{b} mouse strains are similar as assessed through Western, ELISA and immunohistochemical analysis (WESTAWAY *et al.* 1987, 1991; MANSON, MCBRIDE and HOPE 1992), as are the levels of PrP mRNA based both on Northern analysis (WESTAWAY *et al.* 1987) and *in situ* hybridization (MANSON, MCBRIDE and HOPE 1992). Therefore, it seems unlikely that the longer incubation times of Tg 15 mice on a *Prn-p*^{a}/*Prn-p*^{b} heterozygous background were due to reduced amounts of PrP^C in comparison to *Prn-p*^{a} homozygous Tg animals.

Mutations in the PrP gene and susceptibility to prion diseases: The primary structure of PrP undoubtedly has a profound role in prion diseases. In addition to dictating the mouse strain and species barriers to scrapie transmission (CARLSON *et al.* 1989; SCOTT *et al.* 1989), various missense mutations in the PrP gene have been linked to inherited human prion diseases (PRUSINER 1991). Tg mice expressing one of these mutations (codon 101Leu) spontaneously develop neurologic disease similar to scrapie (HSIAO *et al.* 1990); preliminary evidence suggests that the disease is transmissible to other animals (HSIAO *et al.* 1992). In addition to pathogenic mutations, two alleles of PRNP, that may be analogous to incubation time genes, exist in the general population (COLLINGE *et al.* 1990). Approximately 70% of the of the population has methionine at codon 129, while the remainder have a valine at this position. Heterozygosity seems protective against sporadic Creutzfeldt-Jakob disease and may delay age of onset for familial prion disease (PALMER *et al.* 1991; HARDY 1991). This is strikingly similar to the results with some mouse scrapie isolates; for example, *Prn-p*^{a}/*Prn-p*^{b} heterozygous mice inoculated with the 22A scrapie isolate have much longer incubation times than either parent (DICKINSON and MEIKLE 1971). Susceptibility to natural (LAPLANCHE *et al.* 1993) and experimental (GOLDMANN *et al.* 1991) scrapie in sheep also is associated with a missense mutation in the PrP gene.

Multiple mechanisms modulate prion incubation time: The complex scrapie incubation time phenotype can be influenced by a variety of factors, including the efficiency of initiation of the pathogenic process, the rate of prion replication and susceptibility to the neurodegenerative effects of prions. Homology between the primary structures of PrP^C and inoculated PrP^{Sc} can determine the efficiency of disease transmission. Some, but not all, isolates of scrapie prions from *Prn-p*^{a} and *Prn-p*^{b} mice behave differently with respect to the duration and variance of the incubation time depending on the *Prn-p* genotype of the host; this suggests that the amino acid sequence of PrP influences disease transmission (CARLSON *et al.* 1989). This PrP allotype barrier is similar to the species barrier to scrapie transmission (PATTISON 1965); based on results using Tg (HaPrP) mice it seems clear that the species barrier reflects inefficient interaction between nonhomologous PrP^C and PrP^{Sc} (PRUSINER *et al.* 1990; SCOTT *et al.* 1989).

Transgenic mouse studies also indicate that the supply of PrP^C can exert a substantial influence on the incubation time phenotype, with greater amounts of PrP^C expression leading to shorter incubation times (PRUSINER *et al.* 1990). The role of quantitative variations in PrP expression in determining the duration of incubation periods in non-Tg mice is unknown, but is unlikely to account for the dramatic difference between *Prn-p*^{a} and *Prn-p*^{b} mice (WESTAWAY *et al.* 1987, 1991). However, PrP mRNA expression can be

modulated by inoculation with nerve growth factor (MOBLEY *et al.* 1988), raising the possibility that some genes unlinked to *Prn-p* may influence incubation time by altering PrP expression. Although polygenic modulation of prion incubation time clearly exists, and is proposed here to account for putative *Prn-i–Prn-p* recombinant mice, none of these genes have been identified with the possible exception of an *H-2D*-linked locus (KINGSBURY *et al.* 1983).

Although it seems likely that the major influence on scrapie incubation time is an effect of *Prn-p*, definitive assignment of this function to either (or both) the codon 108 or 189 mutations or to linked regulatory elements must await the results of targeted mutagenesis experiments.

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