# Gene targeting studies at the mouse prion protein locus

**Richard C. Moore**

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**Institute of Cell and Molecular Biology**
**University of Edinburgh**
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**Abstract**

Prion diseases are fatal transmissible neurological disorders afflicting a variety of mammalian species and include scrapie in sheep, bovine spongiform encephalopathy in cattle and Creutzfeldt-Jakob disease in humans. The prion protein ($PrP^{c}$) is a normal host-encoded glycoprotein which accumulates as a disease specific protease-resistant isoform ($PrP^{Sc}$) in the brains of infected hosts. In a number of species PrP polymorphisms and germline mutations are associated with the modulation of disease phenotype and the occurrence of familial prion disease.

To investigate the biological consequences of manipulation of the prion protein in mice a flexible two step double replacement gene targeting strategy was developed. This method can be used to generate a series of mouse lines with alterations to the mouse prion protein gene (*Prn-p*). To facilitate gene targeting studies a restriction map of the 129/Ola *Prn-p* locus was constructed and a series of overlapping genomic clones were retrieved from a λ DASH II bacteriophage 129/Ola library.

The double replacement strategy was used to generate PrP deficient mice and mice with subtle alterations to PrP codons 108 and 189. Murine PrP 108L/F and 189T/V dimorphisms give rise to 2 distinct PrP protein allotypes, PrP A and PrP B and these are postulated to be responsible for the control of incubation time following challenge with a wide range of prion inocula. To test this proposal the endogenous 129/Ola PrP A allotype [108L_189T] was converted by gene targeting to encode the PrP-B allotype [108F_189V]. Mice bearing codon 108 and 189 alterations were challenged with mouse adapted BSE isolate 301V. Gene targeting in 129/Ola derived HM-1 ES cells and breeding with 129/Ola mice enabled the investigation of the effect of PrP alterations in the absence of PrP overexpression artefacts or the influence of non-*Prn-p* genes. The dramatic acceleration of incubation time in PrP gtB/gtB mice homozygous for the *Prn-p* $^{a[108F\_189V]}$ gene targeted allele confirmed the major role of codons 108 and 189 in the control of BSE isolate 301V incubation time -and probably other prion isolates. This data provides the strongest evidence yet that the incubation

time control long attributed to the action of different alleles of *Sinc* (*Prn-i)* are determined by PrP codon 108L/F and 189T/V dimorphisms.

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Gordon Hunter once said that scrapie research is not a suitable field for a PhD project. I can only add to this by saying that gene targeting can also be a very precarious way to get a PhD. To combine both fields into one PhD project has been both difficult and time consuming and, on occasion, I have thought that Gordon Hunter was a very wise man indeed. I was doubly fortunate to have Dr. David Melton as a PhD supervisor. He made it possible for me to generate the mice I needed and, by providing me with a research associate position when my BBSRC PhD studentship ran out, enabled me to pursue my intended aim to a satisfying conclusion.

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## Abbreviations

| | |
|---|---|
| A | adenosine |
| ATP | adenosine triphosphate |
| BAP | bacterial alkaline phosphatase |
| BCIP | 5-bromo-4-chloro-3 indolyl phosphate |
| bp | base pairs |
| BSA | bovine serum albumin |
| BSE | bovine spongiform encephalopathy |
| C- | carboxyl terminus (peptide) |
| CIP | calf intestinal phosphatase |
| cm | centimetres |
| cM | centimorgans |
| cDNA | complimentary DNA |
| CJD | Creutzfeldt-Jakob disease |
| CNS | central nervous system |
| d | deoxyribose |
| Da | Daltons |
| DNA | deoxyribosenucleic acid |
| DNase | deoxyribonuclease |
| DTT | dithiothreitol |
| EDTA | ethylenediaminetetra-acetic acid |
| EtOH | ethanol |
| ES | embryonic stem (cells) |
| FCS | foetal calf serum |
| FFI | fatal familial insomnia |
| GCV | gancyclovir |
| GMEM | Glasgows modified Eagles medium |
| GSS | Gerstmann-Straussler-Scheinker disease |
| h | hours |
| HAT | hypoxanthine, aminopterin, thymidine |
| HEPES | N-[2-hydroxyethyl] piperazine -N′-[2-ethanesulphonic acid] |
| HPRT | hypoxanthine-guanine phosphoribosyl transferase |
| HSV | herpes simplex virus |
| HSV-TK | herpes simplex virus thymidine kinase |
| IPTG | Isopropyl β D-thio-galactopyranoside |
| kb | kilobases |
| λ | λ bacteriophage |
| LB | Luria broth |
| LIF | leukaemia inhibitory factor |
| LMP | low melting point (agarose) |
| m | metres |
| M | molar |
| MeOH | methanol |
| ml | millilitres |
| mRNA | message RNA |

| | |
|---|---|
| N | any nucleotide |
| N- | amino terminus (peptide) |
| NCS | newborn calf serum |
| NBT | nitroblue tetrazolium |
| nt | nucleotide(s) |
| OD | optical density |
| OLB | oligo labelling buffer |
| p | plasmid |
| PCR | polymerase chain reaction |
| PIPES | [1,4-piperazinebis-(ethanesulphonic acid)] |
| PMSF | phenylmethylsulphonyl fluoride |
| PNS | peripheral nervous system |
| PrP | proteinase resistant protein or prion protein |
| RF | replicative form (M13) |
| RFLP | restriction fragment length polymorphism |
| RNase | ribonuclease |
| s | seconds |
| ss | single stranded |
| SSC | sodium chloride, sodium citrate |
| SDS | sodium dodecyl sulphate |
| SDS-PAGE | sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SV40 | simian virus 40 |
| STE | sodium/TE |
| TB | terrific broth |
| TE | Tris/EDTA |
| TK | thymidine kinase |
| TRIS | tris(hydroxymethyl)aminomethane |
| ul | microlitres |
| UTR | untranslated region |
| UV | ultraviolet |
| X-Gal | 5-bromo 4-chloro 3-indoyl β D- galactoside |

## Tables



## Figures

# Chapter 1

# Introduction

## Section 1: The biology of prion diseases

### Section 1.1: Introduction

Prion diseases are transmissible degenerative encephalopathies of the central nervous system and occur in a number of mammalian species (Table 1.1, p4). The molecular nature of the infectious agent remains controversial, however there is good evidence that it is associated with $PrP^{Sc}$, a protease resistant isoform of a host-encoded glycoprotein, $PrP^{C}$ (Bolton et al., 1982; McKinley et al., 1983). Prusiner has proposed that $PrP^{Sc}$ may be the major (or sole) component of a novel agent, or prion (Prusiner, 1982a), which may "replicate" in the absence of nucleic acid[1]: if this is correct then prions are without precedent in the history of biology or medicine.

A large and growing body of evidence derived from biophysical, biochemical and transgenic experiments confirm that PrP is of central importance to this disease class (Prusiner, 1997). PrP amino acid sequence variations mediate a number of aspects of these diseases. For example, germline mutations in humans are linked, or associated with, the occurrence of familial prion diseases (Table 1.2, p5) and PrP interspecies sequence variation between hosts is at least one major determinant of the species barrier effect. Mice are the main experimental hosts for a range of prion diseases including BSE, scrapie and Creutzfeldt-Jakob disease. This thesis describes mapping and gene targeted modification of the mouse prion protein gene and, specifically, investigates the role of murine PrP codon 108 and 189 polymorphisms in the control of incubation time following experimental challenge. The fundamental importance of these residues is underlined by the following observations i) despite numerous PrP amino acid sequence differences between mice and natural hosts for these diseases (sheep, cattle, humans), murine PrP dimorphisms at codons 108 and 189 are consistently associated with large differences in incubation time following primary passage with a variety of inocula and ii) mouse adapted scrapie, BSE and CJD isolates retain large differences in incubation times between mice with dimorphic codon 108 and 189 residues. However, the relationship between PrP and mouse genes

[1] However there are alternative models of the infectious agent including the virino hypothesis (Dickinson and Outram, 1988).

*Sinc* and *Prn-i*, which are known to modulate incubation times, remains uncertain. The use of gene targeting to modify PrP codon 108 and 189 sequence dimorphisms is explored in an attempt to delineate the relationship between PrP and these incubation time control genes.

**Section 1.1.2: The spectrum of prion diseases**

Sheep scrapie has been documented in the UK for in excess of 250 years (M'Gowan, 1914) and its transmissibility was demonstrated in 1936 (Cuille and Chelle, 1936). Epidemiological analysis indicates that the spread of natural scrapie is mainly by maternal transmission (Dickinson, 1976), although horizontal transmission also occurs. Related disorders have been described in other species based upon transmission to experimental hosts and clinical and neuropathological similarities to scrapie. For example, transmissible mink encephalopathy (TME) was first described in the 1960's in ranched mink (Hartsough and Burger, 1965) and was subsequently shown to be transmissible (Marsh, 1976; Marsh and Hanson, 1979). Bovine spongiform encephalopathy was first described in dairy cattle in 1987 (BSE: Wells et al., 1987) and was transmitted to mice in 1988 (Fraser et al., 1988). The origin of BSE is presumed to be a scrapie-like agent contaminating meat and bone meal feed supplements (Wilesmith et al., 1988).

Human prion diseases include Kuru (Gadjusek and Zigas, 1957), Gerstmann-Straussler-Scheinker disease (GSS: Gerstmann et al., 1936), Creutzfeldt-Jakob disease (CJD: Creutzfeldt, 1920; Jakob, 1921)[2] and Fatal Familial Insomnia (FFI: Lugaresi et al., 1986). Kuru is an epidemic prion disease transmitted by endocannibalism amongst members of the Fore linguistic group in the highlands of Papua New Guinea (Gadjusek, 1985). Kuru has been responsible for a large number of deaths since 1950 -although the incidence of disease has declined markedly in recent years, partly as a result of government intervention and the erosion of Fore traditional culture. Creutzfeldt-Jakob disease has several epidemiological manifestations: sporadic, familial and iatrogenic. Sporadic CJD has an incidence of

[2] See Creutzfeldt, (1989) and Jakob, (1989) for recent English translations

## Table 1.1:The spectrum of prion diseases

| *Species* | *Disease* | *Comment(s)* | *Distribution* | *Reference* |
|---|---|---|---|---|
| Sheep | Scrapie | Field scrapie; experimentally transmitted to sheep, goats, mice, rats ,hamsters and cattle (Gibbs et al., 1990). | Worldwide | Cuille and Chelle, 1936<br>Wood, Lund and Done, 1992 |
| Goat | Scrapie | Natural cases rare | | Cuille and Chelle, 1939; Pattison and Millson, 1960 |
| Mink | Transmissible mink encephalopathy | Infection in farmed mink; source of infection probably scrapie infected offal. Experimentally transmitted to hamsters. | North America; Europe | Marsh and Kimberlin, 1975; McKenzie et al., 1996 |
| Mule Deer | Chronic wasting disease | Infection of farmed deer probably from scrapie contaminated feed. Experimentally transmitted to other deer. | North America | Williams and Young, 1980; Williams, Young and Marsh, 1982 |
| Elk | Chronic wasting disease | Infection in farmed elk, probably derived from scrapie infected feed. | North America | Williams and Young, 1982 |
| Cattle | Bovine spongiform encephalopathy | UK BSE epidemic mostly in Holstein Friesian dairy cattle; scrapie contamination of feed. Experimentally transmitted to mice (Fraser et al., 1988), pigs (Dawson et al., 1990), monkeys (Lazmesas et al, 1996). | UK; Republic of Ireland; Europe[b] | Wells et al., 1987 |
| Arabian Oryx | a | Infection in a zoo specimen. | UK | Kirkwood et al., 1990 |
| Nyala | a | Infection in a zoo specimen. | UK | Jeffrey and Wells, 1988; HMSO, 1987 |
| Greater Kudu | a | Infection in a zoo specimen. | UK | Kirkwood et al., 1990 |
| Eland | a | Infection in a zoo specimen. | UK | Fleetwood and Furley, 1990 |
| Gemsbok | a | Infection in a zoo specimen. | UK | Jeffrey and Wells, 1988 |
| Domestic cat | a | Infection in >75 domestic cats. Transmitted to mice (Bruce et al, 1994). | UK, Norway | Wyatt et al., 1990; Bratberg,Ueland and Wells, 1995; Willoughby et al., 1992 |
| Cheetah | a | Infection in a zoo specimen | UK | |
| Puma | a | Infection in a zoo specimen | UK | |
| Human | Creutzfeldt-Jakob Disease (sCJD) | Sporadic: $10^{-6}$ incidence; not associated with prion protein (PrP) mutations. Experimentally transmitted to chimpanzees (Gibbs et al, 1968), monkeys, cats and mice | Worldwide | Creutzfeldt, 1920; Jakob, 1921 |
| Human | iCJD | Iatrogenic: transmission following neurosurgery, corneal transplant and dura mater graft. | Worldwide | Gadjusek, 1985; Buchanan et al., 1991;Lane et al., 1994 |
| Human | fCJD | Familial: linked to mutations in prion protein (PrP); autosomal dominant, some have variable penetrance. | Worldwide | for review, see Prusiner, 1994 |
| Human | Gerstmann- Straussler Scheinker- syndrome | Familial disorder tightly linked to mutations in the prion protein (PrP) | Worldwide | Gerstmann et al., 1936; Hsaio et al., 1989 |
| Human | Fatal Familial Insomnia | Familial disorder linked to germline mutation in prion protein (PrP) in association with a common PrP polymorphism. | Worldwide | For review see Gambetti et al., 1993 |
| Human | Kuru | Sporadic CJD spread and maintained by cannibalism; endemic to a remote highland region of Papua New Guinea. Experimentally transmitted to goats, primates and rodents. | Papua New Guinea | Gadjusek and Zigas, 1957; Gadjusek, 1985 |
| Human | CJD new variant (nvCJD) | Unusual CJD variant in UK (20 cases) and France (1 case) with early onset (<49yrs), unusual clinical presentation and neuropathology. $PrP^{sc}$ from nvCJD cases has similar SDS-PAGE banding pattern to BSE $PrP^{sc}$ (Collinge et al, 1996). | UK and France | Will et al., 1996; Chazot et al., 1996 |

***a**, infection associated with UK BSE epidemic; **b**, BSE cases in Switzerland, Portugal, Germany, Italy, Oman and Canada are associated with imports of UK derived feed and cattle ; **i** :iatrogenic; **s**:sporadic; **f** :familial; **nv**:new variant.*

## Table 1.2: Human familial prion diseases associated with PrP mutations

| *Disease* | *Mutation* | *Onset/Duration* *yr:year /m:months* | *Clinical features* | *Neuropathology* | *Reference* |
|---|---|---|---|---|---|
| fCJD | 120bp insertion | 31-45yr / 5-15 yr | Dementia with myoclonus | Spongiosis, neuronal loss and reactive gliosis | Goldfarb et al., 1991a |
| fCJD | 144bp insertion | 22-52 yr/ 1-13 yr | Early personality changes progressing to dementia with ataxia and myoclonus | Widespread atrophy with PrP amyloid plaques | Poulter et al., 1992 |
| fCJD | 168bp insertion | 23-35 yr / >10 yr | Similar to 144bp insertion | Widespread atrophy with spongiosis. | Brown et al., 1992 |
| fCJD | 196bp insertion | 35-54 yr/ 3 m- 13yr | Abnormal behaviour, ataxia, mutism, myoclonus | Widespread atrophy with mild spongiosis. PrP amyloid plaques in cerebrum and cerebellum. | Owen et al., 1992 |
| GSS | 102 Pro-Leu | 15-40yr / 2-10yr | Cerebellar ataxia, extra/pyramidal signs with late onset dementia | PrP amyloid plaques in cerebral and cerebellar cortex., also in white matter of basal ganglia and thalamus. | Hsaio et al., 1989 |
| GSS | 105 Pro-Leu | 42yr / 9yrs | Clumsiness, gait disturbance, decline in intellectual function. | | Kitamoto et al., 1993 |
| GSS | 117 Ala-Val | 35yr/ - | Dementing form<br>Ataxic form | Dementing form (telancephalic GSS): no cerebellar pathology.<br>Ataxic form: widespread cerebellar pathology with PrP amyloid plaques | Doh-ura et al., 1989<br>Mastrianni et al., 1995 |
| Atypical Prion Disease | 145 Tyr -stop [amber mutation] | 38 yr/death at 59 yrs | Slowly progressive Alzheimer's-like disease in a single Japanese woman. | Numerous Kuru plaques and neurofibrillary tangles but little spongiform change. Plaques contained only truncated PrP derived from the mutant allele. | Kitamoto et al., 1993 |
| fCJD | 178 Arg-Asn in phase with 129 Val | 26-56 yr/ 9-51 m | Similar to sCJD. | Spongiosis of cerebral cortex, little thalamic pathology (see FFI). | Goldfarb et al., 1991b |
| FFI | 178 Arg-Asn in phase with 129 Met | 20-71 yr / 6-32 m | Untreatable insomnia, endocrine abnormalities. | Minimal cortical spongiosis. Selective thalamic atrophy; neuronal loss and gliosis in inferior olive. | Goldfarb et al., 1992b; Gambetti et al., 1993 |
| CJD | 180Val-Ile | | Similar to sCJD | Similar to sCJD | Kitamoto et al., 1993 |
| GSS with NFT | 198Phe-Ser | 34-71 yr / 3-11 m | Similar to codon 102 mutation but with early dementia. | Similar to codon 102 mutation but with widespread Alzheimer's type neurofibrillary tangles (NFT) in cerebral cortex. | Hsaio et al., 1992; Dloughy et al., 1992 |
| fCJD | 200Gln-Lys | 35-66yr / 2-41 m | Similar to sporadic CJD . Penetrance estimated at 0.56 | Widespread spongiosis which extends to deep brain nuclei, cerebellum and brain stem. | Goldfarb et al., 1990; Goldfarb et al., 1991c |
| fCJD | 208Arg-His | | | | Mastrianni et al., 1996 |
| fCJD | 210Val-Ile | | | Incomplete penetrance. | Pocchiari et al., 1993 |
| GSS | 217Glu-Arg | | | PrP amyloid plaques and occasional neocortical NFT | Ikeda et al., 1991 |
| CJD | 232Met-Arg | | | Codon 180Ile/232Arg double mutant genotype described in a single Japanese man of 84 years with a sporadic CJD-like presentation (Hitoshi et al., 1993). | Kitamoto et al., 1993 |

FFI: fatal familial insomnia; NFT: neurofibrillary tangles.

$10^{-6}$ and an average onset age of 65 years (Masters et al., 1979; Brown et al., 1987). Approximately 10% of CJD cases (and all GSS and FFI cases) are autosomal dominant familial disorders with tight linkage to mutations in the prion protein gene (See Table 1.2, p5; for review see Prusiner, 1994). A small number of iatrogenic CJD cases have arisen as a result of transmission from contaminated neurosurgical instruments, dura mater grafts (Lane et al., 1994), corneal transplants (Gadjusek, 1985), contaminated cadaver-derived pituitary growth hormone and gonadotrophin (Buchanan et al., 1991). In The UK, much interest has been generated by the possible association between consumption of BSE-infected food products and the recent emergence of a new variant of CJD, (nvCJD) with an earlier age of onset and a novel clinico-pathological phenotype (Will et al., 1996; Chazot et al., 1996).

**Section 1.1.3: Clinical manifestations and neuropathology**

The effects of prion diseases are generally limited to the CNS and the clinical signs are almost entirely neurological. Gross neuropathology can include reduced brain weight, cortical atrophy and enlargement of the ventricles, whilst histological CNS abnormalities include spongiform degeneration, neuronal perikaryal vacuolation, conspicuous neuronal loss, amyloid plaques and astroglial hypertrophy (Bell and Ironside, 1993; DeArmond and Prusiner, 1997). A florid CNS microglial response has been described in CJD and scrapie, which may represent a modified form of inflammatory response (Ironside et al., 1993; Williams et al., 1994). Widespread apoptosis has been described in the brains of sheep, mice and humans with these disorders and may be a major mode of cell death (Fairbairn et al., 1994; Lucassen et al., 1995; Giese et al., 1995).

Natural scrapie in sheep can appear in a number of distinct syndromes including a scratching disorder or as a general paralysis (Pattison and Millson, 1960) and has been attributed to the actions of distinct strains (see section 1.1.5). BSE signs include apprehension, pelvic gait ataxia and hyperaesthesia (Wells et al., 1987; Cranwell et al., 1988; Gilmour et al., 1988) with post mortem diagnosis made from

characteristic vacuolar neuropathology in the medulla oblongata (Wells et al., 1989).

The clinical signs of human prion diseases are variable but include progressive dementia, ataxia and behavioural disturbances. The clinical presentation of Kuru is comparatively uniform and consists of a progressive cerebellar disorder leading to recumbency, often with sparing of higher cortical function, but is invariably fatal within 3 to 12 months of onset (Gadjusek, 1985; Liberski and Gadjusek, 1997). Successful transmission of Kuru[3] to chimpanzees was reported in 1966 (Gadjusek, Gibbs and Alpers, 1966) following an incubation time of 18 months.

Sporadic CJD results in dementia often with cerebellar and visual deficits. Many patients are in their fifth or sixth decade at onset[4]. CJD was first shown to be transmissible in 1968 following transmission to chimpanzees (Gibbs et al., 1968) and has subsequently been transmitted to a wide range of non-human primates. The recently described novel variant of CJD, nvCJD (Will et al., 1996; Chazot et al., 1996), is unusual in that it occurs at a much earlier age than sCJD and has a number of unusual features including prominent early psychiatric manifestations.

GSS generally presents as a progressive cerebellar ataxia with Parkinsonism and nystagmus leading to dementia and has three recognised manifestations: ataxic, telencephalic and dementing with pathologic quantities of neurofibrillary tangles. GSS subtypes are associated with germline PrP mutations at codons 102, 117 and 198 respectively (reviewed by DeArmond and Prusiner, 1997). The first successful transmission of GSS was reported in 1981 (Masters et al., 1981).

Fatal Familial Insomnia is a familial prion disease linked to a PrP germline mutation in association with codon 129 met (Table 1.2, p5) and characterised by intractable insomnia, dysautonomia, multiple endocrine abnormalities and severe selective atrophy of the thalamic nuclei (Lugaresi et al., 1986; Gambetti et al., 1992, 1993) The first successful transmission of FFI was reported in 1995 (Tateishi et al., 1995).

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[3] Hadlow first noted the clinical and histopathological similarities between Kuru and scrapie and suggested that Kuru brain material be tested for transmissibility (Hadlow, 1959).

[4] Although there are a limited number of documented cases of teenagers with sCJD (Berman, Davidson and Becker, 1988; Brown et al., 1985; Britton et al., 1995).

**Section 1.1.4: Prion diseases are transmissible following long incubation times**

The transmission of scrapie and other prion diseases to rodents, especially mice (Chandler, 1961) has enabled the detailed investigation of many features of pathogenesis. The response to experimental inoculation is characterised by a long asymptomatic pre-clinical phase which is followed by a relatively short clinical illness and death. An intriguing feature of scrapie is precise timing of disease onset under controlled conditions: if inbred mice are given the same dose of agent by the same route, all can die within days of each other following a prolonged incubation period of up to 1 year or considerably longer (Scott, 1993). The small standard error and reproducibility of incubation times has enabled the estimation of agent titre by endpoint titration (Chandler, 1961; Dougherty, 1964) and incubation time interval assay (Prusiner et al., 1980; Prusiner et al., 1982b) in mice and hamsters[5]. Intracranial inoculation is commonly used for titration (and thus titres are expressed as i.c. $ID_{50}$) but other routes are available, and include intragastric, intravenous, subcutaneous, intraocular[6].

There is evidence that this precise timing can be a feature of human prion disease -analysis of a number of Kuru cases whose illness is attributable to participation in the same cannibalistic meal[7], has shown that disease can develop within months of each other following an incubation period of 25-30 years (Klitzman, Alpers and Gadjusek, 1984).

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[5] End point titration is a quantal assay which requires the inoculation of groups of susceptible animals (usually mice or hamsters) with tenfold serial dilutions of crude brain homogenate. The titre is determined by the highest dilution at which 50% of the animals develop scrapie and is expressed as $ID_{50}$ or $LD_{50}$ (ID=LD here because these diseases are always fatal). The incubation interval assay estimates the infectious dose based upon the incubation time alone and is based upon observations that the incubation time was related to the dose of agent (Dickinson et al., 1969; Elkund, Kennedy and Hadlow, 1965). Estimates of scrapie titre using this method are very similar to those made by endpoint titration (Prusiner et al., 1982b) and have a number of advantages over end-point titration, most notably being much faster titration experiments and the reduced number of animals required.

[6] Each inoculation route is associated with a different $LD_{50}$ (the effective dose) and gives an estimate of the relative efficiency of each route (Kimberlin and Walker, 1978b; Kimberlin and Walker, 1979). It has also been shown that application of infectious material directly onto skin which has been mildly scarified is as effective a route as intraperitoneal inoculation (Scott, 1993). The most efficient route is via the spinal cord (Kimberlin et al., 1987).

[7] Exposure to the infectious agent is thought to have occurred during cannibalistic mourning rites in which women, young females and males under 6 years eat the lightly cooked tissues of recently deceased relatives. The limited involvement of males in these practices may explain the predominately female incidence of kuru. Kuru is considered to be an epidemic form of CJD (reviewed by Gadjusek, 1977, 1985).

Whilst it is clear that incubation times are influenced by route and dose, incubation times are also determined by scrapie strain properties as illustrated by the large differences noted in the same inbred mouse host for different isolates (Figure 1.1, p12) (Dickinson and Fraser, 1977; Bruce et al., 1991). However, it is clear that host genes modulate the incubation time because the same strain can have very different incubation times in different inbred mouse strains (Table 1.4, p31) (Dickinson, Meikle and Fraser, 1968; Carlson et al., 1986; Carlson et al., 1988; Race et al., 1990). The role of host genotype in incubation time control is the main subject of this thesis and is discussed at length in section 1.3 and thereafter.

**Section 1.1.5: Strains**

Heterogeneity in the clinical presentation of natural scrapie in sheep was long thought to indicate that there may be different "strains" of scrapie. Evidence for the existence of multiple strains was provided upon transmission of scrapie to goats -this gave rise to two distinct clinical syndromes called "drowsy" and "scratching" which could be serially passaged in goats, retaining their distinct properties (Pattison, 1960; Pattison and Millson, 1960, 1961). In the last 30 years many strains of sheep scrapie have been transmitted and propagated in mice (see Figure 1.1, p12; Foster and Dickinson, 1988) and hamsters (Kimberlin and Walker, 1978a; Kimberlin et al., 1989a). A number of other prion diseases with distinct strains have also been transmitted to mice including TME (Marsh and Kimberlin, 1975) and CJD (Mohri and Tateishi, 1989; Kitamoto, Mohri and Tateishi, 1990).

Strains can be differentiated or "typed" on the basis of their different but remarkably reproducible incubation times and neuropathology in inbred mouse strains such as C57 and VM/Dk. Pathological changes in brain can be quantified in standard brain areas and displayed graphically as "lesion profiles" (Fraser and Dickinson, 1968; Fraser and Dickinson, 1973). Lesion profiles are highly reproducible over a large dose range provided the major experimental variables of agent strain, host strain and route of inoculation remain constant. Differing lesion profiles arise from the effects of strain-specific propagation in predilection sites. A number of other strain properties

have been described including differential susceptibility to thermal inactivation (Dickinson and Taylor, 1978) and the ease of transmission to other species (Kimberlin and Walker, 1978a, 1989a). Strains differ with respect to their CNS replication sites (Bruce, McBride and Farquar, 1989) but differ also in the timing and distribution of $PrP^{Sc}$ and infectivity in non-CNS organs such as pancreas and lymphoreticular system (Farquar et al., 1994a; Farquar et al., 1996).

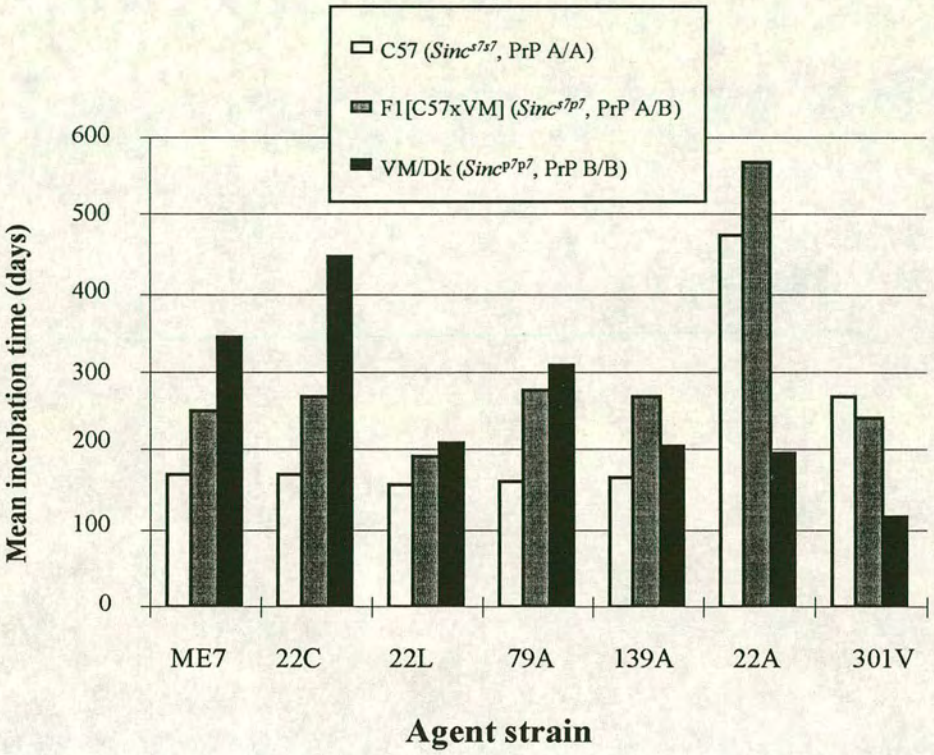
The existence of multiple strains argues that the agent carries biological information capable of specifying strain properties. The biological diversity of scrapie strain characteristics together with the ability to "clone" strains with stable biological properties from mixtures (Kimberlin and Walker, 1978a; Kimberlin, Walker and Fraser, 1989) is also considered by some to be evidence that the agent carries information encoded by a nucleic acid (Bruce, 1993). In contrast to the stability of strain properties, scrapie strain characteristics have also been shown to "mutate" under certain circumstances and this is also considered by some workers to be strong evidence that the scrapie agent has an independent genome (Bruce and Dickinson, 1987) -possibly a nucleic acid. However there is no direct evidence for a nucleic acid component in the agent despite several decades of searching (for review, see Liberski, 1994)[8]. Indeed, efforts to detect scrapie specific nucleic acids have identified a number of host genes differentially expressed during scrapie infection [9].

[8] Although, one must bear in mind Carl Sagan's dictum that "absence of evidence is not evidence of absence.

[9] A number of gene products whose expression is increased during scrapie infection have been identified by subtractive hybridisation and include polyubiquitin C, hsp 70, transferrin, αB crystallin, metallothionein II, vimentin, β2 microglobulin, apolipoprotein E, cathepsin D and sulphated glycoprotein 2 (Duguid and Dinauer, 1989). GFAP, a putative $PrP^{c}$ ligand (Oesch et al., 1990), is also increased during scrapie infection (Diedrich et al., 1987). However, it is likely that many of these genes are involved in tissue response to damage and are not of primary importance to prion diseases *per se* e.g GFAP overexpression is unsurprising given that glial hypertrophy is a feature of prion diseases - indeed GFAP null mice are viable and have a normal scrapie incubation time (Gomi et al., 1995). It has been proposed that Apo E has a significant role in CJD because carriers of the E4 allele have an earlier age of onset and a more rapid clinical progression than those with the E2 allele (Pickering-Brown et al., 1995) however Apo E null mice have unaltered scrapie incubation times and neuropathology (Tatzelt et al., 1996).

**Figure 1.1: Scrapie strains**

A number of TSE or prion strains have been isolated in mice from a range of sources including sheep scrapie and BSE. These have characteristic lesion profiles and incubation times in standard mouse strains such as C57BL, VM/Dk and F1[C57xVM]. Incubation times are controlled by *Sinc*, a gene with two alleles, s7 and p7 (Dickinson, Meikle and Fraser, 1968). The pattern of allelic interaction observed between *Sinc* and the agent is strain dependent. Strains such as 22C, 22L, 79A and 139A are similar to ME7 in that they have short incubation times in *Sinc* $^{s7s7}$ mice. However, in a number of strains (22A and 301V), *Sinc* $^{p7p7}$ mice have the shorter incubation times. Additional complexity is apparent in *Sinc* $^{s7p7}$ mice which reveal a range of dominance patterns including co-dominance with ME7, almost complete dominance with 79A and 301V and overdominance with 22A, 139A and 301C (not shown). Adapted from Bruce et al., 1991 and Bruce et al., 1994.


C57 (Sinc$^{s7s7}$, PrP A/A)
F1[C57xVM] (Sinc$^{s7p7}$, PrP A/B)
VM/Dk (Sinc$^{p7p7}$, PrP B/B)
Mean incubation time (days)
600
500
400
300
200
100
0
ME7
22C
22L
79A
139A
22A
301V
Agent strain

## Section 1.2: The prion protein

### Section 1.2.1: SAF, prion rods and the prion protein, PrP.

Prusiner and co-workers enriched the scrapie agent 100-1000 fold with respect to protein and found a protease resistant protein (PrP) of 27-30kDa copurifying with infectious fractions, called PrP27-30 (Prusiner et al., 1982a). Prolonged proteinase K digestion was found to reduce the agent titre and this was paralleled by the digestion kinetics of PrP27-30, suggesting that this protein was a component of the infectious agent (Bolton et al., 1982; McKinley et al., 1983).

Purification of hamster PrP27-30 to near homogeneity by a combination of detergent extraction, proteinase digestion and sucrose density gradient sedimentation enabled the N-terminal peptide sequence of PrP 27-30 to be determined (Prusiner et al., 1984). This led to the cloning of partial PrP cDNA clones from scrapie infected hamster (Oesch et al., 1985) and mouse brain (Chesebro et al., 1985). Later, the full length PrP cDNA clones were retrieved (Basler et al., 1986; Locht et al., 1986) and it was subsequently found that both infected and uninfected animals have an endogenous gene encoding PrP27-30 (Basler et al., 1986), indicating that PrP 27-30 was not the gene product of a viral pathogen but is the product of the host PrP gene. PrP 27-30 was found to be a protease resistant core derived from full length PrP33-35 kDa by partial proteolysis during purification. Thus it became apparent that both infected and normal animals contained a full length PrP33-35 but that PrP33-35 from diseased animals was resistant to protease digestion and insoluble in detergents. PrP from healthy animals was found to be completely hydrolysed by mild protease digestion and was soluble in non-denaturing detergents. Normal (i.e. protease sensitive) PrP was termed $PrP^{c}$ and the protease resistant infection specific isoform was denoted by $PrP^{Sc}$ -although some authors refer to these PrP isoforms as $PrP^{sen}$ and $PrP^{res}$ respectively (Caughey and Chesebro, 1997).

Unique scrapie-associated fibrils (SAF) were described in scrapie and CJD infected brains (Merz et al., 1981) and are composed of paired helical filaments of 100-1000nm in length. Prion rods were described in preparations of detergent treated PrP 27-30 and are heterogeneous 100-200nm long flattened amyloid rods (Bolton et

al., 1982) which are probably an artefact of the purification procedure. Further analysis of prion rods (Prusiner et al., 1983) and SAF (Hope et al., 1986) demonstrated that PrP27-30 was a major component of these structures.

The *in vivo* relevance of PrP27-30, or rather $PrP^{Sc}$, was confirmed by the observation that antisera raised against PrP 27-30 stained amyloid plaques in the brain of scrapie infected mice (DeArmond et al., 1985). PrP antisera have subsequently been used to detect PrP accumulation in the brains of humans with CJD (Bockman et al., 1985), GSS and Kuru (Brown et al., 1986). $PrP^{Sc}$ has also been described in natural and experimental scrapie in sheep (Rubenstein et al., 1987), BSE (Hope et al., 1988b) and range of experimental murine scrapie strains (Kascsak et al., 1985; Kascsak et al., 1986; Hope et al., 1986; Hope et al, 1988a).

$PrP^{Sc}$ accumulates in the brains of infected animals although PrP mRNA levels do not change throughout the course of scrapie infection (Oesch et al., 1985; Chesebro et al., 1985) suggesting that the accumulation of $PrP^{Sc}$ occurs as a consequence of alterations to protein turnover. Supporting evidence is provided by metabolic labelling studies in scrapie infected mouse neuroblastoma cells which show that, whilst $PrP^{c}$ has a half life of ~3-6 hours, $PrP^{Sc}$ is synthesised slowly (~15hours), is derived from $PrP^{c}$ post translationally (Borchelt et al., 1990; Caughey et al., 1991) and accumulates within the cytoplasm of scrapie infected cells (Taraboulos et al., 1990).

**Section 1.2.2: Tissue distribution and expression levels of $PrP^{c}$**

$PrP^{c}$ is most abundant in the CNS but is found at lower levels in a range of non-CNS tissues in mice, hamsters and sheep (Horiuchi et al., 1995). $PrP^{c}$ is also found in glia (Moser et al., 1995) and in epithelial cells of the choroid plexus (Brown et al., 1990). $PrP^{c}$ has been localised to the synaptic boutons of hamster hippocampus where it colocalises with synaptophysin (Fournier et al., 1995) and has been detected

in the postsynaptic domain of the neuromuscular junction where it colocalises with β-APP (Sarkozi, Askanas and Engel, 1994) suggesting a physiological role in muscle[10].

In situ hybridisation of mouse brain indicates that PrP mRNA is widely expressed, being present at highest levels in the pyramidal neurons of the hippocampus, cerebellar Purkinje neurons and in large neurones in the thalamus and neocortex (Manson, McBride and Hope, 1992). Intermediate levels of mRNA expression are found in cardiac myocytes and lung septal interstitial cells (Oesch et al., 1985). Low levels of PrP mRNA are found in skeletal muscle, spleen (Westaway et al., 1987) kidney and liver (Kretzschmar et al., 1986). PrP mRNA and β-APP have been detected in human muscle macrophages (Askanas et al., 1995). $PrP^c$ is also expressed by lymphocytes (Cashman et al., 1990), follicular dendritic cells in the spleen and in lymph nodes (McBride et al., 1992; Bendheim et al., 1992).

Immunohistochemical detection of $PrP^c$ in adult mouse brain demonstrates that only a subset of cells expressing PrP mRNA actually produce $PrP^c$ (Manson, McBride and Hope, 1992). This discrepancy suggests translational control of PrP expression. The factors involved in the control of PrP expression are poorly defined although NGF has been shown to increase PrP mRNA levels both *in vivo* (Mobley et al., 1988) and *in vitro* (Wion et al., 1988; Lasmesas et al., 1993). Recombinant growth hormone and one of its *in vivo* effectors, insulin-like growth factor-1, can induce PrP expression in PC12 cells[11] (Lasmesas et al., 1993). Cultured human muscle tissue expresses both β-APP and PrP and the mRNA level of both is significantly reduced by glucocorticoids (Askanas et al., 1995).

---

[10] The detection of $PrP^c$ in muscle is interesting given that mice overexpressing hamster, sheep and mouse PrP in a number of tissues, including muscle, develop a late onset neuromyopathy (Westaway et al., 1994b). A necrotising myopathy was described in a wide range of skeletal muscles (but not smooth muscles) which was detectable as early as 90 days of age. This is well before the development of pathology in the PNS and CNS, arguing that PrP overexpression in muscle causes a primary muscle disorder (DeArmond and Prusiner, 1997).

[11] Although it is not known whether the expression of PrP is increased by growth hormone in humans, GH-mediated upregulation of PrP expression in recipients of CJD contaminated growth hormone may have contributed to the the susceptibility of recipients to infection.

**Section 1.2.3: $PrP^c$ structure and function**

The peptide backbone of all mammalian $PrP^c$ molecules studied so far is predicted to be 252-254 amino acids in length. $PrP^c$ is post translationally modified (Figure 1.2, p19) by the removal of a 22 amino acid N-terminal signal sequence following translocation into the endoplasmic reticulum where it is modified by the addition of complex N-linked polysaccharides at 2 asparagine residues (Endo et al., 1989) and a single intrachain disulphide bond (Basler et al., 1986; Turk et al., 1988). Nascent $PrP^c$ is further modified by the addition of a sialated glycosylinositol phospholipid (GPI) anchor at residue 231 (Stahl et al., 1987, 1992) enabling attachment of $PrP^c$ to the surface of neurons (Stahl et al., 1987). A soluble form of $PrP^c$ has been detected in human cerebrospinal fluid (Tagliavini et al., 1992) and there is evidence also of truncated forms of human PrP in brain which are C-terminal fragments from His-111 and Met-112 (Chen et al., 1995).

$PrP^c$ has a number of features including a stretch of 5 gly/pro rich octapeptide repeats between codons 45 and 84 (Liao et al., 1986) and a non-hydrophobic stop transfer effector (STE) sequence (Yost et al., 1990; Lopez et al., 1990) between codons 96 and 112 [12] which determines PrP topology in *in vitro* translation systems - although its *in vivo* relevance remains uncertain. The microheterogeneity of PrP 33-35 observed with SDS-PAGE is attributable to varying degrees of N-linked glycosylation and indeed PNGase F deglycosylated $PrP^c$ runs as a single band of ~16 kDa (Endo et al., 1989; Somerville and Ritchie, 1990).

Sequence data from a wide variety of mammals indicates a high degree of conservation at the amino acid level (Figure 1.3, p21) which, together with the tight spatial and temporal control of PrP expression during embryonic development (Manson et al., 1992), suggests an important role for PrP. However PrP null mice were reported to be fertile and had no overt neurological phenotype (Bueler et al.,

[12] Note that the GSS P102L mutation occurs in this region.

1992). Subsequently a number of other groups have independently generated PrP nulls (Manson et al., 1994a; Moore et al., 1995; Sakaguchi et al., 1995) and a number of differences with wildtype mice have been found and are discussed in chapter 4.

**Section 1.2.4: PrP accumulates in the CNS during infection**

Following the purification of PrP27-30 and the generation of PrP antisera it became apparent that PrP localised to amyloid plaques in scrapie infected brains (Bendheim et al., 1984) and can accumulate to levels ~10-20 fold higher than in uninfected brains (Meyer et al., 1986). PrP immunostaining of normal and infected brains indicated that PrP localisation altered from that of around neuronal cell bodies to staining of the neuropil (DeArmond et al., 1985). A wide variety of PrP immunostaining patterns are now known to occur in prion diseases and it is evident that a number of factors control the localisation of most forms of known neuropathology including agent strain and host genotype (Bruce, McBride and Farquar, 1989; Jeffrey et al., 1994). Extracellular PrP amyloid plaques are a feature of some human and experimental mouse prion diseases. There is good evidence that strains differ with respect to the formation of PrP amyloid plaques, although host genetic control of plaque frequency has been described in the mouse (Bruce and Dickinson, 1985; Carlson et al., 1994).

Co-localisation of PrP immunostaining and neuropathology has been described in a number of prion diseases and the observation that $PrP^{Sc}$ accumulation precedes vacuolation argues for a causative role for PrP (Reviewed by DeArmond and Prusiner, 1997). However, observations in the mouse and in human CJD cases suggest that there is not necessarily a strict association between amyloid plaques and neuropathology (Jeffrey et al., 1994). The level and distribution of $PrP^{c}$ in uninfected rodent brain does not always correspond to infectious titre or to levels of $PrP^{Sc}$ in terminal brains (Kitamoto et al., 1989; Taraboulos et al., 1992). For example, regions which normally produce low levels of $PrP^{c}$ may ultimately exhibit large accumulations of $PrP^{Sc}$ in terminal brains. These observations suggest that cell-type specific factors may be required for the entry and/or replication of agent

**Figure 1.2: PrP primary and secondary structure**

Diagram of $PrP^c$ indicating primary amino acid sequence, secondary structural features and post translational modifications. Hamster PrP is used as an example as it is has been most thoroughly studied of all mammalian PrP molecules. The position of known post translational modifications are marked and include: the removal of N and C terminal signal peptides following translocation into the endoplasmic reticulum, the addition of a GPI anchor at ser 231, addition of complex N-linked glycosylation at asn 181 and asn 197 and the formation of an intrachain disulphide bond between cys 179 and cys 214. A five octapeptide repeat structure is a prominent feature of the N-terminus between residues 51 and 90 and is similar to pro/gly rich repeat structures found in fibrillar proteins such as the collagens and keratins. A number of regions of secondary structure have been predicted including 5 putative α-helices (H1-4 and helix 2.5) (Reviewed by Harrison et al., 1997). Helices 2.5, 3 and 4 have been observed in NMR structures obtained from a fragment of PrP expressed in E.coli (Riek et al., 1996).

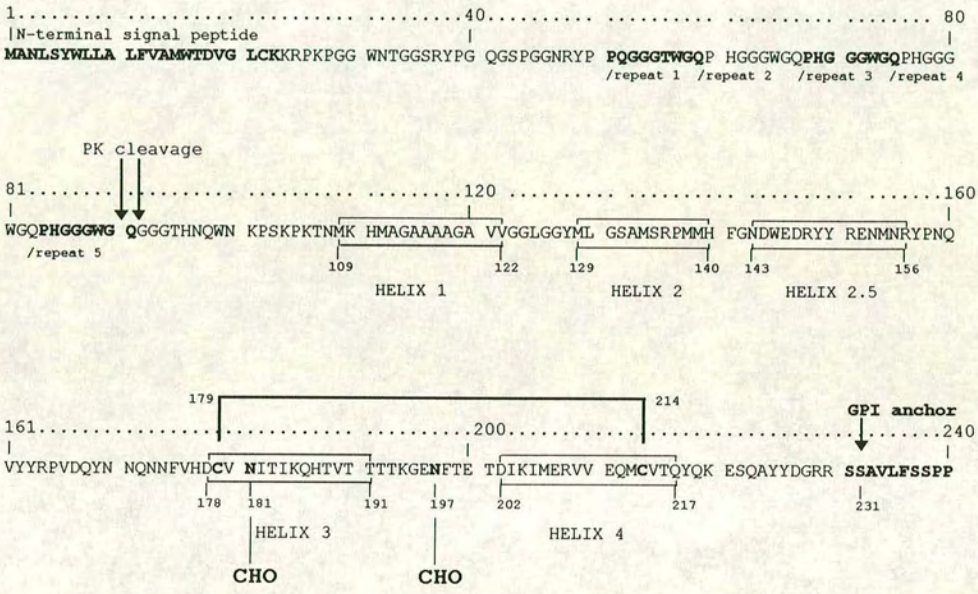

1 40 80
N-terminal signal peptide
MANLSYWLLA LFVAMWTDVG LCKKRPKPGG WNTGGSRYPG QGSPGGNRYP PQGGGTWGQP HGGGWGQPHG GGWGQPHGGG
/repeat 1 /repeat 2 /repeat 3 /repeat 4
PK cleavage
81 120 160
WGQPHGGGWG QGGGTHNQWN KPSKPKTNMK HMAGAAAAGA VVGGLGGYML GSAMSRPMMH FGNDWEDRYY RENMNRYPNQ
/repeat 5
109 122 129 140 143 156
HELIX 1
HELIX 2
HELIX 2.5
179 214
GPI anchor
161 200 240
VYYRPVDQYN NQNNFVHDCV NITIKQHTVT TTTKGENFTE TDIKIMERVV EQMCVTQYQK ESQAYYDGRR SSAVLFSSPP
178 181 191 197 202 217 231
HELIX 3
HELIX 4
CHO
CHO
241 254
VILLISFLIF LMVG
C-terminus signal peptide

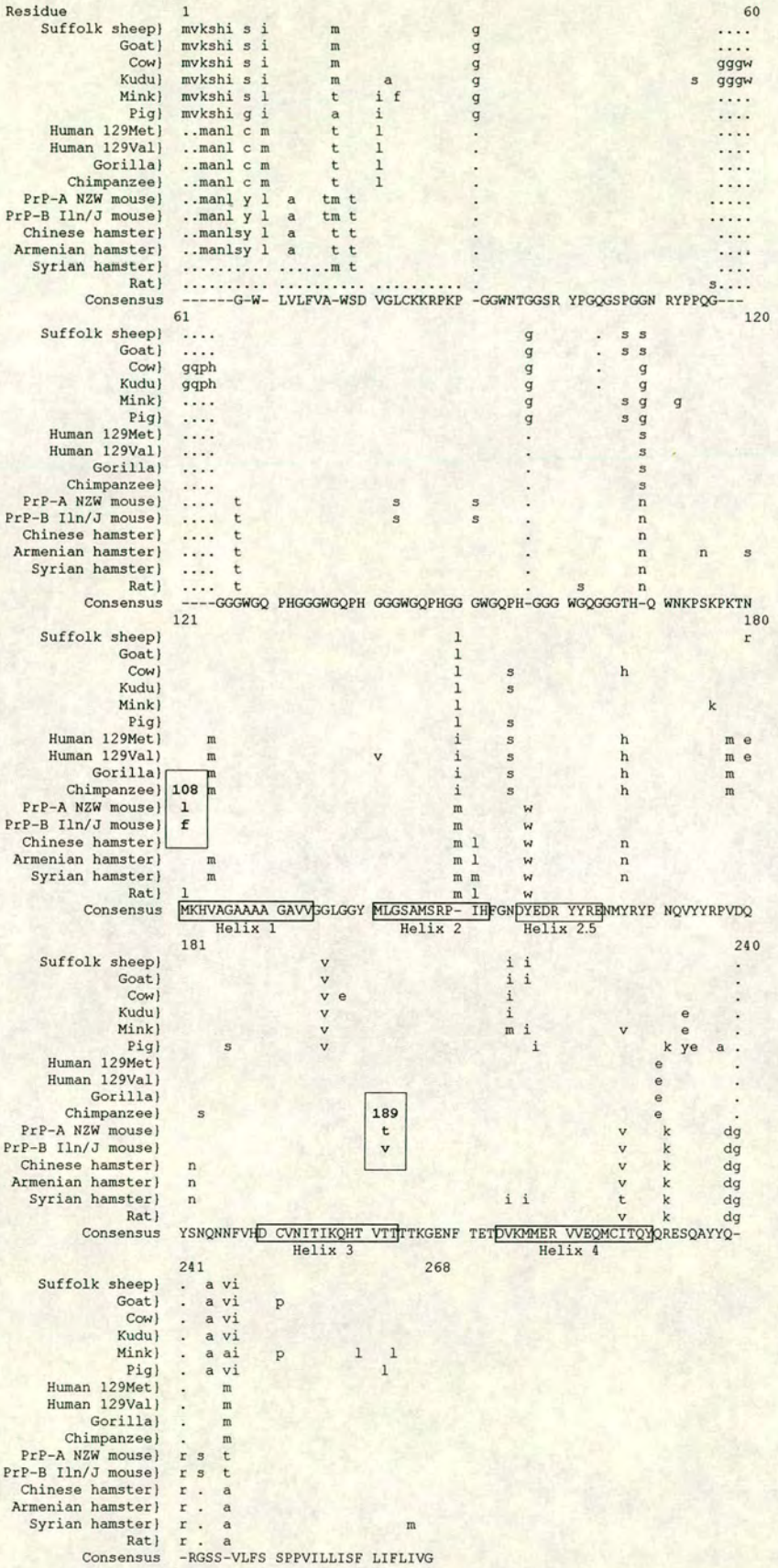
**Figure 1.3: Multiple sequence alignment of PrP amino acid sequences from a number of mammalian species.**

This illustrates the high degree of amino acid sequence conservation of mammalian PrP. Dimorphic mouse codons 108L/F and 189T/V linked to differences in experimental prion disease incubation time control (Westaway et al., 1987) are indicated in bold and are boxed. The position of putative α-helices are indicated by open boxes. Helices 2.5, 3 and 4 have been confirmed by NMR (Riek et al., 1996).

This alignment was generated by the Genetics Computer Group Wisconsin package, gcg8 using translate, pileup then pretty -con -diff (plurality: 8.00, threshold: 1.50, aveweight 1.00). The consensus sequence is indicated in upper case, divergent residues are indicated in lower case and gaps introduced to optimise the alignment are indicated by ".".

Genbank accession numbers of PrP sequences used are as follows:

| | |
|---|---|
| Suffolk sheep | M31313 |
| Goat | X74758 |
| Cow | D10613 |
| Kudu | X74771 |
| Mink | S46825 |
| Pig | L07623 |
| Human (129met allele) | M13899 |
| Gorilla | U08300 |
| Chimpanzee | U08296 |
| PrP-A NZW mouse | M18070 |
| PrP-B ILn/J mouse | M18071 |
| Chinese hamster | M33958 |
| Armenian hamster | M33959 |
| Syrian hamster | K02234 |
| Rat | M202313 |


Residue
Suffolk sheep}
Goat}
Cow}
Kudu}
Mink}
Pig}
Human 129Met}
Human 129Val}
Gorilla}
Chimpanzee}
PrP-A NZW mouse}
PrP-B Iln/J mouse}
Chinese hamster}
Armenian hamster}
Syrian hamster}
Rat}
Consensus
Helix 1
Helix 2
Helix 2.5
Helix 3
Helix 4
108
189
268

(Horiuchi et al., 1995). However, $PrP^{Sc}$ may be transported to such areas from regions expressing high levels of $PrP^{c}$ and producing large quantities of $PrP^{Sc}$. Retrograde transport of infectivity along axons has been described in experimental scrapie models (Kimberlin, Field and Walker, 1983; Fraser and Dickinson, 1985) and may be a common mechanism for the movement of infectivity within the CNS and between the CNS, PNS and peripheral organs.

**Section 1.2.5: PrP gene structure and location**

PrP is encoded by a single copy gene (Liao et al., 1986). Mouse and human PrP are encoded by the *Prn-p* and PRNP loci respectively. *Prn-p* is located on chromosome 2 (Figure 1.4, p24) and PRNP on chromosome 20pter-p12 (Sparkes et al., 1986). *Prn-p* has been mapped between *Il-1a* and *Pax-1* (Carlson et al., 1993) and lies within an area of extensive synteny with the region of human chromosome 20 bearing the human PRNP locus (Siracusa et al., 1990). Six alleles of the murine *Prn-p* gene have been described but most are similar to the $Prn\text{-}p^{a}$ allele (see Figure 1.6a, p32) which spans approximately 23kb. The first PrP gene structure to be elucidated was hamster which was shown to consist of two exons -one upstream untranslated region and one coding exon separated by a large intron (Basler et al., 1986). However, the murine *Prn-p* gene consists of three exons of which, exons 1 and 2 are untranslated, the entire coding region being limited to exon 3. The murine promoter, exons 1 and 2 are sited approximately 18 kb upstream of exon 3. Human, sheep and mouse PrP genes have a GC rich TATA -less promoter bearing consensus binding sites for AP-1, AP-2, Sp-1 and a number of undefined repeats (Westaway et al., 1994a; Herbert Baybutt, personal communication) and are similar to promoters described in so-called "house keeping" genes such as HPRT.

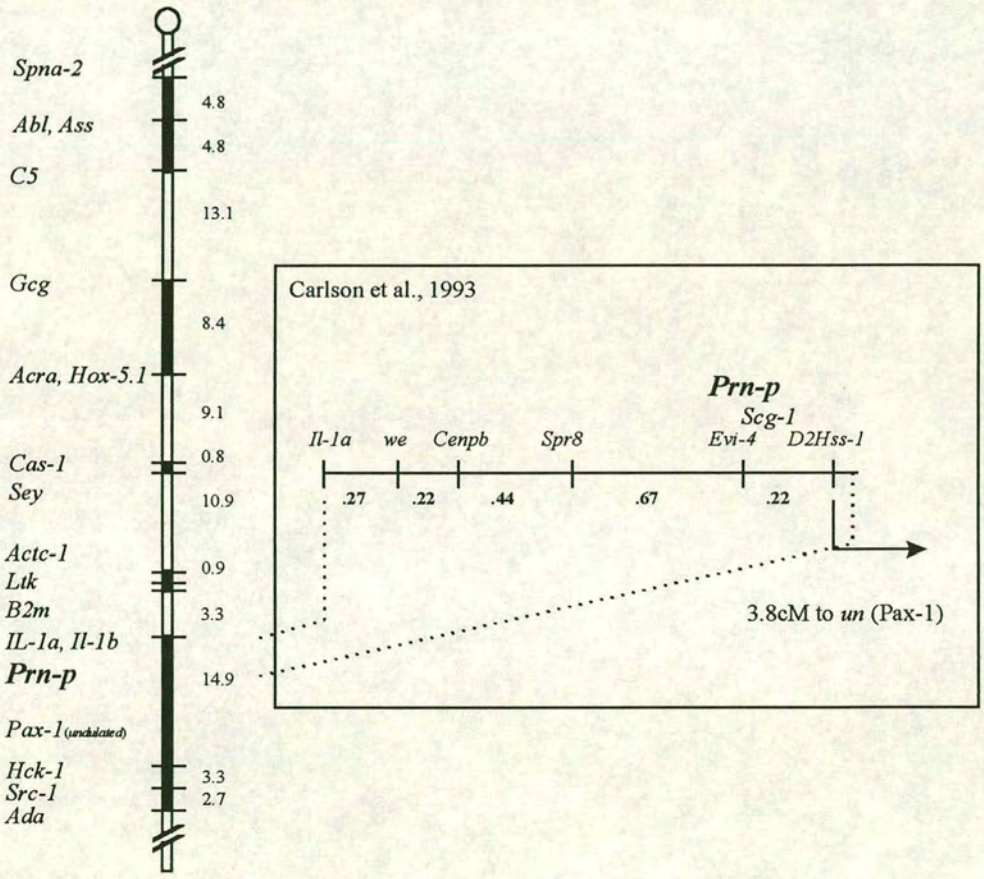
**Figure 1.4: A genetic map of mouse chromosome 2**

A genetic map of mouse chromosome 2 showing the location of the *Prn-p* locus. Areas of extensive synteny with humans shown in black and mapping distances are shown in cM (centimorgans). Mapping data was compiled from Siracusa et al., (1990) and Carlson et al., (1993).

*Note on physical mapping*: Analysis of a YAC spanning *Prn-p* indicates that *Evi-4* is ~300Kb downstream of the *Prn-p* locus (Westaway et al., 1994a).

*Gene abbreviations are as follows*:

*Spna-2*, brain α-spectrin; *Abl*, Abelson proto-oncogene; *Ass*, arginosuccinate synthetase; *C5*, complement component 5; *Gcg*, glucagon; *Acra*, muscle nicotinic acetylcholine receptor a-chain; *Hox 5-1*, homeobox gene 5.1; *Cas-1*, catalase 1; *Sey*, small eye; *Actc*, cardiac actin ; *Ltk*, leukocyte tyrosine kinase ; *B2m*, b2 microglobulin; *Il-1a/b*, interleukin 1a and b; *Prn-p*, prion protein; *D2Hss-1*, anonymous marker; *Pax-1* paired box-containing gene 1(also known as *un* -undulated); *Ada*, adenosine deaminase; *Cenpb*, centromere protein b; *we* wellhaarig; *Evi-4*, ecotrophic virus integration site-4; *Scg-1*, secretogranin 1.


Siracusa et al., 1990
Spna-2
4.8
Abl, Ass
4.8
C5
13.1
Gcg
8.4
Acra, Hox-5.1
9.1
Cas-1
0.8
Sey
10.9
Actc-1
0.9
Ltk
B2m
3.3
IL-1a, Il-1b
Prn-p
14.9
Pax-1 (undulated)
Hck-1
3.3
Src-1
2.7
Ada
Carlson et al., 1993
Prn-p
Scg-1
Il-1a
we
Cenpb
Spr8
Evi-4
D2Hss-1
.27
.22
.44
.67
.22
3.8cM to un (Pax-1)

## Section 1.3: The genetics of incubation time control

### Section 1.3.1: Introduction

Early investigation into the role of genetics in sheep demonstrated a strong genetic influence, however the cost, long generation times, extended incubation times and absence of inbred strains in these species generated the requirement for a rodent model. Following the transmission of sheep scrapie to mice in 1961 (Chandler, 1961) and work performed by Dickinson and colleagues with ME7 and other scrapie strains clearly demonstrated that susceptibility in the mouse was under genetic control (Dickinson and MacKay, 1964).

### Section 1.3.2: Experimental scrapie incubation time control in mice

Dickinson and MacKay investigated the response of a number of inbred and partially inbred mouse strains to ME7 scrapie and found that whilst the incubation time for most strains was between 22 to 25 weeks, one partially inbred strain, VM (or 5M), had a prolonged incubation time of 40 weeks (Dickinson and MacKay, 1964). This suggested that this strain was distinct from the others with respect to genes controlling scrapie incubation time. Following further inbreeding of the VM line they identified a single autosomal gene, *Sinc*, with 2 codominant alleles, s*7* and p*7* (Dickinson, Meikle and Fraser, 1968). In subsequent work with inbred mouse strains it was confirmed that *Sinc* is the major gene controlling the incubation time of mice to a wide range of scrapie isolates (Dickinson and Fraser, 1977; Bruce et al., 1991) and BSE isolates (Fraser et al., 1992; Bruce et al., 1994).

Classical genetic analysis using the Chandler scrapie strain identified a single autosomal gene *Prn-i*, with 2 alleles, N and I, which control scrapie incubation time (Carlson et al., 1986). These workers used I/LnJ (Kingsbury et al., 1983), a mouse strain with a prolonged Chandler incubation time of ~255 days homozygous for the *Prn-i* $^{I}$ allele. In contrast, NZW/LacJ mice with a shorter incubation time of ~113 days were designated as *Prn-i* $^{N}$ homozygotes. The F1 cross of these stains had a long incubation time of ~222 days suggesting that the *Prn-i* $^{I}$ allele was partially dominant.

Important clues to the identity of both *Prn-i* and *Sinc* followed the discovery of linkage of *Prn-i* and *Sinc* to PrP using a *Prn-p* Xba I RFLP (Carlson et al., 1986; Hunter et al., 1987). This Xba I RFLP defined two *Prn-p* variants *Prn-p* $^{a}$ and *Prn-p* $^{b}$ and, in a search for the molecular basis of *Prn-p* allelism, mouse strains with differing Xba I RFLPs were investigated for differences in their PrP gene sequences and expression levels (Westaway et al., 1987). Whilst no significant differences in PrP mRNA or protein levels were apparent, protein coding dimorphisms were found at codons 108 and 189 which gave rise to distinct prion proteins in mice with the *Prn-p* $^{a}$ and *Prn-p* $^{b}$ alleles and were termed the PrP A and PrP B allotypes respectively.

*Prn-p* $^{a}$ homozygous NZW mice with short Chandler scrapie strain incubation times encode the PrP A allotype with leucine and threonine at codons 108 and 189 respectively. The I/LnJ strain with a long Chandler scrapie incubation time are homozygous for the *Prn-p* $^{b}$ allele and encode phenylalanine and valine at codons 108 and 189 (the PrP B allotype).

Although there are a number of *Prn-p* RFLPs which define six alleles (Carlson et al., 1988), only mice with the *Prn-p* $^{b}$ allele encode the PrP B allotype and have long Chandler and ME7 incubation times. A BstE II RFLP provides a marker for the PrP allotype. This RFLP arises from the codon 189 threonine/valine dimorphism. A valine at this position results in loss of the BstEII site, present in mice encoding threonine and expressing the PrP A allotype (Westaway et al., 1987). To date, all mice lacking this exon 3 BstEII site encode the PrP B allotype. Only three mouse strains are known to encode the *Prn-p* $^{b}$ allele and express the PrP B allotype: VM/Dk, ILn/J and P/J. All have long Chandler and/or ME7 incubation times.

### Section 1.3.3: The relationship between *Prn-p* and *Sinc/Prn-i*

Whilst it is likely that *Sinc* and *Prn-i* are identical loci, much effort has been directed towards determining whether the *Prn* complex includes two distinct genes (i.e. *Prn-p* and *Prn-i/Sinc*) or whether *Prn-p* and *Prn-i/Sinc* are in fact the same gene. *Sinc/Prn-i* and *Prn-p* are closely linked (Carlson et al., 1986; Carlson et al., 1988) and in a study of mice with meiotic crossovers near *Prn-p* (Carlson et al., 1993) the

location of *Prn-i* was mapped to between 0.67cM proximal and 0.22cM distal to *Prn-p*.

However, the unambiguous delineation of the relationship between these genes is lacking, not least because tight linkage between *Prn-p* and an incubation time locus only demonstrates a correlative rather than a causal relationship but also because four independent test crosses have yielded small numbers of progeny with discordant PrP genotype and scrapie incubation time phenotype (Carlson et al., 1986; Carlson et al., 1988; Race et al.,1990). In these experiments, *Prn-p* $^{a}$ and *Prn-p* $^{b}$ mice were crossed and the F1 progeny were backcrossed onto a *Prn-p* $^{a}$ strain. In a number of experiments, four F2 mice with a *Prn-p* Xba I RFLP characteristic of the *Prn-p* $^{a/b}$ genotype were observed to have Chandler incubation times characteristic of the *Prn-p* $^{a}$ genotype. These observations could indicate recombination between *Prn-p* and a closely linked (estimated at 4.3+/-1.3 (S.E) cM, Carlson et al., 1993) incubation time control locus. A convincing demonstration of recombination between *Sinc/Prn-i* and *Prn-p* would be highly significant as it would demonstrate that incubation times for murine scrapie, and perhaps other prion diseases, are controlled by a tightly linked but distinct locus from that encoding the prion protein. The lethal nature of the backcrossing experiments has frustrated attempts to determine whether discordant mice are true meiotic recombinants or whether the discordant incubation times are simply due to the reproducible co-segregation of other genes which, in combination, have a large effect upon incubation time.

A different approach to the delineation of the relationship between *Sinc/Prn-i* and *Prn-p* utilised congenic VM/Dk and VM/Dk-*Sinc*$^{s7s7}$ mouse lines (Figure 1.5a, p30). These VM/Dk based mouse lines encode distinct *Sinc* alleles, distinct *Prn-p* alleles, distinct PrP allotypes and have dramatically different scrapie incubation times (Hunter et al., 1987; Hunter et al., 1992). These mice demonstrated the co-transfer of the *Prn-p* $^{a}$ allele and the *Sinc* s7 allele onto the VM/Dk background. However, this cannot provide formal proof for the congruence between *Sinc/Prn-i* and *Prn-p* because these experiments were performed on congenic mice at the 19$^{th}$ backcross. The size of the differential chromosomal segment at this stage of the construction of

any congenic line is around 10cM[13], or roughly 10Mb. Thus the incubation time alteration generated by the transfer of the *Sinc* s7 allele could be due to the effects of a linked gene. Therefore it remains formally possible that *Sinc* and *Prn-p* could still be linked but discrete genes.

Westaway, using a transgenic approach (Figure 1.5b, p30) constructed mice in which a 40Kb cosmid *Prn-p* $^{b}$ transgene from ILn/J mice had been introduced to wild type mice (Westaway et al., 1991). Thus the transgenic mice had two copies of the endogenous *Prn-p* $^{a}$ allele in addition to the *Prn-p* $^{b}$ transgene. The rationale of this approach was based on the observation that in F1 crosses between NZW and ILn/J, *Prn-p* $^{a/b}$ heterozygous mice encoding both *Prn-p* $^{a}$ and *Prn-p* $^{b}$ alleles had Chandler scrapie incubation times almost as long as *Prn-p* $^{b}$ ILn/J mice. This indicated that the *Prn-p* $^{b}$ allele was dominant. Thus if the *Prn-p* $^{b}$ allele was the determinant of long incubation times in ILn/J mice then adding a *Prn-p* $^{b}$ allele onto a *Prn-p* $^{a}$ short incubation time should exert its dominant action by prolonging the scrapie incubation time. Carrying this argument one step further one might imagine that the higher the Tg[*Prn-p* $^{b}$] copy number and the greater the *Prn-p* $^{b}$ (i.e. PrP B) expression level, the longer the incubation time. However the transgenic mice had *accelerated* rather than prolonged Chandler scrapie incubation times.

A number of other transgenic mouse lines with multiple copies of cosmid *Prn-p* transgenes overexpressing PrP have accelerated scrapie incubation times. These include mice overexpressing Syrian hamster PrP (Scott et al., 1989; Prusiner et al., 1990) and the mice overexpressing the PrP A allotype from a *Prn-p* $^{a}$ transgene (Fischer et al., 1996; Telling et al., 1996). This suggests that accelerated Chandler scrapie incubation times are an artefact of PrP overexpression (Carlson et al., 1994).

[13] the size of the differential chromosomal segment in congenic lines is theoretically 200/t cM, where t is the number of backcrosses (Falconer, 1989).

**Figure 1.5: Investigation of the relationship between *Prn-p* and *Sinc/Prn-i*.**

Two experiments exploring the relationship between *Sinc/Prn-i* and *Prn-p* are depicted. *Sinc* congenics **a**). were constructed by repeatedly backcrossing F1[C57xVM/Dk] $Sinc^{s7p7}$ progeny onto VM/DK $Sinc^{p7p7}$ with selection for short incubation times at each of 19 backcross generations (Bruce et al., 1991). *Sinc* congenics enable the comparison of VM/Dk congenic lines in which the genetic variation is limited to the *Sinc* locus. However, after 19 backcross generations the *Sinc* s7 allele from the C57 donor strain carries a ~10cM chromosomal segment bearing multiple linked genes. *Prn-p* Xba I RFLP analysis of these mice (Hunter et al., 1992) demonstrated the co-transfer of the $Prn\text{-}p^{a}$ allele and rapid ME7 incubation times of ~175 days in VM/Dk-$Sinc^{s7s7}$ mice, close to that of C57 mice bearing the *Sinc* s7 allele (~167 days), however this cannot exclude the possibility that *Sinc* and *Prn-p* are distinct but tightly linked loci -they could be as much as much as 10cM distant. A transgenic approach **b**) in which the $Prn\text{-}p^{b}$ allele was introduced onto a $Prn\text{-}p^{a}$ background (Westaway et al., 1991). The dominance of the $Prn\text{-}p^{b}$ allele with Chandler scrapie was expected to result in prolonged incubation times in Tg[$Prn\text{-}p^{b}$] mice, however the transgene actually accelerated Chandler scrapie incubation times (Table 1.3, below). The accelerated incubation time in Tg[$Prn\text{-}p^{b}$] mice may have resulted from selection for a rapidly replicating component from the (uncloned) Chandler isolate[14] (Bruce and Dickinson, 1987), however serial passage from Tg [93 H] in CD-1 mice indicated that the properties of the inoculum were unaltered. This indicates that the accelerated incubation times were the result of raised $PrP^{c}$ expression levels.

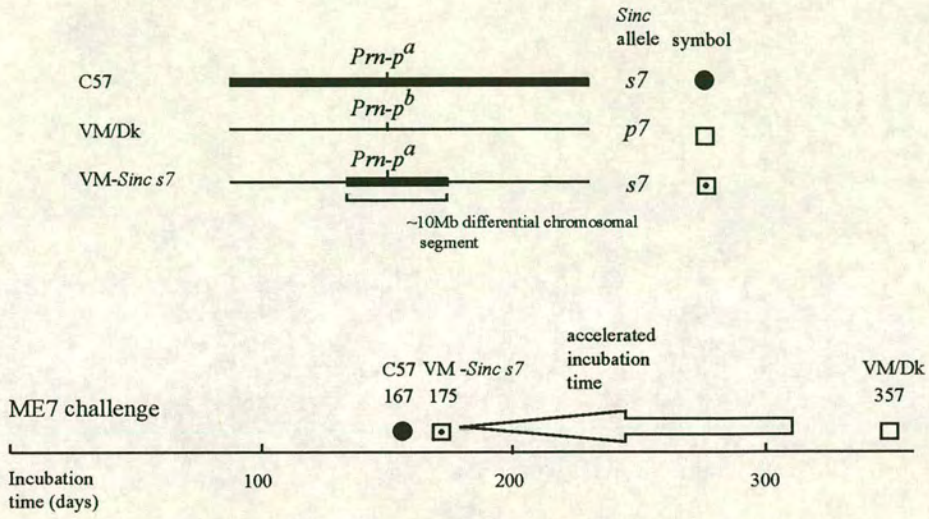
**Table 1.3: Raised expression levels of $Prn\text{-}p^{b}$ accelerate Chandler incubation times.** [Adapted from Westaway et al., 1991]

| Transgenic line | Incubation time[a] (+/-SE days) | $[PrP^{c}]^{b}$ |
|---|---|---|
| Tg[94] | 75 (+/-1.8, n=15) | 8 x |
| Tg [117] | 78.5 (+/-1.9, n=13) | 8 x |
| Tg [93H] | 79.4 (+/-2.9, n=14) | ND |
| Tg [93L] | 96.7 (+/-3.0, n=12) | 2-4 x |
| Non Tg littermates | 125.3-137 (n=61) | 1 x |

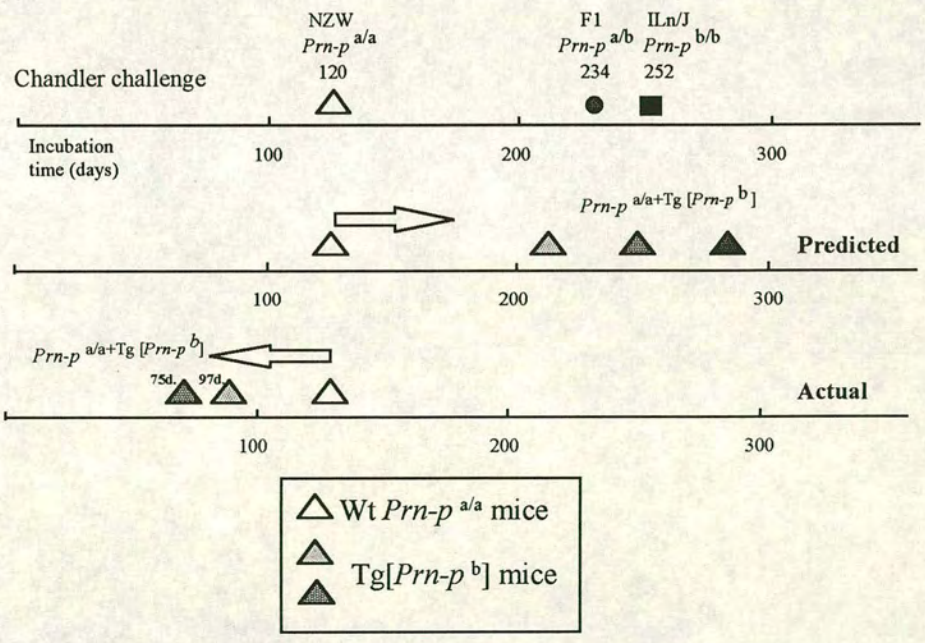
a, incubation time to onset of clinical signs of scrapie;
b, whole brain $PrP^{c}$ expression levels were compared to wildtype mice;
ND, not determined

[14] Bruce et al., (1991) indicated that the Chandler isolate used for a number of incubation time control experiments is a mixture of 139A and 79A and may lead to confusing incubation period results.

**a).**


Sinc allele symbol
Prn-p a
C57
s7
Prn-p b
VM/Dk
p7
Prn-p a
VM-Sinc s7
s7
~10Mb differential chromosomal segment
accelerated incubation time
C57 VM -Sinc s7
167 175
VM/Dk
357
ME7 challenge
Incubation time (days)
100
200
300

**b).**


NZW
Prn-p a/a
120
F1
Prn-p a/b
234
ILn/J
Prn-p b/b
252
Chandler challenge
Incubation time (days)
100
200
300
Prn-p a/a+Tg [Prn-p b]
Predicted
Prn-p a/a+Tg [Prn-p b]
75d.
97d.
Actual
Wt Prn-p a/a mice
Tg[Prn-p b] mice

## Figure 1.6: *Prn-p* polymorphisms

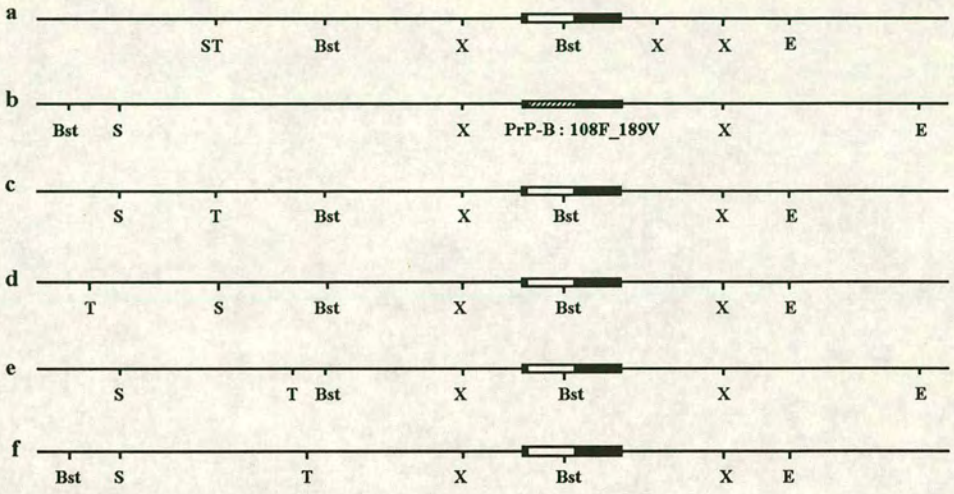
Six alleles of *Prn-p* (*a-f*) have been described on the basis of Xba I, BstE II, Eco RI, TaqI and Sac I restriction fragment length polymorphisms **a)** (Carlson et al., 1988). Xba I RFLPs were used to detect *Prn-p* [a] and *Prn-p* [b] alleles in early linkage experiments (Carlson et al., 1986) but only the short fragment of 3.5Kb is specific for the *Prn-p* [a] allele because a number of mouse strains with both long and short Chandler scrapie incubation times share the larger 5.2 Kb Xba I fragment. The only RFLP which consistently differentiates mice with long and short Chandler and ME7 scrapie incubation times is a BstE II RFLP arising from an exon 3 dimorphism at codon 189. Indeed, the only allele conferring long incubation times with Chandler and ME7 scrapie is the *Prn-p* [b] allele - mice with the other *Prn-p* alleles (*a*, *c*, *d*, *e* and *f*) have short Chandler incubation times (Table 1.4, below; Carlson et al., 1988) and all encode the PrP A allotype. Restriction enzyme abbreviations: Bst, Bst E II; E, Eco RI; S, Sac I; T, Taq I; X, XbaI. **b**). Mice with different incubation times encode distinct prion proteins (Westaway et al., 1987). Codon 108 L/F and 189T/V dimorphisms define two distinct allotypes PrP-A and PrP-B encoding different PrP molecules: PrP 108L_189T and PrP 108F_189V. Despite the RFLPs described within the *Prn-p* locus (Carlson et al., 1988; Figure 1.6a, p32), the only consistent difference between mice with large differences in scrapie incubation times at the outset of this project were these codon 108 and 189 dimorphisms. During the course of this project two variant 3'UTR polymorphisms and some promoter differences have been described between *Sinc s7* and *p7* mice (Baybut and Manson, 1997) have been described.

### Table 1.4: Chandler scrapie incubation times in inbred mouse strains encoding different *Prn-p* alleles (adapted from Carlson et al., 1988).

| Mouse strain | *Prn-p* allele | PrP allotype | Incubation time[a] (days) | SE | n |
|---|---|---|---|---|---|
| C57BL/6BJ | a | A | 143 | 4 | 21 |
| NZW/LacJ | a | A | 120 | 2 | 20 |
| **ILn/J** | **b** | **B** | **322** | 15 | 11 |
| P/J | b | B | 295[b] | 9 | 21 |
| RIIIS/J | c | A | 141 | 3 | 4 |
| MA/MyJ | d | A | 190 | 5 | 6 |
| CAST/Ei | e | A | 216 | - | 1 |
| MOLF/Ei | f | A | 164 | 1 | 7 |

a, incubation time until death; b, incubation time until first diagnosis of scrapie
SE, standard error of the mean; n, number of mice.

**a).**




**b).**

```
          1                                                    50
PrP-B  }
PrP-A  }  MANLGYWLLA LFVTMWTDVG LCKKRPKPGG WNTGGSRYPG QGSPGGNRYP

          51                                                   100
PrP-B  }
PrP-A  }  PQGGTWGQPH GGGWGQPHGG SWGQPHGGSW GQPHGGGWGQ GGGTHNQWNK

          101   108                                            150
PrP-B  }         F
PrP-A  }  PSKPKTNLKH VAGAAAAGAV VGGLGGYMLG SAMSRPMIHF GNDWEDRYYR

          151                                      189         200
PrP-B  }                                           V
PrP-A  }  ENMYRYPNQV YYRPVDQYSN QNNFVHDCVN ITIKQHTVTT TTKGENFTET

          201                                                  250
PrP-B  }
PrP-A  }  DVKMMERVVE QMCVTQYQKE SQAYYDGRRS SSTVLFSSPP VILLISFLIF


          251
PrP-B  }
PrP-A  }  LIVG.
```

**Section 1.3.4: Gene targeting**

These difficulties suggested that the issue of *Sinc*, *Prn-i*, *Prn-p* identity might only be resolved with gene targeting -a technology which can control for both the effect of other genes (by constructing co-isogenic lines) and PrP gene dosage effects.

It was reasoned that dramatic alterations to the incubation time of mice in which the endogenous *Prn-p* [a] allele, expressing the PrP A allotype, had been converted to the PrP B allotype would constitute a definitive test of the proposed congruence of *Sinc*, *Prn-i* and *Prn-p*. Non-*Prn-p* genes have been implicated as significant modulators of incubation times and neuropathology (Bruce and Dickinson, 1985; Carlson et al., 1988; Race et al., 1990; Carlson et al., 1994), therefore genetic variation was controlled by generating co-isogenic 129/Ola mice differing only by targeted codon 108 and 189 alterations (Moore et al., 1995). Gene targeting is discussed in detail in the next section.

**Section 1.4 Gene targeting**
**Section 1.4.1: Introduction**

Gene targeting enables the generation of mice with defined genomic manipulations (reviewed by Moore and Melton, 1997). Much progress has been made within this field in recent years. Formerly it was possible only to delete genes, however a growing number of targeted alterations are now possible -although technically demanding. Such alterations include conditional gene deletion (Gu et al., 1993), subtle alterations such a single base pair changes (Moore et al., 1995) and targeted chromosomal translocations (Smith et al., 1995).

Embryonic stem (ES) cells are derived from the inner cell mass of 3.5 day blastocysts and give rise to the embryo proper (Balinski, 1975). ES cells are pluripotent and can proliferate *in vitro* in an undifferentiated state under the appropriate culture conditions (Martin, 1981; Evans and Kaufman, 1981). Early experiments demonstrated that ES cells could contribute to the germline tissue of chimaeras (Bradley et al., 1984) and that marker genes could be introduced into ES cells and transmitted to the mouse germline via chimeras (Robertson et al., 1986; Gossler et al., 1986). Genetic manipulation of ES cells *in vitro* by gene targeting

makes it possible to transmit a wide range of targeted alterations to the germline of mice (Figure 1.7, p37).

**Section 1.4.2: Early gene targeting**

The first application of gene targeting, as applied to mammalian chromosomal loci, utilised synthetic chromosomal markers providing selectable phenotypes in targeted clones (Lin et al., 1985; Thomas, Folger and Capecchi, 1986). The human β-globin locus was the first endogenous mammalian locus to be targeted (Smithies et al., 1985). It was initially thought that only transcriptionally active genes might be available for homologous recombination -a phenomenon which would severely limit the general utility of gene targeting, especially as applied to ES cells. However, similar targeting frequencies were noted at the β-globin locus in a cell type in which this gene was not expressed.

When DNA is introduced into cells it has a number of fates including nucleolytic degradation, random integration and homologous recombination with chromosomal DNA (for a review see Bollag, Waldman and Liskay, 1989). The main constraint upon the success of gene targeting experiments in higher eukaryotes is the marked preference for random integration of targeting vectors rather than homologous recombination. The ratio of random integration to homologous recombination events in mouse ES cells has been estimated at 1000:1 (Thomas and Capecchi, 1987). To maximise the chances of identification and retrieval of targeted clones, gene targeting is performed with large numbers of cells and selection strategies which enrich for rare homologous recombination events. The main parameters governing targeting efficiency were determined by a series of experiments at the HPRT locus. This locus was well suited for gene targeting experiments as it is possible to select both *for* (with HAT: Szybalska and Szybalski, 1962) and *against* (with 6-thioguanine) HPRT function *in vitro*. Selection of cells for gain or loss of HPRT function is simplified because this gene is X-linked and is therefore hemizygous in male ES cells, thus only one allele need be inactivated to yield a selectable phenotype.

The influence of the length of vector homology has been investigated by a number of workers and the frequency of homologous recombination has been found to be proportional to the length of homology between the vector and target locus (Thomas and Capecchi, 1987; Hasty et al., 1991; Deng and Capecchi, 1992). Most vectors have between 5 and 10 kb of homology and bear a short and a long arm of homology. The short arm facilitates PCR detection of the targeting specific juxtaposition of vector sequence with the target locus (Doetschman, Maeda and Smithies, 1988).

Vectors constructed with regions of homology from a source isogenic with the cell type being targeted provide higher gene targeting frequencies (teReile et al., 1992). The importance of isogenic targeting vectors is now widely acknowledged, but a number of comparisons between the relative efficiencies of insertion and replacement vectors have been reported which do not control for this effect. However a direct comparison has been made at the α2 Na,K-ATPase locus by Askew et al., (1993) and suggests that at this locus insertion vectors have moderately higher targeting frequencies. Targeting vector design is discussed in detail in Hasty and Bradley (1993).

**Section 1.4.3: Gene targeting strategies and selection schemes**

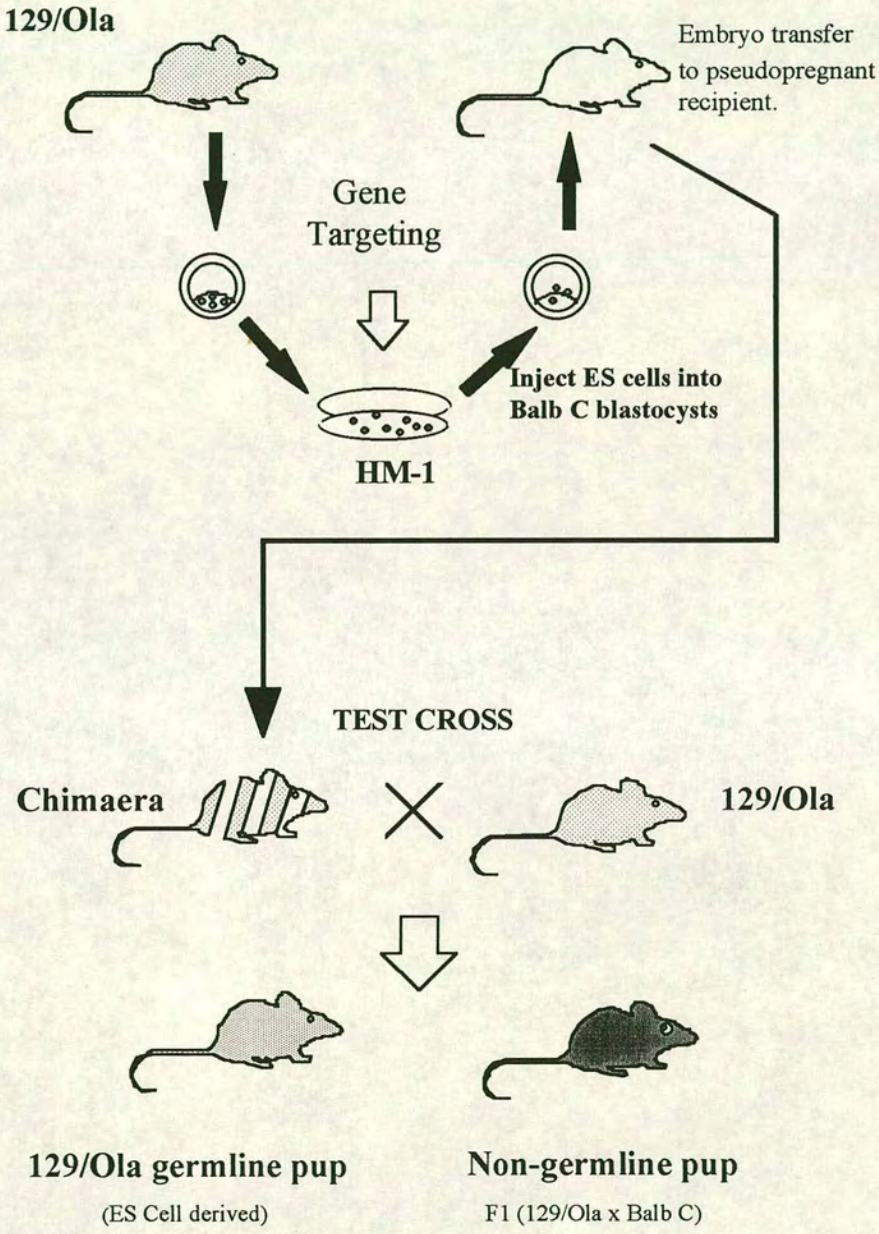
The extension of gene targeting to non-selectable genes required the development of dominant selectable markers and efficient selection schemes. The most widely used selection scheme is the positive-negative strategy (Mansour, Thomas and Capecchi, 1988). Since both the transfection frequency and targeting frequency of ES cells are relatively is low, a positive selection scheme is required to select for cells which have integrated the targeting vector. The most common positive selectable marker in use is the bacterial neomycin phosphotransferase gene (neo) and is used in combination with G418. An HPRT minigene in combination with, HAT and HPRT deficient cells has also been used successfully (Reid et al., 1990; Selfridge et al., 1992; Stacey et al., 1994; Moore et al., 1995). Positive selection schemes select for stable neo transfectants and in practice this means both cells which have

**Figure 1.7: Generation of mice from gene targeted embryonic stem cells**

Most mouse ES lines are derived from 129 substrains. ES cells can be genetically modified *in vitro* then introduced into blastocysts and, following embryo transfer and successful implantation, can generate germline chimeras. Coat and eye colour markers aid the identification of ES cell contribution to chimeras and to germline pups.

Only 50% of germline pups carry targeted alleles because ES cells are usually heterozygous for targeted alterations. Germline transmission of 129/Ola HM-1-ES cell derived gametes is determined by the transmission of 129/Ola coat and eye colour markers (Selfridge et al., 1992): 129/Ola are homozygous for the white bellied agouti allele ($A^w$) and the chinchilla allele ($c^{ch}$) at the albino locus which results in a light brown coat colour. 129/Ola are also homozygous for the recessive allele, pink-eyed dilute ($p$) and have pink eyes.

When used in conjunction with recipient blastocysts which differ from 129/Ola at these loci, it is possible to determine the contribution of ES derived tissue in chimeras. For example, introduction of 129/Ola ES cells into Balb C recipient blastocysts (Balb C mice have a white coat and pink eyes: *c/c*, *P/P*, *A/A*) results in white mice (blastocyst coat colour) and chimeric, 129/Ola derived tisue, shows up against this coat colour as light brown. Following test crosses between chimeras and inbred 129/Ola mice the transmission of ES gametes from germline chimeras is indicated by a chinchilla coat and pink eyes in progeny. Non germline pups have a grey coat and black eyes as they are F1(Balb C x 129/Ola) i.e. they are $c^{ch}/c$, $p/P$, $A^w/A$.


129/Ola
Embryo transfer
to pseudopregnant
recipient.
Gene
Targeting
Inject ES cells into
Balb C blastocysts
HM-1
TEST CROSS
Chimaera
129/Ola
129/Ola germline pup
(ES Cell derived)
Non-germline pup
F1 (129/Ola x Balb C)

undergone the desired gene targeting events and those which have undergone random integration events. As described previously, mammalian cells have a predilection for random integration and it is necessary to use a negative selection step to enrich for homologous recombinants. Selection against non-homologous recombinants with replacement vectors is facilitated by the introduction of negative selectable markers at the termini of regions of homology. Common negative selectable markers include the herpes simplex virus thymidine kinase gene (HSV-TK) and the diptheria toxin A fragment gene (DT-A). Sequence insertion vectors cannot utilise such selectable markers for simple knockouts because all of the vector integrates into the target locus (although they can be used for the run stage of hit and run). However, positive selection markers can be designed to enrich for targeting events by modifying the positive selection marker for expression which may be conditional upon introduction into a transcriptional unit e.g. by using a neo module which is promoterless, poly A-less or contains a splice acceptor site such that neo is expressed as a fusion protein. The main differences between insertion and replacement vectors as used for gene knockouts are illustrated in Figure 1.8 (p40).

### Section 1.4.4: Generating ES cells with subtle alterations

#### Section 1.4.4.1:Introduction

A large number of genes have been inactivated by gene targeting - indeed more than 250 have been reported in the past 6 years. This has enabled the generation of a number of mouse models of human disease. However many disease-causing mutations do not arise from complete loss of function mutations but are caused instead by gain-of-function mutations resulting from subtle alterations such as point mutations, small deletions and repeat amplifications. Thus a major challenge is to develop methods to enable the introduction of such alterations. These procedures are technically very difficult and require prolonged *in-vitro* culture of ES cells -reducing their germ-line potential. There are two main approaches, again utilising sequence insertion (Hasty et al., 1991; Figure 1.9, p42) and sequence replacement vectors

## Figure 1.8: Insertion and replacement vectors for gene knockout

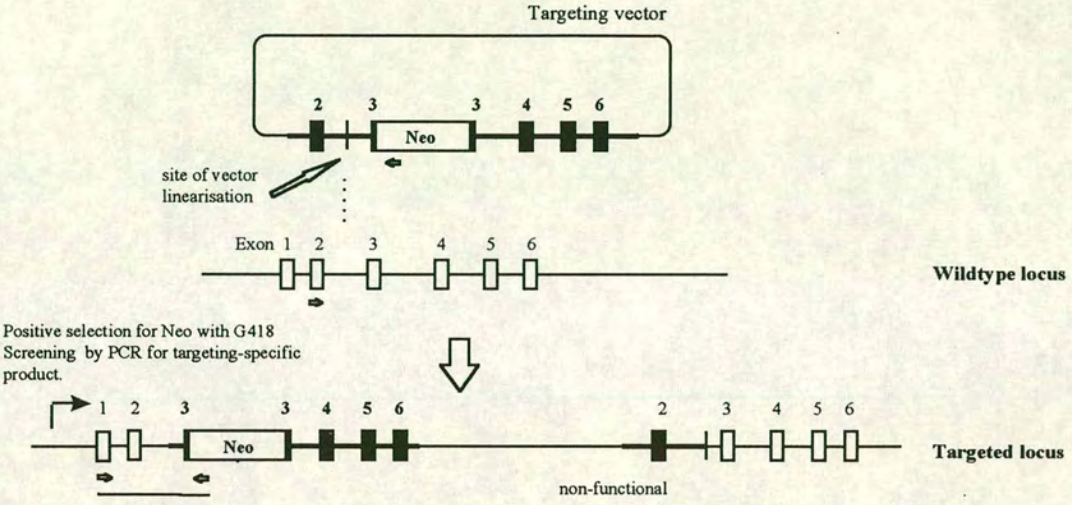
Differences in the design of insertion and replacement vectors for the inactivation of a hypothetical gene by the introduction of neo into exon 3. Targeting vectors consist of 5′ and 3′ flanking sequence homologous with the target locus and a selectable marker gene (e.g. bacterial neomycin phosphotransferase). The neo marker and has the dual role of providing insertional mutagenesis of an essential exon within the target gene and functions as a positive selectable marker for stable transfectants. The main difference between **a) insertion** vectors and **b) replacement** vectors is the site of vector linearisation as this determines the final structure of the targeted allele.

**Insertion vectors a)** are linearised within the region of flanking homology and undergo a single reciprocal recombination event with the target locus, stimulated by a double strand break or gap in the vector homology. The entire vector is integrated into the target locus resulting in duplication of sequence either side of the selectable marker. The degree of enrichment for homologous recombinants is low because there is only positive selection for stable transfectants - which are predominately random integrants[15]. Negative selection modules such as HSV-TK are not possible as they will appear in the targeted locus.

**Replacement vectors b)** integrate following a double reciprocal recombination event resulting in the complete replacement of a region of the target locus with vector sequence. This has a number of advantages, most notably that negative selection against non-homologous recombinants can be used to enrich for homologous recombinants. This is achieved by placing a negative-selectable marker outside the region of vector homology with the target gene. The rationale for this is that regions at the vector termini which are not homologous to the target gene are excluded from the final structure of the targeted locus. Thus negative selectable markers are expressed only transiently, if at all. Conversely, it is likely that a majority of random integrants will be stably transfected with the whole vector and express the negative selectable marker and will not survive counterselection. HSV-TK in combination with the toxic viral thymidine kinase substrate gancyclovir is a popular negative selectable marker. The knockout designs shown here have a number of design flaws: i) in general a more secure null phenotype is observed when the promoter, first exon and first intron are disrupted or deleted, ii) the knockout allele in **a)** and **b)** could still generate a partially functional gene product by splicing around the neo module or by reading through neo to produce a fusion protein -perhaps with novel biological properties.

[15] Good enrichment for homologous recombinants is possible by utilisation of a promoterless or polyA-less neo module but such methods are generally limited to genes expressed in ES cells.

**a).**


Targeting vector
2 3 3 4 5 6
Neo
site of vector linearisation
Exon 1 2 3 4 5 6
Wildtype locus
Positive selection for Neo with G418
Screening by PCR for targeting-specific product.
1 2 3 3 4 5 6
Neo
2 3 4 5 6
Targeted locus
non-functional

**b).**

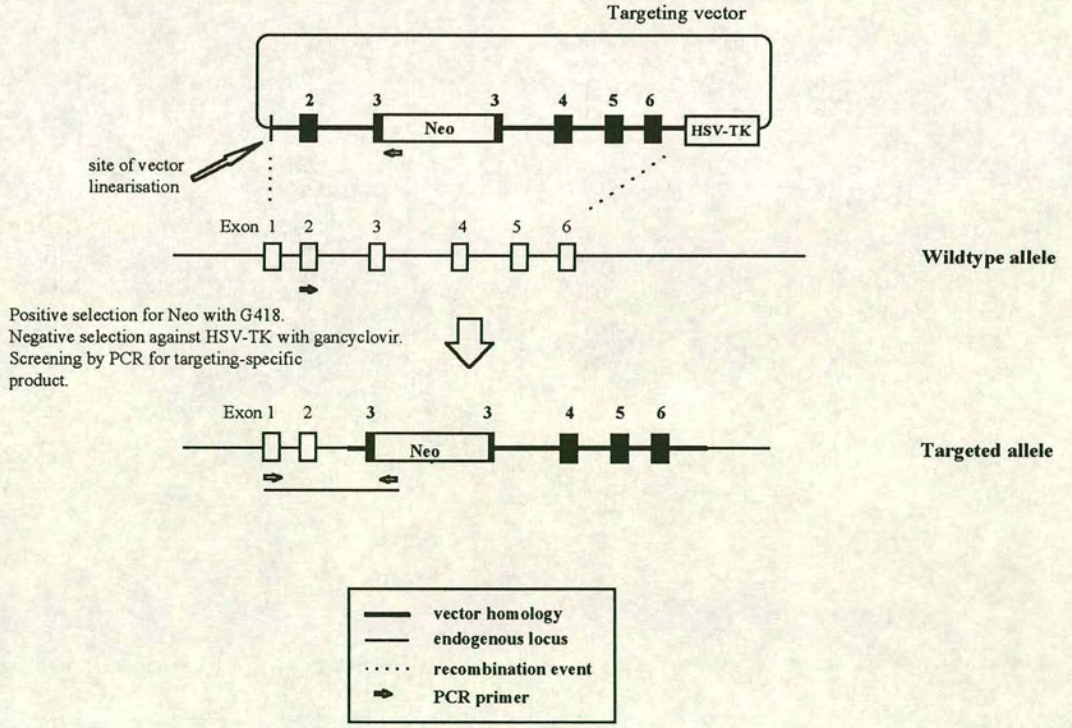
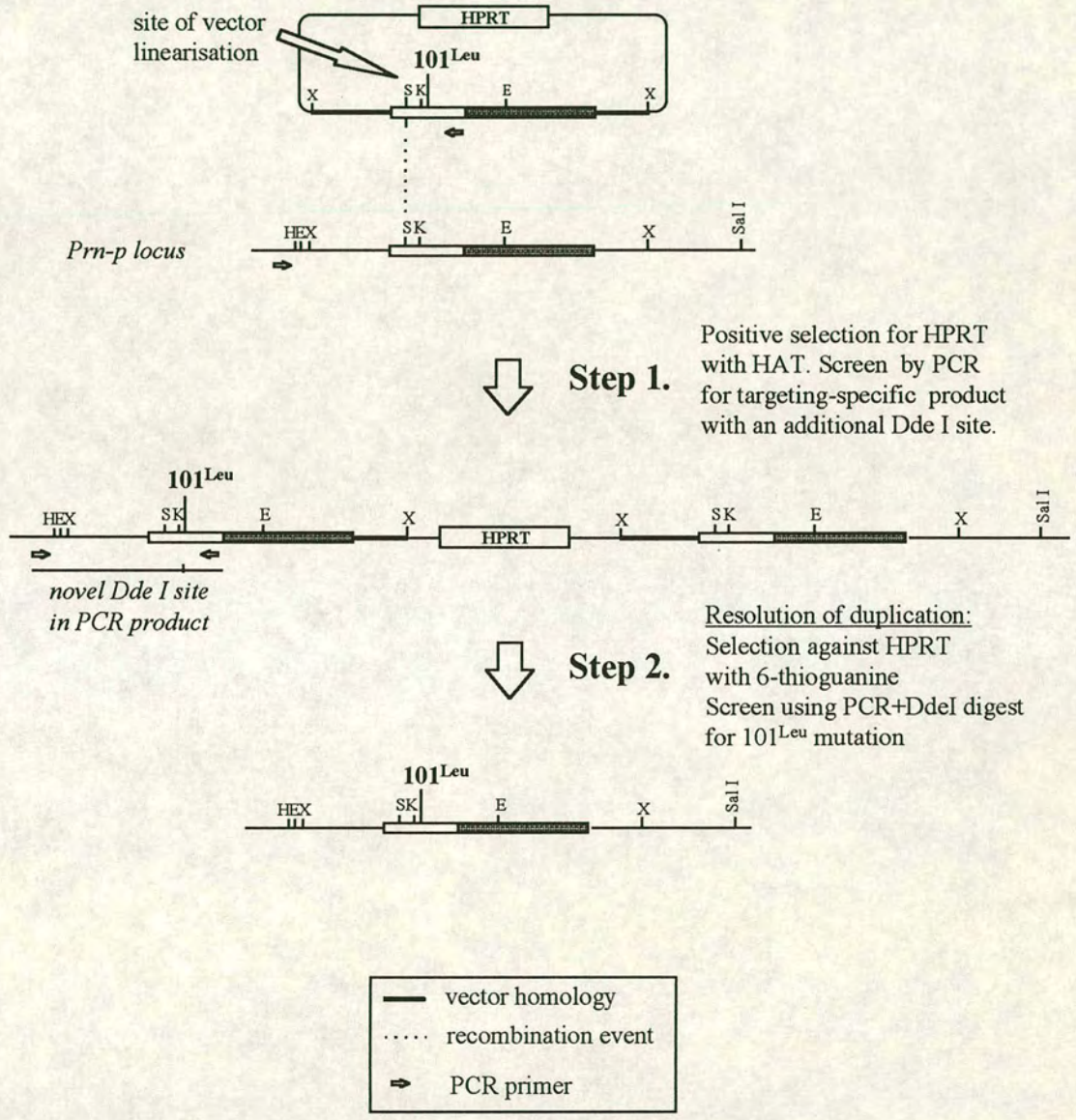

Targeting vector
2 3 3 4 5 6
Neo
HSV-TK
site of vector linearisation
Exon 1 2 3 4 5 6
Wildtype allele
Positive selection for Neo with G418.
Negative selection against HSV-TK with gancyclovir.
Screening by PCR for targeting-specific product.
Exon 1 2 3 3 4 5 6
Neo
Targeted allele
vector homology
endogenous locus
recombination event
PCR primer

**Figure 1.9: Introducing subtle alterations with sequence insertion vectors**

The general method for the introduction of subtle alterations is illustrated by an early attempt to introduce a codon 101 proline to leucine alteration into *Prn-p* exon 3 in HM-1 ES cells (David Melton, unpublished results). Targeting vector homologous sequence is indicated by heavy lines and filled boxes. The site of vector linearisation is indicated by an open arrow. This requires two steps and a positive-negative selection and is known as a "hit and run" strategy (Mansour et al., 1988).

In the first step (the "hit"), an insertion vector is stably integrated resulting in a duplication of exon 3. Targeted ES cells are detected by a PCR screen of cells surviving HAT selection. In the second step (the "run") maintenance of the cells in 6-thioguanine (a toxic HPRT substrate) provides selection against HPRT and enriches for ES cells which have lost the HPRT minigene by resolution of the gene duplication -leaving a single copy gene which bears the desired mutation. In principle ~50% of intrachromosomal recombinants will have retained the 101Leu mutation, however in practice all recombinant clones screened in several experiments had lost the 101Leu mutation. This has been attributed to the proximity of the site of linearisation with the $101^{Leu}$ mutation. Two linearisation sites were examined: Sma I (175bp from mutation) and Kpn I (21bp from the mutation).

This and reports from other workers (Hasty and Bradley, 1993) suggests that the frequency with which the mutation is retained is strongly determined by the distance between the site of linearisation and the site of the mutation -mutations less than 1.5Kb from the site of linearisation are frequently lost. This constitutes a major limitation of sequence insertion vectors -although there have been a limited number of successes. The difficulties experienced with this system led to the adoption of a two step double replacement strategy (Moore et al., 1995).


site of vector
linearisation
HPRT
101Leu
X
SK
E
X
Prn-p locus
HEX
SK
E
X
Sal I
Step 1.
Positive selection for HPRT
with HAT. Screen by PCR
for targeting-specific product
with an additional Dde I site.
101Leu
HEX
SK
E
X
HPRT
X
SK
E
X
Sal I
novel Dde I site
in PCR product
Step 2.
Resolution of duplication:
Selection against HPRT
with 6-thioguanine
Screen using PCR+DdeI digest
for 101Leu mutation
101Leu
HEX
SK
E
X
Sal I
vector homology
recombination event
PCR primer

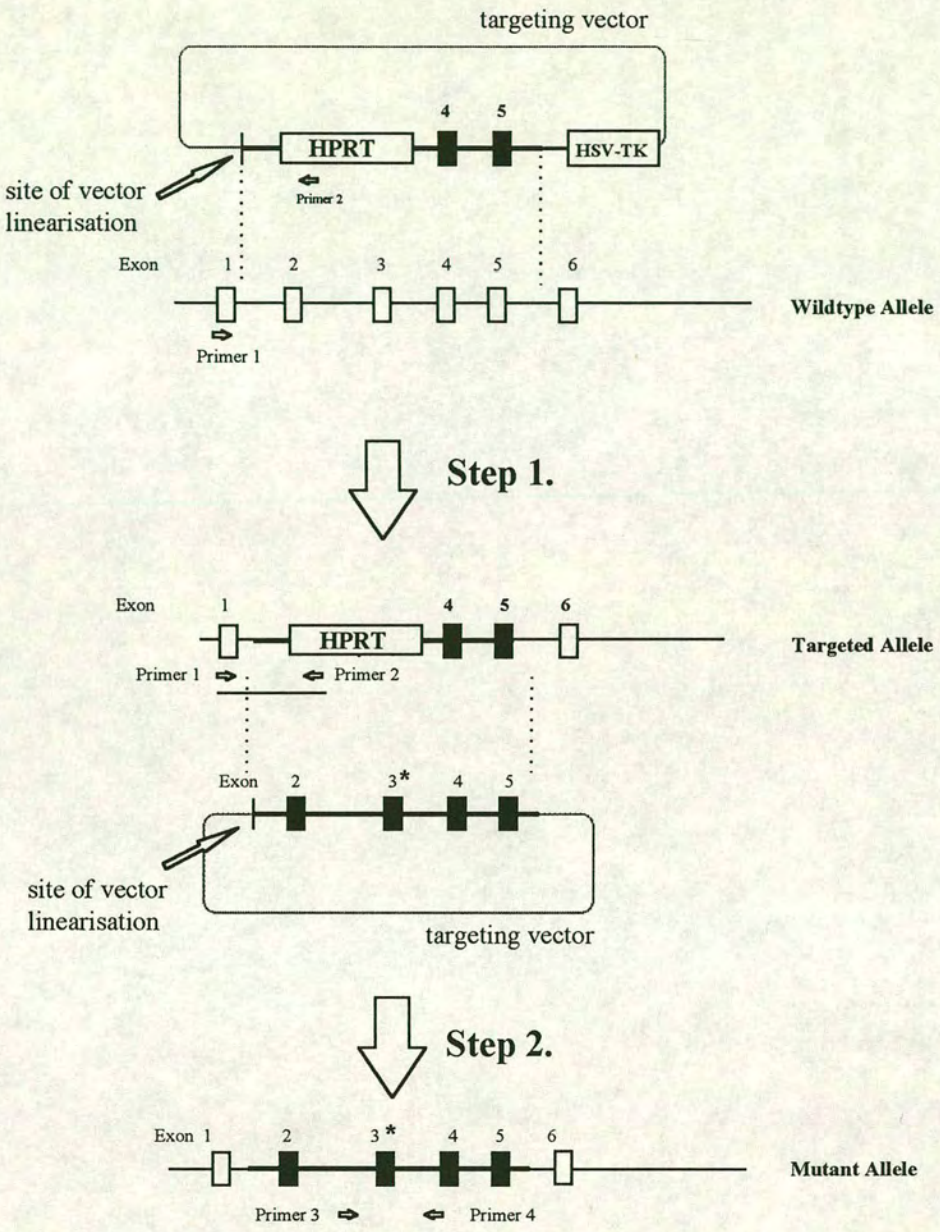
## Figure 1.10: Introduction of subtle alterations with double replacement gene targeting

A two step double replacement strategy for the introduction of a subtle alteration into a hypothetical gene. This method was first suggested by Reid et al., (1990) and is described here using an HPRT minigene for positive selection in HPRT deficient cells in the first step and for negative selection in the second step. Positive-negative selection is required for the first step but negative selection alone is sufficient the enrich for targeting events during the second step because the HPRT minigene is integrated in a known position.

Targeting vector sequence homology to the locus of interest is indicated by heavy lines (introns) and by filled boxes (exons). The site of vector linearisation is indicated by an open arrow. Dotted lines represent the limits of regions of homology and therefore delimit regions capable participating in homologous recombination events. The vector for the first step consists of a short region of 5′ homology, an HPRT minigene driven by a PGK promoter for positive selection, 3′ homology consisting of exons and introns down stream of exon 3 and a HSV thymidine kinase gene outside the 3′ homology, for negative selection in the presence of gancyclovir. The vector for the second step consists of sufficient homologous sequence to reconstruct the disrupted step 1 allele and an engineered mutation (*).

**Step 1.** The vector recombines with the target locus and effectively replaces exons 2,3 and a portion of intron 3 with a HPRT minigene. Targeted ES cells are $HAT^{res}$ + $ganc^{res}$ and ES cell colonies surviving selection are screened for the presence of a targeting-specific PCR product with primers 1 and 2. These ES cells can be used for the construction of null mice or for an almost unlimited number of second steps.

**Step 2.** The disrupted locus is reconstructed with sequences bearing an engineered mutation. Selection against the HPRT minigene with 6-thioguanine is sufficient to enrich for targeted ES cells. 6-thioguanine resistant ES colonies are screened for the desired mutation by a combination of PCR with primers 3 and 4 and a restriction enzyme digest for a mutation-specific RFLP. PCR/RFLP positive colonies are also screened by Southern analysis for the desired targeted gene structure, desired RFLP and the absence of non-homologous recombination events.


targeting vector
4
5
HPRT
HSV-TK
site of vector
linearisation
Primer 2
Exon
1
2
3
4
5
6
Primer 1
Wildtype Allele
Step 1.
Exon
1
4
5
6
HPRT
Primer 1
Primer 2
Targeted Allele
Exon
2
3*
4
5
site of vector
linearisation
targeting vector
Step 2.
Exon 1
2
3*
4
5
6
Mutant Allele
Primer 3
Primer 4
vector homology
endogenous gene
recombination event
exon
subtle alteration
plasmid
PCR primer

(Figure 1.10, p44). Sequence insertion vectors have a number of disadvantages including i) lack of convenient negative selection methods, requiring the laborious screening of many ES colonies to find a targeted colony, i) the frequent loss of the engineered mutation following resolution of duplication and ii) the requirement for repetition of both gene targeting steps for every mutation. A double replacement strategy (first outlined by Reid et al., 1990) can largely overcome these problems.

**Section 1.4.4.2: Double replacement gene targeting**

The introduction of subtle alterations using replacement vectors requires two distinct replacement steps (Wu , Liu and Jaenisch, 1994; Moore et al., 1995) requiring two vectors. The first step results in the targeted introduction of a selectable marker into the desired locus -enabling a null allele to be generated if required (see Figure 1.10, p44). A positive -negative selection strategy can be utilised for this step. The second step requires a replacement vector which consists of intact homologous sequence which bears the desired mutation. Selection is simplified by the use of negative selection against a marker introduced in the first step. The first step need only be performed once and an unlimited number of mutations can be introduced by performing multiple second steps with vectors bearing different mutations. This thesis describes the design and use of a double replacement strategy to introduce mutations into the PrP gene. During this work a number of reports have been published utilising a similar method (Wu, Liu and Jaenisch, 1994) at other loci.

**Section 1.4.5: Gene targeting in HM-1, an HPRT deficient mouse ES line**

The male embryonic stem cell line HM-1 is a HPRT (hypoxanthine-guanine phosphoribosyltransferase HPRT; IMP pyrophosphate phosphoribosyltransferase; EC.2.4.2.8) deficient 40XY ES cell line. HM-1 contributes to the mouse germline at high frequency (Magin et al., 1992). HM-1 cells have undergone a spontaneous deletion of approximately 10kb of sequence 5′ of the HPRT locus which removes the promoter and exons 1 and 2 resulting in loss of HPRT expression from this allele (Thompson et al., 1989). HPRT is X-linked, therefore male ES cells are hemizygous

for this gene. Thus loss of function of one HPRT allele gives rise to HPRT null cells and a selectable phenotype. HPRT catalyses an early step of the purine salvage pathway in mammals. In humans mutations in HPRT leading to loss of HPRT function result in gouty arthritis or Lesch Nyhan disease (Lesch and Nyhan, 1964) depending upon the levels of functional enzyme. However HPRT activity is non essential in rodent cell lines *in vitro*, provided that the main purine synthesis pathway is functional. This feature is very useful as it is possible to select both for and against HPRT activity *in vitro* with medium supplemented with HAT and 6-thioguanine respectively. HPRT deficient ES cells form the basis of an efficient selection system applicable to gene targeting in ES cells (Selfridge et al., 1992). HPRT minigenes can be incorporated into gene targeting vectors and can be used as positive-negative selectable markers in HPRT deficient cells.

**Section 1.4.5: Project Aim**

Linkage and RFLP analysis (Carlson et al., 1986; Carlson et al., 1988; Carlson et al., 1993; Race et al., 1990; Hunter et al., 1987) indicates that the *Prn-p* locus is tightly linked to, and may be coincident with *Sinc* and *Prn-i*. Further evidence for the congruency of *Sinc*/*Prn-i* and PrP was provided by the discovery that mice with different scrapie incubation times differ at dimorphic PrP codons 108 and 189 which give rise to two protein allotypes- PrP A and PrP B (Westaway et al., 1987). This argues that PrP dimorphic residues at codons 108 and 189 are responsible for control of scrapie incubation time currently attributed to the action of incubation time control loci *Sinc* and *Prn-i* loci. Two approaches have been employed by other workers in order to resolve this issue (Westaway et al., 1991; Hunter et al., 1992), but neither have been able to formally demonstrate that *Prn-p*, and its alleles, are responsible for the control of incubation time in the mouse.

Gene targeting on an inbred background can overcome the limitations of previous approaches. This project will attempt to clarify the relationship between scrapie incubation time gene, *Sinc*, *Prn-i* and the dimorphic PrP codons 108L/F and 189T/V. The PrP codon region of 129/Ola ES cells *Prn-p* $^{a}$ allele will be converted by a two step double replacement gene targeting strategy from PrP 108L_189T to encode PrP 108F_189V (i.e. the PrP B allotype) normally expressed from the *Prn-p* $^{b}$ allele. The effect of gene variation in such mice will be controlled by transmitting the targeted PrP allele through the germline directly onto to 129/Ola mice to generate a gene targeted line co-isogenic with wild-type 129/Ola and differing only by the targeted alteration at PrP codons 108 and 189. 129/Ola mice bearing the gene targeted PrP allele will be challenged with a variety of prion strains in order to determine the effect upon incubation time. This will test the hypothesis that the differences in incubation time attributed to *Sinc* and *Prn-i* allelism arise from polymorphisms at PrP codon 108 and 189. As an extension to this main aim, the role of codons 108 and 189 will be explored individually by generating mice which differ from co-isogenic 129/Ola wildtype mice by single PrP codon 108F and codon 189V alterations.

# Chapter 2

# Mapping the 129/Ola *Prn-p* locus

## Section 2.1: Introduction

The generation of mice with gene targeted PrP alterations required a detailed restriction map of the *Prn-p* locus and suitable cloned *Prn-p* DNA isogenic with HM-1, the ES line used for gene targeting. This chapter describes mapping of the *Prn-p* locus in HM-1 ES cells, the retrieval of a set of overlapping *Prn-p* genomic clones from an HM-1 genomic λ bacteriophage library and the subcloning of homologous DNA for targeting vectors.

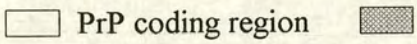
## Section 2.2: 129/Ola mice encode the *Prn-p*$^{a}$ allele and express the PrP A allotype

Restriction mapping of *Prn-p* genes in more than 50 inbred mouse strains (Carlson et al., 1988) has established the presence of six distinct *Prn-p* alleles (*Prn-p* $^{a-f}$) on the basis of restriction fragment length polymorphisms (Figure 1.6a, p32). At the outset of this project no restriction mapping data was available for the *Prn-p* locus in 129/Ola, the mouse strain from which HM-1 ES cells are derived (Magin et al., 1992). A restriction endonuclease map of the exon 3 region was generated (Figure 2.1, p51) using a 1.1Kb SmaI/Eco RI probe (Probe D). This probe was derived from an NZW mouse *Prn-p* exon 3 genomic clone, pUC18N (Westaway et al., 1987). These mapping studies confirmed that 129/Ola encoded the *Prn-p* $^{a}$ allele. This was demonstrated by the presence of characteristic exon 3 restriction fragment length polymorphisms: a single 3.5Kb Xba I fragment and two BstEII bands of 5.2 and 3.2Kb. The Xba I RFLP confirmed that 129/Ola encodes the *Prn-p* $^{a}$ allele as all other known *Prn-p* alleles (b-f) have a larger 5.2Kb exon 3 Xba I fragment (Figure 1.5a, p30). BstEII digests were consistent with both the presence of the *Prn-p* $^{a}$ allele and the PrP allotype (PrP A: 108L_189T). The BstEII RFLP arises from a dimorphic PrP codon 189 residue which can encode threonine (T) or valine (V) as described in Figure 1.5b (p30). Mice expressing the PrP A allotype retain the exon 3 BstEII site but it is absent from mouse strains expressing the PrP B allotype. As described in Section 1.3 (p25), the *Prn-p* $^{a}$ allele is linked to the *Sinc* s7 and *Prn-i* $^{N}$ alleles (Hunter et al., 1993; Carlson et al., 1992). The mapping studies reported here which demonstrate that 129/Ola encode the *Prn-p* $^{a}$ allele, are consistent with the short ME7

**Figure 2.1: A map of the HM-1 *Prn-p* $^{a}$ allele**

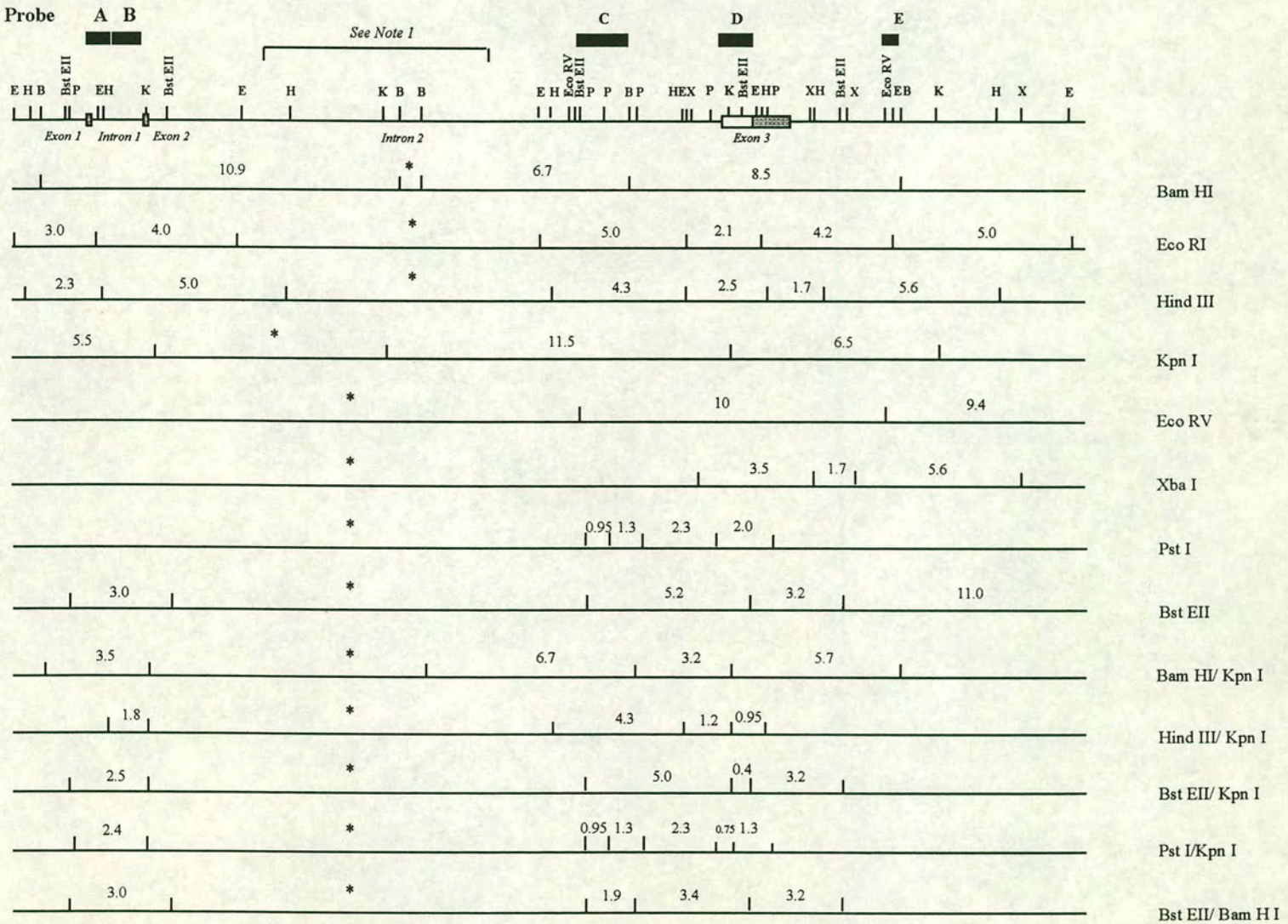
HM-1 genomic DNA was digested with the indicated restriction enzymes and hybridised to DNA probes derived from the *Prn-p* locus. All sizes in Kbp.

Probe A: a 900bp Hinc II fragment derived from pTZE1BK; Probe B: a 1500bp Hinc II/Eco RI fragment derived from pTZE1BK; Probe C: a 1900bp BstEII/EcoRI fragment derived from a λ16-1 10Kb Not I clone in pBluescript (pPrP_Not10); Probe D: a 1100bp Exon 3 SmaI/EcoRI fragment (derived from pUC18N (Westaway et al., 1987), a gift from Jean Manson, BBSRC/MRC Neuropathogenesis Unit, Edinburgh); Probe E: a 700bp Bam HI/Eco RV fragment derived from the 3′ end of p129PrP.




Enzyme abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; X, Xba I; Bst, Bst EII; Sal, Sal I; RV, Eco RV.

***Note 1:*** A number of cloned DNA fragments from this intron 2 region contained repetitive sequence and were not suitable as probes. *Prn-p* regions marked by an asterisk could not be mapped in genomic DNA because of a lack of suitable probes. However, it was possible to map intron 2 in *Prn-p* λDASH II clones (see Figure 2.2, p55).



incubation times of 129/Ola mice (~160 days) and also demonstrates that 129/Ola encode the *Sinc* s7 allele (Manson et al., 1994b).

### Section 2.3: Retrieval and mapping HM-1 *Prn-p*$^a$ clones from a HM-1 λ DASH II genomic library.

A λ DASH II bacteriophage HM-1 genomic library[1] was screened for sequences corresponding to the *Prn-p* locus. This library was constructed from a size selected (10-30Kb) Sau 3A partial digest of HM-1 genomic DNA and ligated to BamHI digested λ DASH II arms (Stratagene). The library was initially screened with a NZW *Prn-p* $^a$ mouse exon 3 1.1Kb Sma I/Eco RI DNA fragment (probe D). Particular attention was given to recombinant λ clones bearing an 8.5Kb Bam HI fragment because the genomic map indicated that this region contained the entire coding region and sufficient flanking homology for targeting vector construction (Figure 2.1, p51).

Primary screening identified 18 putative positive signals of which 10 remained positive to tertiary screening. Single, well isolated plaques were amplified and recombinant λ DNA characterised by restriction endonuclease mapping and Southern analysis with probe D. Not I digestion[2] released a single band from each of the clones (except λ16-1, see below) indicating that each clone was pure and enabling estimation of insert size (range 15-18.5Kb). A Not I digest of clone λ16-1 liberated two fragments of 10.5 and 5.2 Kb (Figure 2.2, p55) and subsequent analysis indicated that it contained a large proportion of *Prn-p* intron 2. Intron 2 is known to contain a Not I site in at least one other mouse strain encoding the *Prn-p* $^a$ allele (Westaway et al., 1994a). All 10 clones contained sequences hybridising to the probe D however four clones (λ5-1, λ6-2, λ14-1 and λ4/3.2) were found to share a common structure which did not fit that predicted from genomic mapping and all appear to have undergone a deletion event truncating the 2.1Kb Eco RI exon 3 fragment to 0.87Kb (data not

[1] a gift from Dr. Thomas Magin

[2] The λ DASH II vector contains Not I sites in its multiple cloning site. This enables Not I excision of the cloned fragment from the λ arms for subcloning or mapping.

shown). These clones were not further characterised. However, λ clones 16-1 and 17-1 were mapped to high resolution by partial digestion and hybridisation to alkaline phosphatase conjugated T3 and T7 oligonucleotides in combination with a chemiluminescent substrate (Stratagene) (Figure 2.2, p55).

Following the recovery of recombinant λ clones containing exon 3 sequence, the library was re-screened for upstream sequence corresponding to the *Prn-p* promoter, exon 1 and exon 2. Screening was performed using a 900bp Hinc II probe (Probe A) derived from the ILn/J mouse strain *Prn-p* exon 1/intron 1 region (pTZE1BK: a gift from Dr. Herbert Baybutt, BBSRC/MRC Neuropathogenesis Unit, Edinburgh). One clone, λ12-1, was retrieved and mapped (Figure 2.2a, p55). Restriction analysis of *Prn-p* λ DASH II clones 12-1 and 16-1 indicated that they overlapped in intron 2. Both contain a number of shared restriction sites including the intron 2 Not I site (Figures 2.2a and b, p55). Clones 16-1 and 17-1 share a considerable degree of overlap in the exon 3 region, although λ 17-1 is the only clone mapped to high resolution which bears the complete 8.5Kb Bam HI fragment (Figure 2.2a, p55). The restriction maps of these clones were consistent with that observed in the genomic locus and indicated that they had not undergone any detectable deletions or rearrangements during cloning. Mapping data was used to construct a contiguous map of the λ clones and is shown in Figure 2.2 d and e (p55).

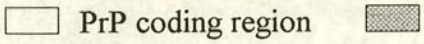
**Section 2.4: Subcloning and mapping of a 8.5Kb BamHI *Prn-p* exon 3 fragment**

Three λ clones (9-1, 14-2 and 17-1) proved to have the complete 8.5kb BamHI fragment spanning exon 3. This 8.5Kb Bam HI fragment was subcloned from λ clone 17-1 into pBluescript SKII+ in both orientations as p129PrP (+/-) and mapped by single and double digestion with restriction endonucleases (Figure 2.3, p57 and Figure 2.4, p59). All targeting vector DNA homologous to the 129/Ola *Prn-p* locus described in this thesis and in Moore et al., (1995), derives from this clone.

The ORF of the exon 3 region was sequenced by the di-deoxy method using double stranded template from p129PrP+ and a variety of exon 3 primers. This confirmed the genomic mapping studies which indicated that 129/Ola encoded the

**Figure 2.2: Restriction analysis of HM-1 *Prn-p* $^{a}$ λDASH II clones**

Restriction analysis of *Prn-p* λ DASH II clones retrieved from a HM-1 ES cell genomic library. Panels a)., b). and c). show the extent of overlap of the three clones. Panels d). and e). show the contiguous map of these clones and their restriction fragment sizes. λ clone 12-1 a).contains the promoter with exons 1 and 2 and a large portion of intron 2; λ clone 16-1 b). contains part of intron 2 overlapping λ 12-1 and exon 3; c). λ 17-1 contains exon 3. The region indicated as a heavy line in c). and d). represent a 8.5Kb BamHI fragment containing HM-1 *Prn-p* $^{a}$ exon 3 which was cloned into pBT to generate p129PrP (Figures 2.3, p57 and Figure 2.4, p59). All sizes in Kbp.




Enzyme abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; X, Xba I; Sal, Sal I; RV, Eco RV.

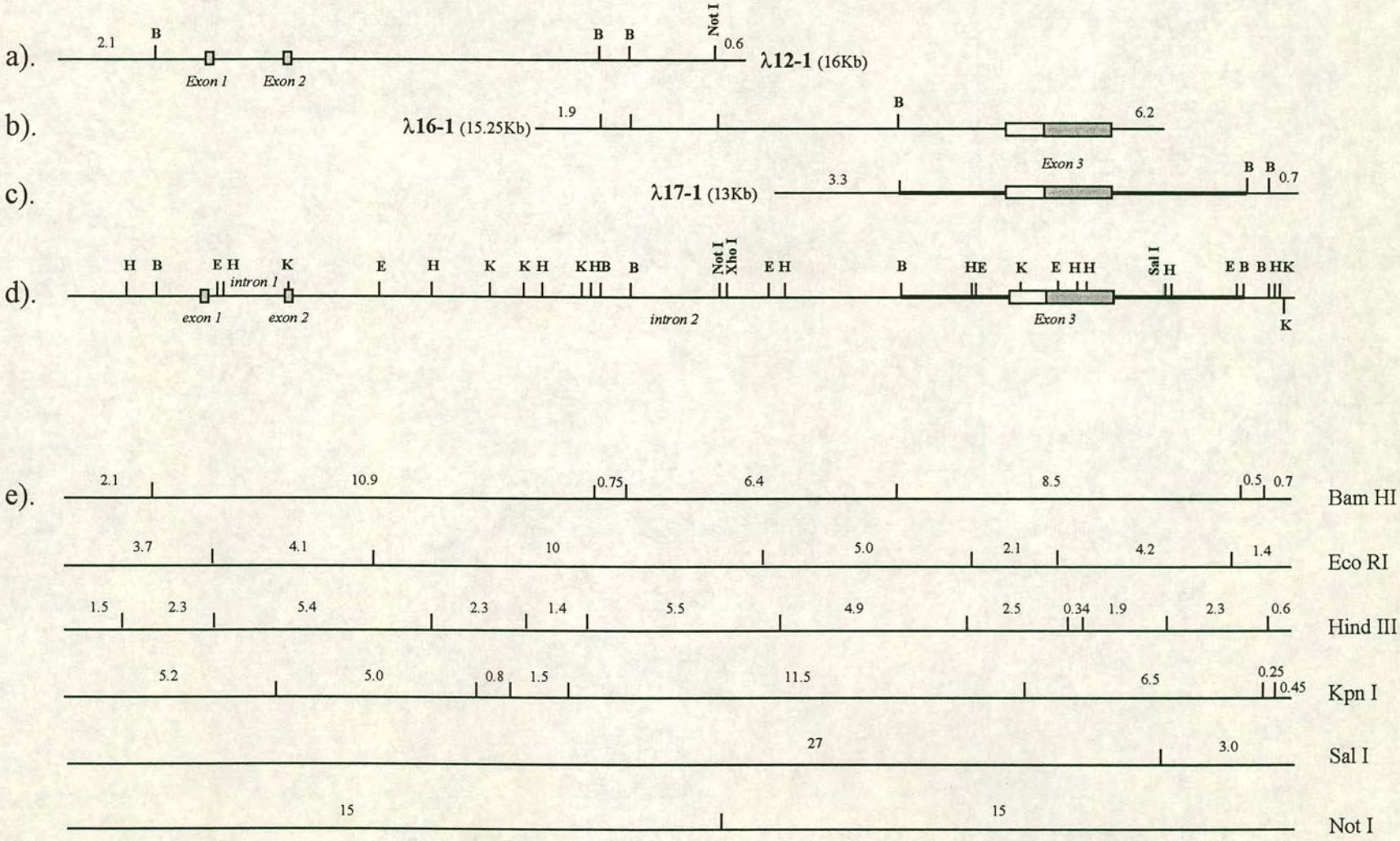
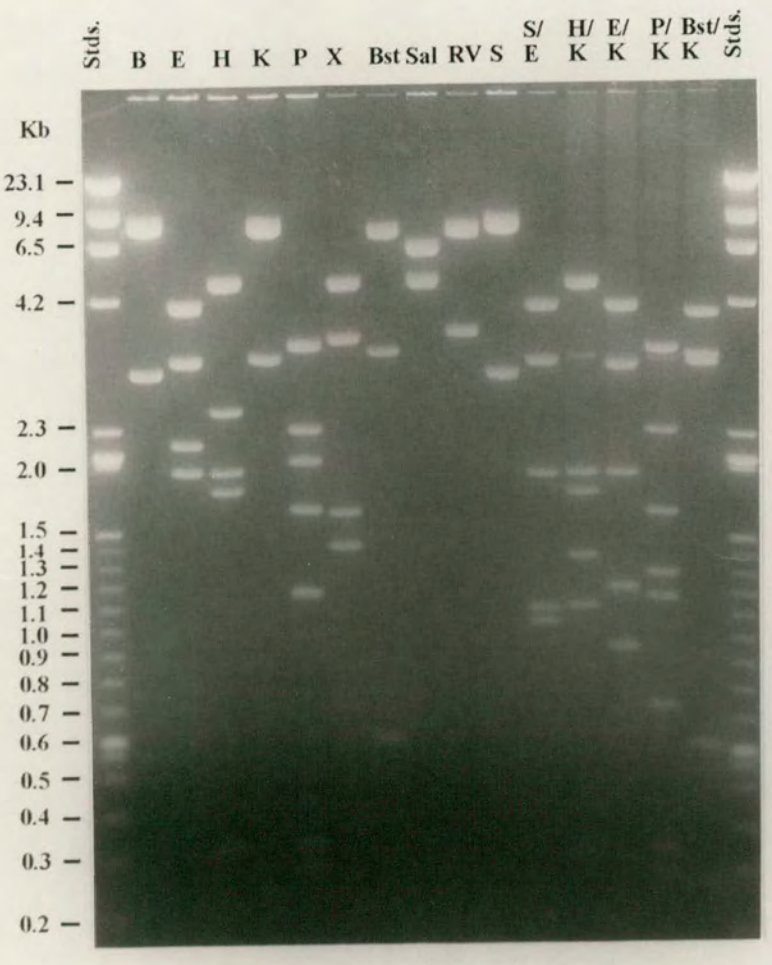

a).
2.1
B
Exon 1
Exon 2
B
B
Not I
0.6
λ12-1 (16Kb)
b).
λ16-1 (15.25Kb)
1.9
B
6.2
c).
λ17-1 (13Kb)
3.3
Exon 3
B
B
0.7
d).
H B E H intron 1 K E H K K H K H B B Not I Xho I E H B HE K E H H Sal I H E B B H K
exon 1
exon 2
intron 2
Exon 3
K
e).
2.1 10.9 0.75 6.4 8.5 0.5 0.7
Bam HI
3.7 4.1 10 5.0 2.1 4.2 1.4
Eco RI
1.5 2.3 5.4 2.3 1.4 5.5 4.9 2.5 0.34 1.9 2.3 0.6
Hind III
5.2 5.0 0.8 1.5 11.5 6.5 0.25 0.45
Kpn I
27 3.0
Sal I
15 15
Not I

**Figure 2.3: Restriction analysis of p129PrP**

p129PrP consists of a 129/Ola mouse *Prn-p* [a] exon 3 8.5Kb Bam HI fragment, derived from λ clone 17-1, cloned into the Bam HI site of pBluescript SKII+ (Stratagene). Single and double digests of p129PrP+ separated on a 1%(w/v) agarose gel stained with ethidium bromide a). blotted and b). hybridised to the 1.1kb SmaI/Eco RI exon 3 probe (probe D). All targeting vector isogenic flanking DNA was derived from this 8.5Kb Bam HI clone.

Enzyme abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; X, Xba I; Bst, Bst EII; Sal, Sal I; RV, Eco RV; S, Sma I.

a).


Stds.
B
E
H
K
P
X
Bst
Sal
RV
S
S/ E
H/ K
E/ K
P/ K
Bst/ K
Stds.
Kb
23.1
9.4
6.5
4.2
2.3
2.0
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2

b).

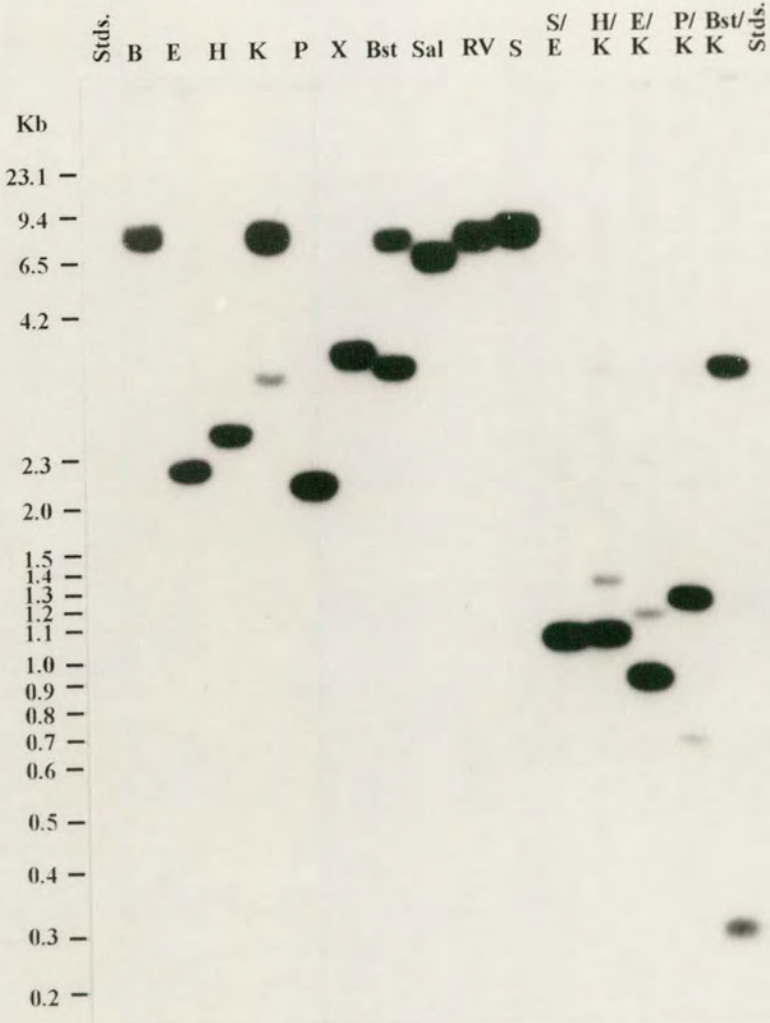
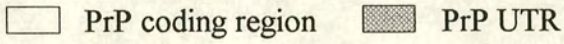
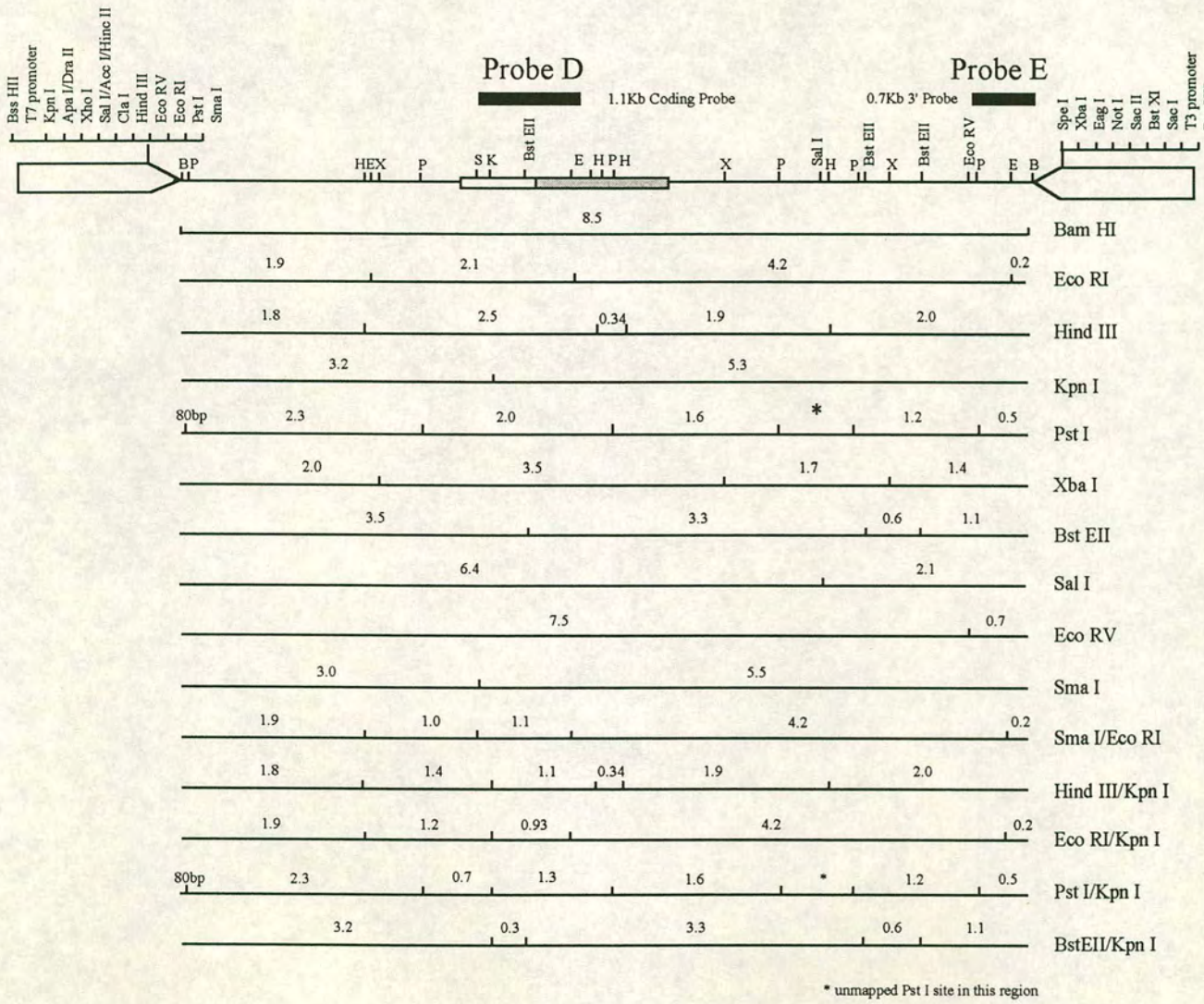

Stds.
B
E
H
K
P
X
Bst
Sal
RV
S
S/ E
H/ K
E/ K
P/ K
Bst/ K
Stds.
Kb
23.1
9.4
6.5
4.2
2.3
2.0
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2

**Figure 2.4: A restriction map of p129PrP**

A restriction map of p129PrP+ determined by single and double digests of plasmid DNA. The positions of the 1.1Kb SmaI/Eco RI (probe D) and 700bp EcoRV/Bam HI (probe E) probes are indicated with filled rectangles. All sizes in Kbp; The region marked * contains unmapped Pst I fragments (~100-200bp). The position of the 5′ Pst I site was confirmed by sequencing.




Abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; X, Xba I; S, Sma I.


Probe D
1.1Kb Coding Probe
Probe E
0.7Kb 3' Probe
Bss HII
T7 promoter
Kpn I
Apa I/Dra II
Xho I
Sal I/Acc I/Hinc II
Cla I
Hind III
Eco RV
Eco RI
Pst I
Sma I
Spe I
Xba I
Eag I
Not I
Sac II
Bst XI
Sac I
T3 promoter
Bss HII
B P H E X P S K Bst EII E H P H X P Sal I H P Bst EII X Bst EII Eco RV P E B
8.5 Bam HI
1.9 2.1 4.2 0.2 Eco RI
1.8 2.5 0.34 1.9 2.0 Hind III
3.2 5.3 Kpn I
80bp 2.3 2.0 1.6 * 1.2 0.5 Pst I
2.0 3.5 1.7 1.4 Xba I
3.5 3.3 0.6 1.1 Bst EII
6.4 2.1 Sal I
7.5 0.7 Eco RV
3.0 5.5 Sma I
1.9 1.0 1.1 4.2 0.2 Sma I/Eco RI
1.8 1.4 1.1 0.34 1.9 2.0 Hind III/Kpn I
1.9 1.2 0.93 4.2 0.2 Eco RI/Kpn I
80bp 2.3 0.7 1.3 1.6 * 1.2 0.5 Pst I/Kpn I
3.2 0.3 3.3 0.6 1.1 BstEII/Kpn I
* unmapped Pst I site in this region

PrP A allotype (PrP 108L_189T). The 129/Ola PrP ORF was identical to that of other inbred mouse strains known to encode the *Prn-p* [a] allele such as NZW (Westaway et al., 1987; Genbank accession No. M18070) and RML (Locht et al., 1986; Genbank accession No. M13685).

**Section 2.5: Discussion**

Comparison of the HM-1 *Prn-p* gene structure with that of published *Prn-p* alleles (Carlson et al., 1988) indicates that 129/Ola encodes the *Prn-p* [a] allele. Studies presented here indicate that this allele spans approximately 23Kb (Figure 2.1, p51), and although the positions of introns 1 and 2 have not been studied in detail, they appear to be 1.8Kb and ~14Kb in length respectively. Mapping studies which show that 129/Ola encode the *Prn-p* [a] allele are consistent with a 160 day ME7 scrapie incubation time (Manson et al., 1994b) and also that 129/Ola mice encode the *Sinc* s7 allele.

The significance of most of the RFLP's differentiating distinct alleles (Figure 1.6 a, p32) is uncertain. However, an exon 3 BstEII RFLP arises from a dimorphism at codon 189 which can encode either threonine or valine (Westaway et al., 1987). Codon 108 is also dimorphic and can encode either leucine or phenylalanine. These dimorphisms are in phase and give rise to distinct PrP protein allotypes, PrP A and PrP B. PrP A encodes 108L_189T and PrP B encodes 108F_189V.

There have been a number of non-coding polymorphisms described in *Prn-p*. A large intron 2 insertion/deletion has been described (Westaway et al., 1994a). Initial investigations of cosmid clones derived from *Prn-p* [a] and *Prn-p* [b] mice indicated that this polymorphism might correspond to a gene or element controlling scrapie incubation time. However, this polymorphism does not consistently correlate with incubation time length.

Polymorphisms have been described in the PrP 3′ UTR where single nucleotide substitutions at nucleotides 444 (a to g) and 1010 (a to g) (H.N. Baybutt-Genbank accession No. X83613) segregate with incubation time length. Recently *Prn-p* promoter polymorphisms have been described in mice with different *Sinc* genotypes, suggesting a potential role in the control of incubation time.

Transgenic mice bearing ~40Kb cosmid based *Prn-p* [a] and *Prn-p* [b] transgenes indicate that they are capable of position independent expression in the CNS (Westaway et al., 1991). However, a *Prn-p* [a] cosmid-based transgene, in which intron 1 had been deleted, did not express PrPmRNA in brain (Fischer et al., 1996). This suggests that this intron contains important expression control elements. Observations with large hamster PrP cosmid transgenes are consistent with this because attempts to use hamster PrP minigenes have failed to express detectable levels of hamster PrPmRNA in brain (Scott et al., 1989). Indeed, mouse *Prn-p* intron 1 contains a large number of consensus binding sites for transcription and other factors (Baybutt and Manson, 1997; Herbert Baybutt, personal communication). Recent evidence suggests that intron 2 does not appear to contain elements essential to widespread CNS expression, but that it may contain one or more elements directing expression to Purkinje neurons (Fischer et al., 1996).

**Section 2.6: Summary**

A genomic map of the 129/Ola mouse prion protein locus was generated, confirming it as the *Prn-p* [a] allele. This map guided studies in which a complete set of λ clones spanning the 129/Ola *Prn-p* [a] allele were isolated. Restriction mapping of these clones confirmed that a set of overlapping clones has been retrieved and enabled the construction of a contiguous map of the whole gene. Comparison of the genomic restriction map with that of the contiguous map of the *Prn-p* λ DASH II clones indicated that the λ clones examined contained no cloning artefacts. A plasmid clone of the 8.5Kb BamHI fragment, p129PrP, was obtained and mapped. This clone contains all the sequence homology necessary for the construction of isogenic targeting vectors.

# Chapter 3

## *Prn-p* exon 3 inactivation in HM-1 ES cells

## Section 3.1: Introduction

A two step gene targeting strategy was used to introduce subtle alterations into the coding region of *Prn-p* exon 3 (Figure 3.1, p66). This chapter describes the first step in this process in which the entire PrP protein coding region was replaced with a selectable marker resulting in an inactivated *Prn-p* allele. *Prn-p* $^{a/-}$ ES cells were used for a second round of gene targeting in which the PrP coding region was reconstructed with PrP sequence bearing engineered point mutations at codons 108 and 189 (Chapter 5, p97). This method has been used to generate a number of ES lines bearing subtle alterations to the endogenous *Prn-p* gene (Moore et al., 1995: Appendix I). *Prn-p* $^{a/-}$ ES cells were also used to construct PrP deficient mice (section 3.6, p78). The analysis of *Prn-p* $^{-/-}$ mice is described in the next chapter (Chapter 4, p84).

## Section 3.2: Construction of the *Prn-p* exon 3 inactivation vector

A replacement type vector was used to inactivate *Prn-p* exon 3 (Figure 3.1, p66). The generic design requirements for replacement vectors are described in Figure 1.8 (p40). The vector used here was designed to introduce a selectable marker into exon 3. This provided a convenient allele structure for the second targeting step (Chapter 5, p97). To this end, the knockout vector was designed to replace a 2.1Kb Eco RI fragment spanning exon 3 with a 2.7Kb HPRT minigene. This generated an allele in which the complete PrP coding region was ablated. The vector consists of three main components: an HPRT minigene, isogenic sequence homologous to regions flanking 129/Ola *Prn-p* exon 3 and a HSV-TK selectable marker.

The HPRT minigene used in this vector (PGK/DWM1) consists of exons 1-9 and introns 7 and 8 from the mouse HPRT locus. The minigene was driven by a mouse *Pgk-1* (phosphoglycerate kinase) promoter (Selfridge et al., 1992). Other minigenes are available which express much higher levels of HPRT however it has been demonstrated that high levels of HPRT expression are not necessary for efficient HAT selection in ES cells (Selfridge et al., 1992). The isogenic flanking DNA was derived from p129PrP, a 8.5Kb genomic clone spanning the exon 3 region (Figures 2.3, p57 and Figure 2.4, p59). A herpes simplex virus thymidine kinase gene (HSV-

TK) located at the terminus of the short arm of homology enabled negative selection against random integrants and therefore enrichment for homologous recombinants. This marker gene is driven by its own promoter and a mutant polyoma virus enhancer, PYF441 (Thomas and Capecchi , 1987).

A three step cloning procedure was used to construct the *Prn-p* exon 3 inactivation vector and is presented in Figure 3.2 (p68). The finished construct was designated vector C and a restriction map is shown in Figure 3.3 (p70).

**Section 3.3: *Prn-p* exon 3 inactivation in HM-1 ES cells**

Approximately $75x10^6$ HM-1 ES cells at passage 14 were electroporated at 800V, 3uF (GenePulser, BioRad) in 0.8ml 1x HBS (21mM HEPES, 5mM D-glucose, 8mM $Na_2HPO_4$, 5mM KCl, 140mM NaCl) with linearised vector C in 2 independent experiments (Table 3.1, p77). Cells were plated in ES medium supplemented with LIF and antibiotics. Approximately 24 hours following electroporation, HAT selection for HPRT activity was added by replacing medium with HAT supplemented medium (Szybalska and Szybalski, 1962). Four days later negative selection against HSV-TK activity was initiated by replacing the medium with medium supplemented with gancyclovir (GCV) and HAT.

**Section 3.4: PCR screening for gene targeted *Prn-p* $^{a/-}$ ES cell clones**

PCR screening for homologous recombinants is provided for by the detection of the targeting specific juxtaposition of HPRT minigene sequence with a region of the *Prn-p* locus outside the targeting vector (Figure 3.4, p72). ES colonies were picked 15-21 days after electroporation and screened by PCR with primer pair G1176 and 262W. Targeted colonies were identified by the presence of a targeting specific band of 2.28Kb (Figure 3.4, p72). Gene targeted ES clones were identified at high frequency from two independent experiments (Table 3.1, p77). To confirm the authenticity of these PCR products, PCR product was blotted and hybridised to [$\gamma$–$^{32}$P]ATP 5′ labelled

**Fig 3.1: A two step double replacement strategy for the introduction of subtle alterations into *Prn-p* exon 3.**

Alterations to *Prn-p* exon 3 were introduced in two distinct steps. In the first step the *Prn-p* exon 3 protein coding region was ablated by targeted replacement with an HPRT minigene. In the second step (described in chapter 5) *Prn-p* exon 3 was reconstructed by replacing the HPRT minigene with exon 3 sequence bearing protein coding alterations at codons 108 and 189. A number of distinct second steps are possible. Three are illustrated in which PrP codon 108F_189V and single codon 108F and 189V alterations are introduced.

For the first step, positive selection for HPRT activity is achieved with HAT supplemented medium. Enrichment, or negative selection against random integrants, is provided by supplementing the ES medium with gancyclovir (GCV).

In the second step, the HPRT minigene is replaced by intact exon 3. Selection against HPRT function with the toxic purine analogue 6-thioguanine is sufficient to enrich for targeting events.

Abbreviations: HAT, hypoxanthine aminopterin and thymidine; HPRT, hypoxanthine phosphoribosyl transferase ; HSV-TK, herpes simplex virus thymidine kinase gene

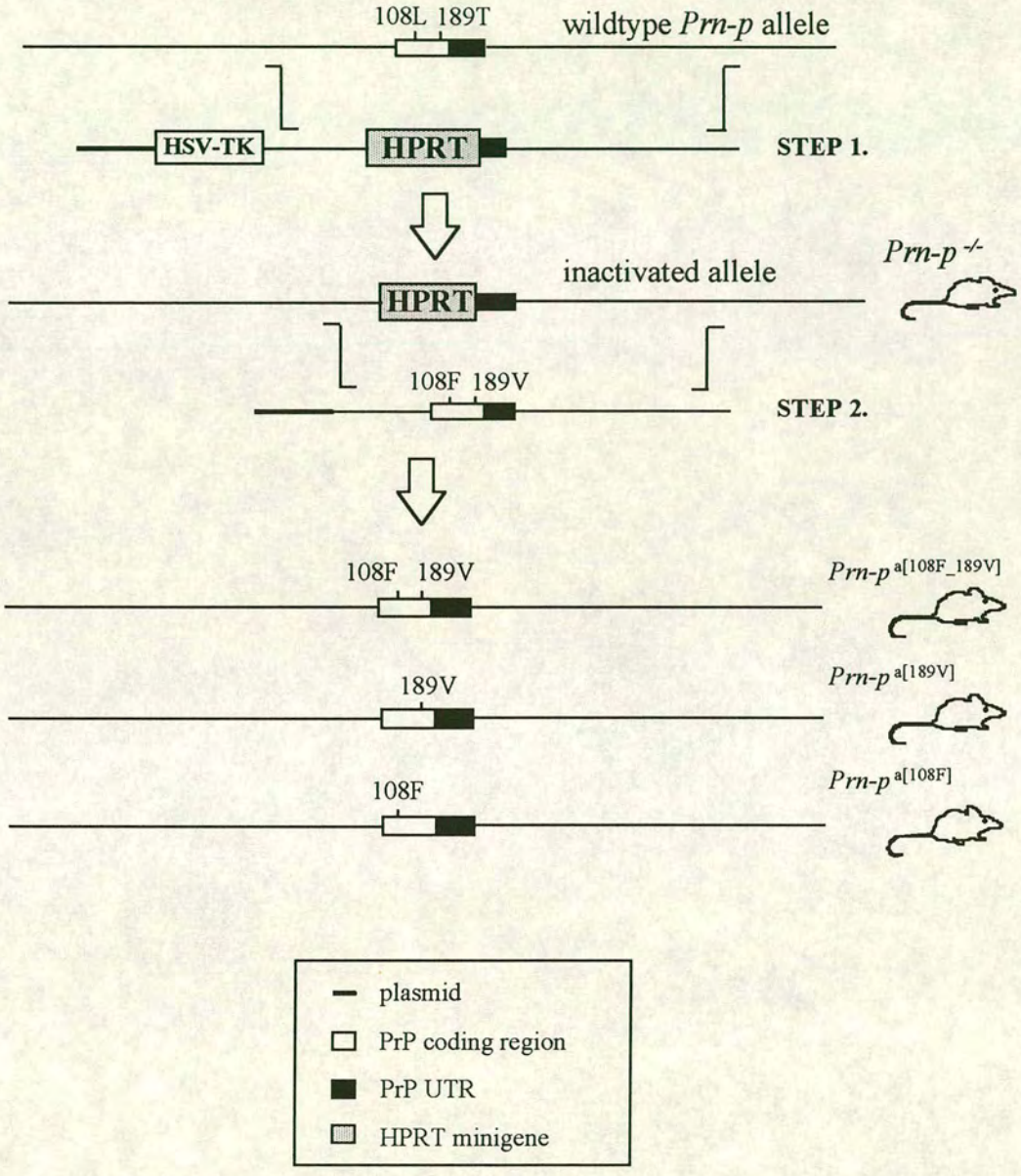

108L 189T
wildtype Prn-p allele
HSV-TK
HPRT
STEP 1.
inactivated allele
Prn-p -/-
HPRT
108F 189V
STEP 2.
108F 189V
Prn-p a[108F_189V]
189V
Prn-p a[189V]
108F
Prn-p a[108F]
plasmid
PrP coding region
PrP UTR
HPRT minigene

**Fig 3.2: *Prn-p* exon 3 knockout vector construction**

The *Prn-p* exon 3 knockout vector was constructed as described below and the final vector (vector C) consists of a mouse HPRT minigene flanked with 1.85 Kb 5′ and 3.7Kb 3′ *Prn-p* homologous sequence. A 2.0 Kb herpes simplex virus thymidine kinase module was placed upstream of the 5′ flanking homology.

**Step 1)**: 1.85Kb of 5' flanking sequence corresponding to a PstI/EcoRI fragment from p129PrP+ and 3.7Kb 3' flanking sequence corresponding to the EcoRI/EcoRV fragment of p129PrP+ were ligated into PstI/EcoRV cut pBluescript SKII+. The resulting construct was designated Vector A.

**Step 2)**: Vector A was linearised with EcoRI and ligated with a 2.7 kb EcoRI PGK/DWM1 fragment bearing the HPRT minigene.

**Step 3)**: Vector B was linearised by Bam HI/Not I double digestion and ligated to a 2kb Bam HI/NotI fragment bearing the HSV-TK gene from pBT/PSP-TK(Xba#1)(this vector was modified by Dr N.Redhead, ICMB, University of Edinburgh). The finished construct was designated vector C and a restriction map is shown in Figure 3.3 (p70). The vector has a unique Cla I restriction site at its 3′ end for vector linearisation. This site is shown in bold.

Key restriction sites are shown: B, Bam HI; E, Eco RI; P, Pst I; RV, Eco RV; N, Not I.

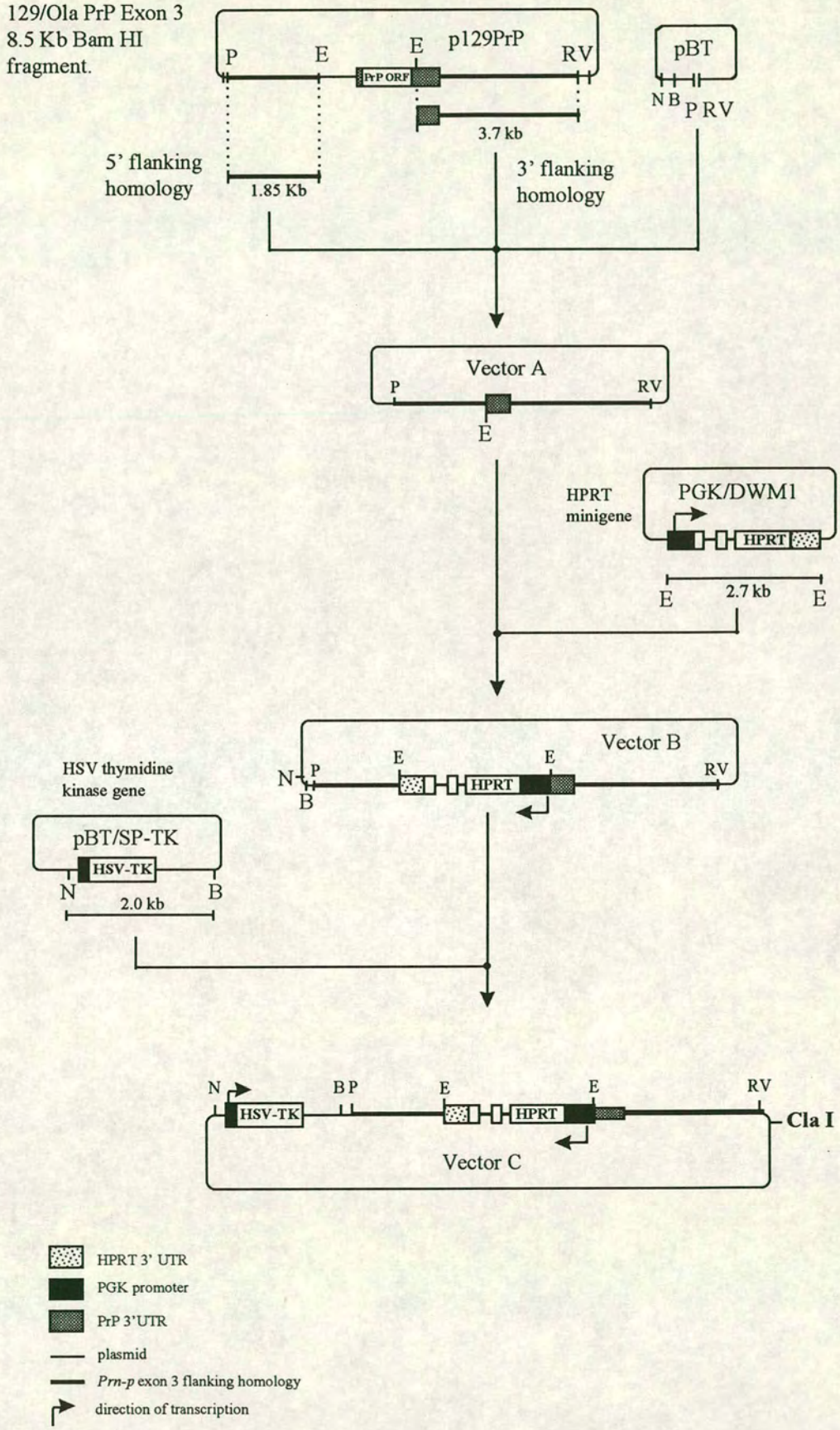
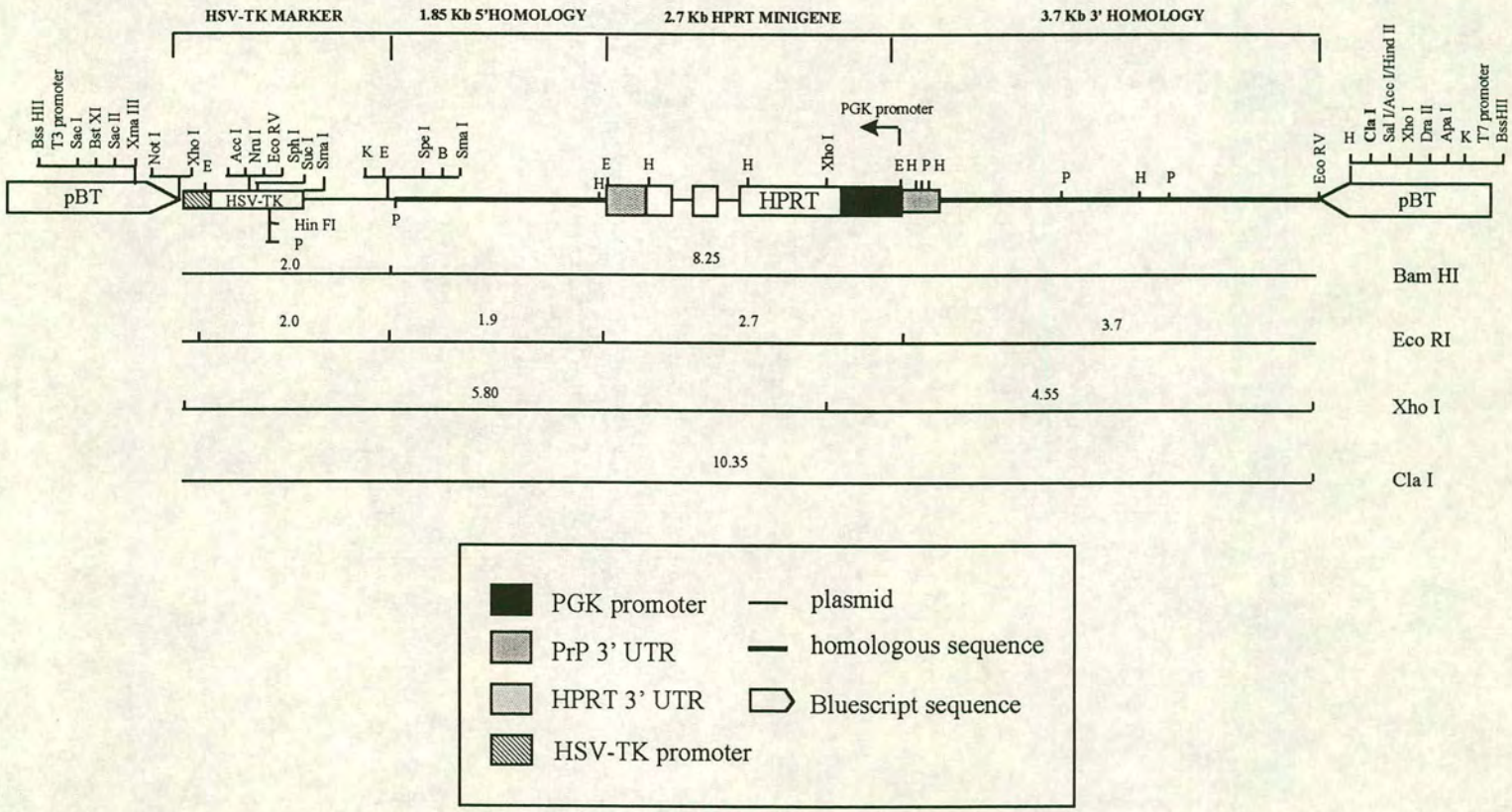

129/Ola PrP Exon 3
8.5 Kb Bam HI
fragment.
P
E
E
p129PrP
RV
PrP ORF
3.7 kb
pBT
N B P RV
5' flanking
homology
1.85 Kb
3' flanking
homology
Vector A
P
RV
E
HPRT
minigene
PGK/DWM1
HPRT
E
2.7 kb
E
Vector B
HSV thymidine
kinase gene
N
P
B
E
E
HPRT
RV
pBT/SP-TK
HSV-TK
N
B
2.0 kb
N
B P
E
E
RV
HSV-TK
HPRT
Cla I
Vector C
HPRT 3' UTR
PGK promoter
PrP 3'UTR
plasmid
Prn-p exon 3 flanking homology
direction of transcription

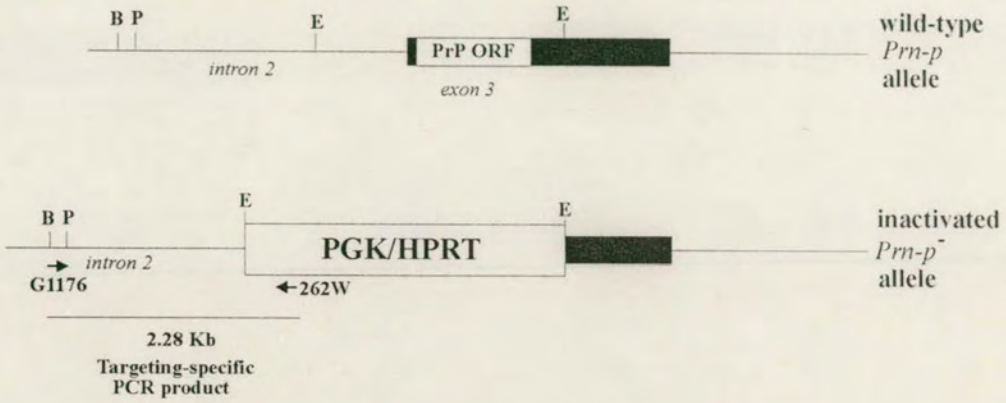
**Figure 3.3: Restriction map of the PrP knockout vector**

Restriction map of *Prn-p* exon 3 knockout vector C. A number of sites have been omitted for clarity.

Restriction enzyme abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P; Pst I. All sizes in Kb.


HSV-TK MARKER
1.85 Kb 5'HOMOLOGY
2.7 Kb HPRT MINIGENE
3.7 Kb 3' HOMOLOGY
Bss HII
T3 promoter
Sac I
Bst XI
Sac II
Xma III
Not I
Xho I
Ace I
Nru I
Eco RV
Sph I
Stu I
Sma I
K E
Spe I
B
Sma I
PGK promoter
Xho I
Eco RV
Cla I
Sal I/Ace I/Hind II
Xho I
Dra II
Apa I
K
T7 promoter
BssHII
pBT
HSV-TK
Hin FI
HPRT
pBT
2.0
8.25
Bam HI
2.0
1.9
2.7
3.7
Eco RI
5.80
4.55
Xho I
10.35
Cla I
PGK promoter
PrP 3' UTR
HPRT 3' UTR
HSV-TK promoter
plasmid
homologous sequence
Bluescript sequence

a).


B P
E
E
PrP ORF
intron 2
exon 3
wild-type
Prn-p
allele
B P
E
E
PGK/HPRT
intron 2
G1176
262W
inactivated
Prn-p-
allele
2.28 Kb
Targeting-specific
PCR product

b).

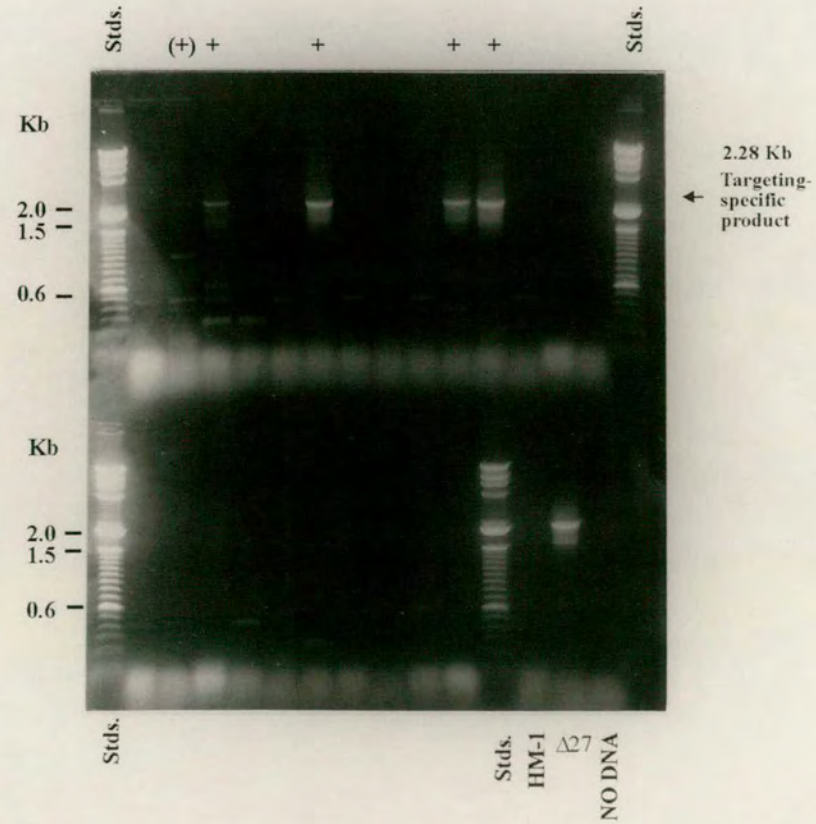

Stds.
(+) +
+
+ +
Stds.
Kb
2.0
1.5
0.6
2.28 Kb
Targeting-
specific
product
Kb
2.0
1.5
0.6
Stds.
Stds.
HM-1
Δ27
NO DNA

**Figure 3.5: *Prn-p* inactivation**

Map of the wild-type *Prn-p* $^{a}$ allele and the inactivated *Prn-p* $^{-}$ allele. The targeted insertion of the HPRT minigene into exon 3 results in the introduction of an Xho I site into the *Prn-p* locus. This is demonstrated by the presence of a 5.2Kb band following Bam HI/Xho I double digestion and hybridisation to a 700bp Eco RV/Bam HI probe (Probe E). The extent of vector homology is indicated by broken lines. All sizes in Kb.

Restriction enzymes: B, Bam HI; E, Eco RI; H, Hind III; P, Pst I.

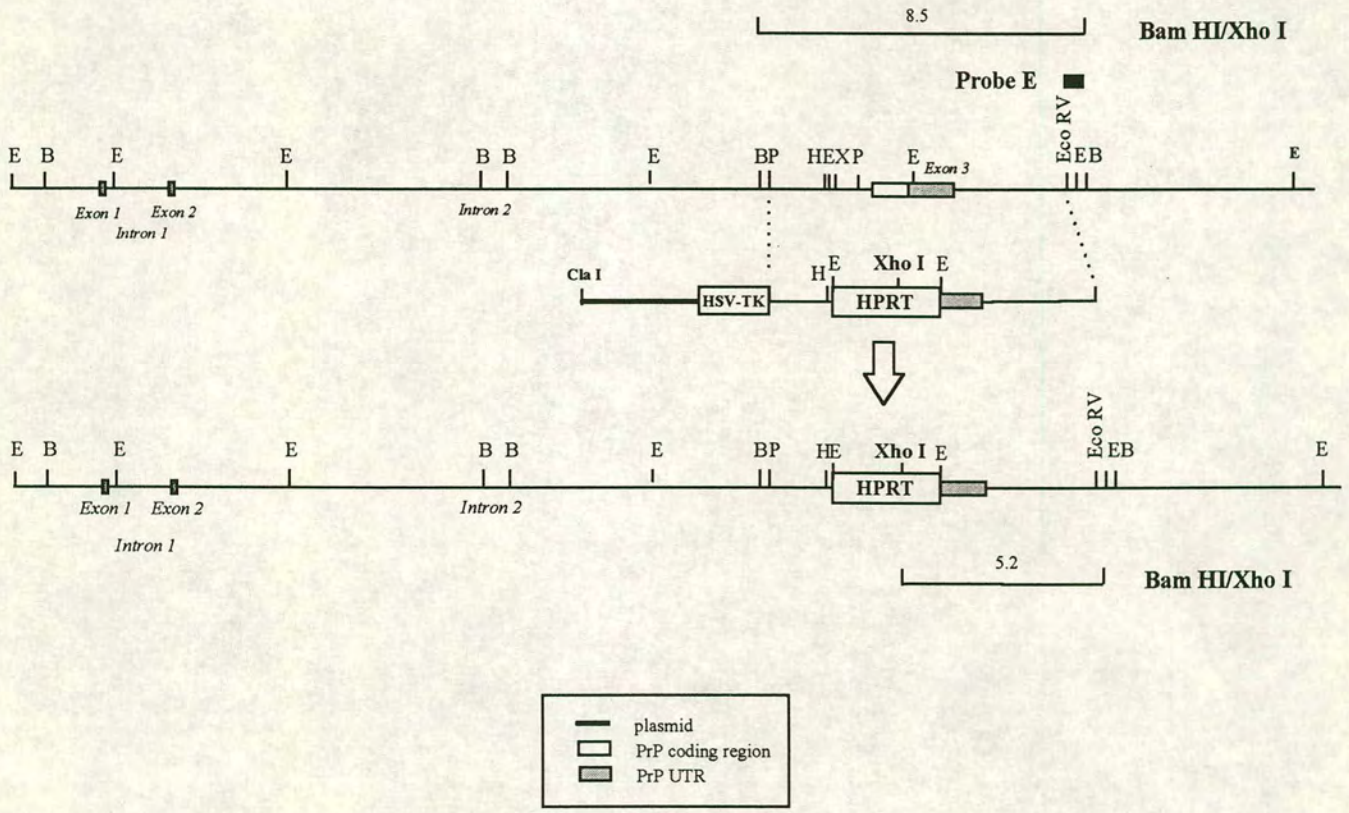

8.5
Bam HI/Xho I
Probe E
Eco RV
E B E E B B E BP HEXP E Exon 3 EB E
Exon 1
Exon 2
Intron 1
Intron 2
Cla I
H E Xho I E
HSV-TK
HPRT
E B E E B B E BP HE Xho I E EB E
Eco RV
Exon 1
Exon 2
Intron 1
Intron 2
HPRT
5.2
Bam HI/Xho I
plasmid
PrP coding region
PrP UTR

**Figure 3.6: Southern analysis of *Prn-p* knockout ES clones**

Southern blot of ES clones bearing an inactivated *Prn-p* allele. Bam HI/Xho I double digestion of ES genomic DNA hybridised to a 700bp Eco RV/Bam HI probe (Probe E). The wildtype *Prn-p* allele does not contain an Xho I site and results in a 8.5Kb fragment whilst the targeted allele contains an HPRT minigene bearing an Xho I site -resulting in a band of 5.2 Kb. Clone 27* is mixed clone in which only a proportion bear the *Prn-p* ⁻ allele. This is a distinct clone from Δ27 in lane 2.

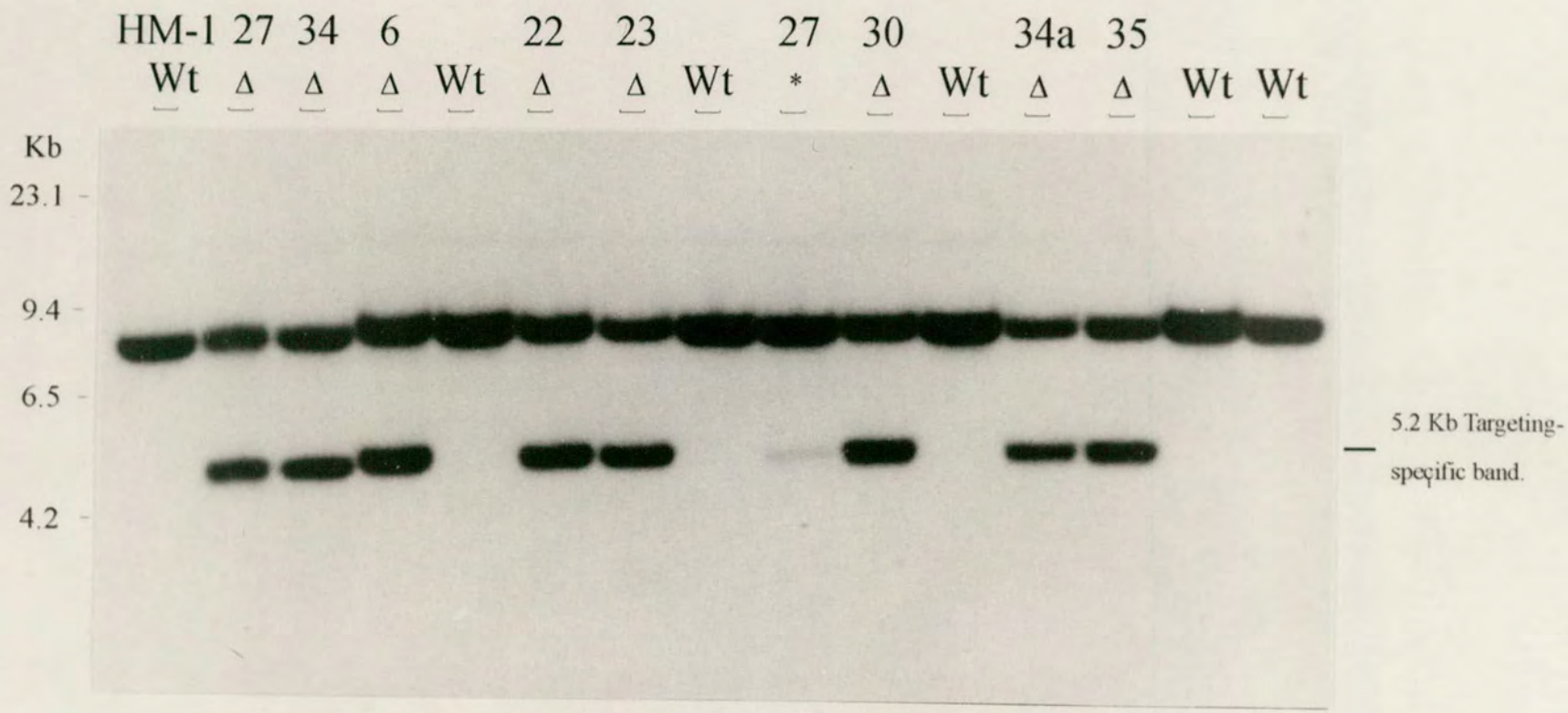

HM-1 27 34 6 22 23 27 30 34a 35
Wt Δ Δ Δ Wt Δ Δ Wt * Δ Wt Δ Δ Wt Wt
Kb
23.1
9.4
6.5
4.2
5.2 Kb Targeting-specific band.

**Table 3.1: Targeting frequencies and germline transmission data for exon 3 knockout ES clones**

| Exp't No. | Date | Linearisation Site | Targeting Frequency[a] | Targeted ES Line | Germline Transmission |
|---|---|---|---|---|---|
| 1 | Oct. 1993 | Cla I | 21/72 | HM-1/Δ27 | Yes[b] |
| | | | | HM-1/Δ34 | Yes[c] |
| 2 | Nov. 1995 | Cla | 6/48 | HM-1/Δ6 | Yes |
| | | | | HM-1/Δ22 | Yes |
| | | | | HM-1/Δ23 | Not tested |
| | | | | HM-1/Δ30 | Not tested |
| | | | | HM-1/Δ34 | Not tested |
| | | | | HM-1/Δ35 | Not tested |

a, Targeting frequency =No.Targeted colonies/No.HAT+GANC resistant colonies.

b, This clone was used in a second step by Dr Nicola Redhead and generated mice with a PrP codon 101Leu alteration linked to Gerstmann Straussler Scheinker syndrome (Moore et al., 1995)

c, This clone was used in a second step and generated mice with a PrP codon 108F and 189V alterations (Moore et al., 1995). This is described in chapter 5.

primer G1177, a 20-mer which hybridises to sequence within the 5′ arm of targeting vector homology. PCR products were also screened by hybridisation to a HPRT cDNA 1.1Kb probe. All PCR products giving rise to the predicted 2.28Kb band hybridised to both G1177 and HPRT cDNA. This confirmed the authenticity of the 2.28Kb products (data not shown). These studies were performed in collaboration with Dr Nicola Redhead, ICMB.

### Section 3.5: Southern analysis of PCR positive ES colonies

PCR positive clones were investigated by Southern analysis for the presence of targeting-specific changes to *Prn-p* restriction fragments (Figure 3.5, p74; Figure 3.6, p76). These clones were also investigated for evidence of additional random integration events. The knockout vector replaced a 2.15Kb EcoRI exon 3 fragment with a 2.7Kb HPRT minigene containing an Xho I site. The targeted introduction of the HPRT minigene into *Prn-p* exon 3 was detected by the introduction of this novel

Xho I site into the predicted position. The wildtype *Prn-p* exon 3 lies within a 8.5Kb Bam HI fragment and targeting-specific changes to restriction patterns around this fragment were detected in Bam HI/Xho I double digested ES genomic DNA. This digest produces an 8.5 Kb band from the wild-type *Prn-p* allele but is truncated to 5.2 Kb by the introduction of the HPRT minigene into the predicted position within exon 3 when hybridised to probe E (Figure 3.6, p76). A single Bam HI digest also produces a 9.1-9.2 Kb band when hybridised to probe E and arises from the replacement of the 2.15Kb Eco RI band with the HPRT minigene, which is approximately 700bp larger (see Figure 3a, lane 2 in Moore et al., 1995 in Appendix I).

Bam HI/Xho I digests were also hybridised to a 1.1Kb HPRT cDNA fragment. This demonstrated, as before, the presence of the HPRT sequence within the predicted position in the 8.5Kb Bam HI fragment spanning exon 3. This probe hybridises to the knockout vector, and the absence of additional banding with this probe, indicated that all of the *Prn-p* $^{a/-}$ ES lines tested had not undergone any detectable random integration events. This is consistent with observations of a number of gene targeting experiments in which additional random integrants are uncommon in ES clones which have undergone a gene targeting event.

## Section 3.6: Testing the germline potential of *Prn-p* $^{a/-}$ ES clones and the generation of PrP deficient mice

The main objective of this research project was the generation of ES cells and mice with gene targeted alterations to PrP codons 108 and 189. This involves a two step double replacement gene targeting method (Figure 3.1, p66) which requires the prolonged *in vitro* maintenance of ES cells, which is known to reduce their ability to contribute to the germline. The suitability of *Prn-p* $^{a/-}$ ES clones for further gene targeting work was assessed by their ability to contribute to the germline.

A number of ES clones with a low frequency of spontaneous 6-thiogunaine resistance were selected for blastocyst injection. Low levels of 6-TG resistance are required for the second gene targeting step. In the second step, a high level of 6-TG resistance would make it very difficult to identify gene targeted colonies amongst the

vast excess of non-targeted ES cells (Stacey et al., 1994).[1] A number of chimeras were generated and were tested for their ability to contribute to the germline. Germline transmission was obtained from chimeras derived from different ES clones (Table 3.1, p77) and were mated with 129/Ola and Balb C stock to generate to *Prn-p* $^{-/-}$ mouse lines. The 129/Ola line was maintained as a pure bred 129/Ola stock without any outbreeding onto other mouse genetic backgrounds. An outbred stock was also generated by crossing germline chimeras with Balb C and then maintaining this stock by random breeding of *Prn-p* $^{-/-}$ mice. Mice bearing the *Prn-p* $^{-}$ allele were analysed for PrP expression and this is discussed in the following chapter (Chapter 4, p84).

**Section 3.7: Discussion**

Four laboratories have independently performed PrP knockouts (Bueler et al., 1992; Manson et al., 1994; Sakaguchi et al., 1995; Moore et al., 1995). The gene targeting frequencies described here, in which HPRT deficient ES cells are used in conjunction with an HPRT minigene, compare favourably with those of other groups (Table 3.2, p82). The final structure of each of the targeted alleles is shown in Figure 3.7 (p81). Bueler et al. (1992) used a vector flanked by 3.0 Kb of 5′ homology and 760bp Kb of 3′ homology. Positive selection for cells stably transfected by the targeting vector was provided by a Neo module driven by a HSV-TK promoter. The low gene targeting frequencies observed in this experiment (1/5,000) are presumably a result of both the short region of 3′ homology and the lack of a means of counterselection against random integrants. This PrP allele has undergone the replacement of codons 4 to 188 with a Neo module. Northern analysis indicates that a substantial quantity of a fusion transcript bearing neo and the remaining 3′ PrP

[1] In the first step a *Prn-p* allele is inactivated by the introduction of an HPRT minigene. This endows HPRT deficient HM-1 ES cells with a HPRT $^{+ve}$ phenotype and resistance to HAT selection. In the second step HPRT is replaced with an intact PrP coding region bearing codon 108 and 189 alterations (Chapter 5). This second step utilises loss of HPRT function, and therefore 6-thioguanine (6-TG) resistance, to enrich for ES cells bearing PrP alterations. However this selection method also permits the survival of all ES cell types which are HPRT $^{-ve}$. Non-targeted HPRT $^{-ve}$ cells are undesirable and may be present in a targeted ES clone as a minor contaminant. These include the original HPRT deficient HM-1 parental clone and *Prn-p* $^{a/-}$ ES clones in which the HPRT minigene has been silenced or deleted. This is discussed by Stacey et al., (1994).

**Figure 3.7: A Comparison of *Prn-p* null allele structures**

Alignment of all extant *Prn-p* null alleles showing the extent of flanking homology, the position and type of selectable markers used and the extent of targeted deletions. All alleles are aligned by a common exon 3 Eco RI site (broken line).

The alleles generated by Moore et al., (1995) and Sakaguchi et al., (1995) result in the complete removal of the PrP protein coding region by the replacement with a selectable marker. This also results in loss of approximately 1Kb of intron 2 sequence. Knockouts generated by Bueler et al., (1992) and Manson et al., (1994a) result in no demonstrable $PrP^c$ expression but do not completely delete the PrP ORF. Bueler et al., (1992) deleted a portion of the protein coding region between codons 3 and codons 188 and introduced a series of stop codons between the Neo module and the remaining 67 PrP C-terminal codons. The null generated by Manson et al., (1994) results in the insertion of a MT-Neo module into exon 3 at codon 95, between the $4^{th}$ and $5^{th}$ N -terminal octapeptide repeat. All sizes in Kb.

Abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; RV, Eco RV; X, Xba I; HPRT, hypoxanthine phosphoribosyltransferase minigene; MT, metallothionein promoter; Neo, neomycin phosphotransferase; ORF, PrP open reading frame; PGK, phosphoglycerate kinase promoter; TK, thymidine kinase promoter.

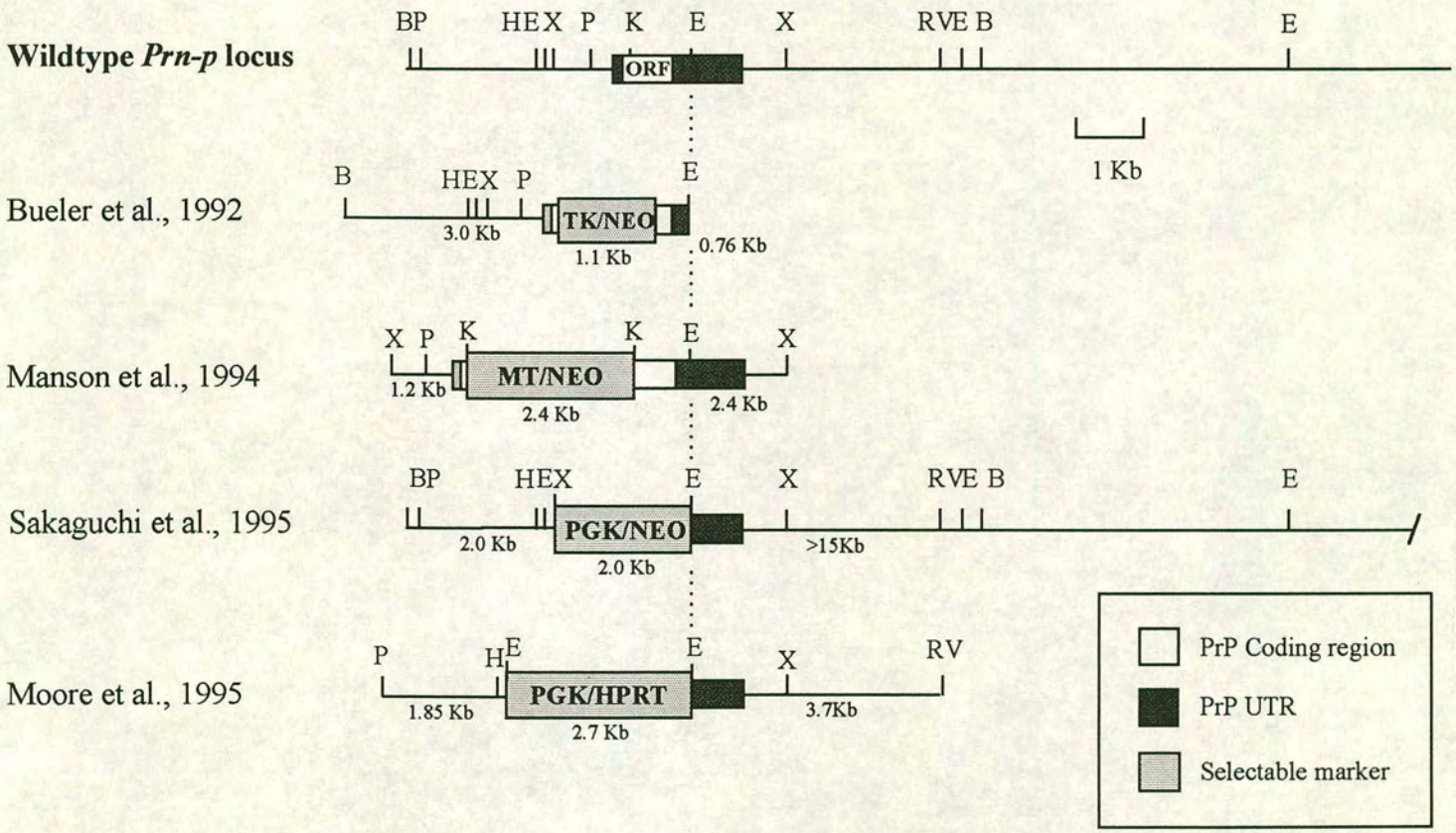

Wildtype *Prn-p* locus
BP
HEX
P
K
E
X
RVE
B
E
ORF
1 Kb
Bueler et al., 1992
B
HEX
P
E
3.0 Kb
TK/NEO
1.1 Kb
0.76 Kb
Manson et al., 1994
X
P
K
K
E
X
1.2 Kb
MT/NEO
2.4 Kb
2.4 Kb
Sakaguchi et al., 1995
BP
HEX
E
X
RVE
B
E
2.0 Kb
PGK/NEO
2.0 Kb
>15Kb
Moore et al., 1995
P
H
E
E
X
RV
1.85 Kb
PGK/HPRT
2.7 Kb
3.7Kb
PrP Coding region
PrP UTR
Selectable marker

**Table 3.2: PrP knockouts**

| Reference | Positive selectable marker/ drug selection | Counter selectable marker/ drug selection | Targeting frequency[a] |
|---|---|---|---|
| Bueler et al., 1992 | TK-Neo/ G418 | Not used | 1/5000 |
| Manson et al., 1994a | MT-Neo/ G418 | HSV-TK/ GCV | 1/800 |
| Moore et al., 1995 | PGK-HPRT/ HAT | HSV-TK/ GCV | 1/6, 1/4 [b] |
| Sakaguchi et al., 1995 | PGK-Neo/ G418 | HSV-TK/ GCV | 1/321 |

a, gene targeting frequency is defined as the number of targeted colonies divided by the number of drug resistant colonies surviving selection screened.
b, data from two independent experiments

| | |
|---|---|
| GCV, | gancyclovir |
| HAT, | hypoxanthine, aminopterin, thymidine |
| HSV, | Herpes simplex virus |
| HSV-TK, | Herpes simplex virus thymidine kinase gene driven by its own promoter |
| HPRT, | hypoxanthine-phosphoribosyltransferase minigene (Selfridge et al, 1992) |
| MT, | metallothionein promoter; |
| Neo, | neomycinphosphotransferase |
| PGK, | mouse phosphoglycerate kinase promoter; |
| TK, | HSV-thymidine kinase |

sequence is expressed from this allele (Weissmann et al., 1993).

Manson et al. (1994a) used a replacement-type vector bearing 1.2Kb of 5′ homology, 2.4 kb of 3′ homology and a MT-Neo module. The higher gene targeting frequency obtained with this vector is probably due to the use of a HSV-TK module in combination with gancyclovir for counterselection. This allele retains the whole PrP coding sequence but the ORF is interrupted by a MT-Neo module inserted into a Kpn I site in exon 3, corresponding to codon 95[2]. Thus PrP sequences upstream and downstream of the Kpn I site are present in the final allele structure. It is therefore formally possible that this allele can still produce low levels of mRNA, either as a fusion transcript with Neo or by splicing around the neo module. However, SDS-PAGE indicates that this allele expresses undetectable levels of PrP -an observation which is consistent with the scrapie resistance of mice generated from ES cells bearing this mutation (Manson et al., 1994b).

[2] PrP codon 95 lies between the 4$^{th}$ and 5$^{th}$ N-terminal repeat.

Sakaguchi et al., (1995) used a large targeting vector with 2.0 Kb of 5 homology and ~15kB of 3′ homology in conjunction PGK/Neo positive selection and counterselection with HSV-TK and gancyclovir. The final allele structure is very similar to that described in this thesis (Moore et al., 1995) and both generate a secure PrP null allele because the entire PrP protein coding region is replaced with a selectable marker.

Extensive homology to the target locus is known to provide higher gene targeting frequencies (Thomas and Capecchi, 1987; Hasty et al., 1991; Deng and Capecchi, 1992), however this cannot be the sole explanation for the high gene targeting frequencies observed in this thesis. The vector described in this chapter uses considerably shorter flanking homology than that used by Sakaguchi et al., (1995). This suggests that the higher gene targeting frequencies for the knockout described in this chapter (Moore et al., 1995) arise from the high efficiency of the HPRT/HAT selection system. This system has been used to inactivate a wide range of genes with similar or even higher gene targeting frequencies and include mouse α-lactablbumin (Stacey et al., 1994), cytokeratin-10 (Porter et al, 1996), ERCCI (Kan-Tai Hsia and David Melton, unpublished data) and DNA ligase I (Bentley et al., 1996).

**Section 3.8: Summary**

A number of ES clones have been generated in which one allele of PrP has been inactivated by the targeted introduction of an HPRT minigene. This allele generates a secure PrP null because all of the PrP protein coding region and part of the 3′UTR has been removed. Clones with low levels of 6-thioguanine resistance were tested for their ability to colonise the mouse germline. A number of clones generated germline chimeras. This demonstrated the generation of a number of *Prn-p* $^{a/-}$ ES clones suitable for the second step of gene targeting. Germline transmission of the *Prn-p* null allele enabled the generation of two lines of *Prn-p* $^{-/-}$ mice. The analysis of these mice is described in the following chapter. The second gene targeting step is described in chapter 5.

# Chapter 4

# Preliminary observations of an adult onset neurological phenotype in PrP deficient mice

## Section 4.1: Introduction

PrP deficient mice were generated as a by-product of the two step double replacement method as described in Chapter 3. No overt biological differences were observed between *Prn-p* $^{-/-}$ , *Prn-p* $^{a/-}$ and wild-type mice during the routine procedures involved in breeding the PrP null line, consistent with published reports of independently produced *Prn-p* $^{-/-}$ lines (Bueler et al., 1992; Manson et al., 1994a). Following the report by Sakaguchi et al., (1996) of an adult onset cerebellar phenotype at 70 weeks, a number of 129/Ola and outbred *Prn-p* $^{-/-}$ mice were aged to determine whether they developed this phenotype. A similar phenotype was observed and is described in Section 4.3 (p88).

## Section 4.2: PrP expression in *Prn-p* $^{a/-}$ and *Prn-p* $^{-/-}$ mice

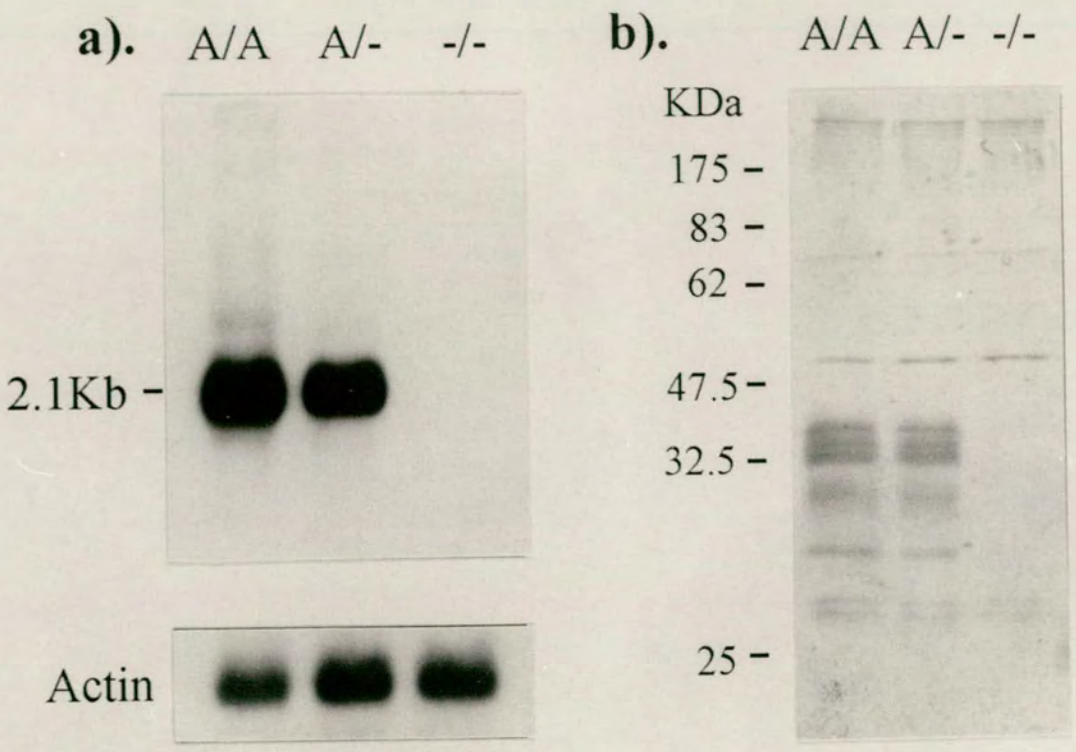
The levels of PrP mRNA and $PrP^c$ in whole adult brain were compared in 129/Ola *Prn-p* $^{a/a}$, *Prn-p* $^{a/-}$ and *Prn-p* $^{-/-}$ mice (Figure 4.1, p87). This revealed that PrP mRNA expression was reduced by ~50% in *Prn-p* $^{a/-}$ brain relative to wild-type 129/Ola *Prn-p* $^{a/a}$ mice and that PrP mRNA was absent from *Prn-p* $^{-/-}$ mouse brain (Figure 4.1, panel a, p87). Western analysis of crude brain homogenates also indicated a ~50% drop in $PrP^c$ expression in *Prn-p* $^{a/-}$ brain and the absence of detectable levels of $PrP^c$ in homozygous nulls (Figure 4.1, panel b, p87). The drop in PrP expression at the level of RNA and protein are consistent with previous reports in independently produced PrP null mouse lines (Bueler et al., 1992; Manson et al., 1994a; Sakaguchi et al., 1995). The scrapie susceptibility of these *Prn-p* $^{-/-}$ mice is currently being tested. The *Prn-p* $^{-/-}$ line generated during the course of this project are referred to as ICMB *Prn-p* $^{-/-}$.

**Figure 4.1: Brain PrP expression in ICMB *Prn-p* $^{a/-}$ and *Prn-p* $^{-/-}$ mice**

Whole brain PrP expression was examined at the level of RNA (panel a) and protein (panel b) in adult male mice.

Panel **a**). Brain PrP mRNA expression was reduced to ~50% of wild-type levels in *Prn-p* $^{a/-}$ mice expressing a single functional *Prn-p* $^{a}$ allele and is absent in *Prn-p* $^{-/-}$ mice homozygous for the *Prn-p* $^{-}$ allele. Northern analysis of whole brain total RNA (10ug) from wildtype *Prn-p* $^{a/a}$ (lane 1), *Prn-p* $^{a/-}$ (lane 2) and *Prn-p* $^{-/-}$ (lane 3) mice. RNA was hybridised to Probe D (mouse PrP exon 3 1.1 Kb SmaI/Eco RI exon 3 ds. DNA fragment).

Panel **b**). $PrP^{c}$ expression in *Prn-p* $^{a/-}$ brain is reduced to ~50% wild-type levels and is absent from *Prn-p* $^{-/-}$ mice. Immunoblot analysis of crude brain homogenate containing 20ug of total brain protein derived from adult *Prn-p* $^{a/a}$ (lane 1), *Prn-p* $^{a/-}$ (lane 2) and *Prn-p* $^{-/-}$ (lane 3) mice. PrP was detected using PrP polyclonal antiserum 1A8 (Farquar et al., 1994b) at a dilution of 1:1000 and mouse antirabbit alkaline phosphatase conjugated IgG (Promega) at a dilution of 1:7500. Alkaline phosphatase activity was visualised with the colorimetric substrate NBT/BCIP (Promega).


a).
A/A
A/-
-/-
2.1Kb
Actin
b).
A/A
A/-
-/-
KDa
175
83
62
47.5
32.5
25

## Section 4.3: A neurological phenotype in 129/Ola PrP deficient mice

### Section 4.3.1: Introduction

Sakaguchi et al., (1996) have reported a fully penetrant cerebellar ataxia in F2[129/Sv x C57BL/6J] *Prn-p* $^{-/-}$ mice with onset at approximately 70 weeks (~490 days). Rotorod tests[1] performed along with age-matched controls indicated a loss of co-ordination beginning at 49 weeks which progressed to a severe ataxia between 80 and 90 weeks. Gross examination of brains from 90 week old *Prn-p*$^{-/-}$ mice revealed that the cerebellum was atrophied and weighed significantly less than that of *Prn-p* $^{a/a}$ and *Prn-p* $^{a/-}$ littermates. Histological examination of *Prn-p* $^{-/-}$ cerebella revealed a marked loss of Purkinje neurons from many, but not all areas of the cerebellum. A thinning of the cerebellar molecular layer was also apparent in 87 week old mice.

The absence of a similar phenotype in other lines of PrP knockout mice of a similar age and of comparable genetic background (personal communication cited in Sakaguchi et al., 1996; Jean Manson, personal communication) suggested that this phenotype might be artefactual rather than the specific effect of PrP deficiency.

Comparison of the structure of all independently generated *Prn-p* knockout alleles (Figure 3.7, p81) revealed that the PrP null allele described in this thesis (Moore et al., 1995) had a number of similarities to that generated by Sakaguchi et al., (1995). They are as follows: i) both alleles delete the entire PrP protein coding region, ii) both alleles delete a 1Kb portion of intron 2 upstream of the protein coding exon and iii) both use the strong PGK promoter to drive expression of the selectable marker. These similarities in knockout allele structure are not shared by the knockouts generated by Bueler et al., (1992) and Manson et al., (1994) which do not develop an ataxic phenotype.

This suggested that the presence or absence of a similar phenotype in the mice generated during the course of this thesis might provide an insight into this issue. A phenotype with some similarities to that reported by Sakaguchi et al., (1996) was observed and the remainder of this chapter is devoted to this issue.

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[1] A test of cerebellum and spinal cord function

**Section 4.3.2: Preliminary observations of a phenotype in ICMB 129/Ola PrP deficient mice**

Groups of male and female inbred ICMB 129/Ola *Prn-p* $^{-/-}$ mice were maintained in excess of 1 year. Thus far, all 129/Ola *Prn-p* $^{-/-}$ mice older than 14 months develop a phenotype similar in many respects to that reported by Sakaguchi et al., (1996). The phenotype was first manifest in a single male and began as a mild gait abnormality and a ruffling of the coat between the shoulder blades at 423 days. Poor coat condition is normally considered a reliable sign of illness in laboratory mice. Many common illnesses of laboratory mice result in rapid deterioration, however this single *Prn-p* $^{-/-}$ mouse was unusual in that its general health did not appear to deteriorate markedly. This mouse was observed frequently and a number of preliminary observations were made: the hind quarters of the mouse became thin and tended to swing laterally when the animal walked -reminiscent of the "mincing gait" reported by Sakaguchi et al., (1996). The mouse lost ~10% of its body weight over a period of three weeks but stabilised thereafter. The gait abnormality progressed slowly over 10 weeks and at 70 weeks the mouse had obvious tremor of the whole body but also of the forelimbs, especially the right foreleg -which it avoided placing weight on when walking. The animal then started to develop signs of respiratory distress and was sacrificed at 496 days. Following initial observations in this mouse, the remaining *Prn-p* $^{-/-}$ mice were observed closely for further abnormalities. These preliminary observations proved to be typical of the phenotype observed in other ICMB 129/Ola *Prn-p* $^{-/-}$ mice aged more than 14 months. Using the above clinical signs as indicators of the phenotype, the mean age of onset was estimated at 442 days[2] or ~63 weeks.

The coat ruffling, proved to be an early indicator of a spinal curvature because many mice eventually developed a kyphosis in the thoracic region between vertebrae T2 and T4 and between T6 and T13 (data not shown). The gait abnormality was progressive and in some mice a marked atrophy of the pelvic girdle musculature was also apparent. The extent to which loss of postural muscle tone contributes to both

[2] Mean age of onset 442 days, SE=5, SD=22 days, n=22

the kyphosis and the gait abnormality is uncertain. The brains of affected animals were examined for signs of neuropathology.

### Section 4.3.3: Neuropathology in ataxic *Prn-p* $^{-/-}$ mice

Examination of H&E stained sections from three ICMB 129/Ola *Prn-p* $^{-/-}$ ataxic mice revealed no obvious morphological abnormalities, however moderate symmetrical vacuolation was apparent in many white matter areas throughout the brain, being most severe in the cerebellar white matter. A reduction in the number of Purkinje neurons was apparent in many cerebellar lobules, with a large proportion of the remaining Purkinje neurons appearing pyknotic or eosinophilic. A comparison was made with available material[3] from age matched 129/Ola wildtype mice and an independently generated line of 129/Ola *Prn-p* $^{-/-}$ mice (Manson et al., 1994a)[4] which do not develop a late onset neurological phenotype (Jean Manson, personal communication). This revealed a comparable degree of vacuolation in both 129/Ola wild-type and NPU *Prn-p* $^{-/-}$ mice[5], indicating that the vacuolar change apparent in 400-500 day old ICMB *Prn-p* $^{-/-}$ mice is age related and does not arise as a result of the PrP knockout.

Purkinje neurons in the 4$^{th}$ and 10$^{th}$ cerebellar lobules were counted[6] in 129/Ola wild-type, NPU *Prn-p* $^{-/-}$ and ICMB *Prn-p* $^{-/-}$ brains (Table 4.1, p91). The extent of Purkinje cell loss in lobule 4 is illustrated in Figure 4.2 (p92). Counting studies revealed that NPU *Prn-p* $^{-/-}$ mice (n=2) and wild-type 129/Ola mice (n=2) had similar Purkinje cell counts in lobule 4 -consistent with the absence of a late onset ataxia in this line of *Prn-p* $^{-/-}$ mice. NPU *Prn-p* $^{-/-}$ mice appeared to have slightly fewer Purkinje cells than wild-type mice in lobule 10. The significance of this observation is

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[3] Space limitations in the animal facility in which the 129/Ola *Prn-p* $^{-/-}$ mice were housed precluded the maintenance of age matched *Prn-p* $^{a/a}$, *Prn-p* $^{a/-}$ and *Prn-p* $^{-/-}$ mice.

[4] Age matched wild-type 129/Ola and *Prn-p* $^{-/-}$ mice were kindly provided by Jean Manson, BBSRC/MRC Neuropathogenesis Unit, Edinburgh. These are referred to as NPU *Prn-p* $^{-/-}$ mice.

[5] All mice had moderate but extensive vacuolar change in the corpus callosum which extended to the lateral ventricles and ventrally to the stria terminalis, cerebral peduncles and pyramidal tract/optic tract. Moderate vacuolation was also observed in the dorsal medulla and thalamus.

[6] These lobules were counted because they are well defined and consistently appear on standard lesion profile sections of the cerebellum.

**Figure 4.2: Purkinje neuron loss in ICMB *Prn-p* $^{-/-}$ mice**

Purkinje neuron frequency in cerebellar lobule 4 in aged wild-type 129/Ola, NPU *Prn-p* $^{-/-}$ (Manson et al., 1994) and ICMB *Prn-p*$^{-/-}$ mice.

Panel a). illustrates the area of lobule 4 used for cell counting in all mice (magnification x2). Purkinje cells were counted along the interface between the granular and molecular layers between points 1, 2 and 3 (indicated). Note: white matter vacuolation is apparent in the region below "1". This is typical of the extent of vacuolar change in all aged mice examined so far.

Panels b). -d). area around "2" in panel a). in the relevant mouse line (magnification x40).

Panel b). 129/Ola wild-type cerebellum showing a complete layer of Purkinje neurons, many of which appear eosinophilic.

Panel c). Normal levels of Purkinje neurons in NPU *Prn-p* $^{-/-}$ mice (Manson et al., 1994). The close similarity in number of Purkinje neurons in wild-type and NPU *Prn-p* $^{-/-}$ mice is consistent with the absence of an ataxia in this line.

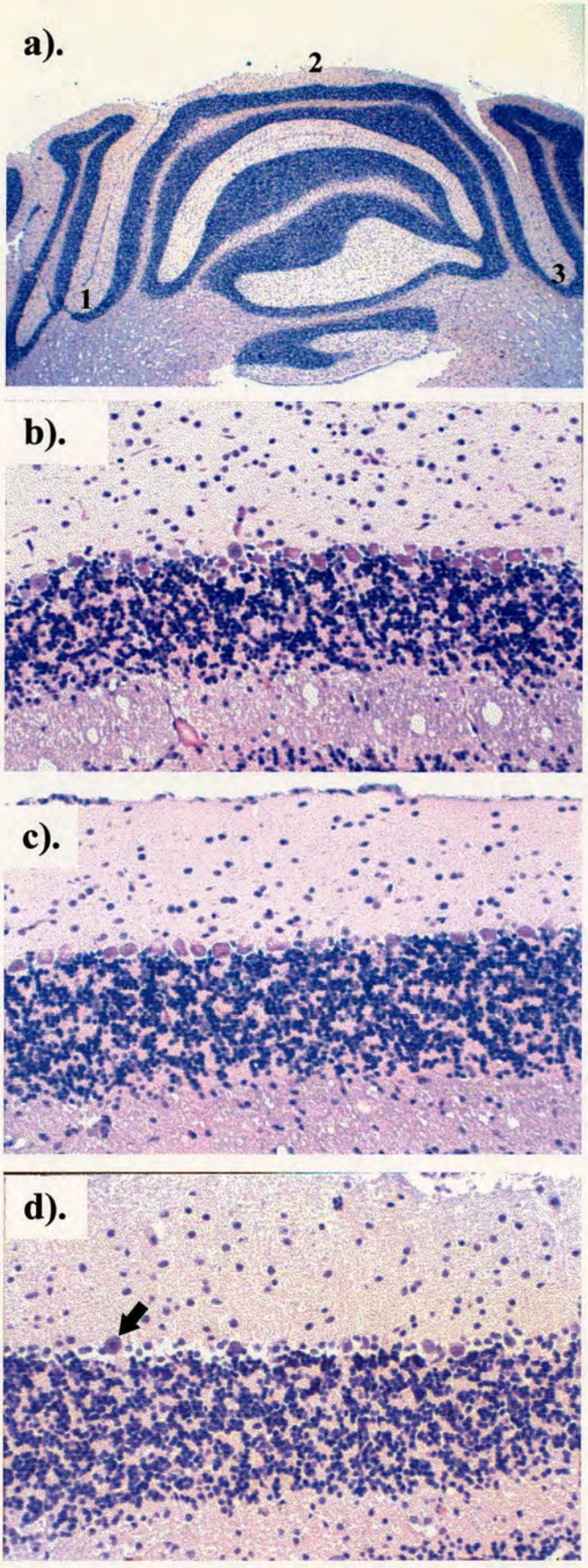
Panel d). ICMB *Prn-p* $^{-/-}$ (Moore et al., 1995) cerebellum with reduced numbers of Purkinje neurons. The arrow indicates one of the few remaining Purkinje neurons.

**Table 4.1: Cerebellar Purkinje neurons are reduced in ataxic *Prn-p* $^{-/-}$ mice (preliminary data)**

| Mouse line¶ | Mouse age (days) | Purkinje cells lobule 4† | Purkinje cells lobule 10† |
|---|---|---|---|
| Wild-type | 550 | 128 | 54 |
| | 550 | 136 | 70 |
| NPU *Prn-p* $^{-/-}$ | 551 | 128 | 33 |
| | 550 | 144 | 39 |
| ICMB *Prn-p* $^{-/-}$ | 517 | 43 | 12 |
| | 517 | 56 | 29 |
| | 496 | 63 | 20 |

¶ All mice used for this study were male.

† This is the average of counts performed on duplicate sections from each mouse.




Wt

NPU
*Prn-p* $^{-/-}$

ICMB
*Prn-p* $^{-/-}$

uncertain given the small numbers of animals examined -the small size of this lobule may also predispose it to sampling variation.

Counting studies based upon a small sample of ICMB *Prn-p* $^{-/-}$ mice (n=3) indicated that these mice had considerably less Purkinje neurons than wild-type 129/Ola and NPU *Prn-p* $^{-/-}$ mice in both lobules 4 and 10.

**Section 4.4: Discussion**

An adult onset phenotype characterised by kyphosis and ataxia is evident in ICMB *Prn-p* $^{-/-}$ mice with a mean onset age of 442 days (±5 SE, n=22). The phenotype is somewhat milder than reported by Sakaguchi et al., (1996) and this is probably because the studies reported here are in mice which are 64-70 weeks old whilst Sakaguchi et al., (1996) reported the phenotype in mice older than 80 weeks.

Examination of the brains of 129/Ola wild-type, *Prn-p* $^{a/-}$ and *Prn-p* $^{-/-}$ mice revealed a degree of age related vacuolation which was not related to PrP deficiency. However, preliminary Purkinje cell counting studies indicate that *Prn-p* $^{-/-}$ mice have a marked reduction in Purkinje cells in two cerebellar lobules -consistent with ataxia in these mice. Future studies with larger numbers of mice are required. The observation of normal levels of Purkinje neurons in lobule 4 in NPU *Prn-p* $^{-/-}$ mice is consistent with the absence of an adult onset ataxia in these mice. Further studies will examine the slightly reduced numbers of Purkinje cells in lobule 10 of NPU *Prn-p* $^{-/-}$ mice. The studies reported here, whilst preliminary in nature, are consistent with those of Sakaguchi et al., (1996) and indicate that the ataxic phenotype arises from the loss of cerebellar Purkinje neurons.

The counting studies reported here also indicated that a large proportion of Purkinje cells in all aged mouse lines were eosinophilic or pyknotic. This indicates that there is an age-related degeneration of this cell type in 129/Ola mice. Future studies will examine the number of Purkinje neurons in these mouse lines at various ages to develop a clearer picture of the age related loss of this cell type and to establish whether ICMB *Prn-p* $^{-/-}$ mice have normal levels of Purkinje neurons earlier in life. Studies with older ICMB *Prn-p* $^{-/-}$ mice may reveal an even more marked loss of

Purkinje neurons. The marked thinning of the molecular layer reported by Sakaguchi et al., (1996) is not apparent in the ICMB *Prn-p* $^{-/-}$ mice studied so far but may arise in older mice.

The adult onset neurological phenotype in ICMB 129/Ola *Prn-p* $^{-/-}$ mice had a mean age of onset of 442 days or ~63 weeks. This is about 7 weeks earlier than reported by Sakaguchi et al., (1996). Potential explanations for this difference may include the benefit of foreknowledge of a possible phenotype, the use of different phenotype scoring criteria and the effects of genetic background. Sakaguchi et al., (1996) used a different 129 ES cell substrain (129/Sv) and crossed their chimeras with C57BL/6J. Their phenotype was subsequently reported in segregating F2[129/Sv x C57BL/6J] crosses. The importance of genetic background as a major modifier of gene knockout phenotypes is well established (for reviews see Gerlai, 1996; Lathe, 1996).

**Section 4.4.1: What causes the *Prn-p* $^{-/-}$ phenotype?**

Sakaguchi et al., (1996) concluded that the ataxic phenotype was the result of the loss of $PrP^c$ function, however the absence of this phenotype in two other independent PrP null lines obliges consideration of alternative explanations. These include: i) an additional mutation in the ES clones used to construct the ataxic *Prn-p* $^{-/-}$ mice, ii) a naturally occurring mutation within the mouse stocks used for the generation of the ataxic nulls, iii) differences in 129 ES cell sub-strain and iv) differences in structure of the PrP null allele. These will be discussed in turn:

The possibility that an additional mutation may have been introduced into the ES cells generated by Sakaguchi et al., (1995, 1996) seems remote given the independent appearance of a similar phenotype in the mice described in this thesis. Similarly, the possibility that the mouse stocks used by Sakaguchi et al., (1996) to generate their *Prn-p* $^{-/-}$ line may have carried a recessive mutation such as *pcd* or *wobbler* seems unlikely given that i) most naturally occurring mutations affecting the cerebellum present with ataxia much earlier in life and ii) the independent appearance of the ataxic phenotype in a separate line of PrP deficient mice co-isogenic with wild-type 129/Ola mice.

Most ES lines are derived from 129 substrains and all groups reporting PrP knockouts have used 129-based ES lines. There is considerable genetic variation between 129 substrains (Simpson et al., 1997) and this is a potential explanation for the presence of a cerebellar phenotype in only 2 of 4 *Prn-p* $^{-/-}$ mouse lines. However, two observations suggest that this is not relevant: i) the knockout reported in this thesis and that reported by Manson et al., (1994a) have utilised related ES lines[7] with a common 129/Ola origin but only one mouse line develops the ataxia and ii) the knockout line reported in this thesis and that of Sakaguchi et al., (1995,1996) both develop ataxia but were generated using distinct ES lines which were derived from different 129 substrains (HM-1: 129/Ola and J1: 129/Sv respectively).

The PrP null allele constructed during this thesis (Moore et al., 1995) and that of Sakaguchi et al., (1995, 1996) are similar in several respects: i) both alleles delete the entire PrP protein coding region, ii) both alleles delete a 1Kb portion of intron 2 upstream of the protein coding exon and iii) both alleles use a strong PGK promoter to drive expression of the selectable marker. This argues that the presence or absence of the ataxic phenotype is associated with these features because PrP null mice which do not display the phenotype have quite different allele structures (Figure 3.7, p81). Manson et al., (1994a) have inserted an MT-Neo module into the Kpn I site of exon 3, retaining all of exon 3 sequence and generating a null allele with a frame shift. Bueler et al., (1992) replaced a large portion (but not all) of the PrP ORF with a TK-Neo module. Both knockouts result in loss of $PrP^c$ expression and both lines are scrapie resistant (Bueler et al., 1993; Prusiner et al., 1993; Manson et al., 1994b). It is possible that the knockout alleles generated by Bueler et al. (1992) and Manson et al., (1994a) generate a fusion transcript reading through the neo gene into the remainder of the PrP coding region and 3'UTR which retains some residual function. Indeed the presence of such a transcript in *Prn-p* $^{-/-}$ brain has been reported in the line generated by Bueler et al. (Weissmann et al., 1993). It is possible that the exon 3 region contains

[7] Manson et al., (1994a) used E14 and Moore et al., (1995) used HM-1. HM-1 was derived from E14TG2a which was in turn derived from E14. The E14 ES line was derived from 129/Ola blastocysts. 129/Ola (full name 129/Ola Hsd) is maintained by Harlan UK and was established from 129/ReJ stock animals from the Jackson Laboratory in 1978 (Simpson et al., 1997).

some sequence with a regulatory function in Purkinje neurons and that Sakaguchi et al., (1995, 1996) and Moore et al., (1995), in deleting the whole coding region, have removed this sequence. In both ataxic lines 1Kb of intron 2 sequence 5′ to exon 3 has also been removed and it is conceivable that this may inactivate an unknown transcriptional unit or control element in this region.

In addition to the structural differences between independent knockout lines there are differences in the selectable modules retained in the targeted allele. Sakaguchi et al., (1995) and Moore et al., (1995) replaced the whole coding region and part of intron 2 with PGK/Neo and PGK/HPRT respectively whilst the knockouts generated by Manson et al., (1994a) and Bueler et al., (1992) were performed by inserting MT/Neo and TK/Neo modules respectively. Both the HSV-thymidine kinase (TK) and metallothionein (MT) promoters are relatively weak whilst the mouse phosphoglycerate kinase promoter is comparatively strong (PGK). It is possible that the insertion of a PGK promoter into the *Prn-p* locus can exert some cis acting influence on the expression of genes/elements within the *Prn-p* locus. It is also possible that such an effect is exerted upon neighbouring genes outside the *Prn-p* locus. There are precedents for dysregulation of neighbouring genes by PGK/neo constructs. For example a PGK/Neo module has been shown to reduce the expression of a number of genes within the granzyme cluster and can act over distances of over 100Kb (Pham et al., 1996).

Further experiments may reveal whether the phenotype is the result of the loss of $PrP^c$ function. To this end, a PrP transgene is currently being bred onto a $Prn\text{-}p^{-/-}$ background (Prof. Stanley Prusiner, personal communication).

**Section 4.5: Summary**

The original report of cerebellar dysfunction in $Prn\text{-}p^{-/-}$ mice (Sakaguchi et al., 1996) was difficult to reconcile with the other lines generated independently in which it did not develop. The observations reported here independently verify the ataxic phenotype. Further studies will be required to establish whether the ataxia in $Prn\text{-}p^{-/-}$ mice arises as a result of $PrP^c$ deficiency or some other cause.

# Chapter 5

# Generation of embryonic stem cells with PrP codon 108 and 189 alterations

## Section 5.1: Introduction

In the first gene targeting step, described in chapter 3, *Prn-p* exon 3 was inactivated by the targeted introduction of an HPRT minigene. This converts HPRT deficient HM-1 cells to an HPRT $^{+ve}$ phenotype. This chapter describes the second step in which the HPRT minigene in such cells was replaced with engineered exon 3 sequence (Figure 3.1, p66). Targeted ES cells revert back to an HPRT$^{-ve}$ phenotype enabling enrichment by selecting against HPRT$^{+ve}$ ES cells with 6-thioguanine, a toxic HPRT substrate.

## Section 5.2: Second step vector construction

### Section 5.2.1: Mutagenesis cassette construction

A mutagenesis cassette was generated by cloning exon 3 sequence containing part of the PrP A ORF into M13mp18. The cloning strategy was adapted to maintain the smallest portion of the PrP coding region in M13mp18 to minimise problems associated with the stability of larger inserts in M13 vectors and to reduce the potential for the introduction of undesired mutations.

A 934bp Kpn I-EcoRI fragment was isolated from p129PrP and cloned into M13mp18. This generated the M13 clone m129K/E$^{wt}$ containing wildtype exon 3 ORF sequence corresponding to the PrP A allotype (PrP 108L_189T). m129K/E$^{wt}$ ssDNA derived from the antisense strand enabled the use of sense mutagenesis oligonucleotides (Figure 5.1, p101).

Prior to *in vitro* mutagenesis by the Kunkel method, double stranded m129K/E$^{wt}$ RF II DNA was passaged through *dut* $^{-}$ *ung* $^{-}$ mutant E.coli strain CJ236. This generated m129K/E$^{Wt}$ ssDNA containing dUTP. The incorporation of dUTP provides biological selection against the parental strand of the heteroduplex following *in vitro* mutagenesis and transformation into a *dut ung* wild type E.coli strain (Kunkel et al., 1987). This method enables the isolation of mutants at high frequency and is described more detail in Section 9.B.10 (p212).

**Section 5.2.2: Introduction of the PrP codon 189V mutation**

m129K/E$^{wt}$ dUTP ssDNA was isolated and 200ng was annealed with 3 pmol of phosphorylated HPLC purified mismatch oligo G6020 (5′ gcagcacacggtc**gt**caccaccaccaaggg 3′). This oligonucleotide introduces a ACC-**GTC** mutation resulting in the substitution of a PrP codon 189 threonine for a valine. The mutant oligonucleotide/daughter strand was extended using T7 DNA polymerase. An aliquot of the reaction product was used to transform *dut ung* wildtype DH5aF′ competent cells. Ten well separated M13 plaques were picked, amplified in 1.5ml liquid cultures and RF II d.s. DNA was screened for the introduction of the codon 189V mutation. The ACC-GTC mutation alters two nucleotides within the exon 3 Bst EII recognition sequence G′GTNACC resulting in loss of this restriction site. Putative mutants, in which the BstEII was lost, were obtained at high frequency (6/10) and confirmed by di-deoxy sequencing (data not shown) using the human PrP primer 508X (a gift from Dr. Otto Windl, BBSRC Centre for Genome Research, Edinburgh). This primer corresponds to a region 200nt upstream of the mouse codon 189 position. All m129K/E$^{189V}$ clones were sequenced between -40nt and +105nt from the codon 189 position and all were found to have sequence indistinguishable from wildtype mouse, other than the desired mutations. A 934bp Kpn-Eco RI fragment from a clone containing the codon 189V mutation was isolated and used to construct the plasmid targeting vector p189V (Figure 5.3, p105). To generate a double mutant p108F_189V targeting vector, a m129PrPK/E $^{189V}$ mutant was used for a further round of mutagenesis to introduce the PrP codon 108F alteration.

**Section 5.2.3: Introduction of the PrP codon 108F mutation**

A CTC-TTC mutation was introduced into the codon 108 position of m129K/E$^{189V}$. Mutagenesis was performed as described above for the codon 189V mutation but with mutagenesis oligonucleotide G6877 (5′ ccaaaaaccaac**t**tcaagcatgtggc 3′). This mutation does not generate a known RFLP[1] but mutants were detected by

[1] The codon 108 mutation results in the loss of a Mnl I site but this enzyme is no longer commercially available.

**Figure 5.1: m129PrPK/E $^{Wt}$ mutagenesis cassette**

The M13-based mutagenesis cassette m129PrP $^{K/E}$. This cassette contains a 934bp KpnI/Eco RI PrP exon 3 fragment and generates ssDNA corresponding to the antisense strand of PrP enabling the use of sense mutagenesis primers.

Restriction enzymes: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; X, Xba I.

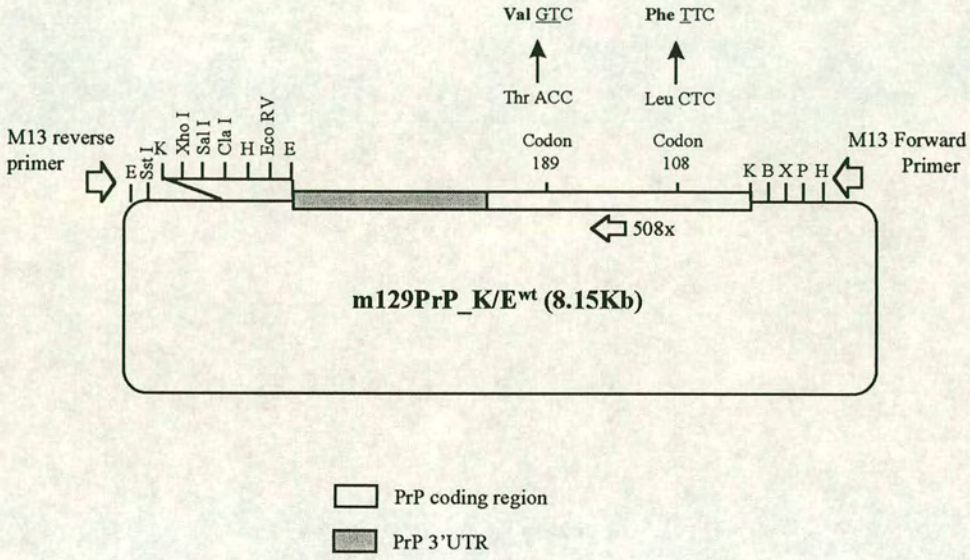

Val GTC
Phe TTC
Thr ACC
Leu CTC
M13 reverse primer
E
Sst I
K
Xho I
Sal I
Cla I
H
Eco RV
E
Codon 189
Codon 108
M13 Forward Primer
K B X P H
508x
m129PrP_K/E^wt (8.15Kb)
PrP coding region
PrP 3'UTR

**Figure 5.2: *In vitro* mutagenesis -screening for the 108F mutation**

Single stranded DNA was sequenced for the 108F mutation with the M13 forward primer. Sample loadings as illustrated. The wildtype control was m129K/E $^{Wt}$. Two of four clones screened were 108F mutants (1 and 4). Sequence surrounding codon 108 is shown for orientation.

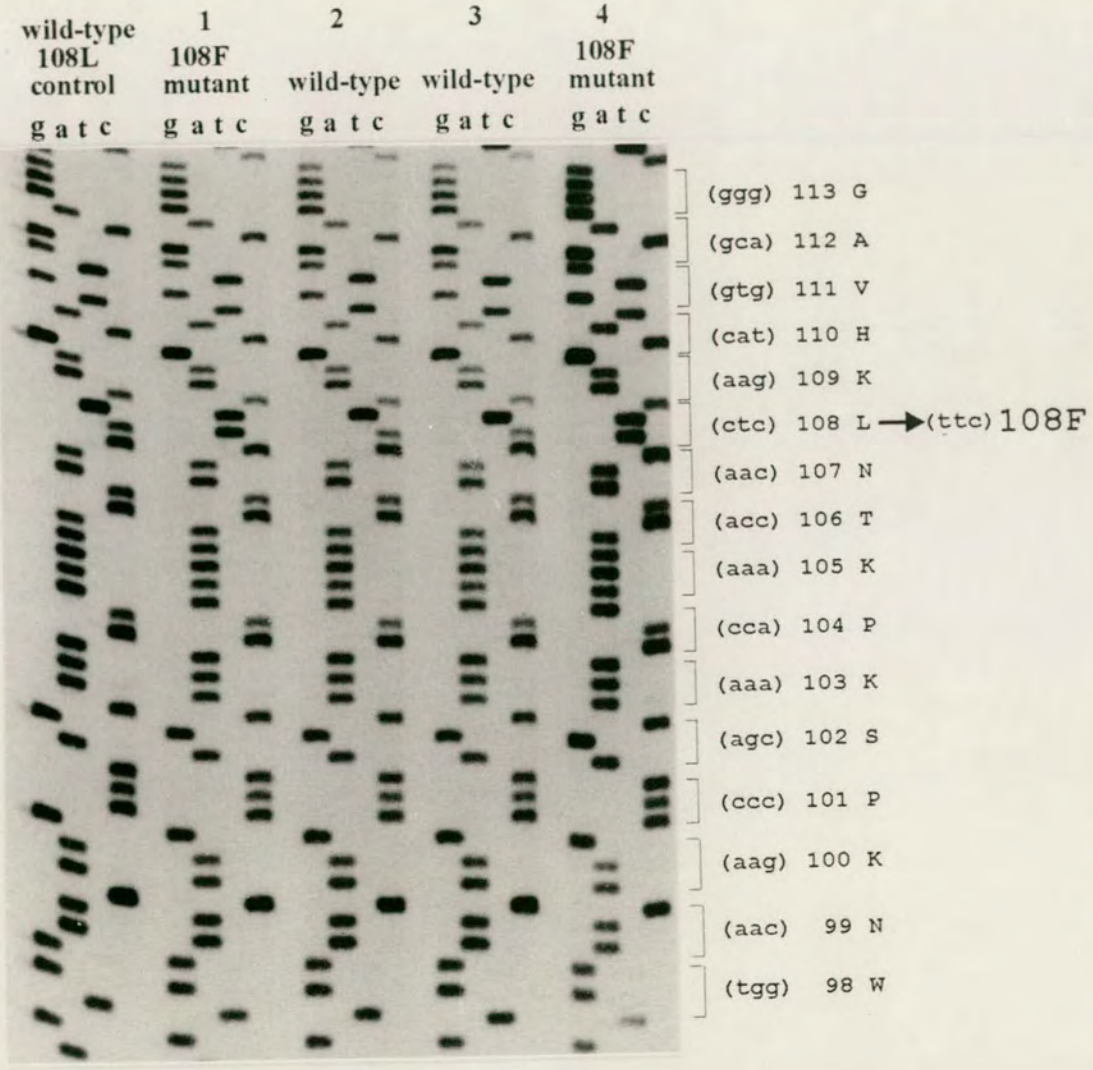

wild-type
108L
control
g a t c
1
108F
mutant
g a t c
2
wild-type
g a t c
3
wild-type
g a t c
4
108F
mutant
g a t c
(ggg) 113 G
(gca) 112 A
(gtg) 111 V
(cat) 110 H
(aag) 109 K
(ctc) 108 L → (ttc) 108F
(aac) 107 N
(acc) 106 T
(aaa) 105 K
(cca) 104 P
(aaa) 103 K
(agc) 102 S
(ccc) 101 P
(aag) 100 K
(aac) 99 N
(tgg) 98 W

**Figure 5.3**: **Construction of targeting vectors p189V and p108F_189V**

Targeting vector p108F-189V was constructed in a single cloning step by ligating a 934bp Kpn/Eco RI fragment from the double mutant m129K/E $^{108F\_189V}$ with a 6.7Kb Bam HI /Eco RI Vector A fragment and a 3.35Kb Bam HI/Kpn I p129PrP+ fragment. Vector p189V was constructed in a similar manner using a 934bp codon 189V mutant fragment from m129K/E$^{189V}$.

Abbreviations: B, Bam HI; E, Eco RI; K, Kpn I; RV, Eco RV.

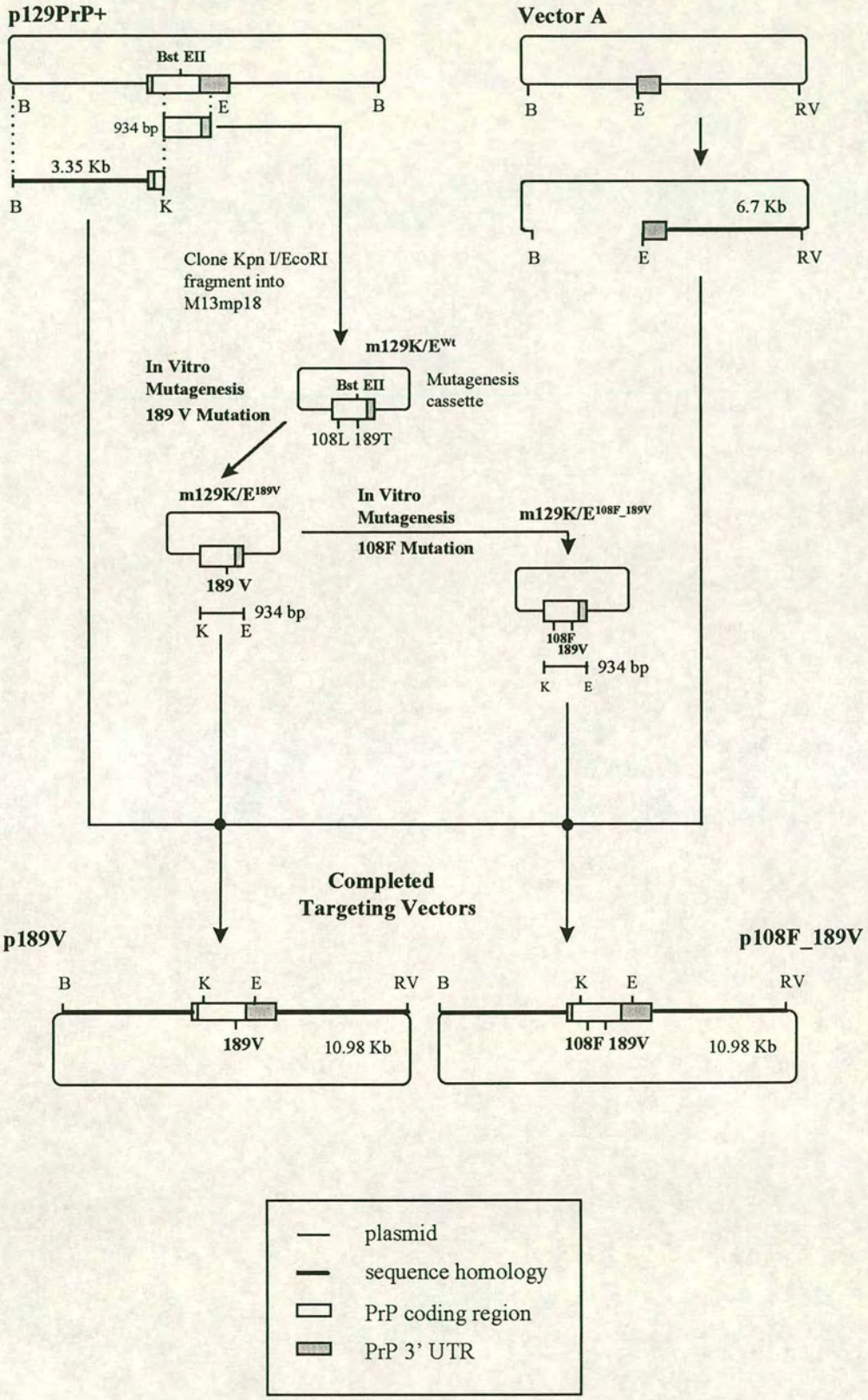

p129PrP+
Bst EII
B
E
B
934 bp
3.35 Kb
B
K
Clone Kpn I/EcoRI fragment into M13mp18
Vector A
B
E
RV
6.7 Kb
B
E
RV
m129K/E^Wt
In Vitro Mutagenesis
189 V Mutation
Bst EII
Mutagenesis cassette
108L 189T
m129K/E^189V
189 V
K
E
934 bp
In Vitro Mutagenesis
108F Mutation
m129K/E^108F_189V
108F
189V
934 bp
K
E
Completed Targeting Vectors
p189V
B
K
E
RV
189V
10.98 Kb
p108F_189V
B
K
E
RV
108F 189V
10.98 Kb
plasmid
sequence homology
PrP coding region
PrP 3' UTR

**Figure 5.4: Construction of targeting vector p108F.**

Vector p108F was constructed in a single cloning step as follows: a 3.4 Kb band containing the PrP 108F mutation was isolated from vector p108F_189V by double digestion with Bam HI and Asp 700I (an isoschizomer of Xmn I). A 704bp band containing the wildtype codon 189T was isolated from p129S/E$^{Wt}$ by Eco RI/Asp 700I double digestion. A 6.7kb Bam HI/ Eco RI fragment was isolated from Vector A. These fragments were ligated and of 7 bacterial colonies screened, 5 contained the desired recombinant, one clone was further investigated by restriction analysis and had the predicted structure. A restriction map of this vector is shown in Appendix II.

Abbreviations: B, Bam HI; E, Eco RI; RV, Eco RV.

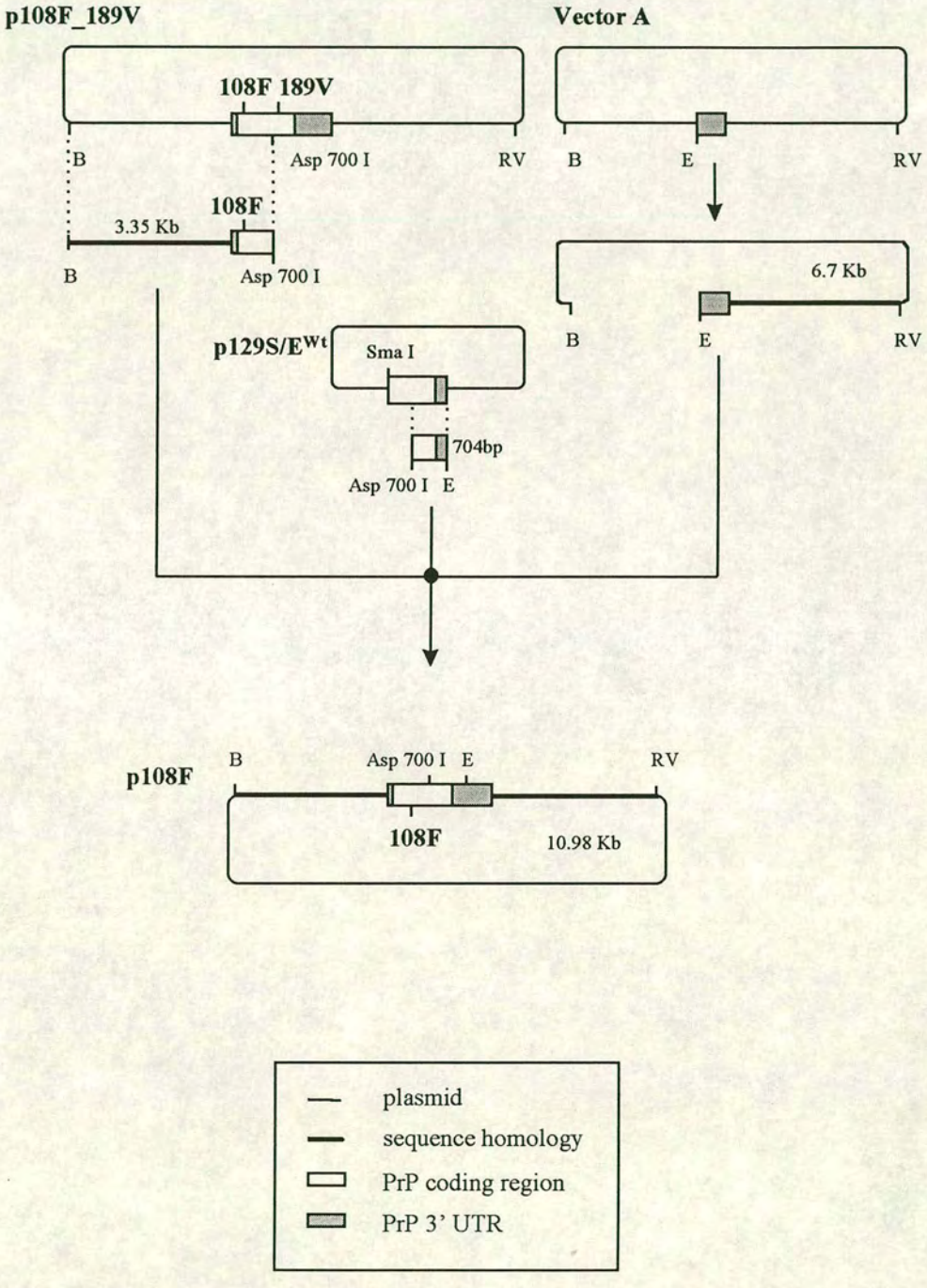
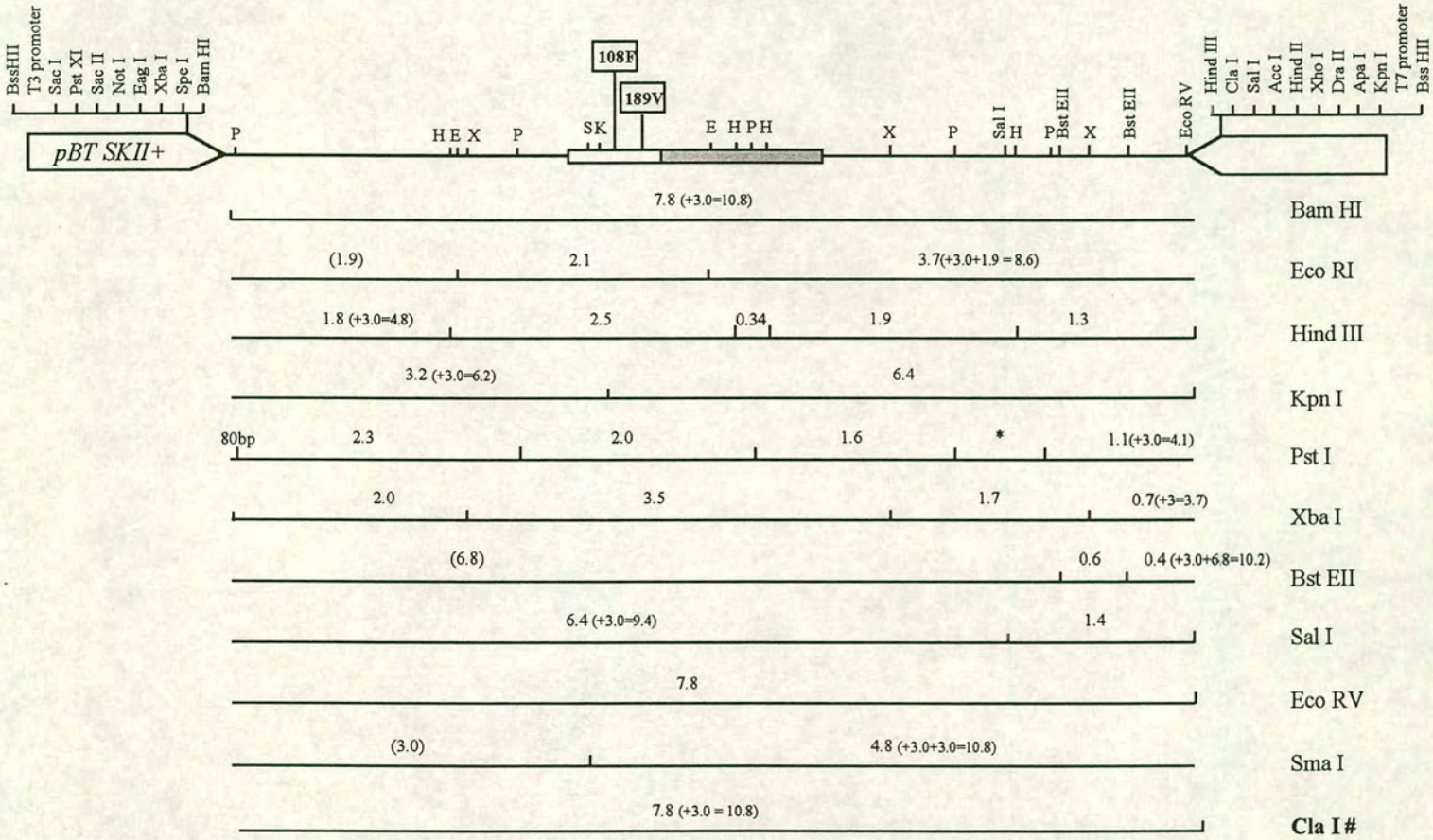

p108F_189V
108F 189V
B
Asp 700 I
RV
108F
3.35 Kb
B
Asp 700 I
Vector A
B
E
RV
6.7 Kb
B
E
RV
p129S/E^Wt
Sma I
704bp
Asp 700 I E
p108F
B
Asp 700 I E
RV
108F
10.98 Kb
plasmid
sequence homology
PrP coding region
PrP 3' UTR

**Figure 5.5: Restriction map of targeting vector p108F_189V**

A restriction map of the completed PrP exon 3 targeting vector p108F_189V. This vector was linearised at a unique 3′ Cla I site prior to electroporation.

Abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; S, Sma I; X, Xba I.




* there is one unmapped Pst site within this region
# unique linearisation site

dideoxy sequencing of ssDNA from small scale cultures (Figure 5.2, p103). Codon 108F_189V double mutants were detected at high frequency: two of four plaques (1 and 4) picked at random contained the desired 108F mutation.

**Section 5.2.4: Targeting vector construction**

Vector p108F_189V was constructed in a single cloning step as shown in Figure 5.3 (p105) using a 934bp KpnI/ Eco RI fragment from m129K/E$^{108F_189V}$. The restriction map of this targeting vector is shown in Figure 5.5 (p109). The construction of vector p108F is shown in Figure 5.4 (p107) and its restriction map is in Appendix II. The complete PrP coding region of the three targeting vectors was sequenced and confirmed the presence of the codon 108 and 189 alterations and the absence of other mutations, deletions or cloning artefacts.

**Section 5.3: Introducing PrP codon 108 and 189 alterations into ES cells**

**Section 5.3.1: Introduction**

As described in the chapter 3, the targeted introduction of the exon 3 inactivation vector results in the replacement of a 2.1 Kb Eco RI *Prn-p* exon 3 fragment with a 2.7 kb HPRT minigene. This endows the HPRT deficient male HM-1 ES cell line with an HPRT$^{+ve}$ phenotype and sensitivity to 6-thioguanine. Thus in the second step it is sufficient to enrich for targeting events by selecting for ES cells which have lost HPRT function. Only *Prn-p* $^{a/-}$ ES lines with a low frequency of spontaneous 6-thioguanine resistance were used for second targeting steps.

**Section 5.3.2: ES cell culture**

The same protocol was used for all second step targeting experiments: $3x10^7$ *Prn-p*$^{+/-}$ ES cells at passage 22-26 were electroporated with 200ug of Cla I linearised second step targeting vector in 800ul of HEPES-phosphate buffered saline, pH 7.05 at 850V, 3u Fd (BioRad Gene Pulser) in sterile BioRad electroporation cuvettes (path length 0.4cm). The cells were then incubated at room temperature for 10 minutes before plating out onto ES medium without selection at a density of $3\text{-}4x10^6$ cells/75cm$^2$ flask into 6-10 independent pools. Cells were maintained for 5-7 days

without selection (to enable decay of HPRT in targeted cells) then plated out onto 90mm dishes at a density of 1-2x$10^6$ cells/dish in ES medium supplemented with 5ug/ml 6-thioguanine. Drug resistant ES colonies were picked 10-14 days later and half of each colony was screened for the targeted alteration. The remainder of each colony was grown in 24 well dishes in ES medium in the absence of selection. PCR positive colonies were expanded and aliquots were frozen for blastocyst injection, if required. Genomic DNA was prepared from each PCR positive ES clone and investigated by Southern analysis for the appropriate *Prn-p* gene structure.

**Section 5.3.3: Detection of PrP codon 108F and 189V alterations**

The exon 3 region of ES colonies surviving 6-thioguanine selection was amplified by PCR and the reaction product screened for the presence of codon 108 and 189 alterations. The codon 108F alteration was detected by allele specific oligonucleotide hybridisation and the codon 189V alteration was detected by the loss of a Bst EII site.

Heat-inactivated proteinase K-treated ES lysates were amplified with PrP exon 3 primers 348M and 411Y generating a 1,327bp product from both wildtype and targeted PrP alleles (Figure 5.6, p113). The codon 189V alteration was detected by the loss of a Bst EII site within the exon 3 PCR product (Figure 5.6, panel a, p113). PCR products from targeted 189V alleles remain undigested as the 189V alteration alters two nucleotides within the sequence G'GTNACC, ablating the BstEII recognition site. The codon 108F alteration is a single nucleotide change (CTC to TTC) and does not give rise to an RFLP with any commercially available restriction enzyme. Therefore PrP exon 3 PCR products were screened for the 108F alteration using allele specific oligonucleotide hybridisation (ASO) with [$\gamma$-$^{32}$P]ATP labelled 108F mutant 19-mer H1160 (5'-gccacatgcttgaagttgg-3': Westaway et al., 1987). This is illustrated in Figure 5.6 (p113).

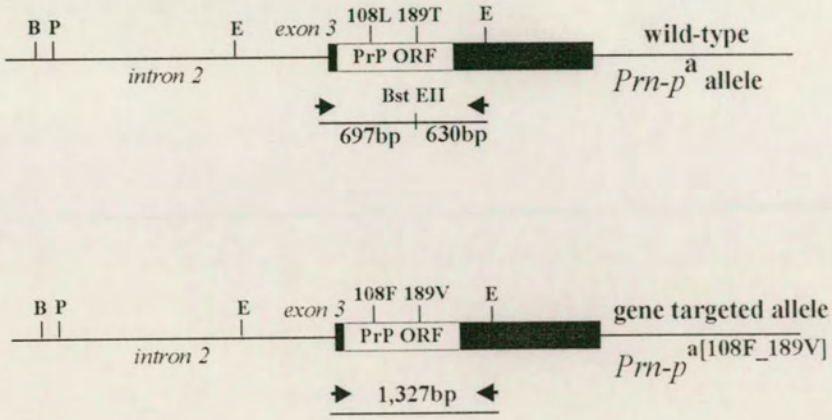
**Figure 5.6: Detection of ES cells with 108F_189V alterations**

Four ES clones with targeted codon 108F_189V alterations are shown: clones14, 18, 44 and 45. Following PCR amplification of exon 3, a 1,327bp PCR product is generated. Reaction products were first screened for the codon 189V alteration (**Panel a**) by digestion with BstEII (20U/tube) at 60°C for 2 hours. Digested PCR products were electrophoresed on a 1.5% agarose gel. PCR products from wildtype alleles have a centrally placed BstEII site and generate 2 bands of 697 and 630bp. PCR products derived from targeted 189V ES colonies do not digest with Bst EII. All clones are heterozygous and have one wildtype and one targeted allele.

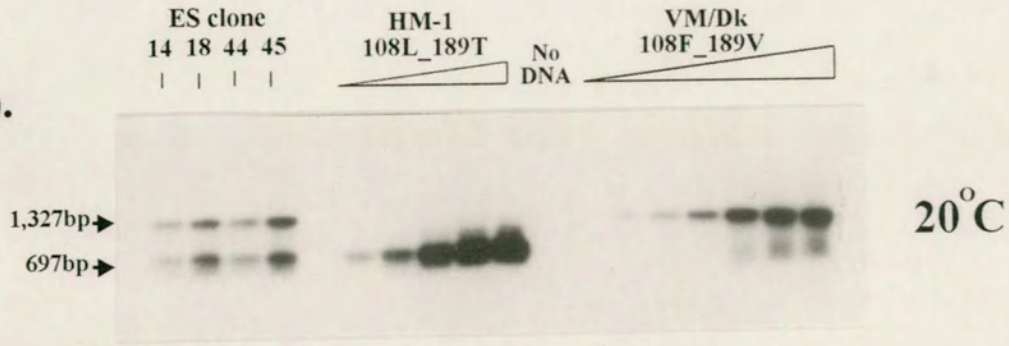
The codon 108F alteration was detected following the transfer of the Bst EII digested PCR product onto nylon membrane. Membranes were hybridised to $\gamma$-ATP$^{32}$ 5′ labelled 108F primer at 37°C and washed at low stringency at room temperature (20°C). The membrane was exposed to provide a control for loading variation between PCR samples (**Panel b**). This illustrates that the 108F oligonucleotide hybridises to 2 bands at low stringency: the larger 1,327bp band is derived from a targeted 189V allele which has lost its Bst EII site and the lower band represents the hybridisation of the 108F mutant oligo to wildtype PCR product under conditions of low stringency. 108F mutant ES clones 14, 18, 44 and 45 were detected by their high signal intensities, relative to controls, following a wash at raised stringency (62°C) (**Panel c**). In experiments involving the targeting vector p108F_189V where both mutations were expected on the same allele, 1,327bp exon 3 PCR products which resisted BstEII digestion and generated a 108F specific signal enabled the physical linkage of codon 108F and 189V alterations on the same allele.

Serial dilutions of positive and negative controls were included in all experiments. Positive controls were generated separately to avoid cross-contamination and consisted of PCR product generated from VM/Dk (PrP B: 108F_189V) mouse genomic DNA. Negative control PCR product was generated from HM-1 (PrP A:108L_189T) genomic DNA template. The detection of single 108F and 189V alterations is described in Moore et al., (1995), Appendix I.

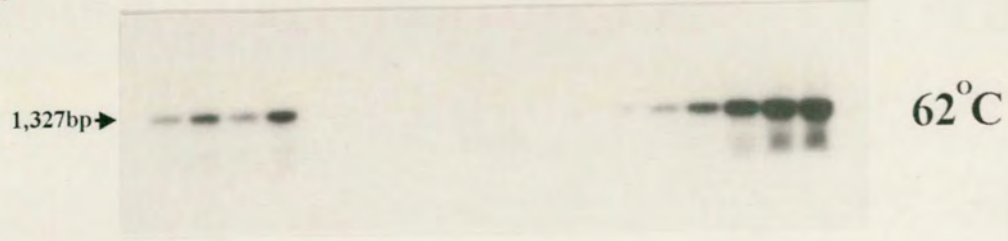
a).


B P
E
exon 3
108L 189T
E
wild-type
PrP ORF
intron 2
Prn-p a allele
Bst EII
697bp
630bp
B P
E
exon 3
108F 189V
E
gene targeted allele
PrP ORF
intron 2
Prn-p a[108F_189V]
1,327bp

b).


ES clone
14 18 44 45
HM-1
108L_189T
No
DNA
VM/Dk
108F_189V
1,327bp
697bp
20°C

c).


1,327bp
62°C

**Section 5.3.4: Southern analysis of PCR positive ES clones**

As described previously, 6-thioguanine resistant ES colonies electroporated with the p108F_189V targeting vector were screened for the 108F and 189V alterations by hybridisation to a [$\gamma$–$^{32}$P]ATP labelled codon 108F oligonucleotide and BstEII digestion respectively. Similar targeting frequencies were observed in two independent experiments using different *Prn-p* ⁻ first step clones (4/60 with HM-1/Δ34 and 4/72 with HM-1/Δ27). A total of eight PCR/ASO positive clones were found to be gene targeted by Southern analysis.

Putative targeted clones were investigated for i) the loss of the HPRT minigene, ii) reconstruction of the targeted *Prn-p* allele and iii) the absence of additional non-homologous recombinants.

The second step replaces the HPRT minigene with *Prn-p* exon 3 sequence bearing codon 108F and 189V alterations. Therefore ES genomic DNA was investigated for the loss of the HPRT minigene by Bam HI/Xho I double digestion (Figure 5.7, panel a, p116). In one experiment, hybridisation to the 3′ probe (probe E) indicated that four of seven 6-thioguanine resistant clones had lost the 5.2 Kb band. These clones had a single 8.5Kb band, characteristic of an intact *Prn-p* exon 3. However, this banding pattern is not diagnostic of the gene targeted introduction of PrP codon 108F_189V alterations as this could also occur as a consequence of gene conversion.

Further investigation by BstEII digestion and hybridisation to an exon 3 coding probe demonstrated that clones 14, 18, 44 and 45 had one allele with the targeted codon 189 T to V alteration and one wildtype allele. This is illustrated in Figure 5.7, panel b (p116). The wildtype allele digests into two fragments of 5.2Kb and 3.2Kb whilst the targeted allele yields a single fragment of 8.4 Kb with the exon 3 coding probe (probe D). Clones 31, 37 and 46 do not have a codon 189T to V alteration and have retained the HPRT minigene. This is consistent with the retention of the 5.2 Bam HI/Xho I band as indicated in Figure 5.7 panel a (p116). No additional bands were observed in any of the gene targeted clones indicating that there were

**Fig 5.7: Southern analysis of ES clones bearing codon 108F_189V alterations**

Panel **a**). Autoradiograph of Bam HI/Xho I double digests of candidate targeted clones 14, 18, 31, 37, 44, 45 and 46 hybridised to the 700bp Bam HI/Eco RV 3′ probe (probe E). Bam HI/Xho I digests of HM-1 and HM-1/Δ34 are included as negative and positive controls respectively. Panel **b**). Autoradiograph of BstEII digested genomic DNA from the clones shown in panel a hybridised to a 1.1Kb Sma I/Eco RI probe (Probe D) with HM-1 and HM-1/Δ34 DNA controls.

Panel **a**) indicates that the gene structure of clones 14, 18, 44 and 45 has been converted from that of the first step HM-1/Δ34 line to that of the HM-1 parental line. This is demonstrated by the loss of the 5.2 Kb Bam HI/XhoI band characteristic of the first step *Prn-p* $^{a/-}$ clone structure. Panel **b**) indicates that in clones 14, 18, 44 and 45 the exon 3 Bst EII site has been lost. This is consistent with the introduction of a PrP codon 189V alteration into one allele and is indicated by the generation of a novel 8.4 Kb BstEII fragment from the 3.2 and 5.2 Kb fragments. This panel, together with those in Figure 5.8 (p118), indicates that there are no additional random integrants in any of the clones shown.

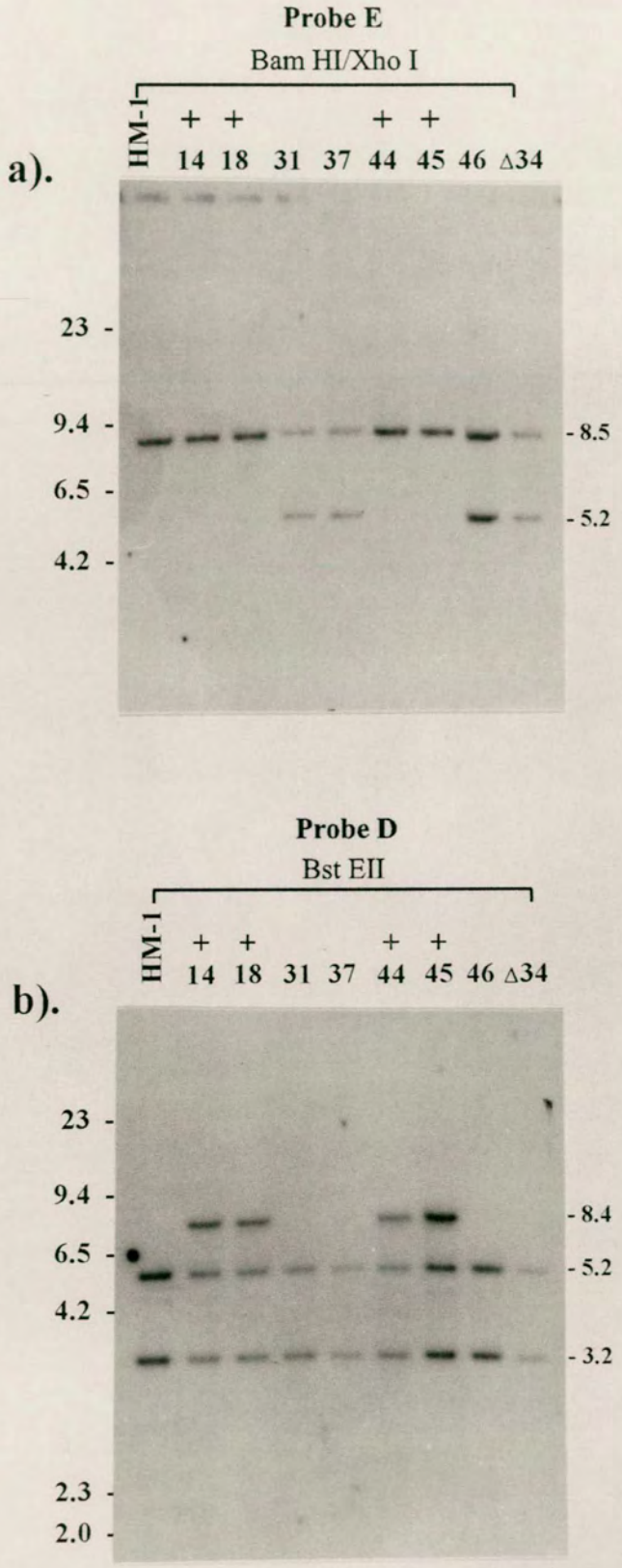
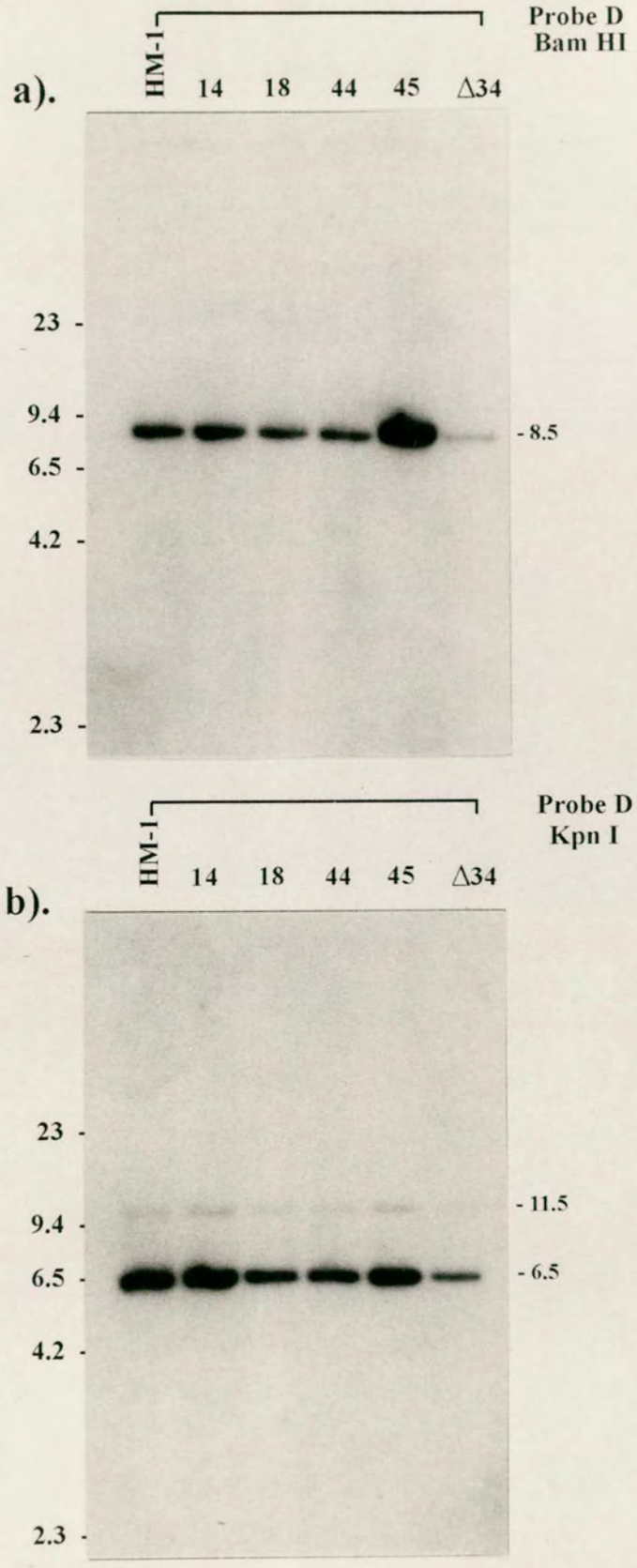

Probe E
Bam HI/Xho I
a).
HM-1
+ +
+ +
14 18 31 37 44 45 46 Δ34
23
9.4
6.5
4.2
8.5
5.2
Probe D
Bst EII
b).
HM-1
+ +
+ +
14 18 31 37 44 45 46 Δ34
23
9.4
6.5
4.2
2.3
2.0
8.4
5.2
3.2

**Fig 5.8: Further analysis of ES clones bearing PrP codon 108F_189V alterations**

Autoradiographs of ES clones with codon 108F_189V alterations, depicted in Figure 5.6. Panel **a**). Bam HI digests of clones 14, 18, 44 and 45 hybridised to the 1.1Kb Sma I/Eco RI coding region probe. HM-1 and HM-1/Δ34 DNA are controls. Panel **b**) Kpn I digests of the same clones shown in panel a). Both panels indicate that the gene structure of clones 14, 18, 44 and 45 has been converted from that of the HM-1/Δ34 line back to that of the HM-1 parental line -consistent with gene targeting. A more detailed analysis of the *Prn-p* structure of clone 45 is provided in Chapter 6 (p125).


a).
HM-1
14
18
44
45
Δ34
Probe D
Bam HI
23
9.4
6.5
4.2
2.3
8.5
b).
HM-1
14
18
44
45
Δ34
Probe D
Kpn I
23
9.4
6.5
4.2
2.3
11.5
6.5

no additional non-homologous recombination events.

Further analysis of clones 14, 18, 44 and 45 by hybridisation of probe D to Bam HI and Kpn I digests confirmed that the restriction pattern around *Prn-p* in these clones was consistent with a wild-type *Prn-p* allele (Figure 5.8, p118). Furthermore, the use of probe D in these studies did not reveal any evidence of random integrants in these clones.

Single PrP codon 108F and 189V alterations were introduced into ES cells using the above procedure, however none of these ES clones generated germline chimeras. Therefore data relating to these experiments is not presented.

## Section 5.4: Discussion

### Section 5.4.1: A two step double replacement gene targeting method

The work presented here and in chapter 3 illustrates that a double replacement gene targeting method can be used to introduce subtle alterations into ES cells. This enables the production of multiple lines of ES cells, and ultimately mice, with defined PrP gene alterations (Moore et al., 1995). The method has a number of advantages over alternative methods such as "hit and run". One important advantage is that no selectable markers are left in the targeted locus after the second step -it is possible that markers might influence the expression of targeted locus in an unpredictable manner. Recently, evidence has emerged from experiments directed at the granzyme gene cluster which show that the PGK/Neo selectable marker can dysregulate genes neighbouring targeted loci up to 100Kb distant (Pham et al., 1996). Given the widespread use of this marker cassette it is possible that this phenomenon might be shared by a number of gene targeted ES lines in which this marker is retained. It is possible that a number of mouse phenotypes which have been attributed to the specific effects of targeted gene deletions or to subtle gene alterations, might instead arise from the effects of the selectable markers. The potential for confusion is likely to be exacerbated by the common arrangement of mammalian genes into gene families in which gene isoforms with similar or complementary functions are arranged into clusters. This has been observed in a number of gene knockouts (Olson et al., 1996).

It is possible that the adult onset neurological phenotype observed in two independently generated lines of PrP nulls (Sakaguchi et al., 1995,1996 and chapter 4 of this thesis) is due to the effect of the selectable marker on neighbouring genes.

These observations suggest that the two-step gene targeting strategy employed here is appropriate to a wide range of applications. This is because, following the second step, the gene targeted allele contains no foreign sequence and differs from the wild-type allele only with respect to the engineered subtle alterations. The evidence for the deleterious effects of selectable markers suggests (Pham et al., 1996; Olson et al., 1996) that future gene knockout vectors should be designed with LoxP (or FRT/FLP recombinase) recognition sites flanking selectable markers to enable their removal from the targeted locus.

A number of parameters are thought to influence the efficiency with which gene targeted clones are retrieved with this two step gene targeting system. The selection system used for the second step enriches on the basis of HPRT function i.e cells expressing HPRT are killed. However this selects for at least three classes of cell: i) HPRT deficient HM-1 cells carried over from the first targeting step as a minor contaminant, ii) cells which have spontaneously lost HPRT function and iii) homologous recombinants. The problems associated with HM-1 carryover can be overcome by generating a number of independent step 1 clones and performing second steps in ES lines with low levels of spontaneous 6-thioguanine resistance (Stacey et al., 1994). Cells which have lost HPRT function can arise for a number of reasons including minigene deletion, gene conversion and methylation of the minigene promoter. The role of methylation in silencing of the HPRT minigene has been explored in step 1 Ligase 1 heterozygous knockout lines which were found to be difficult to retarget. It appears that methylation of the minigene at high frequency results in a high rate of 6-thioguanine resistance which precludes the efficient retrieval of targeted clones in the second targeting step. The Pgk-1 promoter used to drive this minigene is methylated, suggesting that an alternative promoter (HPRT), which is not methylated to the same extent, is more appropriate for use at this locus (D. Melton, unpublished results).

**Section 5.4.2: Recent gene targeting developments**

A number of new reagents have enabled technical improvements to gene targeting strategies. These include the use of viral internal ribosome entry site (IRES) and site-specific recombinases such as the yeast 2u circle FLP recombinase and bacteriophage P1 Cre recombinase. The IRES enables the translation of two distinct mRNA species from the same bicistronic transcript. This enables positive-negative selection to act upon the products of a single transcript produced from a single Neo/HSV-thymidine kinase selectable marker under the control of a shared promoter and poly A addition site. This enables strong and predictable expression of both markers and can be further improved by utilising promoter or polyA trap strategies.

Cre recombinase can mediate recombination between a pair of *LoxP* sites *in vitro* or *in vivo* (Abremski, Hoess and Sternberg, 1983; Gu et al., 1993) resulting in deletion of all intervening sequence. This enzyme can also mediate the insertion of sequence bearing a single LoxP site into chromosomal loci marked with a LoxP site (Sauer and Henderson, 1990). Cre-LoxP can be used in mammalian cells to excise *LoxP* flanked DNA and has been used for a number of gene targeting strategies including conditional gene deletion (Gu, Zou and Rajewsky, 1993) and targeted chromosomal translocations (Smith et al., 1995).

The Cre-*LoxP* system can also be adapted to remove selectable markers from targeted loci enabling the introduction of subtle alterations into genes in a single step (Taki, Meiering and Rajewsky, 1993; Gu , Zou and Rajewsky, 1993).

One group has recently reported the targeted replacement of the entire mouse PrP coding region with the human homologue (Kitamoto et al., 1996). *Lox P* flanked selectable markers were removed efficiently by transient expression of Cre recombinase in fertilised eggs from germline mice (Araki et al., 1995). However this targeted allele retained a single *Lox P* site in the PrP 3'UTR. No expression of human PrP was noted from mice made with these cells -perhaps because the human PrP coding region contained a codon 145 stop mutation

**Figure 5.9: A highly efficient single step PrP gene targeting method**

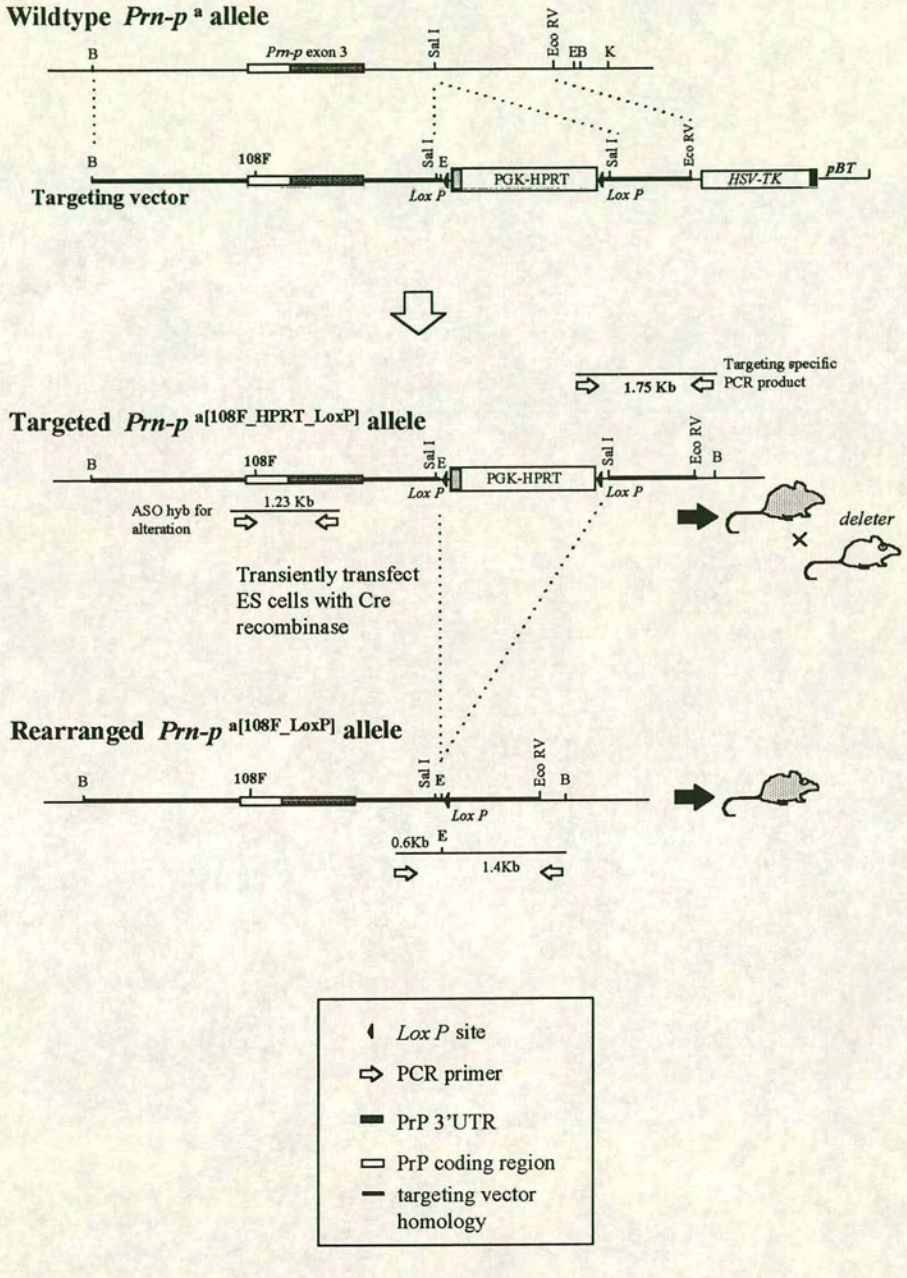
This method is analogous to the first step of the two step method and takes advantage of the high gene targeting frequencies commonly achieved for first steps using the HM-1/HPRT system. The vector is similar to a PrP knockout vector but differs in several critical respects: i) it contains a mutant PrP coding region, ii) the HPRT minigene does not inactivate PrP but inserts downstream of PrP exon 3 and iii) the PGK/HPRT minigene is flanked by Lox P sites, enabling deletion *in vitro* or *in vivo* by Cre recombinase.

Selection for the gene targeting step is provided using positive-negative selection with HAT and gancyclovir as described in chapter 3 for the PrP knockout. Low passage number targeted ES cells can be injected at this stage to make chimeras, however the evidence for the deleterious effects of the PGK/HPRT minigene in mid-adult *Prn-p* knockout mice (Chapter 4) indicates that the selectable marker is best removed. This can be done by mating young adult germline mice bearing this allele with a strain of mouse which expresses Cre recombinase in gametes, such as the *deleter* mouse strain produced in the Rajewsky lab (Scwenk, Baron and Rajewsky, 1996). Unfortunately these mice are of mixed genetic background and the targeted line would lose its co-isogenic status.

However these cells can be subjected to Cre recombinase-mediated deletion of the HPRT minigene *in vitro* and ES cells in which the minigene has been deleted can be used for blastocyst injection, at lower passage than is common in ES clones made using the two step method.

One limitation of this method is that a single LoxP site is retained downstream of PrP exon 3. Suitable controls for this site can be provided by making a line of mice in which the mutation has not been introduced but which have the other features of this allele -these ES cells arise as a by-product of the gene targeting process. One line should suffice as a control for any number of PrP mutations introduced using this method. This method is intended to be complimentary to the double replacement method.

A preliminary experiment to introduce the PrP 108F alteration in ES cells was very encouraging: 59/96 of the HAT/gancyclovir resistant ES colonies were targeted and 40% of them had introduced the 108F alteration. This is one of the highest targeting frequencies gained to date for a subtle alteration in mammalian cells.


Wildtype Prn-p a allele
B
Prn-p exon 3
Sal I
Eco RV
EB
K
B
108F
Sal I
E
PGK-HPRT
Sal I
Eco RV
HSV-TK
pBT
Targeting vector
Lox P
Lox P
Targeting specific PCR product
1.75 Kb
Targeted Prn-p a[108F_HPRT_LoxP] allele
B
108F
Sal I
E
PGK-HPRT
Sal I
Eco RV
B
Lox P
Lox P
ASO hyb for alteration
1.23 Kb
deleter
Transiently transfect ES cells with Cre recombinase
Rearranged Prn-p a[108F_LoxP] allele
B
108F
Sal I
E
Eco RV
B
Lox P
0.6Kb
E
1.4Kb
Lox P site
PCR primer
PrP 3'UTR
PrP coding region
targeting vector homology

(Kitamoto, Iizuka and Tateishi, 1993). Stop codons have been reported to dramatically reduce mRNA levels in a number of human genes associated with disease such as CFTR (Hamosh et al., 1991), β-globin (Baserga et al., 1988) and triosephosphate isomerase (Belgrader et al., 1993). A similar phenomenon was observed in a transgenic line expressing human PrP with a stop codon at codon 143 (Fischer et al., 1996). A number of lines of these mice expressed very low levels of PrP mRNA and no detectable PrP [c].

A broadly similar method was adapted for use with the HPRT/HM-1 system during the course of this thesis and has been used to introduce single PrP codon 108F and 189V alterations into HM-1 ES cells. This single step method appears to be highly efficient and is described in Figure 5.8. It is hoped that these cells will enable the generation of mice with single PrP codon 108F and 189V alterations. Scrapie challenge of such mice may reveal the relative contribution of each of these dimorphisms to the control of scrapie incubation time.

**Section 5.5: Summary**

Eight clones bearing targeted PrP 108F_189V alterations were generated using a two step double replacement gene targeting method. The gene targeted *Prn-p* allele has been named, *Prn-p* [a[108F_189V]] to distinguish it from both wildtype *Prn-p* [a] and *Prn-p* [b] alleles. The germline transmission of this allele and analysis of its CNS expression in adult mice is described in more detail in the next chapter. A number of clones bearing single PrP alterations to codons 108 and 189 were generated but did not transmit to the germline. A modified method has been developed and used successfully to introduce single codon 108F and 189V alterations into ES cells in a single targeting step and blastocyst injections using low passage ES cells are currently underway.

# Chapter 6

# Generation and analysis of mice with PrP Codon 108 and 189 alterations

## Section 6.1:Introduction

This chapter describes the germline transmission of the *Prn-p* $^{a[108F\_189V]}$ allele, analysis of the allele structure and the analysis of PrP expression from this allele at the level of mRNA and protein in adult mouse brain.

## Section 6.2: Germline transmission of the *Prn-p* $^{a[108F\_189V]}$ allele

A number of targeted ES clones bearing the *Prn-p* $^{a[108F\_189V]}$ allele were used for blastocyst injection. Targeted ES clone HM-1/Δ34/BB#45 generated 1 male and 3 female chimeras. One female chimera (No.19) was mated with an inbred male 129/Ola and transmitted the gene targeted allele through the germline[1] to a single male 129/Ola founder. This founder was co-isogenic with 129/Ola.

The HM-1 ES line is HPRT deficient and was derived from E14TG2a[2] (Hooper et al., 1987). E14TG2a and HM-1 share a large HPRT 5′ deletion which has deleted the promoter and upstream exons resulting in a non functional allele (Simon Thompson, PhD thesis; Thompson et al., 1989). HPRT is X-linked, therefore male HM-1 ES cells are HPRT deficient. Although HPRT deficiency in humans causes Lesch-Nyhans disease (Lesch and Nyhan, 1964), HPRT deficiency in mice has few deleterious effects. A number of workers have generated HPRT deficient mice in an attempt to develop an animal model of Lesch-Nyhan disease, however HPRT deficiency causes no overt phenotype in mice unless they are more than 2 years old

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[1] Germline transmission from female chimeras has been observed on a number of occasions in this laboratory (David Melton, personal communication) and by others (Kuehn et al., 1987). Although the HM-1 ES line is male and XY ES cells bias towards the production of male chimeras, female chimeras are not uncommon and can contribute to the germline. XY ES cells do not normally undergo oogenesis in chimeras but often convert the sex of a female recipient blastocyst to a male phenotype enabling spermatogenesis and germline transmission. Sex conversion is not always complete -sometimes resulting in an infertile hermaphrodite. However, germline transmission from a male ES line in a female chimera is presumably due to the loss or silencing of all or part of the Y chromosome such that the ES cell-derived germ cells and oocytes are effectively XO (Papaioannou and Johnson, 1993).

[2] E14TG2a was isolated from the E14 ES line as a 6-thioguanine resistant clone (Handyside et al., 1989).

(Williamson, Hooper and Melton, 1992) or if they are treated with APRT inhibitors (Wu and Melton, 1993).

Given the uncertain effect of HPRT deficiency in mice challenged with scrapie it was considered prudent to ensure that any gene targeted lines derived from HM-1 ES cells did not carry the HPRT mutant allele.

The *Prn-p* $^{a[108F\_189V]}$ heterozygous male founder carried the mutant HPRT allele. All *Prn-p* $^{a[108F\_189V]}$ positive progeny derived from crosses between the founder and 129/Ola inbred stock were screened for the mutant HPRT allele. Tail genomic DNA from 50 *Prn-p* $^{a[108F\_189V]}$ heterozygous pups was screened by BamHI digestion and Southern analysis by hybridisation to a HPRT probe (Konecki et al., 1982)(data not shown). Only mice bearing a wildtype HPRT allele were retained for further breeding. This ensured the elimination of the mutant HPRT allele from this mouse line.

To generate sufficient numbers of 129/Ola mice for 301V challenge, HPRT wildtype, *Prn-p* $^{a[108F\_189V]}$ heterozygous mice were crossed, progeny were PrP genotyped by a combination of PCR and BstEII digestion for the 189V alteration and allele specific oligonucleotide hybridisation for the 108F alteration. Groups of 129/Ola wildtype mice and mice heterozygous and homozygous for the *Prn-p* $^{a[108F\_189V]}$ allele were transferred into quarantine at the BBSRC/MRC Neuropathogenesis Unit, Edinburgh. The first cohort were challenged with 301V and a second set of mice were challenged with ME7[3].

**Section 6.3: Analysis of *Prn-p* $^{a[108F\_189V]}$ allele structure.**

The structure of the wildtype *Prn-p* $^{a}$ and *Prn-p* $^{a[108F\_189V]}$ alleles were compared by restriction mapping using a variety of *Prn-p* probes (Figures 6.1, p130, Figure 6.2, p133). The HM-1 *Prn-p* $^{a}$ allele was compared with that of the germline *Prn-p* $^{a[108F\_189V]}$ heterozygous ES clone (HM-1/Δ34/#BB45), the *Prn-p* $^{a[108F\_189V]}$ heterozygous founder (BB1000) and a mouse homozygous for the *Prn-p* $^{a[108F\_189V]}$ allele (BB1507). VM/Dk DNA was also included and enabled a comparison of the

[3] The ME7 challenge experiment is still underway so the data is not yet available.

structures of the *Prn-p* [a] and *Prn-p* [a[108F_189V]] alleles with that of the *Prn-p* [b] allele and is discussed in Section 6.3.1.

Hybridisation with the intron 1/exon 2 probe (probe B) indicates that a large 10.9Kb Bam HI fragment containing the promoter, exon 1, intron1, exon 2 and part of intron 2 is shared in all 129/Ola lines (Figure 6.1, panel a, p130). An intron 2 probe (probe C) indicates that all 129/Ola samples share a common 6.7Kb Bam HI fragment (Figure 6.1, panel b, p130) and hybridisation to an exon 3 probe (probe D) indicates that all lines, including VM/Dk, share a common 8.5Kb Bam HI fragment (Figure 6.1, panel c, p130).

Hybridisation to BstEII digested DNA with an exon 3 probe (probe D) confirmed the presence of 2 BstEII bands of 5.2 and 3.2 Kb in HM-1 and in all lines encoding the wild-type *Prn-p* [a] allele (Figure 6.1, panel d, p130). In lines heterozygous for the *Prn-p* [a[108F_189V]] allele, these wild-type bands are present in addition to an 8.4 Kb band. The 8.4 Kb band is the sum of the 5.2 and 3.2 Kb wild-type bands and arises from the PrP 189V alteration which ablates a BstEII site within exon 3. A single band of 8.4 Kb is present in DNA homozygous for the gene targeted allele.

Hybridisation of the exon 3 probe (probe D) to a Kpn I digest (Figure 6.1, panel d) (p130) indicates that all lines share a common 6.5Kb exon 3 fragment. Hybridisation of BstEII and Kpn I digests with probe E (Figure 6.1, panel E, p130) extended the analysis downstream of exon 3. This revealed no differences between the 129/Ola DNA samples and indicated that they shared a 11 Kb BstEII and a 6.5 Kb Kpn I fragment.

These mapping studies demonstrate that the *Prn-p* [a[108F-189V]] allele has the structure of a *Prn-p* [a] allele which has been modified by the removal of a single exon 3 Bst EII site[4]. This is based on mapping studies covering in excess of 30 Kb, including 1Kb upstream of the promoter and more than 11Kb downstream of exon 3. The absence of additional banding or of altered banding patterns indicates that there are no

[4] The codon 108F alteration does not result in the gain or loss of a restriction site.

**Fig 6.1: Structure of the targeted *Prn-p* $^{a[108F\_189V]}$ allele**

Southern analysis of genomic DNA from ES cells and mouse tail samples using a variety of restriction digests and *Prn-p* probes B-E.

Panels a)-c). depict Bam HI digests.

Panels d) & e) depict BstEII and Kpn I digests.

Panel a). Bam HI digests hybridised to an intron 1/exon 2 probe (probe B)

Panel b). Bam HI digests hybridised to an intron 2 probe (probe C)

Panel c). Bam HI digests hybridised to an exon 3 probe (probe D)

Panel d). Bst EII and Kpn I digest hybridised to an exon 3 probe (probe D)

Panel e). Bst EII and Kpn I digests hybridised to a probe downstream of exon 3 (probe E)

The same loading order was used in all panels:

"HM-1": 129/Ola HM-1 ES cells, encoding 2 wildtype *Prn-p* $^{a}$ alleles

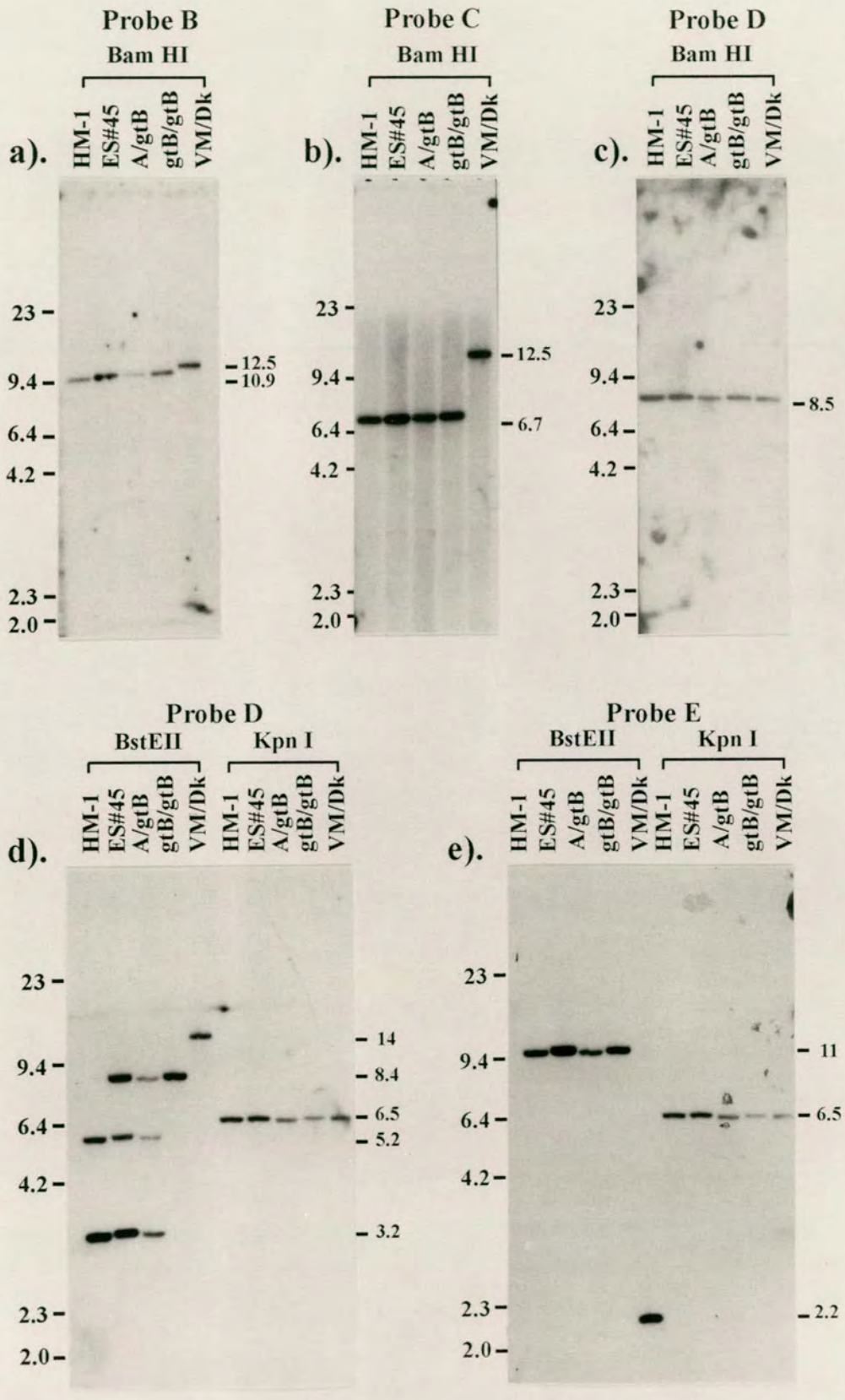
"ES#45": 129/Ola ES clone HM-1/Δ34/BB#45, *Prn-p* $^{a[108F\_189V]}$ heterozygous

"A/gtB": 129/Ola *Prn-p* $^{a[108F\_189V]}$ heterozygous founder (BB1000)[5]

"gtB/gtB": 129/Ola *Prn-p* $^{a[108F\_189V]}$ homozygote

"VM/Dk": VM/Dk *Prn-p* $^{b}$ homozygote

[5] The Gavin Hastings Memorial Mouse or "wee Gav"


Probe B
Bam HI
HM-1
ES#45
A/gtB
gtB/gtB
VM/Dk
a).
23
9.4
6.4
4.2
2.3
2.0
12.5
10.9
Probe C
Bam HI
b).
12.5
6.7
Probe D
Bam HI
c).
8.5
Probe D
BstEII
Kpn I
d).
14
8.4
6.5
5.2
3.2
Probe E
BstEII
Kpn I
e).
11
6.5
2.2

detectable deletions, insertions or other rearrangements in the gene targeted allele. These studies are consistent with the hybridisation studies shown in chapter 5 which indicated that there were no additional non-homologous recombination events in the ES line HM-1/Δ34/BB#45.

**Section 6.3.1: Comparison of *Prn-p* [a] and *Prn-p* [b] alleles**

Comparison indicated that the 129/Ola *Prn-p* [a] and VM/Dk *Prn-p* [b] alleles had a similar general gene structure, although a number of differences were apparent (Figures 6.1, p130 and Figure 6.2, p133). Hybridisation of an exon 3 probe (probe D) to BstEII digests revealed a 14 Kb band in VM/Dk and 2 bands in 129/Ola *Prn-p* [a] (Figure 6.1, panel d). The VM/Dk 14Kb BstEII fragment arises from digestion at a site downstream of exon 3 and an upstream site close to exon 2 (Figure 6.2, p133) whilst the 129/Ola *Prn-p* [a] allele has a BstEII site within exon 3, a site 1.9Kb upstream of the 8.5Kb Bam HI fragment and a third site 3.2 Kb downstream of exon 3. Hybridisation to a probe 3′ of exon 3 (probe E) reveals a BstEII RFLP. Here 129/Ola have a band of 11Kb whilst VM/Dk have a much shorter band of 2.2 Kb (Figure 6.2, panel e).

A number of differences between 129/Ola *Prn-p* [a] and VM/Dk *Prn-p* [b] are apparent in intron 2 (Figure 6.1, panels a and b, p130). Hybridisation to probe B indicates that VM/Dk has a slightly larger Bam HI fragment (Figure 6.1, panel a, p130). This difference probably arises from a polymorphic site within intron 2 rather than at the promoter. This is because other mapping studies performed on a genomic clone containing the *Prn-p* [b] promoter [6] from the ILn/J strain indicates that the Bam HI site immediately upstream of the 129/Ola and ILn/J promoters are shared. It is likely that this is also the case with VM/Dk as it also encodes the *Prn-p* [b] allele. That this RFLP arises from a site within intron 2 is consistent with differences in Bam HI

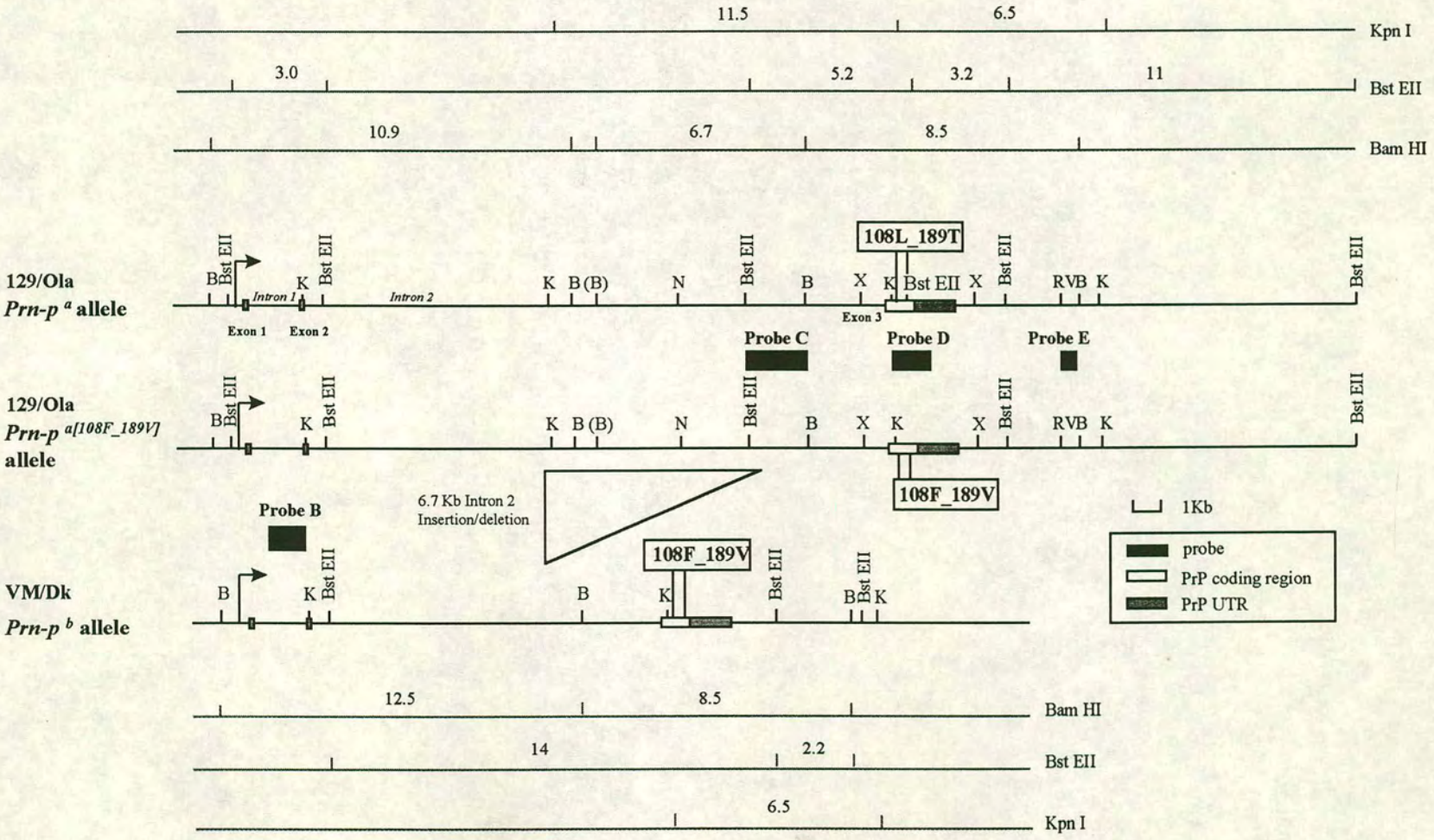
[6] pTZE1B/K, a gift from Jean Manson, BBSRC/NPU Neuropathogenesis Unit, Edinburgh.

**Fig 6.2: A map of *Prn-p* $^{a}$, *Prn-p* $^{b}$ and the targeted *Prn-p* $^{a[108F\_189V]}$ allele**

A restriction map depicting the structure of the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele, the parental 129/Ola *Prn-p* $^{a}$ allele and the VM/Dk *Prn-p* $^{b}$ allele. This map is derived from Southern analysis data shown in Figure 6.1. The main non-coding differences between *Prn-p* $^{a}$ and *Prn-p* $^{b}$ are RFLPs within the introns and a difference in the size of intron 2. The intron 2 size difference arises from a ~6.7Kb insertion/deletion polymorphism (Westaway et al., 1994a).

The promoter is indicated by an arrow and the approximate position of the intron 2 insertion/deletion polymorphism is indicated by the triangle. The endpoints of this polymorphism are unmapped. Probes are indicated by filled boxes over the *Prn-p* region of origin.

All sizes in Kb. Enzyme abbreviations: B, Bam HI; Bst, Bst EII; E, Eco RI; H, Hind III; K, Kpn I; N, Not I; RV, Eco RV; X, Xba I. (B), a Bam HI site detected by mapping λDASHII *Prn-p* clones but which is not detectable in genomic disgests with available probes.


11.5
6.5
Kpn I
3.0
5.2
3.2
11
Bst EII
10.9
6.7
8.5
Bam HI
129/Ola
Prn-p a allele
108L_189T
Bst EII
B Bst EII K Bst EII
Intron 1
Intron 2
K B (B) N Bst EII B X K Bst EII X Bst EII RVB K
Bst EII
Exon 1
Exon 2
Exon 3
Probe C
Probe D
Probe E
129/Ola
Prn-p a[108F_189V] allele
B Bst EII K Bst EII
K B (B) N Bst EII B X K X Bst EII RVB K
Bst EII
108F_189V
6.7 Kb Intron 2
Insertion/deletion
Probe B
1Kb
probe
PrP coding region
PrP UTR
VM/Dk
Prn-p b allele
B K Bst EII
B
108F_189V
K
Bst EII
B Bst EII K
12.5
8.5
Bam HI
14
2.2
Bst EII
6.5
Kpn I

banding apparent with an intron 2 probe (Figure 6.1, panel b, p130). Bam HI RFLPs[7] have been described in a number of mouse strains (Westaway et al., 1994a).

**Section 6.4: Expression from the targeted *Prn-p* $^{a[108F\_189V]}$ allele.**

The PrP allotype expressed from the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele is referred to here as PrP gtB. This distinguishes it from PrP A and PrP B allotypes which are the products of wildtype *Prn-p* $^{a}$ and *Prn-p* $^{b}$ alleles respectively. Mice heterozygous and homozygous for the targeted *Prn-p* $^{a[108F\_189V]}$ allele are referred to as PrP A/gtB and PrP gtB/gtB. Although PrP gtB and PrP B have identical predicted amino acid sequences, this nomenclature acknowledges that they are expressed from different *Prn-p* alleles.

Expression of PrP mRNA from the targeted *Prn-p* $^{a[108F\_189V]}$ allele was initially investigated in ES cells by RT-PCR. Discrimination between PrP A and PrP gtB transcripts was possible by BstEII digestion and codon 108F allele specific oligonucleotide hybridisation. This confirmed expression of PrP gtB transcripts of the predicted size from the targeted allele (data not shown). Following germline transmission of this allele, brain RNA was investigated for PrP transcripts (Figure 6.3, panels a and b, p136). This confirmed expression from the *Prn-p* $^{a[108F\_189V]}$ allele and enabled a comparison with expression levels in mice encoding wild-type *Prn-p* $^{a}$ and *Prn-p* $^{b}$ alleles. Wildtype PrP A/A mice had a PrP mRNA size of 2.1Kb. The size and abundance of this transcript was shared with PrP A/gtB and PrP gtB/gtB mice. Comparison of the above lines with VM/Dk, which express the PrP B allotype from the wildtype *Prn-p* $^{b}$ allele, revealed no apparent differences in PrP mRNA abundance or size. Evidence that *Prn-p* $^{a}$ and VM/Dk *Prn-p* $^{b}$ mice have similar levels of brain PrPmRNA is consistent with previous studies in *Prn-p* $^{a}$ and *Prn-p* $^{b}$ mice (Westaway et al., 1987; Manson, McBride and Hope, 1992). Figure 6.3 (panel a, p136) also indicates that PrP gtB/- mice encoding only one functional *Prn-p* $^{a[108F\_189V]}$ allele have ~50% reduced levels of PrP mRNA.

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[7] This polymorphic region was present on one of the λDASH II clones retrieved from the HM-1 library. It was cloned into pBluescript as a 10Kb Not I fragment, however no further studies have been performed with this clone (pNot10).

**Figure 6.3: PrP expression in adult brain**

Panels a). and b). Northern analysis of male adult mouse brain total RNA Panel c). SDS-PAGE analysis of $PrP^c$ in uninoculated mouse brain.

Panel a) 10ug of total RNA from whole mouse brain hybridised to PrP coding probe D. Panel b). mouse α-actin reprobe of the same membrane shown in a).

Panel c). 12% SDS-PAGE (Laemmli, 1970) of brain homogenates immunoblotted with rabbit anti-mouse PrP antiserum 1A8 at a dilution of 1:1000. All samples contained 50ug of total brain protein. Prestained molecular weight markers, New England Biolabs. All sizes in KDa.

Mouse line abbreviations:

| | |
|---|---|
| PrP A/A: | wild-type 129/Ola *Prn-p* $^{a}$ |
| PrP A/gtB: | 129/Ola *Prn-p* $^{a/a[108F\_189V]}$ |
| PrP gtB/gtB: | 129/Ola *Prn-p* $^{a[108F\_189V]/a[108F\_189V]}$ |
| VM/Dk PrP B/B: | VM/Dk *Prn-p* $^{b}$ |
| PrP gtB/-: | 129/Ola *Prn-p* $^{a[108F\_189V]/-}$ |
| PrP -/-: | 129/Ola *Prn-p* $^{-/-}$ |

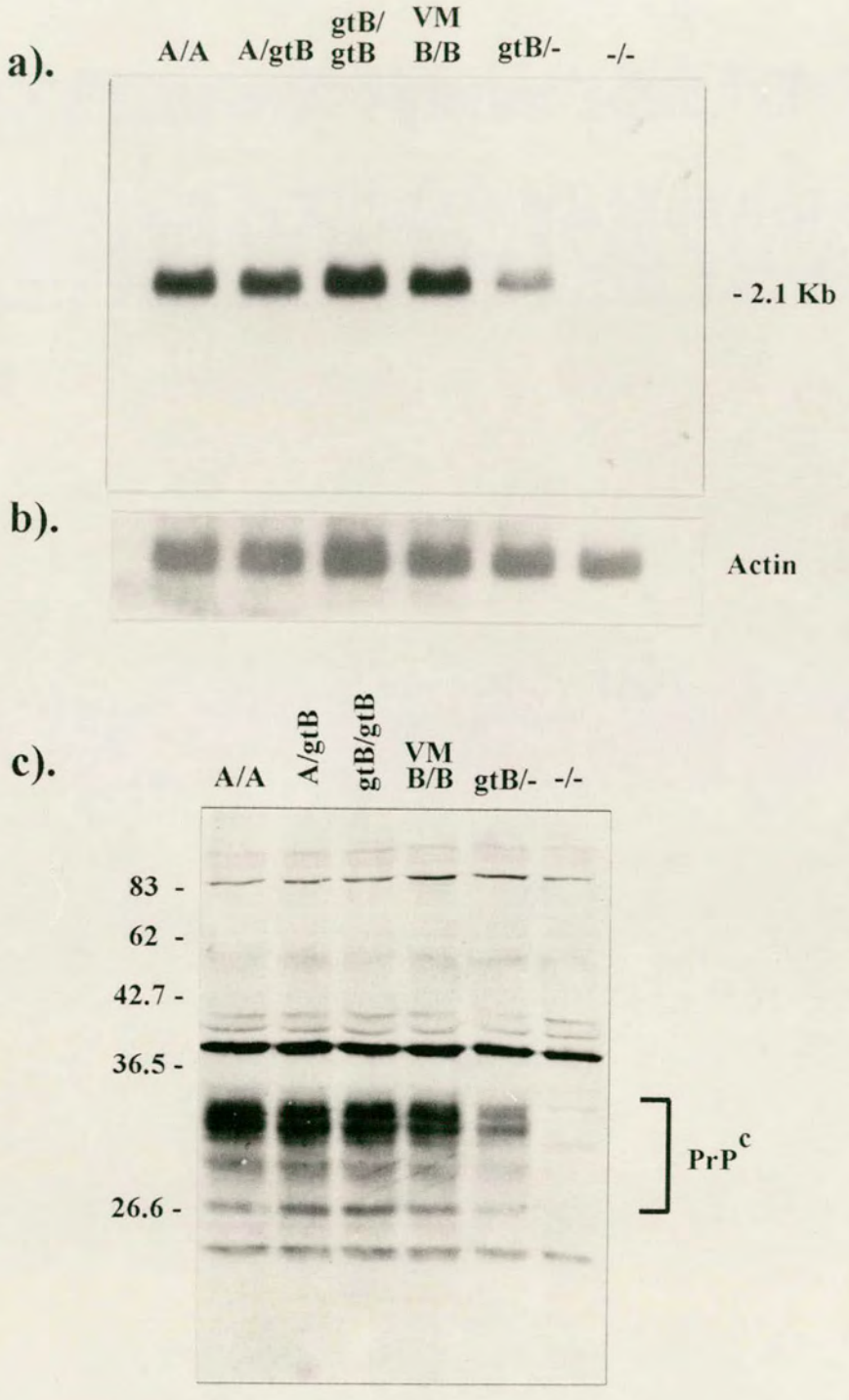
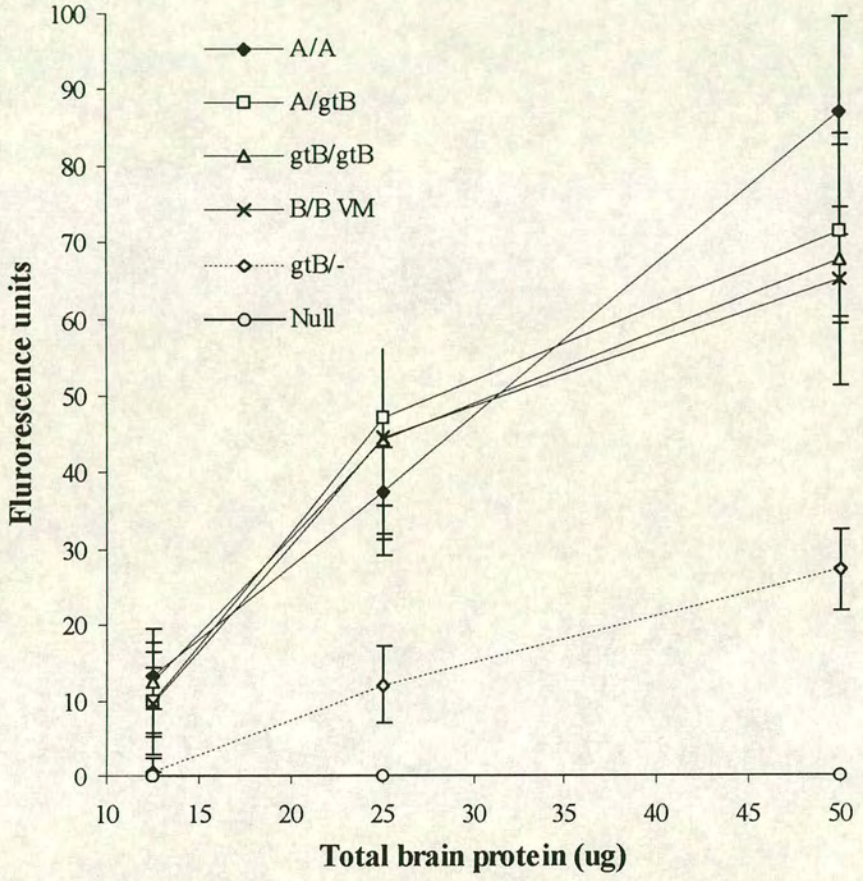

a).
A/A
A/gtB
gtB/ gtB
VM B/B
gtB/-
-/-
- 2.1 Kb
b).
Actin
c).
A/A
A/gtB
gtB/gtB
VM B/B
gtB/-
-/-
83 -
62 -
42.7 -
36.5 -
26.6 -
PrP c

**Figure 6.4: Comparison of brain $PrP^c$ expression levels by densitometry**

Comparison of $PrP^c$ expression levels in wild-type brain and in mouse lines encoding the *Prn-p* $^{a[108F\_189V]}$ allele. Duplicate serial dilutions of crude brain homogenate representing 50ug, 25ug and 12.5ug of total protein samples from 2 age matched adult males were separated by 12%SDS-PAGE (Laemmli, 1970). PrP was visualised by immunoblotting with PrP antiserum 1A8 at a dilution of 1:1000. Bound antibody was detected with an alkaline phosphatase conjugated secondary antibody at a dilution of 1:10,000 (Vistra ECF kit, Amersham) in combination with a fluorescent alkaline phosphatase substrate. The PrP signal was quantitated with a Molecular Dynamics STORM fluorescent imaging system interfaced to a Mackintosh PowerPC. Following background subtraction and correction for the null signal, mean signals (+/- standard error) were plotted against total protein load (ug).


A/A
A/gtB
gtB/gtB
B/B VM
gtB/-
Null
Flurorescence units
Total brain protein (ug)
0
10
20
30
40
50
60
70
80
90
100
10
15
20
25
30
35
40
45
50

The level of $PrP^C$ in uninoculated mice was investigated by western blotting (Burnette, 1981). This confirmed that the targeted *Prn-p* $^{a[108F\_189V]}$ allele was expressed at the level of protein in adult brain and enabled a comparison of $PrP^C$ brain expression levels in wildtype 129/Ola PrP A/A, PrP A/gtB, PrP gtB/gtB mice and VM/Dk PrP B/B mice. PrP immunoblot analysis of 50ug of total protein from crude brain homogenates (Figure 6.3, panel c, p136) indicated that $PrP^c$ brain expression levels in these lines were indistinguishable. Figure 6.3 (panel c, p136) also illustrates that the SDS-PAGE banding patterns appear unaltered in mice expressing PrP gtB from the *Prn-p* $^{a[108F\_189V]}$ allele.

Further studies were performed on whole brain PrP expression by measuring the PrP signal with fluorescence densitometry. PrP immunoblots were performed as before but with serial dilutions of crude brain homogenate containing 50ug, 25ug and 12.5ug of total brain protein. Figure 6.4 (p138) illustrates that this approach revealed no significant differences in the levels of $PrP^c$ expression between 129/Ola PrP A/A, PrP A/gtB and PrP gtB/gtB mice. No significant difference (95% level, Students t-test) was noted between the above lines and PrP B/B VM/Dk mice encoding a wildtype *Prn-p* $^b$ allele. A small difference was noted in the 50ug signal between the PrP A/A and the other lines expressing from two alleles, however this was not significantly different from the others at the 95% level (Students t-test). Conversely, comparison of the PrP gtB/- signal with that of the other lines detected a significant difference (95% level, Students t-test) -indicating that with this method a ~40-50% reduction in expression can be detected.

## Section 6.5: Discussion

### Section 6.5.1: *Prn-p* $^{a[108F\_189V]}$ allele structure

The gene targeted *Prn-p* $^{a[108F\_189V]}$ allele structure was compared with that of the parental *Prn-p* $^{a}$ allele. These studies confirmed that the gene targeted allele had the predicted structure and that there were no apparent deletions or other rearrangements. A number of lines of evidence suggest that deletions within the *Prn-p* locus may have a significant effect upon the expression of PrP. This is illustrated by a series of transgenic lines produced by Fischer et al (1996) in which the PrP transgene was based upon a cDNA construct. Brain PrP expression at the level of mRNA and protein was undetectable in 8 independent lines of *Prn-p* $^{-/-}$/pPrPcDNA transgenic mice. These lines carried a Cos 6.1/ILn/J4 mouse *Prn-p* $^{b}$ transgene modified by the removal of intron 1 and intron 2 but retaining an intact *Prn-p* promoter, exon 1 and 2, fused to exon 3. The absence of PrP expression in a number of independent lines suggests that there are essential expression control sequences in the introns of *Prn-p* . Indeed, a number of *Prn-p* elements necessary for the expression of a reporter construct in mouse neuroblastoma cells have recently been described in the promoter region and intron 1 (Baybutt and Manson, 1997).

Deletions of the *Prn-p* locus can have more subtle effects upon $PrP^{c}$ expression. For example, two independent lines generated by Fischer et al., (1996), *hgPrnpa*19 and *hgPrnpa* 20, in which intron 2 (and some sequence downstream of exon 3) had been removed, exhibited normal PrP expression levels throughout brain but PrP was absent from cerebellar Purkinje neurons -a cell type which normally expresses high levels of $PrP^{c}$. The loss of PrPmRNA and $PrP^{C}$ expression in these lines presumably arises from the removal of 12Kb of intron 2 sequence (or ~15Kb of sequence downstream of exon 3). This suggests that these regions contain one or more control elements necessary to direct PrP expression to cerebellar Purkinje neurons[8].

[8] This is interesting given the widespread death of cerebellar Purkinje neurons in 2 independent lines of *Prn-p* null mice. It may also be added that the absence of a similar phenotype in aged *hgPrna 19* and *hgPrna 20* mice suggests that the cerebellar phenotype is *not* the result of loss of PrP function.

### Section 6.5.2: Comparison of the *Prn-p* $^{a}$ and *Prn-p* $^{b}$ alleles

It is known that *Prn-p* $^{a}$ and *Prn-p* $^{b}$ alleles differ with respect to the PrP allotype expressed (Westaway et al., 1987), however little is known of other differences between these alleles. The *Prn-p* $^{b}$ allele (as derived from ILn/J inbred mice) has been used as the basis of many PrP transgenes (Westaway et al., 1991; Prusiner et al., 1990; Scott et al., 1993) including mice which develop a late onset neuromyopathy (Westaway et al., 1994b). There is little published mapping data for the *Prn-p* $^{b}$ allele in VM/Dk mice, therefore a knowledge of the features differentiating this allele from other mouse *Prn-p* alleles, particularly the *Prn-p* $^{a}$ allele, is desirable. The mapping studies performed here with VM/Dk confirm that this allele shares a short intron 2 sequence with other *Prn-p* $^{b}$ mouse lines (Westaway et al., 1994a). Carlson et al., (1993) has proposed that the *Prn-p* $^{b}$ allele present in P/J, ILn/J and VM/Dk were derived from a common ancestor. The structure of the VM/Dk *Prn-p* allele is very similar to that described in other *Prn-p* $^{b}$ alleles, including the intron 2 polymorphism (Carlson et al., 1986, 1988; Westaway et al., 1994a) and is consistent with a common origin for the *Prn-p* $^{b}$ allele.

The 129/Ola PrP co-isogenic mice described here differ only by 2 PrP amino acids and share a common *Prn-p* structure. This controls for the unknown effects of non-coding polymorphisms within 129/Ola co-isogenic mice.

### Section 6.5.3: PrP codon 108 and 189 alterations do not alter PrP expression levels

The issue of $PrP^{c}$ expression levels is important in experiments concerning incubation time because raised $PrP^{c}$ expression levels have previously been shown to shorten incubation times in transgenic mice (Prusiner et al., 1990; Westaway et al., 1991; Carlson et al., 1994; Telling et al., 1996; Fischer et al., 1996) and reduced $PrP^{c}$ levels are known to extend incubation times in gene targeted mice bearing only a single functional PrP allele (Prusiner et al., 1993; Bueler et al., 1993; Manson et al., 1994b; Sakaguchi et al., 1995).

Comparison of 129/Ola PrP A/A PrP mRNA levels with that of the other lines encoding the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele indicates that they are indistinguishable. The indistinguishable levels of $PrP^c$ in 129/Ola wildtype and VM/Dk mouse brain are consistent with extant reports of indistinguishable brain $PrP^c$ levels in NZW (*Prn-p* $^a$, PrP A/A), ILn/J (*Prn-p* $^b$, PrP B/B) (Westaway et al., 1987) and congenic VM-*Sinc* s7 (*Prn-p* $^a$, PrP A/A) and *Sinc* p7 (*Prn-p* $^b$, PrP B/B) mice (Manson, McBride and Hope, 1992). 129/Ola PrP gtB/- mice encoding a single functional *Prn-p* $^{a[108F\_189V]}$ allele exhibit a ~50% reduction in PrP mRNA levels. This is consistent with several independent reports of mice which express only one functional *Prn-p* allele (Bueler et al., 1992; Manson et al., 1994a; Sakaguchi et al., 1995). The observation of ~50% reduced levels of $PrP^C$ in mice with only one allele argues against a translational control mechanism for the control of PrP levels in brain.

**Section 6.6: Summary**

The targeted *Prn-p* $^{a[108F\_189V]}$ allele was transmitted though the germline and used to generate sufficient numbers of mice for 301V and ME7 challenge. Northern and western analysis of adult brain indicated that this allele was expressed in brain and that its expression level was indistinguishable from wildtype 129/Ola and VM/Dk mice. The data presented here indicates that 129/Ola mice, which normally express the PrP A allotype from the *Prn-p* $^a$ allele, express the PrP B allotype from a gene targeted *Prn-p* $^{a[108F\_189V]}$ allele at levels indistinguishable from non-targeted co-isogenic littermates and VM/Dk mice. This argues that alterations to incubation times and neuropathology in challenged mice are a consequence of the engineered codon 108 and 189 alterations and do not arise as a result of major changes to PrP B expression levels.

# Chapter 7

## 301V challenge of mice with gene targeted PrP codon 108 and 189 alterations

**Section 7.1: Introduction**

The postulated congruence of *Sinc*/*Prn-i* and *Prn-p* argues that incubation times in mice are controlled by PrP dimorphisms at codons 108 and 189[1]. This proposal was tested by 301V challenge of mice in which the endogenous *Prn-p* $^{a}$ allele, encoding the PrP A allotype (108L_189T) was converted by gene targeting to encode the PrP B allotype (108F_189V).

The pattern of allelic interaction known to occur between 301V and *Sinc* alleles is such that VM/Dk *Sinc* $^{p7p7}$ mice have short incubation times of 115-119 days and C57BL/6J *Sinc* $^{s7s7}$ mice have longer incubation times of ~260 days. 301V challenge of wild-type 129/Ola mice gives incubation times of ~227 days, indicating that 129/Ola encodes the *Sinc* s7 allele[2] (Jean Manson, unpublished data).

It was reasoned that if codons 108 and 189 were the molecular determinants of incubation time, conversion of the PrP allotype from PrP A to PrP gtB should also have a corresponding effect on the apparent *Sinc* genotype. Thus the 301V incubation time was predicted to shorten from that of wild-type 129/Ola *Sinc* s7 mouse strain to a ~116 day incubation time characteristic of VM/Dk -a wildtype mouse strain encoding the *Sinc* p7 allele and the *Prn-p* $^{b}$ allele. A dramatic shortening of the 301V incubation time in 129/Ola PrP gtB/gtB mice would constitute good evidence that PrP codon 108 and 189 dimorphisms control incubation time and would support the proposal that *Sinc*, *Prn-i* and *Prn-p* are congruent.

**Section 7.2: 301V origin and properties**

Mice bearing gene targeted PrP alterations were challenged with 301V because this strain has a rapid incubation time in VM/Dk *Sinc* $^{p7p7}$ mice and relatively short incubation times in other genotypes -leading to an experiment of shorter duration than with other agent strains. 301V is a mouse passaged BSE strain derived from a terminal Friesian Holstein cow with pathologically confirmed BSE (Bruce et al., 1994). Primary

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[1] This is because these are the only known features that consistently segregate with scrapie incubation time length (Westaway et al., 1987; Carlson et al., 1986, 1988; Westaway et al., 1994a).
[2] ME7 challenges of this mouse strain also indicate that it encodes the *Sinc* s7 allele (Manson et al., 1994b).

transmission from a number of BSE isolates has clearly demonstrated a major role for the *Sinc* gene in the control of incubation time. Subpassage from a single VM/Dk mouse with an incubation time of 433 days into VM/Dk mice resulted in very short incubation times of 116 (+/-3 SE) days -one of the shortest recorded incubation times in mice expressing normal levels of PrP. Subsequent mouse-mouse passages in VM/Dk have indicated that 301V is stable and it is used here at its 5$^{th}$ passage.

**Section 7.3: 301V challenge protocol**

HPRT wildtype co-isogenic 129/Ola mice heterozygous for the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele (i.e. PrP A/gtB mice) were mated to generate mice of three genotypes: *Prn-p* $^{a/a}$ (PrP A/A), *Prn-p* $^{a/a[108F\_189V]}$ (PrP A/gtB) and *Prn-p* $^{a[108F\_189V]/[108F\_189V]}$ (PrP gtB/gtB) mice. The progeny from this cross were genotyped for PrP codon 108 and 189 alterations before 301V challenge. Groups of 16 mice from each of the three genotypes were challenged by inoculation with 20ul of a $10^{-2}$ dilution of 301V terminal VM/Dk brain homogenate by the intracranial route under halothane anaesthesia. The experiment was performed blind in that the genotype of the 129/Ola mice was unknown to personnel scoring for scrapie signs and at the end of the experiment the code was broken by matching ear notch number with PrP genotype. Postmortem tail samples were PrP genotyped and all corresponded with those obtained before challenge. Control challenges were also set up with VM/Dk, C57BL and F1[C57xVM] mice using the same inoculum under identical conditions.

**Section 7.4: 301V incubation times**

301V challenge of mice encoding the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele demonstrated a critical role for PrP codon 108 and 189 alterations in the control of incubation time (Table 7.1, p146 and Figure 7.1 p148). Control challenges with VM/Dk mice had short incubation times of 119 days (±2 SE, n=12) whilst C57BL/6J and F1[C57xVM/Dk] mice had longer incubation times of 299 days (±4 SE, n=10) and 268 days (±3 SE, n=10) respectively. These incubation times confirm that the inoculum used for the challenge of gene targeted mice was stable and had properties characteristic of 301V.

Wildtype 129/Ola PrP A/A mice had mean incubation times of 244 days (±2.0 SE, n=16). This is close to the 227 day (±3 SE) incubation time previously obtained with 301V challenge of this mouse strain (Jean Manson, unpublished observations) -the small difference in incubation times may be attributable to inter-experimental variation or, less likely, to genetic drift between NPU and ICMB 129/Ola stocks. 301V challenge of 129/Ola PrP gtB/gtB mice homozygous for the *Prn-p* $^{a[108F\_189V]}$ allele demonstrated that alterations to codons 108 and 189 have a dramatic effect upon incubation time. These mice had a mean incubation time of 132.6 days (±1SE, n=16), more than 100 days shorter than wildtype 129/Ola PrP A/A mice. This difference is highly significant ($p$<0.0001, Mann Whitney test) and supports the proposal that PrP codon 108 and 189 dimorphisms (i.e. PrP allotypes A and B) control 301V incubation time.

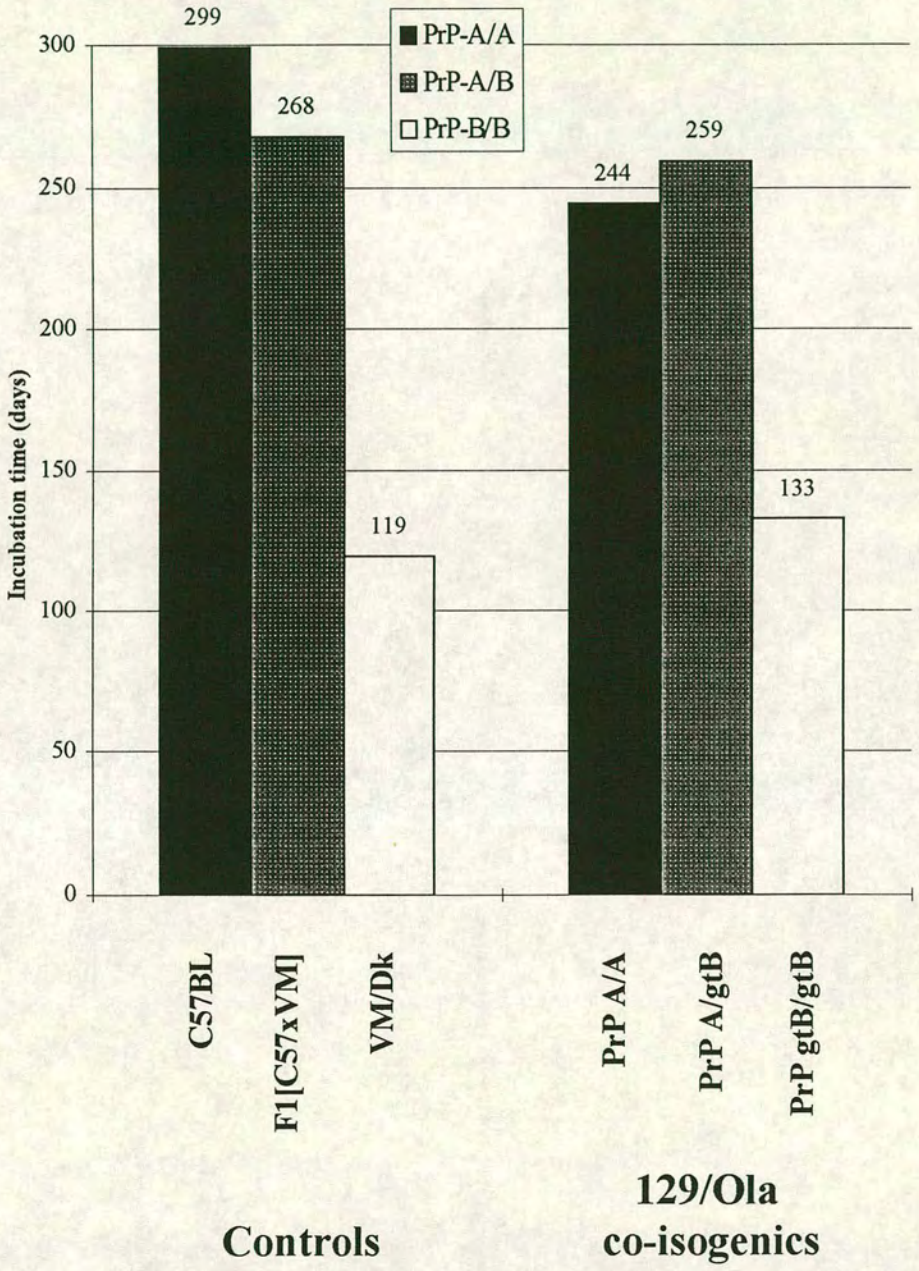
**Table 7.1: 301V i.c. challenge of 129/Ola mice with gene targeted PrP codon 108 and 189 alterations**

| Mouse Strain | *Prn-p* allele | PrP allotype | *Sinc* genotype | Mean Incubation time (days) | SE [a] (days) | SD [b] (days) | n [c] |
|---|---|---|---|---|---|---|---|
| 129/Ola [d] | *Prn-p* $^{a/a}$ | A/A | s7s7 | 244 | 2.0 | 8 | 16 |
| 129/Ola | *Prn-p* $^{a/a[108F\_189V]}$ | A/gtB | - | 259 | 2.2 | 8.5 | 15 |
| 129/Ola | *Prn-p* $^{a[108F\_189V]/a[108F\_189V]}$ | gtB/gtB | - | 132.6 | 0.2 | 0.7 | 13 |
| C57 [e] | *Prn-p* $^{a/a}$ | A/A | s7s7 | 299 | 4 | 12.6 | 10 |
| F1[C57xVM] | *Prn-p* $^{a/b}$ | A/B | s7p7 | 268 | 3 | 9.5 | 10 |
| VM [f] | *Prn-p* $^{b/b}$ | B/B | p7p7 | 119.4 | 2.1 | 7.3 | 12 |

**a**, standard error of the mean
**b**, standard deviation
**c**, number of mice
**d**, 129/OlaHsd, supplied by Harlan UK.
**e**, C57BL
**f**, VM/Dk

**Figure 7.1: 301V Incubation times are dramatically altered by PrP codon 108 and 189 alterations**

Histogram showing the 301V incubation times of 129/Ola PrP A/A, A/gtB and gtB/gtB mice. Incubation times of C57BL/6J (PrP A/A), VM/Dk (PrP B/B) and F1[C57 x VM] (PrP A/B) controls are also shown.


299
268
119
244
259
133
PrP-A/A
PrP-A/B
PrP-B/B
Incubation time (days)
300
250
200
150
100
50
0
C57BL
F1[C57xVM]
VM/Dk
PrP A/A
PrP A/gtB
PrP gtB/gtB
Controls
129/Ola
co-isogenics

The close similarity between the 301V incubation times of 129/Ola PrP gtB/gtB mice and that of VM/Dk PrP B/B mice also supports this proposal (Table 7.1, p146). This is the strongest evidence to date that *Sinc*/*Prn-i* and *Prn-p* are congruent.

The mean incubation time of 129/Ola PrP A/gtB mice was 259 (±2 SE, n=16) days. This is close to incubation times observed in the 301V challenge of F1[C57xVM] mice of 268 (±3 SE, n=10) days. Previous 301V challenges of C57BL (*Prn-p* $^{a}$), VM/Dk (*Prn-p* $^{b}$) and F1[C57xVM] (*Prn-p* $^{a/b}$) mice have shown that F1 *Prn-p* $^{a/b}$ mice have incubation times approximately 20-30 days shorter than C57 *Prn-p* $^{a}$ mice (Bruce et al., 1994). This indicated that the *Prn-p* $^{a}$ allele, or rather the PrP A allotype, is dominant in conferring long incubation times with 301V. It was reasoned that 301V challenge of 129/Ola PrP A/A and PrP A/gtB mice would show a similar incubation time ranking. However, 129/Ola PrP A/gtB mice had 301V incubation times approximately 15 days *longer* than that of PrP A/A 129/Ola mice. This difference is statistically significant ($p$=0.001, Mann-Whitney test) and is slightly different from the relationship noted with 301V challenge of control F1[C57xVM/Dk] PrP A/B mice which had incubation times 31 days shorter than PrP A/A C57BL mice (Table 7.1, p146 and Figure 7.1, p148).

**Section 7.5: Brain $PrP^{Sc}$ levels in 301V challenged mice**

Sedimentable PrP (or $PrP^{Sc}$ 33-35) was isolated from 301V infected brains, immunoblotted and investigated for proteinase K resistance. Loss of solubility and partial resistance to proteinase K are operational definitions of $PrP^{Sc}$ (Meyer et al., 1986). Figure 7.2 (p151) illustrates that sedimentable PrP was evident in all three 129/Ola genotypes. In the upper panel $PrP^{c}$ from uninfected mice is included for reference[3]. Proteinase K resistant PrP (or core PrP 27-30) was apparent in all brains investigated. Proteinase K removes of a ~67 amino acid N-terminal portion of $PrP^{Sc}$ 33-35 (Hope et al., 1986; Somerville, Ritchie and Gibson, 1989) resulting in a downward band-shift (+ lanes). Broadly speaking, the amount of sedimentable PrP in these genotypes was similar,

[3] $PrP^{c}$, as it is soluble, remains in the supernatant following ultracentrifugation and little or no $PrP^{c}$ is isolated with this method. Therefore $PrP^{c}$ from uninfected mice is derived from crude brain homogenates.

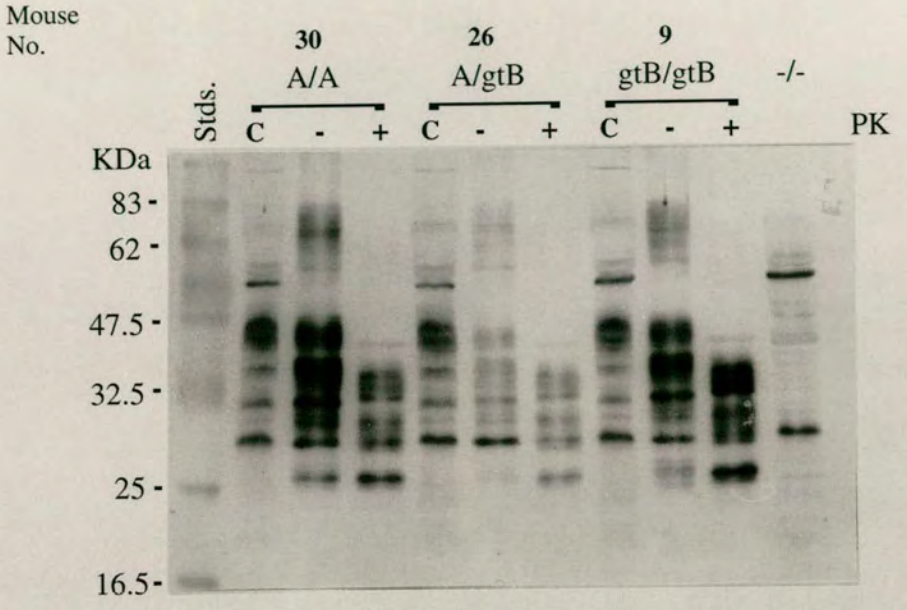
**Figure 7.2: $PrP^{Sc}$ in 301V terminal brain**

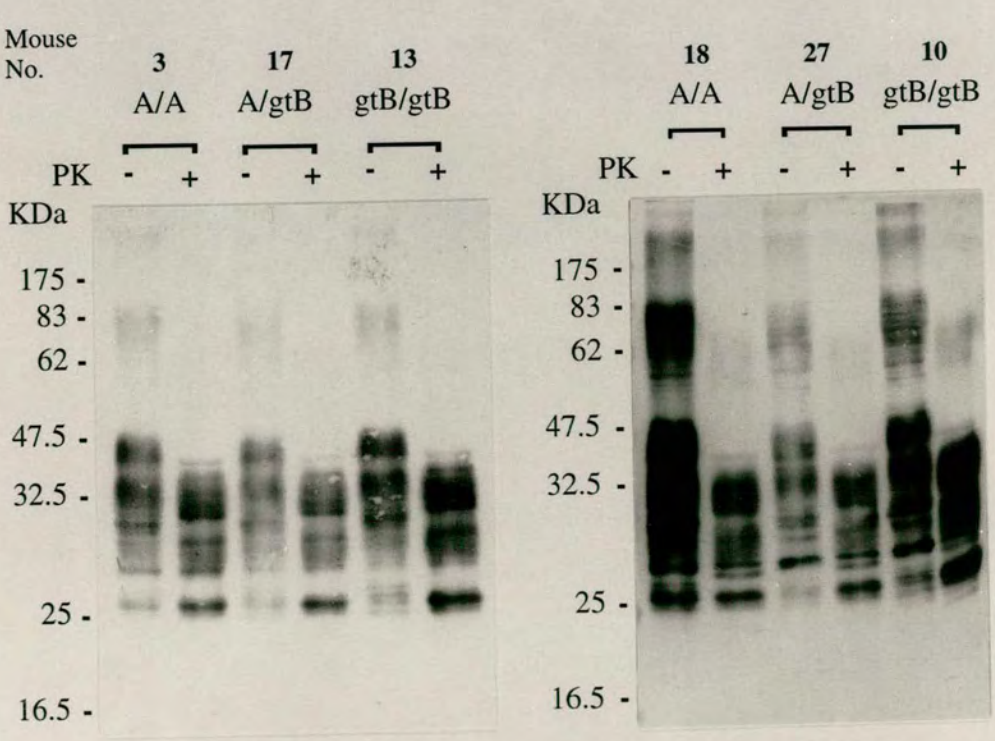
PrP immunoblots of three 301V terminal brains from each 129/Ola PrP genotype. PrP was detected with PrP antiserum 1A8, alkaline phosphatase conjugated IgG and visualised with BCIP/NBT. PK + samples were treated with 50ug/ml proteinase K for 1 hour at 37°C.

**Upper panel**: $PrP^{c}$(C) from uninoculated brain, sedimentable PrP (PK-) and proteinase K resistant PrP (PK+) from 129/Ola PrP A/A, A/gtB and gtB/gtB 301V terminal brains (mice 30, 26 and 9). Uninoculated 129/Ola *Prn-p* $^{-/-}$ mouse brain is also shown (-/-).

**Lower panel (left)**: sedimentable PrP (PK-) and proteinase K resistant PrP (PK+) from 129/Ola PrP A/A, A/gtB and gtB/gtB 301V terminal brains (mice 3, 17 and 13).

**Lower panel (right)**: sedimentable PrP (PK-) and proteinase K resistant PrP (PK+) from 129/Ola PrP A/A, A/gtB and gtB/gtB 301V terminal brains (mice 18, 27 and 10).


Mouse No.
30
A/A
26
A/gtB
9
gtB/gtB
-/-
Stds.
C - + C - + C - +
PK
KDa
83
62
47.5
32.5
25
16.5


Mouse No.
3
A/A
17
A/gtB
13
gtB/gtB
PK - + - + - +
KDa
175
83
62
47.5
32.5
25
16.5
18
A/A
27
A/gtB
10
gtB/gtB
PK - + - + - +
KDa
175
83
62
47.5
32.5
25
16.5

although a number of differences were noted. Figure 7.2 (p 151, upper panel) indicates that although PrP A/A and gtB/gtB mice have similar levels of sedimentable PrP (PK - lanes), PrP gtB/gtB mice have higher levels of $PrP^{Sc}$ (PK + lane). This relationship is also apparent in the other panels within this figure (Figure 7.2, upper and lower panels, p151). That 129/Ola PrP gtB/gtB have similar or higher levels of $PrP^{Sc}$ than 129/Ola PrP A/A mice, despite a >110 day shorter incubation time, indicates that the kinetics of $PrP^{Sc}$ accumulation are faster in this genotype.

PrP A/gtB mice had less sedimentable PrP and proteinase K resistant PrP than the other 129/Ola genotypes and is consistent with the longer incubation times and the reduced neuropathology apparent in some brains areas (see next section).

SDS-PAGE banding patterns sometimes vary between agent strains (Kascsak, Rubenstein and Carp, 1991; Collinge et al., 1996). The minor extent to which the PrP A and gtB allotype influences the PrP SDS-PAGE banding patterns in 301V terminal brains is apparent in Figure 7.2 (p151). It appears that, although the absolute amounts of PrP in each PK- and PK+ lane was subject to some variation, the PrP banding pattern and the ratios of the bands appeared to be relatively invariant -at least in the small numbers of samples analysed here. This indicates that with 301V the engineered PrP codon 108 and 189 dimorphisms have little apparent effect upon the 301V $PrP^{Sc}$ banding patterns at this level of analysis.

## Section 7.6: The influence of PrP codon 108F_189V alterations on 301V neuropathology

### Section 7.6.1: Introduction

The severity, type and distribution of neuropathology is known to be influenced by the strain of agent, inoculation route, host *Sinc* genotype and the effects of unidentified genes (Fraser and Dickinson, 1967, 1968; Fraser, 1976; Bruce et al., 1989, 199; Hecker et al., 1992; Carlson et al., 1994). However, previous attempts to examine the role of PrP A and B allotypes in the control of neuropathology have been unable to exclude the effects of other genes. Genetic variation has been partially excluded in studies with congenic VM/Dk and VM/Dk-*Sinc*$^{s7}$ lines (Bruce et al., 1991) and C57BL/6J *Prn-p* congenic lines (Carlson et al., 1994). However congenic lines differ not

only with respect to their *Sinc* or *Prn-p* alleles, but also with respect to genes closely linked to *Sinc* or *Prn-p*. For example, Carlson has attributed variant 87V scrapie neuropathology [4] in B6.I-1 mice to the effects of a *Prn-p* linked gene between *Spr8* and *Pax-1* (Carlson et al., 1994).

The 129/Ola PrP co-isogenic mice generated during the course of this project enable, for the first time, the investigation of the role of PrP A and B allotype whilst controlling for the effects of other genes. The effects of non-coding *Prn-p* polymorphisms (Westaway et al., 1994a; Baybutt and Manson, 1997) are unknown, however 129/Ola PrP co-isogenic mice share a common *Prn-p* [a] allele which differs only by two amino acids in exon 3. Neuropathological studies performed on 301V terminal brains from these mice revealed that alterations to codons 108 and 189 modified the extent, distribution and kinetics of neuropathological change in a number of brain areas.

**Section 7.6.2: PrP allotype and vacuolar change**

Vacuolar change in 301V terminal brain was assessed in 12 standard brain areas by lesion profiling (Fraser and Dickinson, 1967, 1968). This revealed that PrP codon 108 and 189 alterations were associated with a number of alterations to the lesion profile of 129/Ola mice (Figure 7.3, panel a, p155). Differences between the various 129/Ola genotypes were observed in grey matter areas 1 (dorsal medulla), 4 (hypothalamus), 8 (medial cerebral cortex), 9 ( medial cerebral cortex at the level of the septum) and to a lesser extent in white matter area 2* (mesencephalic tegmentum).

In all of these areas, except area 4 (hypothalamus), 129/Ola PrP gtB/gtB mice had more severe vacuolar change than the other 129/Ola genotypes. The extent of vacuolar change apparent in the dorsal medulla (area 1) is illustrated in Figure 7.4 (panels a-c, p158). Although the cochlear nucleus is not scored as part of the lesion profile system, it is apparent here that 129/Ola PrP gtB/gtB mice have more severe vacuolar change than PrP A/A mice (Figure 7.4, panels d-f, p158).

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[4] This gene influences neuropathology in the thalamus, habenula and raphe nucleus.

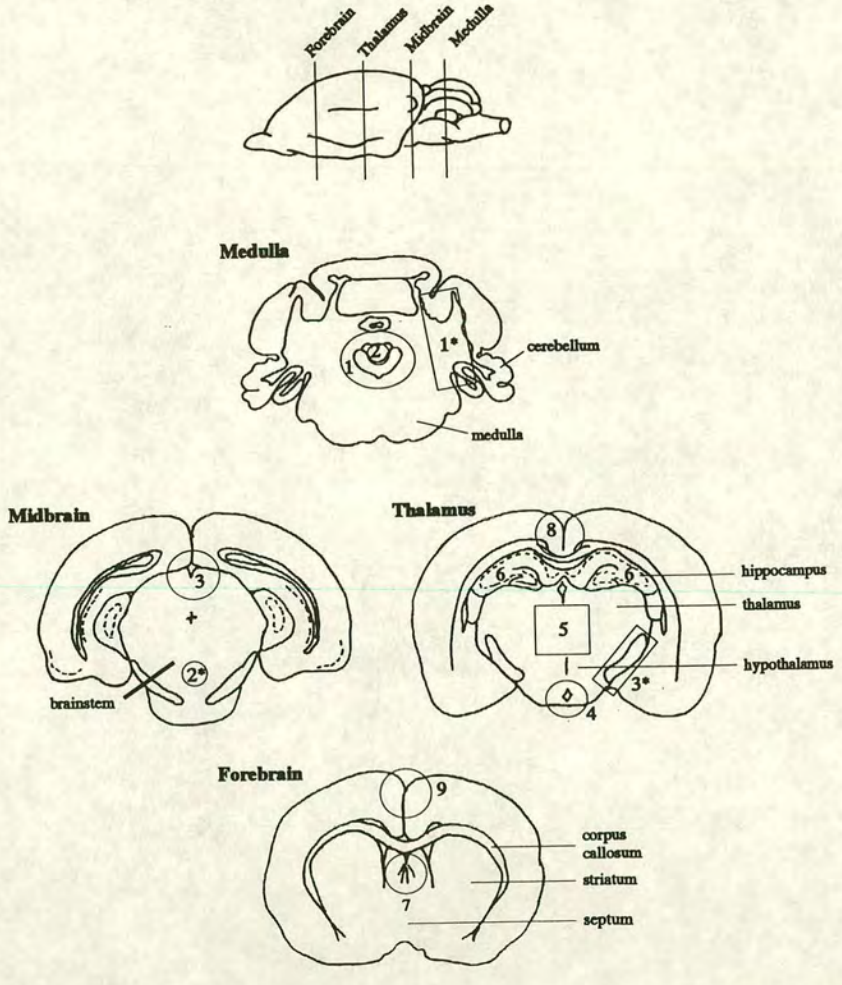
**Figure 7.3: 301V lesion profiles**

Comparison of 301V lesion profiles in 129/Ola PrP A/A, PrP A/gtB, PrP gtB/gtB and VM/Dk mice. Lesion profiles have been superimposed to aid comparison. Panel a). 129/Ola PrP A/A, A/gtB and gtB/gtB lines. Panel b). Comparison of 129/Ola PrP gtB/gtB and VM/Dk PrP B/B.

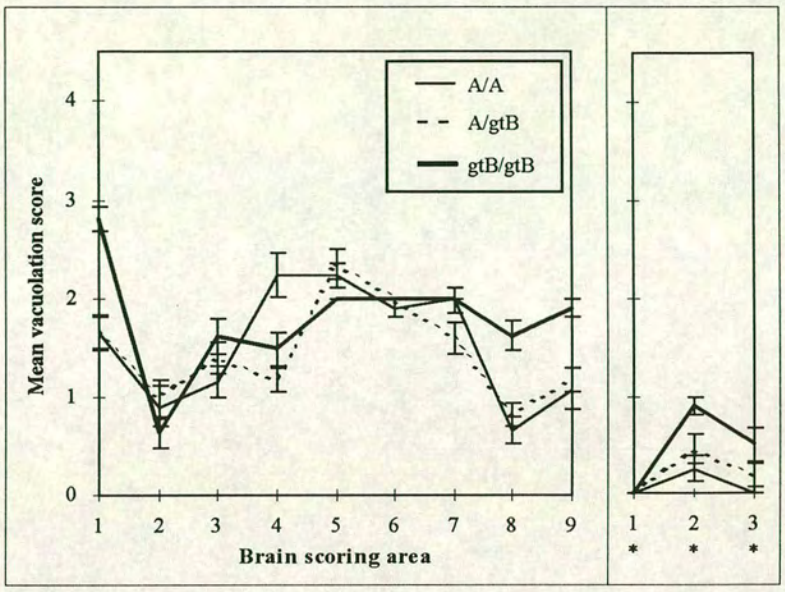
Lesion profiles were constructed by scoring H&E stained 6uM sections for vacuolar degeneration on a scale of 0 to 5 in nine standard grey matter areas and 0 to 3 in three white matter areas as described (Fraser and Dickinson, 1967, 1968). Error bars represent the standard deviation, 10-12 mice were used for each lesion profile.

*Grey matter areas*: **1**, dorsal medulla; **2**, cerebellar cortex; **3**, superior colliculus; **4**, hypothalamus; **5**, medial thalamus; **6**, hippocampus; **7** septum; **8**, medial cerebral cortex at the level of the thalamus; **9**, medial cerebral cortex at the level of the septum.

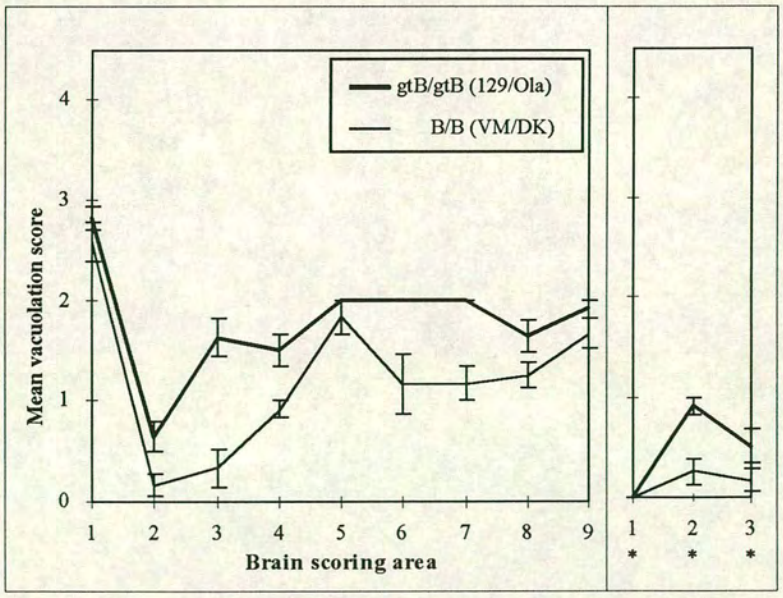
*White matter areas* (*): **1**, cerebellar white matter; **2**, mesencephalic tegmentum; **3**, pyramidal tract.



**a).**


A/A
A/gtB
gtB/gtB
Mean vacuolation score
0
1
2
3
4
1
2
3
4
5
6
7
8
9
Brain scoring area
1
2
3
*
*
*

**b).**


gtB/gtB (129/Ola)
B/B (VM/DK)
Mean vacuolation score
0
1
2
3
4
1
2
3
4
5
6
7
8
9
Brain scoring area
1
2
3
*
*
*

These observations are particularly significant given the short incubation times of 129/Ola PrP gtB/gtB mice and indicates that the PrP gtB allotype can determine more severe vacuolar change in a number of brain areas. It is also apparent that PrP gtB/gtB mice have similar terminal scores to PrP A/A and A/gtB mice despite their short incubation times -this may indicate that the kinetics of vacuolar change are faster in PrP gtB/gtB hosts.

Comparison of 129/Ola PrP gtB/gtB and VM/Dk (PrP B/B) lesion profiles (Figure 7.3, panel b, p155) reveals some areas of shared vacuolar change. Similar scores are apparent in grey matter areas 1, 2, 4, 5, 8 and 9 and in white matter areas 1* and 3*. These similarities suggest that the PrP gtB and PrP B protein direct vacuolar change to common sites in these mice. In a number of areas 129/Ola PrP gtB/gtB mice have more severe vacuolar change. It is unlikely that this represents a difference in the properties of the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele and the wildtype *Prn-p* $^{b}$ allele because examination of Figure 7.3 (panel a, p155) reveals that all 129/Ola genotypes have more severe vacuolar change in these areas -this argues that the differences apparent between 129/Ola gtB/gtB and VM/Dk represent the effects on non-*Prn-p* genes.

The terminal lesion profiles of all three 129/Ola genotypes are similar in a number of areas but in areas where differences are apparent (areas 1, 8, 9, 2*) it is notable that PrP A/A and PrP A/gtB 129/Ola lines have very similar lesion scores. This relationship is consistent with the similar incubation times of PrP A/A and PrP A/gtB mice.

The extent of vacuolar change in the hypothalamus (area 4) is conspicuous in that it is the only area where PrP A/A mice have a higher lesion score than PrP gtB/gtB mice (Figure 7.3, panel a, p155). The fact that PrP A/gtB mice have lower scores here despite incubation times which are longer than PrP A/A mice suggests that greater vacuolar change in the PrP A/A hypothalamus is not merely a non-specific consequence of long incubation times but reflects a difference between the PrP A and PrP gtB allotypes in their ability to promote vacuolar change in the hypothalamus. This area is also unusual in that it is the only area in which 129/Ola PrP A/gtB mice have lower scores than PrP A/A mice -elsewhere they are closely similar. A similar phenomenon is

**Figure 7.4: Vacuolar change in the dorsal medulla and cochlear nucleus**

H&E stained coronal sections of terminal 301V brain. PrP gtB/gtB mice had more severe vacuolar change than the other 129/Ola genotypes in a number of brain areas including the dorsal medulla (panels a-c, scoring area 1) and the cochlear nucleus (panels d-f).

Panels a) PrP A/A b). PrP A/gtB, c)PrP gtB/gtB. Magnification x 40.

Panels d) PrP A/A e). PrP A/gtB, f)PrP gtB/gtB. Magnification x 10.

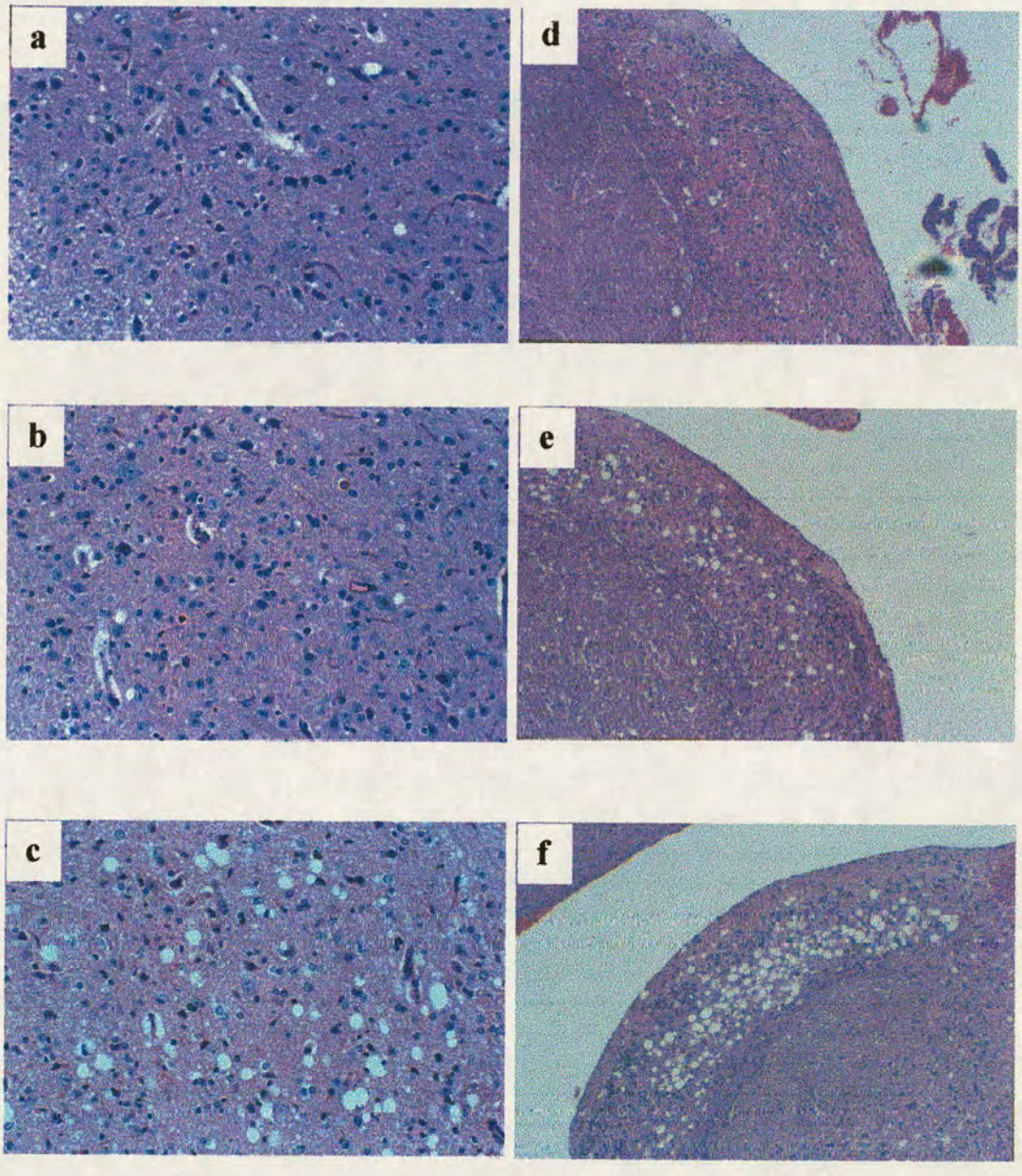
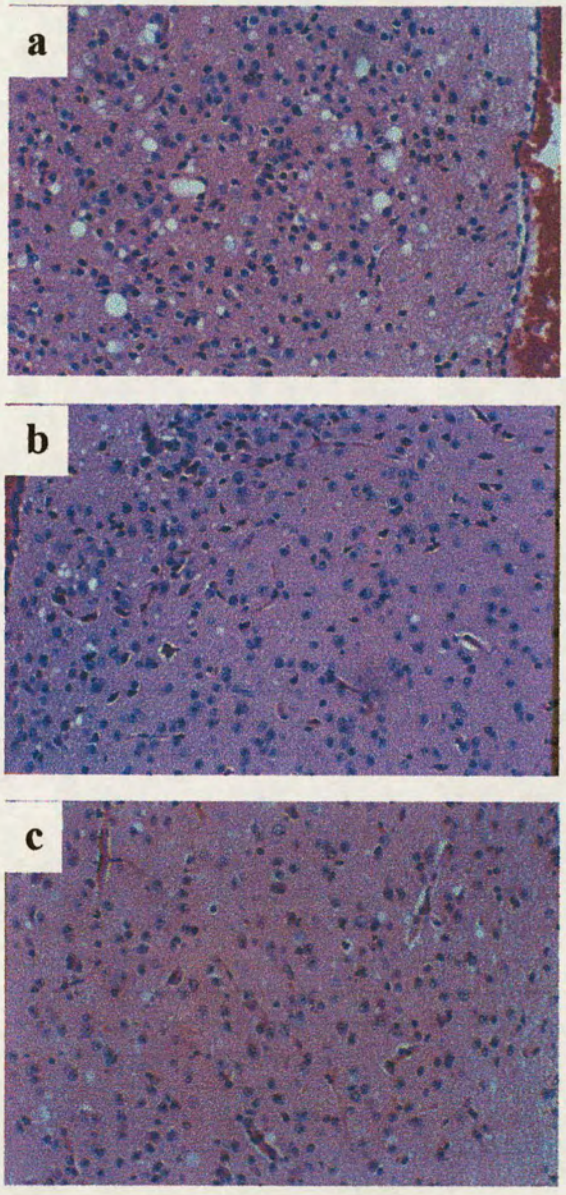

a
d
b
e
c
f

**Figure 7.5: Vacuolar change in the hypothalamus**

H&E stained coronal sections of terminal 301V brain. PrP A/A mice had more severe vacuolar change than the other 129/Ola PrP genotypes in the hypothalamus (scoring area 4).

Panel a) PrP A/A b). PrP A/gtB, c)PrP gtB/gtB. Magnification x 40.


a
b
c

also observed in an independent set of 301V challenges in *Sinc* congenic mice (Dr. Moira Bruce, unpublished data). This suggests that this phenomenon is a reproducible effect which is determined by PrP allotype. This provides further evidence that the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele can reproduce neuropathological features of wildtype *Prn-p* $^{b}$ allele.

**Section 7.6.3: Neuronal loss and PrP accumulation**

301V challenged 129/Ola PrP co-isogenic mice revealed significant differences in the extent of PrP neuronal loss in the hippocampal region (Figure 7.6, p163). PrP A/A mice had severe neuronal loss in the hippocampus, indeed some PrP A/A mice had very little remaining hippocampal structure. Widespread pyramidal cell death was apparent in the CA1, CA2 and CA3 regions. Extensive neuronal loss is also evident in the granular layer of the dentate gyrus. PrP gtB/gtB mice also exhibited severe pyramidal cell loss but had a largely intact dentate gyrus and, in general, the hippocampal architecture was better preserved than in PrP A/A mice. The PrP A/gtB hippocampus had little evidence of cell loss. The extent of hippocampal cell loss in PrP A/A and PrP A/gtB mice was reminiscent of the reduced hypothalamic vacuolar change in PrP A/gtB mice.

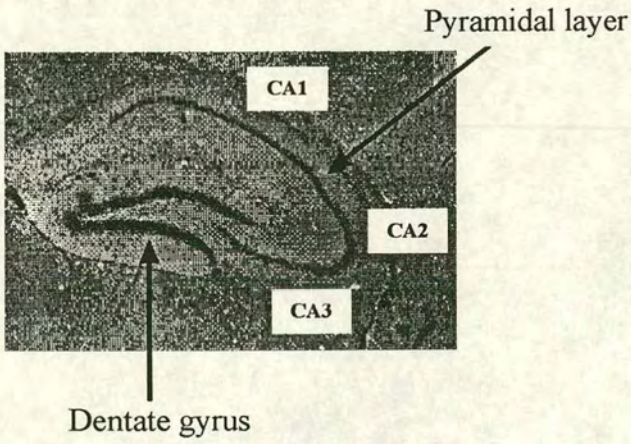
PrP deposition was examined by immunohistochemistry in 301V terminal brains. PrP deposition was most prominent in the hippocampus and the thalamus (Figure 7.7, p165 and Figure 7.8, p167). Although these studies were based on a small number of mice from each genotype (n=4), and some variation was evident between mice of the same PrP genotype, they indicate a role for PrP allotype in the control of PrP deposition. All 129/Ola PrP genotypes had widespread cytoplasmic PrP accumulation in the hippocampus which gave this area a mottled appearance. In all genotypes PrP plaques, when present, were limited to the subcallosal region, specifically the junction between the oriens layer and the alveus.

In addition to a widespread cytoplasmic staining pattern, PrP A/A mice had significant extracellular PrP deposition and numerous prominent regularly spaced PrP plaques in the alveus (Figure 7.7, panel a, p165). The extent of neuronal loss apparent in the PrP A/A hippocampus corresponded well with the extent of PrP deposition.

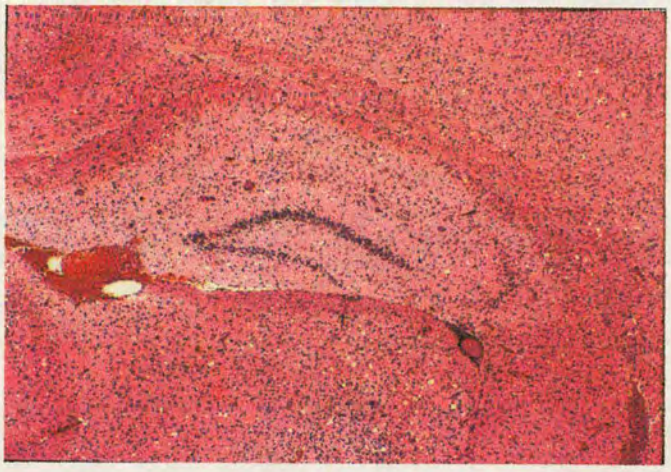
**Figure 7.6: Neuronal loss in the hippocampus**

H&E stained coronal sections of the hippocampus cut at the level of the thalamus. Magnification x 10. Panels a). PrP A/A b). PrP A/gtB, c)PrP gtB/gtB.
Panel a). Extensive neuronal loss throughout the PrP A/A hippocampus, particularly in pyramidal neurons of the CA1, CA2 and CA3 regions. Cell loss was also evident in the granular layer of the dentate gyrus. Panel b). Little neuronal loss was apparent in the PrP A/gtB hippocampus. Panel c) Extensive loss of pyramidal cells in the CA1, CA2 and CA3 regions of the hippocampus in PrP gtB/gtB mice, but not to the extent that was evident in PrP A/A mice. PrP gtB/gtB mice have little cell loss in the dentate gyrus.
Brains were fixed in formal saline and 6μM hippocampal sections cut at the level of the thalamus.

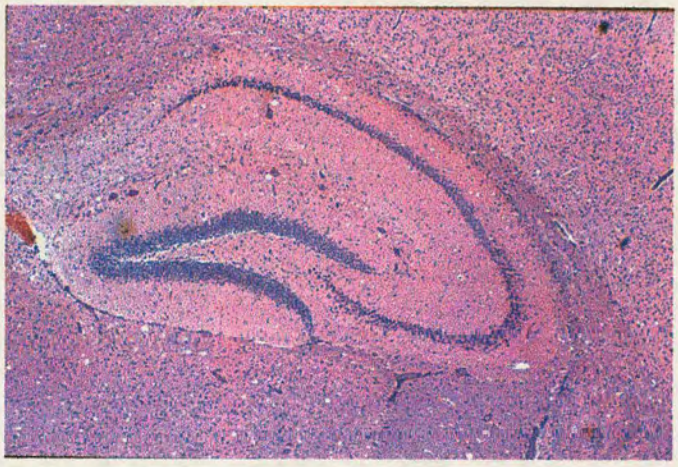
The main features of the mouse hippocampus are shown below:



a).



b).



c).

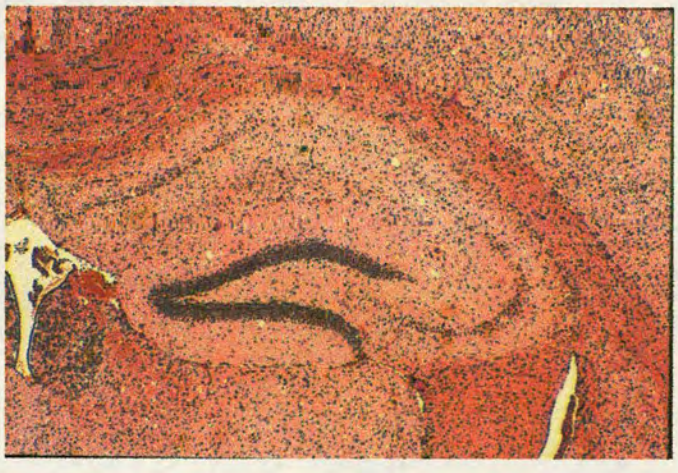


**Figure 7.7: PrP deposition in the hippocampus**

PrP staining in the hippocampus of 301V terminal 129/Ola PrP co-isogenic brain. The subcallosal region is shown in which a PrP allotype-dependent PrP staining pattern was evident. Panels a). PrP A/A b). PrP A/gtB, c). PrP gtB/gtB.

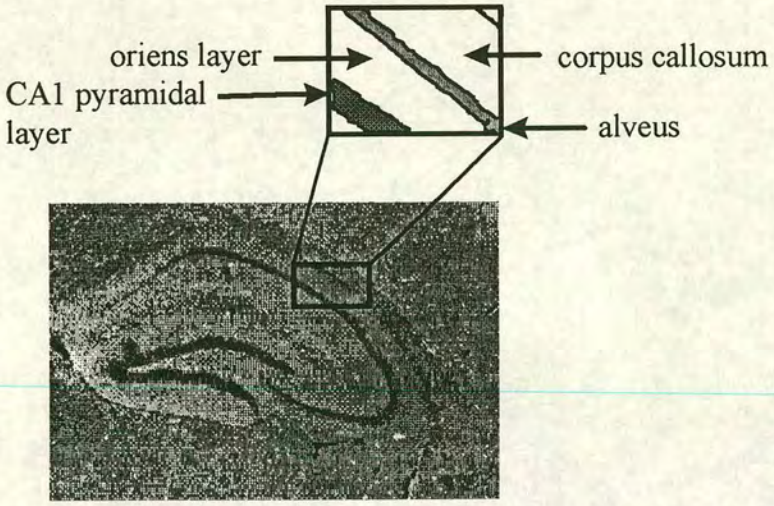
Brains were treated with 98% formic acid, PLP fixed and 6μM coronal sections cut at the level of the thalamus. PrP was detected primary using antiserum 1A8 with the peroxidase-antiperoxidase method and sections were lightly counterstained with haemotoxylin. PrP staining was performed by Patricia McBride. Magnification x40.

Panel a). PrP A/A mice have considerable extracellular PrP staining with frequent large PrP plaques.

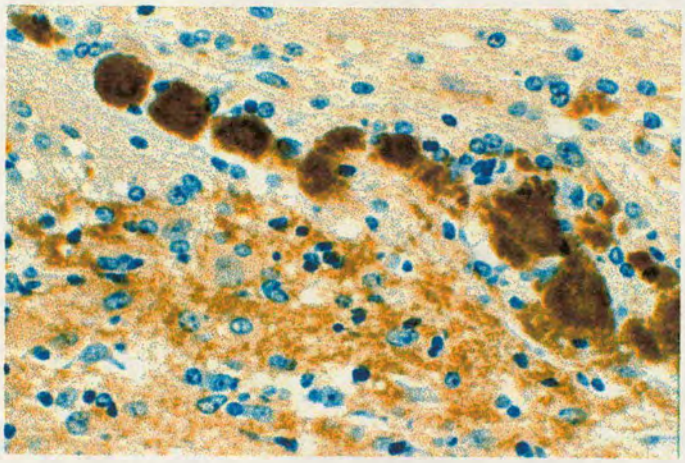
Panel b). PrP A/gtB mice have the longest incubation time but less PrP deposition than PrP A/A mice. A largely intact layer of CA1 pyramidal neurons is evident in the bottom of this panel.

Panel c). Irregular, small PrP plaques were present in the PrP gtB/gtB hippocampus.

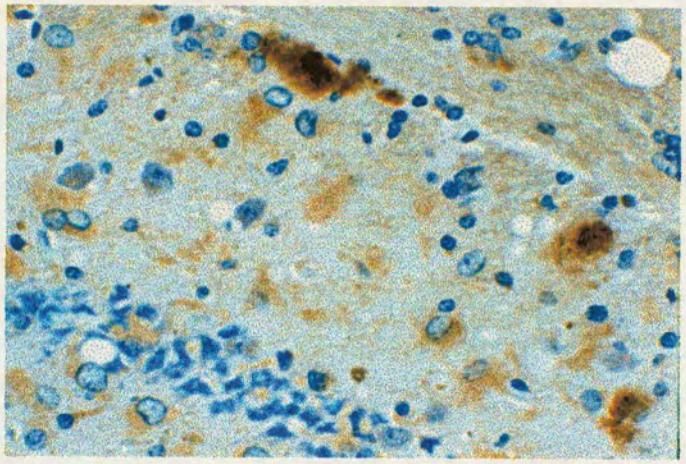
The region of the hippocampus depicted in these panels is indicated below:



a).



b).



c).

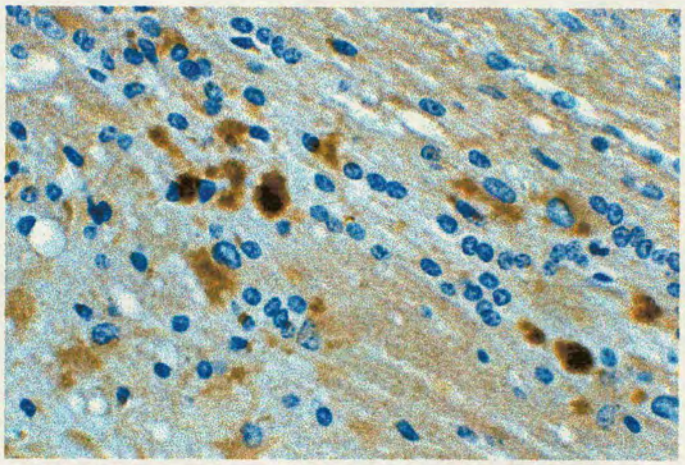


**Figure 7.8: PrP deposition in the thalamus**

PrP staining in terminal 301V brain. PrP allotype was associated with differences in PrP staining in the thalamus, particularly in the dorsal lateral geniculate nucleus. Panels a). PrP A/A b). PrP A/gtB, c). PrP gtB/gtB. Magnification x10.

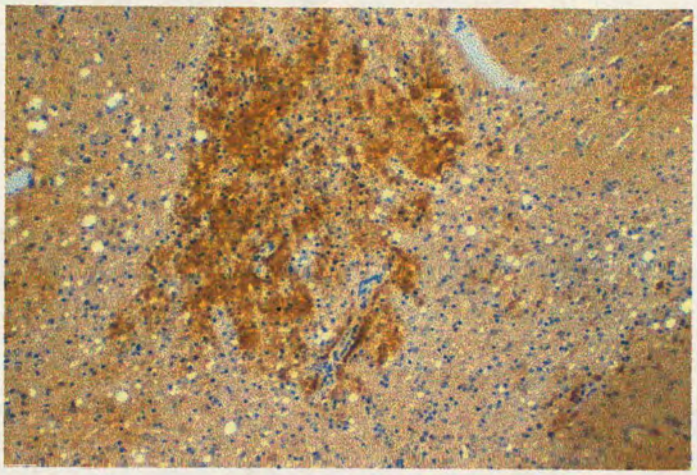
Brains were treated with 98% formic acid, PLP fixed and 6μM coronal sections were stained for PrP using 1A8 a polyclonal PrP antiserum and developed with the peroxidase-antiperoxidase method. Sections were lightly counterstained with haemotoxylin. PrP staining was performed by Patricia McBride.

Panel a). Large amounts of PrP staining was evident in the dorsal lateral geniculate nucleus in PrP A/A brain.

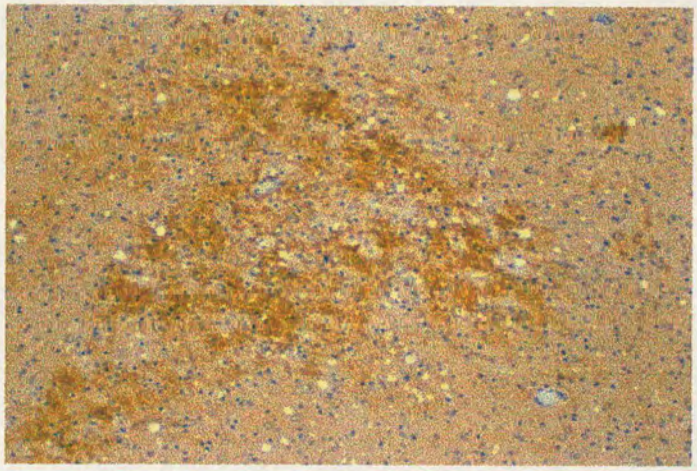
Panel b). PrP A/gtB mice also had PrP deposition in this region but to a lesser extent.

Panel c). PrP gtB/gtB mice had little PrP deposition in this area.

a).



b).



c).

Irregularly spaced PrP plaques were also present to a lesser degree in the PrP A/gtB subcallosal region (Figure 7.7, panel b, p165). Given the longer incubation periods of PrP A/gtB mice this indicates that the severe PrP deposition evident in the PrP A/A subcallosal region is not simply due to a long incubation period but is a property of the PrP A allotype. PrP gtB/gtB mice had occasional small plaques in the alveus but they were smaller and less frequent than in the other genotypes (Figure 7.7, panel c, p165).

In the dorsal and ventrolateral thalamic nuclei, both focal and diffuse PrP deposits were evident in all genotypes although it was apparent that PrP A/A and A/gtB mice had more PrP deposition here than PrP gtB/gtB mice. This was particularly marked in the dorsal lateral geniculate nucleus where PrP A/A mice, and to a lesser extent PrP A/gtB mice, had moderate or large accumulations of PrP, whilst PrP gtB/gtB mice had much less evidence of accumulation here (Figure 7.8, p167).

## Section 7.7: Discussion

### Section 7.7.1: *Sinc/Prn-i* and *Prn-p* are congruent

129/Ola PrP gtB/gtB mice homozygous for the targeted *Prn-p* $^{a[108F\_189V]}$ allele had incubation times of 133 days -more than 110 days shorter than wild-type 129/Ola PrP A/A mice encoding the wild-type *Prn-p* $^{a}$, *Prn-i* $^{N}$ and *Sinc* s7 alleles. The dramatically shortened incubation times of 129/Ola PrP gtB/gtB mice were very similar to the 119 day incubation times of VM/Dk PrP B/B mice encoding the *Prn-p* $^{b}$, *Prn-i* $^{I}$ and the *Sinc* p7 alleles. This data shows that PrP codon 108 and 189 alterations can convert incubation times from that characteristic of mice encoding Prn-p $^{a}$, *Prn-i* $^{N}$ and *Sinc* s7 alleles to those characteristic of mice encoding *Prn-p* $^{b}$, *Prn-i* $^{I}$ and *Sinc* p7 alleles. This demonstrates that PrP codon 108 and 189 dimorphisms are the major molecular determinants of 301V incubation time in the mouse. This is the best evidence yet that *Sinc/Prn-i* and *Prn-p* are congruent and confirms long standing observations that a single gene exerts a major control over incubation times for a number of mouse adapted scrapie, CJD and BSE isolates (Dickinson, Meikle and Fraser, 1968; Carlson et al., 1986, 1988, 1993; Kingsbury et al., 1983; Bruce et al., 1994).

Although the role of PrP codons 108 and 189 are demonstrated here with a single mouse adapted BSE strain (Bruce et al., 1994) it is expected that mice bearing the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele will respond to challenge with other strains in a manner characteristic of the wild-type *Prn-p* $^{b}$ allele. Preliminary observations from ME7 challenge experiments indicate that this is the case.

Transgenic studies have established that increased PrP expression levels accelerate incubation period (Westaway et al., 1991; Prusiner et al., 1990; Telling et al., 1996; Fischer et al., 1996). The PrP immunoblotting studies described in chapter 6 (Figures 6.3 , p136 and Figure 6.4, p138) indicate that gene targeting has not altered the PrP expression levels of mice bearing the gene targeted allele with respect to wild-type 129/Ola and VM/Dk mice. This argues that the dramatic shortening of incubation time is not attributable to raised PrP expression levels in mice expressing the *Prn-p* $^{a[108F\_189V]}$ allele but is due to the specific effect of the engineered codon 108F and 189V alterations.

The 301V incubation times of 129/Ola PrP gtB/gtB and VM/Dk (PrP B/B) mice are very similar and indicate that the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele is capable of specifying the short 301V incubation times normally associated with the *Prn-p* $^{b}$ allele. The 133 day incubation time of 129/Ola PrP gtB/gtB mice is 14 days longer than is observed in 301V challenges of VM/Dk, which are 119 (±2 SE days, n=12) days (Table 7.1, p146). Potential explanations for this slight incubation time difference include the effect of non-PrP genes, the effects of non-coding *Prn-p* polymorphisms and slight differences in the pattern or level of PrP expression between the wild-type *Prn-p* $^{b}$ allele and the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele.

A 14 day difference between 129/Ola PrP gtB/gtB and VM/Dk mice is well within the range of incubation times observed in mice encoding the same *Prn-p* allele. For example, the 301V incubation times of wild-type 129/Ola and C57BL PrP A/A inbred mouse strains, which both encode the *Prn-p* $^{a}$ allele, differ by ~55 days (Table 7.1, p146). A number of non-coding *Prn-p* polymorphisms which differentiate the *Prn-p* $^{a}$ and *Prn-p*$^{b}$ alleles have been described -although the significance of these polymorphisms is uncertain. The dramatic alteration in incubation times reported here in 129/Ola mice encoding a common *Prn-p* $^{a}$ allele, which differs by only two amino acid alterations, indicates that *Prn-p* coding polymorphisms have the major role in the control of incubation time. However, it is possible that non-coding *Prn-p* polymorphisms are responsible for the small difference between the incubation times of 129/Ola homozygous *Prn-p* $^{a[108F\_189V]}$ and VM/Dk *Prn-p* $^{b}$ mice.

As described above, PrP expression levels have a major influence upon incubation times (Prusiner et al., 1990; Telling et al., 1996; Fischer et al., 1996). A role for large differences in PrP$^{c}$ expression is doubtful given the similar levels of brain PrP$^{c}$ expression observed in 129/Ola PrP co-isogenic lines and VM/Dk mice. However, at the level of analysis afforded by PrP immunoblotting it is not possible to exclude that the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele expresses PrP at slightly lower levels than the wildtype *Prn-p* $^{a}$ and/or *Prn-p* $^{b}$ alleles. Even if more detailed analysis can show that the *Prn-p* $^{a[108F\_189V]}$ allele expresses PrP at levels lower than wild-type *Prn-p* alleles, the

main finding of this project (that codons 108 and 189 modulate incubation time) will remain secure. This is because reduced PrP gtB expression levels from the *Prn-p* $^{a[108F\_189V]}$ allele would almost certainly act to prolong incubation times in 129/Ola PrP gtB/gtB mice. If this were the case, the dramatic shortening of 301V incubation times described here in 129/Ola PrP gtB/gtB mice would constitute an underestimate of the biological effects of PrP codon 108 and 189 dimorphism. This would strengthen the case that they control incubation time in mice encoding wildtype *Prn-p* $^{b}$ alleles. When available, a mouse PrP ELISA will be used to compare the whole brain PrP expression levels in mice expressing PrP A from the *Prn-p* $^{a}$ allele, PrP B from the *Prn-p* $^{b}$ allele and 129/Ola mice expressing PrP gtB from the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele.

Although previous studies indicate that the levels of PrP expression in *Prn-p* $^{a}$ and *Prn-p* $^{b}$ mice are indistinguishable (Westaway et al., 1987; Manson, McBride and Hope, 1992), it is formally possible that they *do* differ. A subtle difference in PrP A and PrP B expression levels from these alleles would lead to incubation times which were dependent not only on the differential actions of PrP A and B allotypes but also on expression level effects. A similar argument could be deployed with respect to PrP expression pattern. Such effects could not be fully reproduced in gene targeted mice differing only with respect to PrP codon 108 and 189 dimorphisms.

It is also possible that the *Prn-p* $^{a[108F\_189V]}$ allele differs from the wildtype VM/Dk *Prn-p* $^{b}$ allele in a region outwith the PrP ORF leading to minor expression levels differences *in vivo*. In support of this notion are observations of non-coding polymorphisms within *Prn-p*. The *Prn-p* intron 2 structure of 129/Ola and VM/Dk differ with respect to a cryptic 6.7Kb insertion/deletion (Westaway et al., 1994a) and two PrP 3'UTR polymorphisms have been reported at nt 444 and nt 1010 which differ between *Prn-p* $^{a}$ and *Prn-p* $^{b}$ mice [H.Baybut and J.Hope, Genbank Accession numbers X83612 and X83613]. However, little is known of the effect that these polymorphisms may have upon PrP expression and further analysis will be required to exclude this as a source of slight differences in PrP expression level or expression pattern between mice encoding *Prn-p* $^{a}$ and *Prn-p* $^{b}$.

301V challenge of C57BL (*Prn-p* [a]) and F1[C57xVM] (*Prn-p* [a/b]) mice reveal that F1 *Prn-p* [a/b] mice have incubation times approximately 31 days shorter than C57 mice (Table 7.1, p146). This indicates that the *Prn-p* [a] allele, or rather the PrP A allotype, is dominant in conferring long incubation times with 301V. It was therefore expected that 301V challenges in 129/Ola PrP A/A and PrP A/gtB mice would reproduce this incubation time ranking. Indeed, 129/Ola PrP A/gtB mice had incubation times close to that observed in F1[C57xVM] (Table 7.1, p146) and F1[VM-*Sinc*$^{s7}$ x VM] mice (Table 7.2, p172, below; Moira Bruce, unpublished data), but they were 15 days *longer* than 129/Ola PrP A/A mice.

**Table 7.2: 301V i.c. challenge of *Sinc* congenic mice [a]**

| Mouse Strain/Cross | PrP genotype | PrP allotype | *Sinc* genotype | Mean Incubation time [a] (days) | SE [b] (days) |
|---|---|---|---|---|---|
| VM-*Sinc*$^{s7}$ [c] | *Prn-p* [a/a] | A/A | s7s7 | 248.8 | 5.8 |
| F1 [VM-*Sinc*$^{s7}$ x VM/Dk] [d] | *Prn-p* [a/b] | A/B | s7p7 | 226.9 | 2.1 |
| VM/Dk | *Prn-p* [b/b] | B/B | p7p7 | 117.5 | 1.4 |

**a** This is unpublished data kindly provided by Dr Moira Bruce, MRC/BBSRC Neuropathogensis Unit, Edinburgh; **b**, standard error of the mean; **c**, also known as SV; **d** also known as SVV.

There are a number of potential explanations for this, including the possibility that the *Prn-p* [a[108F_189V]] allele expresses at slightly lower levels than the *Prn-p* [b] allele. Although the expression studies reported here indicate that this is unlikely, it is not inconceivable. If this were the case, it could provide an explanation for the slightly longer incubation times of both 129/Ola PrP gtB/gtB and 129/Ola PrP A/gtB mice. It is more likely that genetic differences between 129/Ola and VM/Dk are responsible for this slight deviation from the predicted incubation time ranking of *Prn-p* [a] and *Prn-p* [a/a[108F_189V]] mice. Indeed, control challenges with *Prn-p* [a/b] (PrP A/B) mice were performed on F1[C57xVM] mice and it is possible that genes linked to *Prn-p* [b] could modify the

incubation time in F1 mice. If this were the case, 129/Ola PrP co-isogenic mice which share a common genetic background would not be able to reproduce this effect.

It is also formally possible that 129/Ola co-isogenic PrP A/A and PrP A/gtB mice reveal, for the first time, a small degree of overdominance which is not evident in challenges with other mouse strains. In support of this is the observation that overdominance was a consistent feature in primary transmissions of BSE to C57BL, VM/Dk and F1[C57xVM] mice and that 301C, a C57BL passaged mouse adapted BSE strain, exhibits overdominance (Table 7.3, p174; Bruce et al., 1994). The reduced pathology evident in PrP A/gtB mice would support this.

### Section 7.7.2: PrP codon 108 and 189 dimorphisms control some aspects of 301V neuropathology

129/Ola PrP gtB/gtB mice had a number of differences in neuropathology that correlated well with their shorter incubation times. The PrP gtB allotype was responsible for raised levels of $PrP^{Sc}$, directed more severe vacuolar change to a number of areas and in many scoring areas appeared to promote more rapid vacuolar change.

Compared to PrP A/A mice, PrP A/gtB mice had a large number of similarities in lesion profile. However, reduced levels of $PrP^{Sc}$ in whole brain and reduced levels of neuropathology in a number of areas were consistent with the slightly longer incubation times of PrP A/gtB mice. Examples of reduced pathology included considerably reduced cell loss in the hippocampus, less vacuolar change in the hypothalamus and reduced PrP deposition in both the thalamus and the hippocampus.

Although PrP A/A mice had more extensive PrP deposition in the thalamus and hippocampus this was not reflected by higher levels of $PrP^{Sc}$ on PrP immunoblots from terminal brains. Indeed, PrP immunoblotting indicated that PrP gtB/gtB had higher levels of $PrP^{Sc}$. This discrepancy may be because impressive regional differences in the extent and degree of PrP deposition are lost when whole brains are homogenised and examined by SDS-PAGE. It is also possible that PrP gtB/gtB brain contains a considerable amount of diffuse PrP which is difficult to quantify by immunohistochemistry.

The incubation time rankings of 129/Ola PrP A/A and 129/Ola PrP A/gtB mice are similar -indicating that the PrP A allotype is dominant. It was notable that in almost all lesion profile scoring areas, PrP A/A and PrP A/gtB mice had similar scores, suggesting that the PrP A allotype is also dominant in its ability to promote vacuolar change in multiple scoring areas. The hypothalamus was conspicuous in that it was the only area in which PrP A/gtB lesion scores were lower than in 129/Ola PrP A/A mice [5]. This may reflect a difference between PrP A and PrP gtB allotypes in their ability to target vacuolar change to this area. The relative sparing of the PrP A/gtB hypothalamus suggests that the PrP gtB allotype can have a "protective" effect. A similar effect was evident in the relative neuronal preservation of the PrP A/gtB hippocampus (Figure 7.6, p163) and in the reduced PrP deposition in the hippocampus (Figure 7.7, p165) and thalamus (Figure 7.8, p167). The reduced pathology in these areas of 129/Ola PrP A/gtB brain may explain their prolonged or "overdominant" incubation times with respect to 129/Ola PrP A/A mice.

It is currently uncertain whether the ranking of the PrP A/A and PrP A/gtB incubation times in 129/Ola PrP co-isogenic mice really reflects overdominance because 129/Ola PrP A/gtB mice have incubation times which are only 15 days longer than 129/Ola PrP A/A mice. As Table 7.3 (below) illustrates, in other challenges where overdominance has been established, F1[C57xVM] PrP A/B mice have incubation times which are at least 60 days longer than parental C57BL PrP A/A and VM/Dk PrP B/B incubation times.

**Table 7.3: Overdominance in various mouse TSE strains**

| | Incubation time (days) | | | |
|---|---|---|---|---|
| Strain | PrP A/A | PrP A/B | PrP B/B | Reference |
| 301C | 207 | 565 | 363 | Bruce et al., 1994 |
| 22A | 474 | 567 | 199 | Bruce et al., 1991 |
| 139A | 166 | 270 | 208 | Bruce et al., 1991 |

PrP A/A= C57BL; PrP A/B=F1[C57xVM]; PrP B/B= VM/Dk. Note: all i.c challenges.

[5] The reduced vacuolar change observed in the 129/Ola PrP A/gtB hypothalamus is also apparent in the lesion profiles of 301V challenged *Sinc* congenic mice (Dr. Moira Bruce, unpublished data).

It has been suggested that overdominance, co-dominance and complete dominance arise from various degrees of blocking effect between the heterologous host PrP allotype and the more productive interactions occurring between isologous host and inoculum donor PrP molecules. Carlson has suggested that overdominance arises from reduced levels of the PrP allotype isologous to the inoculum donor PrP sequence (Carlson et al., 1994). Currently it is unclear which, if any, of these explanations is correct. There is some evidence that a total blocking interaction does not occur in 301V: this is because 129/Ola PrP A/- mice have 301V incubation times of ~320 days (Jean Manson, unpublished data). Although it is difficult to compare incubation times from different experiments, the 244 day incubation time of 129/Ola PrP A/gtB mice reported in this thesis suggests that the gtB allotype is contributing to the shorter incubation period in PrP A/gtB mice (compared to A/- mice). This indicates that the PrP gtB allotype is functional in the presence of the PrP A allotype -arguing against a total blocking effect. This is consistent with Carlson's proposal that overdominance, when it is observed, arises from reduced levels of the allotype isologous to the prion inoculum. However, if this is the case it is unclear why different *degrees* of overdominance are apparent with different strains of agent (Table 7.3, p174). One potential explanation arises from the observation that distinct strains respond differently to reduced levels of PrP isologous to that of the inoculum donor. For example, ME7 incubation times[6] in 129/Ola PrP A/A mice are 160 days and are prolonged to 284 days in PrP A/- mice (Manson et al., 1994) but RML incubation times are prolonged from 143 days (±4 SE) in PrP A/A mice to 426 days (± 18 SE) in the corresponding strain of PrP A/- mice (Prusiner et al., 1993; Carlson et al., 1994). Although these incubation times are taken from different experiments they indicate an role for agent strain in the response to reduced levels of PrP. Thus the different degrees of overdominance, and perhaps other dominance phenomena, may reflect strain-determined responses to reduced levels of the PrP allotype isologous to the donor inoculum.

[6] ME7 and RML passaged in PrP A/A hosts.

**Section 7.8: Summary**

The dramatically shortened incubation times in 129/Ola *Prn-p* $^{a[108F\_189V]}$ homozygotes demonstrate that PrP codon 108 and 189 dimorphisms control incubation time with 301V and argues strongly that *Sinc/Prn-i* and *Prn-p* are congruent.

PrP codon 108F and/or 189V alterations resulted in changes to the neuropathology of PrP gtB/gtB and PrP A/gtB mice. 129/Ola PrP gtB/gtB mice had a number of features which corresponded well with their short incubation times: they had more $PrP^{Sc}$, greater lesion scores in a number of areas whilst the extent of vacuolation in other areas indicated that the rate of vacuolation was increased in PrP gtB/gtB mice.

Compared to PrP A/A mice, PrP A/gtB mice exhibited significantly less vacuolar change in the hypothalamus, less neuronal death in the hypothalamus and reduced PrP deposition in the thalamus and subcallosal region of the hippocampus. These observations were consistent with the longer incubation times of PrP A/gtB mice compared to PrP A/A mice.

# Chapter 8

## Summary

**Section 8.1: Murine scrapie incubation time genetics**

Classical genetic studies performed by Alan Dickinson and colleagues nearly thirty years ago established that experimental scrapie incubation time in mice was under the control of a single gene, *Sinc* encoding two alleles, s7 and p7 (Dickinson, Meikle and Fraser, 1968). Similarly, large differences in Chandler scrapie incubation time between NZW/LacJ and ILn/J inbred mouse strains enabled the identification of *Prn-i*, a gene encoding 2 alleles, N and I (Carlson et al., 1986, Carlson et al., 1988).

Important clues to the identity of both *Prn-i* and *Sinc* followed the discovery of linkage of both genes to the mouse prion protein gene, *Prn-p*, using Xba I RFLP analysis (Carlson et al., 1986; Hunter et al., 1987). A potential molecular basis for *Sinc* and *Prn-i* allelism was inferred following the discovery that mouse strains with large differences in incubation time encode different prion protein allotypes, PrP A and PrP B. PrP allotypes arise from codon 108L/F and 189T/V dimorphisms such that PrP A encodes 108L_189T and PrP B encodes 108F_189V (Westaway et al., 1987). *Sinc/Prn-i* and *Prn-p* are closely linked (Carlson et al., 1986, 1988; Carlson et al., 1993), but the unambiguous delineation of the relationship between these genes is lacking and four independent test crosses have yielded small numbers of progeny with discordant PrP genotype and scrapie incubation times (Carlson et al., 1986, 1988; Race et al., 1990). The lethal nature of these experiments has frustrated attempts to determine whether discordant mice represent meiotic recombination events between *Prn-p* and a linked, but distinct, incubation time control locus, or whether the discordant incubation times are due to the reproducible co-segregation of other genes which, in combination, have a large effect upon incubation time.

A convincing demonstration of recombination between *Sinc/Prn-i* and *Prn-p* would be highly significant and could indicate that incubation times for murine scrapie, and perhaps other prion diseases, are controlled by a tightly linked but distinct locus from that encoding the prion protein. To date, all attempts have failed to formally demonstrate that *Prn-p* allelism is the major determinant of murine scrapie incubation time. One such approach utilised congenic VM/Dk ($Sinc^{p7p7}$) and VM/Dk-$Sinc^{s7s7}$ mouse lines which encode distinct *Sinc* alleles, *Prn-p* alleles, PrP allotypes and have dramatically different scrapie incubation times yet share a common VM/Dk background (Hunter et al., 1987;

Bruce et al., 1991; Hunter et al., 1992). However, such lines could not provide formal proof of congruence between *Sinc/Prn-i* and *Prn-p* because they also differ with respect to ~10cM of chromosomal DNA flanking *Prn-p* which could contain a number of other genes responsible for incubation time control (Hunter et al., 1992). In an attempt to overcome these problems, transgenic mice were generated encoding the PrP B allotype expressed from a *Prn-p* $^{b}$ cosmid transgene but PrP overexpression prevented a formal demonstration that codons 108 and 189 controlled incubation times in these mice (Westaway et al., 1991; Carlson et al., 1994).

**Section 8.2: Gene targeting**

**Section 8.2.1: Introduction**

The difficulties associated with previous attempts to investigate the relationship between *Sinc*, *Prn-i*, *Prn-p* suggested that the issue might only be resolved with gene targeting. It was reasoned that dramatic alterations to the incubation time of mice in which the endogenous PrP A allotype had been converted to the PrP B allotype would constitute a definitive test of the congruence of *Sinc*, *Prn-i* and *Prn-p*. It has also been shown that non-*Prn-p* genes can have a significant effect upon scrapie incubation times and neuropathology (Bruce and Dickinson, 1985; Carlson et al., 1988; Race et al., 1990; Bruce et al., 1991; Carlson et al., 1994) and it was also reasoned that this source of variation might also be controlled by generating co-isogenic 129/Ola mice differing only by targeted codon 108 and 189 alterations (Moore et al., 1995). As an extension to this main aim, it was intended that the role of codons 108 and 189 be explored individually by generating mice which differ from co-isogenic 129/Ola wildtype mice by single PrP codon 108F and codon 189V alterations.

A two step double replacement gene targeting strategy (Moore et al., 1995) was used to alter *Prn-p* $^{a}$ exon 3 in HM-1 (Magin et al., 1992), a 129/Ola ES cell line (Simpson et al., 1997). To facilitate gene targeting, a genomic map of the 129/Ola prion protein locus was generated using HM-1 ES cells. This confirmed that 129/Ola had RFLPs characteristic of the *Prn-p* $^{a}$ allele. The genomic map of *Prn-p* $^{a}$ also guided studies in which a contiguous set of λ DASH II clones spanning the 129/Ola *Prn-p* $^{a}$ allele were isolated from an HM-1 genomic library. A plasmid clone of the 8.5Kb BamHI

fragment, p129PrP, was retrieved and mapped. Mapping studies of p129PrP revealed that this clone contained all the sequence homology necessary for the construction of isogenic targeting vectors. DNA sequencing of the exon 3 region in p129PrP confirmed that 129/Ola mice expressed the PrP A allotype (Westaway et al., 1987; Carlson et al, 1988). These studies were consistent with previous ME7 and 301V challenges in wild-type 129/Ola mice which demonstrated that this mouse strain was *Sinc*$^{s7s7}$ (Manson et al., 1994b; Jean Manson, unpublished data).

**Section 8.2.2: Step 1 -PrP inactivation**

In the first gene targeting step a number of ES clones were generated in which one allele of PrP was inactivated by the targeted introduction of an HPRT minigene. Clones with low levels of 6-thioguanine resistance were tested for their ability to colonise the mouse germline. A number of clones generated germline chimeras, demonstrating their suitability for the second gene targeting step.

Germline transmission of the *Prn-p* null allele enabled the generation of *Prn-p* $^{-/-}$ mice. Ageing of *Prn-p* $^{-/-}$ mice in excess of 14 months revealed an adult onset ataxia with onset 442 days (±5 SE, n=22), similar to that previously described by Sakaguchi et al., (1996). The phenotype was somewhat milder and this could be because these mice were younger than those described by Sakaguchi et al., (1996). Preliminary counting experiments of Purkinje cells in two cerebellar lobules indicated that the PrP nulls made during the course of this thesis had reduced numbers of this cell type. The independent observation of an adult-onset ataxia in these mice verifies the observations of Sakaguchi et al., (1996). Whilst it appears reasonable to conclude that the ataxia arises from the loss of Purkinje cells, it is not apparent why this cell type dies in *Prn-p* $^{-/-}$ mice. Further studies will investigate this issue.

**Section 8.2.3:Step 2 -generation of mice with PrP codon 108 and 189 alterations**

Gene targeting was used to specifically alter PrP codons 108L_189T to 108F_189V, to generate a modified *Prn-p* $^{a}$ allele expressing the PrP B allotype[1]. The targeted *Prn-p* $^{a[108F\_189V]}$ allele differed from the endogenous 129/Ola *Prn-p* $^{a}$ allele only by engineered alterations to codon 108 (CTC-TTC) and codon 189 (ACC-GTC) resulting in L-F and T-V substitutions respectively. The structure of the targeted *Prn-p* $^{a[108F\_189V]}$ allele was investigated by Southern analysis using a variety of *Prn-p* probes and confirmed that the targeted *Prn-p* $^{a[108F\_189V]}$ allele had the predicted structure and had undergone no detectable deletions, insertions or other rearrangements during the gene targeting process.

ES cell clones bearing the *Prn-p* $^{a[108F\_189V]}$ allele were used to construct chimeras. A germline chimera was mated with inbred 129/Ola mice to generate progeny heterozygous for the *Prn-p* $^{a[108F\_189V]}$ allele. All *Prn-p* $^{a[108F\_189V]}$ alleles were maintained on a 129/Ola background, ensuring the co-isogenic nature of these mice with wild-type 129/Ola. This controls for the effects of non-PrP genes and enables changes in incubation time and pathology to be attributed to the specific effects of gene targeted PrP codon 108 and 189 alterations.

The level of PrP mRNA and $PrP^{C}$ in the brains of uninoculated mice was investigated and confirmed the expression of the targeted *Prn-p* $^{a[108F\_189V]}$ allele in brain but also demonstrated that mice encoding a *Prn-p* $^{a[108F\_189V]}$ allele expressed PrP at levels indistinguishable from non-targeted co-isogenic *Prn-p* $^{a}$ littermates. The $PrP^{C}$ SDS-PAGE banding pattern was unaltered in mice homozygous for the *Prn-p* $^{a[108F\_189V]}$ allele and expressing the gene targeted PrP gtB allotype, indicating that manipulation of codons 108 and 189 has no deleterious effect on PrP structure at this level of analysis.

These studies argue that differences in incubation time and neuropathology between wild-type 129/Ola mice and mice encoding the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele are not due to differences in $PrP^{C}$ expression level but to the specific effect of

[1] The gene targeted allele has been named *Prn-p* $^{a[108F\_189V]}$ to distinguish it from wild-type *Prn-p* $^{a}$ and *Prn-p* $^{b}$ alleles.

modified PrP codons 108 and 189. This enabled a formal test of the proposal that PrP codon 108L/F and 189T/V dimorphisms control incubation time.

## Section 8.3: 301V challenge of 129/Ola PrP co-isogenic mice

### Section 8.3.1: PrP codons 108 and 189 control the response of mice to 301V challenge and demonstrate the congruence of *Prn-p*, *Prn-i* and *Sinc*

301V challenges revealed a critical role for PrP codons 108 and 189. 129/Ola PrP gtB/gtB mice homozygous for the targeted *Prn-p* $^{a[108F\_189V]}$ allele had incubation times of 133 days -more than 110 days shorter than wild-type 129/Ola PrP A/A mice encoding the wild-type *Prn-p* $^{a}$ , *Prn-i* $^{N}$ and *Sinc* s7 alleles. The 133 day incubation time was very similar to the 119 day incubation time of VM/Dk PrP B/B mice encoding wild-type *Prn-p* $^{b}$ and *Sinc* p7 alleles. This demonstrates that PrP codon 108 and 189 dimorphisms are the major molecular determinants of incubation time in the mouse and that homozygosity for the *Prn-p* $^{a[108F\_189V]}$ allele can convert incubation times from those of wild-type 129/Ola mice encoding *Prn-p* $^{a}$, *Prn-i* $^{N}$ and *Sinc* s7 alleles to those characteristic of VM/Dk mice encoding wildtype *Prn-p* $^{b}$, *Prn-i* $^{I}$ and *Sinc* p7 alleles.

This confirms that *Sinc/Prn-i* and *Prn-p* are congruent, validating long standing observations that a single gene exerts a major control over incubation times for a number of mouse adapted scrapie, CJD and BSE isolates (Dickinson, Meikle and Fraser, 1968; Carlson et al., 1986, 1988, 1993; Kingsbury et al., 1983; Bruce et al., 1991,1994).

Although the role of PrP codons 108 and 189 are demonstrated here with a single mouse adapted BSE strain (Bruce et al., 1994) it is expected that mice bearing the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele will respond to challenge with other strains in a manner characteristic of the wild-type *Prn-p* $^{b}$ allele. Preliminary observations from ME7 challenge experiments indicate that this is the case.

129/Ola PrP gtB/gtB mice had 301V incubation times that were 14 days longer than VM/Dk mice. This small difference may be attributable to the differing genetic backgrounds of 129/Ola and VM/Dk or to the effects of non-coding *Prn-p* polymorphisms. Indeed, a 14 day difference is well within the range of incubation times observed between different inbred mouse lines encoding the same *Prn-p* allele.

Although a number of non-coding *Prn-p* polymorphisms have been described, the dramatic alteration in incubation times reported here in 129/Ola mice encoding a common *Prn-p* $^{a}$ allele, which differs by only two amino acid alterations, indicates that *Prn-p* coding polymorphisms have the major role in the control of incubation time. Nevertheless, it is possible that non-coding *Prn-p* polymorphisms are responsible for the 14 day difference between the incubation times of 129/Ola PrP gtB/gtB and VM/Dk mice.

Previous 301V challeneges of F1[C57BLxVM] (*Prn-p* $^{a/b}$) mice revealed that they have incubation times which were 20-30 days shorter than C57BL (*Prn-p* $^{a}$) mice. This indicates that the *Prn-p* $^{a}$ allele, or rather the PrP A allotype, is almost completely dominant in conferring long incubation times with 301V. It was therefore expected that 301V challenges in 129/Ola PrP A/A and PrP A/gtB mice would reproduce this incubation time ranking. 129/Ola PrP A/gtB mice had incubation times that were 15 days longer than 129/Ola PrP A/A mice. It is possible that there are small differences in expression levels from these alleles which are below the level of sensistivity afforded by PrP immunoblotting. If this were the case, it could provide an explanation for the slightly longer incubation times of both 129/Ola PrP gtB/gtB and 129/Ola PrP A/gtB mice. It is, however, likely that genetic differences between 129/Ola and VM/Dk are responsible for this slight deviation from the predicted incubation time ranking.

**Section 8.3.2: PrP allotype controls 301V neuropathology**

Although it is well established that agent strain is one of the main determinants of neuropathology (Fraser and Dickinson, 1967, 1968; Fraser, 1976; Bruce et al., 1989, 1991; Hecker et al. 1992; Carlson et al., 1994), the extent to which PrP A and B allotypes control neuropathology remains controversial. Studies with *Sinc* and *Prn-p* congenic lines suggest that PrP allotype can modify pathology, but such studies cannot exclude the possibility that linked genes, rather than *Prn-p*, are responsible. Indeed, Carlson has described a *Prn-p* linked gene which influences neuropathology in a number of brain areas (Carlson et al., 1994).

129/Ola PrP co-isogenic mice provided an opportunity to make comparisons between mice expressing different PrP allotypes whilst controlling for the effects of non-

PrP genes. 301V challenge of these mice indicated that PrP allotype controls the extent and distribution of vacuolar change in many brain areas and the extent of neuronal loss in the hippocampus. Evidence based upon a smaller number of mice indicates that PrP allotype controls the extent and distribution of PrP deposition in the hippocampus and thalamus.

Areas of common vacuolar change between 129/Ola PrP gtB/gtB and VM/Dk mice suggest common lesion targeting sites for the PrP B allotype. Together with the dramatic effect of codon 108 and 189 alterations on incubation time, the alterations to pathology described here argue that the gene targeted *Prn-p* $^{a[108F\_189V]}$ allele can reproduce important features of the *Prn-p* $^{b}$ allele or PrP B allotype. Minor differences in 129/Ola PrP gtB/gtB and VM/Dk incubation times and lesion profiles indicate that genes other than the *Prn-p* influence the pattern and extent of vacuolation in some brain areas. It is also possible that non-coding *Prn-p* polymorphisms can influence incubation time and neuropathology.

**Section 8.4: Future studies in mice with PrP codon 108 and 189 alterations**

Future studies with 129/Ola co-isogenic mice bearing gene targeted alterations to PrP will enable the exploration of the role of PrP sequence in the control of many aspects of prion disease in the absence of PrP gene dosage effects and the confounding effects of other genes. Although the 301V challenges reported here demonstrate that PrP codon 108 and 189 dimorphisms control incubation time, it is not known whether these dimorphisms act singly or together. Future challenges will examine incubation times and neuropathology in gene targeted mice encoding *Prn-p* $^{a[108F]}$ and *Prn-p* $^{a[189V]}$ alleles expressing PrP molecules with individual 108F and 189V alterations. This may reveal whether incubation time is controlled by one or both positions. Challenge of these mice with a variety of strains may also determine whether the relative importance of each PrP codon is fixed or whether it is strain dependent.

# Chapter 9

# Materials and methods

## Section 9A: Materials

### Section 9A.1: Suppliers of laboratory reagents

#### Section 9A.1.1: Restriction endonucleases and other nucleic acid modifying enzymes

Boehringer Mannheim PLC.

GIBCO BRL Life Technologies.

New England Biolabs Inc.

Pharmacia LKB Biotechnology.

#### Section 9A.1.2: Standard laboratory reagents

BDH Chemicals Ltd.

Fisons Chemicals.

GIBCO BRL Life Technologies.

ICN Flow Ltd.

Sigma Chemical Co.

#### Section 9A.1.3: Bacterial media reagents

Becton-Dickinson U.K. Ltd.

Difco Laboratories.

#### Section 9A.1.4: Reagents for mouse embryonic stem cell culture

GIBCO BRL Life Technologies.

ICN Flow Ltd.

Sera-lab Ltd.

Sigma Chemical Co.

#### Section 9A.1.5: Radioactive reagents

[$\alpha$-$^{32}$P]dCTP 3000 Ci/mmol, Amersham Redivue

[$\gamma$-$^{32}$P]dATP 3000 Ci/mmol, Amersham Redivue

**Section 9A.1.6: Antibiotics**

Aminopterin, Sigma Chemical Co.

Ampicillin, Beecham Research Laboratories.

Chloramphenicol, Sigma Chemical Co.

Penicillin G, Sigma Chemical Co.

**Section 9A.1.7: Antisera**

1A8 rabbit anti mouse PrP 27-30 polyclonal antiserum (Farquar et al., 1994), a gift from the BBSRC/MRC Neuropathogenesis Unit, West Mains Road, Edinburgh.

**Section 9A.2: Media, antibiotics and miscellaneous**

**Section 9A.2.1: Bacterial media**

| | |
|---|---|
| Luria Broth (LB): | 10g Bacto-tryptone (Difco), 5g bacto-yeast extract (Difco), 5g NaCl per litre adjusted to pH 7.2 with NaOH. |
| Luria Agar: | As Luria broth with 15 g per litre Difco agar. |
| Terrific Broth (TB): | 12g bacto-tryptone , 24g bacto-yeast extract, 4ml glycerol per 900mls plus 100 mls sterile solution of 0.17 M $KH_2PO_4$, 0.72M $K_2HPO_4$ after autoclaving. |
| 2xYT: | 16g Bacto-tryptone, 10g Yeast extract, 5g NaCl, d.$H_2O$ to 1L. |
| BBL top agar: | 10g trypticase (Baltimore BioLabs), 5g NaCl, 6.5g bacto-agar (Difco), 10mM $MgSO_4$, per litre, adjusted to pH 7.0 with NaOH. |
| M9 minimal agar: | 15g bacto-agar, 100ml 10x M9 salts, 20ml 20% (w/v) glucose, 10ml 0.1M $MgSO_4$, 10ml 1mg/ml Vitamin B1, 10ml 0.1M $CaCl_2$ to 1L with d.$H_20$. |
| 10x M9 salts: | 60g $Na_2HPO_4$, 30g $KH_2PO_4$, 5g NaCl, 10g $NH_4Cl$ to 1L with d.$H_20$. |

**Section 9A.2.2: Antibiotics**

Ampicillin to a final concentration of 100 mg/ml in d.$H_20$ stored in aliquots at -20°C

Chloramphenicol stock: 30mg/ml in 100% ethanol, used at 30ug/ml. Stored in aliquots at -20°C.

**Section 9A.2.3: Miscellaneous**

| | |
|---|---|
| IPTG: | (isopropyl-β-D-thiogalactopyranoside) 100mM stock in d.$H_2O$. Filter sterilised, stored at -20°C. |
| X-gal: | (5 Bromo 4-chloro 3-indolyl β-D-galactoside) 2% stock in dimethylformamide. Stored at -20°C. |
| Proteinase K: | 5mg/ml in d.$H_2O$ (Boehringer Mannheim), stored at 4°C. |
| HAT (100x): | 1M hypoxanthine, 100mM thymidine, 100uM aminopterin, dissolved in d.$H_2O$ and stored at -20°C. |
| gancyclovir: | 2uM stock in PBS, stored at -20°C. |
| Trypsin: | 0.025% (w/v) trypsin (ICN Flow, Cat No. 043-5090), 1mM EDTA, 1%(v/v) chicken serum (ICN Flow Cat No.29-501-49) in 0.8x PBS. |

**Section 9A.2.4: Standard buffers and solutions**

| | |
|---|---|
| 20X SSC: | 3 M NaCl; 0.3 M tri-sodium citrate, pH 7.0 |
| 10X TBE: | 0.9 M Tris-HCl; 0.9 M boric acid, 20 mM EDTA ,pH 8.0 |
| 50X TAE: | 2 M Tris, 1 M glacial acetic acid; 50 mM EDTA, pH 8.0 |
| TE: | 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0 |
| PBS: | 8 mM $K_2HPO_4$; 1.5 mM $KH_2PO_4$, 150 mM NaCl |
| 10x MOPS: | 200mM MOPS, 50mM sodium acetate, 10mM EDTA pH 7.0 |
| Phage Buffer (SM): | 50mM Tris.HCl, pH7.5, 100mM NaCl, 10mM MgSO4, 2% (w/v) gelatine. |
| Denaturing Buffer: | 0.5M NaOH, 1.5M NaCl |
| Neutralising buffer: | 3M NaCl, 0.5M Tris.HCl, pH 7.0 |

**Table 9.1: E. coli strains**

| Strain | Genotype | Reference |
|---|---|---|
| CJ236 | *dut 1, ung 1, thi I, rel AI/pCJ105 ($Cm^r$)* | Kunkel et al., 1987 |
| DH5αF′ | *supE44, ΔlacU169(f80lacZΔM15), hsdR17, recA1, endA1, gyrA96, thi-1* | Hanahan, 1983 |
| JM105 | *thi-I, rpsL, end A, sbc BC, hsdR4, Δ(lac-proAB), [F′ traD36, proAB, $lac^q$ZDM15]* | Yanish-Perron et al., 1985 |
| K802 | *e14⁻(mcrA), mcrB1,hsdR2, galK2, galT22, supE44, metB1* | Raleigh and Wilson, 1986 |
| NM522 | *supE, thi-1, Δ(lac-proAB), Δ(hsdSM-mcrB)5, [F′, proAB, $lac^q$ZDM15]* | Gough and Murray, 1983 |
| XL-1 Blue | *recA1, endA1, gyrA96,thi-1, hsdR17, supE44, relA1, lac [F′, proAB, $lac^q$ZΔM15, Tn 10 ($tet^r$)]* | Bullock, Fernandez and Short, 1987 |

**Table 9.2: Cloning vectors and plasmid constructs**

| Name | Description | Reference |
|---|---|---|
| pBluescript | General cloning vector. | Thummel, 1988 |
| M13mp18 | Cloning vector for in vitro mutagenesis. | Norrander, Kempe and Messing, 1983 |
| λ DASHII | Bacteriophage λ sequence replacement vector. Used for construction of 129/Ola HM-1 genomic library. | Stratagene |
| pHPT5 | Full length mouse HPRT cDNA. cDNA insert used as a hybridisation probe for HPRT genotyping of mouse tail DNA. | Konecki et al., 1982 |
| pUC18 | General cloning vector. | Yanisch-Perron et al., 1985 |
| pUC18N | NZW mouse 8.5kb Prn-p Exon 3 BamHI fragment cloned into pUC18. | Westaway et al., 1987 |
| pUCPrP | 1.1Kb Sma I/Eco RI *Prn-p* exon 3 ORF fragment derived from pUC18N, cloned into pUC18. Used as probe for screening HM-1 λ DASH II for *Prn-p* exon 3 sequences. | D.W. Melton |
| p129S/$E^{wt}$ | HM-1 *Prn-p* exon 3 1.1kb Sma1/EcoRI ORF fragment derived from p129PrP cloned into pBluescript SK II+. | This thesis |
| p129Pst2.0 | p129PrP derived 2.0 kb PstI fragment spanning PrP exon3. | This thesis |
| pBam/RV | p129PrP derived 700bp Eco RV/Bam HI probe fragment cloned into pBluescript. | This thesis. |

**Table 9.2: cont'd. Cloning vectors and plasmids.**

| Name | Description | Reference |
|---|---|---|
| m129PrP$^{wt}$ | HM-1 PrP A ORF 930bp Kpn I/EcoRI fragment from p129S/E$^{wt}$ cloned into M13mp18. Used as a mutagenesis cassette. | This thesis |
| p108F | PrP codon 108 phenylalanine targeting vector (Sinc BA). | This thesis, Moore et al., 1995 |
| p189V | PrP codon 189 valine targeting vector (Sinc AB). | This thesis, Moore et al., 1995 |
| p108F_189V | PrP codon 108 phenylalanine-189 valine targeting vector (Sinc BB). | This thesis, Moore et al., 1995 |
| Knockout Vector A | *Prn-p* exon 3 inactivation vector construction intermediate. | This thesis, Moore et al., 1995. |
| Knockout vector B | *Prn-p* exon 3 inactivation vector construction intermediate. | This thesis, Moore et al., 1995. |
| Knockout vector C | *Prn-p* exon 3 inactivation vector, final version. | This thesis, Moore et al., 1995. |
| PGK/DWM1 | HPRT minigene under the control of mouse phosphoglycerate kinase promoter, cloned into pBluescript. Used as a positive selectable marker in HM-1 cells. | Selfridge et al., 1992 |
| pSP-TK | Herpes simplex virus thymidine kinase under control of HSV-TK promoter with a mutant polyoma enhancer PYF441. | Thomas and Capecchi, 1987 |

**Table 9.3: Embryonic stem cell lines**

| Name | Comments | Reference |
|---|---|---|
| HM-1 | 129/Ola mouse HPRT deficient embryonic stem cell line. | Magin et al., 1992 |
| HM-1/Δ27 (1) | *Prn-p* exon 3 heterozygous knockout (Oct. 1993). | This thesis; Moore et al., 1995 |
| HM-1/Δ34 (1) | *Prn-p* exon 3 heterozygous knockout (Oct. 1993). | This thesis; Moore et al., 1995 |
| HM-1/Δ34 /BB14 | Targeted ES line heterozygous for codon 108F and 189V alterations. | This thesis |
| HM-1/Δ34 /BB18 | Targeted ES line heterozygous for codon 108F and 189V alterations. | This thesis |
| HM-1/Δ34 /BB44 | Targeted ES line heterozygous for codon 108F and 189V alterations. | This thesis |
| HM-1/Δ34/ BB45 | Targeted ES line heterozygous for codon 108F and 189V alterations. | This thesis |

**Table 9.4: Oligonucleotides**

| Oligonucleotide * | Sequence | Note |
|---|---|---|
| G1176 | 5′ aatcgccacctgcattaggg 3′ | PCR primer for detection of PrP exon 3 knockout allele: derived from sequencing the 5′ end of p129PrP between Pst I and BamHI sites. |
| G1177 | 5′ aaaacagaagggaagttctggc 3′ | PCR primer, as above. |
| 262W | 5′ agcctaccctctggtagattgtcg3′ | Located within HPRT cDNA between nt 942-965 (Accession no. J00423) 350nt from 3′ end of HPRT minigene. Used for PCR detection of the HPRT minigene. |
| 348M | 5′ gatgtcaaggaccttcagcc 3′ | Used for PCR of PrP ORF. Corresponds to the *Prn-p* intron 2/ exon 3 boundary. Corresponds to nt 38-58 (Accession no. M18070). |
| 411Y | 5′ aggtgggaggctgttttcca 3′ | Used for PCR of PrP ORF. Corresponds to *Prn-p* exon 3 ORF 3′UTR nt 1297-1277 (Accession no. M18070). |
| A996 | 5′gtggctggggacaaccccat3′ | Used for sequencing. Corresponds to *Prn-p* exon 3 ORF nt 290-310 (Accession no. M18970). |
| E175 | 5′ gacgcagtcgtgcacgaagt 3′ | Used for sequencing. Corresponds to *Prn-p* exon 3 ORF nt 644-624 (Accession no. M18070). |
| E176 | 5′ gaacttcaccgagacccgatg 3′ | Used for sequencing. Corresponds to *Prn-p* exon 3 ORF nt 690-710 (Accession no. M18070). |
| B188 | 5′ gcctagaccacgagaatgcg 3′ | Used for sequencing. Corresponds to *Prn-p* exon 3 ORF nt 916-896 (Accession no.M18070). |
| G7855 | 5′ cctgatcgtgggatgaggg 3′ | Used for sequencing. Corresponds to *Prn-p* exon 3 3′UTR nt 857-877 (Accession no.M18070). |

**Table 9.4: Cont'd. Oligonucleotides**

| Oligonucleotide * | Sequence | Note |
|---|---|---|
| G7856 | 5′ gctctttgcttcaggtccc 3′ | Used for sequencing. Corresponds to *Prn-p* exon 3 3′UTR nt 1043-1063 (Accession no.M18070). |
| 508X | 5′ cgtgaaaacatgcaccg 3′ | Human PrP primer corresponding to mouse *Prn-p* exon 3 ORF nt 555 -571 (M18070) with single basepair T-C nonhomology underlined. A gift from Dr. Otto Windl, BBSRC Centre for Genome Research, Edinburgh. |
| G6020 | 5′ gcagcacacggtcgtcaccaccaccaaggg 3′ | PrP ORF codon 189 threonine to valine mutagenesis oligo. |
| G6877 | 5′ ccaaaaaccaacttcaagcatgtggc 3′ | PrP ORF codon 108 leucine to phenylalanine mutagenesis oligo. |
| H1159 | 5′ gccacatgcttgaggttgg 3′ | PrP ORF codon 108 leucine oligo for allele specific oligonucleotide hybridisation (ASO). |
| H1160 | 5′ gccacatgcttgaagttgg 3′ | PrP ORF codon 108 phenylalanine oligo for ASO. |
| N0252 | 5′ gtccaatttaggagagccaagc 3′ | *Prn-p* exon 2 primer used for RT-PCR in conjunction with exon 3 primer 411Y. |
| M13 (Pharmacia) | 5′ aacagctatgaccatg 3′ | Sequencing oligo. |
| M13 reverse (Pharmacia) | 5′ gtaaaacgacggccagt 3′ | Sequencing oligo. |

* All oligonucleotides were synthesised by the Oswell DNA service, Dept of Chemistry, University of Edinburgh, unless indicated.

## Section 9B: Methods

### Section 9B.1: Bacterial culture

#### Section 9B.1.1: Growth of *E. coli*

*E. coli* was grown in liquid culture in Luria or Terrific broth by inoculation from a single colony. Cultures with volumes greater than 10 ml were grown in conical flasks with a total capacity of 5-10 fold of that of the culture volume to ensure good aeration. Cultures were shaken at 37°C for an appropriate length of time, typically overnight. *E.coli* carrying plasmids were grown on L plates supplemented with the appropriate antibiotic at 37°C (or 30°C to reduce the growth of satellite colonies). *E.coli* cultures inoculated with M13 bacteriophage were always grown at 37°C because pili, required for M13 infection, are not formed below 35°C.

#### Section 9B.1.2: Storage of *E. coli*

For short-term storage (less than 4 weeks), bacterial plates were wrapped with paraffin film and stored at 4°C. Bacterial strains were stored at room temperature as stab cultures: a fresh bacterial colony was used to inoculate a stab culture which was incubated overnight at 37°C with the cap loosened. After overnight incubation the cap was tightened and the bacteria stored at room temperature for up to a year.

For long term storage 900 ml of fresh overnight culture was mixed with 100 ul of glycerol, and stored at -70 °C. Upon recovery, the surface of frozen culture was scraped with a flamed sterile inoculating loop and streaked out onto a Luria broth agar plate with antibiotic, if required. After an appropriate length of incubation at 37 °C, a single colony was picked to propagate a fresh bacterial culture. Bacterial strains TG1 and CJ236 were streaked onto minimal agar and chloramphenicol plates respectively to maintain the F′ episome. DH5αF′ cultures were not plated on minimal media because the F′ in this strain is integrated into the host chromosome and does not require selection.

**Section 9B.1.3: Transformation of *E. coli***

**Section 9B.1.3.1: Transformation with plasmid DNA**

The method generally used was a modification of Nishimura et al., (1990). A 500ul aliquot of an overnight culture of the appropriate *E.coli* strain was used to inoculate 50ml L broth supplemented with $MgSO_4$ (10mM) and glucose (0.2%w/v). This culture was incubated at 37°C until the culture reached mid-logarithmic phase whereupon it was chilled on ice for 10 minutes and pelleted by centrifugation at 1500g for 10 minutes at 4°C. The cell pellet was resuspended in 0.5ml of the above supplemented medium then 2-5ml of medium B was added (30% glycerin, 12% (w/v) PEG 7500, 12mM $MgSO_4$ in LB medium, filter sterilised) and the cells mixed gently. Competent cells were generally split into 100ul aliquots and stored for up to 3 months at -80°C.

Cells were transformed by the addition of approximately 100 pg of plasmid DNA to a 100 ul aliquot of competent cells, followed by mixing and a 15 minute incubation on ice. Cells were heat shocked for 3 minutes at 37°C then placed on ice for a further 1 minute. Then 0.4ml of prewarmed (at 37°C) LB medium was added and the cells incubated for 1 hour in the absence of antibiotic selection. A number of aliquots (usually 100, 50 and 10ul) were then plated to dryness on LB agar 90mm petri dishes, with appropriate antibiotic selection and 30ul of 2% (w/v) X-Gal (in dimethylformamide) and 20ul of 100mM IPTG to aid the identification of recombinants (when appropriate). Plates were incubated at 37°C for at least 20 hours before scoring or picking of colonies. Typical transformation efficiencies were between $1x10^5$ and $1x10^8$ transformants per ug DNA. Recombinant colonies appeared white and non-recombinant colonies were blue using IPTG/X-Gal.

**Section 9B.1.3.2 Transformation of *E.coli* with M13 RF DNA and M13 phage**

Many *E.coli* strains can be transformed with M13 RF DNA using competent cells prepared by the method of Nishimura et al., (1990) (Section 9B.1.31, above). Infection with intact M13 phage is achieved by mixing an aliquot of phage suspension with 200ul of a fresh overnight culture, incubating for 5 minutes and adding 2.5ml of

50°C top agar prior to pouring onto an L-plate. Plaques were picked with a Pasteur pipette and transferred to 1ml TE and the titre determined (~$10^7$pfu/plaque). To determine M13 titre, several serial dilutions were plated out as described. Titre = (No. plaques x 10 x dilution factor) pfu/ml.

Two main bacterial strains were used for M13 work: DH5αF′ and CJ236. *E.coli* DH5αF′ have an integrated F′ and do not require any selection for retention of the F′. However, *E.coli* CJ236 encode the F on a plasmid (pCJ105($cm^r$)) which also encodes a chloramphenicol resistance gene .Therefore CJ236 was streaked out onto an L-plate containing 30ug/ml of chloramphenicol and grown overnight at 37°C.

To make plating cells for use with M13 phage, a single colony was used to inoculate 10ml of LB supplemented with 30ug/ml chloramphenicol and was grown overnight. The $OD_{600nm}$ of the overnight culture was determined and enough cells to yield an $OD_{600nm}$= 0.1 was added to fresh medium and grown until $OD_{600nm}$ = 0.8-0.8 (~2hours). Cells were then pelleted by a short spin at 0°C. The pellet was resuspended in 1ml ice cold 50mM $CaCl_2$, mixed gently, then an additional 20ml 50mM $CaCl_2$ added and the cells stored on ice for 20-30 minutes. Cells were harvested by a short spin as before and resuspended in 4ml 50mM $CaCl_2$.

**Section 9B.2: Nucleic acid isolation**

**Section 9B.2.1: Small scale preparation of plasmid DNA**

Small scale preparations of plasmid DNA were made for screening of bacterial colonies for recombinant plasmids. The method used is a modification of that described by Ish-Horowicz and Burke, (1981). Typically 2 ml of Luria broth or Terrific broth, supplemented with ampicillin to a final concentration of 100 ug/ml, was inoculated with bacteria from a single colony and incubated at 37 °C overnight with vigorous aeration. The culture was transferred to a 2ml Eppendorf tube and bacteria pelleted by centrifugation for 2 minutes at 13K rpm and resuspended in 300 ml of solution P1 (see below). Bacterial cells were lysed by the addition of 300 ml of solution P2 and incubated at room temperature for 5 minutes. Next, 300 ml of solution P3 was added and mixed by gentle inversion. The precipitated complex of

chromosomal DNA, SDS and protein was sedimented by centrifugation (17,000 x g) in a microcentrifuge for 5 minutes. Contaminating proteins in the clarified supernatant were removed by extraction with 1 volume of phenol: chloroform (1:1), vortexed and spun at 13K r.p.m for 2 minutes. The upper, aqueous phase was recovered and 0.6 volumes of isopropanol added and the nucleic acids precipitated with a 10 minute spin. The DNA was washed with 70% ethanol, air dried and resuspended in 50 ml sterile distilled water or TE. Plasmid "miniprep" DNA was then stored at -20°C.

Solution P1: 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 100 mg/ml RNase A
Solution P2: 20 mM NaOH, 1% (w/v) SDS
Solution P3: 2.55 M potassium acetate, pH 4.8

**Section 9B.2.2: Large scale preparation of plasmid DNA**

A single bacterial colony was used to inoculate 50ml of TB containing an appropriate antibiotic and grown overnight at 37°C with vigorous aeration. The bacterial cells were pelleted by centrifugation for 15 minutes at 4 °C in an HB4 rotor in a Sorval RC5 centrifuge at 10,000 rpm. The bacterial pellet was resuspended thoroughly in 10ml of solution P1 (see above) then 10mls of solution P2 was added. The sample was stored at room temperature for 5 minutes, then 10mls of solution P3 added and placed on ice for 10-15 minutes. Following the P3 step, chromosomal DNA, SDS and protein complexes were sedimented by centrifugation at 13,000 rpm in an HB4 rotor for 30 minutes at 25°C. A Quiagen 500 column was equilibrated by the addition of 10ml of buffer QBT. The supernatant was applied onto a Quiagen 500 column through a Whatman filter paper funnel. After washing twice with 30 ml buffer QC, the DNA was eluted with 15 ml buffer QF. DNA was then precipitated by the addition of 15ml (0.6 volumes) of isopropanol and centrifuged as before at 20°C. Pelleted DNA was washed with 70% ethanol, air dried and redissolved in TE or sterile distilled water and stored at -20°C.

Buffer QBT: 750 mM NaCl, 50 mM MOPS, 15% ethanol,
0.15% (v/v) Triton X-100, pH 7.0

Buffer QC: 1M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0

Buffer QF: 1.25 M NaCl, 50 mM MOPS, 15% ethanol, pH 8.2

**Section 9B.2.3: Preparation of bacteriophage λ DNA**

Chloroform was added to the lysed 250 ml culture to a final concentration of 1% (v/v). The culture was transferred to a 250ml centrifuge tube and incubated at room temperature for 30 minutes. Cellular debris was pelleted by centrifugation for 10 minutes at 5,000 rpm in a Sorval GSA rotor. The supernatant (200ml) was transferred to a fresh tube and equilibrated to 37 °C and 400ul buffer L1 (20mg/ml RNase A, 6mg/ml DNase I, 0.2mg/ml BSA, 10mM EDTA, 100mM Tris.HCl pH7.5) added and the sample incubated for 30 minutes to degrade bacterial DNA and RNA. The centrifuge tube was transferred to an ice bucket and 50ml of ice cold buffer L2 (3M NaCl and 30% (w/v) PEG6000 was added and the sample stored for 1 hour on ice (or overnight). Phage were pelleted by a 10 minute spin at 5, 000 rpm in Sorval HB4 rotor. All of the supernatant was removed and the phage pellet resuspended with 9ml of buffer L3 (100mM Tris.HCl, 100mM NaCl, 25mM EDTA, pH 7.5) and transferred to a 50ml Falcon tube. Buffer L4 (4% (w/v) SDS) was added (9mls), the tube mixed gently and incubated at 70°C for 20 minutes then placed on ice. When cool, 9ml of buffer L5 (2.55M potassium acetate, pH 4.8) was added and mixed immediately. The sample was and spun at 10, 000 rpm in a Sorval SS 34 rotor for 30 minutes at 4 °C. A Quiagen tip 500 was equilibrated with 10 ml of QBT (750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton x-100, pH7.0) and the supernatant poured onto the column through a Whatman 3M filter paper funnel. The column was allowed to empty by gravity flow then washed with 30ml of buffer QC (1M NaCl, 50mM MOPS, 15% ethanol, pH 7.0). DNA was eluted with 15ml of buffer QF (1.25M NaCl, 50mM MOPS, 15% ethanol, pH 8.2) into a centrifuge tube and 10.5ml (0.6 volumes) isopropanol added and mixed. DNA was precipitated by a 10 minute centrifugation at

10,000 rpm at 20°C. Pellets were washed twice with 70% ethanol, air dried then dissolved in 100-200ul of TE.

### Section 9B.2.4: Preparation of M13 bacteriophage DNA

#### Section 9B.2.4.1: Isolation of M13 bacteriophage

Plating cells were produced by inoculating 20ml of LB with a single bacterial colony and incubating overnight at 37°C with antibiotic selection for the F′ if required. A 250ml flask containing 50ml of 2xYT medium was inoculated 1ml of overnight culture and grown to an $OD_{600nm}$= 0.3 (1-4 hours). This approximates to $1x10^8$cfu/ml. An appropriate volume of phage stock was added to a molarity of infection of 0.2 phage/cell and incubated with shaking a further 4-6 hours. This culture was transferred to a 50ml centrifuge tube and spun for 15 minutes at 17,000g (12Krpm with a Sorval SS-34 rotor) and the supernatant was transferred to a fresh tube and recentrifuged. The supernatant was transferred to a polyallomer centrifuge tube and 150ug of RNAase A added and incubated at room temperature for 30 minutes. Phage were precipitated by the addition of 0.25 volumes of 3.5M ammonium acetate/20% (w/v) PEG8000, incubated on ice for 30 minutes then centrifuged for 15 minutes at 17,000g (4 °C). The supernatant was carefully removed and the tube drained thoroughly and the phage pellet resuspended with 200ul of high salt buffer (300mM NaCl, 100mM Tris pH8.0, 1mM EDTA), transferred to a 1.5ml Eppendorf tube and held on ice for 30 minutes. Insoluble material was precipitated by a 2 minute centrifugation at 13,000k rpm and the supernatant transferred to a fresh tube. The phage stock was titred as was generally ~$5x10^{11}$pfu/ml.

#### Section 9B.2.4.2: Isolation of single stranded M13 DNA

Single stranded M13 phage DNA was purified as follows: intact phage were precipitated from 1.5ml M13 cultures by the addition of 300ul of 20% (w/v) PEG6000/2.5 M NaCl followed by a 5 minute incubation at room temperature and centrifugation for 10 minutes at 13K rpm. Phage pellets were resuspended in 150ul TE followed by the addition of 8ul of 5% (w/v) CTAB (Hexadecyltrimethyl-

ammonium bromide) and were heated for 5 minutes at 65°C, centrifuged a further 5 minutes and pellets resuspended in 100ul TE/1.2M NaCl. Following a 5 minute incubation at room temperature, 250ul of ethanol was added and ssDNA precipitated by a 10 minute centrifugation. Pellets were washed with 70% ethanol, air dried and resuspend in 30ul of TE. Typically 5ul of 30ul was used for dideoxy sequencing.

An alternative method was sometimes used: 200ul of phage stock (Section 9B.2.4.1) was extracted twice with an equal volume of Tris buffered phenol (pH 8.0), once with phenol/chloroform (1:1) and several times with chloroform until no interphase was visible. M13 ssDNA was precipitated by the addition of 0.1 volumes of 7.8M ammonium acetate and 2.5 volumes of ethanol and was stored at -70°C for 30-60 minutes. The sample was centrifuged 15 minutes and the pellet washed with 90% ethanol, air dried and resuspended in 20ul TE. This method typically yielded several micrograms of M13 ssDNA.

**Section 9B.2.4.3: Isolation of double stranded M13 RF DNA**

The double stranded replicative form of M13 was prepared from a bacterial pellet from an M13 infected culture by following the small scale alkaline lysis method routinely used to isolate plasmid DNA (Section 9B.2.1). Larger quantities of M13 RF DNA was prepared using a large scale plasmid prep in conjunction with a Quiagen column (Section 9B.2.2).

**Section 9B.2.5: Preparation of genomic DNA from embryonic stem cells**

High quality, high molecular weight ES cell genomic DNA, suitable for PCR amplification and Southern analysis was made as follows. A 25cm$^2$ confluent flask of ES cells was washed twice with 5 mls of PBS drained and 5ml of tissue buffer was added (50mM Tris.HCl, pH 8.0, 20mM NaCl, 100mM EDTA, 0.5% SDS, 1mM CaCl2, 0.5mg/ml Proteinase K, 0.1 ug/ml RNase A). The flask was gently agitated for 5 minutes whilst the ES cells were released from the flask prior to transfer to a 50ml Falcon tube. The tube was incubated at 37 °C with gentle agitation for several hours or overnight. The ES cell lysate was extracted twice with 1 volume of

phenol/chloroform (1:1), centrifuged at 3000 rpm for 15 minutes. The aqueous phase was retrieved and extracted against 1 volume of chloroform. High molecular weight DNA was precipitated by the addition of 0.6-1 volumes of isopropanol. The DNA precipitate was retrieved with a wide bore pipette then washed in 70% ethanol and gently resuspended in d.$H_20$ and stored at 4°C.

**Section 9B.2.6: Preparation of genomic DNA from mouse tail samples**

Mice (>3 weeks old) were anaesthetised by intraperitoneal injection of approximately 0.1ml of Hypnorm-Hypnovel and left for 20 minutes for the anaesthetic to take effect. Adequately anaesthetised mice were given an identification mark by earmarking and small samples of tail (0.5-1cm) were removed and placed in a 1.5ml Eppendorf tube with 0.5ml tissue buffer (50mM Tris.HCl, pH 8.0, 20mM NaCl, 100mM EDTA, 0.5% SDS, 1mM CaCl2, 0.5mg/ml Proteinase K, 0.1 ug/ml RNase A) and incubated overnight prior to processing for DNA using the method described in Section 9B.2.5. *Preparation of anaesthetic:* 2ml of Hypnorm (Fentanyl Citrate 0.315mg/ml, 10mg/ml Fluanisone, Janssen Animal Health, Grove Oxford, UK) was added to 4 ml of water and mixed. Two millilitres of Hypnovel (10mg Midazolam, Roche) was added to 4ml of d.$H_20$ and mixed. Both solutions were combined and mixed to yield 12ml of Hypnorm-Hypnovel ready for use. Anaesthetic was stored at room temperature.

**Section 9B.2.7: Preparation of RNA from embryonic stem cells and mouse tissues**

The following method is adapted from Chomoczynski and Sacchi, (1987). Fresh cells or tissue were placed in a sterile polycarbonate centrifuge tube containing 10ml of denaturing buffer (4M guanidium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% sarcosyl, 20mM DTT), homogenised then acidified by the addition of 1ml of 2M sodium acetate, pH 4.0 and mixed prior to the addition of 10ml of water saturated phenol and 3ml of chloroform. The tube was vortexed for 30 seconds and stored on ice for 10 minutes then centrifuged for 10 minutes at 10,000rpm in an HB4

rotor pre-cooled to 4°C. The aqueous phase was transferred to a fresh centrifuge tube and RNA precipitated by the addition of 1 volume of isopropanol pre-cooled to -20°C. The sample was stored at -20°C for at least 20 minutes before a 15 minute centrifugation as above. The RNA pellet was resuspended in 1ml of denaturing buffer, transferred to a 2ml sterile centrifuge tube and reprecipitated by the addition of 1ml of isopropanol and stored at -20°C for 30 minutes. RNA was collected by a further 10 minute spin and the RNA pellet was resuspended in 75% ethanol, centrifuged for 10 minutes and resuspended in sterile TE. RNA was generally stored under EtOH at -70°C.

**Section 9B.3: Quantification of nucleic acids**

**Section 9B.3.1: Estimation of DNA concentration**

The DNA sample was diluted in 1ml of distilled water and the OD of absorbance at wavelengths 260nm and 280nm was measured by a spectrophotometer (Perkin-Elmer, Lambda 15, UV/VIS Spectrophotometer). An $OD_{260nm}$ value of 1.0 represents a concentration of 50 mg/ml for DNA. The ratio $OD_{260nm}/OD_{280nm}$ provides an estimate for the purity of the nucleic acid. A value around 1.8 indicates a pure preparation of DNA.

**Section 9B.3.2: Estimation of RNA concentration**

The RNA sample was diluted in TE and the OD of absorbance at wavelengths 260nm and 280nm was measured by a spectrophotometer (Perkin-Elmer, Lambda 15, UV/VIS Spectrophotometer). An $OD_{260nm}$ value of 1.0 represents a concentration of 40 mg/ml for RNA. The ratio $OD_{260nm}/OD_{280nm}$ provides an estimate for the purity of the nucleic acid. A value around 2.0 indicates a pure preparation of RNA.

**Section 9B.4: DNA manipulation**

**Section 9B.4.1: Digestion of DNA with restriction endonucleases**

Most DNA restrictions were performed using BRL, Boehringer Mannheim or New England Biolabs enzymes and buffers. DNA was digested with approximately 2-

5U of restriction endonuclease per ug of DNA using buffer and temperature conditions recommended by the manufacturer. For double digests involving enzymes with different recommended buffers, the buffers were checked individually in double digests to determine which gave most efficient digestion. If the optimal digestion conditions varied for buffers, the DNA was digested with enzyme at lower salt concentration, the enzyme reaction stopped by heat denaturation or phenol extraction, the DNA precipitated and dissolved in a buffer appropriate for digestion by the next enzyme. Digestion reactions were terminated by addition of DNA sample buffer then heated for 10 minutes at 65°C prior to electrophoresis.

**Section 9B.4.2: Dephosphorylation of plasmid vector termini**

Calf intestinal phosphatase (CIP) is a phosphomonesterase that hydrolyses 3′ and 5′ phosphates from DNA and RNA. It can be used to remove 5′phosphates before end-labelling and to dephosphorylate linearised plasmid vectors before insert ligation. This enzyme can be inactivated by heating for 15 minutes at 75 °C in the presence of EDTA and 10 mM sodium salt. Phenol extraction is not required. The terminal 5′ phosphates were removed from DNA (< 5 pmole ) by treatment with 0.1 U (1 ml) of calf intestinal phosphatase (CIP) (Boehringer Mannheim) in a reaction mixture containing 5 ml 10X CIP buffer (10X: 0.5 M Tris-HCl pH 9.0, 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine) and d.$H_2O$ in a total volume of 49 ml. After incubation at 37°C for 30 minutes, the reaction was terminated by inactivating the enzyme at 75°C for 15 minutes in a solution containing 10 ul of 10X STE (10X: 100 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, pH 8.0) and 2.5ul of 20% (w/v) SDS and d.$H_2O$ in a total volume of 100 ul.

**Section 9B.4.3: Ligation of DNA**

Typically, between 100 and 200 ng of insert DNA was ligated with linearised plasmid vector DNA at an insert to vector molar ratio of 5:1. Ligations were performed in a 10-20ul reaction volume containing 50 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP and 5 % (w/v) PEG8000 and incubated overnight at

15 °C (for blunt ends), or for a minimum of 2 hours at room temperature (for sticky ends). One unit of T4 DNA ligase was used for ligations. Ligation products were then transformed into *E. coli*. (Section 9B.1.3)

**Section 9B.5: Electrophoresis of nucleic acids**

**Section 9B.5.1: Electrophoresis of DNA in agarose gels**

DNA was separated in 0.7-2.0% (w/v) electrophoresis grade agarose with 0.5 mg/ml ethidium bromide in 1X TBE buffer (for Southern blotting and routine analytical gels) or 1X TAE buffer (for preparative gels). Prior to loading, DNA samples were mixed with 1/5 volume of 5x sample buffer (20% (v/v) glycerol, 100 mM EDTA, 0.1% bromophenol blue). Electrophoresis was carried out horizontally across a potential difference of 1-10 V/cm. Bacteriophage λ DNA cut with HindIII and ϕX174 DNA cut with HaeIII were used as size markers. DNA was visualised by short wave UV illumination. Preparative gels were not exposed to short wave UV as this can result in thymidine dimerisation. Instead, the DNA in preparative gels was visualised by long wave UV from a hand held lamp.

**Section 9B.5.2: Electrophoresis of RNA in agarose gels**

RNA samples were electrophoresed through 1.4 % (w/v) agarose gels made with 1x MOPS, 0.66M formaldehyde and 0.5ug/ml ethidium bromide. Samples were prepared by the addition of 10ug of total RNA (in 20ul d.$H_2O$) to an equal volume of FSB (100ul 10x MOPS, 200ul formamide, 120ul de-ionised 37%(w/v) formaldehyde) and one quarter volume of standard sample buffer (20% glycerol, 100 mM EDTA, 0.1% bromophenol blue) was also added and the sample heated for 5 minutes at 65°C then cooled on ice prior to electrophoresis for 300-400V/hours.

**Section 9B.5.3: Recovery of DNA from agarose gels**

DNA was electrophoresed using low melting point agarose in 1X TAE, 0.5 mg/ml ethidium bromide, the desired fragment was visualised by long wave UV illumination. The band of interest was excised and recovered from the agarose by

phenol extraction. The gel slice volume was estimated then 0.5 volumes of TE, 1ul glycogen (Boehringer Mannheim) and 5ul 20% SDS was added to the gel slice and heated to 70°C until the agarose melted (~5 minutes). Liquified agarose was extracted three times with 1 volume of tris buffered phenol (pH 8.0) and once with 1 volume of chloroform. The aqueous phase was retained and DNA precipitated by the addition of 0.1 volume 3M sodium acetate and 1 volume of isopropanol. DNA was pelleted by a 10 minute centrifugation at 13Krpm and the DNA pellet resuspended in d.$H_2O$.

**Section 9B.6: Transfer of nucleic acids**

**Section 9B.6.1: Transfer of DNA from agarose gels to membranes**

The transfer of DNA from agarose gel onto filters for the detection of specific sequences among DNA fragments was initially developed by Southern, (1975) and modified by Smith and Summers, (1980). Genomic DNA was digested with the appropriate restriction enzyme in a total volume of 50-100 ul overnight at 37°C (with the exception of BstEII which was digested for 5 hours at 60 °C under mineral oil) and separated according to size on a 0.7-1% agarose gel (w/v) in 1X TBE and 0.5 mg/ml ethidium bromide. Routinely, genomic DNA was electrophoresed for ~800Vhours then photographed. The gel was then soaked in denaturation buffer (0.5 M NaOH, 1.5 M NaCl) with gentle agitation for 15 minutes. The DNA was transferred to Genescreen Plus nylon membrane (Du Pont; Stevenage, U.K.) by capillary action using denaturation buffer as the transfer medium (Reed and Mann, 1985). Transfer was performed for 12-24 hours then the membrane was neutralised and adherent agarose washed off by incubating for 10 min in 3 M NaCl, 0.5 M Tris-HCl pH 7.0 on a rocking platform. Filters were air dried before use.

**Section 9B.6.2: RNA transfer**

Following electrophoresis, gels were washed three times for 20 minutes in 10xSSC at room temperature to remove formaldehyde and ethidium bromide. Transfer conditions were similar to DNA except that 10xSSC was used as the transfer

buffer. On completion of transfer the membrane was washed in 2x SSC for 10 minutes then baked at 80°C for 2 hours.

**Section 9B.7: Nucleic acid hybridisation**

**Section 9B.7.1: Labelling DNA probes**

DNA labelled to high activities was obtained using the randomly primed DNA labelling method (Feinberg and Vogelstein, 1983). DNA probes were denatured, annealed to random hexamer primers and extended by the Klenow fragment of *E.coli* DNA polymerase I in the presence of [$\alpha$-$^{32}$P]dCTP. Oligonucleotide labelling buffer (OLB) containing nucleotides and random primers is required for this method. It is made by mixing 50 ml Solution A (1.25 M Tris-HCl pH 8.0, 0.125 M $MgCl_2$, 25 mM $\beta$-mercaptoethanol, 0.5 mM each of dGTP, dTTP and dATP), 125 ml Solution B (2M HEPES buffer adjusted to pH 6.6 with NaOH) and 75 ml Solution C (random hexanucleotides $OD_{260nm}$ = 90 in TE). Probes were labelled as follows: 100 ng of d.s DNA was made up to 32 ul with d.$H_2O$ then denatured by boiling for 5 minutes and allowed to cool at room temperature for 10 minutes. The following reagents were then added: 10ml of OLB, 2ul of 10mg/ml BSA, 5ul of [$\alpha$-$^{32}$P] dCTP (3000Ci/mmol) and 10 units of Klenow enzyme. The reaction was incubated at 37°C for 40 minutes or overnight at room temperature.

**Section 9B.7.2: DNA and RNA hybridisations**

Pre/hybridisations were performed in a hybridisation oven (Hybaid Limited) in a total volume not exceeding 30 ml of prehybridisation buffer (6X SSC, 1% SDS, 10% (w/v) dextran sulphate, 100 mg/ml of denatured herring sperm DNA). Blots were prehybridised for a minimum of 1 hour at 65 °C for Southern transfer and at 60 °C for northern transfer. DNA probes were heat denatured in 300ul of 10mg/ml herring sperm DNA and added directly to the prehybridisation solution. Following hybridisation overnight at 65°C, the hybridisation solution was discarded and the blots were rinsed for 5 minutes in 2 X SSC/ 1 % (w/v) SDS at room temperature, followed by a 30 minute wash in 2x SSC, 1% (w/v) SDS at 65°C then a 30 minute wash in

1xSSC, 1% (w/v) SDS at 65°C. Membranes were sealed in a plastic bag and autoradiographed at -80°C, typically overnight. Northern blots were hybridised and washed as above but at 60°C.

**Section 9B.7.3: Autoradiography**

Autoradiography was performed using Cronex X-ray film (DuPont) in a cassette with intensifying screens (Cronex Lightning Plus; Du Pont). Cassettes were exposed at -70°C to facilitate fluorographic enhancement.

**Section 9B.7.4: Stripping probes from membranes**

Radiolabelled probes were stripped from Southern blots by boiling in 0.1X SSC, 1% (w/v) SDS for 10 minutes with gentle shaking. The solution was decanted from the membrane, and the boiling procedure was repeated twice. Blots were autoradiographed to confirm that probe stripping was complete. The membrane was then incubated in pre-hybridisation solution and hybridised as above (Section 9B.7.2).

Northern blots were stripped by washing them 4 times for 5 minutes in boiling 0.01x SSC, 0.01% (w/v) SDS.

**Section 9B.8: Allele specific oligonucleotide hybridisation**

**Section 9B.8.1: 5′ Labelling of oligonucleotides**

Oligonucleotide (10pmol)was 5′ labelled in a 50ul reaction by the addition of 5ul polynucleotide kinase buffer (Promega), 1ul 10mg/ml acetylated BSA (NEB), 1ul (10U) T4 polynucleotide kinase and 5ul [$\gamma$–$^{32}$P]dATP (3000Ci/mmol). The reaction was incubated for 1 hour at 37°C then added directly to the prehybridisation solution and incubated with the membrane.

**Section 9B.8.2: Detection of 108F mutants by differential hybridisation**

Membranes were prehybridised for 30 minutes at room temperature in 10ml prehybridisation buffer (6x SSC, 5x Denhardts solution, 100ug/ml herring sperm DNA). The 5′ end labelled 108F oligonucleotide was added directly to the

prehybridisation solution and hybridised to the membrane for 1.5 hours at room temperature. The membrane was washed for 15 minutes at room temperature in 6xSSC and exposed for 15 minutes at room temperature to provide a control autoradiograph showing the relative signal intensities before washing at raised stringency. The membrane was placed in 6x SSC and washed twice at 62°C for 5 minutes then exposed to X-ray film at room temperature for 15 minutes.

Unambiguous determination of differential hybrids required both positive and negative controls on the membrane. Controls were either PCR products or plasmids: PrP exon 3 ORF PCR product from HM-1 provided a negative control whilst VM/Dk PCR product from the same region provided a positive control for the 108F signal. 108F mutants were characterised by unaltered signal intensities at raised stringency whilst non-mutants had dramatically reduced signals under the same conditions.

Denhardt's solution (50x): 1% (w/v) Ficoll (type 400I), 1% (w/v) polyvinylpyrrolidine, 1% (w/v) BSA (Fraction V, Sigma) in d.$H_2O$.

**Section 9B.9: λ DASH II mouse genomic DNA library screening**

**Section 9B.9.1: Growth of *E.coli* K802 plating cells and titration of phage**

A single colony of *E.coli* K802 from an LB plate was picked and placed in a sterile 250ml conical flask containing 20 mls of terrific broth supplemented with 0.2% (v/v) maltose. The culture was incubated overnight at 37 °C with vigorous aeration. The following day the culture was decanted into a 50ml Falcon tube and the cells pelleted and resuspended in 20ml 10mM $CaCl_2$, 10 mM $MgSO_4$. Plating cells were stored at 4 °C for a maximum of three weeks. To titrate phage, 10ul of an appropriate dilution of phage in LB medium or SM solution (50mM Tris.HCl, 100mM NaCl, 8mM $MgSO_4$, pH 7.5) was added to 100ul of K802 plating cell in a plastic tube, mixed then incubated for 20 minutes at room temperature to allow adsorption of phage by bacteria. Following this, 3mls of molten BBL top agar (50 °C) was mixed with the phage/cells and poured evenly onto an L-agar plate. The plates were left for 10 minutes for the agar to set and plates were incubated overnight at 37°C. Plate lysate stocks were prepared as above but with a phage/bacteria MOI adjusted to

achieve confluent lysis. To lift phage from the L-agar plate, 5mls of SM was added to each plate and placed on a gently shaking table for 30 minutes. The supernatant was poured into a fresh tube, a few drops of chloroform were added to the stock which was then titred.

**Section 9B.9.2: Preparing filter replicas of λ library pates**

Nylon Membranes (Hybond N, Amersham) were placed on the surface of library plates (precooled to 4 °C) for 5 minutes and asymmetrical marks made in the membrane made with an ink soaked needle to facilitate the localisation of positive plaques. Membranes were carefully removed and placed (DNA side up) on Whatman 3M paper pre-soaked with denaturing solution (0.5M NaOH, 1.5M NaCl) for 5 minutes then transferred onto filter paper pre-soaked with neutralising buffer (0.5M Tris.Cl, 1.5M NaCl, pH 8.0) for 5 minutes then transferred onto filter paper pre-soaked with 2xSSC. Filters were air dried for 30 minutes then baked at 80 °C for 1 hour.

**Section 9B.9.3: Pre/hybridisation of filter replicas**

Baked filter replicas were rinsed briefly with d.$H_2O$ and 6xSSC then placed into a sealed plastic hybridisation container and incubated with 100ml prehybridisation buffer (50% formamide, 5xSSC, 50mM $NaPO_4$, pH 7.2, 1% (w/v) SDS, 10 ml 10mg/ml yeast tRNA or 10ml 10mg/ml sonicated herring sperm DNA) for a minimum of 1 hour at 37°C. Following prehybridisation the DNA probe was added and hybridisation carried out for 18 hours at 37°C. Hybridised filters were washed three times for 30 minutes at 55°C in prewarmed 0.1xSSC, 0.1% (v/v) SDS then exposed overnight at -70°C.

**Section 9B.9.4: Picking λ bacteriophage plaques**

Well separated plaques corresponding to putative positive hybridisation signals were picked from L plates with an inverted sterile pipette tip and placed in 1ml of SM into which 1 drop of chloroform was added to inhibit bacterial growth during storage.

To ensure that the phage had diffused out of the agar the plaque suspensions were left for at least 1 hour before further use.

**Section 9B.9.5: Lysis of medium scale λ bacteriophage liquid culture**

Prewarmed LB medium (250 ml) was supplemented with 0.2% maltose (w/v), 10mM $MgCl_2$ and 10ml $CaCl_2$ and inoculated with 500ul of a fresh overnight K802 culture. Several cultures were set up in parallel and inoculated with different amounts of recombinant bacteriophage clones, typically $1x10^9$pfu of plate lysate stock completely lysed a 250ml culture in 6 hours. λ DNA was isolated as described in Section 9B.2.3. The yield from a 250ml culture was generally 150-200ug λ DNA.

**Section 9B.9.6: Mapping recombinant λ bacteriophage clones**

A FLASH Nonradioactive Gene Mapping Kit (Stratagene, Cat No. #200381) was used to generate a high resolution restriction map of λ DASH II recombinant inserts. This method makes use of the T3 and T7 RNA polymerase promoters which flank the polylinker sites of λ DASH II. Following Not I digestion, 100ug of each digested λ DASH II clone was resuspended in $d.H_20$ to a final concentration of 1ug/ul. Then 1ug was digested with 1U of restriction enzyme in the appropriate buffer in a total volume of 10ul. Aliquots of ~250ng (2.5ul) were withdrawn at 2, 5, 10 and 20 minutes and placed on ice and 3ul of 0.5M EDTA added to stop the digestion. Sample buffer was added to all of the samples and they were electrophoresed and transferred to Genescreen+ nylon membrane, neutralised and air dried. Membranes were prehybridised for 10 minutes at 37°C. The AP conjugated T3 or T7 oligonucleotide probe was added to a final concentration of 2nM and hybridised for 1 hour at 37°C. The hybridisation solution was discarded and the membranes washed for 10 minutes with prewarmed (37°C) wash buffer #1 (2xSSC, 1%(w/v) SDS). This wash was then discarded and the membranes were washed for 10 minutes with buffer 2 (2xSSC, 1% (v/v) Triton X-100) for 10 minutes at 37°C. Membranes were then washed twice for 5 minutes at room temperature with assay buffer (0.0096% (v/v) diethanolamine, 1mM $MgCl_2$, pH 10.0) then incubated 10 minutes with substrate

buffer (assay buffer with 0.6% (v/v) CSPD). The membrane was sealed in a plastic bag and exposed to X-ray film for 10 minutes at room temperature.

**Section 9B.10: *In vitro* mutagenesis**

**Section 2B.10.1: The Kunkel method**

Oligonucleotide directed mutagenesis was performed on the M13 clone m129PrP$^{Wt}$ using the method described by Kunkel (Kunkel et al., 1987) in conjunction with a Muta-gene M13 In Vitro Mutagenesis Kit version 2 (Biorad, Richmond USA) and *dut* $^{-}$ *ung* $^{-}$ mutant *E.coli* strain CJ236. Briefly, this method enables the generation and rapid isolation of mutants without the requirement for the subcloning of each mutant. Single stranded template is isolated bearing the PrP ORF region to be mutated and a mismatch primer is annealed and extended by T7 DNA polymerase in the presence of T4 DNA ligase. The heteroduplex is used to transform *E.coli*, plated and plaques picked and screened for the presence of the desired mutation. The efficiency of this method is increased by biological selection for the daughter strand of the heteroduplex. This selection system requires an initial passage of the parental M13 clone in CJ236, a *dut* $^{-}$ *ung* $^{-}$ mutant strain of *E.coli* which lacks both dUTPase and uracil N glycosylase (Kunkel et al., 1987). Following passage in CJ236, ssDNA from m129PrP$^{Wt}$ was used for *in vitro* mutagenesis.

The *dut* $^{-}$ mutation in CJ236 inactivates dUTPase and results in elevated intracellular levels of dUTP which compete with dTTP for incorporation into DNA. A small number of dUTP bases are incorporated into the parental DNA instead of thymine. The *ung* $^{-}$ mutation results in cells which lack uracil N glycosylase -an enzyme which normally removes uracil from DNA. When an M13 clone is passaged through the double mutant strain CJ236, uracil is incorporated into DNA but is not removed. Selection for the daughter strand of the heteroduplex following *in vitro* mutagenesis exploits the semi-conservative nature of DNA replication and was achieved by transforming *dut* $^{+}$ *ung* $^{+}$ *E.coli* strain NM522 with an aliquot of this reaction product. NM522 will not replicate the uracil containing parental strand

efficiently and M13 plaques are preferentially derived from the daughter strand bearing the engineered mutation. Mutation efficiencies are usually between 60-80% and screening by dideoxy sequencing is adequate to isolate mutants. The wildtype clone bearing the PrP A coding region, m129PrP$^{Wt}$, was titred in a wildtype *E.coli* strain (NM522) before and after passage through CJ236 -a large difference in titre was noted confirming selection against dUTP DNA derived from CJ236.

**Section 9B.10.2: Phosphorylation of the mismatch oligonucleotide**

HPLC purified oligonucleotide (200pmol) was phosphorylated using 4.5 units of T4 polynucleotide kinase in 1x phosphorylation buffer (100mM Tris.HCl pH8, 10mM MgCl2, 5mM DTT, 0.4mM ATP) and incubated for 45 minutes at 37°C. The reaction was stopped by heating at 65 °C for 10 minutes and diluted with TE to a oligonucleotide final concentration of 6 pmol/ul.

**Section 9B.10.3: Annealing of the mismatch primer and synthesis of the complimentary strand.**

200ng of dUTP M13 ssDNA template was mixed with 3 pmol of phosphorylated mismatch primer in the presence of 1x annealing buffer (20mM Tris.HCl pH 7.4, 2mM $MgCl_2$, 50mM NaCl) in a total volume of 10ul. This gives a primer:template ratio of approximately 20:1 to 30:1 -higher ratios can result in significant levels of mispriming. A control annealing reaction was performed without primer to determine the rate of endogenous priming. The reactions were placed in a beaker of water prewarmed to 70°C and allowed to cool on the bench to 30°C. Reactions were placed on ice and the buffer was adjusted by the addition of 1ul of synthesis buffer (25 mM dNTP's, 10mM ATP, 100mM Tris.HCl, pH 7.4, 50mM $MgCl_2$, 20mM DTT) followed by 0.5 units of T7 DNA polymerase and 5 units of T4 DNA ligase. The primer was stabilised by the initiation of DNA synthesis on ice for 5

minutes then the primer extended and the complimentary strand synthesised by incubation at 25°C for 5 minutes then 37°C for 30 minutes. Following this the reaction was terminated by the addition of stop buffer (10mM Tris.HCl, 10mM EDTA, pH8). Analysis of the reaction products was performed by gel electrophoresis and an aliquot used for transformation of *E.coli* NM522 to select for the daughter strand of the heteroduplex. Following transformation, 10 well isolated plaques less than 24 hours old were picked and plaque purified to ensure that each plaque derived from a single M13 bacteriophage clone. Plaques were picked and used to inoculate small scale liquid cultures to generate ssDNA template suitable for sequencing and RFII d.s DNA for restriction analysis and subcloning.

**Section 9B.11: DNA sequencing**

**Section 9B.11.1: Annealing of primer to double stranded DNA template**

Template (1-2ug) was denatured for 10 minutes in 40ul of 400mM NaOH then precipitated and resuspended in 10ul of $dH_2O$. Primer (5-10 pmol) was mixed with the denatured template in a total volume of 14ul in 140mM Tris.HCl (pH7.6), 14mM $MgCl_2$ and 23mM DTT and the primer annealed by heating to 65°C for 10 minutes followed by a 10 minute incubation at 37°C.

**Section 9B.11.2: Annealing of primer to single stranded DNA template**

Primer (1-2 pmol) was added to 1.5-2 ug of ssDNA template in 140mM Tris.HCl, 14mM $MgCl_2$ and 23mM DTT, pH7.6 in a total volume of 14ul. The primer was annealed by heating to 60°C for 10 minutes and allowed to cool for 10 minutes at room temperature.

**Section 9B.11.3: DNA sequencing reactions**

Annealed primer/template in a volume of 14ul was incubated for 5 minutes at room temperature following the addition of 3ul of labelling mix (1.375uM dCTP, dGTP, dTTP and 333.5mM NaCl), 1ul of [$\alpha$-$^{35}$S]dATP and 2ul of 1U/ul T7 DNA polymerase (diluted with 20mM Tris.HCl pH7.5, 5mM DTT, 100ug BSA/ml, 5% glycerol). Following the labelling reaction, 4.5ul aliquots were transferred to each of four tubes containing 2.5ul prewarmed (37°C) termination mixes, mixed gently and incubated at 37°C for 5 minutes. Termination reactions were stopped by the addition of 5ul each of stop solution (0.3% (w/v) bromophenol blue, 0.3% (w/v) xylene cyanol, 10mM EDTA (pH7.5) and 97.5% deionised formamide). Reactions were stored at -20°C. Prior to electrophoresis, sequencing reactions were heated to 75-80°C for 2 minutes.

Sequencing reactions were run on 0.2mm 8% (w/v) polyacrylamide gels using a BioRad SequiGen Sequencing Cell at 55-60°C. Gels were transferred to Whatman 3MM blotting paper, covered with Saran wrap and dried for 2-3 hours at 80°C under vacuum. For autoradiography the Saran wrap was removed from the dry gel and placed in a cassette with an intensifying screen and exposed overnight at -80°C. For higher resolution, gels were exposed without fluorographic enhancement at room temperature for 3-4 days. *Gel mix*: 8% (w/v) polyacrylamide was made by adding 50g of urea to 30ml of $H_2O$, 10ml of 10x TBE and 20ml of 40% (w/v) acrylamide/2.105% bis-acrylamide (Easigel, Scotlab). The gel mix was stirred at 37°C for 30 minutes with 50g of Duolite ion exchange resin and filtered through a 0.2uM vacuum filter (Sartorius) and stored at 4°C in foil wrapped bottles until use.

**Section 9B.12: Embryonic stem cell culture and gene targeting**

**Section 9B.12.1: HM-1 culture conditions**

HM-1 ES cells, a feeder independent line (Magin et al., 1992), were cultured in 1x Glasgow's modified Eagles minimum essential medium (GMEM BHK21, Gibco BRL Life Technologies) supplemented with 5% foetal calf serum (Sera-lab, Gibco BRL), 5% newborn calf serum (ICN Flow), non-essential amino acids, 1 mM sodium

pyruvate, 0.1 mM β-mercaptoethanol and recombinant mouse LIF at 1000U/ml as described (Melton, 1997).

**Section 9B.12.2: ES cell culture**

ES cells were passaged by aspirating off the medium, rinsing the bottom of the flask with 1-2ml of trypsin (ICN Flow), aspirating off the trypsin and adding a further 1-2mls of trypsin and placing the flask in a 37°C incubator until the cells had lifted off of the flask (2-3 minutes). ES medium was added to a total volume of 10ml and the ES cells pelleted by a 5 minute spin (13Krpm) at room temperature. The medium was removed and the cells resuspended gently in an appropriate volume of ES medium and an aliquot transferred to a gelatinised (0.1% (v/v) gelatin) tissue culture flask. ES cells were grown in a humidified tissue culture incubator (Forma Scientific water jacketed incubator) at 37°C with 4-8% $CO_2$.

Cells were stored long term in liquid nitrogen. The culture to be frozen was trypsinised as above, spun down, medium removed and placed on ice. Ice cold freezing medium (10% DMSO, 20% (v/v) FCS in LIF supplemented ES medium) was added and the cells gently resuspended in the medium. Cells were frozen at a high density (to maximise cell survival) and 3ml of freezing medium was routinely added to a confluent 25cm$^2$ flask and the cells frozen in 1ml aliquots at -20°C for several hours then transferred to -70°C overnight, then moved into liquid nitrogen for long term storage. ES cells (1ml aliquots) were thawed rapidly by placing them in a 37°C waterbath then quickly transferred to 9mls ES medium and resuspended. The ES cells were pelleted and resuspended in 3-5mls of ES medium and placed in a 25cm$^2$ flask and grown at 37°C as described above.

**Section 9B.12.3: Electroporation of ES cells**

Targeting vector DNA was linearised with an appropriate restriction enzyme prior to electroporation using a BioRad Gene Pulser (BioRad; Richmond, U.S.A.). Cells were suspended in 0.8ml electroporation buffer (21mM HEPES, 5mM D-glucose (dextrose), 8mM $Na_2HPO_4$, 5mM KCl, 140mM NaCl) with 200ug linearised

targeting vector DNA and pulsed with 3 mF, 800 V (Thompson et al., 1989) using Biorad cuvettes (BIO-RAD Gene Pulser Cuvette 0.4cM electrode, gap 50). Cells were incubated at room temperature for 10 minutes before plating.

**Section 9B.12.4: PCR screening ES colonies for targeting events**

Single well isolated ES colonies were removed from culture dishes with a micropipette and placed in a 1.5ml centrifuge tube containing 500ul of ES medium. The colony was broken into a single cell suspension by thorough pipetting and 200-300ul of this cell suspension was placed into a single well of a gelatinised 24 well microtitre dish and 1ml of ES medium added. The remainder was processed for PCR detection of targeting events.

PCR screening for targeting events was performed on crude ES cell lysates as follows: the ES cell sample was spun for 30 seconds at 13,000 rpm on a benchtop microcentrifuge. Supernatants were discarded and 40ul of 1x PCR buffer and proteinase K (10mg/ml) was added and incubated for 1 hour at 65°C. Proteinase K was denatured by heating the samples to 90°C for 25 minutes in a waterbath. The moisture was spun down and 20ul of the heat inactivated ES cell lysate was included in a 50ul PCR reaction. The PCR cycling conditions were adjusted for each primer/template combination. Standard conditions were as follows: 20ul ES cell lysate, 3ul 10x PCR buffer (50mM KCl, 1.5mM $MgCl_2$, 0.01% (w/v) gelatine, 10mM Tris pH8.3, 0.005% (v/v) Triton X-100, 0.005%(v/v) Tween 20), 1.9ul 10mM EDTA, 1ul 5mM dNTPs, 1ul oligo 1 (OD=20), 1ul oligo 2 (OD =20), sterile d.$H_2O$ to 50ul, and overlaid with 50 ul mineral oil. PCR was performed for 35 cycles of 94°C, 30 seconds, 65°C 30 seconds, 72°C 1 minute.

**Section 9B.13: Generation of chimeric mice from gene targeted ES cells**

All procedures performed were as described by Hogan, Constantini and Lacey, (1986). C57BL/6J and Balb C blastocysts were obtained by timed mating females and blastocysts were retrieved 3.5 days after plugging by flushing the uterine horns. Well formed, fully expanded blastocysts were injected with approximately 10 targeted ES

cells following the method described by Wassarmann and Pamphilis, (1993). Injected blastocysts were transferred into the uterine horn of pseudopregnant recipient MF1 females. Pseudopregnant females were obtained by mating them with vasectomised or genetically sterile males.

Chimeras were detected by the appearance of chinchilla coat colour on the background of the coat colour of the recipient blastocyst. Coat colour alleles of the recipient blastocysts were matched with those of the 129/Ola HM-1 ES cells to provide an easily identified coat colour chimaerism (Thompson et al., 1989) as described in Figure 1.7 (p37).

**Section 9B.14: PrP immunoblotting**

$PrP^c$ was detected by immunoblotting with crude brain homogenates in conjunction with rabbit anti mouse PrP27-30 polyclonal antiserum 1A8 (Farquar et al., 1994). Homogenates were prepared as follows: brains were dissected cleanly, weighed and homogenised in a sufficient volume of 0.32M sucrose/20mM PMSF to 10% (w/v). Total brain protein content was determined by the bichinchonic acid method (Pierce) -10%(w/v) homogenates had protein contents close to 1mg/ml. Samples were stored at -70°C prior to use.

Brain homogenates and $PrP^{Sc}$ samples were boiled for 5 minutes with Laemmli sample buffer before separation by 12% SDS PAGE with a 3.3% stacking gel (Laemmli, 1970). NEB broad range pre-stained markers were run with all gels to assess separation and electrotransfer efficiency.

Proteins were electroblotted from acrylamide gels onto PVDF membrane (Immobilon P, Millipore) using a BioRad Transblot SD semi dry electroblotter at 12V for 2 hours then rinsed in PBST. Membranes were blocked for 30 minutes to 1 hour with 5% foetal calf serum, 1%(w/v) BSA in PBST (0.1%(v/v) Tween 20), the blocking solution was discarded and incubated with PrP primary antiserum (IA8 1:1000 in PBST) for 1 hour at room temperature. Membranes were washed three times for 10 minutes in PBST then the secondary antiserum (Promega alkaline phosphatase conjugated goat anti-rabbit IgG 1:7500 dilution) added and incubated for

1 hour. Membranes were washed three times for 10 minutes in PBST, then developed using either a chromogenic method with NBT/BCIP (Promega) or with an enhanced chemifluorescent alkaline phosphate substrate (Vistra ECF Amersham) and visualised with a STORM fluorimager (Molecular Dynamics).

**Section 9B.15: Analysis of 301V terminal brains**

**Section 9B.15.1: 301V Challenge**

Mice were challenged by injection of 20ul of $10^{-2}$ dilution of VM/Dk terminal 301V brain homogenate into the thalamus. All challenges performed during the course of this project used the same 301V inoculum derived from clinically sick VM/Dk brain at its fifth passage. Mice were scored for signs of clinical illness as described (Dickinson, Meikle and Fraser, 1968).

**Section 9B.15.2: Lesion profiling**

Terminal mice were sacrificed by cervical dislocation and their brains fixed in 10% formol saline for 2-3 days followed by decontamination for 1 hour in formic acid (98%). All brains were sectioned coronally at four levels: forebrain, thalamus, midbrain and medulla. Lesion profiles were performed on 6μM H+E stained sections using the method described by Fraser and Dickinson, (1968) in which vacuolar degeneration was scored on a scale of 0 to 5 in nine standard grey matter areas and in three white matter areas.

**Section 9B.15.3: PrP immunohistochemistry**

Brains were removed at the terminal stage of disease and immersion fixed for 48 hours with periodate-lysine-paraformaldehyde (2% paraformaldehyde), followed by decontamination for 1 hour in formic acid (98%). Brains were then dehydrated with alcohols and impregnated in wax during a 7 hour processing cycle. 6μM sections were mounted on slides which had been baked at 200°C for 4 hours and coated with Vectabond. Mounted sections were immunostained by the standard peroxidase-antiperoxidase (PAP) method (Sternberger et al., 1970) using PrP antiserum 1A8 at 1 in

200 dilution (Farquar, et al., 1994). The reaction product was visualised with diaminobenzidine and sections were lightly counterstained with haematoxylin. Controls were carried out using preimmune serum in place of primary antibody.

**Section 9B.15.4: Isolation of sedimentable PrP from 301V terminal brain**

For the analysis of $PrP^{Sc}$ from infected brains, sedimentable PrP was isolated as follows: ½ brains were homogenised in 2ml of brain lysis buffer (10%(w/v) sarkosyl, 0.14%(w/v) $NaH_2PO_4$, pH 7.4) on ice then decanted into 2ml screw capped Eppendorf tubes and centrifuged at 4°C on a benchtop microcentrifuge for 15 minutes at 13,000 rpm. The supernatant was retained and its volume increased to ~3ml with BLB prior to centrifugation at 100,000rpm in a TL120.2 rotor in a Beckman TL100 ultracentrifuge at 4°C for 30 minutes. Samples were flash frozen with liquid nitrogen and stored at -70°C. Samples were also stored for short periods at -20°C.

For the detection of PrP27-30, samples prepared as above were treated with proteinase K (50ug/ml, Boehringer Mannheim) for 1 hour at 37°C and immunoblotted as described in Section 9B.14.

All procedures involving 301V were performed in a class II laminar flow hood in a dedicated room at the BBSRC/MRC Neuropathogenesis unit, Edinburgh.

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# Appendix I

# Publications

# Double Replacement Gene Targeting for the Production of a Series of Mouse Strains with Different Prion Protein Gene Alterations

**Richard C. Moore, Nicola J. Redhead, Jim Selfridge, James Hope[1], Jean C. Manson[1] and David W. Melton***

Institute of Cell and Molecular Biology, University of Edinburgh, King's Buildings, Mayfield Road, Edinburgh EH9 3JR Scotland. [1]BBSRC and MRC Neuropathogenesis Unit, Institute for Animal Health, West Mains Road, Edinburgh EH9 3JF Scotland. *Corresponding author (e-mail: dmelton@srv0.bio.ed.ac.uk).

**We have developed a double replacement gene targeting strategy which enables the production of a series of mouse strains bearing different subtle alterations to endogenous genes. This is a two-step process in which a region of the gene of interest is first replaced with a selectable marker to produce an inactivated allele, which is then re-targeted with a second vector to reconstruct the inactivated allele, concomitantly introducing an engineered mutation. Five independent embryonic stem cell lines have been produced bearing different targeted alterations to the prion protein gene, including one which raises the level of expression. We have constructed mice bearing the codon 101 proline to leucine substitution linked to the human familial prion disease, Gerstmann-Straussler-Scheinker syndrome. We anticipate that this procedure will have applications to the study of human inherited diseases and the development of therapies.**

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Mouse models for human inherited diseases and familial cancers can be produced by gene targeting in embryonic stem cells. The models can be used to study the relationship between the genetic lesion and the development and progression of disease at the molecular, cellular, and whole animal level, to enhance the effectiveness of drug development programs, and to evaluate gene therapies (see ref. 1 for a review of gene targeting).

When DNA is introduced into mammalian cells, it predominantly integrates at random. In gene targeting, homologous recombination between an introduced vector DNA and the chromosomal target locus is used to make alterations to individual mammalian genes. The process is inefficient, so a range of selective techniques have been devised to facilitate identification of gene-targeted clones[2]. If targeting is carried out in cultured mouse embryonic stem (ES) cells, then strains of genetically modified mice can be produced. ES cells are derived from the inner cell mass of mouse blastocysts. They can be cultured for extended periods *in vitro* and still retain totipotency so that, when reintroduced into a host embryo, they will contribute to development, leading to the production of a chimeric animal. If ES cells contribute to the germ line of the chimera, then ES cell-derived strains of mice can be established[3].

Targeted gene inactivation (gene knockout) has been used to produce mouse models for a range of human genetic deficiency diseases[4–7]. However, the generation of models for many human diseases requires more subtle gene alterations. For example, the most common cystic fibrosis mutation, ΔF508, is due to deletion of a single phenylalanine codon from the CFTR gene[8], while the majority of prion protein (PrP) gene mutations associated with inherited transmissible prion disorders are single nucleotide substitutions[9].

Prion diseases in humans are unique in that they can have an etiology which may be both genetic and infectious. Although most cases are sporadic, a subset are familial, being inherited in an autosomal dominant manner, and include familial Creutzfeldt-Jacob disease (CJD), Gerstmann-Straussler-Scheinker (GSS) syndrome, and Fatal Familial Insomnia. While the majority of sporadic CJD cases are not associated with any known genetic lesion, the familial forms of these fatal late-onset neurodegenerative disorders are tightly linked to more than a dozen different point mutations and octapeptide repeat expansions in the PrP gene[10]. In addition, PrP polymorphisms are known to modulate many features of these diseases in humans and mice: a codon 129 Met/Val human PrP polymorphism influences the susceptibility to sporadic CJD[11] and can influence the phenotype of familial prion diseases[12]. Polymorphic residues at codons 108 and 189 in the mouse PrP protein are associated with different experimental scrapie incubation times between inbred mouse strains[13,14].

To investigate the role of PrP mutations and polymorphisms in prion disease etiology in particular and to facilitate the construction of mouse models of genetic disorders in general, it is important that current gene targeting technology be extended to enable the introduction of subtle alterations. Two-step gene targeting procedures have previously been devised to make subtle gene alterations, and some success has been achieved with the "hit and run" method[15], which involves the targeted insertion of a vector containing the gene alteration, followed by resolution of the resulting gene duplication by intrachromosomal recombination, to leave the alteration behind. This method suffers from two disadvantages. First, there is no control over the site of the second intrachromosomal recombination, with the result that the alteration, rather than the endogenous sequence, is often lost and the locus is left unmodified. Second, the whole process must be repeated for every individual gene alteration to be introduced. We have developed an alternative procedure, termed double replacement, which does not suffer from these disadvantages, and have used it to produce mice expressing the human, rather than the mouse form of the milk protein, α-lactalbumin[16]. Here we demonstrate the flexibility of the method by showing how, from a single initial targeting event, double replacement can be used to generate a series of mouse strains with different PrP gene alterations.

## Results

**Double replacement gene targeting.** A double replacement strategy in the HPRT-deficient cell line, HM-1 (ref. 17) was used to introduce subtle alterations into the PrP locus (Fig. 1); in the first step, the entire PrP coding region, located in exon 3, was deleted and replaced with an HPRT minigene, generating an inactivated allele and conferring a HAT selec-

table phenotype. Provision of a Herpes simplex virus thymidine kinase (HSV-TK) gene at the 5′ terminus of the inactivation vector enabled gancyclovir counter-selection and enrichment for homologous recombinants. The second step re-targeted this inactivated allele, replacing the HPRT minigene with exon 3 sequence and giving rise to a 6-thioguanine resistant (6-TG$^R$) phenotype. Reconstitution of the inactivated locus results in a PrP allele indistinguishable from the wild-type allele, with the exception of the introduction of the intended mutation. Multiple second steps were used to construct five ES cell lines bearing different PrP alterations.

**Targeted inactivation of the PrP gene.** Following electroporation of HM-1 cells with the inactivation vector, ES cell colonies surviving HAT and gancyclovir selection were screened by PCR for a targeting-specific product of 2.28 kb. Fourteen of 72 colonies gave the targeting-specific band and positive clones identified by this method were further characterized by Southern analysis (see Figs. 2 and 3): BamHI digestion and hybridization to a 3′ PrP probe yielded a 9.1 kb fragment from the inactivated allele in addition to the 8.5 kb band derived from the wild-type allele. BamHI-XhoI digestion and hybridization to this probe yielded a diagnostic 5.2 kb band from the inactivated allele, in addition to the 8.5 kb band from the wild-type allele. The 5.2 kb band is due to the targeted introduction of a novel XhoI site, present in the HPRT minigene, into the PrP locus.

Two independent knockout clones were chosen for blastocyst injection. Both gave chimeras which transmitted the inactivated allele to their offspring (Table 1). Mice heterozygous for the inactivated allele have been crossed to produce PrP null animals.

**Generation of subtle PrP gene alterations.** Selection for the replacement of the HPRT minigene with altered PrP sequences was achieved in 6-TG. Prior to the second round of targeting, PrP knockout clones were taken out of HAT selection, grown non-selectively for five days and then plated in 6-TG to assess the spontaneous level of 6-TG$^R$. Clones selected for second step targeting had a spontaneous 6-TG$^R$ frequency of $<10^{-6}$. Following electroporation with the second-step targeting vectors, the PrP coding region from surviving 6-TG$^R$ clones was amplified by PCR. PCR products were analyzed for the appropriate RFLP to detect introduced alterations.

**Detection of targeted PrP allele containing 101Leu.** The codon 101 point mutation changes CCC–Pro to CTC–Leu, creating an additional DdeI site within the PCR product from exon 3 (Fig. 4). Eighty-four clones were screened by this method and 10 showed the pattern expected for cells containing one normal PrP allele and one targeted allele containing the 101Leu mutation. Southern analysis of these targeted clones gave the expected wild-type pattern (Figs. 2 and 3). One second-step targeted clone containing the 101Leu allele was used for blastocyst injection and chimeras transmitting the mutant allele were obtained (Table 1). Mice containing the 101Leu allele will be investigated as a model for the human prion disease, GSS.

**Detection of a targeted allele containing 101Leu and the SV40 enhancer.** As an initial screen for the replacement of the inactivated allele using the pGSS101Leu-SV40 vector, sixty 6-TG$^R$ clones were screened for the codon 101Leu mutation (which should be integrated together with the SV40 enhancer). Fourteen were found to be positive and the SV40 enhancer was shown to be present in all of these clones by PCR with SV40 primers. The structure of the targeted allele was confirmed by Southern analysis: the SV40 enhancer contains an SphI site, resulting in a 5 kb targeting-specific band when hybridized to the PrP coding probe (Figs. 2 and 3d).

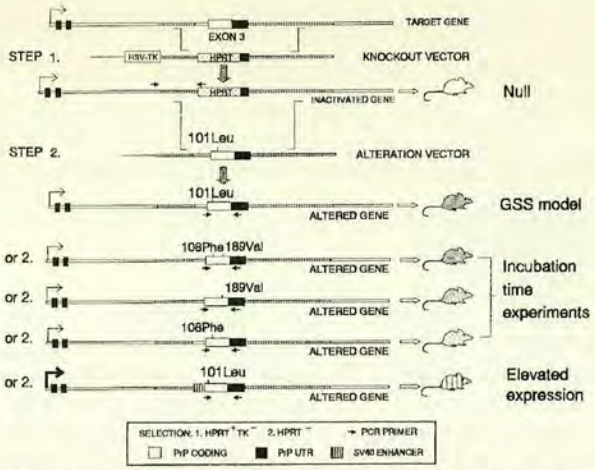


**FIGURE 1. Double replacement gene targeting for the introduction of subtle alterations into the mouse PrP gene. Use of the double replacement strategy in HPRT-deficient ES cells is illustrated. In the first step, the PrP coding exon is deleted and replaced with an HPRT minigene, leading to the production of PrP null animals. In the second step, a range of mouse lines with both subtle PrP coding and transcriptional alterations can be generated. The figure is not drawn to scale. The first two non-coding exons of the PrP gene are located ~18 kb from**

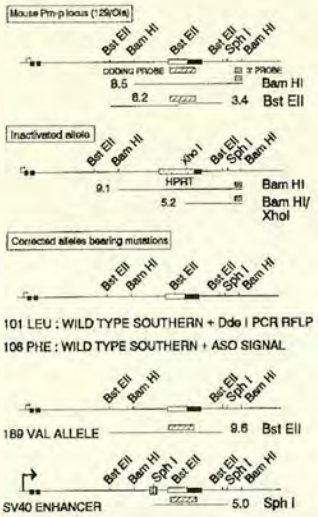


**FIGURE 2. PrP gene structure in wild-type and targeted lines. The structure of the endogenous PrP locus in 129/Ola mice, the inactivated allele and corrected alleles bearing various mutations are shown schematically, using the same conventions as in Figure 1. The location of probes and the sizes of restriction fragments detected in Southern blotting analysis of the different alleles are indicated. ASO, allele-specific oligonucleotide hybridizing to the 108Phe allele.**

**TABLE 1. Gene targeting and germ line transmission frequencies.**

| PrP Alteration | Germ Line Transmission Targeting Frequency[a] | Frequency[b] |
|---|---|---|
| Knockout | 14/72 | 4/7 |
| 101Leu | 10/84 | 3/3 |
| 108Phe-189Val | 4/60 | 1/4 |
| 189Val | 4/72 | * |
| 108Phe | 6/50 | * |
| SV40-101Leu | 8/60 | ND |

[a]Number of targeted clones containing the alteration in the population surviving selection/total number of clones screened. [b]Number of chimeras giving germ line transmission of the targeted allele/total number tested. *Under test. ND = Not determined.

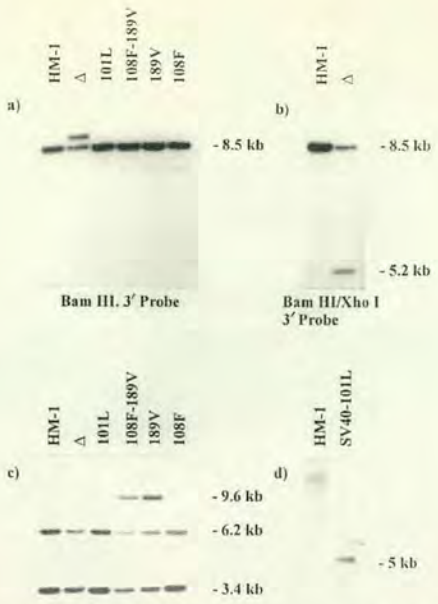


**FIGURE 3. Southern analysis of targeted ES lines. (a) BamHI digest, 3′ PrP probe. Lanes: HM-1, non-targeted ES cell line; Δ, knockout cell line with one PrP allele inactivated; 101Leu, 108Phe-189Val, 189Val and 108Phe, cell lines containing the codon alteration(s) specified. (b) BamHI-XhoI digest, 3′ PrP probe. (c) BstEII digest, coding region probe. (d) SphI digest, coding region probe. SV40-101Leu, cell line with one PrP allele containing an SV40 enhancer element and the 101Leu mutation.**

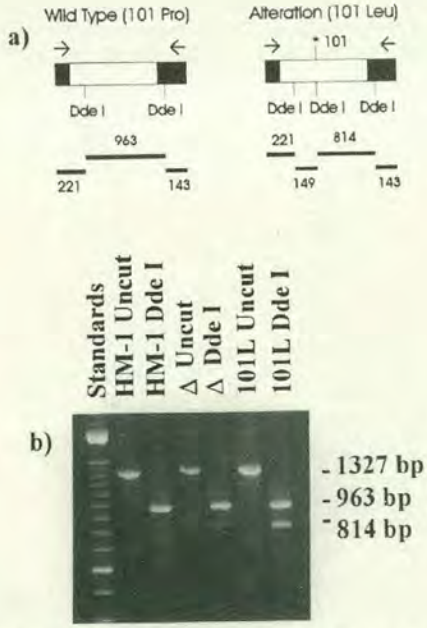


**FIGURE 4. Detection of codon 101Leu targeted allele. (a) DdeI restriction map of PCR products from a wild-type PrP coding exon (101Pro) and an allele containing the 101Leu mutation. Arrows indicate PrP primers. (b) Uncut and DdeI-digested PCR products from PrP wild-type (HM-1) cells, PrP knockout (Δ) cells and cells with one PrP allele containing the 101Leu mutation (101L).**

**Expression of 101Leu alleles.** Northern analysis of ES cell RNA is shown in Figure 5a. Comparison of the relative abundance of the 2.1 kb PrP mRNA shows the expected halving in the knockout cell line compared to HM-1 and restoration in the 101Leu cell line. The 101Leu-SV40 cell line contained an elevated level of PrP mRNA. Figure 5b shows the same filter reprobed with mouse α-actin, confirming the matched loadings of total RNA. To demonstrate expression from the 101Leu alleles, cDNA was synthesized and amplified using primers from mouse exons 2 and 3. PCR products were definitively generated from cDNA, rather than any genomic DNA contaminating the RNA preparation, because the primer positions are separated by approximately 18 kb of intron 2 in the gene[18]. Figure 5c shows the 854 bp PCR product digested with DdeI. Densitometry indicated equal expression of the wild-type (657 bp fragment) and 101Leu alleles (514 bp fragment) in the 101Leu cell line. In the 101Leu-SV40 cell line, densitometry indicated a two-fold increase in the level of expression of the mutant allele above wild type.

**Detection of targeted PrP alleles containing codon 108 and 189 alterations.** The detection of different combinations of codon 108 and 189 alterations required a coupled screening method for determining the ES cell PrP codon 108-189 haplotype of potentially-targeted colonies. PCR product from exon 3 (1,327 bp) was first digested with Bst EII to screen for the codon 189 ACC to GTC alteration, which results in loss of a unique BstEII site and the substitution of Thr with Val (Fig. 6a). In each lane it can be seen that the wild-type allele is digested into 630 bp and 697 bp fragments. This acts as an internal control for BstEII digestion, but also allows spatial separation of wild-type from targeted allele, to enable the demonstration of the physical linkage of both alterations in the 108Phe-189Val targeted allele. The PCR product derived from the 189Val allele does not digest, resulting in three bands: 630bp and 697bp from the wild-type allele and 1327 bp from the 189Val allele. The codon 108 CTC to TTC alteration, resulting in the substitution of Leu with Phe, was readily detected by allele-specific hybridization to a radiolabelled 108Phe oligonucleotide (Fig. 6b).

**Detection of the 108Phe-189Val allele.** Four of the 60 surviving 6-TG$^R$ colonies contained the targeted 108Phe-189Val allele in addition to the normal 108Leu-189Thr allele. Southern analysis indicated the predicted gene structure, with the 189Val alteration leading to the loss of the BstEII site in exon 3 and the generation of a novel targeting-specific band of 9.6 kb (Figs. 2 and 3c).

**Detection of the 189Val allele.** Four of 72 colonies screened by PCR had undergone the targeted alteration to codon 189, as indicated by loss of a BstEII site on one allele (Fig. 6a). Southern analysis confirmed this (Fig. 3c).

**Detection of the 108Phe allele.** Six of 50 colonies surviving 6-TG selection had the targeted single nucleotide alteration to codon 108Phe and hybridized to the 108Phe oligonucleotide (Fig. 6b). The predicted structure of the targeted allele was confirmed by Southern analysis (Fig. 3c).

Chimeras have been produced from ES cells containing the different codon 108 and 189 alterations. Germ-line transmission has been achieved from one 108Phe-189Val chimera, while other chimeras remain under test (Table 1).

## Discussion

We illustrate here a double replacement gene targeting strategy and show that multiple subtle alterations can be rapidly introduced into a gene of interest. This enables a strategic approach to gene targeting programs. It is possible to first generate a null mouse to investigate gene function and/or to generate a model of a gene deficiency disorder, and then target single amino acid or nucleotide alterations into a gene, enabling the construction of mouse models of human disorders arising from single amino acid/nucleotide substitutions. This method can also be used to manipulate the level of endoge-

nous gene expression.

The use of gene targeting for the construction of mouse models of human prion diseases has a number of advantages over pronuclear injection. The latter method is reliant upon gene addition, giving rise to the multiple copy insertion of transgenes which are susceptible to chromosomal position effects and often results in over-expression[19]. Conventional transgenesis has been used to great effect to investigate many features of experimental murine scrapie[20–23]. However, the recent observation that over-expression of wild-type prion protein is pathogenic[24] suggests that gene targeting is more appropriate for the investigation of some aspects of prion disease. A previous conventional transgenic GSS mouse model[23] displayed some of the neuropathology of GSS and suggested the pathogenic nature of the Pro 101 to Leu mutation, however these mice over-expressed mutant prion protein eight-fold and were of mixed genetic background. We have constructed an inbred mouse strain bearing a gene targeted codon 101 Pro to Leu substitution in an attempt to generate a model for human GSS in a mouse expressing normal levels of mutant prion protein in a controlled genetic background.

Past difficulties in generating a mouse model for Alzheimer's disease using pronuclear injection and other methods have led us to view the use of targeted mouse models as potentially a more flexible approach. Although recent work with mice overexpressing mutant APP V717F has demonstrated some of the characteristic neuropathology of Alzheimer's[25], it is currently unclear whether overexpression of genes bearing mutations is a general prerequisite for neuropathology in a short-lived species such as the mouse. Overexpression has certainly provided much biologically useful information[24–27]. Thus, we have also developed a means to increase expression of mutant PrP protein alleles. The targeted 101Leu allele was expressed at a similar level to the normal allele and the targeted incorporation of the SV40 enhancer resulted in an approximately two-fold increase in expression of the 101Leu allele in ES cells. While the enhancer effect is comparatively modest in undifferentated ES cells (the poor function of viral enhancer elements in ES cells is well established), higher expression may occur in the tissues of any mice generated from these ES cells. Targeted introduction of enhancer elements into an endogenous locus will enable overexpression of a given gene, further diminishing the demarcation between gene targeting and conventional transgenesis by pronuclear injection.

Targeted gene replacement can be achieved in a single step by cotransfer of the altered gene and a positive selectable marker[28,29]. However, this neccessarily results in the retention of the marker gene adjacent to the gene replacement, which cannot be considered a subtle gene alteration and could have unforeseen consequences on the expression of the altered gene. The same criticism applies to a two-step targeting procedure involving HPRT selection termed "plug and socket"[30].

Alternative two-step procedures are required for the production of mice with subtle gene alterations. The first of these to be described, involving insertion of the targeting vector and subsequent excision, has been termed "hit-and-run"[15], or "in-out"[31]. Here a targeting vector carrying the desired gene alteration first inserts into the target locus, resulting in a partial gene duplication. In the second step, the duplication is resolved by an intrachromosomal recombination, ideally restoring the locus to its original state, apart from the retention of the subtle alteration. A variety of individual ES cell lines bearing a single subtle alteration at a particular locus have been produced by this technique[31–34], and in one case, mice have also been generated[32]. The method does, however, suffer from two disadvantages: firstly, there is no control over the site of the


**FIGURE 5. Expression of PrP targeted alleles. (a) Northern analysis with PrP coding region probe. Lanes: HM-1, non-targeted ES cell line; Δ, knockout cell line with one PrP allele inactivated; 101Leu and SV40-101Leu cell lines. (b) α-actin probe on the above filter. (c) DdeI-digested PCR products from PrP wild type (HM-1) cDNA, 101Leu cDNA and SV40-101Leu cDNA.**

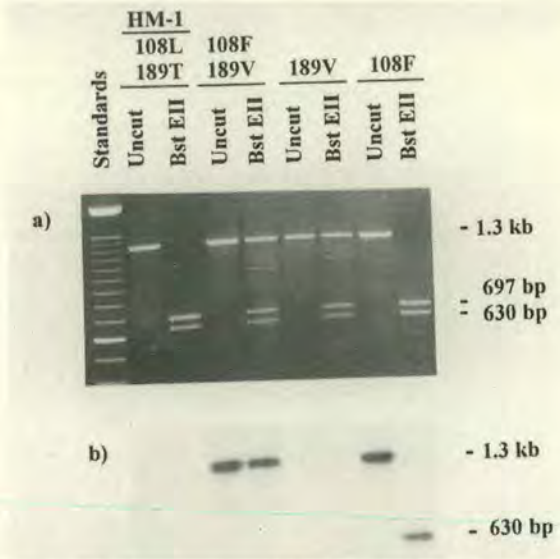


**FIGURE 6. Detection of codon 108 and 189 targeted alterations by BstEII digestion and allele-specific oligonucleotide hybridization to PCR products from ES cell DNA. (a) Uncut and BstEII-digested PCR products. Lanes: HM-1, non-targeted cells, both alleles encoding 108Leu and 189Thr; 108Phe-189Val, 189Val, 108Phe, targeted cell lines with one allele containing the alteration(s) indicated in addition to a second wild type, 108Leu-189Thr allele. (b) Allele-specific hybridization of the gel shown in panel (a) with an oligonucleotide specific for codon 108Phe.**

second, intrachromosomal recombination event, resulting in the frequent loss of the introduced altered sequence, rather than its endogenous homolog, and leaving the locus unmodified; secondly, a completely new round of targeting is required for each subtle gene alteration to be introduced.

The alternative two-step method that we have employed here, double replacement by selection for gain and loss of HPRT expression in HPRT-deficient ES cells, was first proposed by Reid et al.[35]. We have previously exploited this method, utilizing the very efficient HPRT selective systems, to produce mice with an α-lactalbumin locus inactivated by deletion and, in the second step, mice expressing human α-lactalbumin from the murine locus[16]. An equivalent double-replacement strategy, using the less efficient neo-TK marker for double selection, has also been described [36,33] and, in one case, animals containing the subtle alteration have been produced[33]. The double-replacement strategy employed in this laboratory and also in the Jaenisch laboratory[33] involves the deletion of part of the target locus in the first step. This maximizes the likelihood that a complete null phenotype will be exhibited in animals produced from ES cells targeted in the first step, rather than the retention of partial gene activity which can sometimes result from conventional insertional mutagenesis. In the situation described here, the entire PrP coding region was deleted. The key advantage that follows from this strategy is the imposition of control over the site of the homologous recombination occurring during the second targeting step, to maximize the likelihood of the alteration being introduced. In this case, the PrP coding region was deleted in the first step so, in the second step, there was no possibility of recombination between endogenous and vector-derived PrP coding sequences resulting in gene targeting without the introduction of the PrP alteration. Valuable as the above features are, the main attraction of the double-replacement method is that it can be used, from a single initial targeting event, to generate a series of mice with different subtle gene alterations. Here we have realized that potential.

The site of linearization of the inactivation vector was observed to affect the degree of gancyclovir enrichment in the positive-negative strategy employed in the first targeting step. Optimal enrichment was achieved following vector linearization at a site which ensures that HSV-TK 5′ sequence is not exposed to exonucleolytic attack. Selection for 6-TG$^R$ is the basis for the second targeting step in the double-replacement strategy. A high level of spontaneous 6-TG$^R$ in PrP knockout cells from the first step would make it harder to identify second-step clones. Such 6-TG$^R$ could arise from a carry-over of parental HPRT-deficient HM-1 cells during growth of the HAT$^R$ (HPRT-expressing) knockout clones[16], or from physical loss, or loss of expression of the HPRT minigene at the inactivated PrP locus. Only knockout clones showing a spontaneous 6-TG$^R$ frequency of $<10^{-6}$ were used for the second step. Despite this precaution only a fraction of the 6-TG$^R$ clones generated in the second step contained the desired alteration. Of the remainder, some retained HPRT minigene sequences, while others were indistinguishable from starting HM-1 cells and could have arisen either by carry-over or gene conversion. A similar situation has been observed in previous double-replacement targeting[16,33,36]. We consider, however, that the ability to identify five different subtle PrP alterations, while screening only small numbers of clones is proof of the general utility of the method.

## Experimental Protocol

**Isolation of isogenic DNA.** Cloned PrP sequences from HM-1 cells (derived from the 129/Ola strain) were obtained by screening a genomic DNA library (a gift from T. Magin) with a 1.1 kb Sma I-Eco RI exon 3 fragment from mouse strain NZW[14]. An 8.5 kb Bam HI fragment spanning exon 3 was cloned into pBTII SK(+), to give p129 PrP. All PrP sequence in the targeting vectors was derived from this construct.

**Mutagenesis.** A 934 bp KpnI-EcoRI exon 3 fragment, containing most of the PrP coding region, was cloned into M13mp18 to generate a convenient mutagenesis cassette. Oligonucleotide-directed mutagenesis[37] was carried out using a Muta-gene M13 Kit, Version 2 (BioRad). Mutant coding regions were sequenced to confirm the pres-ence of only the desired alteration(s) and used to construct the replace-ment targeting vectors (see below). The oligonucleotides used to introduce the mutations were as follows; codon 101 Pro to Leu (GGAACAAGC**T**CAGCAAACCA), codon 108 Leu to Phe (CCAAAAACCAAC**T**TCAAGCATGTGGC) and codon 189 Thr to Val (GCAGCACACGGTC**GT**CACCACCACCAAGGG). Oligonucleotides were derived from sense strands, with the alterations underlined.

**Construction of targeting vectors.** The knockout vector was constructed in three steps. In the first step, a 1.8 kb PstI-EcoRI restriction fragment and a 3.7 kb EcoRI-EcoRV restriction fragment, serving as 5′ and 3′ flanking PrP homologous sequences respectively, were ligated together into PstI/EcoRV linearized pBTII SK(+), to produce Vector A. An HPRT minigene, PGK/pDWM1[38], isolated as a 2.7 kb EcoRI fragment, was then inserted into the EcoRI site between the two regions of homology in reverse orientation with respect to the PrP promoter. Finally, an HSV-TK gene[39] was inserted as a NotI-BamHI fragment flanking the 5′ PrP homology (Fig. 1). The completed 13.2 kb knockout vector has a unique ClaI site, for linearization prior to electroporation. The exon 3 replacement vectors were constructed from Vector A, p129PrP and the mutagenesis cassette. Initially the region of 5′ homology was excised from Vector A by BamHI-EcoRI digestion. The replacement vector was reconstructed from the residual Vector A backbone, plus a 3.2 kb BamHI-KpnI 5′ homologous fragment, derived from p129PrP, and the KpnI-EcoRI mutagenesis cassette in a single step ligation. Essentially p129PrP is reconstructed with the mutation of interest included and a 0.7 kb EcoRV-BamHI 3′ terminal fragment excised. The linearisation site of the 11.3 kb replacement plasmids was also ClaI. A replacement vector containing the SV40 enhancer was also constructed. Primers, CCGGAATTCCTGGTTGCTGACTAATTGAGAT, with an EcoRI restriction site for the 5′ end and CCCTAAGCTTCCAGCTGTGGAATGTGTGTCAGTTA, with a Hind III restriction site for the 3′ end of the 72 bp repeat region were used to amplify the SV40 enhancer. The cycle conditions (25 cycles) used were: 30 secs at 94°C, 30 secs at 66°C and 30 secs at 72°C. The 5′ BamHI-KpnI region of homology from p129PrP was cloned into pBTII SK(+), and the SV40 enhancer was cloned into the unique EcoRI-HindIII sites to replace an ~100 bp region of intron 2 (Fig. 1). The 5′ region of homology containing the SV40 enhancer was then used for replacement vector construction as above. Five replacement vectors were produced by the cloning method described above; pGSS101Leu, p108Phe-189Val, p189Val, p108Phe and pGSS101-SV40.

**ES cell culture.** The isolation and culture conditions of the HPRT-deficient ES cell line, HM-1, have been previously described[39].

**Gene targeting.** *Step 1: PrP gene inactivation.* HM-1 cells ($5\times10^7$) were electroporated with 200 μg linearised knockout targeting vector DNA in 0.8 ml HEPES-phosphate buffered saline at pH 7.05 (BioRad Gene Pulser, 800 V, 3 μFd) and then incubated at room temperature for 10 mins. Cells were plated at $5\times10^6$, $2.5\times10^6$ and $1.25\times10^6$ cells/90mm dish. Twenty-four hours later HAT selection for HPRT expression was imposed. Four days after electroporation, counterselection (gancyclovir, 2 μM) was added to some dishes. Surviving colonies were fixed and stained, or isolated 11 days after electroporation. Targeted clones bearing the predicted gene structure were used for second step electroporations and for blastocyst injection to generate PrP null mice. Prior to the second step, knockout cells were removed from HAT selection. *Step 2: introduction of subtle alterations.* Knockout cells ($2.5\times10^7$) cells were electroporated as previously with the voltage at 850 V and plated at $4\times10^6$ cells/75 cm$^2$ flask. Cells were grown for seven days (passaging once) before plating at $1\text{-}2\times10^6$ cells/90 mm dish in medium containing 5μg/ml 6-TG. Surviving colonies were isolated twenty one days after the second electroporation step.

**PCR screening for targeting events.** Half the cells (100–1000 cells) from surviving colonies were used to prepare DNA for all PCR analyses[40]. All PCR conditions were those recommended by the suppliers (Gibco BRL). *(i) Detection of PrP knockout:* the primers used to amplify the 2.28 kb PCR product specific for the PrP coding region replacement by HPRT minigene were: AATCGCCACCTGCATTAGGG (intron 2, situated in the 80 bp BamHI-PstI fragment, 60 bp downstream of the 5′ Bam HI site of p129PrP) and AGCCTACCCTCTGGTAGATTGTCG (located within exon 9 of mouse HPRT cDNA sequence, positions 942–965, Genbank accession number J00423) (see Figure 1). The cycle conditions (40 cycles) were: 1 min at 94°C, 1 min at 67°C, 2 min at 72°C (Omnigene, Hybaid). *(ii) Detection of subtle alterations:* the primers used to amplify a 1,327 bp region of the PrP coding exon were: GATGTCAAGGACCTTCAGCC (intron 2, positions 38–57 from NZW sequence, Genbank accession number M18070) and TTGCCTTCAATCAGCTATCG (exon 3, 3′ untranslated region of PrP gene, positions 1290–1271 from NZW PrP cDNA, Genbank accession number M13685). The cycle conditions (35 cycles) were: 30 secs at 94°C, 30 secs at 65°C, 1 min at 72°C. *(iii) Detection of SV40 viral enhancer sequences:* the primers and conditions used to detect the enhancer sequences were the

same as those used originally, except that 35 cycles were used.

**Allele-specific oligonucleotide hybridization.** DNA from ES cell colonies was amplified as described above, digested with BstEII, electrophoresed on a 1.5% agarose gel and Southern transferred to Genescreen Plus membrane (New England Nuclear). The filter was prehybridized for 2 hours at 37°C (in 15 ml of 6 x SSC, 5 x Denhardt's solution, with 100 μg/ml denatured herring sperm DNA). 10 pmol of end-labeled ($^{32}$P) mutant oligonucleotide specific for the codon 108Phe mutation, GCCACATGCTTGAAGTTGG was then added to the prehybridization solution. After 1–2 hours of hybridization, the filters were rinsed in 6 x SSC at room temperature and, as a control for loading and transfer variation between PCR samples, autoradiographed for 15 mins to 1 hour prior to high stringency washing. Filters were then washed at allele-specific stringency: twice for 5 mins each at 67°C in 6 x SSC and then autoradiographed at room temperature for 15 mins to 1 hour.

**Southern hybridization.** Genomic DNA was prepared and Southern hybridization carried out as described[41]. A 0.7 kb EcoRV-BamHI restriction fragment from the 3′ terminus of the 8.5 kb BamHI fragment (3′ probe), and a 1.1 kb SmaI-EcoRI restriction fragment from the coding region of PrP (coding region probe), were used as probes.

**Northern hybridization.** Total RNA was extracted from cultured cells[42] and Northern hybridization carried out as previously described[41] and reprobed with mouse α-actin cDNA[43].

**Reverse transcription.** 10 μg of total ES cell RNA was reverse transcribed as described[38], except that random hexamers (50ng) were included in addition to oligo dT.

**Detection of cDNA sequences.** cDNA produced by reverse transcription of total RNA was analyzed by PCR. The primers used were: GTCCAATTTAGGAGAGCCAAGC (exon 2, positions 63–84 from NZW PrP cDNA sequence, Genbank accession number M13685) and GCCTAGACCACGAGAATGCG (exon 3, positions 915–896 from NZW PrP cDNA) to give a product of 854 bp with cycle conditions (32 cycles): 30 secs at 94°C, 30 secs at 65°C, 1 min at 72°C. When this product is digested with restriction enzyme Dde I, it results in bands of 197 bp and 657 bp. The 657 bp band containing the 101Leu mutation is further cleaved into bands of 143 bp and 514 bp.

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# Transgenic analysis of prion diseases

**Richard C.Moore[1] and David W.Melton**

Institute of Cell and Molecular Biology, University of Edinburgh, Darwin Building, King's Buildings, Mayfield Road, Edinburgh, EH9 3JR, UK

[1]To whom correspondence should be addressed

**Prion diseases are fatal transmissible neurological disorders afflicting a range of mammalian species. Although still controversial, a large body of data suggests that the causative agent may be composed entirely of a small glycoprotein. The brains of infected animals have accumulations of a pathogenic protease-resistant isoform ($PrP^{sc}$) of a normal host-encoded glycoprotein, $PrP^{c}$ or prion protein. A number of lines of biochemical evidence implicate the disease-specific isoform, $PrP^{sc}$, as the transmissible agent and genetic analysis has shown tight linkage between *PrP* gene mutations and polymorphisms and differential susceptibility to prion diseases. Perhaps the strongest evidence for a protein-only model of the agent is that *PrP* gene-ablated mice are resistant to scrapie and that mice with a *PrP* mutation, corresponding to those found in a human familial prion disease, spontaneously develop a transmissible prion disease. This review describes the critical role that transgenic technology has played in the study of the biology of prion diseases and considers the issues raised by this work.**

*Key words:* gene targeting/prion diseases/prion protein/scrapie/transgenesis

## Introduction

Prion diseases are transmissible degenerative disorders of the central nervous system and occur in a number of species (See Table I). Human prion diseases include Kuru (Gadjusek and Zigas, 1957), Gerstmann–Straussler–Scheinker disease (GSS; Gerstmann *et al.*, 1936), Creutzfeldt–Jakob disease (CJD; Creutzfeldt, 1920; Jakob, 1921; see Creutzfeldt, 1989 and Jakob, 1989 for recent English translations) and fatal familial insomnia (FFI; Gambetti *et al.*, 1993). Kuru is an epidemic prion disease transmitted by cannibalism amongst the Fore tribespeople in the highlands of Papua New Guinea (Gadjusek, 1985). Kuru has been responsible for a large number of deaths since 1950, although the incidence of disease has declined markedly in recent years as a result of government intervention. Creutzfeldt–Jakob disease has several epidemiological manifestations: sporadic, familial and iatrogenic. Sporadic CJD has an incidence of $10^{-6}$ and an average onset age of 65 years. Approximately 10% of CJD cases (and all GSS and FFI cases) are autosomal dominant familial disorders with linkage to mutations in the prion protein gene (See Table II; for review see Prusiner, 1994). A small number of iatrogenic CJD cases have arisen as a result of transmission from contaminated neurosurgical instruments, dura mater grafts (Lane *et al.*, 1994), corneal transplants (Gadjusek, 1985) and contaminated cadaver-derived pituitary growth hormone and gonadotrophin (Buchanan *et al.*, 1991). In the UK, much interest has been generated by the possible association between consumption of BSE-infected food products and the recent emergence of an unusual variant of CJD (nvCJD), with an earlier age of onset and a novel clinico-pathological phenotype (Chazot *et al.*, 1996; Will *et al.*, 1996).

## The biological properties of the infectious agent

### *Clinical signs and pathology of the prion diseases*

The effects of prion diseases are generally limited to the central nervous system (CNS). The clinical signs are variable but include progressive dementia, cerebellar ataxia and behavioural disturbances. Gross neuropathology can include reduced brain weight, cortical atrophy and enlargement of the ventricles. Histological abnormalities include bilaterally symmetrical spongiform degeneration of the neuropil, neuronal perikaryal vacuolation, conspicuous neuronal loss, amyloid plaques and astroglial hypertrophy (Bell and Ironside, 1993). A florid CNS microglial response has been described in CJD and scrapie, which may represent a modified form of inflammatory response (Ironside *et al.*, 1993; Williams *et al.*, 1994). Widespread apoptosis has been described in the brains of sheep, mice and humans with these disorders and may be a major mode of cell death (Fairbairn *et al.*, 1994; Giese *et al.*, 1995; Lucassen *et al.*, 1995).

### *Prion diseases are transmissible following long incubation times*

The demonstration of scrapie transmissibility in 1936 (Cuille and Chelle, 1936) and subsequent transmission studies in sheep, goats and other animals established the main biological features of the scrapie agent. The response of all hosts, to

**Table I.** Transmissible spongiform encephalopathies

| Species | Disease | Comments | Geographic distribution | Reference |
|---|---|---|---|---|
| Sheep | Scrapie | Field scrapie; experimentally transmitted to sheep, goats, mice, rats, hamsters and cattle (Gibbs *et al.*, 1990). | Worldwide | Cuille and Chelle, 1936 |
| Mouflon | Scrapie | Naturally occurring case | | Wood *et al.*, 1992 |
| Goat | Scrapie | Natural cases rare | | Cuille and Chelle, 1939; Pattison and Millson, 1960 |
| Mink | Transmissible mink encephalopathy | Infection in farmed mink; source of infection probably scrapie infected offal. Experimentally transmitted to hamsters. | North America; Europe | Marsh and Kimberlin, 1975; McKenzie *et al.*, 1996 |
| Mule Deer | Chronic wasting disease | Infection of farmed deer probably from scrapie contaminated feed. Experimentally transmitted to other deer. | North America | Williams and Young, 1980; Williams *et al.*, 1982 |
| Elk | Chronic wasting disease | Infection in farmed elk, probably derived from scrapie infected feed. | | Williams and Young, 1982 |
| Cattle | Bovine spongiform encephalopathy | UK BSE epidemic mostly in Holstein Friesian dairy cattle; scrapie contamination of feed. Experimentally transmitted to mice (Fraser *et al.*, 1992), pigs (Dawson *et al.*, 1990), monkeys (Lasmezas *et al.*, 1996) | UK; Republic of Ireland; Europe[b] | Wells *et al.*, 1987 |
| Arabian Oryx[a] | | Infection in a zoo specimen | UK | Kirkwood *et al.*, 1990 |
| Nyala[a] | | Infection in a zoo specimen | UK | Jeffrey and Wells, 1988; HMSO, 1987 |
| Greater Kudu[a] | | Infection in a zoo specimen | UK | Kirkwood *et al.*, 1990 |
| Eland[a] | | Infection in a zoo specimen | UK | Fleetwood and Furley, 1990 |
| Gemsbok[a] | | Infection in a zoo specimen | UK | Jeffrey and Wells, 1988 |
| Domestic cat[a] | | Infection in domestic cats. Transmitted to mice (Fraser *et al.*, 1994) | UK, Norway | Wyatt *et al.*, 1993; |
| Cheetah[a] | | Infection in a zoo specimen | UK | Bratberg *et al.*, 1995, |
| Puma[a] | | Infection in a zoo specimen | UK | Willoughby *et al.*, 1992 |
| Human | Creutzfeldt–Jakob Disease (*s*CJD) | Sporadic: $10^{-6}$ incidence; not associated with prion protein (PrP) mutations. Experimentally transmitted to chimpanzees, monkeys, cats and mice | Worldwide | Creutzfeldt, 1989; Jakob, 1989 |
| | *i*CJD | Iatrogenic: transmission following neurosurgery, corneal transplant and dura mater graft | Worldwide | Gadjusek, 1985; Buchanan *et al.*, 1991; Lane *et al.*, 1994 |
| | *f*CJD | Familial: linked to mutations in prion protein (PrP); autosomal dominant, some have variable penetrance | Worldwide | for review, see Prusiner, 1994 |
| | Gerstmann–Scheinker–Straussler Syndrome | Familial disorder tightly linked to mutations in the prion protein (PrP) | Worldwide | Gerstmann *et al.*, 1936; Hsaio *et al.*, 1989 |
| | Fatal familial insomnia | Familial disorder linked to germline mutation in prion protein (PrP) in association with a common PrP polymorphism | Worldwide | For review see Gambetti *et al.*, 1993 |
| | Kuru | Sporadic CJD spread and maintained by cannibalism; endemic to a remote highland region of Papua New Guinea. Experimentally transmitted to goats, primates and rodents | Papua New Guinea | Gadjusek and Zigas, 1957; Gadjusek, 1985 |
| | CJD variant (nvCJD) | Unusual CJD variant in UK (10 cases) and France (1 case) with early onset (<42 years), unusual clinical presentation and neuropathology. Transmissibility to rodents not yet demonstrated | UK; France | Will *et al.*, 1996; Chazot *et al.*, 1996 |

[a]Infection associated with UK bovine spongiform encephalopathy (BSE) epidemic.
[b]BSE cases in Switzerland, Portugal, Germany, Italy, Oman and Canada are associated with imports of UK derived feed and cattle.
*i* = iatrogenic; *s* = sporadic; *f* = familial.

inoculation by a variety of routes, is characterized by a long asymptomatic pre-clinical phase, which is followed by a short clinical illness and death. An intriguing feature of scrapie is that of the long incubation time and precise timing of disease onset: if inbred mice are given the same dose of agent by the same route, all die within days of each other following a prolonged incubation period of up to 1 year or longer (Scott, 1993). This has enabled the estimation of agent titre by incubation time interval assay (Prusiner *et al.*, 1982b) and by endpoint titration (Dougherty, 1964) in mice and hamsters. There is some evidence that this precise timing can be a feature of human prion disease; analysis of Kuru victims, whose illness is attributable to participation in a single cannibalistic funeral rite, has shown that such individuals can develop kuru within months of each other following an incubation period of 25–30 years (Klitzman *et al.*, 1984).

### Agent propagation

Following experimental inoculation with a small initial dose, the agent can propagate to very high titres, well in excess of the original inoculum. The distribution of infectious material is determined by scrapie agent strain and inoculation route. Following i.p. inoculation the agent first replicates in the local lymph nodes and spleen, then spreads to the CNS from the spleen via the splanic nerve and spinal cord (Elkund *et al.*, 1967). Pathogenesis following intracranial inoculation usually results in replication first in the CNS

**Table II.** Human familial prion diseases associated with prion protein mutations

| Disease | Mutation | Onset/duration (years) | Clinical features | Neuropathology | Reference |
|---|---|---|---|---|---|
| *f*CJD | 120 bp insertion | 31–45/5–15 | Dementia with myoclonus | Spongiosis, neuronal loss and reactive gliosis | Goldfarb *et al.*, 1991a |
| *f*CJD | 144 bp insertion | 22–52/1–13 | Early personality changes progressing to dementia with ataxia and myoclonus | Widespread atrophy with PrP amyloid plaques | Poulter *et al.*, 1992; Collinge *et al.*, 1992 |
| *f*CJD | 168 bp insertion | 23–35/>10 | Similar to 144 bp insertion | Widespread atrophy with spongiosis | Brown *et al.*, 1992; Goldfarb *et al.*, 1991a |
| *f*CJD | 196 bp insertion | 35–54/3 months–13 year | Abnormal behaviour, ataxia, mutism, myoclonus | Widespread atrophy with mild spongiosis. PrP amyloid plaques in cerebrum and cerebellum | Owen *et al.*, 1992 |
| *f*CJD | 216 bp insertion | 55/2 | Single patient with ataxia, dementia and myoclonus | | Goldfarb *et al.*, 1992 |
| GSS Ataxic form | 102 Pro–Leu | 15–40/2–10 | Cerebellar ataxia, extra/ pyramidal signs with late onset dementia | PrP amyloid plaques in cerebral and cerebellar cortex, also in white matter of basal ganglia and thalamus | Hsaio *et al.*, 1989 |
| GSS | 105 Pro–Leu | 42/9 | Clumsiness, gait disturbance, decline in intellectual function | | Kitamoto *et al.*, 1993 |
| GSS | 117Ala–Val | 35/– | | Dementing form (telancephalic GSS): no cerebellar pathology. Ataxic form: widespread cerebellar pathology with PrP amyloid plaques | Dohura *et al.*, 1989; Mastrianni *et al.*, 1995 |
| Atypical Prion Disease | 145Tyr–stop [amber mutation] | 38 yr/death at 59 years | Slowly progressive Alzheimer's-like disease in a single Japanese woman | Numerous Kuru plaques and NFT* but little spongiform change. Plaques contained only truncated PrP derived from the mutant allele | Kitamoto *et al.*, 1993 |
| *f*CJD | 178Arg–Asn/129Val haplotype | 26–56/9–51 months | Similar to sCJD | Spongiosis of cerebral cortex, little thalamic pathology | Goldfarb *et al.*, 1991b |
| FFI, Fatal Familial Insomnia | 178Arg–Asn/129 Met haplotype | 20–71/6–32 months | Untreatable insomnia, hallucinations, dysautonomia, ataxia, myoclonus, extra/ pyramidal signs. Endocrine abnormalities | Minimal cortical spongiosis. Selective thalamic atrophy; neuronal loss and gliosis in inferior olive | Goldfarb *et al.*, 1992; Gambetti *et al.*, 1993 |
| CJD | 180Val–Ile | | Similar to sCJD | Similar to sCJD | Kitamoto *et al.*, 1993 |
| GSS with NFT* | 198Phe–Ser | 34–71/3–11 months | Similar to codon 102 mutation but with early dementia | Similar to codon 102 mutation but with widespread Alzheimer's type NFT* in cerebral cortex | Hsaio *et al.*, 1992; Dloughy *et al.*, 1992 |
| *f*CJD | 200Gln–Lys | 35–66/2–41 months | Similar to sporadic CJD Penetrance estimated at 0.56 | Widespread spongiosis which extends to deep brain nuclei, cerebellum and brain stem. | Goldfarb *et al.*, 1990; Goldfarb *et al.*, 1991c |
| *f*CJD | 210Val–Ile | | | Incomplete penetrance. | Pocchiari *et al.*, 1993 |
| GSS | 217Glu–Arg | | | PrP amyloid plaques and occasional neocortical NFT* | Ikeda *et al.*, 1991 |
| *f*CJD | 232Met–Arg and 180Val–Ile | Two Japanese cases with typical sCJD presentation | Typical sCJD pathology. | | Kitamoto *et al.*, 1993 |

*Neurofibrillary tangles.

and later in other organs, although there are examples of strains which exhibit very little non-CNS replication until the clinical stages of disease (Farquar *et al.*, 1996). A significant *PrP* gene dosage effect upon the rate (but not the distribution) of PrP accumulation has also been noted in transgenic mice (see below).

### *There are multiple strains of scrapie*

There is heterogeneity in the clinical presentation of natural scrapie in sheep and this has been attributed to the action of distinct scrapie variants or strains (Pattison and Millson, 1960), for example an isolate of sheep scrapie passaged in goats gave rise to two distinct clinical syndromes called 'nervous' and 'itching' which could be serially passaged in goats, retaining their distinct biological properties (Pattison, 1960). Transmission of sheep and goat scrapie to mice and hamsters has enabled the investigation and description of strain-specific differences in incubation time and neuropathology; the main characteristics used to differentiate scrapie strains.

Multiple strains of scrapie have been isolated, indeed field sheep scrapie isolates have been shown to contain mixtures of strains (Dickinson, 1976). More than 20 mouse and hamster-adapted scrapie strains have been catalogued, some of which have been cloned by several passages at high dilution (Bruce, 1993). Different isolates of human CJD brain material also give rise to distinct incubation times and pathology in inbred mice (Kitamoto *et al.*, 1990), suggesting that different CJD strains exist within the human population.

There is evidence that strain properties can be maintained irrespective of the species (and therefore PrP sequence) through which the agent is passaged (Kimberlin *et al.*, 1989) and such phenomena are cited to support the hypothesis that the scrapie agent carries a nucleic acid genome (Bruce, 1993). In contrast there are examples of dramatic alterations in strain properties (or 'mutation') when scrapie is passaged between species or between mice with different PrP alleles (host-dependent alterations). Paradoxically, this 'mutability' is also considered by some workers to be strong evidence that the scrapie agent has a nucleic acid genome. However there is no direct evidence for a nucleic acid component in the agent despite several decades of searching (for review, see Liberski, 1994). Indeed, artificial scrapie strains with altered host ranges, incubation time and neuropathology can be generated in mice with engineered PrP transgenes (Scott *et al.*, 1993). Despite the undoubted role of PrP sequence differences in the generation and maintenance of strain diversity there remains sound evidence that distinct scrapie strains can be stably maintained in hosts with identical PrP sequences (Bruce *et al.*, 1991; Kascsak *et al.*, 1991; Hecker *et al.*, 1992) and that strains with dramatically different host ranges (Kimberlin and Walker, 1978) can arise from a single host species. This evidence, together with the insights gained from the use of transgenic mice are discussed later in this review.

## The biophysical properties of the agent

### *Resistance to inactivation*

One of the earliest indications that the scrapie agent had unusual physical properties followed the infection of a large number of sheep which had received louping-ill vaccine, which was raised in a flock subsequently found to have scrapie. The contaminated vaccine retained infectivity despite prolonged exposure to formalin (Gordon *et al.*, 1940), and it is now known that formalin pre-treatment can actually render the agent less susceptible to complete inactivation by autoclaving (Bell and Ironside, 1993). Subsequently, resistance has been demonstrated to boiling (Brown *et al.*, 1982), $UV_{260}$ nm (Alper *et al.*, 1967), ashing at 360°C (Brown *et al.*, 1990a) and a wide range of biocidal chemicals (reviewed by Gadjusek, 1985). However, efficient agent inactivation can be achieved by phenol extraction, sodium hypochlorite and sodium hydroxide (Brown *et al.*, 1990b). The resistance to $UV_{260nm}$ is good evidence that the agent is not reliant upon a nucleic acid moitey (Alper *et al.*, 1967; Bellinger-Kawahara *et al.*, 1987).

### *Estimates of agent size*

The first estimates of agent size demonstrated that it could pass though bacterial filters (Wilson *et al.*, 1950), suggesting viral dimensions. However, controversy still surrounds the issue of the size of the agent, because it is held that a small agent of sub-viral size might support a protein-only model of the agent. Various size estimates exist and range from 50–150 kDa, being based upon a number of methods. The agent size has been calculated based upon its inactivation by ionizing radiation (Lea, 1955) and this experiment has been repeated 13 times since it was first performed by Alper *et al.* (1966), with some investigators contending that the agent is within the range of small viruses (Rohwer, 1991), whilst others hold that it is of sub-viral size (Bellinger-Kawahara *et al.*, 1987).

## The prion protein

### *The discovery of the prion protein*

Rapid progress followed the discovery of unique scrapie-associated fibrils (SAF) (Merz *et al.*, 1981) and prion rods in infected brains (Prusiner *et al.*, 1982a). SAF are composed of paired helical filaments of 100–1000 nm in length and are quite distinct in ultrastructure from prion rods which are heterogeneous 100–200 nm long flattened amyloid rods (Bolton *et al.*, 1982). Prusiner *et al.* (1982a) enriched the scrapie agent 100–1000-fold and found a protease-resistant protein (PrP) of 27–30 kDa, co-purifying with infectious fractions. Prolonged proteinase K digestion was found to reduce the agent titre and this was mirrored by the digestion kinetics of PrP27-30, suggesting that this protein was a component of the infectious agent (McKinley *et al.*, 1983). Further analysis of prion rods (Prusiner *et al.*, 1983) and SAF (Hope *et al.*, 1986) demonstrated that PrP27-30 was a major component of these structures and the in-vivo relevance of PrP27-30 was demonstrated by the observation that antisera raised against PrP 27-30 stained amyloid plaques in the brain of infected animals (DeArmond *et al.*, 1985).

Purification of hamster PrP27-30 to near homogeneity by detergent extraction, proteinase digestion and sucrose density gradient sedimentation enabled the *N*-terminal peptide sequence of PrP 27-30 to be determined (Prusiner *et al.*, 1984). This led to the cloning of a 2.1 kb PrP cDNA from scrapie-infected hamster brain (Oesch *et al.*, 1985) and a 2.5 kb cDNA from a scrapie-infected mouse brain (Chesebro *et al.*, 1985). It was subsequently found that both infected and uninfected animals have an endogenous gene encoding PrP27-30 (Basler *et al.*, 1986). This demonstrated that PrP 27-30 is not the gene product of a conventional viral pathogen but is the product of an endogenous gene. PrP 27-30 was found to be derived from the full length form of PrP (33–35 kDa) by partial proteolysis during purification. Thus it became clear that both infected and uninfected animals contained a full length PrP but that PrP from diseased animals was resistant to protease digestion, whilst PrP from healthy animals was completely hydrolysed by mild protease digestion. Normal PrP was termed $PrP^{c}$ and the infection specific-isoform was denoted by $PrP^{sc}$. $PrP^{sc}$ clearly accumulates in the brains of infected animals, but PrP

mRNA levels do not change throughout the course of scrapie infection (Oesch *et al.*, 1985), suggesting that the accumulation of $PrP^{sc}$ during disease does not occur as a consequence of raised rates of transcription or alterations to mRNA levels, but from increased protein stability. This has been corroborated by in-vitro analysis of prion protein metabolism in scrapie-infected mouse neuroblastoma cells, which shows that, whilst $PrP^{c}$ has a half life of ~5 h, $PrP^{sc}$ has a much longer half-life (~15 h) (Borchelt *et al.*, 1990) and accumulates within the cytoplasm (Taraboulos *et al.*, 1990).

### *The prion protein, PrP*

$PrP^{c}$ is a cell surface glycoprotein expressed at highest levels in the brain and at lower levels in a number of non-neural tissues (Oesch *et al.*, 1985), including heart, lung and kidney. The peptide backbone in all mammalian $PrP^{c}$ molecules studied so far is 252–254 amino acids long and is modified by the removal of a 22 amino acid *N*-terminal signal sequence, following translocation into the endoplasmic reticulum, where it is modified by the addition of complex *N*-linked polysaccharides at two asparagine residues and a single intrachain disulphide bond (Turk *et al.*, 1988), in addition to a sialated glycosylinositol phospholipid (GPI) anchor C-terminal (Stahl *et al.*, 1992), to enable attachment of $PrP^{c}$ to the cell surface (Stahl *et al.*, 1987).

Clearly the difference between $PrP^{c}$ and $PrP^{sc}$ is critical to the understanding of prion diseases. These PrP isoforms differ with respect to resistance to protease digestion and $PrP^{sc}$ differs with respect to a number of biophysical properties such as solubility. However $PrP^{sc}$ does not differ from $PrP^{c}$ by any known chemical post translational modification or amino acid sequence (Turk *et al.*, 1988; Stahl *et al.*, 1993). Other modifications such as protein splicing or editing have also been discounted (Stahl *et al.*, 1993). However, there is good evidence that $PrP^{c}$ and $PrP^{sc}$ differ in secondary and tertiary structure. Purification of $PrP^{c}$ and $PrP^{sc}$ under non-denaturing conditions has enabled the structural comparison of normal and disease-specific PrP33-35 (i.e. full length $PrP^{sc}$) isoforms by circular dichroism and infrared spectroscopy. Both methods of analysis demonstrate that, whilst $PrP^{c}$ has low levels of β-sheet and high levels of α-helix, $PrP^{sc}$ is quite different in that it has lowered levels of α-helix and high levels of β-sheet (Pan *et al.*, 1993) and there is evidence that $PrP^{sc}$ is an aggregated folding intermediate (Safar *et al.*, 1994).These observations support the notion that conformational change is a fundamental event in the conversion of $PrP^{c}$ to $PrP^{sc}$.

### *Genetic evidence for the importance of the prion protein*

Although the role of genetics in the susceptibility to field scrapie in sheep had been recognized for some time, the transmission of scrapie to mice (Chandler, 1961) resulted in the discovery of a single autosomal gene, *Sinc*. Two alleles of *Sinc* exist, $s^{7}$ and $p^{7}$. Mice homozygous for these alleles have short and prolonged scrapie incubation times respectively with scrapie strain ME7 (Dickinson and Mackay, 1964; Dickinson *et al.*, 1968), whilst heterozygotes have an intermediate incubation time. *Sinc* alleles also control incubation times with other scrapie strains, BSE and CJD isolates. However, the fact that the incubation time rankings of animals homozygous and heterozygous for different *Sinc* alleles can be reversed with some strains (e.g. 22A and 301V) suggests that the relationship between *Sinc* genotype and incubation time is not simple (Bruce *et al.*, 1991; Bruce, 1993). There is good evidence that the *Sinc* gene and the prion protein gene in mice are tightly linked (Carlson *et al.*, 1986, 1993; Hunter *et al.*, 1987) and may be coincident. PrP is also linked to the sheep *Sinc* homologue, *Sip* (Hunter *et al.*, 1989). Mice, hamsters and sheep with different experimental scrapie incubation times encode distinct prion proteins (Westaway *et al.*, 1987; Lowenstein *et al.*, 1990; Goldmann *et al.*, 1994). Mice with different scrapie incubation times encode different PrP proteins (PrP-A and PrP-B), which differ with respect to codon 108 and 189 dimorphisms (Westaway *et al.*, 1987). However, the formal demonstration that Sinc and PrP are identical and that these dimorphisms control incubation time is lacking. We are currently conducting scrapie challenge experiments on inbred Sinc $s^{7}$ 129/Ola mice (Moore *et al.*, 1995), in which the codon 108/189 dimorphisms have been altered by gene targeting. This may formally resolve the issue of *Sinc*/PrP identity and determine the relative role of codons 108 and 189 in incubation time control.

A common human prion protein codon 129 methionine/valine polymorphism appears to influence susceptibility to sporadic and iatrogenic CJD (Palmer *et al.*, 1991). Further evidence for the importance of the prion protein is provided by the existence of a number of human prion protein mutations which are linked to familial prion disease (See Table II). Differences in the frequency of field scrapie in sheep are also associated with common PrP gene polymorphisms (Goldmann *et al.*, 1994). It is likely that some sheep polymorphisms behave like the human codon 129 met/val polymorphism and influence susceptibility to exogenous infectious agent. It is also possible that some sheep polymorphisms may be similar to the PrP mutations found in human familial CJD kindreds, i.e. some sheep PrP polymorphisms may give rise to sheep scrapie spontaneously in the absence of exogenous agent.

## The transgenic mouse as an experimental model of prion diseases

The remainder of this review deals with the enormous contribution that mouse transgenesis has made to prion disease research. Transgenic mice expressing a variety of PrP genes have been generated by both conventional transgenesis (pronuclear injection) and gene targeting. The relative merits of each method of transgenesis have been elegantly exploited. For example, conventional transgenesis has been used to investigate scrapie incubation times in mice with different wild type hamster PrP gene copy numbers and therefore different PrP expression levels. This has identified the importance of gene dosage effects in the prion diseases. Gene targeting has been used to make PrP-ablated mice, which have demonstrated the absolute requirement of PrP expression to confer susceptibility to prion diseases and to enable prion propagation (Bueler *et al.*, 1992, 1993; Prusiner *et al.*, 1993). The use of gene

targeting to produce mice with point mutations in the absence of overexpression also promises to be very useful (Moore *et al.*, 1995). Future experiments will enable the precise and controlled analysis of the effects of engineered PrP structural alterations on all features of the prion diseases.

### *The species barrier effect arises from PrP amino acid sequence differences between species*

Transmission of scrapie between species can be very inefficient: typically the incubation time for the first passage is long and there may be survivors, but upon subsequent serial passage in the new species the incubation time shortens and stabilizes (Pattison and Jones, 1968; Kimberlin *et al.*, 1989). The reason for the species barrier was unclear for some time, but it was apparent that transmission between closely related species such as Armenian, Syrian and golden hamsters is more easily accomplished than between more distantly related species. Minor differences in the prion protein sequence between these hamster species (Lowenstein *et al.*, 1990) suggested that the species barrier is caused by differences between prion protein sequence. Syrian hamster scrapie strain Sc237 was used to investigate the hamster to mouse species barrier as it causes scrapie in ~ 77 days in Syrian hamsters, but has low pathogenicity for mice which have an incubation time in excess of 600 days (Marsh and Kimberlin, 1975). This effect was thought to be a property of the 16 differences in primary amino acid sequence between mouse and hamster $PrP^c$ and a number of transgenic mouse lines bearing the Syrian hamster PrP gene [*SHaPrP*] were constructed and challenged with hamster scrapie Sc237 to determine whether they became more susceptible to hamster scrapie (Scott *et al.*, 1989). These mice had dramatically accelerated incubation times (Figure 1) and exhibited neuropathology characteristic of hamster Sc237 scrapie, which demonstrated that the species barrier was a manifestation of prion protein gene differences between species.

### *Isologous host–inoculum interactions are favoured over heterologous interactions*

All of the mice used in the above challenges expressed endogenous mouse $PrP^c$ in addition to transgenic hamster $PrP^c$, but produced much larger quantities of hamster $PrP^{sc}$ than mouse $PrP^{sc}$ and also produced much higher levels of hamster prions than mouse prions following Sc237 challenge. However, if these transgenic mice were challenged with a mouse scrapie strain, the transgenic mice developed mouse scrapie and produced mouse $PrP^{sc}$ and prions which were more infectious to mice than hamsters despite expressing much more hamster $PrP^c$ than mouse $PrP^c$ (Prusiner *et al.*, 1990). This was determined by immunoblotting with hamster and mouse- specific monoclonal PrP antisera and was corroborated by scrapie bioassay in non-transgenic mice, HaPrP transgenic mice and hamsters. This showed that these mice could propagate both mouse and hamster prions, but that the source of the scrapie inoculum determined whether mouse or hamster $PrP^{sc}$ and infectivity was generated. This suggests a direct interaction between $PrP^{sc}$ in the inoculum and $PrP^c$ in the host animal, i.e. homologous interactions between $PrP^{sc}$ and $PrP^c$ are

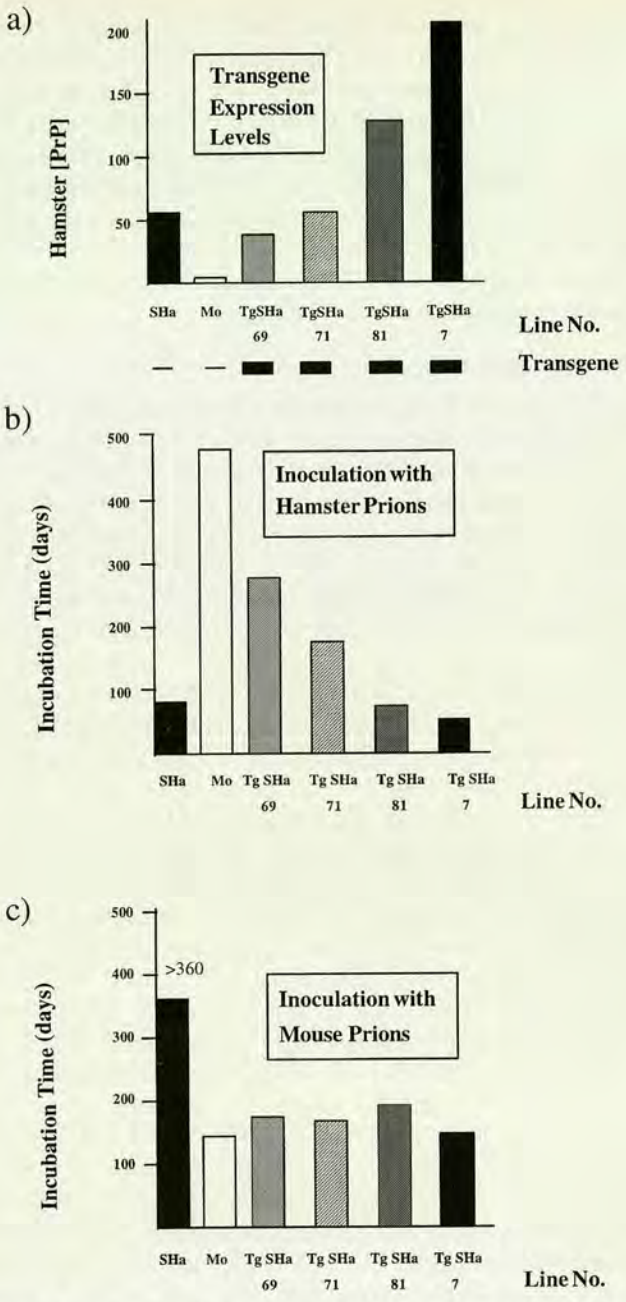


**Figure 1.** Transgenic analysis of the mouse–hamster species barrier. (**a**) Brain levels of hamster host-encoded glycoprotein ($PrP^c$) in Syrian hamsters, non-transgenic mice and a variety of transgenic lines expressing different levels of hamster $PrP^c$. Syrian hamster = SHa; Mouse = Mo; transgenic mouse bearing Syrian hamster PrP transgenes = TgSHa. PrP levels expressed as μg/g brain protein. The relative position of each group is maintained throughout Figure 1a,b,c (adapted from Scott *et al.*, 1989 and Prusiner *et al.*, 1990). (**b**) Hamster scrapie Sc237 challenge of hamsters, mice and transgenic lines. Syrian hamsters have a rapid incubation time of ~77 days, whilst non-transgenic mice are resistant to infection. All transgenic lines are rendered susceptible to hamster scrapie, with an incubation time inversely related to hamster $PrP^c$ expression level. (**c**) Mouse scrapie challenge of hamsters, mice and transgenic lines. Syrian hamsters are resistant to mouse prions, whilst non-transgenic mice and transgenic lines readily succumb to mouse scrapie.

favoured over heterologous interactions. It is also notable that, following inoculation with a mouse passaged scrapie strain incubation times were sometimes slightly prolonged in mice which also expressed hamster PrP (see Figure 1c). One explanation for this is that non-productive interactions between transgene-encoded hamster $PrP^c$ and inoculum-derived mouse $PrP^{sc}$ slightly reduce the effective dose of mouse $PrP^{sc}$ in the inoculum available for isologous interactions with endogenous $PrP^c$ i.e. an excess of heterologous hamster $PrP^c$ molecules can retard the disease process.

### *Prion protein expression levels influence scrapie incubation time*

Transgenic mice bearing hamster PrP transgenes also illustrate that incubation times are influenced by PrP expression levels. Examination of Figure 1a and b shows that scrapie incubation time is inversely proportional to the PrP transgene expression level, thus mice with the highest hamster $PrP^c$ expression levels have a shorter scrapie incubation period than mice expressing lower levels. This demonstrates a gene dosage effect on incubation time and a similar effect is observed in mice overexpressing a mouse PrP-B allele transgene (Westaway *et al.*, 1991) and a PrP-A transgene (Carlson *et al.*, 1994; Fischer *et al.*, 1996; Telling *et al.*, 1996) in which the scrapie incubation time is accelerated. A PrP gene dosage effect is also present in mice with reduced PrP expression levels. PrP null mice express no PrP and are not susceptible to scrapie, whilst heterozygous null mice, expressing only one functional PrP allele and producing ~50% $PrP^c$ levels, have a prolonged incubation time with respect to wild-type littermates (Bueler *et al.*, 1993; Prusiner *et al.*, 1993; Manson *et al.*, 1995).

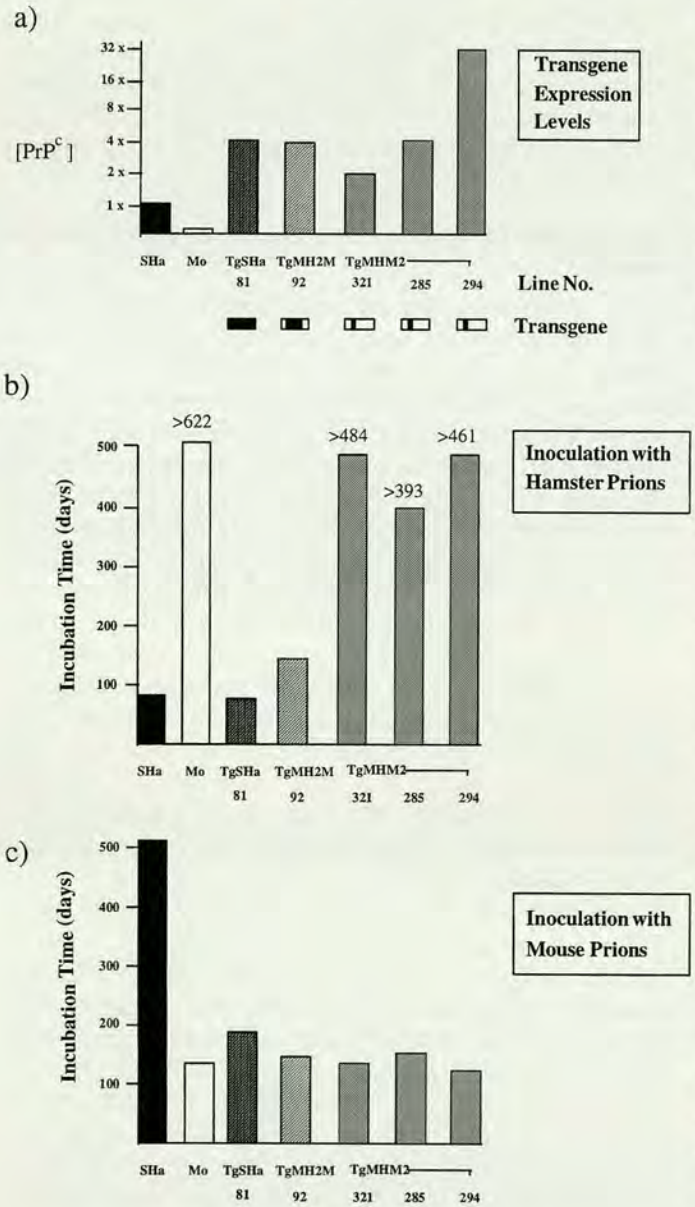


**Figure 2.** The hamster–mouse species barrier is controlled by the central region of PrP (**a**) Transgene expression levels in mice bearing full length hamster and chimeric mouse-hamster PrP transgenes; all expression levels are relative to hamster brain ($PrP^c$)-expression levels. All mice have endogenous mouse $PrP^c$ in addition to transgenes (when present). The type of transgene is indicated below the transgenic line number by a rectangle: filled rectangles represent full length hamster PrP; open rectangles represent wild-type mouse $PrP^c$ and partially filled rectangles represent chimeric mouse/hamster. Tg(MH2M) has five amino acid alterations whilst MHM2 has two alterations (adapted from Scott *et al.*, 1993). (**b**) Hamster scrapie Sc237 challenge of hamsters, wild-type mice and transgenic lines shown in Figure 1a. Syrian hamsters have a characteristically short incubation time whilst non-transgenic mice are resistant to Sc237. Mice with full length hamster transgenes are those shown in Figure 1. Mice with larger hamster regions within the chimeric transgenes encode five hamster amino acid alterations and are susceptible to Sc237 whilst mice with only two hamster alterations [Tg(MHM2)] remain resistant (adapted from Scott *et al.*, 1993). (**c**) Mouse scrapie challenge of hamsters, wild-type mice and transgenic lines. Despite overexpression of chimeric $PrP^c$ in MH2M and MHM2 lines, which have five and two amino acid alterations respectively, the mouse scrapie incubation time is not shortened. This suggests that these alterations have a dramatic effect upon the ability of the chimeric $PrP^c$ to be converted to chimeric pathogenic protease resistant isoform ($PrP^{sc}$) by mouse prions (adapted from Scott *et al.*, 1993).

### *Overexpression of wild-type PrP can be pathogenic*

A number of lines of transgenic mice, bearing high copy number wild-type PrP cosmid transgenes from a number of mammalian species, develop a late onset neurological disorder characterized by spongiform changes in the CNS, a demyelinating neuropathy of the peripheral nervous system and muscular pathology (Westaway *et al.*, 1994b). The myopathy was limited to skeletal muscle groups including diaphragm and intercostal muscles and appeared before PNS demyelination, suggesting that PrP overexpression gives rise to a primary myopathy. CNS pathology included mild focal spongiform change in a number of brain regions. This pathology is similar in some respects to experimental scrapie but differed as follows: the pathology was milder than is generally seen in scrapie, the course was extended and no $PrP^{sc}$ was detected. The absence of $PrP^{sc}$ excludes the simple explanation of scrapie contamination. These observations imply that overexpression of wild-type PrP is sufficient for scrapie-like neuropathology and preliminary transmission experiments indicate that brain material from a number of clinically ill transgenic mice can transmit a scrapie-

like disorder with low efficiency to some hosts. This together, with the transmission from mice overexpressing a GSS101$^{Leu}$ mutant PrP (Hsaio *et al.*, 1990, 1994; Telling *et al.*, 1996) also suggests that not all prions are proteinase K resistant and constitutes further evidence for the de-novo generation of infectious agent in the absence of exogenous prions, good evidence for a protein-only model of the transmissible spongiform encephalopathies (McKinley *et al.*, 1983).

Fischer *et al.* (1996) have also observed a similar phenotype in mice overexpressing PrP-B from a Prn-p$^b$ cosmid transgene, but this phenotype is not seen in two independent transgenic lines which overexpress PrP-A at high levels (Fischer *et al.*, 1996; Telling *et al.*, 1996). This suggests that PrP-B and PrP-A, which differ at codons 108 and 189, have quite distinct properties.

Of course it remains formally possible that the phenotype associated with PrP overexpression is actually caused by overexpression of non-PrP sequences within the cosmid transgenes. It is interesting to note in this regard that both PrP-B expressing lines which develop a late onset phenotype were constructed from the Prn-p$^b$ transgene derived from ILn/J mice whilst the two published examples of absence of pathology in mice overexpressing PrP-A were in high copy number lines constructed with vectors differing in some way from the PrP-B lines: Fischer *et al.* (1996) used a transgene derived from Prn-p$^b$ Cos 6.ILn/J-4 but differed from all those used by Westaway *et al.* (1994b) and the PrP-B overexpressing line because ~12 Kb of intron 2 sequence was removed (within which there may be a Purkinje neuron-specific enhancer element, see Fischer *et al.*, 1996) and Telling *et al.* (1996) used a Syrian hamster PrP transgene (cosSHa.Tet) modified by insertion of the PrP-A ORF. However, modifying effects of the vectors cannot account for the overexpression phenotype because the cosSha.Tet vector was also used for the construction of the high copy number hamster transgenic lines which did develop a late onset phenotype.

The observation of pathology in overexpressing mouse lines raises the intriguing issue of whether some sporadic CJD cases in humans occur as a result of PrP overexpression and whether there is variation within the human population for PrP expression levels. It seems reasonable to conclude that, given the consequences of overexpression in the mouse, humans expressing the prion protein at high levels might constitute a group which are particularly susceptible to environmental prion contamination or to sporadic illness. PrP$^c$ has been detected in the neuromuscular junctions of normal individuals (Askanas *et al.*, 1993) and has been shown to accumulate in the muscles of some individuals with sporadic inclusion–body myositis, hereditary inclusion–body myopathy (Sarkozi *et al.*, 1994) and GSS patients with neuromyopathic changes (Rosenthal *et al.*, 1976; Kretschmar *et al.*, 1992). However, the relevance of PrP in muscles remains unknown as Prn-p$^{-/-}$ mice have normal muscle electrical excitability (Brenner *et al.*, 1992).

### *Chimaeric hamster/mouse prion protein transgenes generate new scrapie strains*

A higher resolution analysis of the hamster-mouse species barrier effect examined the role of five of the 16 amino acid differences between hamsters and mice (Scott *et al.*, 1993). Transgenic mice, bearing chimeric hamster–mouse prion protein transgenes with a variety of hamster-specific amino substitutions, were generated (Figure 2) and this demonstrated that changing five mouse codons (Tg MH2M line: 108/111/138/154/169) to those encoded by the hamster is sufficient to render mice susceptible to hamster prions. Mice with only two hamster substitutions (Tg MHM2 line: 108/111) remain resistant to hamster prions, suggesting that differences at the remaining three of the five codons (138/154/169) are responsible for the majority of the species barrier between mouse and hamster. Mice overexpressing the complete hamster transgene at a similar level [Tg SHaPrP81] develop illness much earlier following inoculation with the same scrapie strain, suggesting that, whilst these amino acid alterations render the mouse susceptible to hamster prions, they synthesize hamster prions somewhat less efficiently than mice bearing the complete hamster sequence.

There is strong evidence that TgMH2M mice (i.e. Tg mice with five hamster substitutions) generate a new scrapie strain (MH2M prions) which has an artificial host range, being infectious to hamsters, Tg(MH2M) mice and wild-type mice. This suggests that it is possible to generate an artificial scrapie strain by manipulating the PrP amino acid sequence, providing evidence that differences in scrapie strains may be a property of PrP structural variation.

### *What have transgenic mice taught us about scrapie strains?*

An interesting but rather neglected property of strains is that the degree of species barrier can differ markedly between scrapie strains (Kimberlin and Walker, 1978). Work with hamsters and some transgenic work has brought this issue into focus and suggests that there are determinants of prion biology other than primary PrP amino acid sequence. If one considers transgenic work on the Sc237 hamster–mouse species barrier it is clear that the barrier effect is due to non-homologies between prion donor and recipient PrP sequences (Scott *et al.*, 1989; Prusiner *et al.*, 1990). However, there is evidence that this is not always the case; Kimberlin and Walker (1978) isolated three scrapie strains in golden hamsters, of which two are very interesting in this regard, 263 K and 431K. 263K was highly infectious to hamsters and propagated to very high titres (incubation time of 60–70 days) but mice were very resistant to this strain, even after observation for 600 days (Marsh and Kimberlin, 1975). However 431K, which was also isolated from golden hamsters, transmitted with relative ease to both hamsters and mice.

Work with Sc237 and mice with chimeric hamster–mouse PrP genes indicates that the majority of the species barrier effect is mediated by three critical PrP residues. One might suggest that the absence of a dramatic species barrier effect between hamsters and mice with 431K may be because the conformation of 431K prions is such that the side chains of these three residues are not involved in (or have a minor importance in) the interaction between hamster 431K PrP$^{sc}$ and mouse PrP$^c$. This suggests that the species barrier and perhaps incubation time may be determined by the interplay

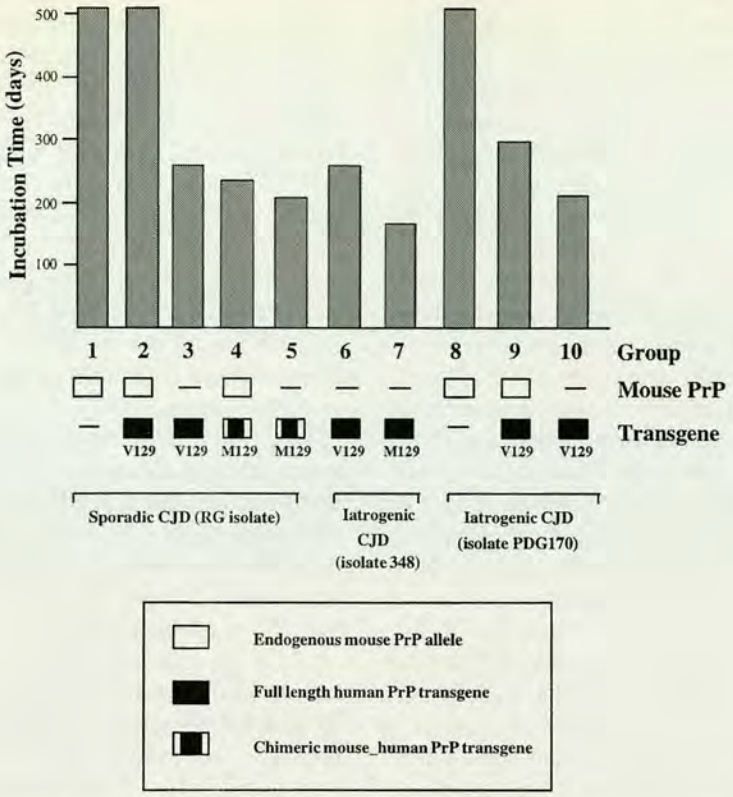


**Figure 3.** The conflicting evidence for Protein-X. A variety of wild-type and transgenic lines of mice, with and without endogenous mouse host-encoded glycoprotein ($PrP^c$), are represented by 10 challenge groups. Groups 1–5 represent mice inoculated with sporadic Creutzfeldt–Jakob disease (CJD; case RG, a codon 129 methionine homozygote). Groups 6 and 7 are challenges with iatrogenic CJD (case 348, a codon 129 methionine homozygote). Groups 8–10 are challenges with an iatrogenic CJD isolate PDG170 (129 methionine/valine heterozygote). Groups 1 and 8 illustrate the resistance of wild-type mice to human CJD prions. The response of group 2 indicates that mice encoding a full length human transgene remain resistant to the RG isolate of CJD. However the incubation time of mice bearing the same transgene is dramatically accelerated by ablation of endogenous mouse *PrP* genes (group 3). This suggests that mouse $PrP^c$ retards the replication of human pathogenic protease-resistant isoform ($PrP^{sc}$) in the mouse. The essential regions of human PrP required to overcome the species barrier between the mouse and human are within the region delimited by codons 88–167 and is illustrated in groups 4 and 5. Comparison of groups 4 and 5 also demonstrates that endogenous mouse PrP no longer retards the propagation of human $PrP^{sc}$ if human PrP is limited to the central region between codons 88 and 167 by use of a mouse–human chimeric transgene. Group 6 illustrates that challenge with another CJD isolate yields similar incubation times in mice devoid of mouse PrP but comparison with group 7 (which express a human PrP transgene encoding methionine rather than valine at codon 129) shows that correct matching of the inoculum and host codon 129 met/val polymorphism can result in faster incubation times. Indeed, the incubation time in group 7 mice is ~100 days shorter despite expressing human PrP at only 25–50% of the levels of the other transgenic lines. This suggests that codon 129 mismatches between inoculum and host PrP significantly retard the propagation of human prions. The data presented in groups 8–10 is derived from challenges performed on the same transgenic mouse lines by Collinge *et al.* (1995) using a different CJD isolate. Mice encoding endogenous mouse PrP in addition to the human transgene (group 9) are susceptible to the iatrogenic CJD isolate PDG170. This is quite different from the response noted by Telling *et al.* with sporadic CJD isolate RG (group 2). This suggests that the effect attributed to Protein-X does not necessarily hold when challenged with a different CJD isolate, although there may be simple technical explanations for this discrepancy.

between both PrP primary structure and other properties of PrP, such as conformation.

### *The prion protein may interact with an unidentified protein: Protein-X*

Given that hamster transgenes overcome the species barrier between mouse and hamster (Scott *et al.*, 1989; Prusiner *et al.*, 1990), one might predict that a mouse expressing human PrP would be susceptible to human prions. A recent series of publications suggests that this is not necessarily the case (Telling *et al.*, 1994) unless the endogenous mouse gene is ablated by crossing the human transgene onto a murine PrP null background (Figure 3) (Telling *et al.*, 1995). This shows that endogenous mouse PrP can dramatically retard the propagation of human $PrP^{sc}$ in the mouse. The authors postulate the existence of a second protein, Protein-X, with which PrP must interact to propagate prions. If one assumes that interaction with Protein-X is essential for the $PrP^c$-$PrP^{sc}$ conversion and that mouse Protein-X interacts with mouse PrP with a much higher affinity than with human PrP, it is likely that endogenous mouse PrP will compete with human PrP for interaction with mouse Protein-X, and this will result in highly inefficient synthesis of human $PrP^{sc}$ in the mouse.

Mice bearing a chimeric mouse–human transgene have a rapid incubation time with CJD prions regardless of the presence of endogenous mouse PrP (compare Figure 3, groups 4 and 5). The chimeric transgene is based upon mouse PrP but has a central region encoding amino acids 96–167 replaced

by the corresponding region of human PrP. This suggests that it is likely that the region of interaction between PrP and Protein-X lies outside the region bearing the human PrP portion of the chimeric transgene. The predicted region of interaction between PrP and Protein-X is in the *C*-terminus. This is based upon two pieces of evidence: (i) the chimeric mouse-human transgenes contain only a central region of human PrP, but the propagation of chimeric prions is not retarded by endogenous mouse PrP; and (ii) it has been shown in scrapie-infected neuroblastoma cells (Rogers *et al.*, 1993) and transgenic mice (Fischer *et al.*, 1996) that large portions of the PrP *N*-terminus are dispensable for the $PrP^{c}$-$PrP^{sc}$ conversion event. The *C*-terminus between codons 215 to 230 contains a number of divergent amino acids, notably a region which is shared by all rodent sequences so far determined, but quite distinct from other mammalian PrP molecules. The conservation of this region between rodents might explain why the effect of Protein-X was not discovered during earlier work with the control of the species barrier between mouse and hamster, i.e. hamster and mouse PrP probably have a similar affinity for mouse Protein-X and thus the propagation of hamster $PrP^{sc}$ in the mouse is not retarded by competition with endogenous mouse PrP.

The postulated existence of Protein-X explains some otherwise confusing observations: Tg(MoPrP-P101L) mice carrying a full-length mouse PrP transgene, with a codon 101 point mutation homologous to the human PrP codon 102 mutation linked to GSS (Hsaio *et al.*, 1989, 1990), develop a spontaneous illness between 50–300 days but mice expressing a full-length human PrP transgene with the same mutation at similar levels do not develop a spontaneous disease after observation for >700 days (Telling *et al.*, 1995). The difference between the two transgenic lines is presumably due to the non-permissiveness of the mouse for the propagation of human $PrP^{sc}$ as described above. It would be predicted from this that the mice with the mutant human transgene will develop illness if the transgene is crossed onto a mouse PrP null background. This work is in progress (Telling *et al.*, 1995).

### Transgenic modelling of human susceptibility to BSE

Transmission of BSE to mice is well established (Fraser *et al.*, 1992; Bruce, 1993; Bruce *et al.*, 1993, 1994) and transgenic mice expressing human PrP at high and low levels have been challenged with BSE to investigate the species barrier between cows and humans to BSE. The preliminary results from this experiment suggest that mice expressing human $PrP^{129Val}$ are not more susceptible to BSE prions than non-transgenic mice (Collinge *et al.*, 1995). However, all transgenic mice used in this study also expressed endogenous mouse PrP, which has been shown to retard the propagation of human $PrP^{sc}$ in mice, at least under some circumstances (see legend to Figure 3) (Telling *et al.*, 1995). In this respect, the BSE challenge response of mice which express human $PrP^{c}$ without endogenous mouse $PrP^{c}$ should resolve this issue, although preliminary results suggest that Prn-$p^{-/-}$/$HuPrP^{129Val}$ mice are not dramatically more susceptible to BSE than mice which also express endogenous PrP.

It should be noted, however, that the codon 129 genotype of the BSE inoculum (cows are 129 met homozygotes) and the human transgene were not matched and this may reduce the efficiency of bovine $PrP^{sc}$–human $PrP^{c}$ interactions. One might predict that if the BSE challenge experiment were repeated using a transgenic mouse strain expressing the human $PrP^{129Met}$ allele, such as Tg (HuPrP440) (Telling *et al.*, 1995), the mice would have a shorter incubation time than mice expressing the $HuPrP^{129Val}$ transgene and provide a quite different picture about the human response to BSE prions. Regardless of the outcome of such work, it is important to remember that mice are not humans and that the consequences of exposure of the human population to BSE prions is difficult to predict (Hope, 1995). The emergence of a new variant of CJD, nvCJD (Will *et al.*, 1996), with a novel clinical and neuropathological phenotype together with the demonstration that macaques challenged with BSE have nvCJD-like neuropathology (Lasmezas *et al.*, 1996) argues persuasively that BSE can be transmitted to humans. $PrP^{sc}$ from BSE cattle brain and nvCJD brain has been shown to have similar sodium dodecyl sulphate–(SDS–PAGE) banding patterns (Collinge *et al.*, 1996) adding further weight to the evidence that nvCJD has arisen from BSE infection of humans. However, the value of SDS–PAGE banding patterns as a means of strain typing prions is controversial not only because very little is known of the diversity of SDS–PAGE banding patterns prevalent amongst CJD cases but because there are examples of strains with very different biological properties which have similar banding patterns in the same hosts (e.g. ME7 and 22L: Kascsak *et al.*, 1991).

### Mouse models of familial prion diseases

Pronuclear injection has been used to generate mice transgenic for the human PrP codon 102 proline to leucine substitution linked to ataxic GSS in humans (Hsaio *et al.*, 1989). Mice overexpressing a mutant mouse MoPrP101Leu transgene 8-fold (the homologous mutation is at codon 101 in mouse PrP) in addition to endogenous PrP-A develop a spontaneous neurologic disorder indistinguishable from scrapie with onset between 50–300 days (average ~196 days). Clinically ill mice had histopathological similarities with GSS including moderate astrocytic gliosis, spongiform degeneration of the cerebrum and brain stem but few PrP amyloid plaques. Brain material from clinically ill $PrP^{101Leu}$ mice transmitted a neurodegenerative disease, providing evidence that a PrP mutation is sufficient to programme the generation of prions *de novo* (Hsaio *et al.*, 1994) in the absence of exogenous agent. These mice transmit disease with low efficiency to both hamsters and low copy number lines of $PrP^{101Leu}$ transgenic mice (which do not normally develop a spontaneous disease). The failure to transmit to wild-type mice suggests that the codon 101 mutation generates a species barrier-like effect. It is possible that the clinical illness was not due to the specific effect of the codon 101 pro–leu mutation, but a result of PrP overexpression similar to that observed in mice overexpressing wild-type PrP-B (Westaway *et al.*, 1994b). The effect of PrP overexpression has recently been addressed and it appears that overexpression of PrP-A (Fischer *et al.*, 1996) at similar levels to the $PrP101^{Leu}$ mutation, in contrast to PrP-B (Westaway *et al.*, 1994b) is not

pathogenic. Breeding the $101^{Leu}$ transgene onto a PrP null background has revealed that endogenous PrP significantly modifies the behaviour of $PrP101^{Leu}$ (Telling *et al.*, 1996): PrP null mice hemizygous for the $101^{Leu}$ transgene have an earlier and more synchronous age of onset at 145 days (homozygotes have disease onset at ~85 days), have more amyloid plaques and transmit more reproducibly to low copy number $PrP^{101Leu}$ transgenic mice.

We have used gene targeting to construct codon $101^{Leu}$ mutant mice in which the mutant allele is under the control of the endogenous mouse PrP promoter in order that the expression of mutant $PrP^{101Leu}$ faithfully reflects that of wild-type PrP and allows the consequences of the mutation to be determined in the absence of overexpression. In contrast to mice which overexpress this mutation we do not observe any neurological illness at ~500 days (R.Moore, D.Melton and J.Manson, unpublished data), suggesting that overexpression of the mutant $PrP101^{Leu}$ is a prerequisite for illness in the mouse, an animal with a short lifetime. The requirement for overexpression to gain a phenotype has also been observed with attempts to model Alzheimer's disease in mice (Games *et al.*, 1995).

A PrP codon 200 glutamate to lysine mutation, responsible for a large number of familial CJD cases in Slovakia (Goldfarb *et al.*, 1990, 1991a,c), has also been introduced into mice as a modified mouse PrP-A transgene but only six of 20 mice developed illness by 744 (±31) days and none of them showed signs of scrapie-like pathology or detectable levels of $PrP^{sc}$ (Telling *et al.*, 1996). The reason for the lack of pathology is unclear but it would be informative to both breed this transgene onto a PrP null background and onto a variety of mouse genetic backgrounds as this can have a significant effect upon the phenotype of neurological disorders in transgenic mice (Hsaio *et al.*, 1995).

Several mouse lines expressing a premature alteration codon (PTC) at mouse codon 143 have been generated (Fischer *et al.*, 1996) in an attempt to model a $145^{stop}$ mutation found in a woman with an Alzheimer's-like illness (Kitamoto *et al.*, 1993). However, the mice expressed very low levels of PrP mRNA and no detectable $PrP^{c}$ and Fischer *et al.* suggest that low levels of $PrP^{c}$ may be present because the truncated peptide may (as it is devoid of the *C*-terminus GPI anchor) be released from the cell surface and be rapidly degraded. An alternative explanation might be that the PTC decreases PrP mRNA stability as has been described with a variety of human genes including *CFTR* (Hamosh *et al.*, 1991), β-globin (Baserga and Benz., 1988), dihydrofolate reductase (Urlaub *et al.*, 1989) and triosephosphate isomerase (Belgrader *et al.*, 1993).

### *PrP knockout mice*

PrP knockout mice have proven to be an invaluable resource and four groups have independently generated PrP knockout mice (Bueler *et al.*, 1992; Manson *et al.*, 1994; Moore *et al.*, 1995; Sakaguchi *et al.*, 1996) by gene targeting in ES cells. The very high amino acid conservation of $PrP^{c}$ between mammals and the presence of a 20 amino acid region that is 100% conserved in all mammalian species and chickens suggests that $PrP^{c}$ has an essential function *in vivo*. However, the first PrP null mice were fertile and had no overt phenotype (Bueler *et al.*, 1992). It is possible that $PrP^{c}$ function is complemented by another protein. However, a growing number of abnormalities have been reported recently in PrP nulls.

An electrophysiological defect has been found (Collinge *et al.*, 1994) which is rescued by human PrP transgenes, demonstrating conservation of function between mouse and human PrP (Whittington *et al.*, 1995). However, another group have been unable to detect these defects in PrP null mice (Lledo *et al.*, 1996), although the authors suggest that this may be due to differences in genetic background. The importance of genetic background as a major modifier of knockout phenotypes is well established amongst workers dealing with the behavioural genetics of organisms such as *Drosophila* (for review of examples in the mouse see Gerlai, 1996).

Recently alterations in the circadian rhythm and sleep patterns have been reported in two lines of PrP nulls (Tobler *et al.* 1996). Kept in a normal light:dark cycle (12 h light:12 h dark), wild-type mice display highly reproducible patterns of activity which can be recorded on running wheels. Being nocturnal, wheel running activity is greatest during the dark cycle and indeed the first half of the dark cycle involves a period of prolonged exercise which trails off and the mice are relatively inactive during the light cycle. When mice are maintained in conditions of permanent darkness, prolonged recording from wild-type mice shows that, whilst absolute activity levels remain the same, the time at which wheel running activity initiates in the 24 h cycle progressively drifts by several min each day. Presumably this is adaptive and enables the mouse to reset (entrain) its circadian activity to changing environmental cues, such as seasonal changes in day length. PrP nulls demonstrated a significantly prolonged circadian period length but even more surprisingly, switching the lights off permanently has little or no effect upon the pattern of wheel running activity. Additionally, when maintained on a normal 12 h dark:12 h light cycle, PrP nulls were more active in the second half of the dark cycle than wild-type mice, whilst electroencephalogram (EEG) analysis suggests that PrP nulls have fragmented sleep patterns and are less tolerant to sleep deprivation. This suggests that a normal function of $PrP^{c}$ may be the control of sleep continuity or intensity. In rodents, lesions in the suprachiasmatic nucleus are known to have an effect upon circadian activity and this area is considered to be the site of the mammalian circadian clock, however there is no published data suggesting that the PrP nulls have a morphological defect in the suprachiasmatic nucleus.

These observations have a number of similarities to FFI, a variant of CJD. FFI has intractable insomnia and selective thalamic atrophy as a major feature (Gambetti *et al.*, 1993). Could it be possible that at least some of the effects of prion diseases are due to loss of normal $PrP^{c}$ function as it is converted into $PrP^{sc}$? It is more likely that this parallel is conincidental and that the insomnia phenotype in FFI is due to the severe thalamic pathology and endocrine abnormalities found in these cases.

A late onset cerebellar ataxia has been reported in one PrP knockout line (Sakaguchi *et al.*, 1996) which develops at ~70

weeks of age, suggesting that PrP may be required for the long-term survival of cerebellar Purkinje neurons. Given that this phenotype has not been reported in other lines of PrP knockout mice, it is important to exclude a number of alternative explanations other than loss of $PrP^c$ function, as the cause of the phenotype. There is the remote possibility that the cerebellar phenotype is due to the inactivation of an unknown gene immediately 5′ to PrP exon 3. The null allele generated by Moore *et al.* (1995) is very similar to that of Sakaguchi *et al.* (1996) and analysis of appropriately aged mice should provide a good comparison.

PrP null mice (Bueler *et al.*, 1992) overexpressing PrP-A from a modified $Prn\text{-}p^b$ transgene in which intron 2 had been removed had PrP expression levels and distribution similar to mice bearing the full length transgene (Fischer *et al.*, 1996) with the notable exception that PrP was no longer expressed in cerebellar Purkinje neurons, a cell type which normally expresses PrP abundantly and suggests that Prn-p intron 2 bears a Purkinje enhancer. These mice do not have an endogenous PrP allele and therefore could be considered to be a Purkinje-specific PrP knockout: the fact that these mice do not develop a late onset cerebellar disorder following observation for >600 days suggests that the loss of PrP expression in cerebellar Purkinje neurons is not the cause of the PrP null phenotype described by Sakaguchi *et al.* (1996).

PrP nulls enable experiments in which the restricted expression of $PrP^c$ from a PrP transgene can help determine the relative roles of various CNS regions and cell types in the propagation of scrapie. The recent demonstration of convincing in-situ hybridization evidence for $PrP^c$ expression in glial cells (Moser *et al.*, 1995) has raised issues about the role of non-neuronal CNS cell types in prion propagation. A PrP null mouse carrying a hamster PrP transgene driven by a neuron specific enolase promoter (Race *et al.*, 1995) developed scrapie, showing that glial expression of PrP is not critical for scrapie, although other experiments will be required to exclude the possibility that non-neuronal CNS cell types may propagate low, but significant, levels of prions or indeed that there are some strains that do propagate in glials.

There is some evidence that glial fibrillary acidic protein (GFAP) is a PrP ligand (Oesch *et al.*, 1990) and is therefore a candidate co-factor for prion propagation *in vivo*. However, GFAP null mice do not have altered scrapie susceptibility (Gomi *et al.*, 1995), suggesting that whilst GFAP may engage in some sort of interaction with $PrP^c$, it is not essential for prion propagation.

The means by which $PrP^{sc}$ accumulation results in cell death is beginning to yield some clues. For instance, primary rat hippocampal cell lines have been shown to die by apoptosis after chronic exposure to a synthetic peptide corresponding to PrP amino acids 106–126 (Brown *et al.*, 1996); a region completely conserved within mammals and also in chicken. This data is particularly interesting because apoptosis seems to be an important means of cell death *in vivo* during prion diseases. The role of $PrP^c$ in this effect is critical because PrP null primary cultures are not susceptible to the toxic effects of this peptide. A similar phenomenon has been shown *in vivo* with brain grafting experiments (Brandner *et al.*, 1996): if brain grafts from $PrP^c$ expressing donors are introduced into the brains of PrP null recipients and the mice inoculated with scrapie, the host mice do not get scrapie but the $PrP^c$ expressing graft does. The characteristic scrapie pathology is clearly evident in the graft but the PrP null host brain is apparently normal. Pathology is strictly limited to graft cells expressing PrP. $PrP^{sc}$ migrates long distances from the graft into the host PrP null brain but does not cause any pathology despite forming PrP amyloid plaques. Thus, there is both in-vitro and in-vivo evidence that $PrP^c$ expression is required for cells to be susceptible to scrapie induced pathology.

## Conclusions

Prion disease research has come a long way in the last 8 years since the first use of mouse transgenesis was reported (Scott *et al.*, 1989). It is now known that the species barrier is controlled in large part by differences in PrP amino acid sequence (and probably structure) between the inoculum and host animal (Scott *et al.*, 1989, 1993; Prusiner *et al.*, 1990). This has been corroborated by in-vitro work which shows that heterologies based upon single amino acid alterations can dramatically alter the efficiency of $PrP^{sc}$ synthesis (Priola *et al.*, 1994; Priola and Chesebro, 1995).

The variable copy number in transgenic mice have shown that scrapie incubation time is not only determined by PrP gene sequences but also by PrP expression levels: mice with reduced PrP expression levels have prolonged experimental scrapie incubation times (Bueler *et al.*, 1993; Manson *et al.*, 1994; Sakaguchi *et al.*, 1996), whilst mice with raised PrP expression levels have shorter incubation times. The extreme end of this spectrum is manifest in a spontaneous scrapie-like (note however that no $PrP^{sc}$ is produced) illness in mice which grossly overexpress wild-type prion protein (Westaway *et al.*, 1994b). The crucial role of PrP is demonstrated by the complete resistance of PrP null mice to prion infections (Bueler *et al.*, 1993). The discovery of the first human prion protein mutation, in a kindred with Gerstmann–Straussler–Scheinker disease (Hsaio *et al.*, 1989), was followed by a transgenic model which developed a similar disease spontaneously which was subsequently shown to be transmissible (Hsaio *et al.*, 1990, 1994). However mice overexpressing a codon $200^{Lys}$ mutant PrP do not develop neurological symptoms (Fischer *et al.*, 1996).

PrP knockout mice have provided clues to the normal functions of PrP, suggesting a role in neurotransmission (Collinge *et al.*, 1994), in the control of some aspects of sleep and circadian rhythm (Tobler *et al.*, 1996) and possibly a role in the long-term survival of cerebellar Purkinje neurons (Sakaguchi *et al.*, 1996).

Much of the mouse transgenesis has provided further weight to the biochemical evidence suggesting that an abnormal isoform of the prion protein, $PrP^{sc}$, is a major component of the transmissible agent (Prusiner *et al.*, 1982a). The prion hypothesis has gained widespread, but not universal, acceptance. Familial prion diseases are linked to PrP germline mutations and it is presumed that mutations can adversely affect the structural stability or the behaviour of mature and

nascent mutant $PrP^c$ peptides to the extent that misfolding events give rise to infectious prions (i.e. $PrP^{sc}$) within the carrier of the mutation. The spontaneous development of a transmissible scrapie-like disorder in a mouse model of the GSS 102 $^{Leu}$ mutation supports this view. However, sporadic CJD represents 85% of all CJD cases but is not associated with PrP mutations or exposure to infectious material, suggesting that sCJD is the result of rare spontaneous $PrP^c$ misfolding events to form prions *de novo* in the absence of exogenous contamination.

### The future

One of the major problems facing proponents of the prion hypothesis is to account for scrapie strain diversity, stability and mutability. The current paradigm of molecular genetics holds that heritable biological information is transmitted by nucleic acids. How can an infectious agent which has distinct strains or isolates encode information in the absence of nucleic acid? There is a growing body of evidence that $PrP^c$ and $PrP^{sc}$ differ in conformation and it is possible that strains may be conformational isomers of $PrP^{sc}$. One approach may be to correlate strain specific properties with distinct biophysical differences between $PrP^{sc}$ molecules. Ideally each strain might represent a distinct conformer (or mixture of conformers) of $PrP^{sc}$. There are a number of examples of $PrP^{sc}$ from different strains with distinct biophysical properties such as differences in protease resistance and SDS–PAGE mobility (Kascsak *et al.*, 1991). As with all things in this field the story is complex and there are examples of $PrP^{sc}$ with similar properties in different strains. It is not understood how different strains may target different cells types. There are numerous $PrP^c$ glycosylation variants and it may be possible that the different lesion profiles found with each strain may arise from the differential infection of neurons which express certain PrP glycoforms. The propagation of $PrP^{sc}$ within such neuronal subtypes may enable the glycosylation patterns to 'breed true' and enable the stable maintenance of the biological properties of a given prion isolate or strain. A good test of this hypothesis is to generate gene targeted mice which have had the glycosylation sites removed, however *in vitro* analysis of glycosylation mutants in neuroblastoma cells suggests that the prion protein accumulates intracellularly (Rogers *et al.*, 1990) and may therefore have a deleterious phenotype in mice, precluding analysis by scrapie challenge.

Clearly, three-dimensional analysis of $PrP^{sc}$ structure should resolve this issue by demonstrating whether different prion strains possess distinct conformations, but progress has been thwarted by technical difficulties crystallizing $PrP^c$ and $PrP^{sc}$. However, a partial NMR structure of $PrP^c$ has been recently published (Riek *et al.*, 1996) and offers some rational basis for the investigation of structural modifications to $PrP^c$.

### Prion diseases and modifier genes

It is clear that, for the foreseeable future, the prion field will focus upon the structural analysis of PrP and, as knowledge accumulates, the in-vivo relevance of structural alteration to PrP will have to be determined in transgenic mice. New prion diseases such as familial progressive subcortical gliosis (Petersen *et al.*, 1995), in which $PrP^{sc}$ is found in the absence of coding region mutations, but with linkage to chromosome 17, point towards a role for other genes in prion diseases. Indeed, several decades of genetic analysis of mouse scrapie incubation time control has shown that there are clearly non-PrP genes involved in the determination of incubation time length and pathology (Kingsbury *et al.*, 1983; Bruce and Dickinson, 1985; Carlson *et al.*, 1994; Westaway *et al.*, 1994a). Similarly, the penetrance of some familial prion diseases associated with protein mutations is significantly $<100\%$ and some individuals within a kindred can present with quite different clinical symptoms and neuropathology despite bearing the same germline PrP mutation (Dohura *et al.*, 1989; Hainfellner *et al.*, 1995; Mastrianni *et al.*, 1995), indicating a role for modifier genes. The identity of PrP ligands such as Protein-X (Telling *et al.*, 1995) and others will also be interesting and their relevance to prion diseases will be tested by gene ablation in mice.

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# Appendix II

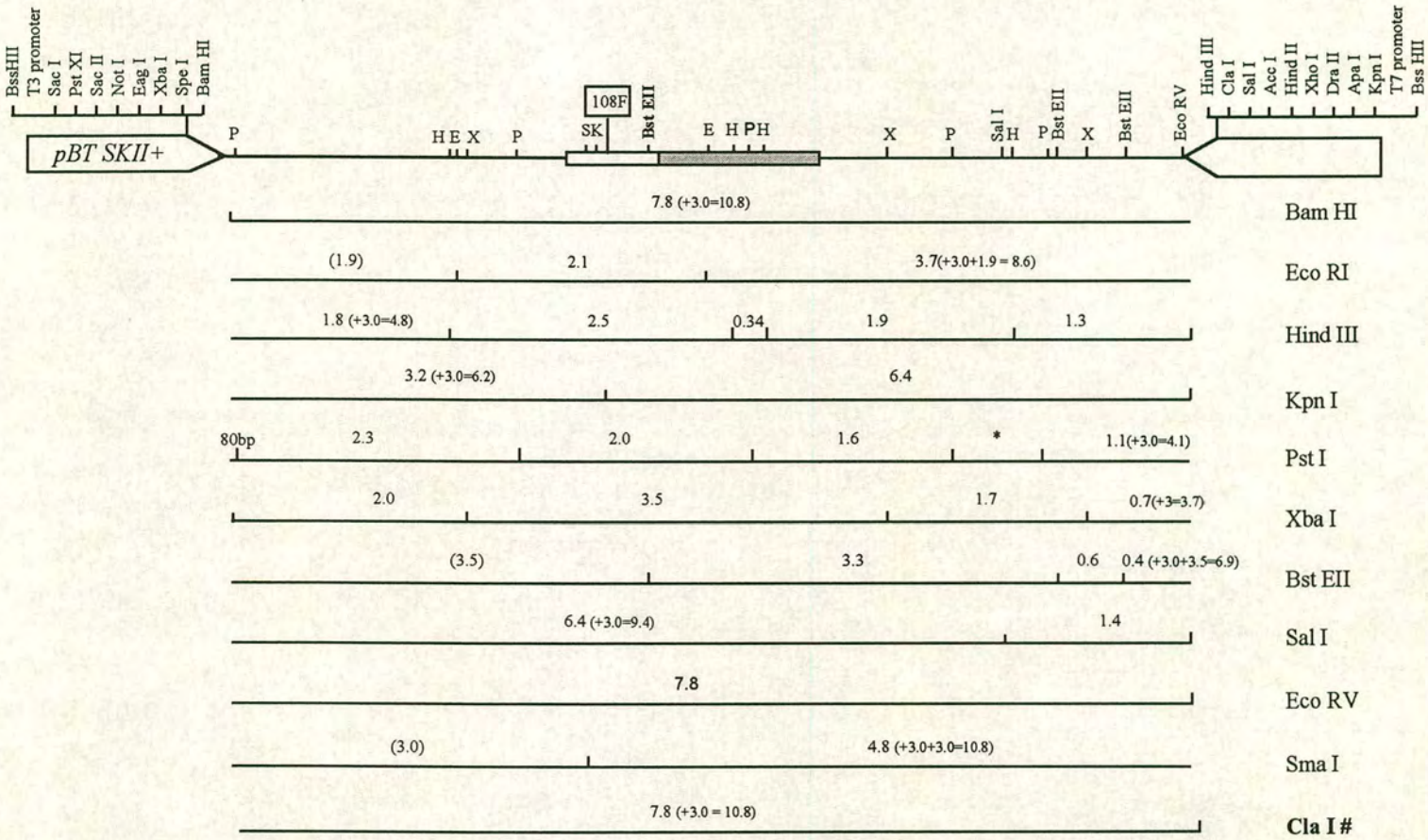
## Restriction maps of targeting vectors p108F and p189V

**Appendix II a: Restriction map of targeting vector p108F**

Map of the completed PrP exon 3 108F targeting vector. This vector was linearised at a unique 3′ Cla I site prior to electroporation.

Restriction enzyme abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; S, Sma I; X, Xba I.

PrP coding region PrP UTR

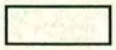


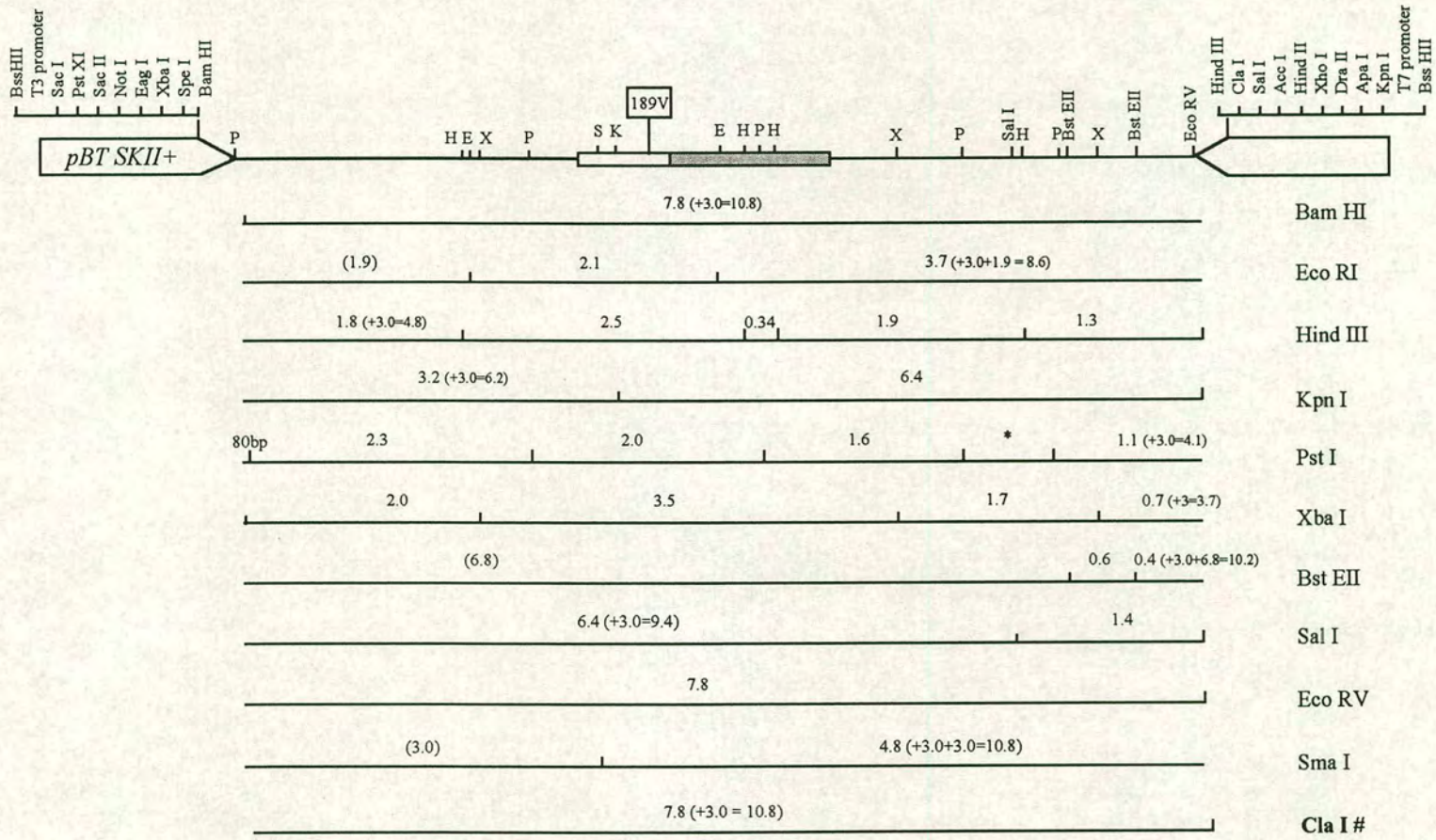

* there is one unmapped Pst site within this region
# unique linearisation site

**Appendix II b**: **Restriction map of targeting vector p189V**

Map of the completed PrP exon 3 189V targeting vector. This vector was linearised at a unique 3′ Cla I site prior to electroporation.

Restriction enzyme abbreviations: B, Bam HI; E, Eco RI; H, Hind III; K, Kpn I; P, Pst I; S, Sma I; X, Xba I.

 PrP coding region  PrP UTR




* there is one unmapped Pst site within this region
# unique linearisation site