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129/Ola Mice Carrying a Null Mutation in PrP that Abolishes mRNA Production Are Developmentally Normal

J. C. Manson,[*,1] *A. R. Clarke,*[2] *M. L. Hooper,*[2] *L. Aitchison,*[1] *I. McConnell,*[1] *and J. Hope*[1]

[1]*Institute for Animal Health, AFRC and MRC Neuropathogenesis Unit, Ogston Building, West Mains Road, Edinburgh, EH9 3JF; and* [2]*CRC Laboratories, Department of Pathology, University of Edinburgh*

Abstract

The neural membrane glycoprotein PrP is implicated in the pathogenesis of the transmissible spongiform encephalopathies; however, the normal function of PrP and its precise role in disease are not understood. Recently, gene targeting has been used to produce mice with *neo*/PrP fusion transcripts, but no detectable PrP protein in the brain *(1)*. Here we report the use of a different targeting strategy, to produce inbred mice with a complete absence of both PrP protein and mRNA sequences. At 7 mo of age, these mice show no overt phenotypic abnormalities despite the normal high levels of expression of PrP during mouse development. The mice are being used in experiments designed to address the role of PrP in the pathogenesis of scrapie and the replication of infectivity.

Index Entries: Gene targeting; PrP gene; null mutation.

Introduction

Transmissible spongiform encephalopathies (TSEs), such as scrapie, BSE, and Creutzfelt-Jakob disease, are associated with alterations in the neural membrane protein PrP. In unaffected animals, the PrP protein is attached to the outer surface of neuronal cells through a glycoinositol phospholipid anchor *(2)*. In infected animals, PrP can be detected in large deposits both in and around cells of the brain, and disease-specific protease-resistant forms of PrP can be isolated from brain and other tissues, such as spleen and lymph nodes *(3)*. The distribution of the PrP aggregates in the brain of scrapie infected mice depends on both the host genotype and the strain of scrapie *(4)*. In contrast, no difference is seen in the localization and amount of PrP mRNA in the brains of uninfected and scrapie-in-

*Author to whom all correspondence and reprint requests should be addressed.

fected mice *(5,6)*, suggesting that a breakdown in the posttranscriptional control mechanism results in the accumulation of PrP during disease.

Allelic forms of the PrP protein differing at residues 108 and 189 have been shown to be closely associated with the gene controlling survival time of mice exposed to scrapie *(7)*. Allelic forms of PrP are also associated with survival time of hamsters *(8)* and sheep *(9)* infected with scrapie. The incidence of Gerstmann-Sträussler-Scheinker disease (GSS) and Creutzfelt-Jakob disease in humans has been shown in some cases to be associated with variant forms of PrP *(10–12)*.

The production of PrP transgenic mice has shown that the type and degree of expression of PrP can selectively alter the incubation period of the disease *(13,14)*. Transgenic mice in which there is expression of high copy numbers of a mutant form of the PrP gene associated with GSS have been shown to have a spontaneous neurodegenerative disease with a similar pathology to GSS *(15)*.

Although PrP is clearly involved in the pathology of the TSEs, its role in the transmissibility of disease and the normal function of PrP has not yet been established. The PrP gene is expressed at high levels in neuronal cells of the adult brain *(6,16)* and at lower levels in other tissues, such as heart, lung, and spleen. PrP mRNA has also been detected during mouse embryogenesis. By 6.5 d, gene expression can be detected in the extraembryonic tissues and by 13.5 d, in the developing central and peripheral nervous system *(17)*. These studies have suggested a role for PrP in promoting neuronal cell differentiation and in maintaining neuronal function in the differentiated neurons. However, the expression of PrP is not limited to neuronal cell populations since it has been detected during kidney development, in the developing tooth bud, and in placenta, amnion, and yolk sac *(17)*, suggesting the role of PrP may be more widespread, perhaps as part of a cell signaling system required for differentiation of specific cells.

In order to address both the normal function of PrP and its role in the TSEs, it is necessary to generate mice lacking normal PrP protein. Beuler and coworkers *(1)* have reported the generation of mice homozygous for a disrupted PrP gene by replacement of part of exon 3 of PrP with a neomycin-resistance gene. The gene targeting was carried out in ES cells from agouti 129/Sv//ev mice. The chimeric mice produced were mated with C57BL/6J females, producing mice of mixed parentage in which the PrP mutant allele is derived from 129/Sv//ev mouse strain and the PrP wild-type allele is linked to loci of C57BL/6J mice. Substantial quantities of fused messenger RNA containing the *neo* and residual PrP sequences were present in the brains of these mice, but PrP protein could not be detected in the brains by Western analysis. No developmental or behavioral abnormalities were detected, and the mice were fertile and produced normal progeny *(1)*. We report here on the use of a different targeting strategy to alter the PrP gene in ES cells derived from the 129/Ola strain of mouse, resulting in the production of inbred 129/Ola mice with no detectable PrP mRNA or protein.

Materials and Methods

Targeting of ES Cells

Embryonic stem cells (E14) derived from strain 129/Ola *(18)* were maintained in 60% BRL conditioned medium *(19)*. 10^7 cells were electroporated in phosphate-buffered isotonic saline with 25 μg of linearized targeting vector, at 800 V and 3 μFd for 0.1 s. The electroporated cells were divided into ten gelatin-coated Petri dishes and cultured in 60% BRL conditioned medium. After 1 d, the targeted cells were selected in 200 μg mL^{-1} G418, and 2 μm ganciclovir were added to the medium after 4 d. Clones were transferred into G418 containing medium after 10 d. Surviving clones were transferred to 6-well culture dishes and grown to confluence. At this point, half of each clone was frozen in liquid nitrogen, and DNA was extracted from the remainder and analyzed by Southern blotting for the presence of the mutated gene.

Generation of Chimeric Mice

F2(C57BL/6xCBA) blastocysts were isolated at d 3.5 and injected with 10–12 ES cells. Seven to eight injected blastocysts were returned to a 2.5-d pseudopregnant foster mother. Pups were scored for chimerism on the basis of light-yellow pigmentation in the coat and were mated at 6 wk of age to 129/Ola females. The progeny were screened for germ-line transmission of the ES cell line by the presence of light-yellow pigmentation. The ES cell-derived offspring were screened for the presence of the mutant gene by Southern analysis of their DNA. Animals heterozygous for the mutant gene were bred, and Southern analysis of genomic DNA prepared from tail biopsies was carried out to identify heterozygous (+/–) and homozygous (–/–) offspring.

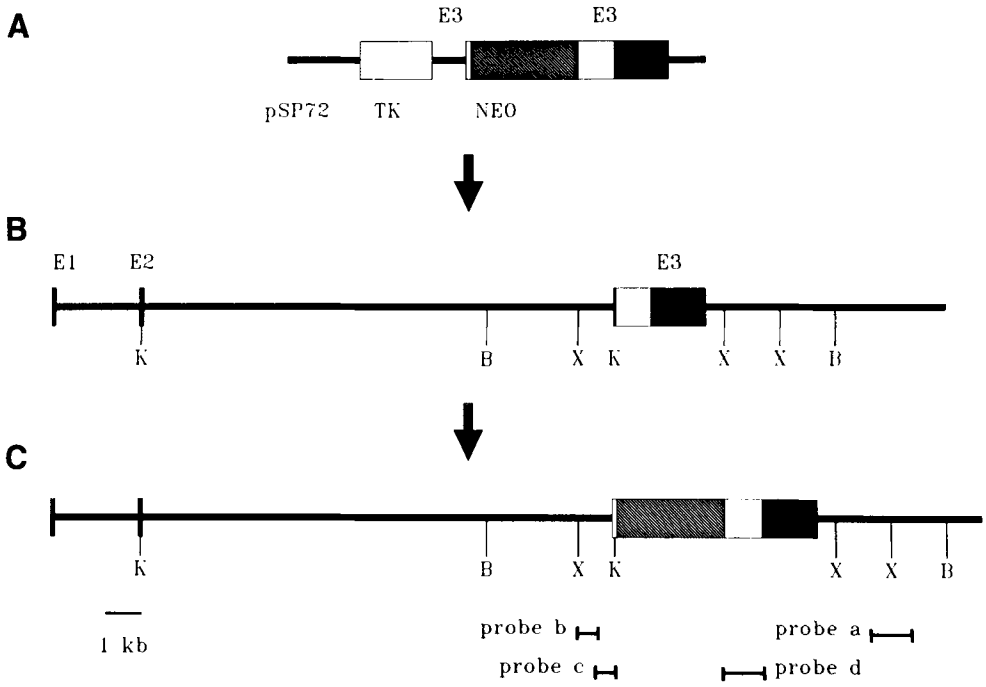

Fig. 1. Targeted mutation of the PrP gene in 129/Ola mouse embryonic stem cells. **(A)** PrP gene targeting construct. The neomycin resistance gene was inserted into a unique *Kpn*I site in exon 3, within a 3.5-kb PrP genomic DNA fragment, to allow for positive selection. The TK gene was added to the 5' end of the PrP to allow for negative selection against random integration. The construct was linearized at a *Bam*H1 site within pSP72 prior to electroporation into ES cells. **(B)** Genomic organization of the gene encoding PrP. The protein coding region is denoted by a white box in exon 3. **(C)** Targeted PrP gene locus. Probe (a) is a probe external to the targeting vector and was used to screen for homologous recombination events by Southern analysis of *Kpn*I-restricted genomic DNA. Probe (b) was used to confirm that the 5' end of the PrP gene was unaltered. Probes (c) and (d) were used to analyze the RNA produced in the mutant mice.

Northern Analysis of Mutant Mice

RNA was prepared from the brains of PrP+/+, PrP+/–, and PrP–/– mice according to Chomczynski and Sacchi *(20)*. Twenty micrograms of total RNA were separated on 1.3% agarose/formaldehyde denaturing gel, transferred to Genescreen plus, and probed with ^{32}P-labeled probes:

1. A 0.7-kb *Pst*I-*Kpn*I restriction fragment containing PrP sequence 5' of the *neo* sequence (Fig. 1, probe c);
2. 1.3-kb *Kpn*I-*Pst*I restriction fragment containing PrP sequence 3' of the *neo* sequence (Fig. 1, probe d); and
3. A 2.4-kb *Eco*RI restriction fragment of the neomycin resistance gene.

Immunoblot Analysis of the Mutant Mice

Membrane extracts were prepared from the brains of PrP+/+, PrP+/–, and PrP–/– mice *(21)*. Proteins were separated by gradient PAGE and immunoblotted using a polyclonal antibody to mouse PrP *(22)*.

Results

Construction of Targeting Vector

The genomic structure of the PrP gene is shown in Fig. 1B. The entire protein coding region is contained within exon 3. To target disruption of the PrP gene, a replacement-type targeting vector *(23)* was constructed. The PrP gene was shown to be expressed at a very low level in mouse embryonic stem cells by Northern blot analysis (Fig. 2). It was therefore decided not to rely on expression of *neo* gene from the PrP promoter for selection in ES cells. A *neo* gene under the control of the mouse metallothioneine promoter was inserted into a unique *Kpn*I site in a 3.6-kb *Xba*I fragment of PrP containing exon 3 and surrounding sequence. The PrP DNA was isolated from a genomic clone of PrP derived from an NZW mouse *(7)*. The targeting vector contained 1.2 kb of PrP sequence 5' to the selectable marker and 2.4 kb of PrP sequence 3' to the *neo* cassette (2.4 kb). To allow for selection against random

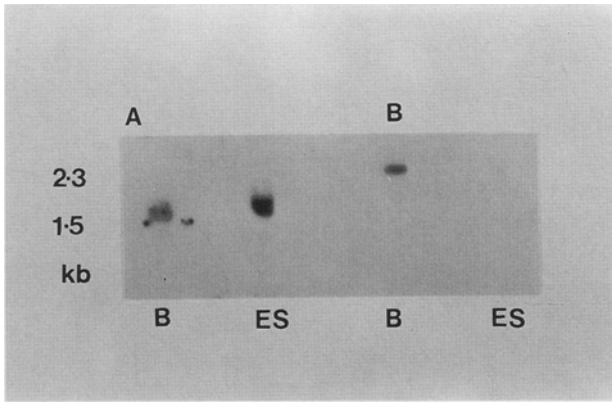

Fig. 2. Northern blot analysis of PrP in mouse ES cells. Total RNA isolated from brain (B) and mouse embryonic stem cells (ES) was separated on a formaldehyde agarose gel, blotted on to Genescreen plus, and probed with **(A)** a HPRT probe and **(B)** a 900-bp *Kpn*I-*Eco*RI fragment from exon 3 of PrP.

integration, a pgk-tk gene cassette *(24)* was added to the 5' end of the genomic sequence (Fig. 1A).

Targeting Mouse Embryonic Stem Cells

The targeting vector was linearized at a *Bam*HI site within the pSP72 polylinker and electroporated into cells of the E14 cell line, derived from strain 129/Ola. These were then subjected to selection as described in Materials and Methods. Enrichment following ganciclovir selection was tenfold. Targeted clones were identified by Southern analysis of genomic DNA digested with *Bam*HI or *Kpn*I, and probed with a *Pst*I fragment (probe a), external to the targeting vector (*see* Fig. 1C). In four separate experiments carried out, homologous recombination was detected in a total of five clones of 400 tested, i.e., a targeting efficiency of 1 in 800 G418-resistant colonies.

Additional Southern analysis was carried out to confirm the structure of the mutant allele. Genomic DNA digested with *Bam*HI, *Hind*III, *Pst*I, *Kpn*I, and *Eco*RI was probed with a 5' probe within the targeting vector, probe b (not shown), and a 3' PrP probe, external to the targeting vector, probe a (Fig. 3A).

Production of Mice Heterozygous and Homozygous for the Mutant Allele

Six ES clones carrying the mutant PrP gene were injected into 4-d-old F2(C57B1/6xCBA) blastocysts to generate chimeric founder mice. Two germ-line chimeras were obtained from one of these clones,

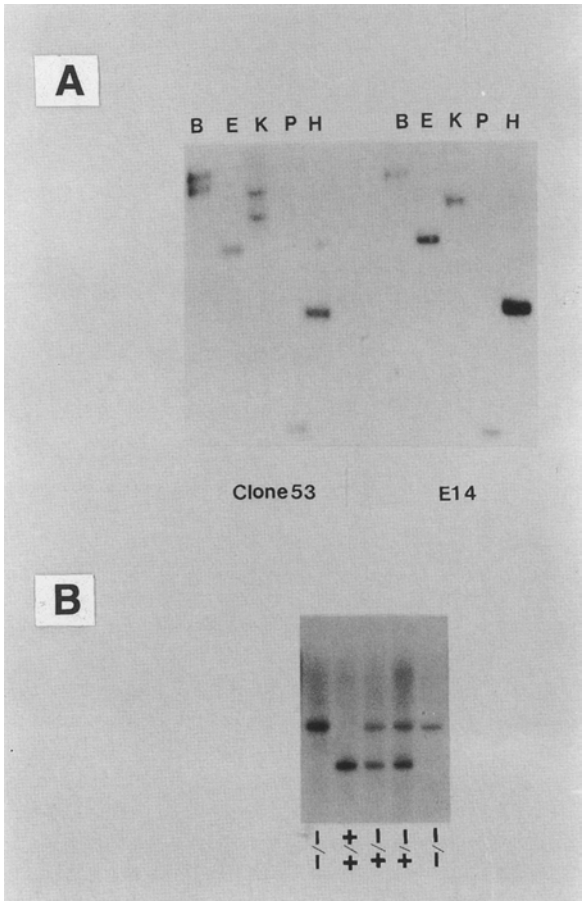

Fig. 3. **(A)** Southern analysis of wild-type and positive clones. Genomic DNA was isolated from E14 cells and from clone 53, digested with *Bam*H1 (B), *Eco*RI (E), *Kpn*I (K), *Pst*I (P), and *Hind*III (H). The fragments were separated on a 1% agarose gel and probed with probe (a). **(B)** Southern analysis of mice containing mutated PrP gene. Genomic DNA was isolated from tail biopsies, digested with *Kpn*I, and DNA fragments separated on a 1% agarose gel. The gel was blotted on to Genescreen plus and hybridized with probe (a).

clone 53. Offspring were analyzed by Southern blotting of their DNA to confirm the presence of the mutant allele. Heterozygous animals (+/−) were crossed to produce mice homozygous for the mutation as determined by Southern analysis of their DNA (Fig. 3B). Chimeras were mated to strain 129/Ola females, and therefore, all experimental animals had an inbred 129/Ola background. The mice heterozygous and homozygous for the mutation are viable, show no obvious abnormalities to date (up to 7 mo), and are fertile.

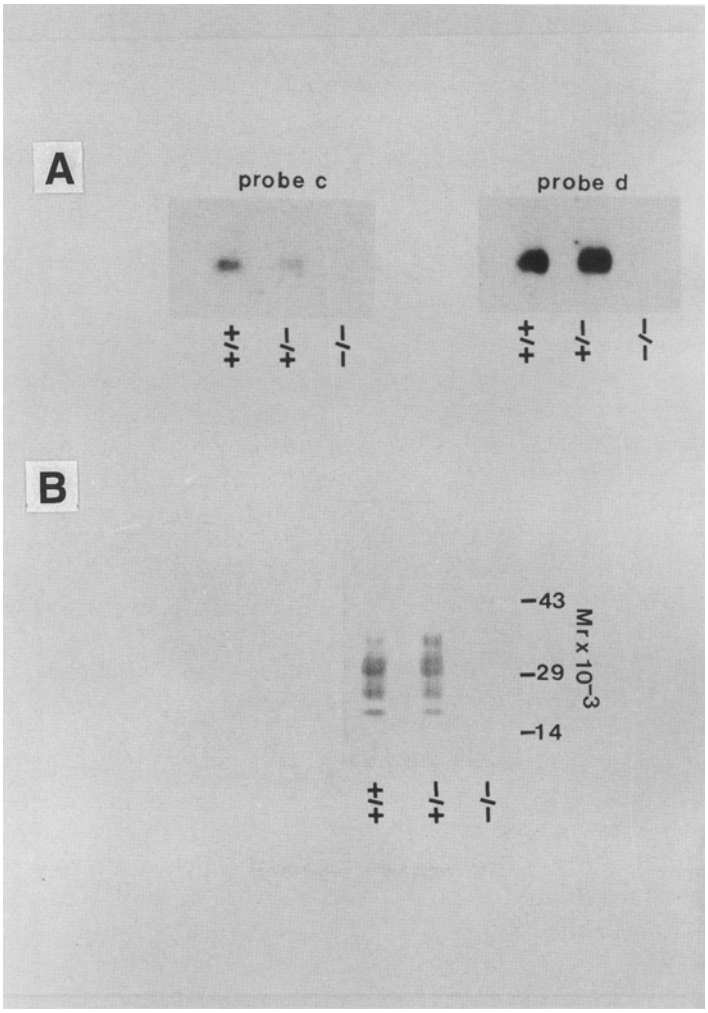

Fig 4. **(A)** Northern analysis of RNA from PrP mutant mice. RNA was isolated from 129/Ola wild-type (+/+), heterozygous (+/–), and homozygous (–/–) PrP mutant mice. The RNA was separated on a 1.3% formaldehyde gel and probed with probe (c) (a PrP probe 3' to neomycin) and probe (d) (a PrP probe 5' to neomycin). **(B)** Western analysis of protein from the brain of PrP mutant mice. Protein was extracted from PrP +/+, PrP+/–, and PrP –/– mice. Extracts were separated by PAGE, blotted on to nitrocellulose, and treated with a polyclonal antibody to PrP.

PrP mRNA Analysis of Mutant Mice

Northern blot analysis was carried out using mRNA isolated from the brains of mice heterozygous and homozygous for the mutant PrP gene, and compared with mRNA isolated from the brain of a wild-type mouse. Three probes were used to analyze the mRNA (Fig. 4):

1. Probe d encoding PrP sequence 3' of *neo*;
2. Probe c, detecting PrP sequences 5' of *neo*; and
3. *Neo* probe (not shown).

Using probes (c) and (d), a 2.4-kb mRNA was detected in the PrP +/+ and +/– mice. This corresponds to the expected size of PrP mRNA. No RNA species hybridizing to either probe (c) or (d) could be detected in the –/– mice (Fig. 4). RNA hybridizing to *neo* was detected in the PrP +/– and –/– mice, but not in the wild-type mice (not shown).

PrP Protein Analysis of Mutant Mice

To determine whether normal PrP was synthesized in the mutant mice, membrane extracts were prepared from the brains of PrP+/+, PrP+/–, and PrP–/– mice. Extracted membranes were subjected to Western blot analysis using a polyclonal antibody to PrP (22). The PrP^c protein is detected in mem-

brane exacts from mouse brain as a broad band in the 20–35-kDa region (22 and Fig. 4). Lower molecular-weight bands can also be seen because of either incomplete processing or degradation of PrP. No bands equivalent to PrP from the wild-type mouse brain were detected in the homozygous mutants, and the bands seen in the heterozygotes were qualitatively indistinguishable from the wild-type PrP.

Discussion

Genetic background is well documented as an important factor in the phenotypic effect resulting from mutant genes. Targeting experiments involving the Hoxb-4 gene have led to different phenotypic effects in different genetic backgrounds *(25)*. This is also clearly seen in the *Min* mouse, which has a mutation in the *apc* gene *(26)* producing different phenotypic effects in different strains of mice owing to the effect of an unlined modifier locus *(27)*. Detailed developmental analysis will establish whether differences in the genetic backgrounds of the PrP mutant mice have led to any phenotypic differences in the absence of PrP.

The ablation of several genes has resulted in a surprising absence of effect in normal development and reproduction. The inactivation of p53 (which is involved in cell cycle regulation) was expected to produce an embryonic lethal effect and instead resulted in normal development and reproduction of the mutant mice *(28)*. Similarly, inactivation of β2 microglobulin *(29)* and of the IB1075 gene, which is normally expressed in the brain *(30)*, has resulted in mice that develop and reproduce normally. There is, therefore, believed to be redundancy that allows the function of the deleted gene to be replaced by another gene. There is no candidate gene to replace PrP. There has been no other gene detected within the mouse genome with any homology to PrP, and we have no understanding of the function for which another protein is required to substitute. If there is indeed no phenotypic effect of abolishing PrP gene expression, the search for a candidate to compensate for the absence of PrP is going to prove challenging.

The ability to produce mice in which the PrP gene has been mutated, preventing the production of PrP protein, allows specific questions on the role of PrP in disease to be addressed. The genetic background of the mice may also be important when both +/– and –/– PrP mutant mice are used for this analysis. *Sinc* is the major gene controlling survival time of mice exposed to scrapie *(31,32)*, and although *Sinc* has been shown to be closely linked to PrP *(33)*, it has not yet be established if they are the same gene. Factors other than *Sinc* genotype also have effects on the disease pathology in infected animals. The production of the inbred strain of mice described here allows analysis of the effect of reduced levels or absence of PrP on scrapie infectivity, without the additional complication of a mixed genetic background.

The mice described here have no detectable PrP RNA or protein. Removal of the 3'RNA sequences appears to have led to the destabilization of the remaining 5' mRNA, suggesting sequences within the 3' region are normally responsible for the stabilization of PrP mRNA. Beuler et al. *(1)* have shown that chimeric RNA sequences can be detected in the brains of their PrP null mice, which contain PrP exon 3 sequences. Several mutations in these sequences have been shown to be associated with the TSEs in humans *(34–36)*. Whether these sequences are important at the RNA or protein level is not known. If interactions at the level of RNA molecules have any relevance in either the replication of infectivity or the disease process, then the different strategies used to produce these mice may produce different results in infectivity studies.

Whether the different genetic backgrounds or different targeting strategies of the PrP –/– and +/– mice affect the normal phenotype, the disease process, or agent replication remains to be established. Nevertheless, the mice described previously *(1)* and here should prove important in understanding the role of PrP in the transmissible spongiform encephalopathies.

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