A Cell Culture Model for the Propagation of Scrapie Prion Infectivity

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Thesis submitted to the University of London for the degree of Doctor of Philosophy 2002

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Abstract

The central event in the pathogenesis of prion disease is the conversion of PrP, the prion protein, from its normal cellular form (PrP$^\text{C}$) to its abnormal conformer (PrP$^\text{Sc}$). In an experimental scrapie prion infection, the initial inoculum of PrP$^\text{Sc}$ is cleared relatively rapidly, thus a critical aspect of disease progression in vivo is presumably the ability of infected cells, for example in the nervous system, to convert their uninfected neighbours to stable expression of PrP$^\text{Sc}$. The mechanisms underlying such cell to cell infection are not understood.

In the present study, the transfer of scrapie prion infectivity was studied in dissociated cell culture. Genetically marked target cell lines were derived by transfection of tagged PrP molecules into uninfected cells under neomycin selection. In initial experiments, target cells were exposed to subcellular preparations of prion rods and were converted to stable production of PrP$^\text{Sc}$.

In order to study cell-mediated infection, a system was established whereby scrapie-infected mouse SMB cells are able to convert uninfected target cells following coculture of the two populations. The mechanism of intercellular prion transmission in this culture system was evaluated in a series of experiments, and was found to be dependent on direct cell to cell contact. This system for establishing cell-based infection is effective and requires significantly less PrP$^\text{Sc}$ than conversion by an infected brain homogenate.

To facilitate the accurate quantitation of conversion at a cellular level, attempts were made to identify a cytochemical marker for PrP$^\text{Sc}$-expressing cells. This search was pursued by assessing the reactivity of a range of PrP antibodies and reagents including plasminogen. Data obtained from such studies should enable the derivation of immunoreagents able to reliably discriminate between PrP$^\text{C}$ and PrP$^\text{Sc}$ at the single cell level.
to my family
Acknowledgements

I am eternally grateful to my supervisor Jeremy Brockes for his continuous guidance, inspiration and advice during the completion of my studies.

I would like to thank my colleagues in the lab, past and present, for their invaluable help and encouragement, in particular, David Drechsel, Anoop Kumar, Yutaka Imokawa and Phillip Gates. A special thanks to Monica Dias, Cristiana Velloso, Sara Morais da Silva, Amy Duckmanton and Tim Landy for their friendship, support and for creating an enjoyable atmosphere in which to work.

I would also like to thank those researchers who contributed cells and reagents, without which, many of these studies would not have been possible. In particular, I thank Chris Bostock, Chris Birkett and Wilfred Goldmann for cells and reagents, and Anthony Williamson, Martin Groeschup and Parmjit Jat for antibodies.

I would like to acknowledge the financial support of the Medical Research Council.
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<th>Description</th>
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<tbody>
<tr>
<td>BrdU</td>
<td>5-bromo-2-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>BSE</td>
<td>bovine spongiform encephalopathy</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob disease</td>
</tr>
<tr>
<td>CLD</td>
<td>caveolae-like domains</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CPB</td>
<td>carboxypeptidase B</td>
</tr>
<tr>
<td>CsCl</td>
<td>caesium chloride</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>Cu</td>
<td>copper</td>
</tr>
<tr>
<td>CWD</td>
<td>chronic wasting disease</td>
</tr>
<tr>
<td>d</td>
<td>days</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dpl</td>
<td>doppel</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>FFI</td>
<td>fatal familial insomnia</td>
</tr>
<tr>
<td>GC</td>
<td>germinal centre</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosyl phosphatidylinositol</td>
</tr>
<tr>
<td>GSS</td>
<td>Gerstmann-Straussler-Scheinker disease</td>
</tr>
<tr>
<td>Gy</td>
<td>Gray</td>
</tr>
<tr>
<td>Ha</td>
<td>hamster</td>
</tr>
<tr>
<td>HCDR</td>
<td>heavy-chain-complementarity-determining region</td>
</tr>
<tr>
<td>Hsp</td>
<td>heat-shock protein</td>
</tr>
<tr>
<td>Hu</td>
<td>human</td>
</tr>
<tr>
<td>LRS</td>
<td>lymphoreticular system</td>
</tr>
<tr>
<td>MBM</td>
<td>meat and bone meal</td>
</tr>
<tr>
<td>Mo</td>
<td>mouse</td>
</tr>
<tr>
<td>Neo</td>
<td>neomycin</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PIPLC</td>
<td>phosphatidylinositol-specific phospholipase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PK</td>
<td>proteinase K</td>
</tr>
<tr>
<td>PNS</td>
<td>peripheral nervous syste</td>
</tr>
<tr>
<td>Prnd</td>
<td>doppel gene</td>
</tr>
<tr>
<td>Prnp</td>
<td>mouse PrP gene</td>
</tr>
<tr>
<td>PRNP</td>
<td>human PrP gene</td>
</tr>
<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>PrP 27-30</td>
<td>protease resistant core of PrP&lt;sup&gt;sc&lt;/sup&gt;</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>cellular isoform of the prion protein</td>
</tr>
<tr>
<td>PrP&lt;sup&gt;sc&lt;/sup&gt;</td>
<td>abnormal, pathogenic isoform of the prion protein</td>
</tr>
<tr>
<td>PS</td>
<td>pentosan sulphate</td>
</tr>
<tr>
<td>SMB</td>
<td>scrapie mouse brain</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>TME</td>
<td>transmissible mink encephalopathy</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TSA</td>
<td>tyramide signal amplification</td>
</tr>
<tr>
<td>TSE</td>
<td>transmissible spongiform encephalopathy</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>Zn</td>
<td>zinc</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction
1.1 THE PRION PROTEIN (PrP) AND TRANSMISSIBLE SPONGIFORM ENCEPHALOPATHIES (TSEs)

1.1.1 Overview
Prion diseases are transmissible neurodegenerative conditions that affect both humans and animals (Prusiner, 1991). Human TSEs are unique in that they may present as genetic, infectious, or sporadic disorders. This group of diseases includes kuru, Creutzfeldt-Jacob disease (CJD), Gertsmann-Sträussler syndrome (GSS), and fatal familial insomnia (FFI) in human beings, as well as scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, and encephalopathies in mink, cats, mule, deer, elk, and several exotic ungulates (Table 1.1). Both animal and human conditions share characteristic histopathological features including spongiform vacuolation, neuronal loss, and astrocytic proliferation which may or may not be accompanied by amyloid plaques (DeArmond and Ironside, 1999). The experimental transmission of scrapie to rodents (Chandler, 1961), followed by the progressive enrichment of brain homogenates for infectivity has resulted in the isolation of a protease resistant protein designated the prion protein (PrP) (Bolton et al., 1982). PrP is a major constituent of infective fractions and is a key component of amyloid deposits found in the brains of some infected individuals. Numerous studies have demonstrated the independence of infectivity on a polynucleotide and have suggested that the infectious agent possesses a protein component (Alper et al., 1967; Latarjet et al., 1970; Prusiner, 1982; Prusiner et al., 1981). Prions are defined as “small proteinaceous infectious particles that resist inactivation by procedures which modify nucleic acids” (Prusiner, 1982). The protease-resistant PrP is of 27-30 kDa and has hence been termed PrP 27-30. Amino acid sequencing of the N-terminus of PrP 27-30 has led to the isolation of the cognate cDNA and PrP27-30 has been demonstrated to be encoded by a single-copy gene, PRNP, on chromosome 20 in humans. PrP 27-30 is derived from a larger molecule of 33-35 kDa known as PrP Sc (denoting the scrapie isoform of the protein) (Oesch et al., 1985). The normal gene product is a protease sensitive protein known as PrP C (denoting the cellular isoform of the protein). PrP Sc is known to be derived from PrP C by a posttranslational process during the manifestation of prion disease (Borchelt et al., 1990; Caughey and Raymond, 1991). Numerous model systems have been established enabling the
mechanisms of disease progression to be conveniently studied. In order to review our present knowledge of TSE pathogenesis, I will start by summarising the prion diseases of humans and animals and what is understood about their molecular and cellular basis, together with some background on the prion protein and the nature of the infectious agent (section 1.1). I will then describe some of the most informative models of prion disease (section 1.2), the cell biology of the prion protein (section 1.3), and finally the experimental focus for this thesis will be discussed (section 1.4).

Table 1.1 The prion diseases of humans and animals

<table>
<thead>
<tr>
<th>Disease</th>
<th>Mode of transmission</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Humans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kuru</td>
<td>infection through ritualistic cannibalism</td>
<td>(Glasse, 1967)</td>
</tr>
<tr>
<td>iatrogenic CJD</td>
<td>infection from tissues transfers HGH etc.</td>
<td>(Brown et al., 1992)</td>
</tr>
<tr>
<td>variant CJD</td>
<td>infection from bovine prions?</td>
<td>(Will et al., 1996)</td>
</tr>
<tr>
<td>familial CJD</td>
<td>germ-line mutations in PRNP</td>
<td>(Pocchiari, 1994)</td>
</tr>
<tr>
<td>GSS</td>
<td>germ-line mutations in PRNP</td>
<td>(Ghetti et al., 1995)</td>
</tr>
<tr>
<td>FFI</td>
<td>germ-line mutations in PRNP</td>
<td>(Chen et al., 1997)</td>
</tr>
<tr>
<td>sporadic CJD</td>
<td>Somatic mutations in PRNP</td>
<td>(Brown et al., 1984)</td>
</tr>
<tr>
<td>Animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>scrapie</td>
<td>infection in genetically susceptible sheep</td>
<td>(Brotherston et al., 1968)</td>
</tr>
<tr>
<td>BSE</td>
<td>infection with prion contaminated MBM</td>
<td>(Wells et al., 1987)</td>
</tr>
<tr>
<td>TME</td>
<td>infection with prions from sheep or cattle</td>
<td>(Marsh et al., 1991)</td>
</tr>
<tr>
<td>CWD</td>
<td>unknown</td>
<td>(Williams and Young, 1980)</td>
</tr>
<tr>
<td>FSE</td>
<td>infection with prion contaminated MBM</td>
<td>(Wyatt et al., 1991)</td>
</tr>
<tr>
<td>exotic ungulate</td>
<td>infection with prion contaminated MBM</td>
<td>(Kirkwood and Cunningham, 1994)</td>
</tr>
</tbody>
</table>

Abbreviations: CJD, Creutzfeldt-Jacob disease; GSS, Gertsmann-Strässler syndrome; FFI, fatal familial insomnia; BSE, bovine spongiform encephalopathy; TME, transmissible mink encephalopathy; CWD, chronic wasting disease; FSE, feline spongiform encephalopathy; HGH, human growth hormone; MBM, meat and bone meal

1.1.2 Human TSEs

1.1.2.1 Sporadic prion disease

Sporadic forms of prion disease, which include most cases of CJD, display no discernable infectious or genetic etiology. They may be attributable to spontaneous conversion of wild-type PrP$^C$ to the PrP$^Sc$ conformation, or to the presence of as yet undetected somatic mutations in the protein that favour its conversion to PrP$^Sc$ (Cohen et al., 1994). The common PrP polymorphism at residue 129, where either methionine or valine can be encoded (M129V), is
thought to be a key determinant of genetic susceptibility to sporadic CJD, the large majority of which occurs in homozygous individuals (Windl et al., 1996). Sporadic CJD occurs in all countries with a random case distribution and an annual incidence of one per million (Collinge, 2001). Onset usually occurs in the 45-75 year age group (mean age 66) with a median illness duration of 4 months (Collinge, 2001).

1.1.2.2 Infectious prion diseases
Iatrogenic transmission of CJD was first suggested in 1974 by a case of CJD in a recipient of a corneal transplant which was derived from a patient who also died of CJD (Duffy et al., 1974). Transmission of CJD has also been reported by inadequately sterilised depth electrodes used for stereotactic electroencephalography recordings (Bernoulli et al., 1977) and also by its presence in human pituitary-derived growth hormone used for the treatment of short stature in a large number of children since the late 1950s (Koch et al., 1985). Small numbers of women were also treated for infertility with human pituitary gonadotrophin over a similar period in a number of countries. More recently, the iatrogenic transmission of CJD by human dura mater grafts has been reported (Thadani et al., 1988). In all of the above cases, recipients were inoculated via a parenteral route either by surgery or by intramuscular injection. The presence of CJD infectivity in human blood is not clearly established. Experimental models have shown that blood can be infectious during the incubation stages of disease, but epidemiologic studies do not support the contention that the administration of blood, blood components, or blood derivatives transmits the disease (Brown, 1995). Since the occurrence of iatrogenic CJD, guidelines have been drawn up in a number countries to minimise the risks of further transmission.

Kuru reached epidemic proportions in the Okapa area of the highlands of Papua New Guinea and affected people of the Fore linguistic group and their neighbours, with whom they intermarried (Alpers, 1987). The epidemic peaked in the early 1960s, and was transmitted during cannibalistic feasts when deceased relatives were consumed by their close relatives and others in the immediate community (Glasse and Lindenbaum, 1992). The epidemic is thought to have originated when a case of sporadic CJD, known to occur at
random in all populations, occurred in a member of this population and was subsequently consumed. The recycling of prions within this relatively isolated population led to a substantial epidemic that became the major cause of death among the children and adult women who ate the brains and internal organs of the deceased. Incubation periods range from 4.5 to 40 years and cases are still occurring, albeit at a very low rate (Collinge, 2001). Following the cessation of cannibalism in the late 1950s, the incidence of the disease has declined.

In 1995 and early 1996, a number of cases of a variant form of CJD (vCJD) were identified which has now affected over 100 patients in Britain, 1 in Ireland, and currently 5 confirmed or suspected cases in France (data obtained from www.mad-cow.org). The age of disease onset ranges from 16-51 years (mean 29 years) in comparison to 66 years for sporadic CJD (Collinge, 2001). vCJD has a prolonged clinical course with a median of 14 months compared to 4 months in sporadic CJD, and vCJD also appears to have a distinctive pathogenesis and neuropathological appearance from sporadic CJD. Epidemiologic evidence implies a causal link between BSE and vCJD (Will, 1998), and this has been supported by other research (Hill et al., 1997). vCJD is associated with PrP$^{\delta\epsilon}$ glycoform ratios that are distinct from those seen in sporadic CJD (Collinge et al., 1996). Similar ratios are seen in BSE in cattle and BSE when transmitted to several other species and the neuropathologic features in macaque inoculated with BSE are similar to vCJD (Lasmezas et al., 1996). Transmission studies have demonstrated that the incubation periods in inbred strains of mice are almost identical in BSE and vCJD (Bruce et al., 1997), and that the anatomic distribution of neuropathology is also very similar in mice following transmission of either BSE or vCJD. Incubation periods and anatomic lesion profiles in vCJD transmissions are also distinct from sporadic CJD. These studies provide strong evidence supporting the proposed causal link. The mechanism of transmission of BSE to the human population remains speculative, although oral transmission is the most likely possibility. All cases to date are homozygous for methionine at PRNP codon 129 (Zeidler and Ironside, 2000), but it is possible that cases of vCJD expressing valine at codon 129 may occur in the future. Whether vCJD will acquire epidemic proportions or be self-limited is difficult to predict at this time point because of the long incubation time of the disease. However, mathematical modelling utilising data
from the first 14 cases has indicated that the possible future number of cases could vary between a few hundred and 80,000 (Cousens et al., 1997). More recent estimations of the epidemic size have suggested that the total number of cases will be around 200 with an incubation time of 17 years (Valleron et al., 2001).

1.1.2.3 Inherited prion diseases
About 10% of the cases of CJD, as well as all forms of GSS and FFI are inherited in an autosomal-dominant fashion (Gambetti et al., 1999). These cases are all attributable to germline mutations in \( PRNP \); over 20 pathogenic mutations have been described in two groups. Point mutations occur in the C-terminal half of the PrP molecule and are associated with CJD, GSS, or FFI. The point mutations can be substitutions e.g. GSS which was first associated with the P102L \( PRNP \) mutation (Hsiao et al., 1989) although it is now recognised as a pathological syndrome associated with several different \( PRNP \) mutations. Another point mutation Y145-STOP, results in the production of a stop codon leading to the expression of a truncated PrP (Ghetti et al., 1995). Insertional mutations, which are associated with CJD, occur in the N-terminal half of the protein and consist of one to nine additional copies of an octapeptide repeat present in a tandem array of five copies in the normal protein. The mutations are presumed to favour spontaneous conversion of the protein to the PrP\(^{Sc} \) state without the need for contact with exogenous infectious agent (Cohen et al., 1994). The codon M129V and codon 219 glutamic acid/lysine (E219K) polymorphisms can profoundly influence the phenotypic characteristics of the disease caused by pathogenic mutations in other positions. On the mutant allele, they modify basic aspects of the disease phenotype, whereas on the normal allele, the codon 129 polymorphism has been shown to influence age at onset and duration of disease. For these reasons, when defining a \( PRNP \) genotype associated with inherited prion diseases, the mutation and modifying polymorphisms on the mutant allele i.e. the haplotype are identified. Approximately 26 disease associated \( PRNP \) haplotypes have been reported, which are associated with distinct phenotypes i.e. age of onset, illness duration, clinical and pathological features. The inherited prion diseases are also characterised by the size of the protease resistant fragments produced and the ratio of the forms that are differentially glycosylated (Monari et al., 1994). Studies with laboratory animals
have also demonstrated that the familial prion diseases are transmissible (Masters et al., 1981).

1.1.3 Animal TSEs
The prototypic prion disease is scrapie, a naturally occurring disease affecting sheep and goats. Scrapie has been recognised in Europe for over 200 years (Brown and Bradley, 1998) and is present endemically in many countries worldwide. The natural mode of transmission for scrapie is unknown, however possible routes include ingestion of infected material (Pattison et al., 1972), entry through skin abrasions (Palsson, 1979), maternal transmission from ewe to lamb (Elsen et al., 1999) and contact with an intermediate host or vector (Clouscard et al., 1995). In mice, it has been shown that TSE development is dependent on the agent strain, the infectious dose, and the host genotype (Bruce et al., 1991) and it appears that the development of scrapie in sheep, chronic wasting disease (CWD), and transmissible mink encephalopathy (TME) follow the same rules. Different sources of scrapie inocula have been demonstrated to have different transmission characteristics for example SSBP/1 and CH1641 in the Cheviot sheep flock (Foster and Dickinson, 1988). Molecular genetic studies have revealed that mutations in the PrP gene coding sequence influence the incubation time of scrapie in mice (Moore et al., 1998) and restriction fragment length polymorphism studies have shown a strong association between the PrP gene and susceptibility to experimental scrapie (Hunter et al., 1989).

CWD is a prion disease of captive and free-ranging mule deer, white-tailed deer, and Rocky Mountain elk and is endemic only in north central Colorado and southeastern Wyoming (Spraker et al., 1997; Williams and Young, 1992). The mode of transmission is unknown (Miller et al., 1998).

TME has occurred as infrequent epidemics among ranched mink and may result from foodborne prion exposure (Marsh et al., 1991). Following a series of experimental infections in Syrian hamsters of TME from a single outbreak, two TME strains produced distinctly different clinical syndromes, and had distinct brain titers (Bessen and Marsh, 1994). These strains were called ‘drowsy (DY)’ and ‘hyper (HY)’. The PrP isolated from hamsters infected with these strains

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was found to differ in sedimentation properties, sensitivity to proteinase K, migration on polyacrylamide gels, immunoreactivity and targeting in the brain, suggesting that the structure of protease resistant PrP determines strain variation (Bessen and Marsh, 1994).

An epidemic of BSE began in Britain in 1986 and has caused the deaths of more than 180,000 cattle, although the total number of infected animals has been estimated at around one million (Anderson et al., 1996). The epidemic appears to have been caused by meat and bone meal (MBM) fed primarily to dairy cattle (Wilesmith et al., 1991). MBM was prepared from the offal of sheep, cattle, pigs, and chickens as a high-protein nutritional supplement. It is presumed that a change in the rendering process in the late 1970s allowed scrapie prions from sheep to escape inactivation and to spread throughout the cattle population from contaminated feed (Prusiner, 1997). An alternative hypothesis, however, is that the BSE epidemic resulted from recycling of rare sporadic BSE cases, as cattle were also rendered to produce cattle feed. While a government-imposed feed ban in 1988 has significantly reduced the incidence of disease, the epidemic has had a major economic and political impact throughout Europe. BSE has since been reported in a number of other countries, with significant epidemics reported in Switzerland, Ireland and Portugal, although over 95% of all BSE cases have occurred in the United Kingdom (Detwiler and Rubenstein, 2000).

Throughout the BSE epidemic, many exotic species such as bovidae and felidae have developed spongiform encephalopathies. The bovidae include greater kudu, nyala, Arabian oryx, Scimitar horned oryx, eland, gemsbok, bison, ankole; the felidae include tiger, cheetah, ocelot and puma (Kirkwood and Cunningham, 1994). The bovid species in which cases have occurred were all fed MBM as a dietary supplement in a manner similar to domestic cattle. Captive wild felids were fed bovine carcass material, including spinal cord, on a regular basis. Several of these cases have been confirmed to be caused by a BSE-like prion strain (Bruce et al., 1994), and it is likely that most or all of these are BSE related. In addition, cases of spongiform encephalopathy have been diagnosed in domestic cats since 1990 (Wyatt et al., 1991). Domestic cats are likely to have been fed pet foods containing bovine tissues and MBM.
1.1.4 PrP gene: structure, organisation and expression

The mouse PrP gene, *Prnp* and human gene, *PRNP*, are located on chromosomes 2 and 20 respectively (Sparkes et al., 1986). The entire open reading frame of all known mammalian and avian PrP genes resides within a single exon (Prusiner and Scott, 1997), which excludes the possibility that PrPSc could arise from alternative RNA splicing. Similarly, the lack of multiple coding exons excludes the possibility of spliced forms of PrP^C with different biochemical and/or subcellular localisations. *Prnp* promoter regions are followed by a short 5' untranslated region (UTR) exon, frequently by a small intron (~2kb), a second 5' UTR exon, a larger intron (>10kb), and an initiator methionine close to the splice acceptor site of the third protein coding exon (Figure 1.1 A). The termination codon is trailed by a 3' UTR of variable length. The PrP gene is expressed at high levels in neurons of the adult brain and has also been detected in astrocytes and oligodendrocytes (Moser et al., 1995). Lower levels of PrP mRNA can be detected in other tissues such as heart, lung, and spleen (Oesch et al., 1985). The PrP protein is present predominantly in brain tissue but also at quite high levels in heart and skeletal muscle, whereas it is barely detectable in liver (Bendheim et al., 1992). Interestingly, PrP^C mRNA has been observed to display a synchronous circadian rhythm of expression at multiple sites within the rat forebrain demonstrating that expression of PrP^C mRNA can vary over a relatively short period *in vivo* (Cagampang et al., 1999).

*Prnd* encodes a novel 179 residue protein designated Doppel (Dpl), which has 25% identity with all known prion proteins and is located 16kb downstream of *Prnp* (Moore et al., 1999). *Prnd* produces two major transcripts of 1.7 and 2.7 kb, as well as unusual transcripts generated by intergenic splicing with *Prnp*. In contrast to PrP, Dpl mRNA is expressed at low levels in the adult CNS and at high levels in testis.

1.1.5 PrP gene: mutations and polymorphisms

Around 10-20% of human prion disease is inherited, and all cases to date have been associated with coding mutations in the PrP gene, of which over 20 distinct types are recognised (Figure 1.1 B) (Collinge, 1997). Five of these mutations have been genetically linked to the inherited human prion diseases (Dlouhy et al., 1992; Gabizon et al., 1993; Hsiao et al., 1989; Petersen et al., 1992;
Poulter et al., 1992). Virtually all cases of GSS and FFI appear to be caused by germ-line mutations in the PrP gene which are not found in the normal population. Such mutations are presumed to be the causal event in initiating these diseases. No such pathogenic PrP gene mutations, however, are present in sporadic and iatrogenic prion disease. The M129V polymorphism status is a key determinant of genetic susceptibility to iatrogenic or sporadic prion diseases, the large majority of which occur in homozygous individuals (Collinge et al., 1991; Palmer et al., 1991; Windl et al., 1996). This protective effect of PRNP codon 129 heterozygosity is also seen in some of the inherited prion diseases (Baker et al., 1991; Hsiao et al., 1992). Apart from M129V, there are several other coding region polymorphisms which have the property of modulating experimental or natural prion diseases (Goldmann et al., 1990; Goldmann et al., 1994; Shibuya et al., 1998; Westaway et al., 1987).

1.1.6 PrP gene: deletion

The first two knockout Prnp\(^{0/0}\) lines were independently generated in Zurich and Edinburgh by homologous recombination (Bueler et al., 1992; Manson et al., 1994a). These Prnp\(^{0/0}\) mice were phenotypically normal, and they generally remained healthy for almost two years. However these mice were subsequently reported to have abnormalities in synaptic physiology (Collinge et al., 1994) and in circadian rhythms and sleep (Tobler et al., 1996). They were completely resistant to prion disease following inoculation and did not replicate prions, although reconstitution of knockout mice with either mouse or hamster transgenes has been observed to restore susceptibility in a species-specific manner (Bueler et al., 1993). A third line of gene-targeted Prnp\(^{0/0}\) mice generated in Nagasaki also developed normally but unlike the Prnp\(^{0/0}\) mice created in Zurich and Edinburgh, they showed progressive ataxia and cerebellar Purkinje cell degeneration at about 70 weeks (Sakaguchi et al., 1996). These mice differ from asymptomatic Prnp\(^{0/0}\) mice in that they carry chromosomal deletions which extend beyond the Prnp ORF into intron 2 at the 5' end, and extending into the 3' UTR of the Prnp mRNA (encoded by exon 3) at the 3' end. These deletions eliminate the Prnp exon 3 splice acceptor site and result in the generation of high levels of Prnd transcripts in the brain by an unusual mechanism involving exon-skipping and intergenic splicing (Moore et al., 1999). Dpl is upregulated in the CNS of the Nagasaki mice that develop late-
Figure 1.1. Overview of the PrP gene region. (A) Diagram of PrP indicating pathogenic mutations causing inherited human prion disease above the line of the human sequence, and below the lines are shown, polymorphic variants of the human, mouse and sheep prion protein, some of which are able to influence the age at onset as well as the disease phenotype. Five octarepeats are shown in grey, the putative regions of secondary structure are labelled H1-H4, the three alpha-helices, A, B and C and the two beta-strands are labelled S1 and S2 (Figure adapted from Prusiner, 1997). (B) The most abundant forms of Pmp and Pmd spliced mRNAs are underlined by the blue lines. Chimeric Pmp/Pmd mRNAs generated by intergenic splicing are present in wild type mice and are overexpressed in some knockout mice. (Figure adapted from Moore et al., 1999).
Figure 1.2. The PrP isoforms. (A) The mouse prion protein consists of 253 amino acids including the N-terminal signal sequence (red) and the C-terminal sequence (yellow) that is removed on addition of a GPI anchor. Following limited proteolysis, PrPSc gives rise to a core of approximately 142 amino acids called PrP 27-30 (Figure adapted from Prusiner, 1998). (B) Western blot of lysates from scrapie-infected and non-infected neuroblastoma cells with and without digestion with protease K and analysed by SDS PAGE. Under these conditions, PrPSc is completely digested, whereas PrPSc gives rise to PrP 27-30 (lane 4).
onset ataxia and Purkinje cell degeneration, but not in the Prnp<sup>0/0</sup> lines that do not develop ataxia, indicating that ectopic expression of this protein may cause cerebellar pathology in Nagasaki mice (Silverman et al., 2000). Two additional PrP knockout lines, Zurich II (Weissmann and Aguzzi, 1999) and Rcm0 (Moore et al., 1999), in which the PrP ORF and its flanking regions were replaced by a loxP sequence and an HPRT cassette, respectively, have confirmed the observations of the Nagasaki line.

### 1.1.7 The prion protein: structure

PrP<sup>sc</sup> can be extracted from affected brains as highly aggregated, detergent-insoluble material that is not amenable to high-resolution structural techniques. Purified fractions contain about 10<sup>5</sup> PrP<sup>sc</sup> molecules per ID<sub>50</sub> unit, the infectious dose at which half of a group of inoculated animals develop disease (Bolton et al., 1982), although it is unclear what proportion of these PrP<sup>sc</sup> molecules are actually infectious. Fourier transform infrared (FTIR) spectroscopy and circular dichroism (CD) studies have shown that PrP<sup>sc</sup> contains about 30% alpha-helical structure and about 40% beta-sheet (Pan et al., 1993). This is in strong contrast to the structural studies of PrP<sup>sc</sup> purified from normal brain which is soluble and has substantial alpha-helical structure (40%) and little beta-sheet content (~3%).

Although the three-dimensional structure of PrP<sup>c</sup> has not yet been determined by crystallographic methods, nuclear magnetic resonance (NMR) solution studies on MoPrP(23-231) (Riek et al., 1997), SHaPrP(29-231) (Donne et al., 1997) and HuPrP(23-230) (Zahn et al., 2000) show that they have essentially the same conformation, as expected from their high sequence identity. After cleavage of the N-terminal signal peptide and removal of the C-terminal peptide, which occurs as a glycosyl phosphatidylinositol (GPI) anchor is added, the mature PrP<sup>c</sup> species consists of an N-terminal region of about 100 amino acids which is flexible and unstructured in the isolated molecule, and a C-terminal segment which is also approximately 100 amino acids in length. The C-terminal domain is folded into a largely alpha-helical conformation (three alpha-helices and a two-stranded anti-parallel beta-sheet) and stabilised by a single disulphide bond linking helices 2 and 3 (Riek et al., 1996). The N-terminal region contains a segment of five repeats of an eight-amino acid sequence (the octa-peptide
repeat region), expansion of which by insertional mutation leads to inherited prion disease. This region contains a tight Cu\textsuperscript{2+} binding site and a second binding site is present upstream of the octa-repeat region (Jackson et al., 2001). It is thought that the unstructured N-terminal region may acquire structure following copper binding. Local differences between the backbone conformations of the three proteins are manifest in helix 3 and the nearby loop between the beta-strand 2 and helix 2 (Zahn et al., 2000). The species variations in the region of helix 3 and the loop are of interest because this surface area has been suggested as a binding epitope for the putative protein X. This has been implicated in the species barrier and is proposed to promote the transition from PrP\textsuperscript{C} to PrP\textsuperscript{Sc} (see section 1.3.3.1).

1.1.8 The prion protein: function
A variety of different methods have been employed to elucidate the function of the prion protein. Its localisation on the cell surface would be consistent with roles in cell adhesion and recognition, ligand uptake or transmembrane signalling. The study of the biochemical and cellular properties of the protein have indicated that mature PrP\textsuperscript{C} undergoes a life-cycle where it is endocytosed and either shuttles back to the cell surface, or is eventually targeted for degradation in lysosomes (see section 1.2.2). The existence of this endocytic recycling pathway suggests that one physiological function of PrP\textsuperscript{C} might be to facilitate uptake of an unknown extracellular ligand, by analogy with receptors responsible for uptake of transferrin and low-density lipoprotein (Harris, 1999a). PrP\textsuperscript{C} becomes attached to the cell surface through a GPI anchor and it is endocytosed via clathrin coated pits (Shyng et al., 1994), although the protein lacks a cytoplasmic domain that can interact with intracellular components of the coated pits such as clathrin and adaptor proteins. A `PrP\textsuperscript{C} receptor' has been proposed to mediate this interaction with intracellular components (Harris et al., 1996). The receptor, which is suggested to be a transmembrane protein, is postulated to have a coated-pit localisation signal in its cytoplasmic domain, is postulated to bind the N-terminal portion of PrP\textsuperscript{C} via its extracellular domain. A similar mechanism seems to be responsible for endocytosis of the receptor for urokinase-type plasminogen activator (Nykjaer et al., 1992).
The observation that PrP binds Cu^{2+} ions has stimulated inquiry into the possibility of a PrP function in copper transport/metabolism or in free radical chemistry. Copper chelators can induce spongiform change in experimental animals (Pattison and Jebbett, 1971) and PrP-null mice have been found to have lower levels of Zn/Cu superoxide dismutase (SOD) activity than wild-type mice (Brown et al., 1997); SOD activity has been shown to mirror the state of copper metabolism (Harris, 1992). Moreover, it has been reported that recombinant PrP possesses SOD activity when refolded in the presence of high concentrations of copper chloride suggesting that PrP could play a direct role in managing cellular toxicity due to free oxygen radicals (Brown et al., 1999a). Endocytosis of PrP is stimulated by the presence of extracellular copper, suggesting that PrP may serve to mediate the uptake of copper into cells from the extracellular environment (Pauly and Harris, 1998). In line with this possibility, binding of copper ions may enhance the affinity of PrP^C for the putative receptor. Disturbance of these functions of PrP by the conformational transition from PrP^C to PrP^S may be involved in prion-related neurotoxicity. It is noteworthy that recombinant PrP^C will also bind manganese and nickel (Brown et al., 2000).

A number of candidate PrP ligands have been identified by in vitro binding studies, including Hsp60 (Edenhofer et al., 1996), 37kDa laminin receptor protein (Rieger et al., 1997), Bcl-2 (Kurschner and Morgan, 1995), glial fibrillary acidic protein (Oesch et al., 1990) and Aplp1 (Amyloid precursor protein-like protein) (Yehiely et al., 1997). However, if it is assumed that PrP^C enters the secretory pathway, is displayed at the cell surface, is internalised in endosomes and is degraded in lysosomes, then some of these proteins appear to be located in incorrect cellular compartments.

A possible role for PrP^C in signal transduction has come from a survey of candidate events induced by antibody mediated cross-linking, using the murine 1C11 neuronal differentiation model (Mouillet-Richard et al., 2000). A Caveolin-1 dependent coupling of PrP^C to the tyrosine kinase fyn is observed, although the nature of the physiological agent or circumstances that might dimerise PrP^C in vivo, as well as the second messenger and the biological response associated with this putative signalling event remain unknown.
Despite these numerous conjectures, the precise cellular function of PrP has remained obscure. It has been suggested that PrP\(^C\) may play a role in regulating apoptosis, with disturbance of normal cellular levels of PrP during infection leading to cell death (Kurschner and Morgan, 1995; Kurschner and Morgan, 1996). If this were the case, then the lack of phenotype in those knockout lines of mice which did not lead to doppel overexpression could be attributed to compensatory adaptations during development. However, the finding that the knockout of PrP in neurons of adult mice has no detrimental effect for up to 15 months post-knockout indicates that the loss of the normal function of PrP\(^C\) is unlikely to be significant in the pathogenesis of prion disease (Mallucci et al., 2002).

1.1.9 The nature of the infectious agent

A wealth of physical, chemical and enzymatic treatments have been used to probe the nature of the infectious agent. In such experiments, inferences drawn from results depend on the loss of infectivity as measured by bioassay. Inactivation of the agent by ionising radiation has given a target size of about 55 kDa, which is smaller than any known virus (Alper et al., 1966). Furthermore, the agent has been shown to be extremely resistant to high doses of ionising radiation at 254 nm suggesting that the mode of replication is independent of the integrity of a nucleic acid moiety (Alper et al., 1967). In agreement with these results, experiments with UV radiation at different wavelengths show no evidence for a nucleic acid component (Alper et al., 1978). Conflicting results have been reached on the chemical composition of the infectious agent. On one hand, the agent exists in a variety of distinct mutable strains which implies the existence of genetic information in a nucleic acid of the infectious particle; on the other hand the finding that the scrapie agent is resistant to both UV and ionising radiation indicates that it is likely to be devoid of nucleic acid. It has therefore been suggested that TSEs are caused by an unconventional virus (Gajdusek, 1977) or virino (viroid-like DNA complexed with host proteins) (Dickinson and Outram, 1979), which could resist treatments modifying nucleic acids, or by a novel pathogen lacking nucleic acids. A vast number of other hypotheses have been put forth for the chemical nature of the infectious agent including a spiroplasma-like organism (Bastian, 1979; Humphery-Smith et al., 1992), a nucleic acid surrounded by a polysaccharide coat (Narang, 1974;
Siakotos et al., 1979), a nucleoprotein complex (Latarjet et al., 1970), and membrane bound DNA (Marsh et al., 1978) to name only a few. More recently, the observation that levels of autoantibodies to brain components as well as antibodies to Acinetobacter calcoaceticus are elevated in animals with BSE (Tiwana et al., 1999), has supported the hypothesis that this disorder may be an autoimmune disease.

Some characteristic features of scrapie infectivity have been determined using endpoint titrations in mice such as the resistance to non-denaturing detergents, nucleases, proteases, and glycosidases (Hunter et al., 1969; Hunter and Millson, 1964; Millson et al., 1976). However, the development of the improved incubation time bioassay in Syrian hamsters (Marsh and Kimberlin, 1975) led to the development of an effective protocol for significantly enriching samples for scrapie infectivity, using a series of detergent extractions, limited digestions with proteases and nucleases, and differential centrifugation (Prusiner et al., 1980b), followed by agarose gel electrophoresis (Prusiner et al., 1980a) or sucrose gradient centrifugation (Prusiner et al., 1982; Prusiner et al., 1983).

Since then, a number of other approaches have been used to explore the nature of the agent. Firstly, infectious prions in highly purified fractions have been subjected to procedures that hydrolyse, modify, denature or shear either proteins or nucleic acids (Riesner et al., 1992). Prions were found to resist inactivation by all procedures that altered nucleic acid (for example nucleases, psoralens, hydroxylamine or Zn^{2+}), whereas procedures that modified or hydrolysed proteins (such as proteases, phenol, SDS or urea) diminished scrapie infectivity. Nucleic acids have also been characterised in fractions enriched for prion infectivity using a wide range of techniques including PCR and differential hybridisation (Riesner et al., 1992). Attempts have been made to identify a nucleic acid unique to scrapie-infected preparations using polyacrylamide gel electrophoresis (Meyer et al., 1991) or return refocusing gel electrophoresis (Kellings et al., 1992) combined with silver staining. These studies have been unsuccessful, but they have given an estimate of the maximum length of a hypothetical scrapie-specific nucleic acid. Based on the assumption that one infectious unit must contain at least one nucleic acid molecule if it is to be essential for infectivity, at a particle to infectivity ratio of 1,
such a polynucleotide would contain approximately 100 nucleotides. Since nucleic acids smaller than 100 nucleotides with a particle to infectivity ratio larger than unity have been identified in highly purified prion preparations, these molecules cannot be excluded as components of infectivity. If such a small nucleotide were essential for infectivity, it is proposed to possess a regulatory function.

The demonstration that scrapie prion infectivity was diminished by procedures altering proteins, along with the apparent independence of infectivity on a polynucleotide, led to the development of the prion concept where prions were defined as "proteinaceous infectious particles that resist inactivation by procedures that modify nucleic acids" (Prusiner, 1982). This narrowed down the list of hypotheses regarding the nature of the infectious agent to those that included a protein, which became known as the prion hypothesis. Purified preparations of the infectious agent were found to contain a 27-30 kDa protease resistant protein, termed PrP 27-30 (Figure 1.2). PrP 27-30 turned out to be the protease resistant core of an abnormal isoform of the prion protein, termed PrP\textsuperscript{sc}. The normal host protein is denoted PrP\textsuperscript{C} and is protease sensitive. One possibility that has been postulated for the prion is that it is composed exclusively of protein, referred to as the protein-only hypothesis (Bolton and Bendheim, 1988; Weissmann, 1991). Four of the most widely accepted hypotheses about the nature of the infectious agent are outlined below.

**Virus hypotheses**

The virus hypothesis demands that PrP\textsuperscript{C} must function as a virus receptor and that pathogenic mutations in PrP\textsuperscript{C} which cause inherited prion disease, act by enhancing the binding of the virus (Figure 1.3 A) (Rohwer, 1991). This would also explain why the length of the scrapie incubation time has been found to be inversely proportional to the level of expression in transgenic mice and why knockout mice are resistant to scrapie. Conversion of PrP\textsuperscript{C} into PrP\textsuperscript{sc} would be brought about by the interaction of the virus with PrP\textsuperscript{C}. The species barrier for infection could be caused by a reduced affinity of a given virus to the PrP\textsuperscript{C} molecule of a different host species. A number of arguments support the contention that the agent is a virus (Prusiner, 1999). Copurification can be explained by the possibility that the virus is tightly bound to PrP\textsuperscript{sc} or possibly
that it has a coat protein that is highly homologous to PrP. The inability to inactivate preparations highly enriched for scrapie infectivity using procedures that modify nucleic acids could be rationaled by the viral genome being protected in some way by PrP$^{Sc}$, from inactivation. In order for the virus and PrP$^{Sc}$ to share structural properties, the virus must acquire a new PrP sequence from each cell it invades. The virus hypothesis readily explains the existence of distinct scrapie strains. The genetic information defining the properties of the various scrapie strains is encoded by a nucleic acid, hence explaining the transmission of mutant prions from FFI and iCJD(E200K) to mice expressing a mouse/human chimeric transgene (MHu2M), which results in the formation of two different PrP$^{Sc}$ molecules. The virus hypothesis is challenged by the claim that in preparations of highly enriched infectivity, the average size of a nucleic acid per infectious unit is not larger than 100 nucleotides.

Virino hypothesis
As a modification of the virus hypothesis, the virino hypothesis postulates that the infectious particle consists of a small nucleic acid coated by a host protein (Dickinson and Outram, 1988). Variations in the nucleic acid sequence could account for the existence of distinct strains. The size of the nucleic could be very small since it does not have to encode for any proteins. The unusual resistance to treatments that destroy or modify nucleic acids could result from the protective effect of a coat. The most likely candidate for the host protein forming the coat of the virino is PrP$^{Sc}$ since an association of infectivity with PrP$^{Sc}$ is suggested by the copurification of PrP$^{Sc}$ with infectivity (Bolton et al., 1982; McKinley et al., 1983; Prusiner et al., 1984).

Protein-only hypothesis
A large body of experimental results argues that prions are composed of PrP$^{Sc}$ molecules and are devoid of nucleic acid. For example, PrP$^{Sc}$ and scrapie infectivity copurify using biochemical and immunological procedures, and the unusual molecular properties of prions and PrP$^{Sc}$ are almost identical (Bolton et al., 1984; Prusiner et al., 1982; Prusiner et al., 1983). Furthermore, levels of PrP$^{Sc}$ are directly proportional to prion titers, and non-denatured PrP$^{Sc}$ has not been separated from scrapie infectivity despite a variety of different attempts (Prusiner, 1999). Further evidence has come from experiments which have
demonstrated that a fragment of PrP\textsuperscript{Sc} formed amyloid \textit{in vitro} and was shown to be a major component of amyloid plaques in some animals and humans with prion disease (Prusiner et al., 1983).

According to the protein-only hypothesis, the species barrier is proposed to result from variations in the PrP sequence and prion diversity is thought to be enciphered within the conformation of PrP\textsuperscript{Sc}. Both FFI and fCJD(E200K) prions can be transmitted to mice expressing the mouse/human chimeric transgene, MHu2MPrP, inducing the formation of MHu2MPrP\textsuperscript{Sc} molecules with different conformations based on the sizes of the protease resistant fragments, yet the amino acid sequence of MHu2MPrP\textsuperscript{Sc} is invariant (Prusiner, 1997; Telling et al., 1996).

Two protein-only models have been proposed for the replication of infectious prion particles, the template-assisted and nucleation-dependent polymerisation models (Figure 1.3 B and C) (Jarrett and Lansbury, 1993; Prusiner, 1982; Prusiner, 1991). According to the template-assisted model, in an uninfected cell PrP\textsuperscript{C} exists in equilibrium in its monomeric alpha helical, protease sensitive form (PrP\textsuperscript{C}) or bound to protein X (denoted PrP*/protein X) (Figure 1.3 B). In this case, PrP*/protein X is inherently more stable than PrP\textsuperscript{C}, but a kinetic barrier precludes the formation of PrP\textsuperscript{Sc}. However, at some threshold level, there would be enough PrP\textsuperscript{Sc} present to form a complex with the PrP*/protein X, creating a replication-competent assembly. The use of cultured cells expressing chimeric PrP genes has demonstrated that binding of protein X to PrP\textsuperscript{C} precedes productive PrP\textsuperscript{Sc} interactions (Kaneko et al., 1997b). The rate determining step is the conformational conversion of PrP*/protein X into PrP\textsuperscript{Sc}, a process which is catalysed by the formation of a PrP*/protein X /PrP\textsuperscript{Sc} heterodimer. Infection with exogenous PrP\textsuperscript{Sc} would facilitate propagation by increasing the concentration of PrP\textsuperscript{Sc} beyond the threshold level, initiating conversion of PrP*/protein X /PrP\textsuperscript{Sc} into a PrP\textsuperscript{Sc}/PrP\textsuperscript{Sc} homodimer and leading to a cascade of subsequent conversions. For inherited prion diseases, the first PrP\textsuperscript{Sc} molecules could be generated as a result of the kinetic barrier to PrP\textsuperscript{Sc} formation being lower for the mutant PrP\textsuperscript{C} than the wild type, and thus mutant PrP*/can spontaneously rearrange to form mutant PrP\textsuperscript{Sc}, allowing replication to precede presumably via the mechanism outlined for infectious disease. Alternatively,
inherited diseases could result from mutations that increase the population of PrP*/protein X. Initiation of sporadic disease may follow from a somatic mutation in the PrP gene, and thus follow a path similar to that for germ-line mutations in inherited disease. Much of the evidence for this model derives from studies in transgenic mice (Telling, 2000).

According to the nucleation-dependent polymerisation model (Jarrett and Lansbury, 1993), in the absence of a PrPSc aggregate large enough to act as an infectious seed, the conversion between PrPSc and PrPc is reversible, but PrPSc monomer is less stable than PrPc (Figure 1.3 C). However, PrPSc seeds, promote the conversion of PrPc by binding to and stabilising the otherwise unfavourable PrPSc conformation. The rate-determining step is the formation of a seed of polymerised PrPSc which, once formed, promotes further polymerisation of PrPSc. The requirement that a seed be formed before conversion is stable predicts certain characteristics of the aggregation process, including the need for a critical protein concentration to be exceeded for the initial formation of a seed, and kinetics displaying a lag phase. In the case of infection, seed is introduced from outside, after which rapid aggregation can begin. Sporadic disorders on the other hand, would depend on seed formation from PrPSc monomers. The equilibrium between PrPc and PrPSc could easily depend on the cellular environment, and be influenced by variables such as pH, or the presence of interacting factors as well as mutations in the PrP gene that favour PrPSc formation. A substantial amount of evidence for this model derives from studies of the in vitro conversion of radiolabelled PrPc to a protease resistant form by denaturation and prolonged incubation with preparations of PrPSc (summarised in section 1.3.2) (Bessen et al., 1995; Kocisko et al., 1994; Kocisko et al., 1996). However, it has also been demonstrated that aggregates of PrPSc do not necessarily contain infectivity (Riesner et al., 1996), which influences me to favour the template-assisted model. Nonetheless, the nucleation and template conversion mechanisms are not considered to be mutually exclusive (Horwich and Weissman, 1997). For example there could be a hybrid mechanism by which the surface of a seed, which is initially formed by a nucleation process, acts as a template to catalyse the conformational change of unconverted monomers.
Unified theory
The unified theory combines the view of the virino hypothesis, which explains the existence of distinct, mutable strains of the infectious agent on the basis of nucleic acid encoded information, with the view of the protein-only hypothesis, which accounts for the resistance of the infectious agent to treatments which alter nucleic acids by postulating that PrP\textsuperscript{Sc} in the infectious preparation is sufficient for infection (Weissmann, 1991). According to the unified theory, the holoprion consists of the apoprion, PrP\textsuperscript{Sc} and the coprion a nucleic acid, which is usually associated with PrP\textsuperscript{Sc} but can also be recruited or exchanged within the host cell. The conjectured coprion is most likely to be a nucleic acid, because it must be susceptible to replication and mutation. In agreement with the protein only hypothesis, propagation of PrP\textsuperscript{Sc} is proposed to occur by the conversion of PrP\textsuperscript{C} into a replica of itself, independently of its association with a coprion. After cell invasion, the coprion undergoes replication by a cellular polymerase, a process which is promoted by or dependent on its association with PrP\textsuperscript{Sc}. The unified theory incorporates most features of the protein-only hypothesis, and therefore adopts the explanations offered by the latter for the species barrier phenomenon and the origin of sporadic and genetically determined prion diseases. The existence of multiple strains can arise as a result of passaging of holoprions through different hosts, which may occasionally lead to an exchange of the nucleic acid. The failure of experiments with UV and ionising radiation, as well as nucleolytic agents, to detect the existence of a nucleic acid component can be attributed to the fact that removal of nucleic acids would not affect infectivity bioassays, which is the criteria upon which these experiments were based.

To date, none of the above models for the propagation of the scrapie agent have been experimentally confirmed or ruled out, and hence the nature of the infectious agent remains controversial. The protein-only hypothesis has gained wide acceptance because a scrapie-specific nucleic acid has not been found. Reports of PrP conversion in a cell free system (see section 1.3.2) have been interpreted in favour of the protein-only hypothesis, nonetheless it cannot be excluded that unidentified components of the infectious preparations were required for the generation of protease-resistant PrP. Some of the properties of PrP\textsuperscript{C} and PrP\textsuperscript{Sc} are summarised in table 1.2.
A Virus hypothesis

B Template-assisted

C Nucleation-polymerisation

Figure 1.3 Models for the conformational conversion of PrP\(^C\) into PrP\(^Sc\). (A) In the virus hypothesis, PrP\(^C\) (red circles) plays the role of a host cell receptor for the virus (wavy black line) which binds to PrP\(^C\) and stimulates its conversion to PrP\(^Sc\) (blue squares). (B) In the template-assisted model, PrP\(^C\) exists in equilibrium with PrP\(^*\), represented by a green hexagon, which is bound to an ancillary factor referred to as protein X. The PrP\(^*\)/protein X complex interacts with PrP\(^Sc\) which results in a conformational change in PrP\(^*\). The end result is two molecules of PrP\(^Sc\) that are free to induce conformational changes in additional PrP\(^*\) molecules. (C) According to the nucleation-polymerisation model, the conversion between PrP\(^C\) and PrP\(^Sc\) is inherently fast and the barrier to conversion lies at the level of the initial nucleation process. Once aggregates of PrP\(^Sc\) form (denoted by a collection of blue squares), they promote the conversion of PrP\(^C\) by binding to and stabilising the otherwise unfavoured PrP\(^Sc\) conformation.
Table 1.2 Properties of cellular and scrapie PrP isoforms

<table>
<thead>
<tr>
<th>Property</th>
<th>PrP&lt;sup&gt;C&lt;/sup&gt;</th>
<th>PrP&lt;sup&gt;Sc&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcellular localisation in cultured cells</td>
<td>Cell surface</td>
<td>Primarily cytoplasmic vesicles</td>
</tr>
<tr>
<td>Secondary structure</td>
<td>Dominated by alpha-helices</td>
<td>Rich in beta-structure</td>
</tr>
<tr>
<td>Protease resistance</td>
<td>Sensitive</td>
<td>Gives rise to resistant core, PrP 27-30</td>
</tr>
<tr>
<td>PIPLC release from membranes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Behaviour in non-denaturing detergents</td>
<td>Soluble</td>
<td>Insoluble</td>
</tr>
<tr>
<td>Synthesis</td>
<td>&lt;0.1 hr</td>
<td>~1-3 hr</td>
</tr>
<tr>
<td>Degradation</td>
<td>~5 hr</td>
<td>&gt;48 hr</td>
</tr>
</tbody>
</table>

Proteinase K resistance is not unique to PrP<sup>Sc</sup>, but is also shared by tNOX, a cell surface NADH oxidase of cancer cells (reviewed by Kelker et al., 2001). tNOX and PrP<sup>Sc</sup> are of similar molecular weight (33-35 kDa), appear to undergo posttranslational modification at the Golgi apparatus and are located at the plasma membrane. Both proteins polymerise into insoluble aggregates and/or form characteristic rod-shaped amyloid and polymerise even in the presence of detergents. Like PrP<sup>Sc</sup>, circular dichroism measurements of recombinant tNOX protein suggest it to be predominantly beta-sheet. Although the two proteins bear no obvious sequence similarity, tNOX, like PrP, is implicated in copper binding and a function in time keeping has been postulated for the constitutive counterpart of tNOX (CNOX). Most interestingly, tNOX appears to impart protease resistance to muscle glyceraldehyde-3-phosphate dehydrogenase indicative of a prion like behaviour.

1.2 CELL BIOLOGY OF PrP
1.2.1 Biogenesis of PrP<sup>C</sup>

PrP<sup>C</sup> is translated from its mRNA in the endoplasmic reticulum along with many other secreted glycoproteins. In the case of Syrian hamster PrP, as the 254 amino acid polypeptide chains are being elongated, the 22 amino acid N-terminal signal sequence is cleaved as is the case for other secretory proteins (Prusiner et al., 1999a). After assembly of the polypeptide chain, a 23 amino acid C-terminal signal sequence is removed and a glycosyl phosphatidylinositol (GPI) anchor is attached to serine at position 231 via an ethanolamine residue (Stahl et al., 1990a; Stahl et al., 1987). While still in the ER, the nascent PrP polypeptide becomes glycosylated. There are two potential N-linked glycosylation sites (Asn-X-Thr) on the PrP polypeptide at positions 181 and 197.
(Endo et al., 1989; Haraguchi et al., 1989). When scrapie infected or uninfected mouse neuroblastoma cells are labelled with $^{[35S]}$-Methionine for short periods, PrP precursors of 25, 28 and 33 kDa are identified differing in their amount of glycosylation; the two larger precursors representing the addition of high mannose glycan chains to one and both of the sites, respectively, to the 25kDa unglycosylated molecule (Caughey et al., 1989). Similar precursors have been observed in a cell free translation system in the presence of microsomes (Hay et al., 1987a). The 28 and 33 kDa PrP precursors labelled in the neuroblastoma cells are post-translationally processed to 30 and 35-41 kDa species within 10–30 minutes as their high mannose glycans are converted to hybrid or complex glycans presumably within the Golgi apparatus (Caughey et al., 1989; Race et al., 1988). The glycans are subsequently sialylated and the GPI anchor also undergoes sialylation (Prusiner et al., 1999a). A single disulphide bond is added between cysteines at positions 179 and 214 (Turk et al., 1988). The mature PrP molecules of 25, 30 and 35-41 kDa are then transported in secretory vesicles to the cell surface, and become attached to the external surface of the plasma membrane by the GPI moiety (Figure 1.4).

### 1.2.2 Trafficking of PrP$^{c}$

Cryo-immunogold electron microscopy studies have established that PrP$^{c}$ is targeted to caveolae-like domains (CLDs) at the plasma membrane (Figure 1.4) (Prusiner et al., 1999a; Ying et al., 1992). The CLDs are detergent-resistant raft complexes which also contain sphingolipids and cholesterol. PrP$^{c}$ in N2a cells is targeted by its GPI anchor to CLDs in the plasma membrane, where it is either initially degraded by proteolytic cleavage to form PrP$^{c}$-II or converted into PrP$^{sc}$ (Taraboulos et al., 1995).

Experiments using neuroblastoma (N2a) cells stably expressing the chicken homologue of mammalian PrP$^{c}$, chPrP, indicate that PrP$^{c}$ constitutively cycles between the plasma membrane and an early endocytic compartment (Shyng et al., 1993). This conclusion is based firstly upon kinetic analysis with chPrP$^{c}$ molecules that have been labelled with membrane-impermeant iodination or biotinylation reagents, indicating that PrP$^{c}$ molecules cycle through the cells with a transit time of approximately 1 hour. After reaching the cell surface chPrP is endocytosed, as evidenced by immunofluorescence microscopy to
track internalisation of antibodies that have been bound to PrP\(^C\) on the cell surface. The localisation of chPrP\(^C\) in N2a cells by immunogold labelling has demonstrated that clusters of molecules are closely associated with clathrin coated pits (Shyng et al., 1994). It has been hypothesised that PrP\(^C\) molecules enter clathrin coated pits after exiting CLDs on the plasma membrane (Gorodinsky and Harris, 1995). The inhibition of internalisation of PrP\(^C\) in the presence of hypertonic sucrose, which disrupts clathrin lattices, indicates that clathrin coated pits mediate the endocytosis of chPrP (Heuser and Anderson, 1989). Double labelling of chPrP and the transferrin receptor, a transmembrane protein known to be concentrated in clathrin coated pits, also shows colocalisation (Shyng et al., 1994).

PrP is also subject to cleavage near residues 108-111 by a specific but unidentified protease, to form PrP\(^C\)-II, suggesting that this posttranslational processing event may be important to the physiological function of PrP (arrow B, Figure 1.5) (Harris, 1999a). The proteolytic cleavage is inhibited by lysosomotropic amines such as ammonium chloride (NH\(_4\)Cl) and chloroquine, and by serine protease inhibitors such as leupeptin. This indicates that the cleavage occurs in a compartment that is both acidic and degradative, properties characteristic of endosomes. Following endocytosis, chPrP is delivered to the endosomes where 95% of the molecules are recycled intact to the cell surface and 5% of the endocytosed molecules are proteolytically cleaved to give PrP\(^C\)-II and amino terminal PrP fragment (Shyng et al., 1993). The C-terminal fragments with a GPI anchor are also returned to the cell surface, while soluble N-terminal fragments are released into the extracellular medium. Some of the membrane-anchored protein is also released into the medium by a second cleavage between the diacylglycerol moiety and the ethanolamine residue of the GPI anchor (arrow A, Figure 1.5) (Stahl et al., 1990a; Stahl et al., 1987). Both cleavages occur relatively slowly in comparison with the half-life of the protein, so that at steady state several different cleavage products in addition to the intact protein can be detected.
Figure 1.4. Synthesis and degradation of PrP in cultured cells. Following synthesis in the endoplasmic reticulum, PrP\textsuperscript{C} (green circles), transits to the cell surface and is targeted by a GPI anchor (green squiggly tail) to caveolae-like domains where PrP\textsuperscript{Sc} formation (blue squares) is thought to occur. PrP\textsuperscript{Sc} synthesis is proposed to require ancillary factor protein X (purple polygon) and the formation of an intermediate complex which subsequently binds existing PrP\textsuperscript{Sc}. Following PrP\textsuperscript{Sc} formation, caveolae-like domains fuse with acidic endosomes where, PrP\textsuperscript{Sc} is cleaved giving rise to PrP 27-30 (blue triangles) which then accumulates in lysosomes. PrP\textsuperscript{C} targeted to clathrin coated pits (green circle with green stem) is endocytosed via clathrin coated vesicles and fails to be converted into PrP\textsuperscript{Sc} (Figure from Prusiner et al., 1999a).
Figure 1.5. Post-translational processing of PrP. (A) The mature protein is anchored to the cell surface via a GPI anchor. Arrows A and B indicate the positions of cleavage sites in PrP\(^C\). Site A lies within the GPI anchor between the diacylglycerol moiety and the ethanolamine residue that is attached to the C-terminal amino acid of the protein. Site B lies near position 110. Arrow C indicates a cleavage site in PrP\(^Sc\) near position 89. (B) A schematic of the core anchor structure indicating the site cleaved by PIPLC. Unlike PrP\(^Sc\), wild-type PrP can be completely released from cells by treatment with PIPLC (Figure adapted from Harris, 1999b).
1.2.3 Post-translational formation of PrP\textsuperscript{Sc}

Pulse chase metabolic labelling studies have indicated that PrP\textsuperscript{C} is synthesised and degraded rapidly, whereas PrP\textsuperscript{Sc} is synthesised slowly by a post-translational process after a lag period of approximately 1 hour, and has a half-life of over 48 hours (Borchelt et al., 1990; Borchelt et al., 1992; Caughey and Raymond, 1991). Studies with the fungal metabolite brefeldin A, which reversibly inhibits PrP\textsuperscript{Sc} synthesis, indicate that PrP\textsuperscript{Sc} synthesis does not occur in the ER-Golgi and that transport along the secretory pathway is required for PrP\textsuperscript{Sc} synthesis (Taraboulos et al., 1992b). Results from other studies with the ionophore monensin demonstrate that PrP\textsuperscript{Sc} precursors traverse the mid-Golgi in the same time frame as PrP\textsuperscript{C}. These PrP molecules continue along the secretory pathway to the cell surface where they become attached to the membrane by a GPI anchor (Borchelt et al., 1992; Caughey and Raymond, 1991). A minority of these molecules are then converted to PrP\textsuperscript{Sc}. The accessibility of the PrP\textsuperscript{Sc} precursor to PIPLC, dispase, biotinylation and surface radioiodination indicate that the precursor resides, at least transiently, on the cell surface (Borchelt et al., 1990; Borchelt et al., 1992; Caughey and Raymond, 1991). Digestion of nascent PrP with PIPLC at 18°C, or with dispase, significantly reduced the synthesis of PrP\textsuperscript{Sc}.

Eighty to ninety percent of plasma membrane PrP\textsuperscript{C} is localised to CLDs and it has been suggested that this may be the site of conversion (Taraboulos et al., 1995). Several lines of evidence point to the formation of PrP\textsuperscript{Sc} in CLDs. Firstly, replacing the GPI anchor addition signal sequence with the CD4 transmembrane carboxy-terminal 62 residues, as well as other transmembrane carboxy-terminal segments, redirects the chimeric molecules to clathrin coated pits and prevents PrP\textsuperscript{Sc} formation (Kaneko et al., 1997a). Additionally, PrP\textsuperscript{Sc} formation is inhibited by lovastatin, which diminishes cellular cholesterol levels, as well as by squalestatin, a more specific inhibitor of cholesterol biosynthesis. Pulse chase studies have indicated that PrP acquires protease resistance after it has been transported to the cell surface via the secretory pathway (Borchelt et al., 1990; Borchelt et al., 1992; Caughey and Raymond, 1991). When ScN2a cells were exposed to dispase immediately after the labelling pulse, no PrP\textsuperscript{Sc} was formed during the subsequent chase period at 37°C (Borchelt et al., 1992). However, when a two hour chase at 37°C was
carried out prior to addition of the dispase, some of the PrP became inaccessible to the dispase in the media and subsequently acquired proteinase K resistance. When ScN2a cells were chased at 18 °C but not at 37 °C, PrP^c could be released by PIPLC digestion, and PrP^Sc synthesis was abolished (Borchelt et al., 1992). At 18 °C, PrP^c remained largely accessible to PIPLC throughout the chase, whereas at higher temperatures PrP molecules probably transit to the surface and are endocytosed too rapidly for PIPLC digestion to be completely effective. Most membrane glycoproteins are transported to lysosomes via endosomes, which act as receptacles for plasma membrane proteins destined for degradation or recycling (Marsh and Helenius, 1980; Stoorvogel et al., 1991; Willingham and Pastan, 1980).

Proteases within the lysosomes then digest susceptible proteins. Evidence for PrP^Sc translocation to lysosomes is supported by studies in N2a cells which reported that although lysosomotropic amines block the digestion of the amino-terminal 90 amino acids of PrP^Sc, they do not interfere with the formation of PrP^Sc (Caughey et al., 1991; Taraboulos et al., 1992b). Since PrP^Sc is not completely degraded in the lysosomes, its conversion to the protease resistant state probably occurs prior to its exposure to proteases within endosomes and lysosomes. Kinetic studies indicate that PrP^Sc acquires protease resistance approximately 1 hour before exposure to lysosomal proteases and digestion of the amino terminus (Taraboulos et al., 1992b). Furthermore, lysosotropic amines do not alter the degradation of PrP^c substantially, which suggests that it might be degraded before reaching lysosomes. Thus PrP^Sc is likely to be formed at the plasma membrane, where the protease- and PIPLC- sensitive precursor is found, or along the endocytic pathway before exposure to proteases.

1.2.4 PrP^Sc accumulation
The details of where PrP^Sc becomes distributed in cells are still being determined. Immunofluorescence methods with antibodies against PrP 27-30 have detected a speckled cytoplasmic signal for PrP^Sc in ScN2a cells but not in control uninfected cells (Taraboulos et al., 1990). The PrP^Sc signal was protease resistant, and in double staining experiments was found to colocalize with ligands of wheat germ agglutinin, a marker for the trans-Golgi network and lysosomes (Virtanen et al., 1980). Immunoelectron microscopy has also
concluded that at least some PrP^sc accumulates within vesicular structures reminiscent of secondary lysosomes, some of which also contained the lysosomal marker acid phosphatase (McKinley et al., 1991). A large portion of nascent PrP^sc has been found to accumulate in CLDs (Vey et al., 1996). The only other location in cell cultures thought to possibly contain PrP^sc is the culture medium, which for some cultures appears to accumulate PrP^sc possibly as a result of excretion from infected cells or as a result of death of infected cells (Schatzl et al., 1997). The tentative conclusion from these studies is that PrP^sc may be widely distributed within infected cells, although improved methods for in situ detection of PrP^sc are needed to establish the relative proportions of the protein in these multiple locations. Whether similar localisation patterns of PrP^sc accumulation will be found in the brains of mammals with prion disease remains to be established. It is unknown whether the accumulation of PrP^sc exerts its effect on neuronal function when it is newly formed in CLDs, or after it is transported into the interior of the cell. Although PrP^sc comes into contact with cellular proteases in N2a cells, it is not completely digested and has a half-life exceeding 48 hours (Borchelt et al., 1990; Caughey and Raymond, 1991). The inability of cells to degrade PrP^sc may be responsible for its steady build-up in the non-dividing cells of the CNS of an infected host. The accumulation of PrP^sc may be harmful and may eventually lead to neurodegeneration.

The cell biology of PrP^sc accumulation that leads to neuronal vacuolation remains unclear. Vacuolation occurs primarily in the region of the synapses and is characterised by focal neuritic swelling, loss of internal organelles, and accumulation of abnormal membranes (Chou et al., 1980; Lampert et al., 1972). One suggestion is that the intracellular accumulation of PrP^sc induces vacuolation of neurons, and as these neurons die, the vacuoles coalesce to form the spongy appearance characteristic of prion disease. Another possibility, based on the fact that PrP^sc accumulates in lysosomes, is that these aberrantly folded molecules trigger dysfunction as they are released into the cytosol (Laszlo et al., 1992). Accumulation of PrP^sc in CLDs may lead to aberrant responses to stimuli mediated through signal transduction systems, which might account for a number of plasma membrane abnormalities that have been detected (Anderson, 1993). A further outcome that could result either from accumulation of PrP^sc in several subcellular compartments, or from abnormal
plasma membrane signalling, is a malfunction in the responsiveness to stress triggered by abnormal PrPSc (Tatzelt et al., 1995).

The reactive astrocytic gliosis that accompanies neuronal degeneration is most easily assessed by glial fibrillary acidic protein (GFAP) immunostaining. During scrapie infection, astrocytes become hypertrophic and exhibit high levels of GFAP mRNA and protein (Mackenzie, 1983), but this is neither specific nor necessary for either the transmission or pathogenesis of disease (Gomi et al., 1995; Tatzelt et al., 1996a). Experiments with a PrP peptide corresponding to amino acids 140-174, denoted P5, have determined that this region of PrP may be involved in the interaction of PrP isoforms with their ligands. One PrP ligand has been identified as GFAP (Oesch and Prusiner, 1992) and it has been proposed that in a scrapie-infected cell, following conversion, the binding of PrPSc to other proteins would trigger its post-translational insertion into the membrane. Translocated PrPSc may then interact with cytoplasmic GFAP, which may in turn lead to intracellular accumulation resulting in cell death.

The existence of two markedly different categories of prion disease, those that resemble scrapie and CJD (characteristics including the accumulation of protease-resistant PrP) and those that resemble GSS (characteristics including abnormal PrP accumulation that is relatively protease sensitive), has raised the possibility that PrP can cause nerve cell degeneration by two distinct mechanisms. Recent discoveries suggest that there are indeed two mechanisms, and that they are related to two different topologies of the PrP molecule: GPI anchored and transmembrane.

1.2.5 PrP topogenesis

Transmembrane isoforms of PrP were initially demonstrated in cell free translation studies of PrP mRNA using rabbit reticulocyte or wheat germ ribosomes in the presence of canine pancreatic microsomes (De Fea et al., 1994; Hay et al., 1987a; Hay et al., 1987b; Lopez et al., 1990; Yost et al., 1990). Whereas most PrP molecules are fully translocated across the ER membrane and are associated with PrPSc attached to the cell membrane via a C-terminal GPI anchor (denoted secPrP), C transmembrane PrP (CImPrP), spans the membrane once via a conserved, hydrophobic segment encompassing residues Ala113 to Ser135
(termed TM1), with the C terminus in the ER lumen and the N terminus accessible to proteases in the cytosol (Figure 1.6 A). The second transmembrane variant, denoted N\textsuperscript{im}PrP, spans the bilayer via the same domain but in the opposite orientation, with the N terminus in the ER lumen and C terminus accessible to proteases in the cytosol. A standard assay is used to distinguish the PrP topologies, using proteases added to the outside of ER-derived microsomal membrane vesicles, which leads to the generation of different sized fragments following enzymatic deglycosylation correlating to regions of the molecule being protected in the ER lumen (Hegde et al., 1998a). The approximate sizes of the fragments generated from \textsuperscript{Sec}PrP, \textsuperscript{Cm}PrP and \textsuperscript{Nm}PrP are 28, 18 and 14 kDa respectively.

The topogenesis of PrP is controlled by unidentified accessory proteins present during the translation process in the ER (Hegde et al., 1999; Hegde et al., 1998b), as well as discrete sequences within the PrP coding region (De Fea et al., 1994; Lopez et al., 1990; Yost et al., 1990). The hydrophobic, potentially membrane spanning region as well as the preceding "stop transfer effector" (STE) hydrophilic domain, spanning residues 104-112, are proposed to act in concert in the formation of different transmembrane forms. Mutations, deletions, or insertions within these domains can alter the relative amounts of each topological form of PrP that is synthesised at the ER (Yost et al., 1990).

Several lines of evidence have implicated \textsuperscript{Cm}PrP in the pathogenesis of prion disease. Transgenic mice have been created that carry PrP mutations that alter the relative ratios of the three topological forms in cell free translation systems (Hegde et al., 1998a). Mice that express transgenes predisposing to an increase in \textsuperscript{Cm}PrP synthesis spontaneously developed signs of neurodegeneration. Topological analysis of the PrP in the brains of the transgenic mice that developed disease was performed following removal of their brains, preparation of intact microsomal membranes and analysis using the topology determination assay described above. These studies revealed the presence of \textsuperscript{Cm}PrP but without detectable protease resistant PrP\textsuperscript{Sc}. Analysis of the state of maturation of the \textsuperscript{Cm}PrP generated in these mice revealed that nearly all had exited the ER and resided in a post-ER compartment. In contrast, non transgenic mice, and those expressing transgenes associated with the exclusive
synthesis of secPrP, failed to show signs of illness and in no instance was CmPrP detected. Expression of the transgenes at different levels in transgenic mice modulates the expression of CmPrP. Mice expressing pathogenic transgenes below a threshold value have undetectable levels of CmPrP expression and fail to develop disease.

In a subsequent study the susceptibility to PrPSc of transgenic mice with similar levels of transgene expression but differing propensities to make CmPrP was assessed (Hegde et al., 1999). These studies revealed that at the time of disease onset, transgenic lines that generated higher amounts of CmPrP were more susceptible to PrPSc, developing disease at a lower level of overall PrPSc accumulation. In addition, following an inoculation with scrapie prions, an inverse relationship between the level of CmPrP expression and the level of PrPSc at disease onset was observed for mice expressing pathogenic mutants, as is the case for mice expressing different levels of wild-type PrP (Manson et al., 1994b). Thus mice with a diminished propensity to form CmPrP had accumulated higher levels of PrPSc at the time of disease onset. These experiments indicate that accumulation of protease resistant PrPSc is not likely to be the proximate cause of disease, rather that subsequent events involving CmPrP are likely to be involved (Hegde et al., 1999). Furthermore, brain tissue from transgenic mice that substantially favour synthesis of PrP in the CmPrP form was not infectious, presumably since these mice can entirely circumvent the requirement for PrPSc in the development of neurodegenerative disease. Lack of transmission provides further support for the hypothesis that neurodegeneration in these genetic prion diseases is caused by CmPrP directly. Additionally, experiments with double transgenic mice which synthesise both mouse and Syrian hamster PrP revealed that following scrapie inoculation, an apparent increase in the levels of CmPrP are detected during the course of accumulation of PrPSc (Hegde et al., 1999).

The alanine to valine substitution at position 117 of PrP, A117V (Hsiao et al., 1991), causing GSS in humans lies in the hydrophobic transmembrane domain of PrP and the pathology associated with these cases of disease shares some features with mice that overexpress CmPrP. Brain tissue from cases of GSS also fails to reveal protease resistant PrP (Tateishi et al., 1990). The mechanism by
which the A117V mutation causes disease has been explored firstly in cell free systems where the mutation significantly favours the synthesis of $^{\text{Cm}}\text{PrP}$ and $^{\text{Nt}}\text{PrP}$ with a concordant decrease in $^{\text{sec}}\text{PrP}$ (Hegde et al., 1998a). Mild proteolysis has revealed increased levels of $^{\text{Cm}}\text{PrP}$ in the GSS(A117V) brain samples however no detectable protease resistant PrP$^{\text{Sc}}$ could be detected, possibly explaining why this disease is not transmissible.

A three stage model has been proposed for prion disease pathogenesis (Figure 1.6 B) (Hegde et al., 1999). In the case of infectious prion diseases, the accumulation of PrP$^{\text{Sc}}$ is initiated by inoculation. Genetic prion diseases could either work by initiating the replication and accumulation of PrP$^{\text{Sc}}$ if the mutation in question results in the spontaneous formation of PrP$^{\text{Sc}}$, with subsequent elevation in $^{\text{Cm}}\text{PrP}$ levels. Such a mechanism seems plausible for the E200K mutation which causes familial CJD (Spudich et al., 1995). Alternatively, other mutations such as A117V in GSS, could directly cause an increase in $^{\text{Cm}}\text{PrP}$ generation. Once formed, $^{\text{Cm}}\text{PrP}$ may be rapidly degraded in the ER or may be able to escape degradation to a post-ER compartment, however the mechanism by which the $^{\text{Cm}}\text{PrP}$ goes on to cause neurodegeneration and the intracellular pathways that are involved remain to be elucidated.

Based on the assumption that these findings of Lingappa and co-workers are correct, it is somewhat surprising that this correlation between $^{\text{Cm}}\text{PrP}$ and prion disease neurodegeneration has not had more impact on the field. One possible explanation for the limited study of the role of $^{\text{Cm}}\text{PrP}$ could stem from the fact that most mutations in the transmembrane domain increase the total proportion of $^{\text{Cm}}\text{PrP}$ to at most 30-40% of the total PrP chains for in vitro translation systems (Hegde et al., 1998a; Hegde et al., 1999), and to only 2% following transfection of cultured cells (Stewart and Harris, 2001). The pathway by which $^{\text{Cm}}\text{PrP}$ causes disease has recently been investigated in a further set of studies (Stewart et al., 2001). In contrast to the results above, these more recent studies demonstrated that $^{\text{Cm}}\text{PrP}$ fails to reach the cell surface after synthesis, and is retained primarily in the ER where it is subject to proteasomal degradation. It was suggested that $^{\text{Cm}}\text{PrP}$ may cause neuronal damage by activating stress-induced signalling pathways that are triggered by the accumulation of misfolded proteins in the ER. It is noteworthy that this study was performed in
Figure 1.6. PrP topogenesis (A) Models of the three topological forms of PrP. CtmPrP exhibits a dual mode of membrane attachment containing both a C-terminal GPI anchor and a transmembrane segment (Figure adapted from Stewart and Harris, 2001). (B) Three-stage model of prion pathogenesis, and possible role of CtmPrP in cell death. Stage 1 represents the formation and accumulation of PrPSc initiated by either inoculation or spontaneous conversion of a mutated PrPC to PrPSc. Stage 2 symbolises events involved in generating CtmPrP either by an unknown process involving PrPSc (dashed lines) or by certain mutations within PrP. Stage 3 represents the unknown events postulated to be involved in CtmPrP mediated neurodegeneration. (Figure adapted from Hegde et al., 1999)
cell culture, unlike the aforementioned studies where brain microsomal membranes were analysed. Furthermore, in the studies of Lingappa and colleagues above, only 20-30% of the PrP molecules were in the CtmPrP form, whereas the cell culture experiments in the later studies were performed using a mutant PrP that is synthesised exclusively in the CtmPrP form. It would be of interest to know the outcome of expressing such PrP mutants in transgenic mice.

1.3 MODELS OF PRION DISEASE

The existence of numerous in vivo and in vitro TSE models has advanced our understanding of many fundamental aspects of disease progression. In order to demonstrate the advantages of such models, I have chosen to review particular examples which have either contributed significantly to our knowledge in this field or have direct relevance to the experiments in my thesis.

1.3.1 Rodent models of prion disease

Mice and hamsters are commonly used in experimental studies to characterise the phenotype and transmissibility of prion disease. Experiments with transgenic mice have contributed to many aspects of the field, including our understanding of the molecular basis of the species barrier. Ectopic expression of PrP in knockout mice has advanced our knowledge of the host cells that are competent for prion replication. Furthermore the availability of transgenic mice overexpressing PrP has allowed an insight into the mechanism of disease pathogenesis.

1.3.1.1 The Species barrier

Transmission of prion diseases between different mammalian species is restricted by a species barrier (Pattison, 1965a). On primary passage of prions from one species to another, not all of the inoculated animals develop disease. Nascent prions synthesised in the new host comprise the same sequence as the host PrP gene and not that of the inoculating PrPsc from the donor. Those animal that do get sick have much longer and more varied incubation periods than those seen with transmission of prions within the same species, where typically all inoculated animals would fall ill within a relatively short, and consistent, incubation period. On second passage of infectivity to further
animals of the new species, transmission characteristics resemble within-species transmissions. Studies with transgenic mice expressing foreign PrP genes have identified three factors that contribute to the species barrier: firstly the difference in the PrP primary amino acid sequence between the donor and recipient species, secondly, the prion strain, and thirdly, the species specificity of an unknown factor, protein X.

Recent data on the transmission of two distinct Syrian hamster prion strains, Sc237 and 139H have demonstrated that the expression of a SHaPrP transgene in mice e.g. Tg(SHaPrP)81, leads to abrogation of the species barrier (Table 1.3) with the transgenic mice being highly susceptible to SHa prions in contrast to nontransgenic control mice (Prusiner, 1998). In addition to the PrP sequence, the strain of prion in the inoculum modified transmission of SHa prions to mice.

**Table 1.3 Influence of prion species and strain on transmission from Syrian hamsters to hamsters and mice**

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Incubation time [days ± S.E.M.] (n/n₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sc237</td>
</tr>
<tr>
<td>SHa</td>
<td>77 ± 1 (48/48)</td>
</tr>
<tr>
<td>Non-Tg mice</td>
<td>&gt;700 (0/9)</td>
</tr>
<tr>
<td>Tg(SHaPrP)81 mice</td>
<td>75 ± 2 (22/22)</td>
</tr>
</tbody>
</table>

(n/n₀= number of diseased animals/number of injected animals, data from Prusiner, 1998).

Further experiments have involved the construction of four lines of transgenic mice expressing different levels of Syrian hamster PrP (Prusiner et al., 1990; Scott et al., 1989). The length of the incubation period after inoculation with SHa prions was found to be inversely proportional to the level of SHaPrP⁰ in the brains of the Tg(SHaPrP) mice, although the levels of SHaPrP⁰ in the brains of clinically effected mice were similar in all four lines. These studies led to the conclusion that the expression of a PrP gene from one species can be both necessary and sufficient to confer susceptibility to prions derived from the species from which the PrP transgene was derived. The fact that most sporadic and acquired CJD cases occur in individuals homozygous for the PRNP polymorphic codon 129 further supports the view that prion propagation...
proceeds most efficiently when the interacting donor PrP\textsuperscript{Sc} and host PrP\textsuperscript{C} molecules are of identical primary structure (Collinge et al., 1991; Palmer et al., 1991).

It has long been recognised that prion strain type affects ease of transmission to another species. Transmission of CJD to wild-type mice is very difficult, resulting in very few, if any of the animals coming down with disease after a prolonged period of time, consistent with the presence of a species barrier (Collinge et al., 1995; Hill et al., 1997). However, transgenic mice expressing only human PrP are highly susceptible to CJD prions, consistent with a complete lack of species barrier (Collinge et al., 1995). In contrast, vCJD prions (with the same primary structure as classical CJD prions) can be transmitted with greater efficiency to wild-type mice, whereas transmission to transgenic mice is relatively less efficient than with classical CJD prions (Hill et al., 1997). An example of a species barrier where the strain component seems to predominate is that of BSE prions, which can be transmitted to a range of species efficiently, while maintaining its transmission characteristics even when passed through an intermediate species with a completely different PrP sequence (Bruce et al., 1994).

Data on the transmission of human (Hu) prions to transgenic mice are summarised in Table 1.4. Mice expressing both Mo and HuPrP are resistant to CJD prions whereas disease could be transmitted to mice expressing only HuPrP (Telling et al., 1995). In contrast, mice expressing a mouse/human chimeric PrP construct, Tg(MHu2M) as well as endogenous MoPrP were susceptible to CJD prions. The results indicate that MoPrP\textsuperscript{C} prevents the conversion of HuPrP\textsuperscript{C} into PrP\textsuperscript{Sc}, suggesting that MoPrP\textsuperscript{C} competes with HuPrP\textsuperscript{Sc} for binding to a cellular component required for conversion to PrP\textsuperscript{Sc}. MoPrP\textsuperscript{C} is hypothesised to have little effect on the formation of PrP\textsuperscript{Sc} from MHu2M because MoPrP and MHu2MPrP have the same amino acid sequence at the C-terminus. The identity of the host-encoded cellular component which facilitates formation of PrP\textsuperscript{Sc} is unknown. The factor is likely to be a protein, hence the designation protein X (Kaneko et al., 1997b; Telling et al., 1995), although there is no evidence that it is actually a protein. Whether protein X functions as a molecular chaperone is not known. Hsp70 mRNA levels have
been shown to increase in scrapie-infected mice (Kenward et al., 1994). Further studies aimed towards elucidating the identity of protein X are summarised in section 1.3.3.1.

### Table 1.4 Evidence for protein X from transmission studies of human prions

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Host</th>
<th>MoPrP gene</th>
<th>Incubation time [days ± S.E.M]</th>
<th>(n/n₀)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sCJD</td>
<td>Tg(HuPrP)</td>
<td>Prnp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>721</td>
<td>(1/10)</td>
</tr>
<tr>
<td>sCJD</td>
<td>Tg(HuPrP)</td>
<td>Prnp&lt;sup&gt;0/0&lt;/sup&gt;</td>
<td>263 ± 2</td>
<td>(6/6)</td>
</tr>
<tr>
<td>sCJD</td>
<td>Tg(MuHuM)</td>
<td>Prnp&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>238 ± 3</td>
<td>(8/8)</td>
</tr>
<tr>
<td>sCJD</td>
<td>Tg(MuHuM)</td>
<td>Prnp&lt;sup&gt;0/0&lt;/sup&gt;</td>
<td>191 ± 3</td>
<td>(10/10)</td>
</tr>
</tbody>
</table>

(n/n₀= number of diseased animals/number of injected animals, data from Prusiner, 1998).

#### 1.3.1.2 Spread of prions through the lymphoreticular system

Prions can enter the body in many different ways, although natural TSE infections are probably most often acquired by peripheral exposure. For example, BSE, vCJD and kuru prions are transmitted as a result of oral administration of infectious material (Bruce et al., 1997; Gajdusek et al., 1966; Hill et al., 1997). Iatrogenic CJD in humans can be transmitted through transplantation of CJD-contaminated tissues or parenteral administration of pituitary derived hormones and gonadotropins (Gibbs et al., 1993). Following peripheral infection of scrapie into mice, infectivity and PrP<sup><6c></6c></sup> rapidly accumulate in lymphoid tissues long before either is detectable in the CNS (Mabbott and Bruce, 2001). Infectivity can accumulate in all components of the lymphoreticular system including lymph nodes and intestinal Peyer’s patches where prions replicate almost immediately after oral administration to mice (Kimberlin and Walker, 1989). The involvement of lymphoid tissues in TSE pathogenesis may be strain-dependent, as BSE prions are hardly detected in the lymphoid organs of cows and sporadic CJD in humans appears to be confined to the nervous tissue (Hill et al., 1999b; Somerville et al., 1997). On the other hand, in vCJD (Bruce et al., 2001; Hilton et al., 1998), most sheep with natural scrapie (van Keulen et al., 1996) and rodents with experimental scrapie (Mabbott et al., 2000; McBride et al., 1992), infectivity accumulates in lymphoid
tissues; abnormal PrP is detected on follicular dendritic cells (FDCs), which are known to express high levels of PrP\(^C\) and are intimately associated with lymphocytes which also express PrP\(^C\). Prions also accumulate on tingible body macrophages within germinal centres (GCs).

A wealth of studies indicate roles for FDCs, macrophages and lymphocytes in scrapie pathogenesis. The role of T and B lymphocytes in pathogenesis was investigated using transgenic and immunodeficient mice with deficiencies in various compartments of the immune system (Fraser et al., 1996; Klein et al., 1997; Klein et al., 1998). Following peripheral infection, deficiencies in the T cell compartment alone have no effect on disease susceptibility or infectivity in the spleen, whereas the accumulation of scrapie infectivity in the spleen and subsequent neuroinvasion are significantly impaired in mice deficient in B cells alone, or in severe combined immunodeficient SCID mice, deficient in both mature B and T lymphocytes. Any genetic defect that impairs the terminal differentiation of B-cell precursors into antibody-secreting cells, completely blocks the colonisation of lymphoid organs by prions. B lymphocytes are known to provide important signals for the maturation and maintenance of other cell types in GCs, for example, tumour necrosis factor-alpha (TNF-alpha) secretion by B lymphocytes is important for maintaining FDC networks (Chaplin and Fu, 1998; Kosco-Vilbois et al., 1997). Sub-lethal whole body gamma-irradiation has no effect on scrapie pathogenesis when administered shortly before or after peripheral scrapie challenge (Fraser and Farquhar, 1987). Since B lymphocytes and T lymphocytes are actively dividing cells, scrapie replication could depend on FDCs which are radioresistant and non-dividing (Brown et al., 1973; Tew et al., 1982) and it is possible that the necessity for B cells could be an indirect one to stimulate FDCs to maintain their differentiated state. Indeed, studies with immunodeficient mice have concluded that prevention of FDC maturation coincides with the absence of detectable infectivity and reduced disease susceptibility in peripherally challenged mice (Brown et al., 1999b; Mabbott et al., 2000). Similar studies with immunodeficient mice suggest that as with natural TSE diseases, some TSE strains target different cell populations in peripheral tissues (Mabbott and Bruce, 2001).
Chimeric mice with a mismatch in PrP expression between FDCs and surrounding lymphocytes have been created by grafting bone marrow from PrP-deficient mice into PrP-expressing mice, and vice versa (Blattler et al., 1997; Brown et al., 1999b; Klein et al., 1998). Following peripheral challenge of these mice with the ME7 scrapie strain, high levels of infectivity accumulated in the spleen only in the presence of PrP expressing FDCs, independently of PrP expression in the donor bone marrow or lymphocytes. In contrast, using the RML scrapie strain, not only did high levels of infectivity accumulate in the spleens of mice with PrP expressing FDCs in the absence of PrP expression by lymphocytes, but also, in the absence of PrP expression by FDCs as long as PrP was expressed by lymphocytes or other haemopoietically derived cells (Blattler et al., 1997; Klein et al., 1998). The fact that high levels of RML scrapie infectivity accumulate in the spleen following reconstitution of PrP-deficient mice with PrP expressing foetal liver or bone marrow cells (Blattler et al., 1997) raises the possibility that RML scrapie strain, unlike ME7, may target both PrP-expressing FDCs and lymphocytes (Mabbott and Bruce, 2001).

Unlike FDCs which have been shown to be a site of accumulating infectivity, tingible body macrophages within GCs are a site of destruction. Intra-lysosomal PrP accumulations have been found in tingible body macrophages in spleens of scrapie-infected mice (Jeffrey et al., 2000). It has been observed that depletion of macrophages before or shortly after peripheral scrapie infection increases the accumulation of infectivity and PrPSc in the spleen, and shortens the incubation period (Beringue et al., 2000). Furthermore, extended in vitro culture with macrophages leads to a decrease in scrapie infectivity (Carp and Callahan, 1982). These data suggest that macrophages act by sequestering scrapie infectivity and impair early scrapie agent replication. FDCs characteristically trap and retain antigens on their surfaces in the form of immune complexes composed of antigen, antibodies and/or the third component of complement. Complement is thought to play a role in the localisation and retention of scrapie to FDCs during the first few days after peripheral infection (Klein et al., 2001; Mabbott et al., 2001), although the mechanism of interaction between PrPSc and complement components is unknown.
The transport of the infectious agent from the site of entry into the body and to the CNS is of crucial importance for the development of neurological disease. Adoptive bone marrow transfer of PrP\(^C\) expressing cells into PrP\(^C\) knockout mice restores replication and accumulation of prions in the lymphatic tissue following peripheral inoculation, but does not restore transport of the agent to the brain (Blattler et al., 1997). This indicates that a non-haemopoietic PrP\(^C\)-expressing tissue is required for neuroinvasion. The arm of neuroinvasion that takes prions from GCs to the CNS probably involves peripheral nerves (which express PrP\(^C\) in humans and animals) belonging to the autonomic nervous system (Baldauf et al., 1997; Glatzel and Aguzzi, 2000; Glatzel et al., 2000). This is supported by the finding that disease and prion replication occur first in the CNS segments to which the sites of peripheral inoculation project (Beekes et al., 1996; Kimberlin and Walker, 1980). Additionally, recent studies with transgenic and knockout mice expressing HaPrP under the control of the neuron-specific enolase (NSE) promoter have confirmed that HaPrP expression in peripheral nerves is sufficient for successful infection of the brain, following oral or intraperitoneal infection (Race et al., 2000). However, since the GCs within lymphoid tissue are poorly innervated, it is not known how infectivity reaches peripheral nerve endings from FDCs. Lymphocytes might be involved in the migration of prions to peripheral nerves. The replication and transport of prions within peripheral nerves could in principle involve both axonal and nonaxonal transport mechanisms. Studies of the rate of transport of infectivity in peripheral nerves in mice has been estimated at 1-2 mm per day (Glatzel and Aguzzi, 2000; Kimberlin et al., 1983) which does not obviously correspond to either slow or fast axonal transport. A mode of transport has been suggested for the PNS whereby PrP\(^{\infty}\) converts localised PrP\(^C\) with directional propagation towards the CNS (Glatzel and Aguzzi, 2000).

A recent study implicates a role for erythroid cells in the progression of prion diseases (Miele et al., 2001). The levels of erythroid differentiation-related factor (EDRF, expressed specifically in cells of the erythroid lineage) are substantially decreased in animals during TSE pathogenesis although the specific means by which this occurs is unknown. Co-expression of EDRF and PrP transcripts in bipotent E/Meg cells (capable of developing along either the erythroid or megakaryocyte lineages) was observed in these studies, and it was suggested
that these cells could play a role in TSE agent replication or disease pathogenesis, and could provide a mechanism whereby prion diseases could affect EDRF expression levels in erythroid lineage cells.

1.3.1.3 Spread of prions in the CNS

Early evidence for the axonal spread of prions has come from studies involving intraocular prion injection in wild-type mice (Fraser, 1982). Scrapie pathology and replication of infectivity occur along the anatomical structures of the visual system, and spread to transsynaptic structures, although again the very slow kinetics of disease development argues against the mode of spread being fast or even slow axonal transport.

Neurografting experiments with knockout mice have been used to demonstrate the dependence of prion spread in the CNS on PrP\(^{\text{C}}\) expression on neural pathways. In initial experiments, brain grafts of PrP-expressing neuroectodermal tissue were placed intracerebrally into scrapie resistant knockout mice (Weissmann et al., 1999). Mice were inoculated with prions intraocularly so as to avoid direct physical trauma to the brain, while still contacting the CNS through the retina. In all experiments, no signs of PrP\(^{\text{Sc}}\) or accumulation or disease was detected in the grafts. In order to rule out the possibility that an immune response to PrP\(^{\text{C}}\) presented in the grafts could neutralise infectivity in the above studies, further experiments were designed. The experiments were repeated using PrP tolerant knockout mice, which are scrapie resistant but overexpress PrP on T-lymphocytes. As before, intraocular prion inoculations did not provoke scrapie in the graft. These experiments concluded that the expression of PrP\(^{\text{C}}\) seems to be necessary for the spread of prions along the retinal projections and within the intact CNS. It has been hypothesised that the spread of prions might proceed along a chain of cells expressing PrP\(^{\text{C}}\), which, when interrupted by PrP\(^{\text{Sc}}\)-lacking cells, prevents propagation of prions to the target tissue (Brandner et al., 1996b).

Further evidence for the transport of PrP\(^{\text{Sc}}\) along axons in scrapie has come from investigations of the rate and pattern of PrP\(^{\text{Sc}}\) accumulation using quantitative Western blot analysis (DeArmond et al., 1987; Jendroska et al., 1991) and the histoblot technique (Taraboulos et al., 1992a). Following unilateral
intrathalamic inoculation of the Sc237 strain into Syrian hamsters, the first brain region in which PrP^c was detectable by Western blot analysis after 14 to 21 days was the thalamus (Jendroska et al., 1991). PrP^c was first detected unilaterally in the thalamus after 7 days by histoblot analysis (Hecker et al., 1992). The progressive spread of disease to other brain regions neuroanatomically interconnected with the thalamus such as the neocortex, and extending to layers where thalamocortical pathways terminate, argues that the PrP^c is transported by axons. Spread of PrP^c to regions not interconnected with the thalamus such as the contralateral thalamus and septum, was detected after 21 and 28 days respectively. The symmetrical identification of PrP^c in homologous nuclei in the contralateral thalamus suggests that PrP^c released from specific populations of neurons into the extracellular space has a propensity to target homologous contralateral neurons. PrP^c also appears to be transported via the cerebrospinal fluid (CSF) to the walls of the lateral ventricles after 7 days where it accumulates, and the septum is one of the regions specifically targeted. Using these techniques, distinct strains of prions have been shown to produce different kinetics and patterns of PrP^c accumulation in Syrian hamster brains. One hypothesis is that such regional differences in PrP^c accumulation could be due to cell-specific replication of prions (Hecker et al., 1992).

1.3.1.4 Prion pathogenesis

The neuropathologic features characteristic of prion disease include spongiform (vacuolar) degeneration of the brain parenchyma, nerve cell degeneration and death, variable reactive astrocytic gliosis, and variable amyloid plaque formation (Prusiner et al., 1990). There are several reasons to believe that these changes are a result of PrP^c accumulation. First, PrP^c is specific for prion diseases and is not found in any other neurodegenerative disorder (Prusiner and DeArmond, 1987; Serban et al., 1990). Second, the only organ to show recognisable pathologic changes is the brain which is where the highest concentrations of PrP^c are found (Oesch et al., 1985). Third, amyloid plaques, which are a characteristic feature of prion disease, have been shown to consist of PrP^c (DeArmond et al., 1985; Prusiner and DeArmond, 1987) and not the beta-amyloid protein of Alzheimer’s disease (Roberts et al., 1988). Finally, there
is a temporal correlation between the accumulation of PrP$_{Sc}$ and the development of pathology within a brain region (Jendroska et al., 1991).

Neuropathologic profiles of spongiform degeneration have also been used to characterise prion strains (Fraser and Dickinson, 1968). FFI prions inoculated into Tg(MHu2M)/PrP$_{0/0}$ mice results in PrP$_{Sc}$ deposition mainly in the thalamus, similarly to FFI in humans (Medori et al., 1992; Telling et al., 1996). In contrast, PrP$_{Sc}$ accumulation in mice inoculated with fCJD(E200K) prions was deposited in many brain regions including the thalamus, the hypothalamus, cerebral cortex, the striatum and spinal cord, similar to the wide distribution of neuropathologic changes in the human disease counterpart. These data support the hypotheses that there are multiple strains of human prions, as there are for scrapie, and their properties remain relatively unchanged following passage from humans to the Tg(MHu2M)/PrP$_{0/0}$ mouse.

Exposure of the brain of a PrP-knockout mouse to scrapie prions does not induce propagation or cause neuropathologic changes, indicating that exogenously derived PrP$_{Sc}$ is not pathogenic by itself (Bueler et al., 1993; Manson et al., 1994b). This is consistent with experiments in knockout mice with PrP-overexpressing brain grafts, which demonstrated that after a scrapie prion inoculation, large amounts of PrP$_{Sc}$ accumulated in the graft, while the surrounding cells remained healthy even though PrP$_{Sc}$ was detected in the surrounding host brain (Brandner et al., 1996a). These studies imply that the conversion of PrP$_{C}$ to PrP$_{Sc}$, and not just the presence of PrP$_{Sc}$, is a requirement for neurodegeneration. It is a consistent finding that PrP$_{Sc}$ is only cytotoxic for cells that express PrP$_{C}$; when a PrP peptide corresponding to residues 106-126, was added to cultured neurons from mouse brain, the cells died (Forloni et al., 1993). In contrast, exposure of cultured neurons from a PrP-deficient mouse to the same peptide had no effect (Brown et al., 1994). These results argue that the expression of PrP$_{C}$ by a cell, rather than the extracellular deposition of PrP$_{Sc}$, is the critical determinant of scrapie pathology. Although PrP$_{C}$ expression is required for disease susceptibility, a number of observations argue that PrP$_{Sc}$ may not actually be highly neurotoxic. Mice with reduced levels of PrP$_{C}$ expression accumulate extremely high levels of PrP$_{Sc}$ in their brains and remain healthy for several months after their wild-type counterparts succumb (Bueler
et al., 1994). In contrast, Tg20 mice, with high levels of PrP<sup>C</sup>, have short incubation periods and produce low levels of PrP<sup>Sc</sup> after inoculation with mouse prions (Fischer et al., 1996). In fact, a gene dosage effect has been reported to determine the time of disease onset in mice following infection with the ME7 strain of scrapie (Manson et al., 1994b). Furthermore, prion diseases in which PrP<sup>Sc</sup> is barely detectable have also been described (Hegde et al., 1998a; Hsiao et al., 1994; Lasmezas et al., 1997; Manson et al., 1999). As detailed in section 1.2.5, it has been suggested that aberrant regulation of protein topology at the ER can result in neurodegeneration (Hegde et al., 1998a; Hegde et al., 1999), and it is possible that C<sup>cm</sup>PrP and PrP<sup>Sc</sup> may contribute independently to disease or may form part of a common biochemical pathway. Nonetheless, the precise cause of neurodegeneration in prion disease remains unclear.

As summarised above, the use of rodent models as an approach to understanding the pathogenesis of prion disease has contributed a wealth of knowledge and has provided an insight into the mechanisms underlying the generation of PrP<sup>Sc</sup> from PrP<sup>C</sup>. Nonetheless, although allowing an in depth study of disease pathogenesis and the molecular basis of strain diversity and the species barrier to be examined in a native environment, these in vivo models are limited in that they do not permit the factors governing propagation to be analysed at the molecular and cellular levels.

### 1.3.2 Formation of protease resistant PrP in cell free systems

Studies in cell-free systems have provided the opportunity to directly investigate the formation of PrP-res under much simpler and defined conditions. These studies have provided an insight into the mechanism of PrP-res formation and the molecular basis for agent replication. Such models have also contributed to our knowledge of the species barrier and the propagation of distinct strains. The various abnormal forms of PrP e.g. PrP<sup>Sc</sup>, PrP<sup>CJD</sup>, PrP<sup>RSE</sup> are considerably more resistant to proteinase K than the normal cellular counterpart (PrP<sup>C</sup> or PrP-sen). These protease resistant prion proteins (PrP-res) also form insoluble aggregates, and have a higher beta-sheet content than PrP-sen.
Initial attempts to generate PrP-res by mixing a unitary ratio of full length PrP\textsuperscript{C} and PrP\textsubscript{Sc} were unsuccessful (Raeber et al., 1992). However subsequent studies involving mixing an excess (>50 fold) of PrP-res purified from scrapie infected brain tissue, with $[^{35}\text{S}]$-labelled PrP-sen, led to the conversion of $[^{35}\text{S}]$-PrP-sen to $[^{35}\text{S}]$-PrP-res (Kocisko et al., 1994; Raymond et al., 1997). This is the inverse of the ratio that probably exists during initiation of prion infection \textit{in vivo}, however, the conversion activity was shown to be PrP-specific, PrP-res dependent and stimulated by the partial reversible unfolding of PrP-res using GdnHCl (Kocisko et al., 1994). About 50\% of the $[^{35}\text{S}]$-labelled PrP\textsuperscript{C} was recovered in complexes, although, the percentage of PrP\textsuperscript{C} that had acquired protease resistance increased slowly and reached 10-15\% after 48 hours. The interaction between PrP\textsuperscript{C} and PrP\textsubscript{Sc} was found to be inhibited by antibodies binding towards the amino terminus of the putative H1 region (see figure 1.1 A).

In other experiments PrP-sen was incubated with a 5000-fold molar excess of certain synthetic PrP peptides e.g. SHaPrP(90-145[A117V]) (Kaneko et al., 1995). Such PrP peptides were able to induce the conversion of PrP-sen into proteinase K resistant complexes that polymerised into fibrils. In these experiments approximately 50\% of the PrP\textsuperscript{C} acquired protease resistance after 48 hours. The amounts of newly formed PrP-res in these experiments are generally too low to test for infectivity in the background of excess PrP-res from the inoculum. However the issue of infectivity has been addressed by the use of a hamster-mouse chimeric MH2M protein (Hill et al., 1999a). The hamster adapted scrapie strain Sc237 is infectious to transgenic mice expressing MH2M PrP\textsuperscript{C} and generates MH2M PrP\textsubscript{Sc} that is infectious to wild-type mice. However, although incubating MH2M PrP-sen with Sc237 prions generated MH2M PrP-res, it was not found to be infectious to mice by bioassay. Although it was unclear from these studies whether the levels of MH2M PrP\textsubscript{Sc} generated \textit{in vivo} for the control experiments were the same as those produced \textit{in vitro}, it seems unlikely that the protease resistant MH2M PrP produced \textit{in vitro} possesses infectivity. It was suggested by these studies that the acquisition of protease resistance by PrP\textsuperscript{C} \textit{in vitro} is not sufficient for the propagation of infectivity, but additional, as-yet-unidentified cofactors may be required.
As discussed in section 1.3.1.1, the difference in sequence between donor PrP\textsuperscript{Sc} and recipient PrP\textsuperscript{C} is one of the factors that contributes to the species barrier and it has been suggested that interactions between PrP\textsuperscript{Sc} and PrP\textsuperscript{C} molecules were required for efficient PrP\textsuperscript{Sc} formation and recipient susceptibility to TSE disease. As expected, cell-free conversion reactions also require PrP sequence specificity. This conclusion was based on experiments using combinations of PrP-res and PrP-sen from a number of species including mice, hamsters, sheep, cattle and human which showed a strong correlation with data on the in vivo transmissibilities of the corresponding agents within or between species (Kocisko et al., 1995). These correlations also extended to in vivo transmission of prions between PrP molecules with different gene polymorphisms (Bossers et al., 1997). Structural differences in PrP-res have been correlated with different strains of TSEs (Bessen and Marsh, 1994). Two examples of hamster-adapted transmissible mink encephalopathy strains are hyper (HY) and drowsy (DY) based on their characteristic phenotype in diseased animals. These strains differ in conformation as a result of differential cleavage by proteinase K. Incubation of HY and DY PrP-res with hamster PrP-sen, induced the strain-specific conversion of hamster PrP-sen into PrP-res.

The cell-free conversion assay has been further adapted to conditions representative of intact brain tissue (Bessen et al., 1997). These in-situ conversion assays involve the incubation of \textsuperscript{35}S-PrP-sen with an infected brain slice, followed by proteinase K digestion of any unconverted PrP-sen. Such experiments reveal that newly formed \textsuperscript{35}S-PrP-res was formed in, and bound to infected, but not uninfected brain slices distributed in a pattern matching pre-existing PrP-res and was not released into the medium. The cell-free conversion assay has also been used to elucidate the mechanism of PrP-res formation (Caughey et al., 1995). Analysis of the physical size of the converting activity indicated that it was associated only with ordered multimers of PrP-res with variable size. The polymerised state of PrP-res also correlates with its resistance to proteinase K, its behaviour in the presence of 2.5-3 M GdnHCl and the presence of infectivity (Caughey et al., 1997; Kocisko et al., 1996). These observations together with the data on in situ conversion are consistent with a nucleated polymerization mechanism of PrP\textsuperscript{Sc} formation and inconsistent with a heterodimer mechanism.
During the incubation period of an *in vivo* infection, minute amounts of PrP<sup>Sc</sup> are responsible for the conversion of the host PrP<sup>C</sup>, which is in excess, into large amounts of aggregated PrP<sup>Sc</sup>. Such events can be more accurately modelled using the sensitive detection method of cyclic amplification of protein misfolding (Saborio et al., 2001). This highly efficient technique is analogous to the polymerase chain reaction, and could offer diagnostic advantages for currently undetectable levels of infectious agent.

### 1.3.3 Cell culture models of prion disease

Considerable efforts have been made to set up tissue culture models for genetic forms of prion disease and cellular cultures supporting the replication of the infectious agent. These models present obvious advantages, including the ability to analyse the biological properties of PrP<sup>C</sup> both at a molecular and cellular level, and the ability to analyse the nature of the infectious agent as well as the factors governing its propagation. Cell cultures expressing significant amounts of PrP (constitutively or after transfection) have allowed the identification of the major steps of intracellular trafficking of PrP (see section 1.2.2). Furthermore, although most PrP binding partners and putative receptors have been demonstrated by yeast two-hybrid approaches (see section 1.1.8), the search has also been pursued in cell culture. These culture models also offer therapeutic advantages in the screening of drugs with diagnostic applications.

#### 1.3.3.1 Infectious prion disease

Several cell types are thought to produce PrP<sup>Sc</sup> following prion infection *in vivo*, including neurons, astrocytes, and cells of the lymphoreticular system (Blattler et al., 1997; DeArmond et al., 1987; Diedrich et al., 1991). To generate prion-infected cell lines, cells are generally incubated in the presence of infectious extracts, either crude brain homogenate, or purified scrapie associated fibrils. However, only some neuronally derived cell lines appear to be susceptible to scrapie prions *in vitro*, including N2a mouse neuroblastoma cells (Butler et al., 1988; Race et al., 1988), PC12 rat pheochromocytoma cells (Rubenstein et al., 1991), T-antigen immortalised hypothalamic neurons (GT1) and the spontaneously immortalised hamster brain cells (HaB), which were reinfected following passage of the cells in culture (Taraboulos et al., 1990) (Figure 1.7 A). It has been established recently that rabbit kidney epithelial cells
overexpressing a transfected copy of the ovine PrP gene became susceptible to scrapie prions. This demonstrates not only that nonneuronal cells are able to replicate the infectious agent but also that a cell line from a different animal origin than the expressed PrP can be successfully infected (Vilette et al., 2001).

It is thought that the infection is caused by the conversion of the endogenous PrP\textsuperscript{C} to the infectious form. Successful cell infection is tested by bioassay after inoculation of the cells into mice brains, as well as by biochemical detection of PrP\textsuperscript{Sc}, which is both quicker and easier to perform. The infected cells display no obvious cytopathology, with the exception of the GT1 cells, a subpopulation of which undergo apoptosis (Schatzl et al., 1997). Furthermore, PC12 cells have a slightly modified phenotype following infection (Rubenstein et al., 1991). It has also been demonstrated that scrapie infection of N2a and hamster brain cells induces abnormalities in receptor-mediated calcium responses, although these abnormalities do not appear to affect the growth or viability of the cells (Kristensson et al., 1993). All the above lines can be cloned and maintained in culture for many passages. I have chosen to review the contributions of several cell lines that have been widely used to investigate the biology of both the normal and pathological isoforms of the prion protein.

1.3.3.1.1 N2a cells
ScN2a cells have proved the most useful cell line for studying the cell biology of prion replication (see section 1.2). This line has been used to determine the subcellular localisation of PrP\textsuperscript{Sc}, as well as the site and kinetics of PrP\textsuperscript{Sc} formation (Borchelt et al., 1990; Borchelt et al., 1992; Caughey and Raymond, 1991; McKinley et al., 1991; Taraboulos et al., 1990). Expression of chimeric PrP proteins in ScN2a cells has demonstrated that specific amino acids are determinant for the passage of a prion strain from one species to another. Interactions between PrP\textsuperscript{C} and PrP\textsuperscript{Sc} are proposed to occur during the de novo generation of PrP\textsuperscript{Sc} (Kocisko et al., 1994) and also when PrP\textsuperscript{Sc} probably interacts with PrP\textsuperscript{C} present on the surface of neurons and astrocytes during prion propagation (Race et al., 1995; Raebet et al., 1997). PrP interactions are implicated in the species barrier to transmission and prolongation of the scrapie incubation period in transgenic mice expressing heterogeneous PrP molecules (Prusiner et al., 1990).
Expression of heterogeneous PrP molecules

Interactions between heterogeneous PrP molecules have been studied in ScN2a cells following the expression of hamster PrP in these cells which results in profound interference of conversion of the endogenous mouse PrP* into PrP^sc (Table 1.5) (Priola et al., 1994; Priola and Chesebro, 1995). In these experiments, PrP was detected using either polyclonal rabbit anti PrP peptide serum (R27) (Caughey et al., 1991) which was reactive with all the PrP molecules used in the series of experiments, or the monoclonal antibody 3F4, reactive with hamster PrP (and recombinant PrP molecules with methionine residues at positions 108 and 111), but not with mouse PrP (Kascak et al., 1987). In subsequent studies, it was established that homology in a single region of mouse PrP from amino acids 112 to 138 is important in the conversion of mouse PrP^c to PrP^sc (Priola and Chesebro, 1995). Expression of mouse PrP molecules with hamster methionine residues substituted at positions 108, 111 and 138, as opposed to the mouse residues Leu, Val and Ile respectively, prevented conversion of these mutant molecules as well as the endogenous mouse PrP^c. However, expression of a mouse PrP molecule with hamster specific methionines at position 108 and 111 and mouse specific Ile (or Leu, the corresponding rat amino acid) at position 138, lead to the detection of a large amount of 3F4-positive PrP^sc. These experiments concluded that residue 138 alone is capable of modulating the conversion process. Additionally, despite conversion of the mutant PrP molecule, there was a dramatic decrease in the total amount of PrP^sc accumulated as detected by R27, indicating that compared to the normal mouse PrP^c, the mutant PrP molecule was preferentially incorporated into PrP^sc.

Function of protein X

As detailed in section 1.3.1.1, studies on the transmission of human prions to transgenic mice suggested that another molecule, provisionally designated protein X, participates in the formation of PrP^sc (Telling et al., 1995). Whether protein X functions as a chaperone is unclear, but it has been observed that cultures of scrapie-infected cells show major differences in the induction of some heat-shock proteins (Tatzelt et al., 1996b; Tatzelt et al., 1995). In N2a cells, both Hsp28 and Hsp72 are highly induced in response to stress, although ScN2a cells have been observed to depart from the normal response, in that they fail to induce these two proteins. It is noteworthy that recently it has been
suggested that these differences may be due to a cloning artefact (Bosque and Prusiner, 2000). Furthermore, Hsp73 is constitutively expressed, is usually found in the cytoplasm, and is translocated to the nucleus in response to stress in normal N2a cells. In contrast, in ScN2a cells, Hsp73 shows an abnormal punctuate staining pattern in the cytoplasm reminiscent of the staining pattern exhibited by scrapie accumulation in cells, and Hsp73 fails to be translocated to the nucleus in response to stress (Tatzelt et al., 1995). Yeast two hybrid analysis has shown that PrP interacts with a number of proteins including Bcl-2 and Hsp60 (Edenhofer et al., 1996; Kurschner and Morgan, 1995). A similar approach has identified the laminin receptor protein as another molecule capable of binding to PrP (Rieger et al., 1997).

Table 1.5 Influence of the expression of hamster PrP and mutant mouse PrP molecules on PrP-res formation in ScN2a cells

<table>
<thead>
<tr>
<th>Background</th>
<th>Position</th>
<th>3F4 Epitope</th>
<th>3F4-positive PrP-res (a.u)</th>
<th>Total PrP-res (a.u)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>108</td>
<td>111</td>
<td>138</td>
<td></td>
</tr>
<tr>
<td>Mouse</td>
<td>Leu</td>
<td>Val</td>
<td>Ile</td>
<td>NA</td>
</tr>
<tr>
<td>Hamster</td>
<td>Met</td>
<td>Met</td>
<td>Met</td>
<td>0</td>
</tr>
<tr>
<td>Mouse</td>
<td>Met</td>
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<td>Mouse</td>
<td>Met</td>
<td>Met</td>
<td>Ile</td>
<td>100</td>
</tr>
<tr>
<td>Mouse</td>
<td>Met</td>
<td>Met</td>
<td>Leu</td>
<td>100</td>
</tr>
</tbody>
</table>

Due to a single amino acid deletion in mouse PrP, homologous residues between mouse and hamster molecules differ by 1 in their numbering. The numbering for mouse residues are used above (data from Priola and Chesebro, 1999).

Experiments with ScN2a cells transfected with chimeric PrP genes have provided evidence for protein X binding to a discontinuous epitope on PrP<sup>C</sup> (Kaneko et al., 1997b). These experiments were based on the findings of the results with transgenic mice and also studies that have shown that the N-terminus of PrP is not required for PrP<sup>sc</sup> formation (Rogers et al., 1993). Seven amino acids distinguish HuPrP from MoPrP between residues 168 and 231. Four of these are close to the GPI anchor, while the remaining three (residues 214, 218 and 219) are within the C-terminal alpha-helix (Huang et al., 1994). A series of recombinant molecules were constructed by replacing these three
mouse residues with their human counterparts. In order to distinguish the recombinant PrP\textsuperscript{Sc} from endogenous wild-type MoPrP\textsuperscript{Sc}, these mutations were introduced into a SHa/Mo chimeric PrP, designated MHM2, that contains the 3F4 epitope (Scott et al., 1992). The conclusions from these studies were first that the substitution of a Hu residue at either position 214 or 218, prevented the conversion of MoPrP\textsuperscript{C} into MoPrP\textsuperscript{Sc}. The side chains of these residues protrude from the same surface of the C-terminal alpha-helix and form a discontinuous epitope with residue 167 and 171 in an adjacent loop. Second, the substitution of a basic residue at position 167, 171, or 218 also prevented conversion. It was suggested that the mechanism of action of these mutant PrP molecules was to bind to protein X and sequester it from functioning in the replication process, thereby acting as dominant negatives.

**Improving susceptibility to infection**

One of the limitations of working with cell lines is their lack of sensitivity to infection, which could be attributable to low levels of PrP\textsuperscript{C} expression (Chesebro et al., 1993). *In vivo* transmission studies with transgenic mice have clearly demonstrated an inverse correlation between the expression level of PrP\textsuperscript{C} in host animals and the incubation time of experimental prion diseases (Manson et al., 1994b). This has been confirmed by experiments with N2a cells, which have revealed that PrP overexpression renders cell lines readily infectable to multiple mouse-adapted scrapie strains (Nishida et al., 2000). In these experiments, stable cell lines were generated by expressing various levels of type A MoPrP in N2a cells and subjecting the cells to ex-vivo transmission of mouse adapted scrapie strains. The scrapie strains used were 22L, 139A and Chandler (obtained from mice with Prnp allotype a/a) and 22A and 87V (obtained from mice with Prnp allotype b/b). The cells were readily susceptible to 2% homogenates of 22L, 139A and Chandler prions (this could be diluted to 0.2% for 22L and Chandler prions), but not to 22A or 87V. The inoculated cells persistently produced PrP\textsuperscript{Sc} for over 30 passages, even without subcloning. As expected, the relative level of PrP\textsuperscript{Sc} production correlated with the level of PrP\textsuperscript{C} expression in the cell lines. The frequency of infection was estimated to be around 13% in the cells with increased levels of PrP expression (as determined by limited dilution subcloning and subsequent PrP\textsuperscript{Sc} analysis), compared to
previous reports for untransfected N2a cells being less than 1% (Race et al., 1987).

Similar results were obtained upon infection of a GT1 subclone, GT1-7, with mouse prions, which expressed PrPSc at even higher levels than the transfected N2a cells. Cell to cell transmission of PrPSc was also demonstrated by incubating cell lysates from the infected cells with noninfected GT1-7 recipient cells which resulted in successful conversion. Experiments with non-neuronal and non-murine cells expressing large amounts of MoPrPSc failed to show infection. This is indicative of an influence of certain tissue- or species-specific factors, other than PrPSc, in the propagation mechanism.

Other attempts to obtain cultures that produce sufficient quantities of PrPSc for biochemical analysis have focussed on identification of sublines of uninfected N2a cultures that are highly susceptible to infection, and hence avoid the requirement for postinfection cloning (Bosque and Prusiner, 2000). Uncloned N2a cultures exposed to exogenous prions produce only low levels of PrPSc and this has been attributed to the fact that only a minor percentage of the cells become infected (Race et al., 1988). Many comparative studies have been based on differences between subclones of ScN2a cells and the cells from which they were derived, and as a result, any observed differences could be artifactual or a result of cloning; thus properties specific to the scrapie infected cells may only be found in a minority of the cells in the parent population (Bosque and Prusiner, 2000). This is illustrated by the differential expression of Hsp72 and Hsp28 in N2a and ScN2a cells (Tatzelt et al., 1995) which was later suggested to be a cloning artefact (Bosque and Prusiner, 2000).

The first step in the generation of N2a cell lines highly susceptible to prion infection involved the creation of a sensitive cell blotting assay that was able to detect de novo generated prions (Bosque and Prusiner, 2000). Following four days of co-culture of ScN2a/N2a cells at a ratio of 1:100, PrPSc could be detected by the blotting assay whereas Western blotting could only detect PrPSc when 10% of the cells were initially infected with prions. Analysis of a number of sublines of N2a cells using this technique typically concluded that 20% of the lines were highly susceptible to infection with RML (rocky mountain
laboratories) prions, 20% of the sublines were resistant, and the remainder had intermediate susceptibilities, despite having a range of levels of PrP<sup>C</sup> production. The results were reproducible for each subline although in continuous culture there was some reduction in the level of de novo generated PrP<sup>Sc</sup> in susceptible sublines. The susceptible sublines were used to establish cultures in which virtually every cell was infected without further subcloning.

These sublines were used to generate a rapid bioassay for prions using either a brain homogenate from a mouse infected with RML prions, or homogenates of ScN2a cells infected with RML prions. Following exposure of susceptible sublines to either prion source and analysis by dot blot after 20 days, this cell culture bioassay system was found to be nearly as sensitive to ScN2a derived prions as is the conventional mouse bioassay following intracerebral prion inoculation. However, the cell culture system was nearly three orders of magnitude less sensitive to brain derived prions compared to ScN2a derived prions, as determined by measurement of the infectious titre of inoculating prion preparations.

A number of possible explanations were proposed to explain these differences in susceptibility (Bosque and Prusiner, 2000). Firstly, N2a cells could be more susceptible to homogenous ScN2a cells than a brain homogenate consisting of a mixture of cell types. A greater effectiveness of in vivo prion clearing for the ScN2a derived prions compared to the brain derived prions could result in lowering of the in vivo infectious titre. Another suggestion was that certain prion inhibitory factors may be present in brain homogenate, which may be more effective with cultured cells than in vivo. It was also suggested that factors in the brain parenchymal milieu could be more effective than the tumour cells in culture at mobilising prions in the brain homogenate. One drawback to the susceptible sublines was that they were only responsive to RML prions and not to other strains, not even to highly concentrated ME7 prions which have an incubation time indistinguishable from the RML strain in CD-1 mice. Although this could be attributed to a lower infectious titre in the ME7 homogenate than the RML homogenate, this observation is consistent with the fact that certain subpopulations of neurons in the brain accumulate more PrP<sup>Sc</sup> with some strains than others (Hecker et al., 1992). The demonstration in these studies that
some sublines of N2a were resistant to scrapie infection, further indicates the existence of cellular factors, other than PrP\(^c\), which can inhibit or promote prion propagation.

**Therapeutic applications**

Scrapie infected neuroblastoma cells also have therapeutic and diagnostic applications, and have been used to search for inhibitors of PrP\(^{Sc}\) formation. The use of ScN2a cells as a model has identified that Congo red, an amyloid-binding dye, potently inhibits the accumulation of PrP\(^{Sc}\), without affecting the metabolism of PrP\(^c\) (Caughey and Race, 1992). It was suggested that Congo red acted by disrupting the conversion of PrP\(^c\) to PrP\(^{Sc}\), or by destabilising PrP\(^{Sc}\) once it had been formed. As a result, ScN2a cells could be incubated with submicromolar concentrations of the dye and within one week the amount of detectable PrP\(^{Sc}\) would be reduced by more than 90%. Another putative mechanism of action for Congo red has been based on the observation that partial denaturation may be required for PrP\(^{Sc}\) to serve as template in the PrP\(^c\) to PrP\(^{Sc}\) conversion. It was proposed that Congo red inhibits prion propagation by binding to and `hyperstabilising' the conformation of PrP\(^{Sc}\) molecules (Caspi et al., 1998).

Other types of molecules have now been demonstrated to decrease the amounts of PrP\(^{Sc}\) produced in cell culture. Pulse chase radio-labelling experiments have identified that Brefeldin A inhibits the synthesis of PrP\(^{Sc}\) in ScN2a cells (Taraboulos et al., 1992b). Pentosan sulphate is one of several polyanionic compounds capable of altering scrapie incubation periods \textit{in vivo} (Diringer and Ehlers, 1991). Pentosan sulphate has been shown to potently inhibit the accumulation of PrP\(^{Sc}\) in ScN2a cells without effecting the metabolism of the endogenous PrP\(^c\) (Caughey and Raymond, 1993; Priola and Caughey, 1994). The inhibition was suggested to be due primarily to the prevention of new PrP\(^{Sc}\) accumulation rather than destabilisation of pre-existing PrP\(^{Sc}\). PrP\(^{Sc}\) accumulation remained inhibited after removal of the pentosan sulphate. Based on the fact that amyloid plaques of prion disease are known to contain sulphated glycosaminoglycans, which have striking similarities to pentosan sulphate, it was hypothesised that the mechanism of action of pentosan sulphate may be to competitively block an interaction between PrP and...
endogenous glycosaminoglycans that is critical for the accumulation of amyloidogenic PrP. Noncytotoxic concentrations of branched polyamines have been observed to rapidly eliminate PrPSc from ScN2a cells (Supattapone et al., 1999). It was reported that such compounds act by stimulating normal cellular mechanisms to destroy PrPSc. Once the cells had been 'cured', they remained free from infectivity during subsequent passaging in normal media for at least three weeks. In contrast, the organophosphate pesticide phosmet, which had previously been proposed to have acted as an environmental trigger to the BSE epidemic (Purdey, 1996a; Purdey, 1996b), has also been reported to cause an increase in PrPSc on the plasma membrane of human neuroblastoma cells in a dose-dependent manner (Gordon et al., 1998). These studies raised the possibility that exposure to phosmet could thereby increase the susceptibility to prion disease.

PrP-derived peptides have also demonstrated a significant effect on cultures of ScN2a cells. A peptide corresponding to hamster PrP residues 119 to 136, which was identical in both mouse and hamster PrP, was able to inhibit PrPSc formation in cultures of ScN2a cells (Chabry et al., 1999). This region of PrP is highly conserved between species, and hence this peptide may have inhibitory effects on PrP conversion in a variety of species. Other therapeutic advances have been made using beta-sheet breaker peptides which have been designed to reverse the PrPSc structure and properties (Soto et al., 2000). These short synthetic peptides were targeted to interact specifically with PrP on the basis of sequence homology, and to induce unfolding of beta-pleated sheet structure due to their ability to adopt the beta-sheet conformation. A 13-residue breaker peptide was incubated in the culture medium of Chinese hamster ovary (CHO) cells overexpressing a mutant PrP corresponding to a familial form of CJD. This mutant PrP is partially protease resistant in these cells. Following incubation, PrP-res concentrations were less than 10% of those seen without addition of the peptide, and no significant effect was seen after incubation with identical concentrations of control peptides. ScN2a cells have also been used in a screen for molecules mimicking dominant negative inhibition of prion replication (Perrier et al., 2000). Sixty-three compounds have been tested for inhibition of
PrP\textsuperscript{sc} formation in these cells and two were observed to inhibit PrP\textsuperscript{sc} formation in a dose dependent manner, and demonstrated low levels of toxicity.

Studies with ScN2a cells have also revealed that antibodies binding to PrP\textsuperscript{C} on the surface of these cells are able to inhibit PrP\textsuperscript{sc} formation in a dose dependent manner, and to cure established infection (Peretz et al., 2001). After 14 days of culture in the presence of 10\(\mu\)g/ml of the most potent antibody, prion replication was abolished in treated cells as assayed by immunoblotting and pre-existing PrP\textsuperscript{sc} was rapidly cleared from the cells. PrP\textsuperscript{sc} remained at undetectable levels after 4 additional weeks of culture in antibody-free medium. Following bioassay in CD-1 Swiss mice, antibody treated ScN2a cells were capable of prolonging the incubation period, compared to untreated ScN2a cells. This is consistent with other experiments on ScN2a cells which reveal that exposure to PIPLC or to the monoclonal anti-PrP antibody 6H4 not only prevents infection of susceptible N2a cells but also cures chronically scrapie-infected cultures, as judged by the long-term abrogation of PrP\textsuperscript{sc} accumulation after termination of treatment (Enari et al., 2001).

The main drawback to most of these compounds is that in vivo, most show a significant affect only if administered long before clinical onset (in some cases with the inoculum) and/or are impractical treatments because of toxicity or bioavailability. The search for therapeutic products is enhanced with more sensitive and quicker techniques than the conventional method of SDS gel electrophoresis followed by Western blotting. New techniques such as the cell blot (Bosque and Prusiner, 2000) mentioned above, ELISA and the slot blot - a sensitive and reliable method for detection of PrP\textsuperscript{sc} (Winklhofer et al., 2001), facilitate high throughput screening for antiprion therapeutic agents and other methods may be developed in the future.

1.3.3.1.2 HaB cells

Another cell type that has been used to study PrP biology is the hamster brain cell line (HaB). HaB cells were obtained from a primary culture of a scrapie-infected Syrian golden hamster brain (Taraboulos et al., 1990). The development of an in situ method for the detection of scrapie infected cells using HaB was based on differences in the properties of PrP\textsuperscript{C} and PrP\textsuperscript{sc}: i) the relative resistance
of PrPSc to proteolysis (Oesch et al., 1985), ii) the differential releasibility of the PrP isoforms from membranes by PIPLC (Stahl et al., 1990b) and iii) the contrasting immunoreactivity patterns of these two proteins (Serban et al., 1990). A number of antibodies were used in these studies including 13A5 and 8C5, which were generated from mice immunised with purified SHaPrP27-30. These antibodies do not react with mouse PrP. Other antibodies used included rabbit polyclonal antisera R017 and R073 raised against denatured purified rods and purified PrP27-30. They react with both hamster and mouse PrP. While both native and denatured PrPc are equally recognised by these antibodies, in many experimental situations PrPSc is not immunoreactive unless it is first treated with protein-denaturing agents (Serban et al., 1990).

These antibodies were initially used to develop an in situ immunoassay for the detection of PrPSc in cultured cells grown in 96 well plates (Taraboulos et al., 1990). N2a, ScN2a, HaB and two clones of ScHaB: ScHaB-4 and ScHaB-4-C4 (the isolation of which will be described below) were used in these studies. Cells were fixed, permeabilised and in some cases were treated with proteinase K. Cells were then incubated with 6 M GdnHCl and an ELISA was performed with the aforementioned PrP antibodies. After limited proteinase K digestion, the PrP signal was reduced in all the cells tested reflecting the presence of PrPSc. GdnHCl denaturation of the proteinase K digested cells restored the immunoreactivity of the scrapie-infected cells but not in uninfected cells. The GdnHCl-dependent signal was found to correlate with PrPSc. These results were confirmed using PIPLC to verify that the proteinase K, GdnHCl signals detected by ELISA were specific for PrPSc. When the cells were digested with PIPLC before fixation for the ELISA assay, the PrPSc signal was strongly reduced, whereas no significant change was observed in the GdnHCl-dependent signal of the proteinase K-digested, scrapie infected cells, as expected for PrPSc.

Based on the results from the ELISA, the immunofluorescent detection of PrPSc was performed on these cells (Taraboulos et al., 1990). An intense intracellular signal appeared in GdnHCl-treated scrapie infected ScHaB-4-C4 and ScN2a cells, but not in scrapie-infected cells not treated with GdnHCl or in uninfected cells. The guanidine-dependent PrP signal in the scrapie infected cells was
resistant to PIPLC and proteinase K digestions, as expected for PrP\(^{Sc}\). By immunofluorescence, PrP\(^{Sc}\) was detected as a diffuse fluorescence on the cell membrane and did not appear to interfere with the intracellular PrP\(^{Sc}\) signal. These results were confirmed by confocal microscopy which revealed that PrP\(^{Sc}\) was found primarily within the intracellular space although weak fluorescent signals were observed on the plasma membrane. The PrP\(^{Sc}\) immunostaining was subsequently compared to the intracellular pattern of wheat germ agglutinin, a marker for the trans-Golgi network (Virtanen et al., 1980) and considerable overlap was observed (Taraboulos et al., 1990).

A colony assay for PrP\(^{Sc}\) was described to define the optimal conditions for PrP\(^{Sc}\) accumulation (Taraboulos et al., 1990). To analyse the temperature sensitivity of PrP\(^{Sc}\) synthesis, HaB cells were infected with purified hamster rods and cells were seeded at low density onto 10 cm dishes and grown at either 37.5°C or 34°C until the formation of sizable colonies occurred. Cells were lysed and transferred in situ onto nitrocellulose membranes and PrP\(^{Sc}\) was measured after limited protease digestion and GdnSCN denaturation. Analysis of the number of PrP\(^{Sc}\) containing colonies in the plates revealed a dramatic increase in the percentage of infected colonies formed at 34°C relative to 37.5°C. It was suggested that this could be a result of the cell division rate exceeding the rate of PrP\(^{Sc}\) synthesis at the higher temperature thus effectively diluting PrP\(^{Sc}\) from the culture. Following infection of HaB cells and subsequent cloning at 34°C, approximately 15% of the clones produced PrP\(^{Sc}\) and infectivity as analysed by ELISA. There was a strong correlation between the PrP\(^{Sc}\) signal and the infectivity of subclones as exemplified by the ScHaB-4 subclone. After one year in culture, ScHaB-4 cells appeared to lose their PrP\(^{Sc}\) as assayed by immunofluorescence. A PrP\(^{Sc}\) positive subclone of these cells designated H3 also appeared to loose its PrP\(^{Sc}\) content after a few months in culture. These cells were further subcloned to give ScHaB-4-C4 which were also used in the above experiments. This apparent instability in the level of PrP\(^{Sc}\) in these HaB cell lines limits their suitability as a long-term culture model for prion disease which could explain their limited use over the last decade.
1.3.3.1.3 GT1 cells

Another cell line used for the study of prion diseases are GT1 cells, which are gonadotropin-releasing hormone (GnRH)-secreting neurons, generated by targeted tumorigenesis in transgenic mice, using immortalisation with SV40 T antigen under the control of the GnRH promoter (Mellon et al., 1990). A subclone was stably transfected with the trkA receptor to render the cells responsive to nerve growth factor (GT1-trk) (Zhou et al., 1994). It was reported that GT1-trk as well as other GT1 subclones are highly susceptible for propagation of scrapie prions (Schatzl et al., 1997). Following treatment of GT1-trk cells with NGF, cells were infected with RML prions and immunoblotting revealed the presence of PrP\(^{\text{Sc}}\). De novo synthesised PrP\(^{\text{Sc}}\) was distinguished from traces in the inoculum by performing metabolic labelling of the cells, and treating the lysates with proteinase K, followed by immunoprecipitation. This procedure revealed that the infected cells were synthesising radiolabelled PrP\(^{\text{Sc}}\).

Indirect immunofluorescence combined with guanidine isothiocyanate denaturation demonstrated intracellular accumulation of PrP\(^{\text{Sc}}\) in the cytoplasm of these cells as punctuate fluorescent signals. Parental GT1-trk and ScGT1-trk cells were analysed by bioassay in CD-1 Swiss mice. Mice inoculated with ScGT1-trk cells had incubation times ranging from 134 to 188 days whereas none of the mice inoculated with GT1-trk cells developed scrapie. The infectious titre of the ScGTK-trk cells remained stable with passaging indicating that prion infectivity was produced de novo and was not the result of the inoculum.

The spread of prions from cell to cell was demonstrated by exposing uninfected GT1-trk cells to the conditioned medium from cultures of ScGT1-trk cells, which resulted in the transfer of infectivity as assayed by immunoblotting. Analysis of the expression of several genes in GT1-trk and ScGT1-trk cells failed to show any differences. This provides evidence against the possibility that a major selection event resulted from prion infection. Scrapie infection was shown to reduce the viability of GT1-trk cells and also diminish their proliferation. A number of cytopathological changes were produced in the infected cells, a finding that has not been demonstrated in other infected cell lines. The changes closely mimicked the spongiform degeneration observed in vivo in prion disease, with the form of cell death resembling an autophagic
subtype of apoptosis. Treatment with NGF was found to significantly reduce the morphological and biochemical features of autophagic cell death and improved cellular viability.

1.3.3.1.4 PC12 cells
The rat pheochromocytoma cell line, PC12 has been used to study replication of the scrapie agent (Rubenstein et al., 1984). In response to NGF, these cells exhibit a number of neuronal properties including morphological differentiation, electrophysiological responsiveness and neurotransmitter synthesis. For infection, NGF-treated cultures are firstly exposed to 139A scrapie brain homogenate, serially passaged and harvested during subsequent weeks for analysis by incubation period assay (Rubenstein et al., 1984). In these studies, replication of the agent was analysed by injecting infected cells into mice and measuring the time to disease onset. An inverse relationship was observed between the time interval from infection to cell harvesting and the level of infectivity which was attributed to replication of the agent in vitro. These findings were subsequently extended with the analysis of the cellular conditions necessary for agent replication (Rubenstein et al., 1990). The removal of NGF was observed to cause a gradual dedifferentiation of PC12 cells, which resulted in the inability of scrapie to replicate. The scrapie infectivity detected in PC12 cultures was reported to be cell-associated and not released into the medium and comparison to mock-infected cultures revealed no changes in morphology.

In light of these results, it was suggested that any scrapie-induced effects would have to be associated with non-vital cellular functions. Indeed, studies on the neurotransmitters in 139A infected PC12 cells demonstrated that the adrenergic pathway was unchanged but the activity of the cholinergic pathway-related enzymes were decreased (Rubenstein et al., 1991). The effects of the 139A scrapie strain on the cholinergic system appeared to be dose-dependent and were first detected prior to the detection of scrapie agent replication in the cells. The cell culture system was further used to examine differences in the replication of different mouse strains which demonstrated that the 139A strain consistently yielded 100-1000-fold higher levels of infectivity than the ME7 strain (Rubenstein et al., 1992) and no replication was observed for hamster
263K or rat 139R prions. Infectivity changes and enzymatic changes were compared in PC12 cells infected with ME7 and N2a cells infected with Chandler scrapie. These studies suggested that other factors in addition to strain replication were involved in the significant changes in neurotransmitter levels in cultures exhibiting low infectivity titres. Although PC12 cells are not widely used, it is interesting that they are susceptible to mouse, but not rat, prions (Rubenstein et al., 1992).

Scrapie-infected cultures have provided an insight into the infectious manifestation of prion diseases. However, the small number of persistently infected cell lines, low levels of *in vitro* susceptibility to infection and the restriction of infected cell lines to certain prion strains has hindered these studies.

### 1.3.3.2 Inherited prion disease

Several cell culture systems have been set up to model familial prion diseases. To model these disorders, cultured cells have been transfected to express PrP molecules carrying disease specific mutations. The cell types most widely used in these studies include CHO, PC12, N2a, M17 and 3T3 (Figure 1.7 B). These cell lines have largely contributed to our comprehension of the pathogenic processes occurring in TSEs and their applications are summarised in more detail below.

#### 1.3.3.2.1 CHO and PC12 cells

A large amount of data on the metabolism of mutant PrP molecules has been contributed by studies in Chinese hamster ovary (CHO) cells stably transfected with MoPrP molecules with six different mutations which are pathogenic to humans: PG11, P101L, D177N/Met128, D177N/Val128, F179S/Val128, E199K and as a negative control M128V, the non pathogenic mutation equivalent to the human M129V (Daude et al., 1997; Lehmann et al., 1997; Lehmann and Harris, 1995; Lehmann and Harris, 1996a; Lehmann and Harris, 1996b; Lehmann and Harris, 1997). In these studies it was observed that MoPrPs carrying pathogenic mutations acquire all the biochemical characteristics of PrPSc including detergent insolubility, protease resistance and failure to be released from the membrane following cleavage of the GPI anchor with PIPLC.
These conclusions were based on the following experiments. Following lysis in buffer containing non denaturing detergents such as TritonX-100 and centrifugation at 265,000 x g, most of the wild-type PrP remained in the supernatant consistent with its solubility under these conditions. The majority of the PrPs carrying pathogenic mutations were found in the pellet (E199K was more soluble than the other mutants), whereas the M128V MoPrP behaved like the wild-type protein.

Further experiments investigated the protease resistance of the MoPrPs which revealed that like PrP\textsuperscript{Sc}, treatment of the mutant PrPs with 3.3 \(\mu\)g/ml proteinase K for 10 minutes resulted in the production of a PrP 27-30 fragment, while under the same conditions, wild-type PrP and the M128V mutant were completely degraded. It is nonetheless noteworthy that the relative protease resistance of PrP\textsuperscript{Sc} is much higher than that of the mutant PrPs. Although both PrP isoforms are attached to cell membranes via a GPI anchor, PrP\textsuperscript{c} but not PrP\textsuperscript{Sc} can be released using the bacterial phospholipase PIPLC, which cleaves the diacylglycerol portion of the GPI anchor (Figure 1.5) (Stahl et al., 1990b). Upon treatment of the CHO cells (expressing glycosylated and GPI anchored mutant PrPs on their surface) with PIPLC, approximately 5% of the PrP molecules carrying pathogenic mutations were released from the membranes (although approximately 50% of the E199K mutant was released, consistent with its higher solubility) whereas approximately 90% of wild-type and M128V PrP were released. This resistance to PIPLC-mediated release has been attributed to the GPI anchors becoming physically inaccessible to the phospholipase as part of their conversion to the PrP\textsuperscript{Sc} state (Harris, 1999b), since denaturation in SDS renders the GPI anchors of the mutant PrPs more susceptible to cleavage. It was further hypothesised that the same structural changes that render the polypeptide chain of PrP\textsuperscript{Sc} partially inaccessible to proteinase K may also render it inaccessible to PIPLC. This effect presumably involves a change in the conformation of the polypeptide chain, but it may also be related to aggregation or polymerisation of the protein. A number of other similarities were identified between the mutant PrPs and PrP\textsuperscript{Sc} including slow metabolic generation and turnover (Lehmann and Harris, 1996b), differences in the proteinase K cleavage site among the different mutant PrPs as well as
differences in their glycosylation pattern which is analogous to the strain-specific characteristics of natural TSE isolates (Lehmann and Harris, 1996a).

This system has also been used to identify intermediate steps in the conversion of mutant PrPs to the PrP\textsuperscript{sc} state by measuring the kinetics with which the three aforementioned PrP\textsuperscript{sc} related properties develop in pulse chase labelling experiments (Daude et al., 1997). These studies have elucidated that the conversion process proceeds in three distinct steps: i) acquisition of PIPLC resistance, which is thought to occur in the ER during or soon after completion of the polypeptide chain ii) acquisition of detergent insolubility, presumably reflecting aggregation of PrP molecules and iii) acquisition of protease resistance. The two latter steps are likely to take place subsequent to delivery of the protein to the cell surface either on the plasma membrane or in an endocytic pathway. There is evidence that CLDs might be involved in the acquisition of detergent insolubility and proteinase resistance since artificially constructed transmembrane forms of mutant PrP, which are excluded from these rafts, never develop these two properties (Harris, 1999b). Based on the evidence that oligosaccharide chains can modify the efficiency of the conversion process (DeArmond et al., 1997) and may serve as markers for diverse prion strains (Collinge et al., 1996), the role of N-glycans in PrP metabolism was explored (Lehmann and Harris, 1997). This was done by expressing, in transfected CHO cells, mouse PrP molecules that were abnormally glycosylated either by synthesis in the presence of the drug tunicamycin, or by altering one or both of the consensus sites for N-glycosylation. These studies suggested that PrPs carrying glycosylation site mutations display all the biochemical properties of PrP\textsuperscript{sc}, and that wild-type PrP synthesised in the presence of tunicamycin exhibits a subset of these properties to a limited extent.

Normal prion protein contains five copies of an eight-amino acid region and PrP molecules with six or more copies of this repeat region are all associated with disease in familial CJD. Recently, the biochemical properties and effects of a PrP molecule carrying a nine-octapeptide insertion expressed in the neuronal cell type PC12 has been analysed (Chiesa and Harris, 2000). In accord with the results obtained in the CHO cells above, this mutant designated PG14 acquires scrapie like properties when stably expressed in PC12 cells, including detergent
insolubility, protease resistance, and resistance to cleavage of its GPI anchor using PIPLC. Furthermore, a detergent-insoluble and PIPLC resistant form of PG14 was reported to be released spontaneously into conditioned medium. The relevance of cell culture models to an \textit{in vivo} system has recently been investigated. Transgenic mice expressing the mouse homologue of PG14 exhibited a slowly progressive neurological disorder accompanied by the accumulation in the brain of mutant PrP molecules displaying the biochemical characteristics of PrP\textsuperscript{Sc} (Chiesa et al., 1998).

1.3.3.2.2 N2a and 3T3

Further studies have investigated the biochemical properties of PrP with insertional mutations expressed in different cell types (Priola and Chesebro, 1998). In these studies, the effect of increasing numbers of the repeat region on aggregation and protease resistance was examined with recombinant hamster PrP molecules expressed in N2a cells and \textit{Y2/PA317} cells (derived from NIH 3T3 mouse fibroblasts). The cell surface expression of hamster PrP with 5 (wild-type), 7, 9, or 11 copies of the octapeptide repeat was first assessed following expression of these molecules in the \textit{Y2/PA317} cells and treating the cells with PIPLC. As the number of repeats increased, the amount of monomeric HaPrP released by PIPLC decreased demonstrating that in these cells, aberrant cell surface expression of GPI-linked HaPrP was not just an intrinsic property of the PrP amino acid sequence but could also be dependent upon the cell type in which the mutant was expressed.

In further experiments the aggregation state of the mutant proteins was assessed by centrifugation of detergent lysates. In general, as the number of repeats increased so too did the tendency of the protein to aggregate. In \textit{Y2/PA317} cells, monomeric proteins with 5, 7, 9 or 11 copies of the repeat aggregated to a greater extent than the same constructs in N2a cells. These results suggested that the extent of aggregation was not only a consequence of the HaPrP sequence but was also a consequence of the cell-type in which the
protein was expressed. Analysis of the proteinase K resistance of the mutant PrP molecules demonstrated that in both cell types, as the number of repeats increased above 7, the resistance to low concentrations of proteinase K increased. All of the constructs in Ψ2/PA317 cells were more resistant to proteinase K treatment than the same constructs expressed in N2a cells. These data correlated with in vivo observations that increasing numbers of the repeat motif are associated with an earlier onset of familial CJD. In contrast to the studies in CHO cells described above, these experiments with N2a cells demonstrated that two mutants were susceptible to PIPLC while still showing increased aggregation and proteinase K resistance. These studies concluded that whereas increased aggregation and increased proteinase K resistance correlate with increasing numbers of octapeptide repeats, sensitivity to PIPLC may be a consequence of cell type. It remains possible that expression level could at least in part explain the higher levels of aggregation and proteinase K resistance in fibroblast cells compared with neuroblastoma cells, since in these experiments the fibroblast cells expressed higher levels of recombinant PrP protein.

1.3.3.2.3 M17 cells
Another study has focussed on the metabolism of mutant PrP in transfected M17 human neuroblastoma cells expressing constructs reproducing the FFI and CJD128 genotypes (Petersen et al., 1996). These molecules share the same D178N mutation in PRNP, however the amino acid present at position 129 of the mutant PrP in all FFI patients examined to date is methionine, whereas the corresponding amino acid in all CJD128 patients is a valine (Goldfarb et al., 1992). Expression of D178N mutants in M17 cells leads to the synthesis of the three high mannose PrP glycoforms in amounts comparable to control cells transfected with the corresponding wild-type molecules. However, in contrast to controls, these three forms are all decreased in amount at or after they have passed the trans Golgi compartment and have been transported to the cell surface. This decrease appears to preferentially affect the unglycosylated form which is vastly under represented at the cell surface.

The synthesis and degradation of mutant PrP was subsequently investigated in a series of pulse chase experiments involving treating the cells with PIPLC after
the chase to remove the GPI-anchored protein from the cell surface. The cell lysate (intracellular PrP) and the medium (cell surface PrP) were immunoprecipitated and analysed by SDS-PAGE (Petersen et al., 1996). These studies concluded that mutant PrP molecules, especially the less modified glycoforms, were inefficiently transported through the secretory pathway and this was more severe in cells expressing the FFI 129M,D178N sequence than cells expressing the CJD127 129V,D178N sequence. In light of these results, the stability and transport characteristics of the two mutant PrP molecules were investigated in a further series of pulse chase experiments with tunicamycin and brefeldin A. In control cells, transport to the cell surface of unglycosylated PrP^C was relatively unaffected by tunicamycin, whereas in tunicamycin treated cells expressing either of the D178N mutants, the mutant PrP molecules were barely able to reach the cell surface and were undetectable inside the cell shortly after synthesis. These observations led to the conclusion that the lack of N-glycans destabilised the mutant PrP molecules consistent with the under representation of these unglycosylated mutant PrP molecules on the cell surface.

Experiments with brefeldin A revealed that in control cells, all PrP^C glycoforms stably exist in the ER-Golgi compartment and are not transported to the cell surface. However in the cells expressing the 129M,D178N mutant, the unglycosylated molecule is degraded or glycosylated at a slightly greater rate than the unglycosylated PrP^C in the control cells, whereas it is more stable in the cells expressing 129V,D178N. These data indicate that the D178N mutation is unstable and that this instability is partially corrected by N-glycosylation. Hence, while the glycosylated forms are synthesised and transported to the cell surface, the unglycosylated form remains unstable and is degraded before it reaches the cell surface. In contrast to the data above regarding expression of mutant PrP molecules in CHO cells, the mutant PrP molecules on the surface of the M17 cells were readily released by PIPLC and were fully digested with proteinase K, although the reason for this discrepancy remains unknown.

Upon examination of the brain of an FFI patient with the D178N mutation, the unglycosylated mutant PrP was present at one-third of the amount of unglycosylated PrP^C expressed by the normal allele, consistent with the
conclusion that the unglycosylated form of the 129M/D178N mutant is also unstable in the brain cells of the FFI patients. This experimental model has now been extended to other mutations that are linked to CJD or GSS. Point mutations which have been studied, that are clustered in the regions of N-glycosylation and near the disulphide bridge, all show effects on the glycoform ratio of the prion protein (Parchi et al., 1999).

Overall, comparable results have been obtained by the authors regarding the cell culture models of inherited prion disease summarised above. Despite some differences, these models have demonstrated that PRNP mutations cause profound and early changes in the metabolism and physicochemical properties of mutant PrP molecules. However the test of whether the mutant PrPs synthesised in cultured cells represent authentic PrPSc will be to determine whether these proteins are infectious in animal bioassays. Useful information has also been obtained with cell cultures derived from PrP deficient animals. It has been shown that PrP expression correlates with cell susceptibility to oxidative stress, enzymatic activities like Cu/Zn superoxide dismutase or copper incorporation (Brown et al., 2001; Brown et al., 1998; Pauly and Harris, 1998). In the neuronal cell line 1C11, PrP has been implicated in a Fyn Src kinase and caveolin dependent signalling pathway (Mouillet-Richard et al., 2000).

Despite some limitations discussed above, it is apparent that the cell culture approach to prion disease offers advantages over in vivo models. Many of the questions addressed in the culture models, such as the subcellular localisation and biological properties of PrP isoforms, cannot be addressed using the rodent models. Furthermore, analysis of the cell to cell spread of infectivity and the effects of mutations on the biochemical properties of PrP are also not feasible using animal models. Clearly, the combination of both in vivo and in vitro studies has provided a fundamental insight into the central issues regarding the pathogenesis of prion disease.

1.3.4 Yeast and fungal prions
The generality of the prion concept has been extended to non-mammalian species with the identification of non-Mendelian genetic elements in
Figure 1.7. Cell culture models of prion formation. (A) Several lines of cultured cells have been used to model the infectious manifestation of prion disease following infection with purified prion preparations from infected rodent brains. (B) In order to model familial prion disease, cultured cells have been transfected with PrP constructs expressing mutations associated with inherited prion disease. N2a are mouse neuroblastoma cells, HaB are spontaneously immortalised hamster brain cells, PC12 are rat pheochromocytoma cells, GT1 are T-antigen-immortalised hypothalamic neurons, CHO are Chinese hamster ovary cells, M17 are human neuroblastoma cells and 3T3 are transformed mouse fibroblasts.
Saccharomyces cerevisiae called [PSI] and [URE3] and also [Het-s] in the filamentous fungus Podospora anserina.

1.3.4.1 [PSI]

Nonsense codons in yeast, as well as in other organisms, can be read through (suppressed) by mutant tRNAs. Nonsense suppression by tRNA mutation was found to be more efficient in some strains, named [PSI+] and less efficient in others, called [psi-] (Cox, 1965). Sup35p is the yeast counterpart of eRF-3, a factor involved in translational termination in eukaryotic cells (Stansfield et al., 1995; Zhouravleva et al., 1995). [PSI+] is thought to be a conformational isoform of Sup35 (just as PrPSc is a conformational isoform of PrP^C). Yeast cells with a mutation in Sup35p have the same phenotype as cells with [PSI+]. However this is a recessive mutation and is passed on in Mendelian ratios whereas [PSI+] is passed on in a non-Mendelian manner and is dominant. Yeast cells can be reversibly cured of [PSI+] by growing them on a protein denaturant, such as 1-5 mM guanidine hydrochloric acid for 7-8 generations (Tuite et al., 1981). Overexpression of Sup35 can cause [PSI] formation de novo, due to overproduction of Sup35p, although the mechanism of this phenomenon is not entirely clear (Derkatch et al., 1996). Like mammalian prions, SUP35 is required for [PSI] maintenance and cell viability. The amino-terminal 253 amino acids of Sup35p (the prion forming domain) is required for [PSI] maintenance while the carboxy-terminal 432 amino acids are necessary for translation termination and growth (Ter-Avanesyan et al., 1993). Although Sup35p is soluble in [psi-] cells, in [PSI+] strains it is assembled into insoluble aggregates, which are probably inactive in translational termination, leading to translational readthrough (Patino et al., 1996; Paushkin et al., 1996). Furthermore, Sup35p in [PSI+] strains shows increased proteinase resistance, although the level of resistance is significantly lower than for PrPSc, and Ure2p in [URE3] strains (section 1.3.4.2) (Patino et al., 1996; Paushkin et al., 1996). It has been shown that fusion constructs between the prion forming domain and green fluorescent protein causes formation of fluorescent aggregates in Psi+ but not Psi- strains (Patino et al., 1996). Yeast cells contain a heat shock protein (Hsp104) which protects against heat and other stresses and is responsible for induced stress tolerance (Sanchez and Lindquist, 1990). Over-production or inactivation of Hsp104 caused the loss of [PSI] and an intermediate amount of the chaperone Hsp104
was required for the maintenance of the [PSI] factor (Chernoff et al., 1995). Strain variations in [PSI+], which have different stabilities and strengths of nonsense suppression, are proposed to be due to different conformational states of Sup35 (Derkatch et al., 1996). Purified Sup35 protein has a tendency to form highly ordered beta-sheet-enriched fibres in vitro. The resulting fibres were remarkably similar to the amyloids formed by the mammalian prions (Glover et al., 1997; King et al., 1997). Preexisting fibres greatly accelerated formation of the new fibres by the newly added soluble protein, thus reproducing themselves in a prion-like fashion.

1.3.4.2 [URE3]

Yeast cells growing on a rich source of nitrogen such as glutamine shut down the transcription of genes needed for using a poor nitrogen source such as allantoate. Ure2p is one of the proteins that mediates this repression by blocking the transcriptional activator Gln3p and other positive transcription factors related to nitrogen metabolism, apparently by binding to Gln3p at its site of action on the DNA (Blinder et al., 1996). [URE3] results in loss of this repression and is thought to be a prion of Ure2p (Lacroute, 1971). [URE3] segregates in a non-Mendelian fashion during meiosis and is efficiently cured by growth of cells on rich medium containing 5 mM guanidine hydrochloric acid by an unknown mechanism, but it can reform de novo (Cox et al., 1988; Wickner, 1994). Overexpression of Ure2p can increase the frequency with which [URE3] arises by 100-fold (Wickner, 1994). [URE3] strains show the same phenotype as Ure2 mutants (Drillien et al., 1973) and Ure2 is needed for the propagation of [URE3] (Wickner, 1994). Ure2p is more resistant to proteinase K digestion in extracts of [URE3] strains than in extracts from wild-type strains (Masison and Wickner, 1995). The N-terminal part of Ure2p is responsible for prion formation and propagation and has been denoted the prion domain, and the carboxy-terminal part has been named the nitrogen regulation domain (Masison and Wickner, 1995). The prion domain of Ure2p, like that of Sup35p, can form amyloid in vitro, suggesting that amyloid formation is the basis of these prion diseases (Wickner et al., 2000).
1.3.4.3 [HetS]

When two fungal colonies grow together their advancing processes, called hyphae often fuse to form heterokaryons (Begueret et al., 1994). These heterokaryons have two types of nuclei, one from each parent. For hyphal fusion to occur cells judge each other for compatibility based on whether they have the same alleles at certain genetic loci and in *Podospora anserine* these are the *het* loci. One example of such a locus is the *het-s* locus, with alleles *het-s* and *het-S*. Strains with the same *het-s* alleles are compatible for fusion, but when *het-s* and *het-S* strains meet, the hyphae of the two strains fuse, the heterokaryon cells die and form a barrier between the two colonies, preventing further fusion and this is called heterokaryon incompatibility. Cells with the *het-s* genotype have either [Het-s] or [Het-s*] phenotype. Strains with the [Het-s] phenotype are compatible with other *het-s* strains, but [Het-s] strains show the usual incompatibility with *het-S*. Clones with [Het-s*] show a neutral phenotype and can fuse with either *het-s* or *het-S* strains. [Het-s] behaves as a non-Mendelian genetic element. This is based on the finding that following heterokaryon fusion between [Het-s] and [Het-s*] strains, the [Het-s] trait spreads throughout what was the [Het-s*] colony, including into regions that do not have the nucleus of the [Het-s] parent. It has also been shown that the [Het-s] phenotype is located in the cytoplasm. Mating female [Het-s] and male [Het-s*] strains produced meiotic progeny, all of which were [Het-s]. Whereas, if male [Het-s] strains are crossed with female [Het-s*] strains, almost all the progeny are [Het-s*]. The meiotic progeny of mating *het-s* [Het-s] with *het-S* strains included *het-S* segregants and *het-s*[Het-s*] progeny, but none that were *het-s* [Het-s]. In this effect, the meiotic cross efficiently cured the [Het-s] non-Mendelian genetic element. However, among cells that had lost the [Het-s] trait via meiosis, the trait could arise again at low frequency, representing reversible curing of [Het-s]. Overproduction of the *het-s* protein increases the frequency at which [Het-s*] strains become [Het-s] (Coustou et al., 1997). Strains without the *het-s* protein cannot propagate the [Het-s] phenotype. Deletion mutants of *het-s* are capable of carrying out hyphal fusion, but they have a neutral phenotype. These mutant strains are also unable to propagate the [Het-s] trait (Coustou et al., 1997). Further support for [Het-s] being a prion comes from the finding that Het-s protein is more resistant to protease in [Het-s] strains than in [Het-s*] strains.
(Coustou et al., 1997). This [Het-s] system is unique in that the putative prion carries the normal cell function of the het-s protein.

1.4 FOCUS OF EXPERIMENTS FOR THIS THESIS

Following intracerebral inoculation of rodents during the infectious manifestation of prion disease, the majority of the inoculum is rapidly dispersed, attributed to the extreme vascularity of the brain, whereas only 10% of the inoculum actually remains in the brain (Prusiner et al., 1999b). During disease progression, when the titer of prions reaches a critical threshold critical level, the animals develop signs of neurologic dysfunction. The length of the interval from inoculation to reaching the threshold prion concentration is referred to as the incubation period. In prion diseases incubation periods appear to span from several months to decades, during which prions presumably propagate slowly from the site of inoculation and spread throughout the brain. It is not known how prions spread from one cell to another. One possibility could involve facilitated release of prions from an infected cell followed by uptake by a neighbouring uninfected cell. Another possibility is that PrP\(^{\text{Sc}}\) on the surface of one cell acts as a receptor for PrP\(^{\text{Sc}}\) on the surface of an infected cell, thus representing a mechanism requiring direct cell-cell contact.

Peripheral inoculation studies demonstrate that prions spread at a rate of 1–2 mm per day which is not obviously consistent with either fast or slow axonal transport (Glatzel and Aguzzi, 2000; Kimberlin et al., 1983). It has been suggested that the capacity of intranerval spread of prions is modulated by PrP\(^{\text{C}}\) availability in the nerve (Glatzel and Aguzzi, 2000). A mode of transport has been suggested for the PNS involving the conversion of PrP\(^{\text{C}}\) by adjacent PrP\(^{\text{Sc}}\) along a chain of cells which express PrP\(^{\text{C}}\) and are capable of supporting prion replication, as has been proposed for the CNS (Brandner et al., 1996b). Although prions appear to spread primarily to regions that are neuroanatomically connected following intrathalamic inoculation in rodents, some patterns of PrP\(^{\text{Sc}}\) accumulation suggest that diffusion through the extracellular space, as well as the cerebrospinal fluid, may also feature in the spread of prions within the CNS (Hecker et al., 1992).
The focus of the experiments described in chapter 3 was to establish a robust system in dissociated cell-culture that would allow me to analyse the propagation of the PrP conformational change between scrapie-infected donor cells and neighbouring uninfected target cells. In order to facilitate the detection of newly synthesised prions I introduced a tagged PrP molecule into uninfected target cells and attempted to assay its conversion to PrP$^{\text{sc}}$ by subcellular preparations of prion rods. In further experiments, attempts were also made to convert the endogenous mouse PrP in the target cells. In chapter 4 this system was used to establish an assay for cell-based infection which allowed me to investigate the efficiency of conversion, and to elucidate the cellular requirements for the manifestation of stable prion infection. Fundamental details of the conversion mechanism were also identified, in particular the requirement for cell contact in the cell based transfer of scrapie prion infectivity. The aim of the experiments described in chapter 5 was to establish a single cell cytochemical assay capable of distinguishing an infected from an uninfected cell. This would facilitate the accurate quantitation of the conversion efficiency in the present culture system, as well as many further studies on infection at the cellular level.
Chapter 2

Materials and Methods
2.1 CELL CULTURE

2.1.1 Cell lines and media

Scrapie mouse brain cells (SMB), pentosan sulphate treated SMB cells (PS) and ham-1 cells were obtained from Dr. C. Birkett, I.A.H., Compton. SMB cells were originally derived by culture from a brain obtained from a mouse clinically-affected with scrapie and have been subsequently cloned (Birkett et al., 2001; Clarke and Haig, 1970; Haig and Clarke, 1971). PS were obtained by growth of SMB cells in the presence of 1 μg/ml pentosan sulphate for 14 generations (Birkett et al., 2001). Ham-1 cells were derived by stable transfection of SMB cells with hamster PrP. These cells were grown as monolayers in plastic tissue culture flasks in Medium 199 (M199, GibcoBRL), supplemented with 10% Newborn Calf Serum (NBCS, GibcoBRL), 5% Foetal Calf Serum (FCS, Firstlink) and 50 i.u./ml penicillin, 50μg/ml streptomycin. The cells were grown in a humidified atmosphere of 7.5% CO₂ at 37°C. Growth medium was changed every 3-4 days. In culture, these cells have a doubling time of approximately 2-3 days hence the cells were passaged at a 1:3 ratio every 7 days.

The cell line SA80BR (obtained from Dr. W. Goldmann, NPU, Edinburgh) was derived by explant culture from ovine brain and was grown in Dulbecco’s Modified Eagles Medium (DMEM, GibcoBRL), supplemented with 10% FCS (GibcoBRL), heat inactivated at 56°C for 45 minutes and centrifuged at 2000 rpm (719 x g, in a Harrier refrigerated centrifuge) for 10 minutes, and 50 i.u./ml penicillin, 50 μg/ml streptomycin. Cells were grown on gelatine-coated flasks at 37°C in an atmosphere of 7.5% CO₂. Growth medium was changed every 3-4 days and the cells were passaged at a 1:3 ratio every 10 days.

2.1.2 Coating flasks with gelatine

Sterile tissue culture flasks (80 cm²) were coated by spreading 2 ml of gelatine (0.75% w/v porcine skin gelatine in water, Sigma), onto the surface and air-drying for 2 hours in the hood. The flasks were washed twice with phosphate buffered saline, pH 7.4 (PBS, GibcoBRL) prior to cell plating.

2.1.3 Cell trypsinisation and passaging

Trypsin-EDTA (0.5%) was obtained from GibcoBRL and diluted 1:10 in either Hanks’ Balanced Salt Solution (HBSS, GibcoBRL), for murine cell culture or
Chapter 2

0.02% versene (GibcoBRL), for ovine cell culture, to make a 1x working solution.

Cells were washed once with 2-5 ml of HBSS for murine cells or with PBS for ovine cells. 2 ml of 1x trypsin was added per 162 cm² flask and cells were returned to the incubator for 3 minutes. Trypsin was inactivated with the addition of 4 ml of the appropriate culture medium and 2 ml of the cell suspension was added to 18 ml of fresh culture medium in each of three 162 cm² flasks.

2.1.4 Cell counting
After trypsinisation, the cells were resuspended in 2-5 ml of media and counted using a haemocytometer. The number of cells in the four outer chambers (each with a volume of \(10^{-4}\) ml) was counted and the average was multiplied by \(10^4\) to estimate the number of cells present per ml of medium.

2.1.5 Cell freezing
Cells were trypsinised and centrifuged at 600 rpm (65 x g), for 5 minutes and the pellets were resuspended in complete medium containing 10% Dimethyl Sulfoxide (DMSO, Sigma). Aliquots of 1 ml were transferred to Nunc CryoTube vials which were transferred immediately to -70°C storage. After 24 hours, the vials were transferred to liquid nitrogen for long term storage.

2.1.6 Cell thawing
Cell vials were defrosted at 37 °C and the DMSO concentration was diluted with the addition of complete media. Cells were centrifuged at 600 rpm for 5 minutes and the pellet resuspended in 2 ml of fresh medium. Cells were transferred to 80 cm² flasks containing 8 ml of complete medium.

2.1.7 Labelling cells with fluorescent tracker dyes
Cells were labelled using either 5 \(\mu\)M Syto 64 (Molecular Probes) for 30 minutes, 2 \(\mu\)M PKH26 (Sigma) for 5 minutes or 10 \(\mu\)M Cell-Tracker Green (Molecular Probes) for 30 minutes, each according to the manufacturers' instructions.
2.1.8 Irradiation of cells

Cells were trypsinised and centrifuged to pellet. 1.0 ml of medium was added to the pellet which was transferred to a 1.5 ml screw cap eppendorf tube. Cells were irradiated under a 212 kilovolt source (General Electric, emitting a dose of 4.51 Gray (Gy)/minute at 30 cm from the source), for 2 minutes and 13 seconds to obtain a dose of 10 Gy. After irradiation, cells were centrifuged, replated and returned to the incubator.

2.1.9 BrdU labelling

Cells were labelled by the addition of 10 mM 3-bromo-2-deoxyuridine (BrdU) to the culture medium for 6 hours prior to fixation and subsequent processing (see section 2.9.5).

2.1.10 Metabolic labelling of PrP<sup>sc</sup>

Cells were grown to near confluence in two 80 cm<sup>2</sup> flasks, changed to methionine-free M199 for 1-2 hours, and then to methionine free M199 with 1 mCi of [<sup>35</sup>S] – methionine (37 TBq/m mole, Amersham). After 6-8 hrs, the medium was changed to normal M199 growth medium for 24-48 hours, the cells were washed, lysed and processed to give PrP<sup>sc</sup> pellets as detailed in section 2.6. Each pellet was solubilised in 0.1 ml 4 M guanidine isothiocyanate, 0.1 M acetic acid for 15 minutes at room temperature, prior to precipitation with 4 volumes -20°C methanol. The methanol pellet was dissolved in 0.5 ml of detergent-lipid-protein complexes (DLPC) buffer (see section 2.11.3) with 1mM PMSF and divided into two for reaction (1 hour at room temperature) with either control monoclonal antibody to digoxigenin (5 μg/ml), or a mixture of 6H4 and R2 (obtained from Dr. R. A. Williamson, Scripps Institute, La Jolla, CA) monoclonal anti-PrP antibodies, (each at 2.5 μg/ml). Immune complexes were collected by rotating overnight at 4°C with Eupergit beads (Fluka) derivatised with sheep anti-mouse IgG (Sigma) according to the manufacturer’s instructions. The beads were washed in DLPC buffer, then boiled in N-glycanase incubation buffer containing 0.5% SDS, 5% mercaptoethanol, prior to addition of Nonidet-P40 (NP40) to 2% and 5 mU of N-glycanase (Glyko). After overnight incubation at 37°C and addition of 1x sample buffer, the samples were electrophoresed on a 12% gel (see section 2.7.1) which was analysed by autoradiography and imaging. The recovery of [<sup>35</sup>S]-radioactivity was
monitored throughout the preparation and was comparable for the different cell types (approximately 1% in DLPC fraction prior to immunoprecipitation, relative to cell lysate). The N-glycanase digestion was included because it increased the sensitivity of detection of radioactive PrP after SDS gel electrophoresis.

2.1.11 [³H]- Leucine incorporation assay

Cells to be analysed were grown to 50% confluence in 35 mm dishes. Cells were either fixed with 1% Paraformaldehyde, 0.05% glutaraldehyde or control live cells were assayed. 10 μCi/ml [³H]-leucine (1.6 TBq/mmol specific activity, Amersham) was added to the cell culture medium for 6 hours. Cells were washed three times with fresh media, once with PBS and fixed with -20°C methanol for 5 minutes. After washing with distilled water samples were left to air-dry overnight. Cells were coated with Ilford K5 emulsion pre-warmed to 50°C diluted 1:2 with water and the dishes were air-dried and kept in the dark for 24 hours. The cells were developed with 20% Phenisol (Ilford) for 6 minutes, washed with water and fixed with 20% Hypam rapid fix (Ilford) for 6 minutes. Cells were finally rinsed with water and observed. The grain density was determined on cells and also on background areas.

2.2 DNA PREPARATION

2.2.1 Construction of PrP mutants

The hamster-mouse chimeric molecule was made in the lab by Mr. P. Gates. A 224 bp sequence from mouse PrP (MoPrP) was inserted into a hamster (ham) background to reproduce the chimeric construct, (SP66) of Priola and Chesebro (Priola and Chesebro, 1995) using standard molecular biology techniques (Ausubel et al., 1998). Briefly, hamster PrP, (originally in the pEE6 expression vector) was subcloned into vector pUC18. Using PCR mutagenesis, a NaeI restriction site was introduced into MoPrP (in the pIAH1 vector). MoPrP was cut with NaeI and EcoRI to yield a MoPrP sequence (from nucleotide 436 to the 5' end) which was subcloned into the NaeI-EcoRI site of pUC18-Ham PrP (to generate pUC18-hamster-mouse). The same protocol was used to insert the BstEII to 3’ end of ham PrP into the pUC18-hamster-mouse backbone to give pUC18-hamster-mouse-hamster. This complete construct, named HMH was subsequently subcloned into the pEE6 expression vector, containing the gene for...
neomycin resistance. The DNA sequence was confirmed and the purity checked by agarose gel electrophoresis (Sambrook et al., 1989).

MWY PrP was constructed in the lab by Mr. P. Gates, by introducing a single amino acid substitution at position 144 of Mo-PrP-pIAH1 from a tryptophan to a tyrosine using PCR mutagenesis. The plasmid pIAH1 also contained the neomycin resistance gene.

2.2.2 Bacterial transformation
Plasmid DNA was used to transform Epicurian Coli XL2 Blue Ultracompetent Cells (Stratagene) that have a maximum transformation frequency of $5 \times 10^8$ transformants/μg of plasmid DNA. Transformation was carried out according to the manufacturer’s recommendations. Typically, 0.1-50 ng of DNA was used to transform 100 μl of bacterial cells. 50 and 100 μl aliquots of the transformation mixture were spread onto 1.5% Bacto-Agar (Difco)-NZCYM (GibcoBRL) plates containing 100 μg/ml ampicillin. The plates were incubated at 37°C overnight. A single colony was used to inoculate 5 ml of Luria-Bertani media (LB) containing 100 μg/ml ampicillin, and the culture was incubated with shaking at 37°C for 10 hours, and subsequently inoculated into 250 ml LB containing 100 μg/ml ampicillin and shaken overnight.

2.2.3 CsCl purification of plasmid DNA
The following day, 250 ml of fresh ampicillin-containing media was added to the overnight culture and incubated for a further 5 hours. Preparation and purification of plasmid DNA were by alkali lysis and equilibrium centrifugation on a CsCl gradient (Sambrook et al., 1989). The solution containing plasmid DNA and CsCl 1.55 g/ml was introduced into Beckman pollyallomer centrifuge tubes, heat sealed, and equilibrated overnight at 80,000 rpm at room temperature using a Beckman TLN100 rotor. The lower band containing supercoiled plasmid DNA was collected and a second equilibration was performed at 100,000 rpm for 4 hours at room temperature. The ethidium bromide was removed with water-saturated-butanol extraction and the DNA was precipitated and resuspended in Tris-EDTA (TE).
2.2.4 Qiagen Minipreps
Plasmid DNA was purified from the 250 ml overnight culture using the standard Qiagen plasmid purification protocol. The technique briefly entailed alkali lysis of bacteria followed by binding of plasmid DNA to Qiagen Anion-Exchange Resin. Plasmid DNA was eluted in a high salt buffer and then concentrated and desalted by isopropanol precipitation.

The DNA concentrations were determined spectroscopically by measuring the $A_{260}/A_{280}$ ratio and the purity checked by horizontal agarose gel electrophoresis (Sambrook et al., 1989).

2.3 TRANSFECTIONS
Cells were plated out in 60 mm dishes at 50-80% confluence 24 hours before transfection.

2.3.1 Calcium phosphate transfection
Cells were transfected according to standard procedures (Ausubel et al., 1998). A DNA CaCl$_2$ mixture was prepared by mixing 10 µg of DNA, 50 µl of 2.5M CaCl$_2$ and water to 500 µl. 500 µl of transfection buffer was added to a clear falcon tube. The DNA/CaCl$_2$ solution was added dropwise to the transfection buffer whilst vortexing. The mixture was left to stand at room temperature for 30 minutes to precipitate. The solution was mixed thoroughly and 500 µl was added dropwise to 2 ml of medium in a 60 mm dish of cells. After 6 hours the medium was changed and the cells were returned to the incubator.

2.3.2 TransFast transfection
Cells were transfected according to the manufacturer’s guidelines (Promega). Briefly, for transfection of cells in a 60 mm dish, 5 µg of plasmid DNA was mixed with 2 ml of SFM and TranFast Reagent to 1:1 lipid:DNA ratio. After incubation of the mixture at room temperature for 10-15 minutes, the mixture was vortexed and added to the dish of cells. Cells were incubated with the mixture for 30 minutes in the incubator, replaced with 4 ml of fresh growth media and returned to the incubator.
For transient transfections, cells were harvested by lysis 7 days after transfection. For stable transfection, cells were allowed to grow for 5 days before passaging in medium containing 0.35 mg/ml Geneticin (G418 sulphate, GibcoBRL). G418-containing medium was replaced every 3-4 days for 6-8 weeks. Isolated colonies were picked using a cloning cylinder and expanded in individual wells of a 4 well plate. At confluency, cells were transferred to a 35 mm dishes and the clones were expanded.

2.3.3 T-antigen transfection
Viral producer lines, SV40Tag (wild-type T antigen) and gag-myc (myc) (obtained from Dr. P. Jat, L.I.C.R., UCL) were plated in 10 cm dishes and grown for exactly 12 hours in culture medium. The medium containing viral proteins was collected and filtered through a 0.45 μm filter. PS cells were transfected at 40-80% confluence in 10 cm dishes by the addition of 2 ml of filtered viral media in the presence of 8 μg/ml polybrene. Cells were incubated for 2-2.5 hours followed by the addition of 8 ml of fresh viral media for a further 48-72 hours. Cells were passaged either at a 1:5 ratio into media containing 0.35 mg/ml G418 or split 1:15 into normal culture media. The culture medium was changed every 3-4 days for 3-4 weeks. Individual clones were picked using a cloning cylinder and transferred to individual wells of 4 well dishes and were subsequently expanded.

2.4 INFECTION OF TARGET CELLS WITH SCRAPIE PRIONS

2.4.1 Brain homogenate preparation
Brains were weighed and homogenised in PBS 10% (w/v) using a hand held dounce, and homogenates were aliquotted and stored at -70°C. Immediately before use, the homogenates were thawed and dispersed by sonication in a Soniprep ultrasonic disintegrator.

2.4.2 In vitro infection
79A Scrapie brain homogenates were diluted to 2% (w/v) in culture medium. Where appropriate, the 2% homogenates were further diluted with medium prior to incubation with the cells. Cells to be infected were grown to approximately 75% confluence in 35 mm culture dishes and exposed to 0.55 ml of 2% brain homogenate for 5 hours at 34 °C with a 5% CO₂ supply. After 5
hours, 4 ml of media was added for a further 18 hours, cells were washed, grown to confluence and the cultures were passaged. At various intervals, cells were harvested by lysis and assayed for PrP\textsuperscript{Sc} by Western blotting.

For infections to be analysed by colony lift assay, cells were plated at a density of 10\(^3\) to 10\(^4\) cells per 10 cm dish and the medium changed every 3-4 days for 6-8 weeks. Once colonies had reached 1mm in diameter, cells were lysed onto Immobilon-P transfer membranes (Millipore) and processed for PrP\textsuperscript{Sc} as detailed in section 2.7.3.

2.5 INFECTION OF TARGET CELLS WITH SMB CELLS
2.5.1 Coculture of target cells with live SMB cells
For infection by coculture, SMB and target cells (140 k each) were mixed in an 80 cm\(^2\) flask and cultured for 7 days. The confluent cultures were passaged at 1:2 in media containing 0.35 mg/ml G418. Cells were expanded in G418-containing media and passaged at confluence for at least 14 days. Cells at different passages were harvested by lysis and assayed for PrP\textsuperscript{Sc} by Western blotting. For experiments with cocultures for 1, 3 or 5 days, the number of cells plated out was scaled up, to be confluent at the end of the coculture period. For some cocultures, cells were plated out at different ratios, however the number of target cells was kept at 140 k.

2.5.2 Coculture of target cells with live SMB cells using inserts
Cells were plated onto the two growth surfaces of 9.6 cm\(^2\) dishes with translucent polyethylene terephthalate (PET) inserts (4.2 cm\(^2\), 0.4 micron high pore density; Becton Dickinson catalogue no. 3090). Typically, 3.3 x 10\(^4\) cells were plated on the dish, and 1.5 x 10\(^4\) cells on the insert. In some cases G418-medium was introduced when the SMB cells had attained high density so that the HMH cells were exposed to the products of G418-induced cell death. One week after coculture, target cells were trypsinised and propagated in medium containing G418 for 14 days, washed, lysed and processed for PrP\textsuperscript{Sc} (see section 2.6).
2.5.3 Incubation of target cells with conditioned medium from SMB cells
Fresh medium was added to a nearly confluent flask of SMB cells for two days. Medium was removed and filtered through a 0.45 μM filter to remove dead cells and debris. Filtered, conditioned medium was mixed 1:1 with fresh culture medium and incubated with target cells for 7 days with one change of medium. Cells were washed, lysed and processed for PrP^sc (see section 2.6).

2.5.4 Coculture of target cells with fixed SMB cells
200k SMB cells were plated into an 80 cm² flask. Cells were washed twice with PBS, fixed for 5 min at room temperature with a mixture of 1% paraformaldehyde (PFA, Sigma) and 0.05% glutaraldehyde (grade 1, Sigma) in PBS. Cells were washed twice with PBS, growth media was added and cells were returned to the incubator for 48 hours to remove any residual fixative. 200k target cells were plated onto the flask with the fixed SMB cells and cultured for 7 days. Cocultures with fixed SMB cells were generally compared to parallel cocultures with fixed PS, and live SMB cells. For fixed cell cocultures for shorter times, the number of target cells added was scaled up to be confluent at the end of the coculture period. Target cells were trypsinised and replated for two days prior to cell lysis and processing for PrP^sc. Alternatively, target cells were propagated in medium containing G418 for 14 days prior to assay for PrP^sc.

2.5.5 Bullseye cocultures
A 10 mm length of Tygon tubing (6.4 mm diameter) was heated to approximately 60°C onto a glass slide using a heated plate. Whilst hot, the tubing was transferred onto the centre of a 35 mm tissue culture dish and left to cool. It was important not to melt the tubing onto the dish in order to avoid leaving behind residual plastic. Cells were plated into the centre of the tube and in the surrounding dish. After 3-5 hours, the tubing was peeled away and the cells returned to the incubator for 2-14 days. At various times, cells were lysed onto immobilon membranes and processed for PrP^sc as detailed in section 2.7.3.

2.6 PROCESSING OF CELLS FOR ANALYSIS
2.6.1 PrP isolation from cultured cells
Cells were washed twice with PBS and lysed; typically, 1 ml of lysis buffer was added per 162 cm² flask. Cells were scraped and the lysate transferred to an
Cell debris was removed by centrifugation at 1000 x g, 4°C for 5 minutes in an eppendorf refrigerated centrifuge. For PrP<sup>C</sup> analysis, 10% of each lysate was removed and methanol precipitated by the addition of 9 volumes of -20°C methanol and transferred to -20°C storage for at least an hour. Precipitated proteins were centrifuged at 1000 x g, 4°C for 15 minutes. Pellets were resuspended in 100 µl of 1x sample buffer. For PrP<sup>Sc</sup> analysis, the remaining 90% of the lysate was incubated with 30 µg/ml proteinase K for 1 hour at 37°C. Digestion was stopped with the addition of phenylmethyl sulphonyl fluoride (PMSF) to 1 mM and incubated on ice for 30 minutes. Samples were centrifuged at 365,000 g<sub>av</sub> for 15 minutes at 4°C in a Beckman ultracentrifuge using a TLA 120.1 rotor. The pellets were solubilised in 30 µl of 1x sample buffer prior to electrophoresis.

### 2.6.2 BCA assay

Protein concentrations were determined by colorimetric detection using the BCA (bicinchoninic acid) protein assay reagent kit (Pierce), based on the biuret reaction. A set of protein standard (ranging in concentration from 0 to 250 µg/ml) were prepared by diluting a stock of 2 mg/ml BSA in sample buffer. Samples to be assayed were diluted 1 in 100, also in sample buffer. The BCA working reagent was prepared by mixing 50 parts of reagent A with 1 part reagent B. 10 µl of each standard and unknown samples were pipetted into the appropriate wells of a 96 well microtitre plate. Sample buffer was used for the blank wells. 200 µl of the working reagent was added to each well and the plate was mixed on a shaker for 30 seconds and incubated at 37°C for 30 minutes. Absorbances at 562 nm were measured using an MRX Microplate Reader, and the total protein concentrations of the unknown samples were estimated.

### 2.7 ASSAYS FOR PrP<sup>Sc</sup> DETECTION

#### 2.7.1 Western blotting

Western blotting was performed according to standard procedure (Sambrook et al., 1989). Briefly, samples were heated to 100°C for 5 minutes in a heated block. Protein electrophoresis was performed using Novex pre-cast tris-glycine 12% polyacrylamide gels (Invitrogen). Samples were electrophoresed at 200 V for 50 minutes in tris-glycine SDS running buffer (Invitrogen). Proteins were blotted onto nitrocellulose membranes (Schleicher and Schuell, BA85 Protran
cellulosenitrate). Transfer was for 3 hours at 150 mA in transfer buffer. Membranes were washed in tris buffered saline, 0.1% Tween20 (TBST) and blocked in non-fat dry milk TBST (NDM-TBST) for 10 minutes each. The blots were reacted with 6H4 monoclonal anti-PrP (PrionicsAG) at a concentration of 0.5µg/ml in NDM-TBST overnight at 4°C. Blots were washed 5 times for 10 minutes in TBST, reacted with horseradish peroxidase conjugated rabbit antimouse antibody (HRP Rb anti-mouse, Dako) diluted 1:5000 in NDM-TBST for 2 hours at room temperature and washed five times for 10 minutes with TBST. For Western blots performed using biotinylated secondary antibodies, proteins were detected by probing with horseradish peroxidase conjugated to streptavidin (TSA kit, Perkin Elmer), diluted 1:500 in tyramide signal amplification (TSA) buffer (see section 2.9.6.9). Proteins were visualised using the Enhanced Chemiluminescence (ECL) kit (Amersham), a light emitting, non-radioactive detection system for the horseradish peroxidase enzyme, according to the manufacturer’s instructions, and developed on Kodax Imaging film.

2.7.2 Quantitation of PrPSc by image analysis
Images of the blots were captured with a Fujifilm Luminescent Image Analyser using LAS 1000 Image Reader Software. Images were exported to Image Gauge V3.01 (Science Lab 97) for quantitative analysis. The area of interest tool was used to create a box surrounding the bands to be analysed as illustrated in figure 2.1 A. The box was duplicated to create boxes of equal size for all the lanes under comparison. After instructing the computer to quantitate the data, an arbitrary value was assigned to the total pixel intensity within the selected areas (Figure 2.1 B, column AU). Within every experiment, a control sample was run for example of non-infected target cells (Figure 2.1 A, lane 4). The pixel value for this area was subtracted from all other samples to correct for background. The corrected pixel values were divided by the box size to quantitatively compare PrPSc levels between lanes. All units were arbitrary.

2.7.3 Immobilon lift assay
For each dish of cells to be processed, one piece of immobilon-P transfer membrane (Millipore) and two pieces of 3MM blotting paper were cut to the desired size. Cells were washed twice with PBS. Membranes were soaked in
Figure 2.1 Quantitation of PrP<sub>Sc</sub> by image analysis. A) The area of interest function was used to draw a rectangle (blue) around the bands of interest. A first box was drawn and the duplicate function was used to create additional boxes of equal size which were placed over the the bands in the adjacent lanes. After instructing the computer to quantitate the information, a table of data was generated as illustrated in B. In this example, the background (BG) sample is in lane 4. To correct for background, the pixel intensity (AU) for box 4 was subtracted from that in lanes 1 to 3 to give the data in column AU-BG. These values were divided by the box area (Area [px2]) to give an arbitrary value for the amount of PrP<sub>Sc</sub>, (A-B)/px2.

<table>
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<th>No.</th>
<th>AU</th>
<th>Area (px2)</th>
<th>AU-BG</th>
<th>AU-BG % of Grp</th>
<th>AU/px2</th>
<th>(A-B)/px2</th>
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methanol, rinsed in TBS and soaked in lysis buffer. The membrane, backed with a piece of 3MM paper, also pre soaked in lysis buffer was placed directly onto the cells and pressed down gently. After 5 minutes, a second piece of 3MM paper was added on top and pressed down firmly. The three layers were then carefully lifted off the dish and the membrane was placed, cell side up in TBST.

For PrP<sup>sc</sup> analysis, membranes were incubated in TBST containing 30 µg/ml proteinase K for 1 hour at 37°C with gentle agitation. PMSF was added to 3 mM and was left to incubate at room temperature for 30 minutes with rocking. Membranes were washed three times for 10 minutes each in TBST. To enhance PrP<sup>sc</sup> immunoreactivity where appropriate, membranes were incubated in 3 M guanidine isothiocyanate solution (GdnSCN) in TBST for 10 minutes and washed three times for 10 minutes each in TBST. Membranes were blocked in NDM-TBST for 10 minutes prior to immunodetection. Membranes were incubated with either 6H4 at 0.5 µg/ml or L42 diluted 1:10 in NDM-TBST, at 4°C overnight. After washing membranes five times each for 10 minutes in TBST, they were incubated in HRP Rb anti-mouse for 3 hours at room temperature. After washing five times for 10 minutes in TBST, membranes were developed by ECL.

2.7.4 Dot blots
Samples to be analysed were serially diluted and 2 µl of each was pipetted onto Immobilon membranes. Typically, between 20 ng and 20 pg of protein was blotted. After air drying for an hour, membranes were washed once with TBST and blocked with NDM-TBST each for 10 minutes. From this stage, membranes were treated as the Western blots (section 2.7.1). After the final washing step, proteins were visualised using ECL.

2.8 IN SITU HYBRIDISATION
2.8.1 Neo probe
A 760 bp antisense RNA probe was generated from the SPT18-Neo vector (Roche). This vector was linearised with PvuII and transcribed in vitro using T7 RNA polymerase to generate an antisense neo riboprobe. The RNA probe was labelled with digoxygenin using the DIG RNA labelling kit (Roche).
2.8.2 In situ hybridisation
Cells were fixed with freshly prepared 4% PFA in PBS for 10 minutes. To reduce endogenous alkaline phosphatase background, the fixed cells were incubated in PBT at 70°C for 15 minutes prior to probe hybridisation. The antisense neo riboprobe was hybridised to the cells at a final concentration of 1 μg/ml in hybridisation buffer overnight at 55°C. Dishes were washed first with washing solution 1, then with washing solution 2, both washes were for 20 minutes at 55°C. The cells were then reacted with affinity purified alkaline phosphatase–labelled sheep _-digoxigenin (Roche) at a concentration of 375 mU/ml and left overnight at 4°C. To develop, the cultures were treated with the standard BCIP/NBT substrate (Promega) in developing solution according to the manufacturer’s instructions.

2.9 IMMUNOFLUORESCENCE
2.9.1 Washing coverslips
13mm glass coverslips (thickness 1, BDH) were soaked in concentrated nitric acid for 2 hours, rinsed several times in distilled water and washed twice with methanol. Coverslips were finally baked at 150°C for 4 hours.

2.9.2 Laminin coating of coverslips
Coverslips were incubated with 20 μg/ml Poly(D)lysine (Sigma) in PBS for 30 minutes, washed in M199 and coated with 20 μg/ml laminin (Sigma) in M199 for 1 hour.

2.9.3 Cholera toxin coating of coverslips
Coverslips were incubated with 20 μg/ml Poly(D)lysine (Sigma) in PBS for 30 minutes, washed in PBS and coated with 1 mg/ml cholera toxin B subunit (Sigma) in PBS at 4°C overnight.

2.9.4 Labelling of proteins
Proteins were biotinylated or digoxigenin-labelled using the Roche labelling kits according to the manufacturer’s instructions. 1 mg of purified protein in PBS was mixed with 30 μg of biotin-7-NHS, or 47 μg of DIG-NHS solutions in DMSO for 2 hours at room temperature with gentle stirring. The pure labelled proteins were separated from excess label using either a spin column (30,000
MW size exclusion resin in PBS, Molecular Probes) or by dialysis against PBS. Labelling efficiency was assessed by Western blotting of 10 μg of labelled product. For biotinylation reactions, blots were probed with HRP-streptavidin and developed with liquid 3,3'-diaminobenzidine (DAB) substrate kit (Zymed), according to the manufacturer’s instructions. For DIG labelling reactions, blots were probed with alkaline phosphatase labelled sheep anti-DIG (Roche) at 1.5 U/ml in 3% BSA, 0.1% Tween20 in PBS for 30 minutes and detected using BCIP/NBT (Promega), also used as outlined in the manufacturer’s protocol.

2.9.5 Immunocytochemistry
Cells were washed twice with PBS and fixed with either -20°C methanol for 5 minutes or with 0.5% paraformaldehyde, 0.05% glutaraldehyde for 5 minutes at room temperature. Where appropriate, fixed cells were permeabilised with 0.2% Triton-X100 in PBS for 3 minutes, washed twice with PBS and treated with 6 M guanidine hydrochloride, 50 mM Tris HCl, pH 7.4 for 10 minutes. Cells were rinsed 3 times with PBS and blocked with 10% goat serum in PBS (PBS-GS) for 30 minutes. Primary antibody incubations (BrdU and Pab101), were for either 1 hour at room temperature or overnight at 4°C. After three 10 minute washes with PBS-GS, secondary antibodies were added for 1 hour at room temperature, followed by three 10 minute washes in PBS-GS. For BrdU staining, a 10 minute hydrolysis in 2 N HCl was performed prior to the blocking step in PBS-GS. Nuclei were stained with Hoechst 33258 at 1 μg/ml for 2-5minutes and cells were counterstained with 10 μg/ml Fluorescein conjugated cholera toxin (Sigma) for 2-3 minutes, washed and finally mounted in 90% glycerol in 200 mM Tris-HCl buffer (mounting medium).

2.9.6 Live cell Immunofluorescence
Cells were grown on laminin coated coverslips. At confluence, cells were washed once with M199/Hepes and incubated with block (or BSA block where appropriate) for 10 minutes on ice.

2.9.6.1 PIPLC digestions
Cells grown as monolayers were incubated with 2 U/ml PIPLC (Glyko) in BSA block and incubated for 1 hour on ice. Cells were washed 4 times with block, for 10 minutes on ice.
2.9.6.2 Protease digestion
Cells grown as monolayers were incubated with 20-1000 μg/ml of either proteinase K, dispase, papain, or trypsin (Sigma) in M199/Hepes for between 10 and 60 minutes on ice. Cells were washed 4 times with block over 10 minutes on ice.

2.9.6.3 Digestions in suspension
For PIPLC treatment and protease digestions performed in suspension, cells were trypsinised, pelleted at 600 rpm for 5 minutes and the pellet resuspended in either BSA block or M199/Hepes. Cells were incubated with either PIPLC or protease at 4°C whilst rotating. Block was then added and cells were re-centrifuged and the pellet resuspended in complete medium. The cells were then plated out onto cholera toxin coated coverslips on ice for 2-3 hours.

2.9.6.4 Cell staining
Live cells were incubated with PrP antibodies (6H4, 3F4, R2, R26, 15B3, anti CRD, Fab b12, PrP-Fabs 119-136, 121-144 and 121-158) diluted to 20 μg/ml in block for 1 hour on ice, and washed 4 times with block. Biotinylated plasminogen and fibrinogen were used at 250 μg/ml in BSA block, for 1 hour on ice. In some experiments, 0.1 M lysine was added to the BSA block and was also included in the washes. Cells were washed 4 times over 10 minutes with BSA block. In parallel experiments, cells were pretreated with 100 U/ml carboxypeptidase B, (Porcine pancreas, Calbiochem) in BSA block for 30 minutes at 37°C, prior to labelling with the biotinylated proteins.

2.9.6.5 Culture of cells with primary antibodies
Cells were grown on coverslips in the presence of 5-10 μg/ml primary antibodies for between 15 minutes and 48 hours. Cells were washed three times with M199/Hepes and once with block.

2.9.6.6 Incubation of cells with PIPLC
Cells were grown on coverslips with the addition of 2 U/ml PIPLC to the culture medium for 24 hours. Cells were washed 4 times with block.
2.9.6.7 Secondary antibodies
Secondary antibodies, either biotinylated goat anti-mouse (Jackson) or biotinylated goat anti-human (Pierce), diluted 1:100 in block were incubated with cells for 1 hour on ice.

2.9.6.8 Fixation
Ethylene Glycol-bis (succinic acid N-hydroxy-succinimide ester, Sigma), EGS was made to 50 mM in DMSO. Cells were washed once with PBS and fixed by the addition of 1 mM EGS diluted in HBSS, followed by 1 mM EGS diluted in extract buffer, each for 10 minutes at 37°C. Cells were washed 4 times with TBST.

2.9.6.9 Tyramide signal amplification (TSA)
To enhance fluorescent signals, TSA was employed using the Cyanine 3 TSA system (Perkin Elmer). To minimize the background, endogenous peroxidase activity was quenched by incubation of the fixed cells with 1% H$_2$O$_2$ in PBS for 30 minutes at room temperature. Cells were again washed 4 times with TBST and incubated with streptavidin conjugated horseradish peroxidase diluted 1:500 into TNB buffer (supplied in kit) for 30 minutes at room temperature. After washing 4 times with TBST, cells were incubated in fluorophore tyramide (amplification reagent) diluted 1:50 in amplification diluent (supplied in kit) for 5 minutes. Cells were washed 4 times and nuclei were routinely stained with Hoechst 33258 at 1 μg/ml for 2-5 minutes. Coverslips were finally washed in TBST and mounted.

2.9.7 Microscopy
Cells were observed under bright field illumination using phase contrast optics or under epi-illumination using standard fluorescein (excitation 450-490 nm, emission between 515-565 nm), rhodamine set (excitation 538-558 nm and emission above 590 nm), and UV (excitation 390-420 nm and emission above 425 nm) filter sets on either a Zeiss Axiophot 2 upright microscope (for image capture of fixed, mounted cells) or Zeiss Axiovert S 100 inverted microscope (for image capture of live cells). The upright microscope was connected to a CV-12 cooled monochrome digital camera and the inverted microscope was connected to an FDI cooled monochrome digital camera (both from Photonic
Sciences, UK). 12-bit images were collected with Image Pro Plus software (Media Cybernetics, USA). The individual grey scale images from each channel were converted to 8 bit images and merged to obtain 24 bit colour images. These images were exported to Adobe Photoshop 4.0 (Adobe Systems, USA) for colour correction and contrast enhancement and printed on a Kodax digital science 8650 PS dye sublimation printer.

2.9.8 Quantitation of antibody binding
The CV12 digital camera was used to take high magnification images of cells under 63x magnification. Between 10 and 20 images were acquired for cells on each coverslip. The intensity of fluorescence was quantitated as a measure of antibody binding to cells in different experimental conditions, and following various treatments. As illustrated in figure 2.2, there were regions on the coverslips where cells were sparse or had retracted, which had to be avoided in the analysis. For this reason, the area of interest (AOI) tool was used to isolate an area covering a cell from the rest of the image enabling only the pixels within the AOI to be focused on. The histogram command was used to display the intensity profile and statistics for the pixel values in the AOI. The average (mean) intensity value for the AOI was noted and entered into an excel spreadsheet. Approximately 30-50 measurements were made for each coverslip and an average was taken. Within every experiment, as a negative control, a coverslip of cells was processed in parallel, without the inclusion of a primary antibody or using a control primary antibody. The average of the pixel intensities for the negative control could be subtracted to correct for background fluorescence.

2.10 IMMUNOPRECIPITATION
2.10.1 Coupling antibodies to magnetic beads
Pierce ImmunoPure Goat Anti Human IgG F(ab')2 was coupled to Tosyl-activated paramagnetic Dynabeads (Dynal, M-280) according to the manufacturer's protocol. Briefly, 1 mg antibody was diluted in 1.7 ml coupling buffer (0.1 M Na-phosphate, pH 7.4). 1.7 ml Dynabeads were washed twice with PBS. Beads were rocked in coupling buffer containing antibody for 24 hours at 37°C. The beads were washed twice in buffer D for 5 minutes at 4°C.
Figure 2.2 Quantitation of surface PrP on live cells. The fluorescent intensity of the cells was quantitated by using the area of interest function to draw square over an area of cell to be analysed (yellow box). The histogram function was operated to display data relating to the pixels within the selected area. The mean value displayed represents the average pixel intensity value (arbitrary units) for the area. For each coverslip, approximately 30-50 measurements were recorded and were entered into an excel spreadsheet. Measurements were also taken from cells from which the primary antibody had been excluded or which had been incubated with a control primary antibody. These control values were recorded for background subtraction.
and once in buffer E for 4 hours at 37°C. Beads were finally washed once in buffer D for 5 minutes at 4°C and stored in buffer D.

2.10.2 Preparation of samples for immunoprecipitation
Normal mouse brain and 79A scrapie infected mouse brain, 10% (w/v) in PBS were mixed with an equal volume of TBS, 1% NP40, 1% DOC. Diluted brain samples were homogenised with a dounce and sonicated as in section 2.4.1. Normal brain samples were cleared by centrifuging at 10,000 x g 4°C 15 minutes and 79A samples, were centrifuged at 500 x g, 4°C 15 minutes. For immunoprecipitation of PrP from SMB and PS cells, lysates were made as outlined in section 2.6.1. After the initial centrifugation step (1000 x g, 4°C for 15 minutes), lysates were treated for immunoprecipitation in the same way as the brain homogenates.

For immunoprecipitations performed on digested PrP<sup>Sc</sup>, 79A homogenates or SMB lysates were treated with either 50 µg/ml proteinase K or water for control non digested samples and were incubated at 37°C for 1 hour. Protease activity was inhibited by the addition of 1 mM PMSF and samples were left on ice for 30 minutes. The protein concentrations of the samples were then determined spectroscopically by measuring the A<sub>260</sub>/A<sub>280</sub> ratio.

2.10.3 Standard immunoprecipitation protocol
Immunoprecipitations were typically performed by incubating 1 mg of protein samples with 5 µg of PrP or control antibodies or 100 µg of plasminogen, for 2 hours at room temperature under continuous mixing. Beads, either goat anti-human coupled dynabeads for antibodies Fab b12, PrP-Fabs 119-136, 121-144, 121-158, sheep anti-mouse coupled dynabeads (Dynal M-280) for 6H4 or, ImmunoPure Immobilized Streptavidin agarose beads (Pierce) for plasminogen were washed 3 times with washing buffer. For the magnetic beads, all washes were performed using the Magnetic Particle Concentrator (MPC, Dynal). The agarose beads were washed by vortexing for 15 seconds and collecting the beads by centrifugation at 1000 rpm (in a bench top microcentrifuge) for 5 minutes. Beads were added to the samples and incubated overnight at 4°C under continuous mixing.
2.10.4 Alternative immunoprecipitation protocol

Washed beads were incubated with primary antibodies or plasminogen in assay buffer for 1 hour at room temperature and washed three times with washing buffer. For immunoprecipitations with plasminogen, half of the beads were then incubated with 0.1 M lysine in assay buffer and again washed three times with lysine containing washing buffer. Beads, coupled to proteins of interest were then mixed directly with 1 mg of protein samples in assay buffer and incubated overnight at 4°C.

Beads were washed three times with 1 ml washing buffer and once with 1 ml TBS. Finally beads were sedimented by centrifugation at 1000 rpm for 5 minutes, supernatant discarded and 20 µl sample buffer was added. Samples were heated to 100°C and centrifuged at 1000 rpm for 5 minutes to pellet the beads prior to Western blotting.

2.11 REAGENTS

2.11.1 Cells and brain homogenates

SMB, PS, Ham-1 cells, all brain homogenates, mouse and hamster PrP were kindly provided by Dr. C. Bostock and Dr. C. Birkett (I.A.H., Compton) with whom we have collaborated. SA80BR cells were provided by Dr. W. Goldmann (NPU, Edinburgh). Viral producer lines, SV40Tag and gag-myc were obtained from Dr. P. Jat, L.I.C.R., UCL)

2.11.2 Antibodies

Mouse monoclonal PrP antibodies used were 6H4, R26, and 15B3 (Prionics AG) and 3F4 (provided by Dr. C. Birkett, I.A.H., Compton ), used at 20 µg/ml. Recombinant mouse PrP antibody R2, human Fab b12, and PrP-Fabs 119-136, 121-144, 121-158 were provided by Dr. R. A. Williamson (Scripps Institute, La Jolla, CA) with whom we have collaborated and were used at 5-20 µg/ml. Polyclonal rabbit anti-cross reacting determinant (anti-CRD, Glyko) was used at 10 µg/ml. Wild type T antigen antibody pAb101 a gift from Dr. Parmjit Jat, and PrP antibody L42, provided by Dr. Martin Groschup were hybridoma culture supernatants. BU-20, the IGg1 BrdU antibody (Amersham) was used at 3.4 µg/ml.
Secondary antibodies were FITC or TRITC conjugated goat anti-mouse, rabbit anti-mouse, or swine anti-rabbit (Dako) or subclass specific FITC, TRITC (used at 10 μg/ml), or biotin conjugated goat anti-mouse (Jackson) or goat anti-Human IgG F(ab')2 (Pierce) (used at 6 μg/ml)

2.11.3 Solutions and Buffers

Miscellaneous buffers

LB (Luria-Bertani Medium)
10 g bacto-tryptone
5 g bacto-yeast extract
10g NaCl
in 1 litre H₂O

Tris-EDTA (TE)
10 mM Tris-HCl, pH 8.0
1 mM EDTA

Transfection buffer, pH 7.1
136 mM NaCl
5 mM KCl
11.2mM glucose
208 mM HEPES
1.4 mM Na₂HPO₄

DLPC buffer
20 mM Tris-HCl, pH 7.5
150 mM NaCl
2% sarkosyl
0.4% L-alpha-Lecithin (hen egg) PC

Dry a 0.1 mg aliquot of diethyl-ether or ethanolic solution of PC in glass tube under nitrogen. Carefully add 25 ml TBS-sarkosyl. Seal and sonicate at between 20-25°C in a cup-horn sonicator until clarified.
**In situ hybridisation buffers**

**PBT**
0.1% Triton X-100
in PBS

**Hybridisation buffer (10ml)**
50% formamide
2.5 ml SSC (20 x)
1 mg/ml tRNA
0.1 mg/ml Heparin
0.1 ml Denhardt's (100 x)
0.1% Tween20

**Washing solution 1(10ml)**
50% formamide
2.5 ml SSC (20 x)
1% SDS

**Washing solution 2 (10ml)**
50% formamide
1 ml SSC (20 x)
1% SDS

**AP buffer (10ml)**
100 mM Tris pH 9.5
100 mM NaCl
50 mM MgCl₂
0.1% Tween20

**Developing solution (10ml)**
1 g PVA (hydrolysed, MW 31,000 – 50,000, Aldrich)
10 ml AP buffer
33 μl BCIP (50 mg/ml)
66 μl NBT (50 mg/ml)
**Western blot buffers**

*Lysis buffer*
10 mM Tris-HCl, pH 8.0
100 mM NaCl
10 mM EDTA
0.5% NP40
0.5% sodium deoxycholate

*Proteinase K*
1 mg/ml in H₂O

*Phenyl methyl sulphonyl fluoride (PMSF)*
200 mM PMSF
in MeOH

*2 x sample buffer*
100 mM Tris-HCl, pH 6.8
4% SDS (electrophoresis grade)
0.2% bromophenol blue
20% glycerol

*Transfer buffer, pH 8.3*
39 mM glycine
48 mM Tris base
0.07% SDS (Biorad, electrophoresis grade)
20% methanol (analytical grade)

*Tris buffered saline (TBS)*
50 mM Tris-HCl, pH 8.0 base
200 mM NaCl

*TBS-Tween20 (TBST)*
25 mM Tris-HCl, pH 8.0
100 mM NaCl
0.1% Tween20
nonfat dry milk in TBST (NDM-TBST)
25 mM Tris-HCl, pH 8.0
100 mM NaCl
0.1% Tween20
0.5% nonfat dry milk (marvel)

**Immunofluorescence buffers**

M199 Hepes
25 mM Hepes, pH 7.5
in Medium 199

**Serum block**

25 mM Hepes, pH 7.5
5% sheep serum
in Medium 199

**BSA block**

25 mM Hepes, pH 7.5
0.5% BSA
in Medium 199

**Extract buffer**

50 mM Hepes, pH 7.5
1 mM EGTA
4% PEG6000
0.5% Tween20

**Immunoprecipitation buffers**

0.1 M Na-phosphate buffer pH 7.4 (coupling buffer)
2.62g NaH₂PO₄·H₂O
14.42g Na₂HP04.2H₂O
in 1 litre water
Buffer D  
0.1% w/v BSA in PBS

Buffer E  
200 mM Tris-HCl, pH8.5  
0.1% BSA

Assay Buffer  
50 mM Tris-HCl, pH 8.0  
200 mM NaCl  
3% NP40  
3% Tween

Washing Buffer  
50 mM Tris-HCl, pH 8.0  
200 mM NaCl  
2% NP40  
2% Tween
Chapter 3

Isolation and characterisation of cell lines capable of propagating scrapie prion infectivity
3.1 INTRODUCTION

Following peripheral inoculation of scrapie prions, infectivity is detected at relatively early stages in the lymphatic organs, but access to the CNS occurs via peripheral nerves (Glatzel et al., 2001; Kimberlin and Walker, 1988). Despite the wealth of data attained from numerous models of prion disease, many of the steps involved during disease progression remain to be elucidated. Prion diseases can be transmitted to laboratory rodents which have been used extensively to study these conditions. However the cost and complexity of these in vivo studies has led to the establishment of in vitro models of prion disease. Cell culture models provide the opportunity to analyse, at both the molecular and cellular levels, the biological requirements for establishing a scrapie prion infection. Analysis of the mechanism of intercellular propagation of the PrP conformational change in culture depends on the availability of scrapie-infected donor cells and highly infectable target cells lines capable of propagating infectivity.

Historically, propagation of scrapie prions has been established in cultured cells as early as 1970 with the isolation of the scrapie mouse brain (SMB) cell (Clarke and Haig, 1970). SMB cells were originally derived from the brain of a mouse infected with the Chandler strain of mouse scrapie and were shown to be of mesodermal origin (Haig and Clarke, 1971). They have subsequently been cloned (Birkett et al., 2001) and display no obvious cytopathology. SMB cells stably express PrP\textsuperscript{C} and PrP\textsuperscript{Sc} as assayed by Western blot (Birkett et al., 2001) (and this thesis) and display an invariant level of scrapie infectivity through over 100 passages as assayed by bioassay of cell extracts in mice (Clarke and Haig, 1970; Haig and Clarke, 1971). They were found to maintain an infectious titre of approximately $10^3$ LD\textsubscript{50} units /10\textsuperscript{5} cells. The permanently cured cell line PS was derived by growth of the SMB cells in the presence of the polyanionic glycan pentosan sulphate (Birkett et al., 2001). The mode of action of pentosan sulphate is uncertain although a direct interaction with PrP\textsuperscript{C} during the curing process has been implicated. Pentosan sulphate has been proposed to increase the rate of endocytosis of PrP\textsuperscript{C} in mouse neuroblastoma cells causing a subcellular redistribution of PrP\textsuperscript{C} from the plasma membrane to the cell interior, thereby inhibiting the production of PrP\textsuperscript{Sc} (Shyng et al., 1995) or just direct binding preventing conversion. PS cells stably express PrP\textsuperscript{C}, no detectable
PrP\textsuperscript{Sc}, and no infectivity as determined by mouse bioassay (Birkett et al., 2001). They have been reported to be susceptible to \textit{in vitro} infection with 22F, 139A and 79A scrapie prion strains (Birkett et al., 2001). Infection appeared to be stable with PrP\textsuperscript{Sc} expression remaining constant over 50 passages following infection with 22F without requiring subcloning. Phase contrast micrographs of SMB and PS cells are shown in figure 3.1.

The study of \textit{de novo} prion synthesis following challenge of target cells with mouse prions requires that all traces of the inoculum be removed by dilution prior to assaying for PrP\textsuperscript{Sc}. In order to allow newly synthesised prions to be detected at earlier times, I constructed further target cell lines by transfecting the PS cells with tagged PrP molecules. The rationale behind this was to allow newly synthesised tagged-prions to be distinguished from mouse prions present in the inoculum. One tagged construct was based on a recombinant mouse/hamster molecule used in a study of the specific PrP amino acids required for the conversion process (Priola and Chesebro, 1995). This chimeric PrP molecule contains the central 75 amino acids of mouse PrP encoding amino acid residues 112 to 187, although only residues 112 to 138 are important in the conversion of mouse PrP\textsuperscript{C} to PrP\textsuperscript{Sc} (Priola and Chesebro, 1995). Mouse PrP and hamster PrP differ at only 3 of the 75 amino acids in this region and as discussed in section 1.3.3.1, mouse residue 138 alone is capable of modulating the conversion process. The chimera is in a hamster PrP background and hence expresses the hamster specific 3F4 epitope which is not present in mouse PrP.

Transient expression of this chimeric molecule in scrapie infected mouse neuroblastoma cells resulted in conversion to 3F4-positive PrP\textsuperscript{Sc}, although, this interfered with the conversion of the endogenous mouse PrP molecules. Such interference is commonly observed following expression of heterogeneous PrP molecules in scrapie infected cells.

A second construct used in this series of experiments was based on the reactivity of a novel monoclonal antibody, L42, which recognises PrP from a broad range of mammalian species including sheep, cattle and humans, but does not bind to mouse, hamster or rat PrP (Vorberg et al., 1999). The L42 epitope is located in the vicinity of amino acid 144 of mouse PrP and antibody binding requires the presence of a tyrosine residue at this site. In the rodent
Figure 3.1 SMB and PS cells in culture. Phase contrast micrographs of live SMB and PS cells at approximately 50% confluence, 3 days after plating. Note that the SMB cells form tightly packed epithelial-type clusters whereas the PS cells are more loosely dispersed. Scale bar equals 100 μm.
species mentioned above, the presence of a tryptophan in this position prevents antibody binding. Mutant mouse PrP molecules encoding a tyrosine at position 144 were generated by site-directed mutagenesis and stably expressed in mouse neuroblastoma cells. These epitope-tagged molecules were strongly recognised by L42 and after translation they were correctly processed and translocated to the cell surface.

The focus of the experiments described in this chapter were to isolate and characterise cell lines capable of propagating scrapie prion infectivity. The two aforementioned epitope tagged PrP constructs were generated and transiently expressed in SMB cells to investigate their conversion to PrP^Sc. The constructs were then stably expressed in PS cells and their localisation and expression were analysed by immunofluorescence and Western blotting. The stably transfected cell lines were exposed to scrapie infected brain homogenates and conversion of the endogenous wild-type and tagged PrP molecules to PrP^Sc was investigated.

3.2 RESULTS
3.2.1 Assays for PrP detection

3.2.1.1 Detection of PrP isoforms by Western blot

Cultures of SMB and PS cells were distinguished by standard biochemical procedures dependent on two properties of PrP^Sc: its relative resistance to proteolysis, and its insolubility in non-denaturing detergents. Cultures of SMB and target cells were lysed and PrP^C and PrP^Sc were isolated as outlined in section 2.6. A typical result of preparations assayed by Western blot is illustrated in figure 3.2 A. The antibody used to probe the blot, 6H4 reacts with native PrP^C (amino acids 144-152) from a wide range of mammalian species (Korth et al., 1997), but only recognises PrP^Sc following denaturation after which it refolds into a PrP^C-like conformation. Before proteinase K digestion, the PrP content of the SMB cells is a complex mixture of PrP^C and full length PrP^Sc. Following digestion, approximately 67 amino acids, are cleaved from the N-terminus of PrP^Sc i.e. residues 21-88, generating its protease resistant core, PrP 27-30. In the PS cells, only PrP^C is present and this is completely digested following protease treatment (Figure 3.2 A). The most abundant PrP^C isoform is diglycosylated (35-37 kDa), followed by a mixture of monoglycosylated
isoforms with either glycosylation site occupied (30-33 kDa) and a small amount of the unglycosylated form (26 kDa). It is noteworthy that in SMB cells, the majority of the PrP\textsuperscript{Sc} is unglycosylated and monoglycosylated, whereas the least abundant PrP\textsuperscript{Sc} glycoform is unglycosylated. Western blotting analysis has led to an estimate of \(20\%\) of the PrP in SMB cells being present in the PrP\textsuperscript{Sc} conformation.

**3.2.1.2 Surface PrP detection by live cell immunofluorescence**

Surface PrP was visualised by live cell immunofluorescence and in early experiments, traditional methods involving fluorescent labelled secondary antibodies were used for PrP detection. However due to the limited amount of PrP present on the cell surface, the signal to noise ratio obtained was in the order of 2:1. In order to enhance the fluorescent signals, tyramide signal amplification (TSA) was employed in the immunofluorescence procedure which increased the signal to noise ratio to approximately 8:1 as determined by image analysis. The amplification procedure uses horseradish peroxidase (HRP) to catalyse the deposition of cyanine 3-labelled tyramides immediately adjacent to the immobilised HRP enzyme. This is accomplished by using a biotin labelled secondary antibody followed by streptavidin-HRP. The cyanine 3 fluorophore can be detected on the cell surface by immunofluorescence microscopy. An example of PrP staining as detected using the 6H4 antibody on the surface of SMB and PS cells is shown in figure 3.2 B. The PrP staining presents as a punctate fluorescent red signal across the cell surface and is consistently 5-10 fold more intense at cell junctions as determined by image analysis. A low level of background staining can be detected in control cells processed in parallel with omission of the 6H4 antibody. Since the live cells are reacted with the antibody at 4°C (see Methods), intracellular PrP is not detected. Furthermore, 6H4 would not bind to PrP\textsuperscript{Sc} on the surface of the SMB cells since prior denaturation was omitted.

**3.2.2 Isolation of cell lines**

**3.2.2.1 Derivation of HMH1 and HMH8**

The hamster/mouse chimeric construct was generated as detailed in section 2.2.1 and named HMH. This molecule contains the central 75 amino acids of mouse PrP and is in a hamster PrP background which contains the hamster
specific 3F4 epitope which allows discrimination between the chimeric molecule and endogenous mouse PrP (Figure 3.3 A) (Priola and Chesebro, 1995). Following transfection of HMH into PS cells and neomycin selection, four independent cell lines were isolated which differed in their level of expression of the chimeric molecule as assayed by Western blotting of cell lysates and live cell immunofluorescence with antibody 3F4. The cell line with the highest HMH expression level was named HMH8, in contrast line HMH1 expressed no detectable HMH protein as assayed by both methods. Although some variation in the level of HMH expression was evident between individual HMH8 cells, no decrease in the overall level of HMH protein expression could be detected over 30 passages. Both lines express the neo gene as detected by in situ hybridisation (see Chapter 4) and can be maintained in G418. An example of the immunofluorescent detection of HMH on the surface of live HMH8 and HMH1 cells using the 3F4 antibody is shown in figure 3.3 B and quantitation of the expression levels of the four isolated cell lines is shown in figure 3.3 C. The control cell lines used are Ham-1 cells derived by stable expression of hamster PrP in SMB cells and, as a negative control, non transfected PS cells. Lines HMH8 and HMH1 were selected for further studies.

3.2.2.2 Derivation of MWY23 and MWY24 cell lines
The second construct was generated by a single amino acid substitution from a tryptophan to a tyrosine at position 144 of mouse PrP by site directed mutagenesis and named MWY PrP (Figure 3.4 A). Transfection of this construct into PS cells and neomycin selection led to the isolation of 3 independent cell lines stably expressing MWY PrP as determined by Western blotting of cell lysates and live cell immunofluorescence with antibody L42. Immunofluorescence micrographs of one line MWY24 and control non transfected PS cells are shown in figure 3.4 B and the results obtained from quantitation of the expression levels of the 3 cell lines and control PS cells are shown in figure 3.4 C. All transfected lines appear to express stable levels of the mutant protein and are neomycin resistant. The MWY23 and MWY24 cell lines expressed the highest levels of MWY PrP and were selected for further experiments. The PrP expression characteristics of each of the aforementioned cell lines are summarised in table 3.1.
The scrapie-infected SMB cell and its cured derivative, the PS cell, were as described (Birkett et al., 2001). PS cells were transfected with the pEE6 plasmid (Stephens and Cockett, 1989) expressing the chimeric mouse hamster PrP SP66 construct (Priola and Chesebro, 1995), referred to here as HMH, as well as the neo selectable marker. HMH8 transfectants were selected with G418 and expressed HMH PrP (as detected both by immunofluorescence on live cells and by Western blotting with antibody 3F4 (Kascsak et al., 1987)), and neo\(^R\) as detected by in situ hybridisation. HMH1 cells expressed neo\(^R\) but no detectable HMH protein. PS cells were also transfected with the pIAH1 plasmid expressing the mutated mouse PrP, MWY, as well as the neo selectable marker. MWY23 and MWY24 cells were selected with G418 and expressed MWY PrP (as detected both by immunofluorescence on live cells and by Western blotting with antibody L42 (Vorberg et al., 1999)). Neo\(^R\) in MWY23 and MWY24 cells is inferred, but has not been demonstrated by in situ hybridisation. The column headed 'Mouse PrP\(^{Sc}\)' refers to the status of PS, HMH1, HMH8, MWY23 and MWY24 cells prior to infection.

### Table 3.1 PrP expression characteristics of the cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Mouse PrP(^C)</th>
<th>Mouse PrP(^{Sc})</th>
<th>HMH-PrP</th>
<th>MWY-PrP</th>
<th>Neo(^R)</th>
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<td>+</td>
<td>-</td>
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Figure 3.2 Detection of PrP isoforms. A) Cell lysates were treated with or without proteinase K and the proteins were separated on a 12% polyacrylamide gel and analysed by Western blotting. Variable glycosylation gives rise to three glycoforms; di-, mono-, and unglycosylated PrP. Treatment with proteinase K completely digests all the PrP in the PS cells consistent with PS cells expressing only PrP^C. SMB cells contain a mixture of PrP^C and PrP^Sc. Treatment of SMB cells with proteinase K digests the N-terminus of PrP^C giving rise to a subset of bands of lower molecular weight representing the protease resistant core of PrP^Sc, PrP 27-30. Note that in SMB cells, the PrP^Sc is predominantly unglycosylated and monoglycosylated whereas the least abundant PrP^C glycoform is unglycosylated. B) Immunofluorescence micrographs of PrP on the surface of SMB and PS cells. Live cells were incubated with monoclonal PrP antibody 6H4, followed by biotinylated goat α-mouse. The signal was increased by tyramide signal amplification (TSA) using the cyanine 3 fluorophore (see Methods) detected as a punctate red staining on the cell surface. Note that the intensity of fluorescence appears stronger at cell junctions (arrowed). Cells processed in parallel with omission of the primary antibody are shown as negative controls. Scale bar equals 30 μm.
Figure 3.3 Construction of HMH PrP expressing cell lines. A) Illustration of the HMH construct showing the positions of the 75 amino acid mouse fragment and the hamster specific 3F4 epitope encompassing methionines at positions 109 and 112. Hamster PrP has a single amino acid insertion at position 54 as compared with mouse PrP hence homologous sequences differ by 1 in their numbering. Also shown are the signal sequence (red), C terminal GPI addition sequence (yellow), N-glycosylation sites and the disulphide bond. B) Live cell immunofluorescence of two cell lines generated following transfection of HMH PrP into PS cells and G418 selection. Live cells were incubated with antibody 3F4 followed by biotinylated goat α-mouse and tyramide signal amplification as before. Note that HMH PrP can be detected on the surface of the HMH8 cells with variation in the expression level between individual cells. HMH1 cells do not express detectable levels of HMH protein as assayed by immunofluorescence and also Western blotting. Scale bar represents 30 μm. C) Quantitative image analysis (see Methods) was used to compare the surface fluorescent intensity of the four isolated cell lines, control Ham-1 cells expressing hamster PrP and non-transfected PS cells. Note that HMH8 cells express the highest levels of HMH protein whereas HMH1 cells are negative. The error bars represent the standard deviation from the mean. The measurements of the mean fluorescent intensity were estimates from 47 Ham-1 cells, 52 HMH1 cells, 48 HMH8 cells, 52 HMH10 cells, 49 HMH11 cells and 47 PS cells.
Figure 3.4 Construction of MWY PrP expressing cell lines  

A) Diagram of MWY PrP showing the single amino acid substitution at position 144 in mouse PrP from a tryptophan to a tyrosine which is detected by antibody L42.  

B) Live cell immunofluorescence of MWY24 cells derived by stable expression of MWY PrP in PS cells and control non-transfected PS cells. Live cells were incubated with antibody L42 followed by biotinylated goat α-mouse and tyramide signal amplification as before. Note the strong expression of MWY in line MWY24 relative to non-transfected cells. Scale bar represents 30 μm.  

C) Quantitative image analysis (see Methods) was used to compare the surface fluorescence of three isolated cell lines and control non-transfected PS cells. Note that MWY23 and MWY24 are the highest expressers of MWY protein whereas PS cells are negative. The error bars represent the standard deviation from the mean. The measurements of the mean fluorescent intensity were estimates from 53 MWY18 cells, 50 MWY23 cells, 49 MWY24 cells and 53 PS cells.
3.2.3 Transient expression of constructs in SMB cells

3.2.3.1 Conversion of HMH PrP\textsuperscript{c} to PrP\textsuperscript{sc}

Initial experiments were aimed at elucidating whether these constructs could be converted to PrP\textsuperscript{sc} following their transient expression in SMB cells. HMH was transfected into SMB cells and assayed after 1 week for the formation of 3F4-positive PrP\textsuperscript{sc} by Western blotting as shown in figure 3.5 A. Taking into consideration the amount of protein loaded per lane, the level of HMH expression in transfected SMB cells (lane H\rightarrow S) was estimated to be around twice the level of the stable cell line HMH\textsubscript{8} (H). The PrP is predominantly diglycosylated with very little unglycosylated similar to the glycoform pattern observed for mouse PrP\textsuperscript{c} in PS cells. The transfected cells were observed to express 3F4-positive PrP\textsuperscript{sc} indicating that the chimera could be converted by the endogenous mouse PrP\textsuperscript{sc} in the SMB cells whereas control untransfected SMB cells (S) are 3F4-negative for PrP\textsuperscript{sc}. Approximately 2\% of the HMH protein was estimated to be in the PrP\textsuperscript{sc} conformation which is quite low relative to approximately 10-30\% for the endogenous mouse protein.

3.2.3.2 Conversion of MWY PrP\textsuperscript{c} to PrP\textsuperscript{sc}

MWY PrP was also converted to PrP\textsuperscript{sc} following transient expression in SMB cells and the results of the transfection as assayed by Western blotting are shown in figure 3.5 B. The MWY PrP in transfected cells (M\rightarrow S) is expressed at a comparable level to MWY PrP in the stable cell line MWY\textsubscript{24} (M). L42 does not recognise mouse PrP hence SMB cells (S) are negative with this antibody. However a sharp band is visible at around 31kDa in the SMB cells, the nature of which and its absence in the corresponding position with the 6H4 antibody suggest that this band is unlikely to be PrP-related. The transfected cells also express L42-positive PrP\textsuperscript{sc} (M\rightarrow S) indicating that transient expression of this mutant molecule leads to its conversion to PrP\textsuperscript{sc} by the endogenous mouse PrP\textsuperscript{sc} in the SMB cells. Similarly to the HMH PrP\textsuperscript{sc} above, all three PrP\textsuperscript{sc} isoforms could be detected in the L42-positive PrP\textsuperscript{sc}. Approximately 3\% of the total L42-positive PrP appears to be converted consistent with the results above with HMH. Analysis of the blot with 6H4 which detects both mutant and wild-type molecules shows that as expected, the total amount of PrP\textsuperscript{sc} is higher in transfected cells than the untransfected SMB cells. Furthermore, in agreement with the studies with HMH, no difference could be detected between the total
amount of 6H4-positive PrP<sup>Sc</sup> in SMB cells transiently expressing MWY PrP (M→S) and non transfected SMB cells (S), since the amount of converted mutant protein was small relative to the total amount of endogenous PrP<sup>Sc</sup>. This also confirms that conversion of the tagged PrP molecules did not interfere with conversion of the endogenous PrP.

3.2.4 Infection by brain homogenates

Target cells were infected with brain homogenates in which PrP<sup>Sc</sup> is present in the form of prion rods. Initial experiments were aimed at reproducing previous experimental results on the conversion PS cells (Birkett et al., 2001). PS cells were exposed to 79A scrapie prions followed by passaging and analysis of PrP<sup>Sc</sup> by Western blotting as outlined in figure 3.6. Following infection, PS cells were observed to express PrP<sup>Sc</sup> after 10 generations and 5 passages, PS(10) and after 12 generations and 6 passages, PS(12) (Figure 3.7 A). Infected PS cells were not observed to display any differences in growth rate or morphology to non-infected PS cells. The level of PrP<sup>Sc</sup> expression in cells appeared to be stable as evidenced by passaging, indicating that this was due to de novo synthesised PrP<sup>Sc</sup>. Furthermore, the mobility of the unglycosylated band in infected brain homogenate (lane B) runs characteristically above the equivalent band in SMB cells (lane S) and infected PS cell samples (lanes PS10 and PS12) further confirming that this was not a result of carryover from the original inoculum.

These observations were repeated for the HMH1 target cells. The results are shown in figure 3.7 A with infected HMH1 cells expressing PrP<sup>Sc</sup> at 8 generations and 4 passages after infection, H1(8) and also at 12 generations and 6 passages after infection, H1(12). These results were reproducible and consistently led to conversion of mouse PrP in the target cells to approximately 10% of the level of an equivalent number of SMB cells. Mock infections with normal mouse brain homogenates failed to induce conversion of the target cells (not shown). Attempts were also made to infect HMH8 cells with mouse prions. The detection of 3F4-positive PrP<sup>Sc</sup> was not observed indicative of a lack of conversion of the HMH molecule, however the endogenous mouse PrP in HMH8 was converted to a similar level as that observed for PS and HMH1 cells (not shown).
Figure 3.5 Transient expression of epitope-tagged PrP in SMB cells. A) Conversion of HMH PrP<sup>C</sup> to PrP<sup>Sc</sup> following transfection into SMB cells. The plasmid expressing HMH PrP was transiently transfected into SMB cells. After 6 days the cells were lysed, and 10% of the lysate was processed for PrP<sup>C</sup> and 90% for PrP<sup>Sc</sup> (see Methods) followed by Western blotting as detected by antibody 3F4 which recognises the chimeric molecule, but not the endogenous mouse protein. Samples were derived from Scrapie-infected hamster brain homogenate, HB, which is predominantly diglycosylated following protease digestion; H MH8 cells, H8; SMB cells, S; and transiently transfected SMB cells, H —► S. Note the presence of 3F4-positive PrP<sup>Sc</sup> (lane H —► S) reflecting conversion of the chimeric molecule. B) Conversion of MWY PrP<sup>C</sup> into PrP<sup>Sc</sup> following transfection into SMB cells and analysis after 6 days. Cells were lysed and processed for PrP<sup>C</sup> and PrP<sup>Sc</sup> as before and Western blotted using antibodies L42 which recognises only the mutant PrP molecule and 6H4 which recognises the mutant and wild-type PrP. Samples were derived from MWY24 cells, M; SMB cells, S, which produces an unidentified discreet band at around 31kDa; and SMB cells transiently expressing MWY PrP, M —► S. Note the expression of L42-positive PrP<sup>Sc</sup> (lane M —► S) reflecting conversion of the MWY PrP. A sample of Scrapie-infected mouse brain homogenate is present as a further control, B.
Infection of PS cells was also analysed following growth of individual colonies in a 10 cm plate for 6 weeks after infection, followed by lysis onto an immobilon membrane and assaying for the percentage of PrP^Sc-positive colonies. Plating 10^4 cells into a 10 cm plate led to the formation of approximately 100 colonies indicating that individual colonies were not derived from single cells but from cooperative growth of several founder cells. Approximately 50% of the colonies formed in the plate of 79A scrapie infected cells were PrP^Sc-positive. No PrP^Sc-positive colonies were observed in plates of mock infected cells (Figure 3.7 B).

The dependence of conversion of mouse PrP in target cells on the dilution of the infectious brain homogenate was analysed by exposing target cells to serial dilutions of 2% brain homogenate. An example of such a titration is shown in figure 3.8 A. Brain homogenate dilutions of 10 fold or greater were unable to induce conversion of mouse PrP^Sc in the HMH1 cells. The results, as quantitated by image analysis, are displayed in figure 3.8 B. The steep drop in conversion ability between the 2 and 10 fold dilution of the homogenate indicates a requirement for high concentrations of prion rods for infection. This illustrates a lack in sensitivity of HMH1 cells to conversion by prions in this form.

The possible conversion of MWY PrP was investigated by exposing MWY24 cells to 79A scrapie brain homogenate. PrP^Sc preparations from two passages of the cells are shown in figure 3.9. MWY24 cells at 6 generations and 3 passages after infection (M(6)) and MWY24 cells at 10 generations and 5 passages after infection (M(10)) both show the expression of L42-positive bands that migrate predominantly between 30-40 kDa. These bands are also detected by the 6H4 antibody and are present at a lower level in control uninfected MWY24 cells (M). Antibody 6H4 detects both mouse PrP^Sc as well as mutant PrP, and the presence of the MWY PrP obscures the identification of whether the endogenous mouse PrP has been converted. The appearance of these bands was also detected in PrP^Sc preparations from line MWY23 (not shown).

3.3 DISCUSSION
The results presented in this chapter demonstrate firstly that two recombinant PrP molecules are able to undergo conversion to PrP^Sc after transfection into mouse scrapie infected cells, albeit with low efficiency. Conversion of these
Figure 3.6 Procedure for the infection of target cells with scrapie prions. Target cells were exposed to a 2% scrapie brain homogenate as detailed in Methods and passaged after 5 days. PrPSc production was either analysed in successive passages of target cells by Western blotting using antibody 6H4 which recognises mouse prions. Alternatively, cells were plated out at a density of $10^4$ per 10 cm dish and incubated for 6-8 weeks until the formation of discreet colonies. Cells were subsequently lysed onto an immobilon membrane and processed for PrPSc.
Figure 3.7 Infection of target cells with 79A scrapie prions. A) PS and HMH1 cells were exposed to infectious mouse brain homogenate and passaged. PrP$_{\text{Sc}}$ samples were derived from Scrapie-infected mouse brain homogenate, B; SMB cells, S; PS cells at 10 generations after infection, PS(10); PS cells at 12 generations after infection, PS(12); control uninfected PS cells, PS; HMH1 cells at 8 generations after infection, H1(8); HMH1 cells at 12 generations after infection, H1(12); control uninfected HMH1 cells, H1. The mobilities of monoglycosylated and unglycosylated bands are indicated by the empty and solid arrowheads respectively. B) PrP$_{\text{Sc}}$ analysis by colony lift. PS cells were exposed to 79A Scrapie-infected and control uninfected mouse brain homogenates and were subsequently plated out in a 10 cm plate and left to grow. Cells were lysed onto an immobilon membrane and processed for PrP$_{\text{Sc}}$ (see Methods). Note that approximately half of the colonies that formed in the infected plate were PrP$_{\text{Sc}}$ positive compared to none for the control plate.
Figure 3.8 Titration of 79A scrapie brain homogenate on HMH1 target cells.
A) Cells were exposed to varying dilutions of a 2% 79A Scrapie mouse brain homogenate, passaged and assayed for PrPSc by Western blotting after electrophoresis of 75 μg protein/lane. Samples were derived from SMB cells, S; 1, 2, 10, 100 and 1000 are serial dilutions of the 2% brain homogenate; and non-infected HMH1 cells, H1. B) PrPSc expression of samples from the Western blot in A were quantitated by image analysis (see Methods). Note that no PrPSc signal is detected for dilutions of 10-fold or more.
Figure 3.9 Infection of MWY24 cells with 79A scrapie prions. Cells were exposed to scrapie infected brain homogenate, passaged and processed for PrPSc. Samples were derived from SMB cells, S; MWY24 cells at 6 generations after infection, M(6); MWY24 cells at 10 generations after infection, M(10); and uninfected control MWY24 cells, M. A sample of scrapie mouse brain homogenate is shown as a further control, B. Note the appearance of L42-positive bands in the infected MWY24 cells with slightly different mobility and glycoform pattern to the PrPSc in SMB cells. The level of the signal in infected MWY24 cells is more strongly detected using 6H4 than L42. Upon increased exposure of the blots, protease resistant bands also appear in the uninfected controls as detected by both PrP antibodies, although the reason for this is unclear.
molecules did not interfere with the metabolism of the endogenous mouse PrP. Possible explanations for this low level of conversion include the fact that since SMB cells also express endogenous mouse PrP, the two different PrP molecules could be competing for conversion to PrP\textsuperscript{Sc}. Since no decrease can be detected in the synthesis of mouse PrP\textsuperscript{Sc}, the endogenous molecules appear to be more favourably converted. This is in contrast to other studies which demonstrated that although transient expression of a mouse/hamster chimeric molecule equivalent to HMH in mouse neuroblastoma cells led to conversion to 3F4-positive PrP\textsuperscript{Sc}, these molecules interfered with conversion of the endogenous mouse protein (Priola and Chesebro, 1995). These differences could be cell type related or could be attributed to a higher level of expression achieved using the retroviral expression vector for the neuroblastoma cells.

The conversion of the endogenous mouse PrP was observed to occur at a similar level in PS, HMH1 and HMH8 cells, although exposure of the HMH8 cells to mouse prions did not lead to conversion to 3F4-positive PrP\textsuperscript{Sc}. HMH protein, like the endogenous mouse protein, is present on the surface of the cells as determined by immunofluorescence and is correctly glycosylated as shown by Western blotting, indicating that localisation and trafficking are appropriate. Furthermore, conversion of HMH8 upon transient expression in SMB cells indicates that the chimeric PrP\textsuperscript{Sc} is potentially able to adopt the PrP\textsuperscript{Sc} conformation. The lack of stable conversion of HMH by the scrapie brain homogenate could again be attributed to more favourable conversion of the endogenous mouse PrP. In light of the conversion of the endogenous mouse PrP by exogenous scrapie prions, a single cell assay capable of distinguishing a scrapie-infected from non-infected cell would facilitate the quantitation of the conversion efficiency. In the absence of such an assay, it is only possible to conclude that infection by scrapie prions leads to conversion of endogenous mouse PrP to approximately 10% of the level of the equivalent number of SMB cells as estimated by Western blot analysis.

Whether this fraction corresponds to 10% of the cells being converted to an equivalent level to SMB or all the cells converted to a fraction of the level of SMB remains to be elucidated. Half of the 79A challenged PS cells observed by the colony lift assay had been stimulated to express PrP\textsuperscript{Sc} which is consistent with previous attempts to infect PS cells which reported that 27% of the resulting colonies were PrP\textsuperscript{Sc} positive (Birkett et al., 2001). This estimate is much
higher than similar attempts to convert neuroblastoma cells which have only a low frequency of PrP\textsuperscript{Sc} stimulation with only 1% of cells becoming infected when challenged in vitro (Butler et al., 1988; Race et al., 1988). If the PS cells were able to survive at clonal density, then 100 cells could be plated, and this would ideally lead to the formation of 100 discrete colonies. Despite attempts to achieve clonal growth of the cells (including introduction of T-antigen and myc into PS cells) and maintenance of the cells in conditioned medium, clonal growth was unfortunately not attained. PS cells required plating of at least 10\textsuperscript{4} cells into a 10 cm plate which led to the formation of only 100 individual colonies. This inability of the PS cells to survive at clonal density interferes with the accuracy of the quantitation for this assay.

Analysis of the titration of the 79A scrapie brain homogenate on HMH1 cells shows that the maximum dilution that could be used to infect a 10 cm\textsuperscript{2} dish was equivalent to a 1% homogenate in 500 \textmu l and further dilution gave no detectable PrP\textsuperscript{Sc}. In comparison, recent attempts to convert a subline of N2a cells in a 2 cm\textsuperscript{2} dish with RML scrapie prions reported that dilutions as low as 0.1% in 20 \textmu l consistently produced detectable PrP\textsuperscript{Sc} on cell blots (Bosque and Prusiner, 2000), an increase in sensitivity of approximately 50-fold. Furthermore, the RML brain homogenate was reported to have a lower infectious titre than the 79A homogenate. A combination of the increased sensitivity of the cell blot over the Western blot and increased susceptibility of this N2a subline to prion infection could be responsible for the lower sensitivity in the HMH1 cell system. This apparent lack of sensitivity could be attributed to the heterogeneous nature of the crude brain homogenate. The homogenate could contain prion inhibitory factors which might effectively lower the sensitivity of the cells to in vitro infection.

The origin of the bands detected upon infection of MWY24 cells with mouse prions remains to be elucidated. One possibility is that they are derived from spontaneous conversion of MWY PrP to a protease resistant form in these cells, which is amplified following exposure to infectious mouse prions. The stronger detection of these bands with 6H4 could be attributed to a greater binding efficiency of this antibody. However the molecular weights of these bands and the glycoform pattern is not characteristic of authentic mouse PrP\textsuperscript{Sc} in these
cells. Whether this spontaneous conversion is indeed the case would require a number of stable cell lines to be derived and continually assessed over successive passages to see if these observations are reproducible.

The studies reported in this chapter have led to the establishment of readily infectable target cell lines capable of sustaining scrapie prion infectivity throughout multiple passages. The focus of the experiments in chapter 4 will be to employ these cell lines to study the intricate mechanism of prion propagation between cultured cells.
Chapter 4

Cell-mediated infection of target lines in dissociated cell culture
4.1 INTRODUCTION

The complex mechanism of prion neuroinvasion following peripheral inoculation involves the early accumulation of prions in the lymphoreticular system (LRS) followed by access to the central nervous system (CNS) via peripheral nerves (Beekes et al., 1998; Glatzel et al., 2001; Kimberlin and Walker, 1988; Race et al., 2000). Despite the partial elucidation of the cellular requirements for invasion of prions into lymphoid tissues, many aspects of the disease process remain enigmatic. To what extent does spread of infection around the body involve the migration of infected cells, prions passing from infected to adjacent uninfected cells, or transport over considerable distances through or along nerves? In an experimental scrapie infection, the initial inoculum of PrP$^\text{Sc}$ is cleared relatively rapidly (Prusiner et al., 1999b), and subsequent propagation of the infection must depend on the ability of infected cells to convert uninfected target cells to stable production of PrP$^\text{Sc}$. The mechanism underlying such cell-based infection is not understood. Within the CNS, it is unknown whether the spread is partly or exclusively via neurons and which types of cell become infected (Raeber et al., 1997).

The assumption that spread of prions occurs by axonal transport rests mainly on the demonstration of diachronic spongiform changes along the retinal pathway following intraocular infection (Fraser, 1982). These studies revealed that scrapie prions could travel over a considerable distance along a nerve pathway. However alternative possibilities such as spread of infection via cell-to-cell contact in epineural glial cells, or diffusion in the extracellular space cannot be ruled out. Experiments concerning the kinetics of disease development argue against the hypothesis that prions follow fast or even slow axonal transport. The overall rate of spread of scrapie within the CNS has been estimated at around 0.5-2 mm per day (Beekes et al., 1996; Kimberlin et al., 1983). Further studies regarding the spread of prions within the CNS have been based on intraocular inoculation of prions into Prnp$^{0/0}$ mice containing PrP-overexpressing neurografts (Brandner et al., 1996b). These experiments resulted in a lack of graft infection even in the absence of an immune response to PrP, demonstrating that PrP$^\text{C}$ appears to be necessary for the spread of prions along the retinal projections and within the intact CNS. This led to the proposal that the intracerebral propagation of prions to target tissue is dependent on the
presence of an uninterrupted chain of PrP<sup>C</sup>-expressing cells capable of supporting prion replication. Similarly, the rate of spread of prion infectivity in peripheral nerves has been estimated at around 1-2 mm per day (Glatzel and Aguzzi, 2000; Kimberlin et al., 1983). Furthermore PrP<sup>C</sup> availability in the nerve was observed to modulate the capacity for intranerval transport, but not the velocity (Glatzel and Aguzzi, 2000). These results were indicative of a mode of transport in which PrP<sup>C</sup> localised on the PNS is converted by adjacent PrP<sup>Sc</sup> molecules in a ‘domino’ fashion, similar to the model proposed for the CNS.

It has been proposed that infection can spread more easily along some neuronal pathways than others (Kimberlin and Walker, 1988). Furthermore, it has been suggested that fatal clinical disease develops after infection has reached certain clinical target areas (CTA) in the CNS but only when scrapie replication there has caused sufficient functional damage. As a result, the site of invasion of the CNS determines the neural pathways by which the CTA are reached and hence effects the rate of spread of infection. The occurrence of plateau concentrations of infectivity in both the CNS and LRS are indicative of restrictions on the scrapie replication process, and can be attributed to a finite number of cells that can support replication, and a limitation on the net replication process in individual cells (Kimberlin and Walker, 1988). These cells are most likely to be follicular dendritic cells in the LRS, and in the CNS neurons are probably responsible (as discussed in Introduction). Furthermore, the cell-to-cell spread of scrapie may be restricted at the cellular interface between the LRS and the nervous system (Kimberlin and Walker, 1988). These limitations are most probably responsible for the slowness of disease progression, and may be closely related if replication is needed before infection can spread to other cells.

The mechanisms underlying cell-to-cell infection have not been studied directly. Since some PrP<sup>Sc</sup> is expressed at the cell surface and is accessible to biotinylation (Lehmann and Harris, 1996a; Vey et al., 1996; Walmsley et al., 2001), it is possible that cell-to-cell contact might play a role in intercellular conversion. Another possibility is that infected cells produce extracellular forms of PrP<sup>Sc</sup> which then act on target cells. The studies described in chapter 3 concerned the isolation of genetically marked target cells which were converted to stable production of mouse prions following exposure to subcellular
preparations of prion rods containing aggregates of PrP\textsuperscript{Sc}. This chapter addresses some of the questions outlined above by establishing an assay for cell-based infection. The focus of the experiments was to elucidate the mechanism by which infected cells are able to convert uninfected cells to stable production of PrP\textsuperscript{Sc}. The rationale behind the experimental design was that since the target cells are closely related to the infected SMB cells, this should minimise constraints in cell-based infection. The efficiency of conversion and its dependence on cell contact were also investigated in order to determine the cellular requirements for propagation of the infectious agent.

4.2 RESULTS

4.2.1 Infection by live SMB cells

In order to assay for cell-based infection, in this case the ability of SMB to stably convert its cured derivatives, the infected cells were plated out with an equal number of target cells (HMH1, HMH8, MWY23 or MWY24) and cocultured for 1 week as outlined in figure 4.1. The two cell types divided and associated to form close packed monolayers without evidence of cell sorting as evidenced by labelling one population with a fluorescent cell-tracker dye prior to coculture (Figure 4.2). Following coculture, conversion of the HMH and MWY PrP molecules, was assayed directly by Western blotting of PrP\textsuperscript{Sc} preparations using antibodies 3F4 and L42 respectively. Conversion of the endogenous mouse PrP in the target cell lines was assayed following their selective re-isolation by 2 weeks of G418 treatment to kill the neo-sensitive SMB cells, followed by PrP analysis using antibody 6H4 which recognises prions from a wide range of species including mice, hamsters and sheep (Korth et al., 1997).

4.2.1.1 Assay for conversion of HMH PrP

Initial experiments involved attempts to convert the chimeric HMH molecule in HMH8 cells following coculture with SMB cells. Development of such a straightforward assay would allow many aspects such as the timing of the conversion process to be addressed without the need to counterselect against the SMB cells. After 1 week of coculture, cells were lysed and processed for PrP\textsuperscript{Sc} followed by analysis by Western blotting and detection using the hamster specific 3F4 antibody which recognises HMH but not mouse prions, and 6H4 which recognises both PrP molecules (Figure 4.3 A). No 3F4-positive PrP\textsuperscript{Sc} was
detected from the coculture (lane 1H8:1S) indicating a lack of conversion of the HMH molecule. Analysis of the Western blot with 6H4 led to the detection of PrP\textsuperscript{Sc} from the same sample (lane 1H8:1S). This 6H4-positive PrP\textsuperscript{Sc} is likely to have been derived from the mouse PrP\textsuperscript{Sc} in the SMB cells, as well as by any newly synthesised mouse PrP\textsuperscript{Sc} generated in the HMH8 cells during coculture. This experiment was repeated 9 times and on no occasion was conversion of HMH PrP detected.

### 4.2.1.2 Assay for conversion of MWY PrP

Attempts were also made to assay for conversion of the mutant MWY PrP in MWY23 and MWY24 cells following 1 week of coculture with SMB. Cells were lysed and processed for PrP\textsuperscript{Sc} as before, followed by Western blotting using antibodies L42, which recognises only the mutant PrP, and 6H4 which detects the mutant and wild-type PrP molecules (Figure 4.3 B). Analysis of the blot with L42 revealed the presence of a discreet band at around 23kDa from the coculture between SMB and MWY23 (lane 1M23:1S), which was not reminiscent of the presence of PrP\textsuperscript{Sc} (see figure 3.9 for putative MWY PrP\textsuperscript{Sc}, synthesised following infection by scrapie prions). Upon extended exposure of the blot, the presence of this band was also apparent in the control MWY23 cells. The identity of this band is unknown. Analysis of the same coculture with 6H4 revealed the presence of PrP\textsuperscript{Sc} (lane 1M23:1S). This signal was likely to be derived from the mouse PrP\textsuperscript{Sc} in the SMB cells. The control MWY23 cells (M23) also demonstrate the presence of low levels of 6H4-positive PrP\textsuperscript{Sc} the origin of which also remains to be established (see figure 3.9). The results from the coculture between SMB and MWY24 cells were less clear. L42-positive protease resistant bands could be detected from the coculture (lane 1M24:1S) and control MWY24 cells (lane M24) migrating between 22-34 kDa. Additional bands of greater intensity could also be detected using the 6H4 antibody. The origin of these bands is also unknown. The MWY PrP does not appear to have been converted in these experiments.

### 4.2.1.3 Assay for conversion of endogenous mouse PrP

In light of the preceding negative results obtained for conversion of HMH and the unclear results with MWY PrP, I attempted to investigate whether the endogenous mouse PrP in the target cell lines could be converted following
Figure 4.1 Schematic of the coculture procedure. SMB cells (red) and target cells (blue) were cocultured for 7 days and divided in culture to form a close packed monolayer. Conversion of tagged PrP molecules was either assayed directly by cell lysis and processing for PrPSc (see Methods) followed by analysis with either antibody 3F4 for HMH PrP or L42 for MWY PrP. Alternatively, the conversion of the endogenous mouse PrP was assayed following selective re-isolation of target cells by G418 treatment. Production of mouse PrPSc was assayed in successive passages using antibody 6H4; incorporation of [35S]-methionine into PrPSc was assayed by metabolic labelling of cultures, followed by immunoprecipitation with PrP antibodies 6H4 and R2.
Figure 4.2 Coculture of SMB and HMH8 cells. A) Phase contrast micrograph of SMB and HMH8 cells after 6 days of coculture, with tracker-labelled SMB cells identified by an asterisk. Note the close apposition of the two cell types. B) Fluorescence micrograph of A showing the SMB cells labelled in red as a result of prior incubation with Syto 64 cell tracker dye. The staining intensity was observed to persist for at least 21 days and no dye transfer between cells was observed in initial experiments. Scale bar equals 50 μm.
Figure 4.3 Conversion of tagged PrP molecules is generally not observed following 7 day coculture with SMB cells. A) SMB and HMH8 cells were cocultured at a 1:1 ratio for 7 days, lysed, processed for $\PrP^{Sc}$ and Western blotted using antibodies 3F4 which recognises only hamster PrP (hence also binds to the chimeric HMH PrP) and 6H4 which recognises both hamster and mouse PrP. Samples were derived from scrapie-infected hamster brain, HB; recombinant hamster PrP, R; SMB cells, S; cocultured SMB and HMH8 cells, 1H8:1S; HMH8 cells, H8; scrapie-infected mouse brain, B. Note the absence of 3F4-positive $\PrP^{Sc}$ from the coculture. The 6H4-positive $\PrP^{Sc}$ detected in the coculture is derived from mouse $\PrP^{Sc}$ in the SMB cells as well as any conversion of the endogenous mouse PrP in the HMH8 cells following coculture.

B) SMB cells were cocultured with either MWY23 or MWY24 cells for 7 days and processed for $\PrP^{Sc}$ as before. The Western blot was analysed using antibody L42, which recognises the mutant MWY PrP, and antibody 6H4, which recognises both mutant and wild-type mouse PrP. Samples were derived from SMB cells, S; cocultured SMB and MWY23 cells, 1M23:1S; MWY23 cells, M23; cocultured SMB and MWY24 cells, 1M24:1S; MWY24 cells, M24. Note the appearance of a discreet band from the SMB/MWY23 coculture, which upon increased exposure also appears in the MWY23 lane. This presence of this band is not characteristic of $\PrP^{Sc}$ and its identity, and the origin of the additional bands detected from the MWY24 cells and the cocultured SMB/MWY24 cells remain to be established. The exposure time for the L42 blot is 5-fold longer than the 6H4 half.
coclulture with SMB cells. After 7 days of coculture the cells were passaged in G418 for 2 weeks to remove all the neo-sensitive SMB cells from the coculture, thus allowing selective re-isolation of the neo-resistant target cells. This selection step was included so that production of PrP\(^\text{Sc}\) could be detected using the 6H4 antibody, having removed the mouse prions present in the SMB cells.

The efficiency of G418-induced SMB cell death was confirmed by labelling SMB cells with the PKH26 cell-tracker dye and also by in situ hybridisation of populations with an antisense probe to the neo transcript. Following 1 week of coculture the presence of PKH26 labelled SMB cells (red) could be detected along with unlabelled HMH8 cells (Figure 4.4 A). After G418 treatment, no labelled cells could be detected demonstrating the efficient removal of all the SMB cells, and also that no dye transfer between cells had occurred. Parallel cocultures which were not subjected to G418 treatment demonstrated that the dye intensity could persist for at least 21 days. I found that 10 days of exposure to the antibiotic was sufficient to kill the SMB cells but for all the studies in this series of experiments, cells were passaged in G418-containing media for a minimum of 14 days prior to analysis.

G418-induced death of the SMB cells was further demonstrated by in situ hybridisation (Figure 4.4 B). Following hybridisation of populations with an antisense probe to the neo transcript, approximately 10,000 cells of each type were counted. All control HMH8 cells were labelled whereas all control SMB cells were negative. The HMH8 cells re-isolated from the coculture with SMB cells were also all positive, indicating the removal of the SMB cells by G418. The efficiency of SMB cell removal was quantitated by counting the number of neo-negative cells in the re-isolated population. I estimated that less than 1 cell in 1000 was an SMB cell after neo selection.

Conversion of the endogenous mouse PrP in the re-isolated target cells was assayed following coculture with SMB cells for 7 days followed by 2 weeks in G418. The selected populations were generally passaged at least 3 more times (8-10 generations) in medium containing G418 prior to analysis. Cells were lysed, processed for PrP\(^\text{Sc}\), Western blotted and newly synthesised mouse prions were detected using antibody 6H4. This assay was repeated 14 times and
consistently led to the detection of mouse PrP\textsuperscript{Sc} in the target cells as demonstrated with HMH8 and HMH1 in figure 4.5 A. The coculture resulted in conversion of mouse PrP in HMH8 cells (lane 1H8:1S) and two independent cocultures with HMH1 cells at different ratios (lanes 1H1:1S and 1H1:3S). The coculture procedure was robust and consistently led to conversion of the target cells to approximately 30% of the level in the equivalent number of SMB cells assayed in parallel, and this was maintained after prolonged drug selection.

Since conversion of the target cells appeared to be stable, the signals detected were unlikely to be due to carryover from the PrP\textsuperscript{Sc} in the SMB cells, however, conclusive evidence for conversion came from the biosynthetic incorporation of a radioactive amino acid into PrP\textsuperscript{Sc}. Following coculture and selection, cells were pulse labelled in medium containing [\textsuperscript{35}S]-methionine followed by a chase in normal medium. The PrP\textsuperscript{Sc} was isolated from detergent extracts by proteinase K digestion followed by ultracentrifugation, and denatured with acidic guanidine isothiocyanate (see Methods). Radiolabelled PrP was renatured and immunoprecipitated, followed by deglycosylation and analysis by gel electrophoresis (Figure 4.5 B). A radiolabelled band at 21kDa was specifically immunoprecipitated from SMB cells (lanes 1, 2) and from HMH8 cells re-isolated from a coculture (lanes 3, 4), but not from control PS cells (lanes 5, 6). These results were reproducible and were confirmed following coculture of HMH1 cells with SMB. These studies demonstrated that the target cells were converted to stable expression and synthesis of PrP\textsuperscript{Sc} by coculture with SMB.

Similar attempts were made to convert the endogenous mouse PrP in MWY23 and MWY24 cells following coculture and G418 treatment. Re-isolated target cells were lysed, processed for PrP\textsuperscript{Sc} and Western blotted (Figure 4.6 A). Production of MWY prions was detected using antibody L42, and mouse prions were detected using 6H4, which also detects MWY PrP. Low levels of L42-positive PrP\textsuperscript{Sc} could be detected from the coculture between MWY23 and SMB cells (lane 1M23:1S) demonstrating conversion of the mutant molecule. These bands were similar to those seen in figure 3.9 following infection of MWY24 cells with a brain homogenate. This signal was more intense with 6H4, and control MWY23 cells were negative (lanes M23). The results obtained from the
coculture between MWY24 and SMB cells were confusing since although PrP\textsuperscript{sc} signals could be detected from the cocultured cells (lanes 1M24:1S), equivalent bands of lower intensity were detected from the control MWY24 cells (M24) detected with both PrP antibodies as were seen earlier in figure 3.9. It appears that the MWY PrP molecule in MWY23 cells is converted to PrP\textsuperscript{sc} in these experiments, although it cannot be ruled out that the endogenous mouse PrP may have also undergone conversion.

Further cocultures were performed between the neo-resistant, PrP\textsuperscript{-}-expressing, ovine cell line SA80BR (provided by Dr. W. Goldmann, NPU, Edinburgh) and SMB cells. These experiments were performed to assess whether the SA80BR cells were susceptible to infection by SMB. Following coculture and selective re-isolation, cells were lysed, processed for PrP\textsuperscript{sc} and Western blotted using antibody 6H4 to detect ovine prions. No production of ovine PrP\textsuperscript{sc} was observed in repeated attempts (Figure 4.6 B) and this is likely to be due to the species barrier between sheep and mice. This experiment served as a good negative control, providing further evidence that all SMB cells are killed after G418 treatment.

### 4.2.2 Efficiency of infection by live SMB cells

The quantitative dependence of the interaction was analysed by varying the input ratio of SMB to target cells, while keeping the number of target cells constant. After one week of coculture, the SMB cells were removed by antibiotic selection for two weeks as before, and the level of PrP\textsuperscript{sc} in the target cells was determined by Western blotting using antibody 6H4 (Figure 4.7 A). The maximum expression of PrP\textsuperscript{sc} was achieved at around a unitary ratio, and increasing the number of SMB cells to a ratio of 0.2 (target/SMB) led to a characteristic decrease in expression. Conversion of the target cells was detected at a ratio of 20, but was little over background was observed at a ratio of 100. The results, as quantitated by image analysis, are illustrated in figure 4.7 B.

Infection by scrapie prions (Chapter 3) and by live SMB cells (above) are not strictly comparable since the source of the scrapie prions in the brain homogenate used to directly infect the target cells, and the strain used to infect
the mouse from which SMB cells were originally derived are different. Furthermore, the time scale for infection using the two methods also differ. However, since the target cells used are the same, and assuming that for both experimental designs, the amounts of PrP\textsuperscript{Sc} used were on a linear part of the conversion curve, the two methods can be compared. The amounts of PrP\textsuperscript{Sc} that were required for conversion of target cells using SMB cells and a brain homogenate were estimated by comparative Western blotting. These studies indicated that conversion of a dish of target cells by SMB cells required approximately 2,500-fold less PrP\textsuperscript{Sc} than conversion by a brain homogenate.

4.2.3 Evidence for contact dependence of SMB cell infection

4.2.3.1 Analysis of infectivity in SMB cell conditioned medium

As discussed in the introduction, a previous report on GT1 cells demonstrated that conditioned medium from infected cells could transfer prions to uninfected cells (Schatzl et al., 1997). This encouraged me to analyse if SMB cells could convert the target cells by releasing or generating PrP\textsuperscript{Sc} in the medium. In order to investigate this possibility, medium was conditioned for 2 days by SMB cells at high density, and then incubated with HMH1 cells for 7 days. PrP\textsuperscript{C} and PrP\textsuperscript{Sc} samples were prepared from cell lysates, followed by Western blotting using antibody 6H4 (Figure 4.8 A). Exposure of HMH1 cells to conditioned media did not lead to conversion of the endogenous mouse PrP in the HMH1 cells (lane CM). Parallel cocultures were performed with live SMB cells which as expected induced conversion of the target cells (lane 1H1:1S). In repeated attempts, SMB-conditioned medium did not induce any detectable conversion to PrP\textsuperscript{Sc} in the target cell lines. The activity of live SMB cells is therefore unlikely to depend on the presence of stable infective components, such as PrP\textsuperscript{Sc}, in the medium.

4.2.3.2 Analysis of insert cocultures

Another possible means through which SMB cells could conceivably infect target cells is via the release of either an unstable activity, or PrP\textsuperscript{Sc} in membrane exovesicles (Denzer et al., 2000) which might not be recovered adequately in conditioned medium. This possibility was investigated by coculturing SMB and target cells by bringing them into close proximity but not into contact. This was achieved by coculturing one population on a polyethylene terephthalate track-etched insert membrane with high density pores of 0.4 \( \mu \text{m} \) diameter, while the
Figure 4.4 Selective re-isolation of target cells. A) Merged phase and fluorescence micrographs of a coculture between SMB and HMH8 cells. SMB cells were labelled with PKH26 cell tracker dye and cocultured with HMH8 followed by neo selection. Note that 7 days after coculture approximately half of the cells are labelled whereas following neo selection none are labelled indicating that there are no longer any SMB cells present. Scale bar represents 100 μm. B) Labelling of neoR cells by in situ hybridisation. Cells were hybridised in parallel with an antisense probe to the neo gene showing that all HMH8 cells are labelled whereas none of the SMB cells are labelled. Note that all cells are labelled following hybridisation to HMH8 cells isolated by neo selection after coculture with SMB. Scale bar equals 50 μm.
Figure 4.5 Production and synthesis of PrPSc in HMH target cells after coculture with SMB cells. A) SMB cells were cocultured with either HMH8 or HMH1 for 7 days followed by neo selection. PrPSc production was analysed by Western blotting using antibody 6H4 which recognises mouse PrP. Samples were derived from scrapie-infected mouse brain, B; SMB cells used for coculture, S; HMH8 cells re-isolated by neo selection from a coculture between HMH8 and SMB at a 1:1 ratio, 1H8:1S; HMH8 cells used for coculture, H8; HMH1 cells re-isolated from a coculture at 1:1 (HMH1:SMB) ratio, 1H1:1S; HMH1 cells re-isolated from a coculture at 1:3 (HMH1:SMB) ratio, 1H1:3S; HMH1 cells used for coculture, H1. B) Synthesis of radiolabelled PrPSc following metabolic labelling of cultured cells with [35S]-methionine (see Methods). Lanes 1 and 2 are from SMB cells used in the coculture; lanes 3 and 4 are from HMH8 cells re-isolated from a 1:1 coculture with SMB cells; lanes 5 and 6 are from control PS cells. Note the 21kDa deglycosylated band present in SMB cells and cocultured HMH8 cells, but not in control PS cells.
Figure 4.6 Coculture of SMB cells with MWY23, MWY24 and SA80BR cells. A) MWY23 and MWY24 cells were cocultured with SMB cells for 7 days re-isolated from the coculture by two weeks in G418, lysed, processed for PrPSc and Western blotted using antibodies L42 which recognises the mutant MWY PrP and 6H4 which recognises both mutant and wild-type mouse PrP. Samples were derived from SMB cells, S; MWY23 cells re-isolated from a coculture at 1:1 (MWY23/SMB) ratio, 1M23:1S; MWY23 cells, M23; MWY24 cells re-isolated from a coculture at 1:1 (MWY24/SMB) ratio, 1M24:1S; MWY24 cells, M24. Note the appearance of L42-positive bands from the MWY23/SMB coculture which could reflect conversion of MWY PrP since control MWY23 cells are negative. The mobility and glycoform pattern of these bands differ from that of PrPSc in SMB cells. The presence of 6H4-positive PrPSc from the same coculture could also reflect conversion of the endogenous mouse PrP in MWY23 cells. The identity of the bands present in the MWY24 cells, which are more intense for the cocultured MWY24/SMB cells remains to be established. B) SA80BR cells were cocultured with SMB at a 1:1 ratio for 7 days, re-isolated from the coculture by two weeks in G418, lysed and processed for PrPC and PrPSc. Samples were derived from control SMB cells; two independent cocultures of SA80BR and SMB cells, 1SA80:1S; and control SA80BR cells. Note the absence of PrPSc from either of the cocultures reflecting firstly, that SMB cells are unable to induce conversion of ovine PrP in SA80BR cells, and furthermore, the complete removal of the SMB cells during the neo selection.
Figure 4.7 Dependence of PrPSc production on the cell ratio in the coculture. A) HMH1 cells were cocultured with SMB so as to vary the ratio over a 500-fold range, followed by selective re-isolation of HMH1 cells, processing for PrPSc and analysis by quantitative Western blotting at 60 μg protein/well. Samples were derived from SMB cells, S; lanes 2-8 were from cocultures at 0.2:1 to 100:1 (HMH1/SMB) ratios; HMH1 cells, H1. B) Quantitation of the data in A by prior image analysis of the Western blot. The decrease in PrPSc level seen at the lowest ratio of HMH1:SMB was reproducible and did not appear to reflect any toxic effect of higher cell density.
other population was cultured on the bottom surface of the dish and shared the medium with cells on the insert (see Methods and Figure 4.8 B). In order to address the possibility that SMB cell death was responsible for conversion of target cells during the period of G418 selection of cocultures, G418 was added to the medium of parallel insert cocultures to expose target cells to the products of dying SMB cells. A control coculture between SMB and HMH8 cells in contact was performed in parallel. When cells were either cocultured in contact for 7 days followed by G418 selection (lane v), or were cocultured separately and assayed after a comparable time (lanes i, ii, iii, iv), the separated configuration did not give detectable conversion, that is less than 10% of the level of PrP\textsuperscript{Sc} observed for the contact case. The lack of detectable conversion even in the insert cocultures exposed to G418 rules out the possibility that the products of dying SMB cells is responsible for conversion and the results supports the importance of close apposition or cell contact in cell-based infection.

### 4.2.4 Infection by aldehyde-fixed SMB cells

In order to directly investigate the dependence of conversion on cell contact, cocultures were performed with fixed infected cells. The motivation behind the fixation of the SMB cells was two-fold. Firstly, fixed cells are essentially dead, so this would result in a simplification of the functions of the infected cells. Secondly, following fixation and extensive washing, the infected cells would not be able to liberate PrP\textsuperscript{Sc} by facilitated release. SMB cells were fixed in a mixture of buffered paraformaldehyde and glutaraldehyde (see Methods), and then washed for 48 hours to remove the fixatives as outlined in figure 4.9. The cells were dead as assayed by staining with vital dyes, and the cells did not incorporate a radiolabelled amino acid into protein as detected by autoradiography (Figure 4.10). Live and fixed SMB cells were stained in parallel with trypan blue for 5 minutes. The dye was excluded from the live cells (A) and was only taken up by the fixed cells (B). Protein synthesis was also compared in live and fixed SMB cultures by monitoring uptake of [\textsuperscript{3}H]-leucine. After development and fixation, more grains were visible in the live cells (C) and very few in the fixed cells (D), demonstrating that protein synthesis was abolished in the fixed cells, indicating that they were all dead. Quantitation of the number of grains per unit area on cells and on empty areas of the dish
revealed that no grains over background levels were detected in all the fixed cells. The grain count for the fixed cells was equivalent to approximately 5% of the level in live cells.

The state of PrP\textsuperscript{Sc} was investigated by fractionation of fixed cells. Little or no PrP was detected in the high speed pellets from fixed SMB cells after detergent lysis and proteolysis (Figures 4.11 B and 4.12 A, lanes F) whereas PrP\textsuperscript{Sc} was found as aggregates in the initial low speed pellets (Figure 4.12 A, lane P). PrP\textsuperscript{Sc} isolated from live SMB cells was processed in parallel as a control (Figures 4.11 B and 4.12 A, lanes S). Target cells were plated onto the fixed and washed SMB cells for varying times and came into close apposition or partial overlap with them (Figure 4.11 A).

4.2.4 Analysis following 2 day propagation period
The target cells were cocultured with an identical number of fixed SMB, or control fixed PS cells for 5, 24 or 96 hours as outlined in figure 4.9. After trypsinisation, the target cells were replated for 2 days, lysed and processed for PrP\textsuperscript{Sc}. A typical fixed cell coculture with HMH1 cells is shown in figure 4.11 B. Although the cells were propagated for 2 days and samples from an entire 80 cm\textsuperscript{2} flask were processed for each time point, the signals detected on the Western blot were consistently weak. Upon increased exposure of the blot, non-specific bands could be detected even in control HMH1 cells (lane H1). PrP\textsuperscript{Sc} was detected at low levels only from the 96 hour coculture with fixed SMB cells (lane S96). This experiment shows that the PrP\textsuperscript{Sc} signal cannot be accounted for by carryover from the fixed cells. This would in any event be unlikely because of the evidence for effective cross linking of PrP\textsuperscript{Sc} as illustrated in figures 4.11 B and 4.12 A.

4.2.4.2 Analysis following 14 day propagation period
In light of the low signal levels obtained after replating the cells for 2 days, it seemed probable that the level of conversion could be increased if the protocol was modified to extend the propagation time post-coculture. HMH1 cells were cocultured with an identical number of either live or fixed SMB cells for 1, 3, 5, or 7 days, after which the target cells were trypsinised and propagated in medium containing G418 for 2 weeks. Cells were lysed and processed for PrP\textsuperscript{Sc}
followed by Western blotting (Figure 4.12 A). The signals from the live cell cocultures for 1, 3, 5 and 7 days (L1, L3, L5 and L7) were consistently stronger than the corresponding signals from the fixed SMB cell cocultures for 1, 3, 5 and 7 days (F1, F3, F5 and F7). An increase in conversion with time was observed for both contexts. The signals detected were higher than those obtained following a 2 day propagation period consistent with a requirement for amplification following the coculture period. No detectable conversion was observed after 1 day of coculture with fixed SMB cells, and no conversion was observed in parallel experiments with fixed, washed PS cells in place of SMB cells. The results as quantitated by image analysis demonstrate that for each time point, the level of conversion observed for fixed cells was approximately 10% of the level for live cells (Figure 4.12 B). These results demonstrate that dead, fixed SMB cells retain substantial activity in converting target cells to stable production of PrP$.4.2.5$ Infection by irradiated SMB cells

It has been suggested that neurons may be the most important cells for the spread of scrapie in the PNS and CNS (Kimberlin and Walker, 1988). Since SMB is an actively dividing cell, I wanted to determine if cell division was required for propagation in the cell cocultures. This was addressed by a series of irradiation studies. SMB and HMH1 cells were exposed to different doses of X-irradiation and replated. After 7 days, BrdU incorporation was used to assess DNA synthesis in the cultures relative to unirradiated controls (Figure 4.13 A). A dose of 10 Gy was effective at reducing DNA synthesis in SMB and HMH1 cells by approximately 7- and 4- fold respectively. Upon analysis of the 10 Gy irradiated cells in culture, the density of the cells appeared stable and a high proportion of multinucleated cells were visible. This was a clear indication that entry into S phase as well as to mitosis were blocked hence a dose of 10 Gy was used for all subsequent experiments. Higher doses such as 15 Gy were found to be severely detrimental to cell morphology and viability.

$4.2.5.1$ Assay for conversion of endogenous mouse PrP

In initial experiments, both SMB and HMH1 cells were irradiated and cocultured at high density for 7 days (see Methods). During the subsequent two week neo selection, the majority of the HMH1 cells in the coculture died along
Figure 4.8 Evidence for contact dependence of SMB cell infection. A) HMH1 cells were incubated with medium conditioned by SMB cells for 7 days and processed for PrPSc. Samples were derived from scrapie-infected mouse brain, B; SMB cells, S; marker lane showing artifactual band, M; control coculture of SMB and HMH1 cells, 1H1:1S; HMH1 cells exposed to conditioned medium from SMB, CM; HMH1 cells, H1. Note the absence of PrPSc production in the HMH1 cells exposed to SMB-conditioned medium. B) Appearance of PrPSc is prevented by separation of infected and target cells. In (i) to (iv) the SMB and HMH8 cells were separated during coculture by growth on different surfaces of a culture dish with an insert of pore size 0.4 μm. Note that one population grows on the insert and the other on the bottom of the dish and that the medium is shared. In (ii) and (iv), G418 was added to the medium in order to kill SMB cells during the coculture. In (v), HMH8 and SMB were cocultured as before. After coculture, the HMH8 cells were propagated in G418 for (i) to (iv), or selected from the coculture (v) and processed for PrPSc. Samples were derived from SMB cells, S; cocultures (i) to (v); HMH8 cells, H8; marker lane, M.
Figure 4.9 Schematic of the fixed SMB cell coculture procedure. SMB cells (red) were fixed with a mixture of paraformaldehyde and glutaraldehyde (see Methods) and washed extensively for 2 days. Target cells (blue) were plated onto the fixed SMB cells and cocultured for up to 7 days during which the target cells divided and overlapped the SMB cells. Target cells were trypsinised, replated and either assayed directly for PrP<sup>Sc</sup> after 2 days or propagated in G418 for two weeks prior to analysis.
Figure 4.10 Demonstration that fixed SMB cells were dead. Cell viability was determined by trypan blue exclusion and incorporation of radioactively labelled leucine into protein. A) live and B) fixed SMB cells were incubated with 0.4% trypan blue. Note that the vital dye is only taken up by the fixed SMB cells. Scale bar represents 50 µm. C) live and D) fixed SMB cultures were exposed to [3H]-leucine for 6 hours, fixed and developed. Note that for the fixed SMB cells, the grains are at the same density on the cells as on the background, demonstrating that no protein synthesis was occurring in the fixed cells, which indicates that they were all dead. Scale bar equals 30 µm.
Figure 4.11 Coculture of target cells with fixed SMB cells. A) Association of fixed SMB and live target cells labelled with cell tracker dyes. SMB cells were stained with Syto 64 cell tracker dye (red), plated, fixed and washed (see Methods) and HMH1 cells were labelled with Cell Tracker Green and were plated onto the fixed SMB cells for three days. Note that the HMH1 cells extend over the upper surface of three of the SMB cells giving a yellow zone of overlap. Scale bar equals 50 μm. B) Infection of target cells by fixed SMB cells. Following coculture of HMH1 cells with either fixed SMB or PS cells for 5, 24 or 96 hours, HMH1 cells were propagated for 2 days and assayed for PrPSc by Western blotting. Samples were derived from fixed SMB cells, F showing that fixation leads to cross linking of PrPSc; live SMB cells, S; cocultures between HMH1 and SMB for 5, 24 and 96 hours, S5, S24, S96 respectively; cocultures between HMH1 and PS for 5, 24 and 96 hours, P5, P24, P96 respectively; HMH1 cells, H1. Note the appearance of PrPSc from the coculture between fixed SMB and HMH1 cells for 96 hours.
Figure 4.12 Conversion of HMH1 cells by fixed SMB cells. SMB cells were fixed and washed as before and cocultured with HMH1 cells for 1, 3, 5 or 7 days followed by trypsinisation of the HMH1 cells, propagation in G418 for 2 weeks, processing for PrPSc and quantitative Western blotting at 80 μg protein per well. Samples were derived from the low speed pellet fraction from SMB cells showing aggregates, P; PrPSc fraction from fixed SMB cells after proteinase K digestion and ultracentrifugation, F, note that no PrPSc is visible; PrPSc fraction from control live SMB cells, S; HMH1 cells, H1; L1, L3, L5, and L7 were derived from control cocultures with live SMB cells for 1, 3, 5, and 7 days respectively, note the appearance of PrPSc even from a 1 day coculture; F1, F3, F5, and F7 were derived from cocultures with fixed SMB cells for 1, 3, 5, and 7 days respectively, note that the amount of PrPSc increases with extended length of coculture period. Note that the levels of PrPSc production from the fixed SMB cell cocultures were approximately 10% of the levels from the corresponding live cell cocultures. Parallel cocultures with fixed PS cells did not induce any conversion of the target cells. The gel is representative of 3 experiments.
with the SMB cells. This was apparently not due to a loss of neo-resistance in the
dissociated HMH1 cells, since propagation of irradiated HMH1 cells in parallel
without neomycin treatment also resulted in cell death. In contrast, the viability
of irradiated SMB cells appeared stable for several weeks in culture. In light of
these results, unirradiated HMH1 cells were cocultured with either irradiated
or unirradiated SMB cells for 1 week followed by 2 weeks in G418 as before.
The cells were lysed, processed for PrP\textsuperscript{Sc} and Western blotted (Figure 4.13 B).
The coculture with irradiated SMB cells (lane 1H1:1S\textsubscript{10 Gy}) led to a comparable
level of conversion as the control coculture with unirradiated SMB cells (lane
1H1:1S). These results demonstrate that irradiated SMB cells remain fully
capable of converting target cells to stable production of PrP\textsuperscript{Sc}.

4.2.5.2 Bullseye cocultures

Attempts were made to analyse the propagation efficiency of the PrP
conformational change across a lawn of irradiated cells in culture by
establishing a 'bullseye' coculture configuration (Figure 4.14). The intention of
the experiment was to plate irradiated SMB cells in the centre of a dish as a
focal point of infectivity, and to surround them with irradiated target cells.
Monitoring the propagation of infectivity across the dish could be used to
assess the timing of the event. The target cells selected for this series of
experiments were HMH1, HMH8 and MWY23—the latter was selected over
MWY24 due to the higher background associated with these cells as shown in
previous experiments. The protocol was from Dr. G. Dunn, KCL and Dr. D.
Zicha, ICRF, personal communication. After irradiation, SMB cells were plated
in the centre of a small Tygon plastic tube (6.4 mm diameter) which had been
melted onto a 35 mm dish. The cells were allowed to sit down before the
irradiated target cells were plated around them and also left for 3-5 hours. The
tubing was then removed and the cells were cocultured for 2-14 days. During
the first 1 to 2 days, both cell populations migrated to fill the gap (0.8 mm)
created by the tube wall. The cells were subsequently lysed onto an immobilon
membrane and processed for PrP\textsuperscript{Sc} (see Methods). A processed lift from a 5 day
bullseye coculture between SMB and HMH1 cells as detected by 6H4 is
illustrated in figure 4.14. Since the 6H4 antibody recognises mouse prions, the
black zone in the centre of the lift is largely attributed to PrP\textsuperscript{Sc} in the SMB cells.
Figure 4.13 Conversion of HMH1 cells following coculture with irradiated SMB cells. A) DNA synthesis in SMB and HMH1 cells was analysed following exposure of the cells to different doses of X-irradiation and measuring BrdU incorporation after 1 week. Note that a dose of 10 Gy was effective at reducing DNA synthesis in SMB and HMH1 cells by approximately 7- and 4-fold respectively. The error bars represent the standard deviation from the mean. Individual measurements were estimates from 190 to 210 cells. B) HMH1 cells were cocultured with 10 Gy irradiated SMB cells for 1 week, re-isolated by 2 weeks in G418 and processed for PrPSc. Samples were derived from scrapie-infected mouse brain, B; SMB cells, S; control coculture between HMH1 and non irradiated SMB cells, 1H1:1S; coculture between HMH1 and irradiated SMB cells, 1H1:1S10Gy; and HMH1 cells, H1. Note that irradiated SMB cells are able to convert HMH1 cells to a similar level as non-irradiated SMB cells.
**Figure 4.14 Schematic of the bullseye coculture procedure.** SMB and target cells were exposed to a 10 Gy dose of X-irradiation and replated for five days to recover. SMB cells were plated in the centre of a piece of Tygon plastic tubing and target cells in the surrounding dish and cocultured for 2 to 14 days. Cells were subsequently lysed onto an immobilon membrane and processed for PrPSc by treatment with proteinase K, PMSF, guanidine isothiocyanate, incubation with 6H4 and development. Note the appearance of the black zone in the centre of the filter contributed to by the PrPSc in the SMB cells.
The difficulties associated with the establishment of this assay are discussed below.

4.3 DISCUSSION

The focus of the experiments described in this chapter were to establish a system in dissociated cell culture to study the cell-based transfer of scrapie prion infectivity. This was achieved using the scrapie-infected SMB cell line and derivatives of its cured counterpart, the PS cell. One of the main observations from these studies was that the target cells were converted to stable expression and synthesis of mouse PrP

\[ \text{PrP}^{\text{Sc}} \]

by coculture with SMB. The absence of conversion of the chimeric PrP in HMH8 cells is likely to be attributed to more favourable conversion of the endogenous mouse PrP as discussed in the previous chapter concerning infection with scrapie prions. The results regarding the MWY23 and MWY24 lines are suggestive of low levels of conversion to PrP^{Sc} in these cells, which are amplified by coculture with SMB. Confirmation of this possibility would require the isolation of further cell lines expressing MWY PrP and careful analysis of their PrP expression levels over multiple successive passages.

Analysis of the efficiency of infection by live SMB cells revealed that the maximum level of conversion attained was to approximately 30% of the level in the equivalent number of SMB cells assayed in parallel. In the absence of a single cell assay to identify PrP^{Sc} positive cells, it is not clear whether this represents conversion of 30% of the cells to the SMB level, or conversion of a higher percentage to a level below that of SMB. However, this conversion efficiency by live SMB cells is comparable to the 10% level reported in chapter 3, for infection of target cells with subcellular preparations of prion rods containing aggregates of PrP^{Sc}. Although infection by the two methods is not strictly comparable, it is noteworthy that conversion of target cells by SMB cells required approximately 2,500-fold less PrP^{Sc} than conversion by a brain homogenate, as determined by comparative Western blotting. These differences in specific activity between PrP^{Sc} in subcellular aggregates and intact cells could possibly be explained in light of earlier studies of prion liposome complexes. End point titration studies have revealed that an approximately 100-fold increase in infectivity is associated with the dispersion of prion rods into
liposomes (Gabizon et al., 1988). Further experiments revealed that PrP 27-30 in liposomes was rapidly taken up by cultured cells whereas prion rods remained as extracellular masses. These results, although not strictly comparable to the present system, suggest that the presentation of monomeric or oligomeric PrP\textsuperscript{Sc} embedded or anchored in a lipid membrane is more effective for converting target cells than the presentation of aggregates in suspension.

The mechanism of cell-based infection was initially investigated by experiments with conditioned medium from the infected cells. It is conceivable that SMB cells might generate stable infective components in the medium which could be transferred to the target cells. The finding that conditioned medium had no activity indicates that this extracellular pathway does not operate in this particular system. This was further supported by the absence of detectable conversion during the insert cocultures where the two populations were cocultured separately. It is noteworthy that although not all PrP\textsuperscript{Sc} rods would be able to pass through the 0.4 \(\mu\)m diameter pores of the insert, the role of released PrP\textsuperscript{Sc} in conversion is ruled out by the conditioned medium experiments. On the other hand, exovesicles released by the infected cells would still be able to pass through the pores and contact the target cells. While the possibility of a short range diffusible signal cannot be ruled out, the results support the importance of close apposition in cell-based infection. In order to investigate the requirement for cell contact as well as cell viability, I decided to inhibit the activities of live cells which could conceivably induce conversion of the target cells and examine the ability of dead infected cells to initiate infection. It is plausible that live cells could induce conversion through the facilitated release of GPI-anchored PrP\textsuperscript{Sc} followed by its insertion into target cell membranes. Another remaining possibility is the active release of membrane exovesicles which could contain PrP\textsuperscript{Sc}, since they are involved in a variety of physiological processes in a number of cell types (Denzer et al., 2000). One classical property of scrapie infectivity is its resistance to conditions of aldehyde fixation which kills both cells and conventional infectious agents (Pattison, 1965b). In light of suggestions that the effects of paraformaldehyde fixation are potentially reversible (Pearse, 1980), I decided to permanently fix the SMB cells with a mixture of paraformaldehyde and glutaraldehyde. These fixation conditions were effective at cross-linking the PrP\textsuperscript{Sc} in the infected cells as shown
in figure 4.12 A. The experiments with the fixed cells demonstrated that SMB was unable to induce conversion after 1 day of coculture followed by a 14 day propagation period, but the level of conversion in the target cells increased as the period of initial coculture was extended. This is consistent with the results from the live SMB cell cocultures, where the signals also intensified with increased length of coculture period. Furthermore, the extent of conversion was higher than the levels attained following a 2 day propagation period. It seems likely that an extended propagation period is required in order to detect newly synthesised PrPSc in target cells following coculture with fixed SMB cells.

The activity of fixed SMB cells is indicative of a templating mechanism, whereby PrPSc on the infected cell is able to act in trans to convert PrPc on the surface of a target cell. The two cell types have to be in contact for a minimal length of time for this initial stage to lead to conversion. The conversion could be amplified by the cis interaction between newly formed PrPSc in the target cell and other PrPc molecules in the plane of the membrane. During the subsequent propagation period, infected target cells could also act as prion donors, converting neighbouring target cells in a similar manner. This is analogous to the production of protease-resistant PrP in cell-free systems where the formation of PrPc/PrPSc complexes precedes the gradual increase in the percentage of PrPc that acquires protease resistance.

During the course of an experimental infection, prions propagate slowly from the initial site of inoculation causing neurologic dysfunction in certain areas of the brain once threshold levels have been reached. The duration period for replication in the brain can span several months (Kimberlin and Walker, 1988). A mechanism that is dependent on cell contact would in general be expected to propagate at a much slower rate than a mechanism involving diffusible extracellular forms of PrP. Neurons are the highest expressers of PrP mRNA and are considered to be the most important cell type for the spread of prions in the body (Kimberlin and Walker, 1988; Kretzschmar et al., 1986). Taking into account the fact that neurons are non-dividing cells, I decided to model in vivo prion propagation by establishing the bullseye coculture assay for analysis of prion transfer across a monolayer of irradiated cells. Several problems were associated with the bullseye coculture experiments. Firstly, since previous
experiments revealed that the SMB cells were unable to convert the chimera in HMH8, I decided to use the 6H4 antibody to detect conversion of the mouse PrP in the HMH1 and HMH8 target cells. As expected, 6H4 recognised the mouse prions in the SMB cells, detected as a black circle in the centre of the blots as illustrated in figure 4.14. I then decided to look for an increase in diameter of this region of infectivity with time in parallel cocultures. The results of the earlier coculture experiments suggested that the levels of conversion after 1 day were low, and increased with extended coculture period. Bearing this in mind, it was conceivable that only 1 target cell diameter could be infected per day, and this could probably take longer since intracellular propagation is also required to generate a decent signal. For these reasons, the time required to notice an increase in the size of the infected region could be several weeks. However, no increase was detected over a 14 day period, after which the viability of the cell monolayer became compromised. It is possible that the antiproliferative antibiotic mitomycin C may be more suitable than irradiation in these experiments for maintaining SMB cells. In further experiments, MWY23 cells were cultured with SMB cells and assayed for conversion of MWY PrP using antibody L42. Although the L42 antibody did not recognise the inoculating mouse prions and in theory would allow any detected signal to be attributed to newly synthesised mouse prions, low background signals were detectable all over the blots. Since any signals generated from de novo synthesised PrP\textsuperscript{Sc} would be expected to be low, the background signal on the blot obscured the possible detection of conversion. Attempts were made to reduce the background by increasing the concentrations of proteinase K, altering the constituents of the block, and using lower concentrations of antibodies but the background levels were unavoidable. For these reasons, I decided to discontinue these studies which were also relatively time consuming, and focus on the other experiments detailed above. It is nonetheless possible that these cultures may be a fruitful avenue for future investigation of the mechanism of cell-based propagation.

The principal conclusion from the present chapter is that an infected cell is capable of converting its neighbours to stable expression and synthesis of PrP\textsuperscript{Sc}. The mechanism of conversion in this system is dependent on cell-to-cell contact, as demonstrated by the evidence above. Since the activity of fixed cells can
account for approximately 10% of the activity of live cells, it remains possible
that other mechanisms may also play a role in the cell-based transfer of scrapie
prion infectivity. It is also possible that all the observed activity is due to a
contact dependent mechanism, and this is decreased by fixation. The target cells
in this study were converted to approximately 30% of the level in the SMB cells
used to induce the infection. In light of these findings, a single-cell assay
capable of distinguishing a PrPSc-expressing cell from an uninfected cell would
allow more comprehensive quantitation of the conversion efficiency.
Chapter 5

Search for a PrPSc-specific cytochemical marker
5.1 INTRODUCTION
A number of cell types chronically infected with scrapie prions display no obvious cytopathology, as exemplified by the SMB cell described in the previous chapters. A significant question with regard to such infected cultures is whether the conversion that has been detected represents uniform infection of most or all of the cells in the culture, or rather the relatively greater infection of fewer cells. The development of a method for determining the frequency and levels of conversion in cultured cells requires the establishment of a reliable assay for detecting the presence of scrapie agent. Profound alterations in stress proteins have been recorded in scrapie-infected N2a cells (Tatzelt et al., 1995) and a subpopulation of infected GT1 cells exhibit autophagic vacuolation and apoptosis (Schatzl et al., 1997), yet these changes are not found consistently, and are not characteristic of all cell types capable of sustaining prion infectivity. As a result, the detection and quantitation of scrapie infectivity in tissue culture has remained largely dependent on animal bioassays (Prusiner et al., 1999b). To obtain this information, cultures are typically subjected to limited dilution cloning to establish cultures from single cells and these cultures are analysed by infecting mice with cell aliquots or extracts. Such studies, apart from being slow, tedious and relatively expensive, cannot render the detailed information obtained from plaque assays used in virology. However, a remarkable convergence of biochemical, immunologic, pathologic, and genetic data accumulated over the last two decades convincingly argues that PrP^Sc is a major and necessary component of the infectious prion particle (Prusiner, 1999; Scott et al., 1989; Westaway et al., 1989). On this basis, further detection systems based on either the proteinase K resistance or detergent insolubility of PrP^Sc have been developed to detect its presence in cells and tissues. The detection of protease-resistant PrP in cell cultures offers a method of monitoring the presence of infectivity in given samples.

The most extensively used of these techniques is the Western blot (Bendheim et al., 1984) which in this system requires the preparation of at least 10^7 cells to generate a detectable signal on a Western blot. Based on the assumption that on a typical Western blot I am able to detect 10ng of PrP^Sc, and using the fact that the limit of detection corresponds to approximately 10^5 LD_{50} units (Prusiner and Scott, 1997), it can be calculated that 10ng of PrP^Sc is equivalent to
approximately $10^{13}$ moles, or $10^{10}$ molecules of PrP$^\text{Sc}$. Since the signal generated from $10^{10}$ molecules corresponds to $10^5$ LD$_{50}$ units, it can be estimated that 1 LD$_{50}$ unit corresponds to $10^5$ molecules. As discussed in Chapter 3, rodent bioassays have led to the estimate that 100 SMB cells also constitute 1 LD$_{50}$ unit (Clarke and Haig, 1970; Haig and Clarke, 1971), hence if $10^5$ molecules of PrP$^\text{Sc}$ are present in 100 cells, each SMB cell should contain around 1000 molecules of PrP$^\text{Sc}$. This minimum requirement for $10^7$ cells (at 1 LD$_{50}$ per 100 cells) corresponds to approximately $10^6$ LD$_{50}$ units which, as noted above is the detection limit for a Western blot. PrP 27-30 has also been detected using histoblots (Taraboulos et al., 1992a) and dot blots (Serban et al., 1990). However, the foregoing immunoassays are relatively insensitive compared to bioassays. More recently, several other methods have been developed to detect the presence of PrP$^\text{Sc}$ in infected cells including the cell blot (Bosque and Prusiner, 2000), the slot blot (Winklhofer et al., 2001) and ELISA which are quicker and more sensitive than Western blots. Nonetheless, the lack of single cell resolution with all of these assays restricts their suitability in quantitative applications.

The development of a rapid \textit{in situ} assay for the detection of scrapie infected cells has also been hindered by difficulties in the generation of PrP-specific antibodies. This restriction is attributed to immune tolerance to PrP$^\text{Sc}$ which is invoked to prevent immune reactivity with normal cellular PrP. The majority of the antibodies that have been raised to date in either Prnp$^{0/0}$ mice or by immunising hosts with PrP isolated from non-homologous species, react equally well with native and denatured PrP$^\text{C}$, but relatively poorly with non-denatured PrP$^\text{Sc}$ after limited proteolysis catalysed by proteinase K (Taraboulos et al., 1990). However PrP$^\text{Sc}$ immunoreactivity is greatly enhanced by exposure to protein-denaturing agents such as chaotrophic salts and formic acid (Serban et al., 1990). One of the most widely used monoclonal PrP antibodies is 3F4 which recognises SHa but not Mo PrP (Kascak et al., 1987) (Figure 5.1). Recombinant antibodies have also been recovered following immunisation of knockout mice with PrP 27-30 dispersed into liposomes for example R2, which reacts with non-denatured PrP 27-30 (Peretz et al., 1997). Attempts have further been made to develop PrP$^\text{Sc}$-specific antibodies, the availability of which could provide a starting point for the development of an effective \textit{in situ} assay.
A previous attempt to develop an immunofluorescent assay for PrP$^{\text{Sc}}$ positive cells was based on PIPLC treatment of live cells after which they were fixed with formaldehyde, permeabilised with Triton X-100, incubated with proteinase K, denatured with guanidine hydrochloride and processed for the detection of PrP (Taraboulos et al., 1990). These studies revealed an intense intracellular signal in scrapie-infected cells which colocalised with markers of the Golgi apparatus. One drawback to these studies is that fixation often introduces artefacts which could invalidate results, hence it was not surprising that these colocalisation findings were not consistently detected in all the subclones examined. A recent attempt to assess the susceptibility of cultured cells to \textit{in vitro} infection involved growth of infected cells at clonal density for several weeks, and calculation of the percentage of PrP$^{\text{Sc}}$-expressing colonies produced using a colony-lift blotting procedure (Birkett et al., 2001). One limitation to this method is that a number of cell types fail to grow at low density, hence clonal growth is almost impossible to attain. For these reasons the colony-lift procedure does not give a true estimate of the frequency of infection.

In the previous chapters, I demonstrated the establishment of an assay to study the transfer of scrapie prion infectivity between cultured cells. The target cells were converted to approximately 30% of the level of expression observed in extracts of an equivalent number of SMB cells, as assayed by Western blotting. In the absence of a single cell assay to identify PrP$^{\text{Sc}}$-positive cells, it was not clear if this represented conversion of 30% of the cells to the SMB level, or conversion of a higher percentage to a level below that of SMB. The focus of the experiments described in this chapter was to identify a PrP$^{\text{Sc}}$-specific marker that could be applied to live cells. This search was pursued using a number of recently generated PrP antibodies on infected SMB and uninfected PS cells, as well as selective removal of PrP$^{\text{C}}$ using a combination of proteases and PIPLC as a means to distinguish the two cell types. The ability of the plasma protein plasminogen to discriminate between PrP$^{\text{C}}$ and PrP$^{\text{Sc}}$ (Fischer et al., 2000) was also investigated in an attempt to establish this assay. The development of such a system would in theory allow infection efficiencies to be quantitated more accurately.
5.2 RESULTS

5.2.1 Comparison of SMB and PS cells using PrP antibodies

Initial attempts to develop an assay for distinguishing SMB from PS cells involved the putative PrP\textsuperscript{Sc}-specific antibody, 15B3 (Korth et al., 1997). The specificity of this antibody was first assessed by immunoprecipitation using lysates from the two cell types, however, there was no indication that 15B3 recognised PrP\textsuperscript{Sc} or even PrP\textsuperscript{C}. These results were confirmed by testing the reactivity of the antibody with live cells by immunofluorescence, however, no significant binding to either SMB or PS cells was observed.

5.2.1.1 Optimisation of PrP\textsuperscript{C} digestion conditions

As demonstrated in Chapter 4, the requirement for cell to cell contact in the cell-based transfer of scrapie prion infectivity reflects the importance of PrP\textsuperscript{Sc} localised on the plasma membrane in this culture system. For this reason, I decided to focus on surface rather than internal PrP\textsuperscript{Sc}. In the absence of a PrP\textsuperscript{Sc}-specific antibody, a considerable effort was made to optimise conditions for the digestion of PrP\textsuperscript{C} from the surface of cells using a combination of proteases and PIPLC. The rationale behind this approach was that following removal of PrP\textsuperscript{C} from SMB and PS cells, PrP antibodies would be reactive with PrP\textsuperscript{Sc} remaining only on the surface of the SMB cells. Due to the availability of the 3F4 antibody, the removal of PrP\textsuperscript{C} was first optimised using Ham-1 cells which were derived by transfecting SMB cells with hamster PrP. A range of proteases including proteinase K, papain, dispase and trypsin were tested along with PIPLC treatment. The concentrations, incubation times and temperatures for these reagents were all varied in an effort to define conditions for maximal removal of PrP\textsuperscript{C} from these cells. After treatment, the live cells were typically incubated with 3F4 followed by biotinylated goat anti-mouse and tyramide signal amplification. Proteinase K digestion resulted in extensive loss of cells from the coverslips and compromised the structural integrity of those cells that remained, hence this protease was avoided in further experiments. Maximal removal of PrP\textsuperscript{C} was achieved using 2U/ml PIPLC followed by 0.5 mg/ml dispase, each for 1 hour on ice. Using these conditions, 95% of the surface hamster PrP was removed from ham-1 cells as detected by antibody 3F4 (Figure 5.2). The intensity of staining was typically 5-10 fold higher at cell junctions as determined by image analysis. The results of this series of experiments as
Figure 5.1 Epitopes for the PrP antibodies used in these studies. Monoclonal antibody 15B3 recognises three distinct linear peptide sequences highlighted in purple. The 3F4 epitope comprises two methionines at positions 109 and 112 in hamster and human PrP but does not detect murine PrP since the corresponding amino acids are a leucine and valine respectively. The 6H4 antibody recognises amino acids residues 144-152 of PrP from a broad range of mammalian and rodent species. Recombinant monoclonal Fab R2 recognises an epitope comprising residues 225-231. PrP-Fabs were also generated by grafting mouse PrP sequences corresponding to amino acids 119-136, 121-144 and 121-158 into the HCDR3 of a control IgG Fab, b12.
quantitated by analysis of approximately 50 cells per coverslip are illustrated in figure 5.3. Whereas hamster PrP was removed from the surface of the ham-1 cells relatively efficiently, the results with 6H4 reveal that removal of mouse PrP is more complicated. Following treatment of the cells with dispase alone, the 6H4 signal consistently increased suggesting that protease digestion led to exposure of epitopes that were hidden in non-treated cells. Treatment with both PIPLC and dispase could at best remove only 60% of the 6H4-positive PrP^C.

### 5.2.1.2 Comparison of PrP on the surface of SMB and PS cells

Having defined optimal digestion conditions using the ham-1 cells, the effectiveness of this treatment on SMB and PS cells was investigated. To increase access of the reagents to cell-junctions, the cells were pre-treated with 5 mM EGTA for 5 minutes. After incubation of PS cells with PIPLC and dispase, the signal was decreased to 40% of the level in non-treated cells. However, SMB cells characteristically retain tight associations even following EGTA treatment, hence even low dispase concentrations caused the majority of the cells to peel off the coverslip in a sheet. As a result, SMB and PS cells were treated with EGTA and PIPLC alone and stained with antibody R2, which recognises both native PrP^C and PrP^Sc, followed by tyramide signal amplification (Figure 5.4). Using these defined conditions, only 50% of the PrP^C could be removed from the surface of the PS cells (A, non-treated; B, treated), hence when compared to SMB (C, non-treated; D, treated), no apparent differences could be detected between the treated cells. I also attempted to improve the effectiveness of PrP^C removal after treatment by increasing the salt and bicarbonate concentrations in the washing buffers, but the level of PrP^C digestion remained the same. In an effort to achieve more effective digestion, PS cells in suspension were incubated with PIPLC followed by dispase, after which they were plated onto cholera-toxin coated coverslips and allowed to adhere. The rationale behind the use of coverslips treated with cholera toxin, is that the toxin binds to ganglioside, G_M1, a ubiquitous glycolipid cell surface receptor (Heyningen, 1974). These digestion conditions were only marginally more effective than the monolayer treatment with the PrP signal from the PS cells decreasing by approximately 65% (Figure 5.5).
5.2.2 Analysis of the PrP\(^{\text{Sc}}\)-specificity of plasminogen

5.2.2.1 Immunoprecipitation of PrP 27-30 from brain homogenate by plasminogen

Plasminogen has been identified as a PrP\(^{\text{Sc}}\)-binding protein and it has been proposed that this binding activity is retained by the kringle domains which specifically bind to lysine residues of interacting proteins (Fischer et al., 2000). It has been further suggested that this binding can be competed by free lysine. The PrP\(^{\text{Sc}}\) specificity of plasminogen was initially investigated by immunoprecipitation from proteinase K digested, 79A scrapie infected brain homogenate. Beads were coated with either biotinylated-plasminogen or biotinylated-BSA, and half the coated beads were incubated with lysine before addition of the brain homogenate. Precipitates were analysed by Western blotting using antibody 6H4 (Figure 5.7). PrP 27-30 was specifically precipitated by plasminogen (lane PL) and this was prevented following preincubation of the beads with lysine (lane PL+L), in conformation of the results of (Fischer et al., 2000)

5.2.2.2 Analysis of plasminogen cell labelling by immunofluorescence

The specificity of plasminogen was used to develop an immunofluorescence assay for PrP\(^{\text{Sc}}\)-expressing cells. Live SMB and PS cells were incubated with either biotinylated-plasminogen alone, or in the presence of lysine, followed by tyramide signal amplification (Figure 5.8). The signals generated following incubation with plasminogen were similar for both cell types (A, PS cells; C, SMB cells) and the signals decreased to background levels when lysine was added with the plasminogen (B, PS cells; D, SMB cells) indicating that there was no preferential binding to PrP\(^{\text{Sc}}\) on SMB cells. Cell-surface proteins with C-terminal lysine residues have been proposed to function as plasminogen binding sites, and alpha-enolase has been identified as a prominent
Figure 5.2 Removal of 3F4-positive hamster PrP from the surface of Ham-1 cells. Live Ham-1 cells were treated with PIPLC followed by dispase. Surface PrP was detected using the hamster specific 3F4 antibody followed by biotinylated goat α-mouse and tyramide signal amplification. Note the diminution of the signal in the treated cells reflecting the removal of hamster PrP. The signal was estimated to decrease by 95% as determined by image analysis. Scale bar equals 30 μm.
Figure 5.3 Removal of PrP from the surface of Ham-1 cells using various treatments. Live Ham-1 cells were treated with PIPLC and/or dispase as detailed in Methods. PrP was detected by reaction with either antibody 3F4 (which recognises only hamster PrP, green bars) or 6H4 (which recognises both hamster and mouse PrP, red bars), followed by biotinylated goat α-mouse and tyramide signal amplification. Note that approximately 90% of the hamster PrP is effectively removed from the cells by these treatments. In contrast, the 6H4 epitope is largely retained following treatment reflecting the limited release of mouse PrP from these cells. Control samples analysed in parallel were either not treated with PIPLC or dispase (no treatment) or had the primary antibody incubation step omitted from the subsequent detection procedure (Control). The error bars represent the standard deviation from the mean. Individual measurements for each condition were averages from 45 to 50 cells.
Figure 5.4 Removal of mouse PrP from the surface of SMB and PS cells following treatment with EGTA and PIPLC. Live cells were incubated with EGTA for 5 minutes followed by treatment with PIPLC. Surface PrP was detected by reaction with antibody R2 (which recognises both native PrpC and PrpSc), followed by biotinylated goat α-mouse and tyramide signal amplification as before. A) non-treated PS cells; B) treated PS cells; C) non-treated SMB cells; D) treated SMB cells. Note that in the non-treated cells the intensity of PrP staining was higher at cell junctions whereas staining was more uniform in the treated cells. The fluorescent intensity of the treated PS cells was estimated to be 50% of the level in non-treated cells as determined by image analysis. Scale bar equals 30 μm.
Figure 5.5 Removal of mouse PrP from PS cells by digestion in suspension. Live cells in suspension were incubated with PIPLC followed by dispase before plating onto cholera toxin coated coverslips as detailed in Methods. PrP was detected by incubation with antibody 6H4 followed by biotinylated goat α-mouse and tyramide signal amplification. Note that the cells stay rounded up and fail to spread. The fluorescent intensity of the treated cells was estimated to be 35% of the level in non-treated cells as determined by image analysis. Scale bar equals 30 μm.
Figure 5.6 Intracellular PrP staining of fixed SMB and PS cells. Live cells were incubated with PIPLC, fixed, permeabilised with triton and treated with dispase. Intracellular PrP was detected using antibody R2 followed by tyramide signal amplification. Note that no significant differences in the staining can be detected between the two cell types. Scale bar equals 30 μm.
representative of this class of receptors (Miles et al., 1991). The enzyme carboxypeptidase B which removes exposed C-terminal lysines, has been observed to abolish the ability of alpha-enolase to bind plasminogen in some cell types (Hawley et al., 2000), hence it is possible that pre-treatment of cells with this enzyme could improve the PrP\textsuperscript{Sc}-specificity of the signal. Cells were treated with carboxypeptidase B, followed by incubation with digoxigenin-labelled plasminogen, anti-digoxigenin-HRP and tyramide signal amplification as before. The results as quantitated by analysis of 30 cells per coverslip are shown in figure 5.9. In both experiments, plasminogen reacted with both SMB and PS cells to an equivalent extent, and this binding was inhibited in the presence of lysine. Carboxypeptidase B treatment did not appear to reduce binding of plasminogen to other cell-surface proteins since plasminogen reacted to the same extent with PS cells in both experiments. Similar results were obtained when the cell labelling experiments were performed using fibrinogen, which has also been proposed to interact with disease-associated PrP (Fischer et al., 2000). As a result, no differences could be detected between SMB and PS cells using these proteins.

5.2.3 Analysis of the specificity of PrP-Fabs
In a further attempt to distinguish the SMB from PS cells, I used a series of recombinant PrP-Fabs (prepared in the lab of Dr. R. A. Williamson, with whom we have collaborated in the characterisation of these antibodies). These Fab fragments had been generated by grafting mouse PrP sequences corresponding to amino acids 119-136, 121-144 and 121-158, into the heavy-chain-complementarity-determining region (HCDR3) of an IgG antibody, Fab b12, a human recombinant antibody specific for the envelope glycoprotein of HIV-1 (Figure 5.10) (Moroncini et al.). These sequences were selected based on a series of antibody curing experiments (Peretz et al., 2001), and correspond to the region of PrP\textsuperscript{C} that is implicated in heterodimeric association with PrP\textsuperscript{Sc} during prion propagation. Fab b12 was selected as the recipient molecule for grafted PrP sequences since the parental antibody possesses a relatively long HCDR3 that projects vertically about 20Å from the surface of the antigen binding site (Saphire et al., 2001). These PrP-Fab molecules were constructed based on the rationale that transplanting these sequences into an antibody could impart specific recognition of PrP\textsuperscript{Sc}. 
Figure 5.7 Immunoprecipitation of PrP 27-30 from 79A scrapie brain homogenate by plasminogen. Beads coupled to proteins of interest were either added directly to proteinase K digested brain homogenate or preincubated with lysine before addition to the homogenate. Precipitates were analysed by Western blotting using antibody 6H4. Samples were derived from scrapie brain homogenate, B; marker lane, M; BSA control, BSA; plasminogen, PL; and control beads with no protein, Ctrl, and for each condition beads were either incubated alone or with lysine (+L). Note that PrP 27-30 is only immunoprecipitated by plasminogen and that this interaction is prevented following preincubation of the beads with lysine.
Figure 5.8 Plasminogen labelling of SMB and PS cells. Live PS cells (A and B) and SMB cells (C and D) were reacted with biotinylated plasminogen, followed by incubation with HRP-streptavidin and tyramide signal amplification as before. In B and D, 0.1M lysine was added to the cells with the plasminogen and was present in all the subsequent washes. Note that the intensity of staining in the two cell types is similar and furthermore that the signals are slightly lower in the presence of lysine. Scale bar equals 30 μm.
Figure 5.9 Labelling of SMB and PS cells with plasminogen. Live SMB and PS cells were incubated with DIG-labelled plasminogen alone (+PL) or in the presence of lysine (+PL+L) followed by anti-DIG-HRP and tyramide signal amplification. In a parallel experiment, cells were preincubated with carboxypeptidase B (+CPB). Note that there is a marginal increase in fluorescent intensity for both cell types following addition of plasminogen and that in each experiment the signal decreases in the presence of lysine. Control samples were processed in parallel without the addition of plasminogen or lysine (none). The error bars represent the standard deviation from the mean. Individual measurements for each condition were averages from 45 to 50 cells.
5.2.3.1 Immunoprecipitation of \( \text{PrP}^{\text{sc}} \) and \( \text{PrP} \ 27-30 \) from brain homogenate by \( \text{PrP-Fabs} \)

The specificity of the Fabs was initially investigated by immunoprecipitation on proteinase K digested scrapie brain homogenate, or normal brain homogenate as a control. Precipitated PrP was detected by Western blotting using 6H4 (Figure 5.11). The PrP-Fabs (121-144, 119-136 and 121-158) did not appear to react with \( \text{PrP}^{\text{C}} \) from the normal brain homogenate, although a faint signal in the region of diglycosylated PrP was visible with Fab 119-136 (Figure 5.11 A). As a positive control, 6H4 was used to precipitate \( \text{PrP}^{\text{C}} \) and as a negative control, Fab b12 was included which did not react with \( \text{PrP}^{\text{C}} \). In contrast, each of the PrP-Fabs immunoprecipitated PrP 27-30 from infected brain homogenate, and again the reaction with Fab b12 was negative (Figure 5.11 B). Fab 121-158, which precipitated PrP-27-30 with greatest efficiency, was next evaluated for reactivity with full-length \( \text{PrP}^{\text{sc}} \), using plasminogen (PL) as a positive control. Fab 121-158 precipitated \( \text{PrP}^{\text{sc}} \) from undigested infected brain homogenate whereas the parental Fab b12 was negative (Figure 5.11 C). It is also noteworthy that these PrP-Fabs did not react with either \( \text{PrP}^{\text{C}} \), \( \text{PrP}^{\text{sc}} \) or PrP 27-30 when used to probe Western blots of mouse brain homogenate (data not shown). These results indicate that grafted PrP sequence composed of residues 121-158 endows specific antibody recognition of \( \text{PrP}^{\text{sc}} \) which is dependent on its native conformation, and that this epitope is retained in PrP 27-30.

5.2.3.2 Immunoprecipitation of PrP 27-30 from cell lysates by PrP-Fabs

The specificity of PrP-Fab 121-158 was further investigated in a series of immunoprecipitation experiments using lysates from SMB cells. Precipitates were again analysed by Western blot using antibody 6H4 (Figure 5.12). Fab 121-158 did not bind \( \text{PrP}^{\text{C}} \) in undigested SMB lysate but specifically precipitated PrP 27-30 in digested lysate. However, unlike the immunoprecipitations from scrapie brain homogenate above, no full length \( \text{PrP}^{\text{sc}} \) was precipitated from SMB cells using this antibody.

5.2.3.3 Reaction of PrP-Fabs with live cells

The preferential binding of the PrP-Fabs to \( \text{PrP}^{\text{sc}} \) and PrP 27-30 over \( \text{PrP}^{\text{C}} \) was subsequently investigated by immunofluorescence. Live SMB and PS cells were stained with the PrP-Fabs followed by biotinylated goat anti-human and
tyramide signal amplification. 6H4 staining was included as a positive control. The results as quantitated by analysis of 30 cells per coverslip are illustrated in figure 5.13. None of the PrP-Fabs significantly reacted with either cell type to levels above background. In an extension of these experiments, cells were pretreated with combinations of proteases and PIPLC to exposes epitopes that may be hidden. However no significant binding of the PrP-Fabs to either SMB or PS cells was observed.

Based on the proposed competition between PrP\(^C\) and the PrP-Fabs for the same epitope on PrP\(^\Sc\) (Moroncini et al.), I then attempted to favour binding of the PrP-Fabs, by incubating them in the growth media of the SMB and PS cells. The rationale behind this series of experiments was that PrP\(^C\) molecules on the cell surface are internalised and recycled back to the cell surface with a transit time of approximately 60 minutes (Shyng et al., 1993). Accordingly, during this period, PrP-Fabs present in the growth media could bind uncomplexed PrP\(^\Sc\) on the cell surface before PrP\(^C\) reappears. PrP-Fab 121-158 and control Fab b12 were added to the media of SMB and PS cells for between 2 and 48 hours followed by the detection of antibody binding by immunofluorescence as before. The result of a typical experiment following incubation of cells with two doses of 5 \(\mu g/ml\) of the Fabs over 24 hours is illustrated in figure 5.14 A. A marginal increase in fluorescent intensity above background was observed for both SMB and PS cells following incubation with PrP-Fab 121-158 and no apparent differences could be detected between the two cell types. The results as quantitated following analysis of 30 cells per coverslip are illustrated in figure 5.14 B. Incubation of cells with antibody 6H4 was included as a positive control. It was evident that while Fab 121-158 could bind PrP 27-30 from SMB cell lysates, no binding to live cells was observed.

5.3 DISCUSSION

The intention of the studies described in this chapter was to establish a single cell cytochemical assay that could be used to distinguish infected cells expressing PrP\(^\Sc\) from control uninfected cells. Initial attempts to selectively remove PrP\(^C\) from the surface of ham-1 cells were encouraging with approximately 95% of the molecules being released. The residual cell-associated PrP may be located inside the cell and thus inaccessible to PIPLC. An
Figure 5.10 Construction of the PrP-Fab molecules. A) Schematic illustration of mouse PrP 121-158 peptide replacing Fab b12 HCDR3 sequence to yield PrP-Fab 121-158. The N-terminal Val residue and 4 C-terminal residues (Tyr-Met-Asp-Val) of the original b12 HCDR3 are retained; two Gly residues are added to each flank of the grafted PrP sequence. B) Modelled structure of Fab 121-158 generated by grafting the NMR structure of mouse PrP 124-158 into the crystal structure of IgG1 b12.
Figure 5.11 Immunoprecipitation of PrP\textsuperscript{Sc} and PrP 27-30 from 79A infected mouse brain homogenates. A) Normal mouse brain homogenate was incubated with PrP-Fabs 121-144, 119-136, 121-158 and control Fab b12. Antibodies were precipitated with polyclonal goat α-human IgG F(ab')\textsubscript{2} linked to paramagnetic beads. Precipitates were analysed on a Western blot for the presence of PrP using antibody 6H4. Cross-reaction of the secondary antibody with the precipitating Fabs produces bands at approximately 50 kDa. PrP\textsuperscript{C} is detected in a sample of normal mouse brain homogenate and is specifically precipitated by the control antibody 6H4. Note that no PrP\textsuperscript{C} is detected following immunoprecipitation with the PrP-Fabs or the control Fab. B) Scrapie-infected brain homogenates digested with proteinase K was reacted with the PrP-Fabs 121-144, 119-136, 121-158 and control Fab b12 followed by immunoprecipitation and analysis as in A. A sample of PrP 27-30 from the digested homogenate was run as a control. Note that equivalent PrP bands are present following immunoprecipitation with the PrP-Fabs but not from the control reaction with Fab b12. C) Non-digested scrapie-infected brain homogenate was reacted with PrP-Fab 121-158 and control Fab b12 followed by immunoprecipitation and analysis as above. Full length PrP\textsuperscript{Sc} from the homogenate was loaded as a control. Note that PrP\textsuperscript{Sc} is efficiently precipitated by Fab 121-158, but not by control b12. PrP\textsuperscript{Sc} precipitated by plasminogen is also shown.
Figure 5.12 Immunoprecipitation of PrP 27-30 from SMB cell lysates. Proteinase K digested and control non-digested lysates from SMB cells were incubated with PrP-Fab 121-158 or control Fab b12. Antibodies were precipitated with polyclonal goat α-human IgG F(ab')2 linked to paramagnetic beads. Precipitates were analysed on a Western blot for the presence of PrP using antibody 6H4. Samples of cell lysates were loaded as controls. In the absence of proteinase K treatment, neither Fab 121-158 nor Fab b12 recognised either PrPc or PrPSc. Note the presence of PrPSc from the digested SMB lysate which was reacted with Fab 121-158, and not from the control immunoprecipitation. Cross-reaction of the secondary antibody with the precipitating Fabs produces bands at approximately 50 kDa.
Figure 5.13 PrP-Fabs staining of SMB and PS cells. Live cells were incubated with PrP-Fabs 121-144, 119-136, and 121-158 followed by biotinylated goat α-human and tyramide signal amplification. Parallel samples were incubated with either 6H4 as a positive control, or Fab b12, a DIG monoclonal antibody, or no primary antibody (Ctrl) as negative controls. Note that none of the PrP-Fabs give a significant level of staining over background with either PS or SMB cells. The error bars represent the standard deviation from the mean. Individual measurements for each condition were averages from 45 to 50 cells.
Figure 5.14 Incubation of PrP-Fab 121-158 in the culture media does not allow SMB and PS cells to be distinguished. A) Cells were cultured in the presence of either PrP-Fab 121-158 or control Fab b12 for 24 hours, washed, incubated with biotinylated goat α-human followed by tyramide signal amplification. Note that for both SMB and PS cells, the signals following incubation with PrP-Fab 121-158 were similar and were marginally higher than the levels obtained with the control Fab. Scale bar equals 30 μm. B) Quantitation of the data in A by image analysis of approximately 50 cells per sample. Parallel cultures were incubated with antibody 6H4 as a positive control and standard culture media was used as a negative control (ctrl). The error bars represent the standard deviation from the mean. Individual measurements for each condition were averages from 45 to 50 cells.
alternative explanation may be that a subset of PrP molecules exist that are resistant to PIPLC release due to acylation of inositol hydroxyl groups (Mayor et al., 1990). However, these optimised conditions were only able to remove approximately 60% of the mouse PrP from PS cells. This less effective digestion of the mouse molecules could reflect an additional association of mouse PrP with other molecules in the plane of the membrane such as with mouse PrP, whereas the transfected hamster PrP molecules in ham-1 cells are likely to be uncomplexed, or could simply adopt a conformation whereby the GPI anchor is more susceptible to cleavage by PIPLC, or the protein to protease digestion. After treatment of SMB and PS cells, there was also some variability in the fluorescent intensity between individual cells which probably reflects slight heterogeneity in the populations. As a result, when treated SMB and PS cells were compared, the average levels of staining were similar, hence the cells could not be distinguished by this procedure.

The inability to discriminate between the two cell types could in theory be overcome if conditions were established to effectively digest PrP without dislodging the cells from the coverslip. After treatment, the cells tend to adhere better to plastic rather than coated glass coverslips, hence one possibility may be to culture the cells on plastic coverslips, which, despite their unsuitability for immunofluorescence applications due to their autofluorescence, can be used for immunocytochemistry. After treatment and antibody incubations, a chromogenic substrate could be used in the tyramide signal amplification procedure, although the sensitivity may not be as high as with the current protocol. Alternatively, in view of the highly effective removal of the transfected hamster PrP in ham-1 cells, it is possible that the desired assay could be established using a pair of infected and uninfected cell lines expressing hamster PrP, or possibly using other pairs of infected and uninfected cell lines.

The results with plasminogen demonstrate that a number of other receptors are present on the surface of SMB and PS cells which precluded the specific detection of PrP on SMB cells. In agreement with the results from the immunoprecipitation experiments using brain homogenate, the plasminogen binding activity on live cells was competed for with lysine. However, carboxypeptidase B treatment failed to reduce the binding of plasminogen to
SMB and PS cells in these experiments. Several distinct molecules appear to contribute to plasminogen binding to cells, although, the subset of plasminogen receptors responsible for enhancing plasminogen activation expose C-terminal lysines on the cell surface that are sensitive to proteolysis by carboxypeptidase B (Hawley et al., 2000). Different cell types appear to have distinct sets of molecules that constitute the population of cell surface profibrinolytic plasminogen-binding proteins. Furthermore, of the cell membrane-associated plasminogen binding proteins with C-terminal lysines present on a given cell type, only a small fraction expose a C-terminal lysine that is accessible to carboxypeptidase B on the cell surface. These finding are likely to explain why carboxypeptidase B pre-treatment in these studies did not appear to alter plasminogen binding to SMB and PS cells. The marginal increase in signal for both PS and SMB cells following labelling with plasminogen, together with the fact that all binding was competed for with lysine, implies that the observed interactions were between plasminogen kringle domains and genuine plasminogen binding sites, hence although plausible, it is unlikely that the PrP<sup>Sc</sup>-specificity could be improved in this system, even with the use of the isolated kringle domains of plasminogen.

The experiments with PrP-Fab 121-158 revealed that whereas both PrP<sup>Sc</sup> and PrP 27-30 could be immunoprecipitated from scrapie-infected brain homogenate, no full length PrP<sup>Sc</sup> was precipitated from SMB cell lysates. A proposed explanation for these differences has been based on the ratio of PrP<sup>C</sup>:PrP<sup>Sc</sup> in the two samples (Moroncini et al.). Since the ratio in SMB cells is approximately 4:1, but is likely to be less than 1 in a clinically affected mouse brain, it is plausible that in the cell lysate, all the existing PrP<sup>Sc</sup> would be bound to PrP<sup>C</sup>, precluding the PrP-Fab from binding to its epitope on PrP<sup>Sc</sup>. Conversely, in the brain homogenate, due to the stochiometric excess of PrP<sup>Sc</sup> over PrP<sup>C</sup>, the PrP-Fabs would be able to bind a proportion of uncomplexed PrP<sup>Sc</sup>.

Recent studies have revealed that monoclonal antibodies binding the first alpha-helix of PrP<sup>C</sup> (residues 145-155) potently inhibit prion propagation in scrapie infected cells (Peretz et al., 2001), presumably by preventing heterodimeric association of PrP<sup>C</sup> with PrP<sup>Sc</sup>. Consistent with these results, the
most effective PrP-Fab for precipitating disease-associated PrP in these studies was Fab 121-158, containing sequence composed of the first alpha helix of PrP\(^{C}\). These studies suggest that this region of PrP\(^{C}\) may be a critical component of the PrP\(^{C}\) - PrP\(^{Sc}\) replicative interface. However, the finding that both PrP-Fabs 119-136 and 121-144 also precipitated PrP 27-30 to a lesser extent implies that helix A is not imperative for specific recognition of abnormal PrP isoforms.

Despite the specific recognition of PrP\(^{Sc}\) observed in the immunoprecipitation experiments using brain homogenates, I was unable to establish conditions for distinguishing live SMB from PS cells using the PrP-Fabs. It is possible that the inability of PrP-Fab 121-158 to generate a detectable PrP\(^{Sc}\)-specific signal from live SMB cells could reflect differences in the conformation of PrP\(^{Sc}\) as aggregates in suspension, compared to monomeric or oligomeric PrP\(^{Sc}\) attached to the SMB cell surface. In view of the proposed lack of uncomplexed PrP\(^{Sc}\) available on the cell surface, it also seemed plausible that either incubation of the PrP-Fabs in the culture media, or pre-treatment of the cells with PIPLC and/or proteases to remove PrP\(^{C}\) prior to antibody incubation, would have facilitated the establishment of the assay. However, pre-treatment of the cells with proteases and PIPLC did not appear to expose epitopes for the PrP-Fabs. Furthermore, in contrast to the direct staining of the cells with the PrP-Fabs where no binding could be observed to either of the cells, following incubation of the antibodies in the growth media, PrP-Fab 121-158 was observed to bind to both SMB and PS to a similar extent. The signals generated as a result of culturing both SMB and PS cells the in the presence of PrP-Fab 121-158 appear to be genuine, whereas only background levels could be detected using the control, Fab b12. It appears that this PrP sequence (amino acids 121-158) binds specifically to an epitope present on both cells, that is not accessible when the cells are stained with the antibodies for an hour at 4°C. It is possible that the construction of additional Fabs containing truncated and mutated PrP sequences may possess higher affinity for PrP\(^{Sc}\), thus increasing the specificity of the signal and decreasing the background.

The estimated proportion of PrP\(^{Sc}\) in the SMB cells is between 10-30%, however, the actual amount present on the cell surface is unknown. The results of the fixed cell cocultures described in chapter 4 demonstrate that a reasonable
percentage of PrP\textsuperscript{Sc} must reside at the surface although its availability may be limiting. It is thus conceivable that the PrP antibodies and plasminogen are capable of recognising PrP\textsuperscript{Sc} in the conformation that is present on the cell surface, but the levels of PrP\textsuperscript{Sc} present in SMB may be too low to generate a detectable signal. Accordingly, this assay could possibly be facilitated if the levels of PrP\textsuperscript{Sc} in the SMB cells were increased. In SMB cells, the least abundant PrP\textsuperscript{C} glycoform is unglycosylated, whereas the major PrP\textsuperscript{Sc} glycoform is unglycosylated, hence the synthesis of PrP\textsuperscript{Sc} in SMB cells may be limited by the availability of unglycosylated PrP\textsuperscript{C}. It is therefore possible that treatment with drugs which inhibit N-glycosylation such as tunicamycin could be a useful means of increasing the proportion of PrP\textsuperscript{Sc} in these cells prior to analysis.

Although I was unable to establish a procedure whereby an infected cell, stably expressing PrP\textsuperscript{Sc}, could be distinguished from a normal uninfected cell, the results obtained from these studies, particularly the experiments with the PrP-Fabs, provide a starting point from which further efforts should be directed in the search for a PrP\textsuperscript{Sc}-specific cytochemical marker.
Summary
Prion diseases are fatal neurodegenerative disorders that have attracted enormous attention, not only for their unique biological features, but also for their impact on public health (Prusiner, 1998). Neuropathologically, these disorders produce a characteristic spongiform degeneration of the brain, as well as deposition of amyloid plaques (DeArmond and Ironside, 1999). The infectious agent may be composed exclusively of a protein molecule designated PrP^{Sc}, a conformationally altered form of a normal, host-encoded membrane glycoprotein, called PrP^{C} (Prusiner, 1997). It has been proposed that PrP^{Sc} acts autocatalytically as a template in converting PrP^{C} molecules to the abnormal conformer (Prusiner et al., 1999a). Prion diseases therefore exemplify a novel pathogenic mechanism based on a self-propagating change in protein conformation.

Defining the mechanisms underlying the generation of PrP^{Sc} from PrP^{C} and the process by which this conformational change is propagated have become central issues in understanding the pathogenesis of prion diseases. To date, a number of different experimental approaches have been undertaken to address these issues both in vivo and in vitro (see Introduction). Rodent models have highlighted the relatively slow progression of these disorders (Manolakou et al., 2001). This has also been reflected by the slow acquisition of protease resistance observed for PrP^{C} in cell-free conversion systems (Bessen et al., 1995; Kocisko et al., 1994). These studies suggest a slowly propagating conversion process, the mechanism of which was the focus for this thesis, and this was investigated in the context of cell culture.

The principal conclusion from this study is that a scrapie prion-infected cell is able to convert a neighbouring uninfected cell by a process that is dependent upon cell contact. This finding was supported by several lines of evidence, in particular, that dead, fixed infected cells retain substantial activity in converting target cells to stable production of PrP^{Sc}. This activity of fixed infected cells is indicative of a templating mechanism, whereby PrP^{Sc} on the infected cell is able to act in trans to convert PrP^{C} on the surface of the target cell. Levels of conversion may be amplified by the cis interaction between PrP^{Sc} and further PrP^{C} molecules in the plane of the target cell membrane, via either the template-assisted or the nucleation polymerisation models. This templating mechanism
could play a role in the high levels of infectivity associated with scrapie agent adsorbed to stainless steel surfaces (Zobeley et al., 1999).

One of the intriguing features of prion diseases is why they are so slow, particularly when no known host responses are induced to slow them down. A mechanism that is dependent on cell contact thus bears potential relevance to the time course of prion infections in vivo. Cell to cell contact may be involved in the propagation of prions through the lymphoreticular system. Perhaps in the peripheral nervous system, cell contact could be required for the progressive conversion of Schwann cells along the sheath. Consistent with this hypothesis, in vitro and in vivo studies have demonstrated that PrP$^C$ expression is mainly localised at the cell membrane of Schwann cells (Follet et al., 2002). However, since the activity of fixed infected cells in the present analysis can account for only a fraction of that observed with live cells, alternative possibilities such as diffusion through the extracellular space, as well as the cerebrospinal fluid, may also feature in the spread of prions within the CNS. The fixed SMB coculture system described in chapter 4 has demonstrated the requirement for an initial contact period of greater than 1 day and a subsequent propagation step of around 2 weeks. More precise estimates of the timing for the templating and amplification steps are necessary to gain an insight into the time course of disease progression.

Another important observation from these studies was that cell-mediated infection of target cells required significantly less PrP$^Sc$ than infection by a brain homogenate. This finding is consistent with similar attempts to infect an N2a subline with RML prions (Bosque and Prusiner, 2000). A number of hypotheses have been put forth to explain these observed differences, which I believe is attributable to acute differences in the conformation of PrP$^Sc$ as aggregates in suspension, versus presentation of monomeric or oligomeric PrP$^Sc$ anchored in a lipid membrane. It would be of interest to establish whether the converted target cells, like the donor SMB cells (Clarke and Haig, 1970; Haig and Clarke, 1971), are indeed infected as determined by rodent bioassay.

The ability to convert target cells in this study to stable production of PrP$^Sc$ raises fundamental questions such as the percentage of cells that are infected
and the levels of conversion attained. I attempted to address these issues in a series of cell-labelling experiments. Present work taking place in the laboratory is aimed at pursuing the cell labelling approach, by screening a panel of engineered PrP-Fab fragments in the search for a PrP\textsuperscript{Sc}-specific cytochemical marker. A PrP\textsuperscript{Sc}-specific marker that unequivocally discriminated between an infected and an uninfected cell would have many applications in prion research. Such a reagent would facilitate immunohistochemical detection of PrP \textit{in situ} and could have clinical applications in the early diagnosis of prion diseases in both humans and animals.

In view of the fact that neurons are non-dividing, and that during the course of an experimental infection, prions propagate through a tissue from an initial focus, I have also begun to address the propagation of the PrP conformational change across a stable monolayer of cells. The 'bullseye' system described in chapter 4 offers an opportunity to analyse the mechanisms underlying cell-contact dependent infection in a context that is intermediate between dissociated cells and complex tissue architecture. The precise cellular identity of SMB is unknown, although it has been suggested to be of mesodermal origin (Haig and Clarke, 1971). A relevant future direction for these studies would be to extend them to primary neural or glial cells, and other clonal cell lines might be used to substitute for the target cells analysed in the present study. This should not only provide a further insight into the slow progression of \textit{in vivo} prion infections, but may also provide insights into the differential susceptibility of cells to infection (Race, 1991). A still closer approximation of \textit{in vivo} events could be attained by extending these studies to the complex cytoarchitecture of a brain slice. Rodent brain slices can be manipulated in culture, and following local infection by scrapie prions, propagation across the tissue could be monitored using sensitive PrP\textsuperscript{Sc} detection techniques like the histoblot (Taraboulos et al., 1992a), although once again a single cell assay would be most appropriate.

Several of the aspects investigated in this study reflect the variable properties of prion-susceptible cell lines. Unlike the GT1 cells (Schatzl et al., 1997), SMB cells and the infected target lines display no obvious cytopathology and do not release PrP\textsuperscript{Sc} into the medium. SMB cells have been maintained in culture for
over 150 passages with a constant infectious titre (Clarke and Haig, 1970; Haig and Clarke, 1971), and to date, no spontaneously 'cured' subline has been isolated. SMB cells thus possess a high degree of competence as a host cell for scrapie, unlike some of the unstable subclones derived from HaB cells (Taraboulos et al., 1990). PS cells have been reported to be susceptible to a number of mouse adapted scrapie strains (Birkett et al., 2001 and this thesis), a feature exhibited by a limited number of N2a sublines (Bosque and Prusiner, 2000; Nishida et al., 2000). Infected PS cells were reported to express scrapie strain-related PrP^Sc glycoforms, which could provide an early indication of strain-specific reproduction prior to the method of biological strain phenotyping (Birkett et al., 2001). Furthermore, estimates of the frequency of infection for PS cells were higher than those reported for N2a cells (Race et al., 1987). It appears that the susceptibility of a cell to infection and its ability to sustain the replication of that infection are governed by separate factors. It is possible that a library transfection approach could be used to identify factors that may influence the susceptibility of a cell to infection. Clearly, the SMB/PS culture system provides a valuable tool for advancing our knowledge of the fundamental aspects of intercellular prion transmission.

The design of the fixed SMB coculture system allows for target cells to be brought into contact with dead infected cells for varying lengths of time from 2 hours up to 7 days. After trypsinisation and propagation, the target cells can be analysed to identify any changes that may be acutely associated with scrapie-infection. One possible consequence of infection is a weakening of the cellular resistance to reactive oxygen species with a resultant increase of free oxygen radicals. Scrapie-infected neuronal cells (Milhavet et al., 2000) as well as neurons from PrP-knockout mice (White et al., 1999) show a dramatic reduction in the activities of glutathione-dependent and Cu/Zn-superoxide dismutase (SOD) antioxidant systems, together with an increased sensitivity to oxidative stress. Increased levels of oxidative stress markers have also been demonstrated in scrapie-infected brains (Guentchev et al., 2000) as well as in the brains of PrP-knockout mice (Wong et al., 2001). It could be straightforward and informative to analyse the timing of these changes in relation to target cell infection, using the present culture system, by assaying SOD activity in the target cells after coculture with SMB cells for varying times.
It has recently been demonstrated using the murine 1C11 neuronal differentiation model that PrP<sup>C</sup> is bound to caveolin-1 and, by recruiting Fyn kinase, can participate in signal transduction events connected to cell differentiation (Mouillet-Richard et al., 2000). It is hence possible that conversion of PrP<sup>C</sup> to PrP<sup>Sc</sup> in a target cell could affect its association with caveolin-1 and thereby influence subsequent signal transduction events. The timing concerning the modification of these events in relation to the infection of the target cells could be analysed using the fixed SMB coculture system.

The relatively slow progression of prion diseases can be contrasted with that of viral infections, which, unlike the former, often elicit a profound immune response. Many viruses, for example those that cause rabies and herpes, exploit the anatomical connections provided by peripheral nerves, and reach the CNS by axoplasmic transport (Gillet et al., 1986; Smith et al., 2001). Cell to cell contact has been demonstrated in culture to be responsible for the spread of at least one strain of measles virus (Firsching et al., 1999). In this system, mixture of measles virus-infected cells with uninfected cells, led to a very fast and efficient contact-mediated infection of the target cells, with virtually all cells infected after 24 hours. Similarly, the pathogenesis of foot-and-mouth disease in pigs displays a rapid progression of infection, with a peak of viraemia levels reported as early as 3 days after exposure to the virus, at which time clinical signs were already evident (Alexandersen et al., 2001). In contrast, the rate of spread of scrapie prions within the CNS is comparable to the rate of slow anterograde axonal transport of cytoskeletal proteins, which travel at around 0.25-3 mm/day depending on the type of neuron and other factors (Ackerley et al., 2000; Nixon, 1998). With limitations on the cell to cell spread of infection that have been proposed, coupled with restrictions on the scrapie replication process (Kimberlin and Walker, 1988), the slow pathogenesis of prion disorders can be justified.

From the perspective of inherited and sporadic neurodegenerative disorders, prion disease could share a similar pathogenic mechanism with other disorders that cause dementia. One of the hallmarks of Alzheimer’s disease is the extracellular cerebral deposition of amyloid in neuritic plaques which precedes paired helical filament tau formation and neurofibrillary tangles (Selkoe, 1999).
In Alzheimer's disease, the main amyloid protein component is a 39-42 amino acid beta-amyloid peptide (A beta), which is a proteolytic breakdown product of the amyloid precursor protein (APP), a large membrane spanning protein, whose normal function is unclear (Selkoe, 1998). It is supposed that aggregation of A beta into amyloid is a prerequisite for neurotoxicity. Similarly, Huntington's disease is associated with aggregates of huntingtin protein fragments (Gutekunst et al., 1999). Normal huntingtin protein does not self aggregate, but increased length of a polyglutamine stretch in the protein causes a change in its structural properties, and as such, mutated huntingtin has an increased tendency to aggregate. It is unknown whether the cytoplasmic or nuclear accumulation of huntingtin contributes to neuronal toxicity. Although amyloid aggregates associated with Alzheimer's disease and prion disorders are predominantly extracellular, many other dementia related aggregates have intracellular locations (Lovestone and McLoughlin, 2002). Furthermore, in contrast to the other disorders, the formation of amyloid in Alzheimer's is regulated by other proteins such as the presenilins and apolipoprotein E (Lovestone and McLoughlin, 2002). A fundamental point of difference, however, arises from the transmissibility of the prion disorders, which has not been demonstrated for Alzheimer's disease (Godec et al., 1994).

To date, the definite diagnostic test of prion disease in humans relies on post-mortem neurohistopathological examination of brain tissue. Obviously it would be extremely useful to diagnose the disease during its progression. More recently, a highly sensitive immunoblotting procedure has been developed for the detection PrP$_{Sc}$ in vCJD tissues (Wadsworth et al., 2001), although more high throughput procedures may be developed in the future. A protease resistant PrP isoform has also been detected in the urine of prion-infected individuals, a finding which may have useful applications for early diagnosis of the disease (Shaked et al., 2001). Active immunisation with A beta has recently been shown to decrease brain A beta deposition in transgenic mouse models of Alzheimer's disease (Schenk et al., 1999) and certain peripherally administered anti-Abeta antibodies have been observed to mimic this effect (Bard et al., 2000; DeMattos et al., 2001). Antibodies directed against Huntington's disease protein have also been reported to reduce aggregate formation (Heiser et al., 2000). Although the PrP-Fabs used in the present studies have the disadvantage of a short half-life,
whole-antibody molecules prepared from the Fabs may have useful in vivo applications in targeting prion disease.

Cultured cells expressing PrP\textsuperscript{Sc} have provided a useful tool for screening potentially therapeutic products which could interfere with PrP\textsuperscript{Sc} propagation, such as Congo red and pentosan sulphate (Caughey and Raymond, 1993) (see Introduction). However, these compounds have been shown to have limited effects in animal models of prion disease (Ingrosso et al., 1995; Ladogana et al., 1992). An alternative approach could be to block disease transmission by interfering with the interaction between PrP\textsuperscript{Sc} on the infected cell and PrP\textsuperscript{C} on the surface of the target cell. The potential importance of the culture system described in this thesis, is that it offers a favourable opportunity to analyse the timing of prion propagation under defined conditions, and to study the cellular and molecular aspects of intercellular prion transmission.


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