

***In vitro* analysis of C-terminal mutations of the murine PrP gene.**

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I declare that the work contained in this thesis is my own, except where otherwise stated.



for

WILL RAMSBOTHAM

Abstract

Scrapie, CJD and BSE are transmissible neurodegenerative disorders producing alterations in physical — chemical properties of the host protein PrP. In uninfected animals PrP is a membrane bound glycoprotein found in brain and other tissues. In disease PrP accumulates as protease resistant deposits in and around cells. This disease specific form of PrP (PrP^{sc}) also differs from the normal form (PrP^c) by its greatly increased turnover time in cell culture and its inability to be released from scrapie infected cells by the action of PIPLC (Borchelt *et al.*, 1990; Caughey and Raymond, 1991). The amount and distribution of PrP mRNA is identical in the brains of uninfected and infected mice suggesting that the accumulation of PrP in disease is due to post-transcriptional alterations in PrP synthesis. PrP undergoes three major post-translational changes, N-terminal cleavage, N-linked glycosylation and the cleavage of a C-terminal peptide with the subsequent addition of a phosphatidylinositol (GPI-anchor), which is responsible for the attachment of the protein to the plasma membrane (Stahl *et al.*, 1987). Most PrP^{sc} appears to have an intact GPI-anchor although it cannot be released from tissue culture cells by the action of PIPLC whereas PrP^c is released into the medium (Stahl *et al.*, 1990b). However, 15% of PrP^{sc} from hamster brain has been shown to be truncated at amino acid 228, these proteins having no GPI-anchor (Stahl *et al.*, 1990a). The significance of these truncated proteins has not yet been elucidated. It is possible that the presence of a small amount of an abnormal, truncated protein could act as a seed for a conformational change that would result in the conversion of PrP^c to PrP^{sc} (Prusiner, 1991). This project sets out to investigate the differences between the cellular processing of the wild type GPI-anchored protein and a mutant protein where addition of the GPI has been prevented. The mutant is investigated to show that the engineered mutation does indeed give rise to a GPI-less form and the size, glycosylation status,

immunoreactivity and cellularlocation of the two proteins are investigated firstly in a cell-free system and secondly in tissue culture cells. The question of whether PrP^{sc} can be produced when the GPI-anchor is absent will be addressed by subsequent studies of these mutations in transgenic mice.

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Abbreviations

Frequent abbreviations used in this text:

1°	Primary
2°	Secondary
Amp	Ampicillin
Approx.	Approximately
Chp.	Chapter
CJD	Creutzfeld-Jacob Disease
GSS	Gerstmann-Straussler Syndrome
DEPC	Diethylpyrocarbonate
DPM	Dog pancreas membranes
ds	Double stranded
FFI	Fatal familial insomnia
GPI	Phosphatidylinositol-glycolipid
GSS	Gerstmann-Straussler Syndrome
Min	Minute/s
Oligo.	Oligonucleotide
PCR	Polymerase chain reaction
Pers.commun.	Personal communication
RRL	Rabbit reticulocytelysate
RT	Room temperature
Sec	Seconds
ss	Single stranded

CHAPTER ONE:

Introduction

A group of transmissible neurological diseases collectively known as the transmissible degenerative encephalopathies (TDE's) has proved a difficult problem for molecular biologists due to the undefined nature and unusual properties of the infectious agent. The best documented example of this group is Scrapie in sheep, which has been recognised for over two hundred years, but for which the causative organism has never been isolated. More recently the emergence of bovine spongiform encephalitis (BSE) (Wells et al., 1987) has excited public concern, as has a spate of cases of Creutzfeldt-Jacob disease (CJD), a human TDE, in children treated with contaminated human growth hormone (Collinge et al., 1991). The nature of the infectious agent has proved controversial because of the failure to detect a disease specific nucleic acid and its resistance to procedures that normally destroy nucleic acids (Prusiner, 1982; Alper, 1985). Purification of the infectious fraction leads to the concentration of a disease specific, protease resistant form of a normal membrane protein PrP, and attempts to purify this protein from infected brains concentrates infectivity (Merz et al., 1981; Chesebro et al., 1985; Oesch et al., 1985; Lochter et al., 1986). This has led some workers to postulate an infectious protein theory, whereby a disease specific protein is able to self-replicate in the absence of a disease specific nucleic acid (Prusiner, 1982). Opposed to this is the evidence that numerous, (at least 20), different strains of the scrapie agent exist (Bruce et al., 1991), which differ in their incubation period characteristics, pathology and clinical features, depending on the genotype of the host. These strains undergo mutation in a manner analogous to that seen for a nucleic acid containing agent. Also, genetic susceptibility seems to have a role to play in the disease, as illustrated by differences in incubation times in mice of different genetic backgrounds in experimental scrapie (Hunter et al., 1987) and as some forms of CJD and Gerstmann-Straussler syndrome (GSS) in humans

arise in a heritable fashion (Goldfarb et al., 1992a, b). Thus any theory of infectivity has to be able to account for all these differing properties.

In the past ten years much of the work in this field has concentrated on the analysis of the involvement in scrapie of the PrP protein (for review see (Hope and Manson, 1991). PrP is a membrane glycoprotein that exists in two forms: a normal cellular isoform PrP^C, a 33-35kD protein that is located at the cell surface and is completely protease sensitive, and PrP^{Sc} which is partially protease resistant, giving rise to PrP 27-30kD and accumulates intracellularly in the brains of scrapie infected animals. Intraneuronal vacuolation is the most common pathological lesion, with some strains giving rise to amyloid plaques made up of PrP protein. When infected brains are extracted with detergent PrP occurs as rod-shaped fibrils called scrapie associated fibrils (SAF). It has been shown that the primary structure of the PrP gene is the same in both infected and uninfected brains, that the entire open reading frame (ORF) is contained on one exon and that usually only one species of mRNA is observed (Basler et al., 1986; Goldmann et al., 1990) Also, levels of mRNA expression do not differ between infected and uninfected brains as shown by *in situ* hybridization (Manson et al., 1992a). This rules out differences at the level of transcription or major RNA splicing as a mechanism to account for the differences between PrP^C and PrP^{Sc}. These physico-chemical differences are believed to result from some post-transcriptional or post-translational change.

Both isoforms have been shown to have a 22 amino acid N-terminal ^{cleavable} signal peptide (Basler et al., 1986; Hope et al., 1986; Turk et al., 1988) Both have also been shown to have their two predicted N-linked glycosylation sites occupied by complex glycans (Caughey et al., 1989; Borchelt et al., 1990; Somerville and Ritchie, 1990) It has been shown by tunicamycin treatment (Taraboulos *et al.* 1990) and also by site-directed

mutagenesis of the linkage sites (Rogers et al., 1990) that this glycosylation is not necessary for the production of PrP^{Sc} in scrapie infected neuroblastoma cells (ScN₂a), so differential glycosylation cannot account for the differences between the two isoforms.

The third post-translational modification of PrP is the cleavage of a C-terminal peptide and the addition of a phosphatidylinositol-glycolipid (GPI) anchor. The objective of this project was to examine the role of the GPI anchor in the normal PrP protein and to investigate if the absence of this modification would lead to accumulation of the protein in a manner similar to that seen in the disease state. This was to be achieved by the production of site-directed mutations in the PrP gene such that the signal sequence for GPI addition would be deleted ^{followed by} the examination of these mutations in *in vitro* systems. Detailed experimental design is given in Chp.s 3, 4 and 5. To help understand the significance of this method of membrane attachment in relation to PrP a brief outline of current knowledge of GPI anchors is given below.

GPI-membrane anchors

Many membrane proteins are anchored to the cell surface by hydrophobic interactions of the protein with the lipid bilayer (Lingappa, 1991b). The possibility that some membrane associated proteins might be covalently linked to glycolipids was first raised by the release from cell membranes of proteins such as placental alkaline phosphatase (PLAP), 5'-nucleotidase and acetylcholinesterase (AChE), by the action of bacterial phosphoinositol-specific phospholipase-C (PIPLC). Due to the abundance of this type of membrane attachment in the variant-surface glycoprotein (VSG) of *Trypanosoma brucei* (up to 1×10^7 copies per cell), much of the initial determination of the chemical structure of GPI anchors was carried out using this protein (Ferguson 1988) Ever increasing numbers of eukaryotic proteins

(up to 150 are now known, (Englund, 1993)) in a number of different cell types of disparate function and origin, have been found to utilize this mechanism of membrane anchorage.

Structure and Biosynthesis.

The GPI-anchoring units in these proteins share a common core structure consisting of an ethanolamine-phosphate, a glycan, consisting of three mannose (Man) residues, and a non-acetylated glucosamine attached to a phosphoinositol ring and lipid (see Fig.1). They are linked to the carboxy-terminus of the polypeptide via an amide bond. A substantial body of work has shown that the core structures of these GPI-moieties are preassembled in the rough endoplasmic reticulum (RER) (Cross, 1990; Amthauer et al., 1993) and are transferred *en bloc* to the nascent polypeptide within a couple of minutes of their luminal translocation (Cross, 1990). The addition of the GPI moiety is concomitant with the removal of a hydrophobic carboxy-terminus amino acid sequence, which may act to initially anchor the newly synthesized peptide to the membrane. The enzyme(s) responsible for this transamidation reaction have not yet been identified. As an increasing number of mammalian anchor structures are elucidated variations to the common core structure have been discovered. Many non-VSG GPIs, including PrP, contain a second ethanolamine phosphodiester linked to one of their Man residues and many GPIs are substituted with branching glycans which may be protein, tissue and species-specific (Cross, 1990). VSG GPIs undergo a fatty acid remodelling to myristate in the Endoplasmic Reticulum after their addition (Masterson et al 1989), but, as yet, this has not been shown to be the case for mammalian anchors.

The normal isoform of PrP (PrP^C) and the scrapie isoform (PrP^{Sc}) have both been shown to contain a GPI anchor (Stahl et al., 1987) by a combination of PIPLC release and chemical analysis of the C-terminus. A more extensive

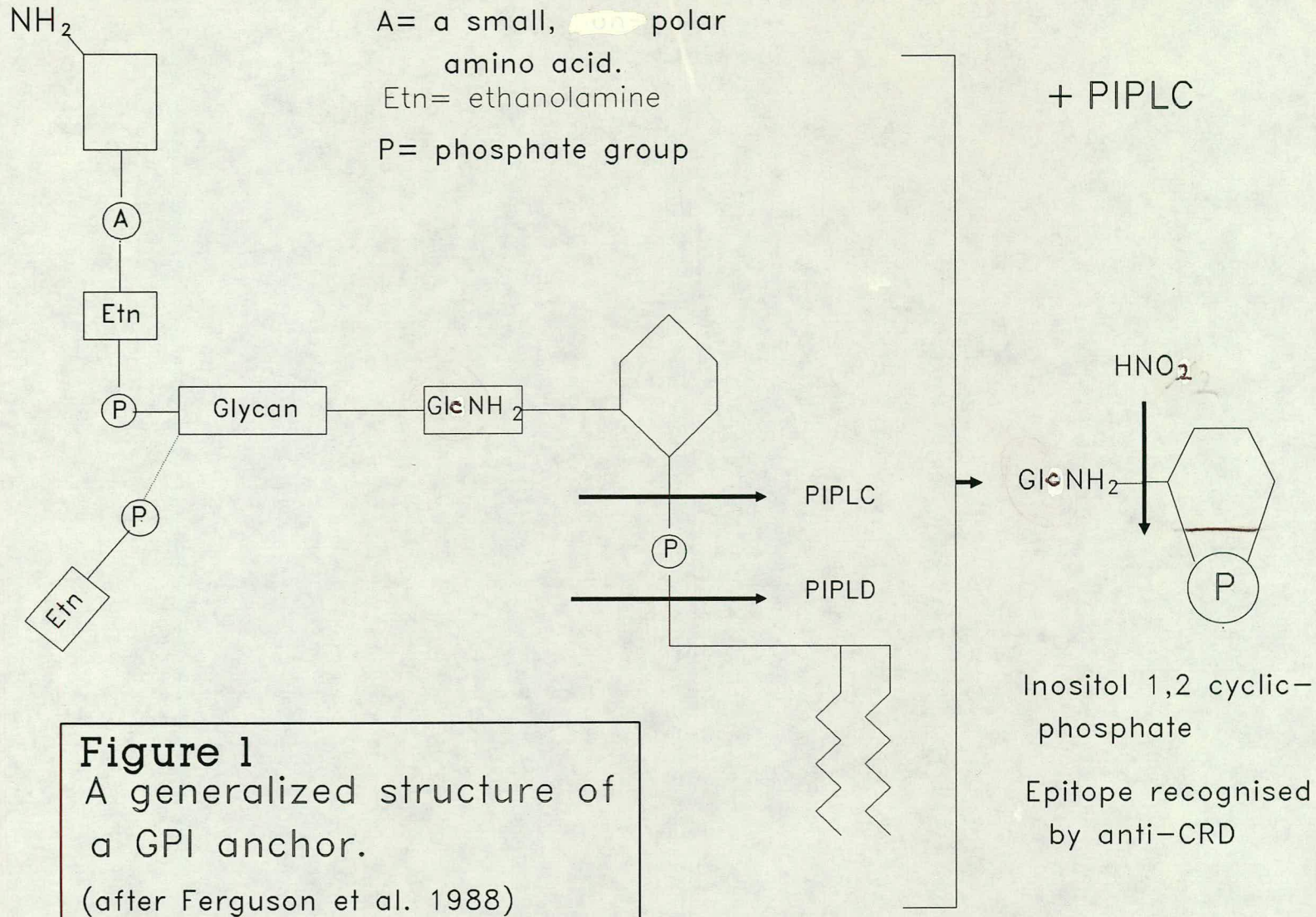


Figure 1
 A generalized structure of a GPI anchor.
 (after Ferguson et al. 1988)

analysis of the PrP^{Sc} anchor has been performed (Stahl et al., 1991, 1992, 1993), which has shown that there are six different glycoforms, which differ by the presence of additional hexose and sialic acid residues. Two of the glycoforms contain N-acetylneuraminic acid, which has not previously been reported as a component of any GPI (Stahl et al., 1992). Also, all the observed glycoforms contain two ethanolamine residues per moiety and contain stearic acid as part of their lipid anchor. Structural studies using mass spectrometry and Edman sequencing have shown no differences between the GPI anchors of PrP^C and PrP^{Sc} in their posttranslational chemical modification (Stahl et al., 1993). The anchor is attached at Ser 231 in the Syrian hamster PrP (Stahl et al., 1990a), but the attachment site for other species has not yet been determined. It has been observed that 15% of PrP^{Sc} extracted from hamster brains is truncated at amino acid 228 (Stahl et al., 1990a) and that these proteins do not contain a GPI anchor. The significance of this finding is not yet understood.

Release by PIPLC

Release from membranes by the action of PIPLC has often been used as an indication of GPI-anchorage. However, PIPLC resistance does not preclude the presence of a GPI, as some GPI-anchored proteins including human decay accelerating factor (DAF) and acetylcholine esterase (AChE) are resistant to cleavage, due to the presence of palmitic acid esterified to a position on the inositol ring (Ferguson and Williams, 1988, Walter et al., 1990, Roberts et al 1988). This site has been identified for the procyclic acidic repetitive protein (PARP) of *T. brucei* (Ferguson, 1992), where it is esterified roughly equally to the 2- and 3- hydroxyls of the inositol. Many mammalian anchor precursor intermediates are acylated on their inositol moieties, but this acylation has been removed from the mature precursors, as judged by their sensitivity to PIPLC (Urkaze et al. 1992). This (Puoti and Conzelmann 1993.)

modification has not been observed on PrP^C and PrP^{Sc} GPI anchors (Stahl et al., 1992, 1993). It is not known if this is the only substitution that will prevent PIPLC cleavage. Experiments in tissue culture cells have shown that most PrP^C is released from the surface of neuroblastoma cells by the action of PIPLC, whereas PrP^{Sc} is not (Borchelt et al., 1990; Taraboulos et al., 1990). If, however, the PrP^{Sc} is first denatured before PIPLC treatment the GPI anchor is then released by the action of the enzyme. This result led to the postulation that the conformation or aggregation state of PrP^{Sc}, or its position in the cell blocks the access of the enzyme to the C-terminus (Stahl et al., 1987). PrP^{Sc} has been found to have an intracellular location by immuno-confocal laser microscopy (Taraboulos et al., 1990).

Cross Reactive Determinants (CRD)

Treatment of many mammalian GPI-anchored proteins with PIPLC (but not PIPLD), produces a "cross reacting determinant" (CRD) that is found in anti-VSG sera raised in rabbits (Ferguson and Williams, 1988). The reaction of anti-CRD sera is of lower affinity with other proteins than with VSG, but can be used as an additional indication of GPI presence. Further treatment of the PIPLC-treated protein with nitrous acid removes the inositol 1,2 cyclic phosphate and destroys reactivity indicating that this moiety may be the recognised epitope (Zamze et al 1988).

PrP released from the surface of tissue culture cells by PIPLC reacts with anti-VSG sera (Borchelt et al., 1990).

Precursor biosynthesis pathway

Much work has been done recently to elucidate the pathway of GPI precursor synthesis. A stepwise model of precursor synthesis has been proposed (Tartakoff and Singh, 1992 ^{England 1993}). These steps have been worked out by a combination of *in vitro* methods using trypanosome membranes and

radiolabelled sugar residues (Masterson et al., 1989; Menon et al., 1990; Tartakoff and Singh, 1992) and studies of a series of murine T-cell lymphoma mutants deficient in Thy-1 expression (Tartakoff and Singh, 1992). Inhibitors of precursor synthesis include mannosamine (Lisanti et al., 1991), which inhibits addition of the third Man residue, PMSF(phenylmethane^{sulphonyl}fluoride) (Masterson and Ferguson, 1991), which inhibits addition of ethanolamine phosphate, fluoroglucose (Cross, 1990), which inhibits Man addition and certain myristic acid analogues which competes with myristate addition in trypanosomes (Cross, 1990). Simply stated, the GPI moiety is assembled by the sequential glycosylation of phosphatidylinositol (PI), followed by the addition of phosphoethanolamine. Transfer of radiolabelled GPI precursor to VSG has been observed in a cell free system (Mayor et al. 1991).

Disease associated precursor synthesis defects

A general deficiency in GPI biosynthesis has been implicated in at least one human disease, paroxysmal nocturnal hemoglobinuria (PNH) (Rosse, 1990). This is a rare disorder of bone marrow stem cells that is thought to develop through the clonal proliferation of an abnormal hematopoietic cell altered by somatic mutation. PNH-affected cells are characterized by an increased susceptibility to complement lysis. Three of the membrane proteins involved in the regulation of the complement cascade (DAF, HRF and CD59) are missing from the surface of blood cells in patients with PNH, and a common structural feature of these three is their GPI-anchorage (Plough et al., 1992). At least seven other GPI-anchored proteins have been shown to be absent from the cell surface in this disease. One of these, LFA-3, also exists in a transmembrane form, but only its GPI-linked form shows impaired expression. Normal levels of mRNA for the above proteins are present in the

cells, indicating that there is an underlying fault in the biosynthesis of the glycolipid precursor (Rosse, 1990; Plough et al., 1992). Recent work has shown that an early step in GPI precursor biosynthesis is deficient in B lymphocyte cell lines established from PNH patients (Takeda et al., 1993) and that this deficiency results from a somatic mutation in PIG-A, a gene that takes part in the synthesis of N-acetylglucosaminyl phosphatidylinositol (GlcNAc-PI), an early intermediary in the GPI precursor biosynthetic pathway (Miyata et al., 1993).

Transfer of glycolipids to protein

Sequence requirements

GPI-anchor addition involves the coordinated removal of a carboxy-terminus amino acid sequence from the nascent chain, followed by the addition of the preformed anchor to the new carboxy-terminus. This substitution is a very early posttranslational event (Bangs et al., 1985; Ferguson et al., 1986) and takes place in the endoplasmic reticulum (Conzelmann et al., 1988). GPI-addition is directed by a signal at the carboxy-terminus of the protein, but as no primary sequence homology between different GPI-anchored proteins has been identified the signal is thought to be of a general nature.

Cell-free and tissue culture studies with PLAP, DAF, Fc1R111, Qa-2 and others have defined features which govern GPI-anchorage (Berger et al., 1988; Waneck et al., 1988; Bailey et al., 1989; Kodukula et al., 1991a; Moran and Caras, 1991). These include a hydrophobic tail of a minimum length at the very C-terminal end of the primary amino acid sequence and, 10-12 residues amino terminal to this sequence, the presence of a small non-polar amino acid at the actual attachment site (Micanovic et al., 1990; Moran and Caras, 1991). Also, the absence of a recognisable possible cytoplasmic domain at the C-terminus seems to be a prerequisite of GPI

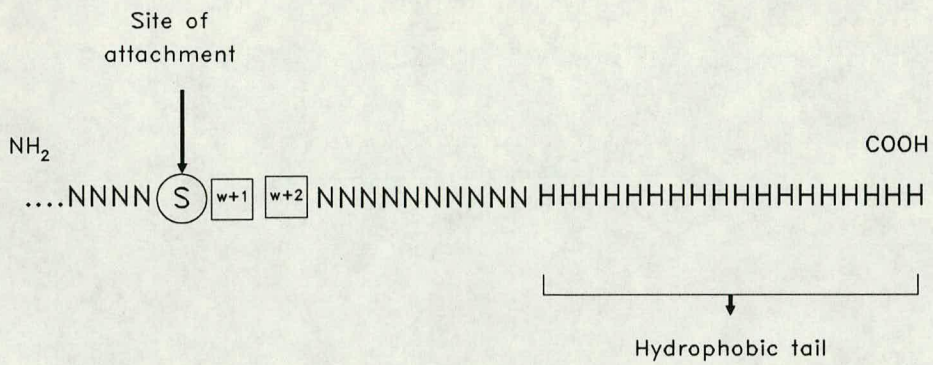
addition (Englund, 1993).


Hydrophobic domain

It has been shown, (Berger et al., 1988), that for PLAP in COS cells a hydrophobic tail of at least 17 residues is necessary for the protein to become GPI-anchored. Truncation mutations which contained 13 or less hydrophobic residues failed to become anchored and were secreted into the medium (Berger et al., 1988). The exact amino acid content of the hydrophobic region is not important as proteins having different hydrophobic sequences substituted for their own are, nevertheless, GPI-anchored. Replacement of the hydrophobic region with a hydrophilic sequence, however, leads to the protein being secreted. The length of the hydrophobic sequence has recently been shown to have the same significance in cell-free system (Kodukula *et al.* 1992).

The cleavage attachment site

The nature of the amino acid residue at the attachment site has also been shown to be a specific requirement for GPI-addition (Micanovic et al., 1990). PLAP mutants containing what these authors designated category 1 amino acids (glycine, alanine, serine, cysteine or asparagine) at their attachment site were anchored normally at the plasma membrane and exhibited alkaline-phosphatase activity, whilst those containing category 2 amino acids (glutamic acid, glutamine, proline, tryptophan, leucine, valine, phenylalanine, threonine, methionine and tyrosine) at their attachment sites showed very



S= a small,  polar amino acid
 N= any amino acid
 H= hydrophobic amino acids
 w+1, etc. amino acids as described in the text

Generalized signal for GPI attachment

Figure 2

reduced GPI-anchorage and little or no alkaline phosphatase activity associated with the cells. A general trend towards having a category one amino acid at positions one and two of the cleaved peptide has also been observed, with position $w + 2$ (two amino acids amino-terminal from the attachment site, Fig.2) having a much more stringent requirement than position $w + 1$ (Fig. 2). A similarity between N-terminal signal peptidase and the C-terminal transamidase with respect to the required amino acids at the cleavage site has been noted (Ferguson and Williams, 1988; Kodukula et al., 1993). It has been suggested for signal peptidase that there is a requirement for particular amino acids to occupy positions -1 and -3, and that these amino acids represent sites of interaction with N-terminal signal peptidase (Von Heijne, 1990). Kodulkula et al. suggest that the positions w and $w + 2$ are sites of a similar interaction between the protein and the transamidase (Kodukula et al., 1993) It has been shown that the immunoglobulin heavy chain binding protein (BiP), a protein known as a molecular chaperone, binds to nascent PLAP and it is suggested that it acts to hold the protein in an appropriate conformation for interaction with the transamidase to take place (Amthauer et al., 1993).

The general rules presented here have been tested by constructing synthetic GPI signals using unrelated sequence elements that do not normally function in GPI-anchoring and testing their efficacy in cell culture, and it appears that they do indeed act as GPI addition signals (Moran and Caras, 1991).

Functions

Our knowledge of the basic biochemical structure of the GPI anchor has increased greatly in the past few years, yet it is still difficult to assign a particular function to it, as the proteins attached in this way have diverse physiological functions, where, indeed, these functions are known at all (Cross, 1990). The fact that the cell should choose such a complex

mechanism of anchoring suggests that there may be good reasons, that may vary for different proteins, cell types and stages of development. A number of cell-cell adhesion molecules in the central nervous system (CNS) such as Thy-1, TAG-1, F3/F11, N-CAM and P-31 possess GPI anchors (Faivre-Sarrailh and Rougon, 1993) and are selectively expressed in time and place. Often expression is restricted to a subset of neurons and can be axonally or dendritically specific. The function of the PrP protein is unknown, but it is known to possess a GPI anchor (Stahl et al., 1987) and expression is known to vary between different cell types and stages of development (Manson et al., 1992b).

Cellular location

Most of the GPI anchored proteins thus far identified are located on the extracellular surface of the plasma membrane (Lisanti et al., 1990a). There are some exceptions to this rule which have an intracellular location, such as GP-2 in the zymogen granules of the exocrine pancreas (Fukuoka et al., 1992) but these all tend to face the lumen of an intracellular vesicle rather than the cytoplasm. Truncation mutations of GPI anchored proteins have been shown to be secreted in *in vitro* systems (Berger et al., 1988; Bailey et al., 1989; Amthauer et al., 1992), and conversely, a normally secreted protein can be relocated to the plasma membrane by the inclusion of a GPI addition signal (Moran and Caras, 1991). Thus a GPI-anchoring signal leads to the asymmetrical distribution of the anchored protein.

Apical sorting in polarized cells

This seems to have particular importance in polarized cells, such as epithelial cells, where the presence of a GPI anchor has been implicated as a signal for sorting of proteins to the apical membrane (Lisanti et al., 1989a; Brown and

Rose, 1992). In a number of different polarized cell lines such as Madin-Darby canine kidney (MDCK) and Caco-2 cells GPI anchored proteins are selectively enriched in the apical membrane whilst depleted or absent in the basolateral membrane (Lisanti et al., 1990a, Garcia *et al.* 1993). The addition of a GPI signal sequence to the normally basolaterally sorted VSV G protein (Brown, et al., 1989) causes its relocation at the apical surface. It has been suggested that this apical sorting may result from the association of the apical protein to glycosphingolipids in the trans-Golgi network (TGN) (Brown, and Rose, 1992). Experiments to support this hypothesis have shown that apically sorted proteins, such as influenza virus HA, are soluble in detergents such as TX100 and CHAPS when in the ER, but become insoluble on reaching the TGN (Simons and van Meer, 1988). Basolateral proteins, however, do not become insoluble. It has been proposed that this insolubility is due to the association of the proteins with glycosphingolipids, which have also been shown to have detergent insolubility properties and that these mixed clusters might be packaged into apically directed vesicles (Simons and van Meer, 1988; Brown, and Rose, 1992; Hannan et al., 1993). In a similar fashion the GPI-anchored protein Thy-1 has been found to be sorted to the axonal membrane of rat hippocampal neurons and not to the dendritic membrane (Dotti et al., 1991), suggesting the possibility of a similar sorting mechanism to that in epithelial cells and the possible existence of tight junctions in neurons. However, other work implies that Thy-1 exhibits both axial and dendritic expression in other cell types (Morris et al., 1985; Xue et al., 1990), although it is possible that the dendritic form may exist in the transmembrane state. In general the distribution of GPI-anchored adhesion molecules in neurons is complex and seems to depend on the cell type, particular protein and differentiation state (Faivre-Sarrailh and Rougon, 1993). This regulation could be achieved by differences in the structure of the GPI-anchor or by regulation of microtubule-based transport vesicles by the modification of

cytoskeletal components during neuronal maturation.

Exclusion from clathrin coated pits

Many different membrane receptors that participate in receptor mediated endocytosis achieve internalization of their ligands via the formation of endosomes from clathrin coated pits (Brown et al., 1983). The clustering of receptors in these pits is promoted by their cytoplasmic tails (Brodsky, 1988). It has been shown that many GPI-anchored proteins, including Thy-1, 5'-nucleotidase and the folate receptor, are excluded from these pits. It is expected, therefore, that GPI-anchored proteins, having no cytoplasmic domain, undergo endocytosis via a clathrin-independent pathway. Recently it has been shown that transmembrane and GPI-anchored forms of the same protein are endocytosed by different pathways (Keller et al., 1992; Schell et al., 1992). GPI-anchored CD4 was endocytosed via non-coated microinvaginations of the plasma membrane. These invaginations have now been named caveolae and GPI anchored proteins have been shown to be clustered in them (Anderson et al., 1992; Rothberg et al., 1992). Different proteins have shown different mechanisms of internalization, with 5'-nucleotidase (Van den Bosch et al., 1988) and DAF (Tausk et al., 1989) clearly becoming visible in internalized vesicles, whereas the folate receptor remains associated with caveolae at the plasma membrane, where it internalizes folate by a process named potocytosis (Anderson et al., 1992; Rothberg et al., 1992). Caveolae have a specialized lipid composition (Anderson et al., 1992), and although it has not been directly demonstrated to contain glycosphingolipids their similarity to apically sorted vesicles from the TGN has been further supported by the discovery of a protein that is present in both, VIP21, ^{also called caveolin} (Dupree et al., 1993). Whether this or other proteins recycle from the plasma membrane to the TGN has not yet been shown. PrP has been shown by pulse-labelling experiments in

mouse neuroblastoma (N₂A) cell lines to recycle from the cell surface (Caughey et al., 1991) and has also been found in caveolae (Anderson et al., 1992a).

Lateral mobility

One consequence of lipid anchoring is an inherent increase in mobility in the plane of the membrane. Diffusion coefficients in the order of $4 \times 10^{-9} \text{ cm}^2/\text{sec}$ are observed for Thy-1 (Ishihara et al., 1987) alkaline phosphatase (Noda et al., 1987), DAF (Thomas, J. et al., 1987), and PH-20 (Phelps et al., 1988). These are lower than expected for freely diffusing lipid probes ($0.5-1 \times 10^{-9}$), but much higher than those for transmembrane glycoproteins ($0.5-6 \times 10^{-10}$). However, these proteins also contained a significant immobile fraction, suggesting some heterogeneity in structure or secondary interactions. Some GPI anchored proteins may need a high degree of mobility for their function (like AChE for deactivation of acetylcholine at the synapse), whilst others may require a high degree of immobility (like the trypanosomal VSG). Insertion of VSG into BHK cells did not increase its mobility, implying that it is not the tight packing of these molecules in the trypanosomal cell surface that retards it, but some other intermolecular interaction (Cross, 1990). PH-20, a GPI-anchored guinea pig sperm protein, diffuses at varying rates at different stages of sperm maturation (Phelps et al., 1988).

In general, although there is a wide variation in the diffusion properties of GPI-anchored proteins, their lipid anchorage gives them the ability to diffuse at a rate closer to that of lipids than that of integral membrane proteins. There is no documented evidence on the lateral mobility of PrP other than the existence of a "capping" effect that is seen with fluorescence labelled anti-PrP probes on the surface of N₂a, PC12 and hamster primary brain cells (Stahl et al., 1987)

Release of proteins from the cell surface

The GPI-anchor is the substrate for a number of different enzymes (proteases, glycosidases and phospholipases). The actions of these enzymes could possibly result in GPI-specific anchor degradation and consequent release of the attached protein from the membrane. Such an enzyme mediated release mechanism could be regulated or constitutive. Soluble forms of many of these proteins have been reported, both *in vivo* and from tissue culture cells (Low and Saltiel, 1988; Lisanti *et al.*, 1990b; Tagliavini *et al.*, 1992b). Some of these arise from differential splicing of mRNAs to produce non-GPI-anchored proteins, as shown for N-CAM, Qa-2 and DAF (Lisanti *et al.*, 1990b) whilst others are released as a result of certain stimuli. As the PrP open reading frame is all contained on one exon (Basler *et al.*, 1986) there is no evidence to suggest that differential splicing gives rise to a soluble form. However, soluble forms of PrP are released from N₂A cells (Caughey *et al.*, 1988, 1989; Borchelt *et al.*, 1993) and isolated from human cerebrospinal fluid (CSF) (Tagliavini *et al.*, 1992b). PrP that is shed from tissue culture cells does not contain a CRD epitope (Prusiner, 1991) and it is believed that most if not all of the GPI-anchor is removed from these proteins (Borchelt *et al.*, 1993).

The observation that free GPI molecules are hydrolysed in response to insulin action led to the question if a similar reaction could be seen with GPI-anchored proteins. Several such proteins were shown to be released from tissue culture cells in response to insulin, including 5'-nucleotidase (Klip *et al.*, 1988) and alkaline phosphatase (Romero *et al.*, 1988). A reduction in the number of PLC releasable proteins from the surface of insulin sensitive BC3H1 cells was observed when the cells were preincubated with insulin (Lisanti *et al.*, 1989b). Only some of the GPI anchored proteins seemed to be affected, with levels of others remaining the same. This led to the speculation that hormone sensitive and insensitive structural pools of GPI

anchor exist. It is already known that structural modifications of the GPI, such as the esterification of palmitic acid to the inositol ring, can play a role in susceptibility to enzymatic degradation (Roberts et al., 1988, Ferguson 1992). This is one mechanism whereby proteins could be differentially released by hormone action. Similarly, AChE is released from certain areas of the brain and adrenal chromaffin cells by a variety of stimuli (Greenfield, 1984) and Qa-2 is released from concavalin-A stimulated T-cells (Soloski et al., 1986). Serum levels of carcinoembryonic antigen (CEA) are increased in disease, leading to the idea that loss of apical polarity and subsequent basolateral expression, coupled with enzymatic degradation could account for this increase (Lisanti et al., 1990b). It is possible to check whether these soluble proteins have been released by the action of PIPLC by determining whether or not they react with the CRD of *T. brucei*, as only GPI molecules so cleaved will contain the reactive determinant (Hooper and Bashir, 1991).

Signal transduction

A possible role for GPI-anchoring in the process of signal transduction has been postulated as a result of findings involving the activation of lymphocytes by treating them with antibodies against GPI-anchored proteins on their surfaces (Robinson et al., 1989; Cashman et al., 1990). PrP is found on the surface of lymphocytes and this surface abundance is increased by cell activation. Also polyclonal antibodies to PrP suppress mitogen induced activation (Cashman et al., 1990). These findings may implicate a role in signalling for PrP. Transgenic mice expressing either GPI-anchored or transmembrane Qa-2 have been produced (Robinson et al., 1989) and it was shown that only T-cells from the former could be activated by Qa-2 specific antibodies, thus implying that the GPI-anchor is essential for activation. Antibody-mediated modulation of cell activation is also

observed for other GPI-anchored proteins such as TAP-1, Thy-1, Qa-2 RT-6, and DAF (quoted in Cashman 1990). The finding that some GPI-anchored proteins associate with protein tyrosine kinases (Stefanova et al., 1991; Thomas and Samelson, 1992), and the well documented involvement of free GPI in secondary messenger systems (Saltiel et al., 1986; Eardley and Koshland, 1991) increase the possibility that the GPI-anchor has some sort of signal transduction function. How the GPI-anchored proteins, which are located exclusively on the outer leaflet of the plasma membrane activate intracellular tyrosine kinases is not yet known but it has been postulated that transmembrane linker proteins mediate the interaction between the two (Brown, 1993). The possibility also exists that GPI-anchored proteins do not directly bind to transmembrane linker proteins, but that these transmembrane proteins are associated with specialized lipid clusters which GPI-anchored proteins also have an affinity for, and that this association is close enough for a kinase bound to the cytoplasmic domain of the transmembrane protein to interact with the GPI-anchored protein (Brown, 1993). Lipid structures have been coprecipitated with GPI-anchored protein/tyrosine kinase complexes in a number of different cell lines (Brown, and Rose, 1992, Horejsi 1992, Lublin quoted in Brown 1993). The association of GPI-anchored proteins, including PrP, with caveolae (Anderson et al., 1992) leads to speculation whether they can become transiently internalized by the process of potocytosis. The role of such internalization in signaling is not known.

CHAPTER TWO:

General Methods

Chemicals

All chemicals were of molecular biology grade and were obtained from BDH, Gibco-BRL, Sigma, and Boehringer-Mannheim and stored as per the manufacturers instructions. Restriction endonucleases and other enzymes were obtained from Gibco-BRL, Boehringer-Mannheim and Promega.

All water was double-distilled, except water used for tissue culture which was tissue culture grade from a Millipore Mill-QUF system

Bacteria

All bacteria used were laboratory attenuated strains of *E.Coli* and are listed in Table 1.

Table 1.

Strain	Phenotype
CMK 603	thr, leu, thi, supE, recBC, T ₁ ^R , T ₅ ^R , r ⁻ , m ⁺ , lacZΔM15 lacY, F' lac ^{q1} lacZΔM15 pro ⁺
Mut-L	F', dam ⁻ ,
GM242	F', dam3, recA1, Sin2, trr1, leuB6, proA2, his 4, metB1, lacY1, galK2, ara14, tsx33, thi1, deoB6, supE44, rpsL260 (Str')

Techniques of DNA manipulation

Small scale DNA preparations

Small scale DNA preparations were carried out by the alkaline lysis method of Birnholm and Dolby ,(Birnholm and Dolby, 1979) as described below.

- 1) 3ml shaking overnight bacterial cultures were grown at 37° in L-broth (Gibco-BRL) supplemented with Ampicillin (Amp) to 0.1µg/ml.
- 2) 1ml of this was centrifuged in a minifuge at 13,000 rpm for 1min. The supernatant was discarded and the pellet resuspended in 0.1ml lysis solution (lysozyme 2mg/ml, 25mM Tris.Hcl pH 8.0, 10mM EDTA pH 8.0, 50mM glucose) by vortexing.
- 3) Samples were left at 0°C for 30min.
- 4) 0.2ml alkaline SDS (sodium dodecylsulphate) solution (NaOH 0.2N, SDS 1%) was added. Samples were then mixed by inversion and left on ice for a further 5 min.
- 5) 0.15ml of high salt solution (Na acetate pH5 3M, tRNA 300µ/ml) was then added and samples left on ice for 30-60min.
- 6) The thick white precipitate that forms at this stage was removed by centrifugation for 5min and the supernatant removed to a fresh tube.
- 7) DNA was precipitated by the addition of 1ml of ethanol, leaving at -20°C for 30 min and centrifugation at 13,000 rpm for 2 min. A further precipitation in 0.1ml 0.1M Na acetate pH 6, 0.2ml ethanol at -20°C for 5min was performed and DNA was recovered by centrifugation at 13,000 rpm for 2min, the supernatant discarded and the pellet dried and resuspended in 50µl of TE (appendix 1).

Large scale DNA preparations

Large scale DNA preparations were carried out by a modification of the alkaline lysis method of Birnholm and Dolby (Birnholm and Dolby, 1979), as described by Sambrook (Sambrook et al., 1989)

- 1) The bacterial pellet from an overnight 500ml culture was resuspended in 18ml of solution 1 (50mM glucose, 25mM Tris.HCl pH 8.0, 10mM EDTA pH 8.0).
- 2) 2ml of a freshly prepared solution of lysozyme (10mg/ml) in 10mM Tris.HCl pH 8.0 was added, followed by 40ml of freshly prepared solution 2 (0.2N NaOH, 1% SDS). The contents of the bottles were thoroughly mixed by inversion and stored at room temperature (RT) for 5-10min.
- 3) 20ml of ice cold solution 3 (5M potassium acetate 60ml, glacial acetic acid 11.5ml, H₂O 28.5ml) were added, the contents of the bottles mixed well and left on ice for 10min.
- 4) The bacterial lysate was centrifuged at 4000rpm for 15min at 4°C in a Beckman JS-21 centrifuge and the rotor allowed to stop without braking.
- 5) The supernatant was carefully removed and filtered through four layers of cheesecloth into a 250ml bottle.
- 6) A 0.6 volume of isopropanol was added, the contents mixed and allowed to stand at room temperature for 10 min.
- 7) The nucleic acids were recovered by centrifugation at 5000rpm for 15min at RT. The supernatant was decanted and the pellet dried and then resuspended in 3ml of TE pH 8.0.
- 8) This DNA was then purified by equilibrium centrifugation in CsCl-ethidium bromide gradients as follows. Caesium chloride (CsCl), was added to the DNA to a final concentration of 1mg/ml, then 0.1ml/ml of a stock solution

of 5mg/ml ethidium bromide solution and the remaining volume of the tubes filled with a 1mg/ml solution of CsCl. This solution was centrifuged either in a Beckman NVT65 vertical rotor at 57,000rpm for 16hours, or a Beckman 70.1Ti fixed angle rotor at 37,000rpm for 48hours in heat sealable 20ml Beckman polyallomer tubes in a Beckman L8 ultracentrifuge. The closed circular plasmid DNA was visualised by UV illumination and separated from contaminating chromosomal DNA by side puncture of the tubes.

9) Ethidium bromide was removed by extraction with TE-saturated butan-1-ol and CsCl removed by dialysing against at least 5x1 litres of TE at 0°C, the final dialysis being left overnight.

10) DNA concentration was determined by the optical density observed at a wavelength of 260nm in a quartz cuvette in a Beckman DU64 spectrophotometer.

Digestion of DNA with restriction endonucleases

CsCl banded or mini-prep DNA was digested with 3 units of enzyme per μg in the presence of the appropriate restriction buffer at 37°C, in a reaction volume of 20-30 μl for at least 1 and usually 3 hours. The enzyme Bcl1 was incubated at 50°C and Sma 1 at 30°C as these are the optimum temperatures for these enzymes. Double digests were performed in whichever buffer was optimal for the digest, with salt levels being adjusted before digestion with the second restriction endonuclease was carried out. Enzymes and buffers were obtained from Gibco-BRL and Boehringer-Mannheim.

Enzymes were inactivated either by heat treatment appropriate for the particular enzyme or by phenol/chloroform extraction and ethanol precipitation of the DNA.

Phenol/Chloroform extraction and Ethanol precipitation

Where necessary phenol/chloroform extraction was carried out by adding an

equal volume of TE-equilibrated phenol to the sample, vortexing it for 2-5 min in a minifuge and removing the upper aqueous layer to a fresh tube, followed by a similar extraction with chloroform. Ethanol precipitation was carried out by the addition of one tenth volume of either 3M sodium acetate pH 5 or 5M potassium acetate to the sample along with three volumes of 100% ethanol. The tubes were mixed and stored at -20°C from 1 hour to overnight. DNA was recovered by centrifugation in a minifuge for 15min, and the pellets washed with 70% ethanol and dried briefly before resuspension in either TE (Appendix 1) or H₂O as required.

Agarose electrophoresis

Agarose gels were prepared using high grade agarose from Gibco-BRL . Usually, 0.8% gels were used, with 1-3% gels being used when smaller fragments were being examined. TAE buffer (appendix 1) was used to make the gels and as a running buffer. Gels were run at varying voltages according to their size, but generally from 50-100 volts for two to three hours or at 25 volts overnight, on a Bio-Rad horizontal gel apparatus. Low melting point agarose was also supplied by Gibco-BRL and gels were prepared in a similar manner and run overnight at 25 volts. 3 μ l of agarose gel loading buffer (appendix 1) was added to each sample before loading and 1 μ g of an HindIII digest of lambda used as size markers.

Gels were stained for 15 min in a 1 μ g/ml solution of ethidium bromide and destained for 30min in double distilled water before photographing on a Polaroid MP-4 Land Camera .

Recovery of DNA fragments from gels

A number of different techniques were used to recover fragments of DNA from gels. For random priming of cDNA (see below) fragments were isolated by carefully cutting the appropriate band out of a low melting point gel with a sterile scalpel blade and then weighing the piece of gel and

incubating it in 3ml/g of H₂O at 100°C for seven minutes. The obtained solution was stored at -20°C until required (Wieslander, 1989). For cloning and the recovery of PCR products two methods were used. Fragments were separated on agarose gels as above.

1) These fragments were then cut out and centrifuged at 13,000rpm for 1 min. through a piece of blotting paper in an eppendorf ~~the~~ ^{of which} bottom had been pierced with a needle, allowing the expressed buffer to be collected in another eppendorf (Weichenhan, 1991). It was not necessary to phenol/chloroform the DNA which could be recovered by ethanol precipitation.

2) A piece of DEAE membrane (Schleicher & Schull, NA45 0.45µm) was placed in front ^{of} and behind ~~the~~ the appropriate fragment and the electrophoresis was continued until the band had run onto the membrane (Dretzen *et al.*, 1981). DNA was eluted from the membrane by heating it to 65°C for 15 min in elution buffer (10mM Tris, 1mM EDTA, 2.5M NaCl pH 8). This was then extracted once in phenol , once in chloroform and then ethanol precipitated as described above.

Ligations

In general fragments that were to be used in ligations were ethanol precipitated after extraction from gels before inclusion in the ligation reaction.

A sample ligation reaction would contain:

Vector DNA,	xµg,
Insert DNA,	3x molar excess over
vector,	
T4 DNA ligase buffer x5,	4µl,
T4 DNA Ligase (1u/µl),	1µl,

H₂O to a final volume of 20μl.

Incubate overnight at 9°C.

Making competent cells

Bacteria were made competent for the transformation of plasmid DNA by two methods, one of which was designed to prepare a stock of frozen competent cells and one to prepare highly competent cells.

1) Calcium chloride method:

This was based on the method of Cohen (Cohen et al., 1972). Starting with a fresh plate of bacteria a 3ml overnight ^{culture} was grown up. This was used to inoculate approximately 100ml of L-broth in a sterile 250ml conical flask. The culture was incubated at 37°C with gentle shaking and the optical density measured at various times by comparing it to that of uninfected L-broth at 560nm in a Beckman DU64 spectrophotometer until it reached an OD of 0.25. At this stage the cells should be replicating exponentially. When the required cell density was achieved further growth was inhibited by placing the cells on ice for ten minutes.

The cells were collected by centrifugation at 4000g for 10min and resuspended in 1/2 x volume of ice-cold, sterile 0.1M calcium chloride (CaCl₂). They were then mixed gently and left on ice for 15 min. The centrifugation step was repeated and the cells resuspended in 1/25 the original volume of 0.1m CaCl₂. They were mixed gently and left on ice for four to twelve hours before use.

2) Freezing method:

This was carried ^{out} in much the same manner as the previous method, except that buffers containing cryoprotectants were substituted for CaCl₂.

After the first centrifugation cells were resuspended in 1/3 volume of RF1 (appendix 1) and after the second centrifugation in 1/12.5 of RF2 (appendix

1).

This was followed by a 15 min incubation on ice, after which the cells were aliquoted into freezing vials and flash frozen in liquid nitrogen. Frozen competent cells were stored at -70°C and used within three months of manufacture.

Transformation of bacteria with plasmid DNA

DNA or ligation mixes were diluted or divided into the desired quantities and aliquoted into sterile 13ml round-bottomed Falcon tubes (Becton and Dickinson). $200\mu\text{l}$ of competent cells were then added and mixed gently. The mixture was left on ice for 30 min and then heat shocked in a 42°C water bath for 45 sec. The tubes were returned to ice for 2 min. $800\mu\text{l}$ of L-broth was added to each tube and they were placed at 37°C for 45-60 min to allow the cells to express their *Amp* resistance gene. The cells were plated onto L-Agar plates containing ampicillin or other appropriate plates by spreading with a sterile spreader and incubated overnight at 37°C .

Transfer of DNA to nitrocellulose and nylon filters

Agarose gels were run, stained and photographed as described above. A number of different methods were used during the course of these experiments.

1) Dry blotting

Gels were soaked for 2 x 15 min in 0.25M HCl,
rinsed in distilled water,

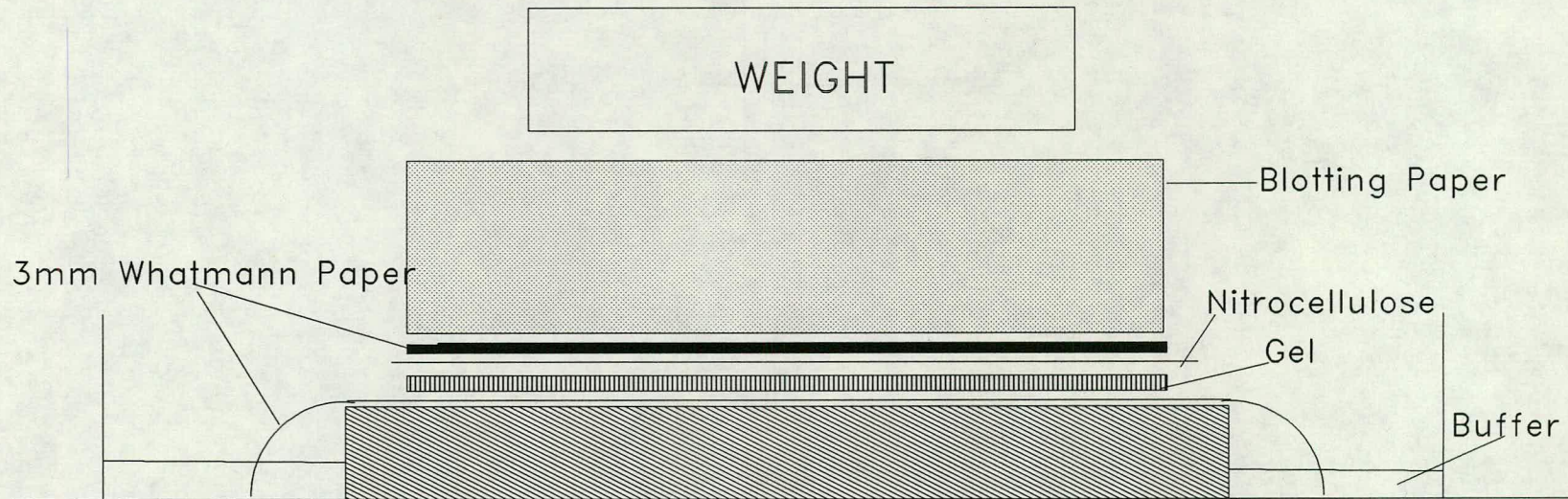
soaked for 2 x 15 min in 0.5N NaOH, 1.5 M NaCl,

soaked for 2 x 30 min in 1M NH_4Ac , 0.02 N NaOH, pH 8.

While this is being done a gel sized piece of nitrocellulose (Schleicher & Schull PH 79) or nylon membrane (Genescreen, Hybond) was soaked in the

Figure 3.

Southern Blot Set-up Procedure



third buffer, along with four gel-sized pieces of Whatmann 3mm paper. The gel was then sandwiched as shown in Figure 3 except that the buffer was omitted, with a suitable weight being placed on top of the glass plate and the whole blot being covered with cling-film and left overnight at RT. When the filter was removed from the blot it was washed in 2 x SSCP for 2 min and air dried before baking at 80°C (nitrocellulose), or crosslinking with UV light (Hybond membranes) to fix the DNA to the filters.

2) Southern blots

Southern blots were performed using a buffer reservoir (Southern, 1975) and Genescreen (Du Pont) or Hybond membrane exactly as described for Northern blots (see below).

3) Colony blotting

This was carried out by the methods of Wallace (Wallace et al., 1980) and Suggs (Suggs et al., 1981). Colonies or plaques were picked off plates and streaked onto squared, numbered nylon filters (Hybond) on the surfaces of appropriate plates. These were allowed to grow up overnight at 37°C. Corresponding master plates were also streaked out at the same time. The filters were treated as follows:

1) 3 sheets of Whatmann 3mm paper were soaked in denaturation solution (0.5M NaOH, 1.5M NaCl). The filters were placed face up on this for 5 min. The colonies lysed.

2) Using flat edged forceps the filters were transferred to the surface of Neutralizing buffer-soaked paper (1M Tris.HCl pH 7.5,) and left for 5 min.

3) The filters were transferred to 2x SSCP (appendix 1) soaked paper and left for 5 min.

4) The filters were then transferred to dry filter paper and allowed to air dry for > 1 hour.

5) They were wrapped in Saran wrap, and placed face down on UV illuminator for 2 min to fix the DNA.

Random priming of cDNA fragments

This was performed essentially as described by (Sambrook et al.,1989). Suitable restrictions were performed to obtain the required fragment containing an appropriate amount of DNA and these fragments were separated on low-melting point agarose (Gibco-BRL) at 25 volts. The fragment of interest was cut out with a scalpel and treated as follows:

- 1) 3ml of H₂O per g of agarose was added.
- 2) The tubes were boiled in a water bath for 7 min and left at 37°C for 10 min to 1 hour.
- 4)The following were mixed:

DNA (20-50ng)	35.5 μ l
OLB buffer (appendix 1)	10 μ l
³² P dCTP (Amersham 10 μ C/ μ l)	5 μ l
Klenow fragment (5u/ μ l)	1 μ l

- 5) This mixture was incubated at RT for at least 4 hours and preferably overnight.
- 6) 200 μ l of 50 μ g/ml herring sperm (HS) DNA was added.
- 7) One phenol extraction was performed.
- 8) Probes were ethanol precipitated and air dried, and resuspend in 100 μ l TE. The probes could be stored at -20°C at this stage.
- 9) 1 μ l of each probe was taken and counted in a scintillation counter

Labelling of oligonucleotides

This was performed as described by Sambrook (Sambrook et al., 1989).

1) Oligos were diluted to 20-40ng in a 4 μ l volume.

2) The labelling reaction was prepared as follows:

Oligo		4 μ l
10x Polynucleotide kinase buffer		2.5 μ l
³² P dATP		30 μ C
Polynucleotide Kinase		1 μ l
H ₂ O	to	25 μ l

and incubated at 37°C for 1 hour.

3) 200 μ l herring sperm (HS) DNA (50 μ g/ml) and 400 μ l Ethanol were added, and the probe precipitated at -20°C, recovered by centrifugation and air dried.

5) The probe was resuspended in 100 μ l of TE.

Southern hybridization with labelled fragments

Filters were pre-hybridized overnight in sealed plastic bags or in hybridization tubes and hybridized over the following night as follows.

Prehybridization buffer:

20x SSPE		2.5ml
Formamide(BDH 40%w/v)		5 ml
50x Denharts solution (appendix 1)		0.9ml
BSA (100 mg/ml)		0.1ml
Denatured HS DNA (100 μ g/ml)		0.1ml
10% SDS		0.1ml
H ₂ O	to	10 ml

HS DNA was denatured by boiling for 10 min before adding to the prehybridization mix.

Incubations were overnight at 42°C.

For hybridization the probe was added to this buffer and incubations carried out overnight at 42°C.

After hybridization was complete the probes were collected and stored for decay and background radiocativity washed off with sequential washes as below. The filters were monitored with a Geiger counter between washes.

- 1) 2x SSPE, 0.1% SDS 15 min, 37°C.
- 2) 2X SSPE, 0.1% SDS 15 min, 55°C.
- 3) 2X SSPE, 0.1% SDS 15 min, 65°C.
- 4) 1X SSPE, 0.1% SDS 15 min, 65°C.
- 5) 0.5 X SSPE, 0.1% SDS 15 min, 65°C.

The filters were sealed in plastic, covered with X-OMAT (Kodak) X-ray paper stored, at -70°C and developed after an appropriate length of time.

Southern hybridization with oligonucleotides

Filters were sealed into plastic bags or hybridization tubes and pre-hybridized overnight at 37°C.

Pre-hybridization buffer:

20X SSCP	15ml
10% SDS	0.5ml
50X Denharts	1.0ml
HS DNA (5mg/ml)	1.0ml
H ₂ O	to 50ml

Techniques of RNA manipulation

Total RNA isolation from tissue culture cells

For all RNA manipulations gloves were worn at all times, glassware was baked at 200°C for 4 hours, tips autoclaved and water treated with DEPC, (appendix 1). Total RNA was isolated from tissue culture cells using RNazol B (Biogenesis Ltd.), a single-step preparation method, as per the manufacturers instructions.

- 1) Each 35mm dish of cells was washed in phosphate buffered saline (PBS, Gibco-BRL tissue culture grade) and then overlaid with 1ml of RNazol B. The cells start to lyse immediately and RNA was solubilized by passing the lysate up and down through a pipette.
- 2) Samples were transferred to Eppendorf tubes and 100µl of chloroform added to each. The tubes were shaken vigorously and stored at 4°C for 5 min.
- 3) The aqueous and phenol-based phases were separated by centrifugation at 13,000rpm for 15 min and the aqueous phase removed to a fresh eppendorf.
- 4) An equal volume of isopropanol was added and the samples stored at 4°C for 15 min or longer.
- 5) RNA was recovered by centrifugation at 13,000rpm for 15 min, followed by a 70% ethanol wash.
- 6) Samples were briefly air dried and resuspended in an appropriate volume of H₂O.

Agarose gels for RNA

Small aliquots of RNA were run on 0.8% agarose gels in exactly the same manner as DNA samples in order to visualise ribosomal bands and thus indicate the quality of the RNA preparation.

Formaldehyde gels for RNA

RNA was separated on formaldehyde gels as described by Sambrook *et al.* 1989 which were made as follows:

- 1) 1.95g of agarose was dissolved in 109ml of H₂O (for a 1% gel in a 150ml cast).
- 2) The agarose was cooled to 60°C and 15 ml 10x MOPS (Sigma) added.
- 3) 26ml formaldehyde solution (BDH) was added and the gel poured immediately (N.B. in a fume hood).

Samples were prepared as follows:

- 1) An equal volume of formamide sample buffer (appendix 1) was added to the RNA sample.
- 2) This was heated to 60°C for 5 min and snap cooled on ice.
- 3) 0.25 volume of Ficoll-dye-EDTA (FDE)(appendix 1) was added, and the sample mixed and loaded onto the gel immediately.

Gels were run overnight at 25 volts.

Northern blotting

Northern blots of formaldehyde gels were done as follows , as described by Seed (Seed, 1982), with the substitution of nylon membrane for nitrocellulose.

- 1) The gel was washed in 2x 500ml of H₂O for 15 min each to remove the formaldehyde.
- 2) The gel was then washed for 2x 10min in 10x SSC (appendix 1).
- 3) A piece of Genescreen (Du Pont) was cut to the exact dimensions of the gel, and this soaked first in H₂O, then in 10x SSC.
- 4) Step 3 was also done with 4 sheets of Whatmann 3mm paper.
- 5) The gel was sandwiched as shown in Fig.1 and 10x SSC used as the

reservoir buffer.

6) After blotting overnight the filter was allowed to air dry for at least 1 hour and then baked at 80°C for 2 hours.

Northern hybridization with labelled fragments

Filters were pre-hybridized overnight at 65°C in a Hybaid-maxi hybridization oven.

Pre-hybridization buffer:

Dextran-sulphate	2.5g
20x SSC	7.5ml
H ₂ O	to 20 ml

The buffer was vortexed hard to dissolve dextran sulphate and incubated at 65°C to get rid of bubbles.

0.25ml of sonicated single-stranded salmon sperm ^{DNA}(SSSSS, Stratagene) was added, which had been heated to 100°C for 5 min.

For hybridization, the pre-hybridization buffer was removed from the hybridization tube, the denatured probe added and the 20ml buffer replaced in the tube. Filters were hybridized overnight at 65°C, and washing steps performed as above for southern hybridizations with the exception that SSC was used instead of SSPE.

Developing of autoradiographs

All autorads were developed for 1 min with AGFA G150 developer and fixed for 1 min in AGFA G350 fixer at room temperature (RT), washed and air dried.

Techniques of manipulating phage and phagemids

Plating of phages

All manipulations with phage and phagemids were carried out as described by Sambrook et al., 1989. Phage were plated out into bacteria that contained an F' episome and thus were competent for the production of single stranded phage DNA. Routinely, *E. Coli* strain CMK (see Table 1) were used. Double-stranded phage DNA (see below) was transformed into competent cells by the same method as plasmids, except that no incubation at 37°C was required, but 200µl of a bacterial overnight ^{culture} and 3 ml of melted top agar (appendix 1) was added and the mixture quickly mixed and plated onto minimal plates (appendix 1). This gives rise to plaques or holes in the bacterial lawn that result from the production of single-stranded phage that replicate and lyse their host cells. The dilution that gave the best separation could then be used for picking single plaques.

Alternatively, if a stock of single stranded phage was available it was possible to streak this solution, onto the surface of a minimal plate, and then to pour onto this 3ml of top agar (appendix 1) to which 0.5ml of an appropriate bacterial culture had been added, pouring from the more dilute side of the streak to the more concentrated side. Plaques appeared after 8-10 hours of incubation at 37°C.

Small scale preparation of double stranded M13 DNA

- 1) A single plaque was picked with a sterile toothpick from a plate prepared as above into 3ml of an appropriate bacterial culture that had been grown to OD 0.3.
- 2) This was then grown up with vigorous shaking at 37°C for a further 4.5 hours.
- 3) 1ml aliquots were centrifuged at room temperature (RT) for 5 min and the supernatant removed and either kept at 4°C for use in single-stranded

preparations (see below) or titrated and kept at 4°C for use as a stock of single stranded phage.

4) The pellet was used to prepare the RF (replicative form ie. double stranded) form of the phage by the same method used for plasmid mini-preparations (Birnholm and Dolby, 1979) and above.

Large scale preparation of double stranded M13 DNA

1) A dilution of the phage was plated out as described above. Also a 3 ml overnight ^{culture} of an appropriate bacteria was grown up.

2) This overnight ^{culture} was used to inoculate 20 ml of L-broth and this was grown to OD 0.3.

3) Plaques were picked with a sterile toothpick and placed in 3ml aliquots of this culture. This was incubated with shaking for 6 hours at 37°C and then stored overnight at 4°C.

4) On this day a large overnight ^{culture} of appropriate bacteria (approx. 20ml) was set up.

5) This overnight ^{culture} was diluted 1/40 with fresh L-broth to a volume of 500ml in sterile conical flasks and grown to OD 0.3. 1ml of the six hour culture was inoculated into each 250ml flask and incubated at 37°C with shaking for 4.5 hours.

6) This culture was then taken off and prepared as a normal plasmid preparation. This method produces the "RF" form of the phage, which behaves exactly as an ordinary double-stranded plasmid.

Propagation of the helper phage M13K07

The helper phage M13K07 was propagated by plating out serial dilutions and then single plaques were picked into media containing kanamycin (70µg/ml). The resultant supernatant was collected and stored at 4°C.

Preparation of single stranded M13 DNA

- 1) An overnight ^{culture} of an appropriate bacterium was diluted with fresh L-broth and grown to an OD of 0.3 at 560nm wavelength.
- 2) This was divided into 3ml aliquots and a single plaque picked into each. The tubes were incubated with vigorous shaking at 37°C for 4.5 hours exactly as for the mini-RF prep.
- 3) 0.8ml of the supernatant was carefully removed and placed in a fresh eppendorf ^{tube} where it could be stored at 4°C overnight if necessary. A second centrifugation of 5 min was usually performed to ensure that all of the bacterial pellet had been removed.
- 4) 200µl of 20% polyethyleneglycol (PEG 6000, BDH) was added and the mixture allowed to stand at RT for 30 min.
- 5) It was then centrifuged in a microfuge for 5 min.
- 6) The supernatant was taken off, with a second brief ^{centrifugation} being used to ensure that all the PEG 6000 was removed. The pellet was resuspended in 100µl of TE (appendix 1).
- 7) 50µl of phenol was added and the mixture vortexed for 10 min, then allowed to stand at RT for 10 min, after which the 10 min vortex was repeated.
- 8) After a 1 min ^{centrifugation} in a microfuge the aqueous layer was removed to a fresh tube and the single stranded DNA precipitated by the addition of 10µl of 3M sodium acetate pH 5 and 250µl of ethanol, followed by storage at -70°C for 5 min and centrifuging in a microfuge for 10 min. The pellet was washed once with 1ml of ethanol and then air dried.
- 9) The DNA was resuspended in 50µl of TE and stored at -20°C.
- 10) Single stranded DNA was visualized by the addition of 1µl of 2% SDS to 5µl or 10µl aliquots and then running on 0.8% agarose gels as used for double stranded DNA.

Production of single stranded DNA from phagmids

This was carried out according to the methods of Vieira and Messing, 1987.

- 1) A fresh bacterial colony containing the phagmid was suspended in 3ml of L-broth containing Amp in a 15ml sterile tube.
- 2) The helper phage M13KO7 was added to a final concentration of 2×10^7 pfu/ml (plaque forming units), and the culture incubated with shaking at 37°C for 1.5 hours.
- 3) Kanamycin was then added to a final concentration of 70µg/ml and the incubation at 37°C allowed to continue overnight.
- 4) 1ml aliquots of this culture were centrifuged and the single stranded prep. performed as for ordinary phages.

Analysing insert orientations in M13

If different M13 DNAs contain identical DNA sequences inserted in opposite orientations they are able to hybridize to each other, forming a DNA structure with a higher molecular weight than either of the parental DNAs (Howarth et al., 1981). This larger molecule can be recognised by agarose gel electrophoresis.

- 1) The phage were grown up in a suitable bacteria as described above for 6.5 hours.
- 2) 1ml aliquots were taken and centrifuged for 5 min.
- 3) 20µl of the supernatant was taken and to this 1µl of 1% SDS and 3µl of agarose loading buffer (appendix 1) were added .
- 4) This was incubated at 65°C for 1 hour.
- 5) Samples were loaded onto gels and run as for normal agarose gels.

Analysis of DNA

Sequencing

All sequencing was done using the method of Sanger, 1977 and the United States Biochemical (USB) Sequenase version 2.0 kit and using single stranded templates prepared either from M13 or from phagmids as described above. The buffers for the sequencing reaction were all supplied by USB. Gels were run on a Life Technologies sequencing tank, model SA.

Preparation of glass plates for pouring of polyacrylamide gels:

Plates were washed in H₂O and methanol and dried. In earlier experiments gels were dried directly onto the glass plates, whilst in later experiments the gels were removed from the plates and dried onto 3mm Whatmann paper. In the first case one plate was coated with two layers of a solution of Bind-silane (Pharmacia) (appendix 1), a silica based substance which causes the gel to adhere to the glass plate, whilst the other plate was treated with Repel-silane (Pharmacia), which causes the plate to repel the gel and facilitates separation of the plates without damage to the gel. These operations were carried out in a fume hood, the plates allowed to dry and were then wiped over once with a damp tissue. For gels that were to be transferred to paper the Bind-silane step was omitted. 1mm spacers and a sharks-tooth comb were used and the edges of the plates sealed with PVC tape.

Polyacrylamide gels:

7% or 8% gels were prepared from a 40% stock solution as described in appendix 1, poured into the assembled plates taking care to avoid the formation of bubbles and allowed to polymerize for at least 1 but usually 2-3 hours at RT. Excess polyacrylamide was removed from the comb bed and the gel warmed by running it for 30-40 min at 2000V before loading the

samples. TBE (appendix 1) was used as a running buffer and gels normally run at 2000V. Gels were run for 1.5-3 hours depending on how close or far from the primer the required sequence lay.

Sequencing reaction:

One tenth of a single stranded (ss) DNA preparation, as described above, in a volume of 5 μ l was used in each sequencing reaction. Sequencing oligonucleotides were at least 12 and usually 15 or more nucleotides long and were commercially produced on an Applied Biosystems Oligonucleotide Synthesizer (Oswell DNA Services, Dept. of Chemistry, Edinburgh University). The components of the annealing step are listed below.

SS DNA	5 μ l
Primer	1 μ l (2pmole)
5 x sequencing buffer	2 μ l
H ₂ O	2 μ l

Reactions were heated to 65°C in a hot block for 3 min and then removed from the heat and cooled slowly to less than 35°C. After this the tubes were stored on ice and used within 4 hours.

While the annealing reaction was cooling each of the four dideoxynucleotidetriphosphates (ddNTPs), A, C, G, and T were aliquotted at 2.5 μ l in colour coded tubes. The labelling mix was diluted 1:10 in sterile H₂O and the Sequenase enzyme diluted 1:8 in the buffer provided. The labelling reaction was assembled as described below.

template/primer	10 μ l
0.1 M dithiothreitol (DTT)	1 μ l
diluted labelling mix	2 μ l
³⁵ S dATP (Amersham)	0.5 μ l
diluted Sequenase	2 μ l

When sequence very close to the primer was required 1 μ l of the manganese buffer supplied by the manufacturer was also included at this stage. The above components were mixed and incubated at RT for 2 min exactly. Immediately the 2 min incubation was finished 3.5 μ l aliquots of the reaction were added to each of the four previously prepared ddNTP tubes, mixed and incubated at 37°C for a further 5 min. (ddNTPs were stored as aliquots at -20°C and thawed and warmed to 37°C for at least 1 min prior to use.) This termination reaction was stopped by the addition of 4 μ l of loading buffer (USB) to each tube, mixing and storing on ice until use. Before loading the samples were heated to 85°C for 5 min.

Drying the gels:

After the gel had run for the required length of time the plates were removed from the electrophoresis apparatus and carefully levered apart. The gel, still attached to one plate, was then washed gently in 10% acetic acid for 20 min to remove the urea. In the experiments where Bind-silane was used the gel was then dried directly onto the plate by baking it in an 80°C oven for 20 min. When Bind-silane was not used the gel was carefully transferred to 3mm Whatmann paper and dried on a vacuum slab gel drier (Hoeffer Scientific) for 30 min at 80°C. Dried gels were exposed to Kodak X-OMAT film overnight at RT.

Manipulation of proteins

SDS-page electrophoresis

Proteins generated using *in vitro* translation (Chp. 4) or isolated from tissue culture cells (Chp.5) were examined for size heterogeneity by SDS polyacrylamide gel electrophoresis (SDS-page) (Laemmli, 1970). A Bio-Rad "Protean" mini-gel system was used throughout. Plates were routinely

cleaned in chromic acid to ensure removal of any contaminating proteins, and then rinsed thoroughly in H₂O and finally washed with methanol. 15% slab gels, with 7 or 8% stacking gels were generally used and the ingredients for their formulation are given below:

	<u>15% Separating gel</u>	<u>7% Stacking gel</u>
H ₂ O	2.7ml	2ml
30% acrylamide	5.55ml	600 μ l
Gel buffer	2.85ml	888 μ l
10% APS	168 μ l	56 μ l
Temed	7.5 μ l	10 μ l

These were mixed and poured immediately.

APS = ammonium persulphate

The 30% acrylamide solution was prepared by adding:

Acrylamide	30g	
Bisacrylamide	0.8g,	H ₂ O to 100ml.

Buffer compositions are listed in Appendix 1.

Before loading samples were diluted 1:5 in final sample buffer (FSB, appendix 1) and heated to 90°C to denature the proteins. A Tris-glycine running buffer was used (appendix 1) and gels run at 200 volts for 45-50 min or until the dye-front had run off the gel.

Gel drying

Gels bearing radiolabelled proteins were dried onto 3mm Whatmann paper on a vacuum slab gel drier (Hoffmann Scientific) for 2 hours and exposed to X-OMAT X-ray film (Kodak) at RT for varying periods of time.

Some of these gels were first fixed and treated with the fluorographic

reagent Amplify (Amersham) according to the manufacturers instructions, and consequently exposed at -70°C instead of RT.

Immunoblotting

Gels that did not bear radiolabelled proteins were transferred to nitrocellulose paper and probed with anti PrP primary and gold conjugated secondary antibodies as described below.

Transfer

1) 2x6 pieces of 3mm Whatmann paper and 1 piece of nitrocellulose were cut to the exact size of the gel and briefly soaked in semi-dry blotting buffer (appendix 1).

2) These were loaded onto a semi-dry transfer cell (Bio-Rad) in this order:

6x Whatmann paper

Nitrocellulose

Gel

6x Whatmann paper

taking care to exclude all air bubbles.

3) A current of 2mA /cm² was passed through the cell for 1 hour.

4) The cell was unloaded and the filter allowed to dry.

Blotting

All steps were performed at RT.

1) Filters were washed in blocking solution with shaking for 2 hours + .

Blocking solution: Bovine Serum Albumin (BSA) 0.5g
Natural Goat Serum (NGS) 0.5ml
PBST (appendix 1) to 25ml

2) The blocking solution was poured off and primary (1°) antibody solution (Farquhar *et al.* 1989) added.

1° antibody solution: Antibody IB3 (1/1000) 25µl



PBST to 25ml.

This was allowed to wash at RT with shaking for at least 2 hours or overnight.

3) Filters were washed 4 x 5 min with PBST.

4) 2° antibody solution was added and allowed to wash with shaking for at least 2 hours.

2° antibody solution: Antibody (Biocell)	100µl
Gelatin (Cambio)	250µl
NGS	100µl
PBST	to 25ml.

5) Filters were washed 2 x 5 min with PBST and 2 x 15 sec with H₂O.

6) Silver enhancement of the 2° antibody was performed according to the manufacturers instructions (Biocell). 7ml initiator and 7ml enhancer were mixed and the filter washed in this for 30-40 min. This step was repeated after a brief water wash if necessary.

8) The gel was washed in 2 X 50ml H₂O for 30 min dried and photographed.

CHAPTER THREE Site-directed Mutagenesis

Introduction

As described in Chp.1, for various different GPI-anchored proteins the removal of the C-terminal signal sequence prevents attachment of the GPI and the proteins are completely translocated across the membrane (Berger et al., 1988; Bailey et al., 1989). Experiments with PLAP and DAF have found that a minimum of thirteen hydrophobic amino acids left at the C-terminus were necessary for GPI attachment (Berger et al., 1988; Caras and Weddell, 1989). Therefore mutated PrP genes were designed which contained early stop mutations which should give rise to truncated proteins in which varying numbers of the C-terminal hydrophobic amino acids were removed (Fig. 4). Whether these mutations also prevented GPI-addition to the PrP protein was investigated in in vitro systems.

Oligonucleotide-mediated site-directed mutagenesis

Oligonucleotide-mediated site-directed mutagenesis was chosen as the method to make these mutations because it can be used to introduce specific alterations in the DNA to the level of one base pair and thus alter individual codons in the DNA coding sequence (Sambrook et al., 1989). Two variations of the method were used, where in each case the mutated oligo is used as a template to amplify the DNA using different mechanisms to achieve this amplification. The first of these is the double primer method, which is an adaptation of the original method of Hutchinson et al. (Hutchinson et al., 1978) which uses amplification in filamentous phage. The second involves the use of mutagenic oligonucleotides (oligos) and the polymerase chain reaction (PCR). (Saiki et al., 1985; Mullis and Faloona, 1987) to amplify the mutated DNA.

Table 1: Oligonucleotides used in SDM

Oligo.no.	Sequence
968	GCA CAA GAA CTG GGG CAG GAG AAA CAG
162	CTC CTC CCC TCC TG A*TC ATC CTC CTC ATC
063	TTA CGA CGG GAG ATA G*GA TCC AGC AGC ACC
902	AAT GCT GCC CTC TAT C*CT AGG TCG TCG TGG
873	CAT G*AA TCC TGC AGA TCA GTC ATC <u>ATG</u> GCG A
874	GCA CCC <u>TAC TCC</u> CTC CGG AAG_AGA T*CT CGA

Bases in bold represent insertions into the normal PrP sequence.

In oligo 873 the start codon is underlined, and the position of the EcoR1 site marked with an asterix.

In oligo 874 the normal PrP stop codon is underlined and the position of the Xba1 site marked with an asterix.

Oligos 063 and 902 are from the same position on opposite strands of DNA for the PCR site-directed mutagenesis and the asterix represents the position of the engineered BamH1 restriction site.

In oligo 162 the asterix represents the position of the engineered Bcl1 restriction site.

Figure 4.

PREDICTED C-TERMINUS AMINO ACID SEQUENCE FOR
PrP PROTEIN PRODUCED FROM WILD-TYPE AND MUTANT DNA

221 SQAYYDGRRS S⁺⁺⁺STVLFSSPP⁺⁺ VILLISFLIF⁺⁺⁺⁺ LIVG⁺⁺⁺ pSKPr251

221 SQAYYDGRRS S⁺⁺⁺STVLFSSPP⁺⁺ V pSKPr241

221 SQAYYDGR pSKPr228

+ = hydrophobic amino acid

S

= putative GPI attachment point

_____ = GPI attachment signal sequence

Design of oligonucleotides

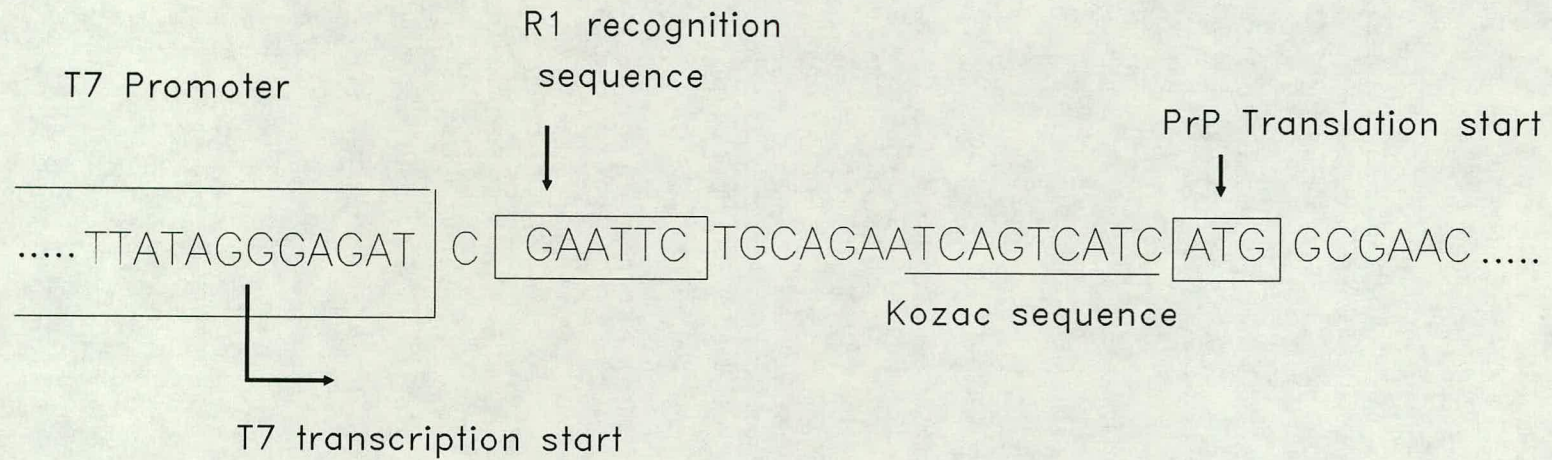
Careful design of the mutagenic oligonucleotides can greatly increase the frequency of positive mutants obtained. Firstly the oligo must be made from the strand complementary to that produced by the single stranded (ss) phage. It must be sufficiently long to anneal, preferably 25 or more nucleotides in length and be free from palindromic sequences. Also the oligo should be tested for homology to non specific sequences in the vector to avoid spurious priming. The substitution or insertion should be placed as close as possible to the centre of the oligo leaving the flanking arms perfectly matched.

Two such oligos were designed, incorporating these features, which introduced early stop codons into the PrP gene sequence (Table.1). An additional feature that these oligos incorporated was that they introduced a unique restriction site into the DNA which could subsequently be used to identify phage carrying the mutation (Fig.5). The first mutation (stop 241) introduces two mismatches, inserts a BclI site and should result in the removal of eleven hydrophobic amino acids. The second (stop 228) introduces two mismatches, inserts a BamHI site and should remove all seventeen hydrophobic amino acids. This should also remove the putative site of attachment (Ser 231) for the GPI anchor. In this case the mutated protein should be truncated at the same position as the 15% of PrP^{Sc} identified by Stahl et al (Stahl et al., 1990a).

Flanking oligos for use in the 2^o PCR reaction, (oligos 873 and 874), were at least 30mers, containing 21 and 22 bases of homology with either end of the PrP ORF, and 9 non-homologous bases which contained a unique restriction site at their ends (Table.1). The 5' oligo, (873), was designed to conserve the sequence immediately 5' to the start codon as this contains the consensus sequence for the correct initiation of translation (Kozac, 1983) (Fig.6).

The sequences of the mutant oligonucleotides are listed in Table 1.

pSVK3/PrP Junction Figure 6



"Double primer" method

This involves the use of filamentous phage vector. The fragment to be altered is cloned into M13 or one of its modified derivatives (M13mp18/19, Gibco-BRL) and single-stranded DNA templates are then prepared to which an oligonucleotide containing the desired mutation can be annealed. The 5' end of the oligonucleotide is phosphorylated to prevent its displacement by the 5'-3' exonuclease activity of the Klenow fragment and a second, non-phosphorylated universal primer, is also annealed to the template. The Klenow fragment of DNA polymerase 1 and T4 ligase are then used to complete the double stranded DNA molecule. This is then transfected into a repair negative (Dam-) strain of *E. Coli*. (to avoid correction of the mutation by the bacterium's repair mechanisms), where the mutated strand is used as a template for the production of ss phage particles containing the mutation. The strain of *E. Coli* used is called Mut-L (Chp.2, Table 1). The resulting plaques are then screened for the presence of the mutation using the mutated oligonucleotide as a probe in a differential hybridization experiment. Once a potential mutant has been selected it must first be sequenced in its entirety to ensure that no spurious mutations have been inserted, whereupon it may ^{be} reinserted into its original position in the gene of interest. The experimental procedure is outlined below.

1) A 3.6Kb fragment of the PrP gene from the NZW strain of mouse (Westaway et al., 1987) was cloned into the Xba1 site in the multicloning region of the vector M13mp18 (BRL) and the subsequent clone called Mp18NX3.6.

1) The recombinant phage was grown in an *E. Coli* strain that contains an F' epitope, (M13 infect via the sex pili), *E. Coli* NM522 and CMK were used (Chp.2).

2) High quality ss DNA was prepared from the above as described in Chp.2.

3) The mutagenic oligo was synthesized by Oswell DNA Services at Edinburgh Univerisity on an Applied Biosystems synthesis machine and

HPLC purified.

4) The mutagenic oligo was phosphorylated with T4 polynucleotide kinase:

Mutagenic oligo	200pmole
10xT4 kinase buffer	2 μ l
10mM ATP	1 μ l
T4 kinase	4units
H ₂ O	to 20 μ l

Incubate 37°C, 1 hour.

Heat to 68°C, 10 min to stop enzyme.

5) Mutagenic and universal primers were annealed to ss recombinant template:

universal primer (17mer)	10pmole
ss template	0.5pmole
phosphorylated mutant oligo	10pmole
10xPE1 buffer (see below)	1 μ l
H ₂ O	to 10 μ l

Mix.

Heat to 20°C >T_m, (T_m=4[G + C] + 2[A + T]), in this case 62°C, 5 min.

T_m=dissociation temperature

Allow to cool slowly to RT.

6) Whilst this was cooling PE3 mixture was prepared:

10XPE2 buffer (see below)	1 μ l
dNTPs 2mM	1 μ l
10mM ATP	1 μ l
T4 ligase	5units
Klenow fragment	2.5 units
H ₂ O	to 10 μ l

This was stored on ice until needed.

7) 10 μ l of PE3 was added to reaction mixture (step 5) and incubated at

16°C, 6-15 hours.

8) Serial dilutions of the reaction mix were made and transfected into Dam⁻ *E. coli*

<u>10xPE1 buffer</u>		<u>10xPE2 buffer</u>	
Tris.HCl pH7.5	200mM	Tris.HCl pH7.5	200mM
MgCl ₂	100mM	MgCl ₂	100mM
NaCl	50mM	DTT	100mM
DTT	10mM		

Screening plaques for positive mutants by differential hybridization.

At an appropriate dilution the reaction mix from above gave rise to well spread out plaques that could be picked without cross-contamination. To screen these plaques for the presence of the mutation the method of differential hybridization was used, where the mutant oligonucleotide was used as a probe. Small synthetic oligonucleotides which are hybridized to a piece of complementary DNA will dissociate over a sharp range as the temperature increases (Wallace et al., 1980; Suggs et al., 1981). The dissociation temperature T_d (°C) of a perfectly matched duplex between a synthetic oligonucleotide up to 20 bases long and single stranded template can be calculated from the equation:

$$T_d = [2x \text{ number of A,T base pairs}] + [4x \text{ number of G,C base pairs}].$$

The T_d for the mismatched duplex will be less than that for the perfectly matched duplex. When the filter is washed at close to the calculated T_d the probe should selectively dissociate from the wild type DNA. The temperature of the wash had to be raised a few degrees higher than the calculated T_d for oligonucleotides greater than 20 bases.

Colonies that appeared positive from hybridization were picked and grown up for DNA extraction and the DNA was analysed using the enzyme for which the unique site had been engineered.

Results of "double priming" experiments

Stop 241 mutation:

Differential hybridization gave rise to two potential mutants and these were tested for the presence of the mutation by both restriction with Bcl1 and sequencing of the mutation site. Bcl1 will only recognise DNA that is unmethylated, and thus it was necessary to first transfect the phage into a methylase negative strain of *E.Coli* (GM242 Chp.2, Table 1) and prepare plasmid DNA from these bacteria. When this was done one of these clones appeared to contain the mutation and the other did not. This result was confirmed by sequencing the region of the mutation for both clones (Fig.7) and the ORF of the one containing the correct mutation was subsequently sequenced in its entirety. No spurious mutations were located. As the NZW PrP mouse gene has no suitable sites for cloning flanking its ORF it was necessary to use the polymerase chain reaction (PCR) to add restriction sites to the 5' and 3' ends before cloning into an expression vector was possible. This process is described in detail in the next section.

Stop 228 mutation:

Although the second mutation, stop 228, repeatedly gave rise to what looked like positive mutants in different experiments, on subsequent digestion with BamH1 they did not contain the unique restriction site that the mutation incorporates. This was confirmed by sequencing the site of the mutation in these possible mutants (Chp.2), which all proved to resemble the wild-type. As repeated efforts using this strategy did not produce the stop 228 mutant a different strategy involving PCR was undertaken.

Oligonucleotide-mediated site-directed mutagenesis by PCR

PCR (Saiki et al., 1985; Mullis and Faloona, 1987) is a process whereby two

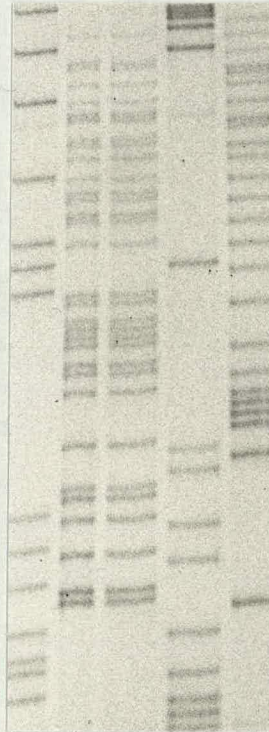
Figure 7a. The presence of specific mutations is confirmed by sequence data.

Wild-type (pSVPr251)

Bcl1 mutant (pSVPr241)

T G C A

A C C G T



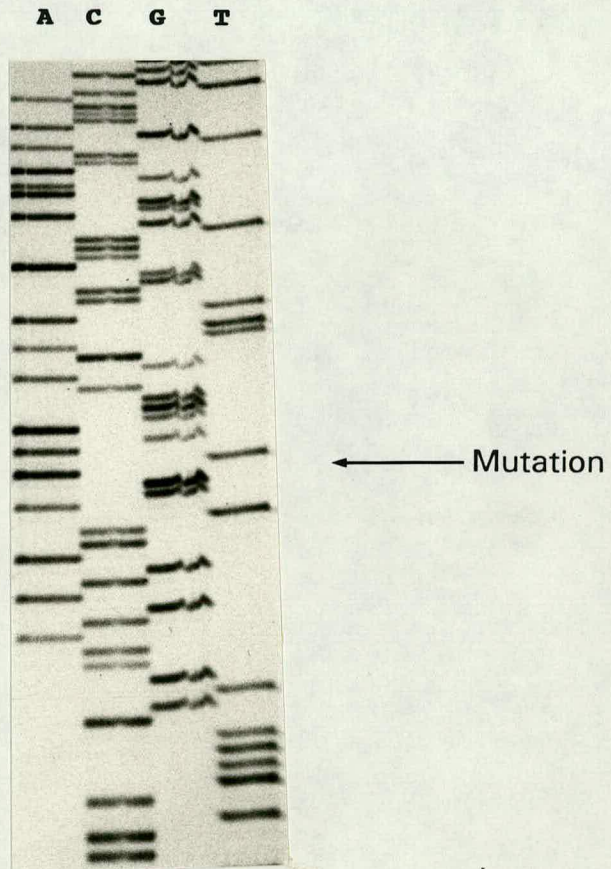
← Mutation

Unmutated sequence: C CCC TCC TGT CAT CCT CCT CAT C

Mutated sequence: C CCC TCC ATG ATC ATC CTC CTC A

Figure 7b. The presence of specific mutations is confirmed by sequence data.

BamH1 mutant (pSVPr228)



Unmutated sequence: **A CGA CGG GAG AAG ATC CAG CAG**

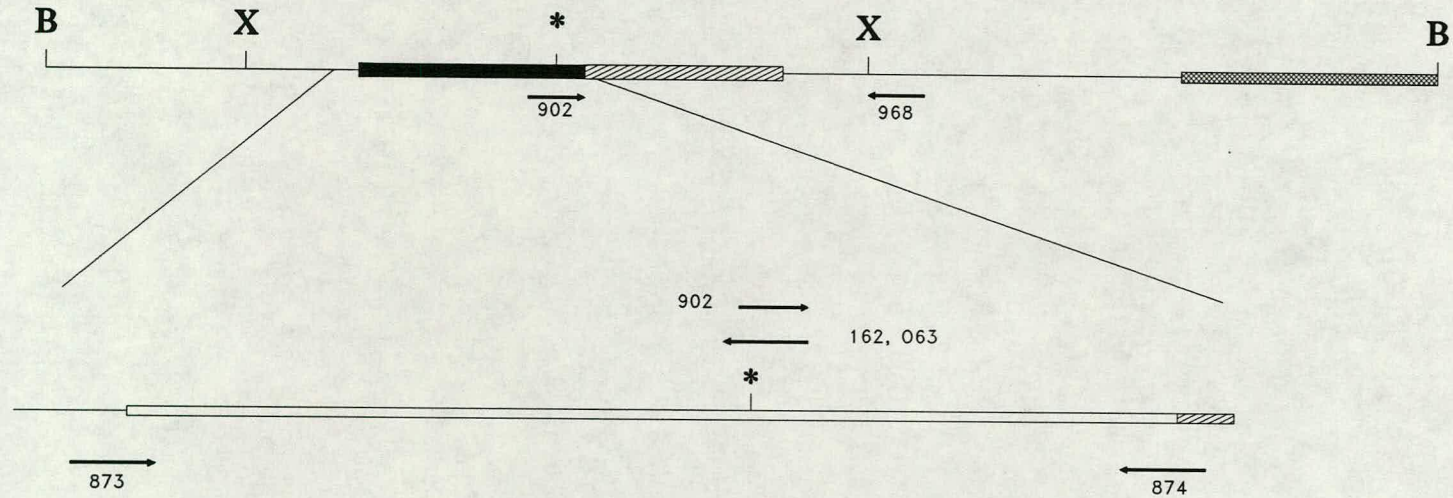
Mutated sequence: **A CGA CGG GAG ATA GGA TCC AGC**

synthetic oligos are used as primers to amplify a sequence of interest. The primers anneal at either end of the sequence of interest, one to each strand, and multiple rounds of 3' extension by DNA polymerase, denaturation and reannealing is used to achieve amplification. It is possible to incorporate changes into the primers that will then be amplified giving rise to a mutated fragment of DNA. The limitation of this strategy has been that such mutations can only be placed at the ends of the amplified fragment, as this is where the primers are. This problem has now been overcome by the use of more than one round of PCR involving two different sets of oligos in a process termed overlap extension (Higuchi et al., 1988; Ho et al., 1989; Horton et al., 1989).

Oligos containing the desired mutation are synthesised to opposite strands of the target DNA (Fig.8), and two separate PCR reactions are performed, the double stranded product of each having the desired mutation at one end. These two products are then used as the template for a further round of PCR, using two unmutated flanking oligos as primers (873, 874, Table 1). On denaturation and reannealing the mutated ends will bind as they are complementary and extension will continue until a full length fragment is created between the two flanking primers. Thus the mutation can be placed in the centre of a clonable fragment of DNA which can then be reinserted into the gene of interest.

In the mouse PrP gene the restriction sites at the 5' and 3' ends of the ORF are unsuitable for cloning and it is difficult to isolate a fragment of the gene that contains just the ORF (Locht et al., 1986). For this reason it was decided to use the secondary PCR reaction to introduce useful restriction sites at the 5' and 3' ends of the ORF to facilitate its insertion into an expression vector. This was done by adding these sites onto the ends of otherwise homologous primers flanking the ORF (Table 1). Thus the final PCR product consists of an 850bp PrP fragment containing the 228 stop mutation and having an EcoR1 site at its 5' end and an Xba1 site at its 3'

Figure 8



- * = site of mutation
 - 063, 902 = BamH1
 - 162 = Bcl1
 - ORF = ORF
 - 3' non-coding region = 3' non-coding region
 - 503 = EcoR1
 - pUC18 = pUC18
 - 874 = Xba1
 - intron = intron
- oligos are numbered in order of production

end which can be cloned directly into an expression vector. The oligos were designed to leave intact the nine bases 5' of the start site as these encode the consensus sequence (Kozac 1983) for efficient initiation of translation (Fig.6). The fragment was sequenced to ensure that no spurious mutations were introduced by the PCR reaction.

Experimental procedure

Primary PCR was performed on Mp18NX3.6 which had been linearized with Sal1, phenol/chloroform extracted and ethanol precipitated and resuspended in TE (Chp.2). Each reaction contained:

Template DNA	200pg
Oligos	400ng(of each)
10x Cetus PCR buffer	5 μ l
10mM dNTPs	4 μ l
H ₂ O	to 50 μ l
Taq.polymerase(Cetus)	0.5 μ l

Components were added in the order listed, mixed, spun briefly and overlaid with a couple of drops of mineral oil to prevent evaporation. Controls included a reaction with no DNA and a reaction with no enzyme. The cycling parameters used were:

Denaturation	94°C, 2min.
Annealing	60°C, 2min.
Extension	71°C, 3min.

30 cycles of amplification were performed and a Techne thermocycler was used throughout.

Products were examined by agarose gel electrophoresis (Chp.2).

Results of PCR mutagenesis experiments

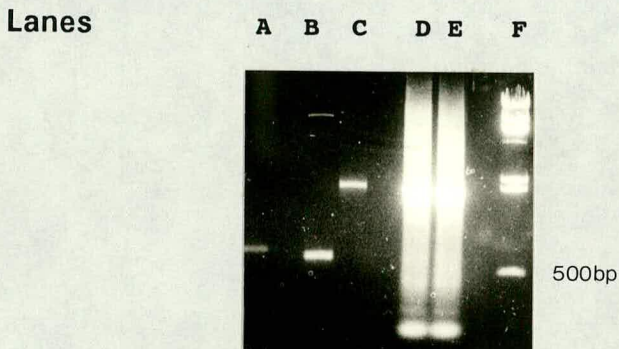
The primary (1°) round of PCR gave rise to two products which corresponded to the predicted sizes (Fig.9). Oligo 968 was used in this 1° round in preference to oligo 874 as the distance between the site of the mutation and the end of the ORF is too small to yield a retrievable fragment in this instance (Fig.8). Thus the expected sizes were 2.2Kb for the 968/063 reaction and 700bp for the 902/873 reaction (oligo 902 is exactly complementary to oligo 063) (Fig.9). The 2.2Kb reaction did not work every time, giving either the correct sized band or no band at all. This may be due to a number of causes, including limitation of components or a difference in the ideal parameters for a fragment of this length. However, each fragment was isolated from agarose gels and together used as the template for the secondary (2°) PCR reaction. For this reaction oligos 873 and 874, which respectively added the EcoR1 site to the 5' end and the Xba1 site to the 3' end of the ORF, were used, giving rise to an 850bp fragment. This fragment should contain a unique BamH1 site near to its 3' end if the mutation has been incorporated. A number of fragments were obtained which did not contain this site and which resembled the wild type. This was explained by examining the isolated fragments used as template in the 2° reaction. One of these was found to contain some plasmid contamination ie. traces of the unmutated plasmid template used for the 1° reaction which was being used as a template instead of the mutated fragment. Subsequent re-isolation of the mutated fragment meant that the 2° reaction proceeded as planned and a fragment containing the 228 stop mutation and BamH1 site was obtained.

PCR addition of restriction sites

As described above PCR was used to add unique restriction sites to the ends of the 2° PCR product of the mutagenesis experiment. Exactly the same process and the same oligos (873 and 874) were used to add restriction

sites to the ends of the ORF of the 241 mutation obtained from the double primer method and also to the wild type PrP ORF, so that this could be used as a comparative control. All PCR fragments were hybridized with a probe containing the coding region of PrP to verify their authenticity according to the methods described in Chp.2.

Figure 9. Agarose gel electrophoresis of 1° and 2° PCR fragments.



Two rounds of PCR were performed to introduce the BamH1 mutation into the PrP ORF and at the same time engineer suitable restriction sites for cloning onto the ends of the fragment. The first round(1°) produced two fragments, one of 2Kb and one of 700bp, each having the mutation at one end. The second round used these fragments as a template to produce one 850bp fragment with the mutation at its centre and EcoR1 and Xba1 sites at its 5' and 3' ends respectively. 2° PCR 850bp fragment (Lane A), 1° PCR 700bp fragment (LaneB), 1° PCR 2KB fragment (Lane C), 1° PCR 2KB fragment (Lane D,E-before gel isolation), λ Hind 111 size markers.

Cloning of PCR fragments into expression vector

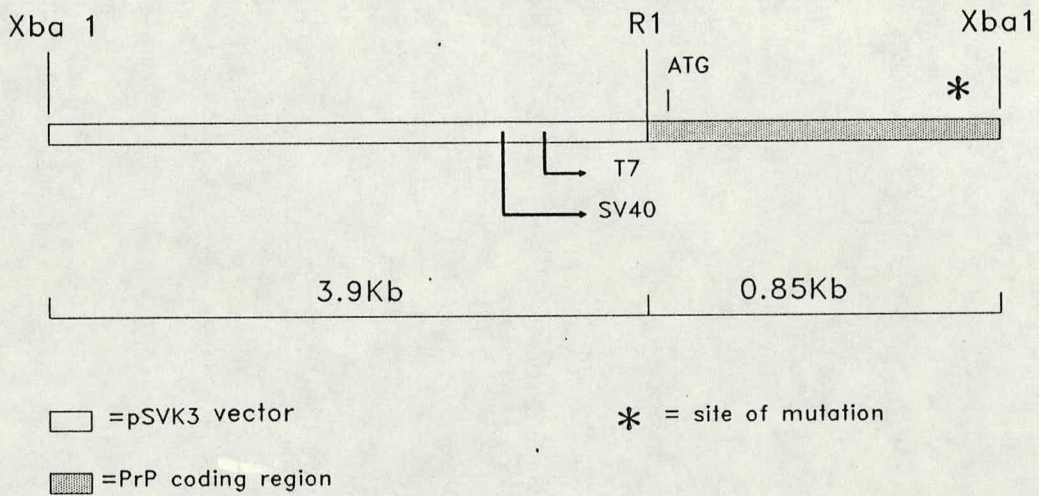
In vitro transcription involves combining all the necessary components for correct RNA processing. This is usually under the control of a very strong promoter, often a bacteriophage promoter, ie.T7, SP6. These are particularly useful as bacteriophage RNA polymerase is extremely specific

for its own promoter sequence and thus will give no detectable transcription from other prokaryotic or eukaryotic promoters; it is extremely strand specific and does not tend to initiate or terminate at nicks in double stranded DNA template molecules (Kreig and Melton, 1987). A splice site and polyadenylation site must be provided in the vector for correct processing of mammalian RNA. *In vitro* transcripts of protein coding sequences can act as functional mRNAs with a stability and translational activity equivalent to that of natural mRNAs (Kreig and Melton, 1987). Thus *in vitro* transcription can be used to examine the mRNAs produced by the mutated PrP genes and compare them to the mRNA of the unmutated gene transcribed in the same system.

The expression vector that was chosen is a member of the pSV family called pSVK3 (Mongkolsuk, 1988). This vector is a phagemid, having an origin of replication for F1 and can therefore be used to produce ss as well as ds DNA. It contains the T7 promoter upstream of a comprehensive multiple cloning site (MCS), the SV40 early polyadenylation site and the SV40 small T antigen splice sites. For transient expression it has the SV40 early promoter. Thus once the DNA of interest is cloned into the multiple cloning site a single vector can be used for *in vitro* transcription/translation experiments, transient expression and sequencing.

Two PCR fragments containing the correct mutations and one containing the wild-type with convenient EcoR1/Xba1 restriction sites at their ends were produced as described above. These were cloned into the MCS of pSVK3 to give rise to the clones pSVPr241 ,pSVPr228 and pSVPr251 respectively (Fig.10).

pSKPr228/241



pSKPr251

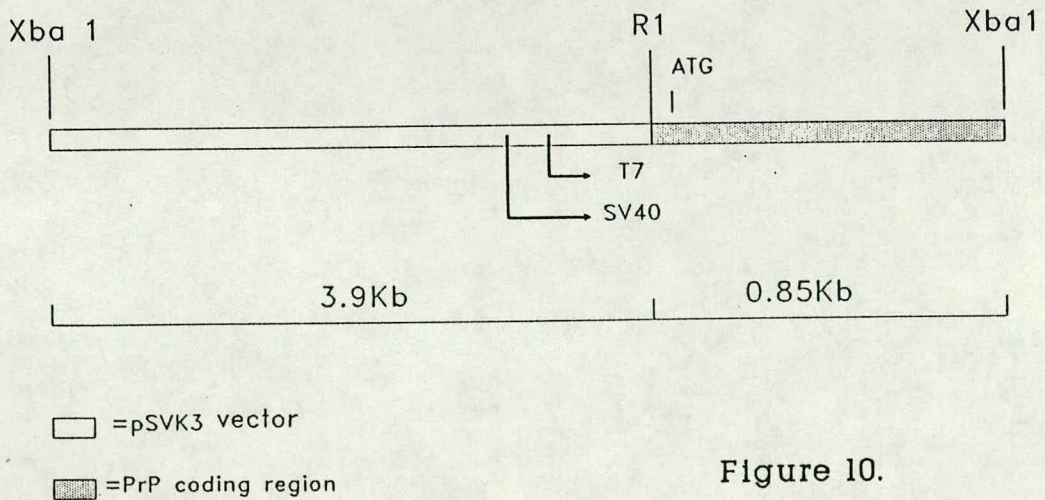


Figure 10.

Verification and sequencing of PCR products

Both mutations underwent a round of PCR to facilitate cloning as described above. Therefore it was necessary to sequence the entire open reading frame of both to check for spurious mutations. As this was now contained in a phagemid it was possible to make ss DNA and perform the sequencing according to established methods (Chp.2). Both mutant clones contained the intended codon changes in their C-termini (Fig.7). pSVPr241 contained no other changes in its ORF. Similarly, the wild-type clone contained no mutations in its ORF. However, on sequencing, an additional mutation was found in pSVPr228.

This was a G to A change at base pair 510, which leads to a Arginine to Lysine change at codon 135. As both of these are basic amino acids and an initial transcription/translation experiment detected no difference at the protein level between this and the product of the other mutant (see Chp.4), due to constraints of time it was decided to continue using this clone instead of repeating the mutagenesis and cloning stages.

CHAPTER 4: Cell-Free Transcription and Translation of mutants

Introduction

We owe much of what we know about the topogenesis of membrane bound and secretory proteins to the use of cell-free and whole-cell systems to study simple model polypeptides (Lingappa, 1991a). Such systems provide a much simplified environment in which to observe the fate of polypeptides of a specific sequence.

The Rabbit Reticulocyte Lysate System

Rabbit reticulolysate (RRL) is prepared from New Zealand white rabbits injected with phenylhydrazine (Pelham and Jackson, 1976). The reticulocytes are purified to remove contaminating cells and then lysed. The lysate is treated with micrococcal nuclease to destroy endogenous mRNA and reduce background translation to a minimum. The lysate contains the cellular components necessary for protein synthesis: tRNA, rRNA, amino acids and initiation, elongation and termination factors. Further optimization of the system has been achieved by the addition of i) hemin to prevent inhibition of initiation, ii) phosphocreatine kinase and phosphocreatine as an energy generating system, iii) potassium acetate and magnesium acetate at levels recommended for the translation of most mRNAs and iv) a mixture of tRNAs to expand the range of mRNAs that can be translated.

Processing events such as signal peptide cleavage, core glycosylation and GPI addition can be examined by adding canine microsomal membranes (DPMs) to a standard translation reaction.

The RRL system has been used previously to produce recombinant PrP (Hay et al., 1987a, b), and has more recently been shown to possess all the necessary biosynthetic machinery for GPI addition (Fasel et al., 1989). This system enabled us to characterise the mutant protein in the absence of any

endogenous background protein. As discussed previously, truncation mutants of normally GPI-anchored proteins, in which the COOH-terminal signal peptide has been deleted are predicted to be secreted, and this has been shown for several proteins (Berger et al., 1988; Bailey et al., 1989).

The two truncation mutations of the PrP gene, which have the GPI addition signal sequence deleted, as well as the full-length wild type gene have been cloned into an expression vector. This vector, pSVK3, contains the bacteriophage T7 promoter directly behind a multiple cloning site, and by the *in vitro* addition of T7 RNA polymerase, ribonucleotides and a capping analogue can be used to produce mature mRNA. This mRNA can then be used in the rabbit reticulolysate (RRL) system, in the presence of DPMs, to produce fully processed proteins *in vitro*.

***In vitro* transcription:-**

Preparation of DNA templates:

CsCl banded DNA (see chp.2) was used to produce linear template DNA by digesting 10 μ g overnight in the presence of excess Xho1 restriction enzyme (40 units). This enzyme produces a 5' overhang as recommended for run-off RNA transcription. Aliquots of the reaction were run on agarose gels to ensure that restriction had gone to completion. The remainder of the reaction was phenol/chloroform extracted and ethanol precipitated, followed by two washes with 70% ethanol. The DNA was resuspended in RNase free water (DEPC treatment, appendix 1) at a concentration of 1 μ g/ml.

Transcription reaction:

In vitro transcription was carried out using T7 RNA polymerase as outlined below. Reaction constituents were added in the order listed below to prevent the possible precipitation of DNA by the spermidine in the buffer.

T7 RNA polymerase buffer x1 (BRL and Promega),

10mM DTT,
50u RNAsin (Promega),
0.5mM ATP, CTP, UTP, 0.05mM GTP,
0.5 mM m⁷G^(5')ppp^(5') (Pharmacia),
5μg linearized DNA,
40u T7 polymerase.

The mixture was incubated for two hours at 40°C .

After 30 min 1μl of 8mM GTP was added.

After 1 hour an additional 1μl of T7 RNA polymerase was added.

After the incubation was complete 1-5μl of the reaction volume was run on an agarose gel to check for RNA production, whilst the rest of the reaction was extracted once with phenol/chloroform and chloroform respectively, and then ethanol precipitated and washed with 70% ethanol as before.

The RNA was resuspended in 5-10μl of RNase free water, depending on the amount produced and stored at -70°C.

1μl of this was used per translation reaction. The DNA template was not removed as its presence had no effect on the translation reaction.

For the production of uncapped RNA the cap analogue was omitted from the reaction and GTP included at 0.5mM at the outset of the reaction.

***In vitro* Translation**

In vitro translation was performed using a Promega RRL kit as per the manufacturer's instructions. The standard reaction was normally supplemented with DPMs to facilitate the co-translational processing of the proteins produced. ³⁵S methionine was used to label the newly synthesized proteins and thus enable small quantities of product to be visualized by

fluorography. A standard reaction contained:

17.5μl RRL,
0.5μl 1mM amino acids (minus methionine),

2 μ l ³⁵S methionine (1200Ci/mmol) at 10mCi/ml,
2.2 μ l RNase free H₂O
1.8 μ l DPMs,
1 μ l RNA substrate in H₂O,
to a total volume of 25 μ l, and was incubated at 30°C for 90min.

RNA substrate was heated to 65°C for 10 min and cooled immediately on ice prior to addition to prevent the formation of secondary structures.

5 μ l aliquots of the translation reaction were added to 20 μ l of final sample buffer (FSB) and 5-10 μ l of this run on 15% polyacrylamide slab gels on a Bio-Rad mini-gel apparatus as described in Chp.2.

Reactions performed without DPMs were done in essentially the same manner except the volume of all reagents was doubled.

Negative control reactions were performed by adding H₂O in an equal volume instead of the RNA substrate.

Coupled *in vitro* Transcription and Translation

Whilst this work was in progress coupled *in vitro* Transcription and Translation systems became commercially available (Promega). These systems greatly simplify the process of *in vitro* protein synthesis as they do away with the necessity of performing separate transcription and translation reactions and greatly reduce the number of steps and the amount of handling necessary, with a consequent reduction in the chance of RNA degradation taking place.

The saving in time is also considerable as it is no longer necessary to produce linear DNA templates as supercoiled plasmid DNA can be used directly as a template. This system is also reported to produce significantly more protein in a 1-2 hour reaction than the conventional one (Promega technical bulletin). For these reasons some of the later experiments used the Promega TnT coupled system in preference to the conventional one. A standard TnT reaction is described below:

TnT RRL	12.5 μ l
TnT reaction buffer	1 μ l
TnT T7 RNA polymerase	0.5 μ l
Amino acids (minus methionine), 1mM	0.5 μ l
³⁵ S methionine (1,000Ci/mmol) at 10mCi/ml	2 μ l
RNAsin (40u/l)	0.5 μ l
DPMs	2.5 μ l
DNA substrate	1 μ g

H₂O to 25 μ l.

Incubate at 30°C for 90 min.

SDS-page electrophoresis

All *in vitro* synthesised proteins were examined by SDS-PAGE electrophoresis on a Bio-Rad mini-gel system, dried and exposed to X-ray film as described in the General Methods section (Chp.2).

Protease protection assays

5 μ l aliquots of the translation reaction were incubated with 1 μ l of the protease proteinase K (1mg/ml), in the presence or absence of a 1% solution of the detergent Triton X-100, to a final volume of 10 μ l, at 0°C for 1 hour. Prior to use, the Proteinase K was diluted from a -20°C stock of 20mg/ml and warmed to 37°C for 15 min. The reaction was stopped by the addition of PMSF to a final concentration of 2mM. 20 μ l of FSB was added and 10 μ l of this sample subjected to SDS-page electrophoresis.

Immunoprecipitation

Proteins were immunoprecipitated from the translation reaction mix by the use of a polyclonal anti-mouse SAF antibody, IB3, raised in rabbits (Farquhar et al., 1989). 10 μ l of translation reaction was diluted in 400 μ l of lysis buffer (10mM TrisHCl pH7.4, 100mM NaCl, 10mM EDTA, 0.5% Sodium

Deoxycholate, 0.5% Nonidet P-40, 1mM NEM, 1mM phenylmethane sulphonyl flouride (PMSF)).

Two volumes (800 μ l) of TBST (see appendix 1) and antibody 1B3 to a dilution of 1/1000 (1.2 μ l) were added. This mixture was incubated at 4°C with rocking for three hours or overnight. 100 μ l of 10% w/v Protein A-Sepharose solution (made up in TBST) was then added and the incubation continued at room temperature for a further hour, also with gentle shaking. The Sepharose beads were collected by spinning for 20 sec in a microfuge and the excess liquid carefully drawn off using a syringe attached to a 23g or smaller needle. This minimised the loss of beads during the washing step. Five washes of 100 μ l of TBST were performed with the beads being recollected each time by brief centrifugation in a microfuge. This was sufficient to remove any residual unbound protein. After the final wash the bound proteins were released from the beads by washing in 8 μ l of (0.5%SDS, 50mM NaCl) and incubating at 90°C for 10 min. As much of the sample as possible (10 μ l) was then, on addition of loading buffer, loaded directly onto a SDS-polyacrylamide gel and dried and examined as previously described.

Deglycosylation procedures.

To examine if the higher molecular weight bands observed in the *in vitro* synthesised proteins were the result of glycosylation at the predicted N-linked glycosylation sites the proteins were treated with an enzyme, PNGase F, which cleaves off N-linked glycans from the peptide backbone. This was done by first immunoprecipitating the proteins as described above, with the exception that a different elution buffer was used that contained a reduced amount of SDS (0.2% SDS, 25mM NaCl) and this remaining SDS was captured into micelles by the further addition of 100mM NaPO₄ and 10% NP40 to a final volume of 50 μ l. This sample was split into two aliquots of 25 μ l to one of which 5 μ l of enzyme (1 unit) was added and to the other 5 μ l

of water and both samples were incubated overnight at 37°C. This procedure protected the enzyme from the denaturing effects of the SDS by complexing it into micelles with the NP40. Ovalbumin was used as a control for assaying enzyme activity.

Sodium carbonate extraction

Various procedures were used to try to determine if it was possible to release the *in vitro* synthesised proteins from membranes by the actions of sodium carbonate extraction and/or sonication and membrane sedimentation. Sodium carbonate extracts peripherally attached or secreted proteins, which should then be found in the supernatant fraction, but leaves integral membrane proteins attached to the membrane.

Carbonate extraction (Perara and Lingappa, 1985):

Translation reactions (25 μ l) were split into two aliquots and diluted to 2.5 ml with either ice cold 0.1M Na₂CO₃ pH 11.5 or an isotonic solution (0.25M sucrose, 5mM MgCl₂, 1mM DTT, 50mM triethanolamine pH 7.4, 0.1 M KCl). Samples were then incubated at 0°C for 30 min and then centrifuged at 50,000 rpm for 1 hour at 2°C and the supernatants removed. Pellets were resuspended in 1% Triton buffer as above, and then both pellet and supernatant of the carbonate extracted sample had their pH adjusted with 10% acetic acid. The supernatants were then methanol precipitated as before.

Sonication and pH:

Translation reactions (25 μ l) were made up to 1ml with Tris.HCl, either pH 8 or 10, and subjected to 3x10 sec bursts of sonication with a Kontes micro-sonic cell disrupter, and left on ice for 30 min. Samples were then centrifuged in a Beckman benchtop ultracentrifuge (TL100) at 50,000 rpm for 1 hour at 2°C. Supernatants were taken off and precipitated at -20°C

with four volumes of methanol, whilst pellets (the membrane fraction) were resuspended in a 100mM Tris.HCl pH.8, 1% Triton X-100 solution, before both were run on SDS-PAGE.

TX114-extraction and PIPLC treatment

Differential solubilization in detergents can be used to separate proteins which have an overall hydrophobicity from hydrophilic proteins. Detergents with low critical micellar concentrations (ie. Triton X-100, Triton X-114 and Nonidet P-40) have been shown to be relatively ineffective at solubilizing substantial amounts of GPI-anchored proteins, whilst detergents with high critical micellar concentrations (ie.CHAPS) solubilized substantial amounts of GPI-anchored proteins (Hooper and Turner, 1988). The action of bacterial phosphoinositol-phospholipase-C (PIPLC) cleaves the GPI anchor from the protein, thus greatly reducing its overall hydrophobicity. These two results can be combined to separate and identify GPI anchored moieties from those which do not contain a GPI-anchor and those that contain membrane spanning regions (Hooper and Bashir, 1991). Thus, a protein that partitions into the detergent phase on TX-114 extraction and subsequently shifts into the aqueous phase on PIPLC digestion can be identified as a GPI-anchored protein.

TX-114 extraction was performed by a modified version of the method of [redacted] (Bordier, 1981) . For extraction without PIPLC treatment, two translation reactions were pooled (50 μ l) and diluted up to 200 μ l in (0.1M Tris.HCl pH 7.4, 0.5M NaCl , 10mM EDTA) and briefly sonicated. Membranes were sedimented by centrifugation at 100,000g for 1 hour at 2°C and the supernatant taken off and discarded. The pellet was resuspended in 200 μ l of extraction buffer (10mM Tris.HCl pH 7.4, 150mM NaCl, 1% TX-114) and sonicated for 20 sec, before being left on ice for 30 min. The samples were centrifuged for 10 min at 2,000rpm in a bench top

Beckman GS-6R centrifuge at 0°C and the supernatants collected and centrifuged further at 55,000 rpm for 1 hour at 2°C in a Beckman benchtop ultracentrifuge TI100.3 rotor. The pellets from this centrifugation were stored at 4°C to be examined in later experiments, whilst the supernatants were subjected to phase separation at 30°C. This was achieved by carefully layering the supernatant on to 300µl of 6% sucrose buffer (10mM Tris.HCl pH 7.4, 6% sucrose, 150mM NaCl, 0.06% TX-114) and immersing this in a 30°C waterbath for 10 min. This temperature is above the cloud point of TX-114 and causes a microscopic phase separation to occur. Macroscopic separation is achieved by centrifugation in a microfuge for 5 min. A detergent layer, a sucrose layer and an aqueous layer separate out. The two were pooled and this and the detergent layer both were methanol precipitated and run on SDS-PAGE.

TX-114 extraction after PIPLC digestion required small modifications of the technique. 50µl of translation reaction was diluted and membranes pelleted as before but a different extraction buffer (0.05 M Tris.HCl pH 7.4, 10mM EDTA, 0.1% TX-114) containing a lower concentration of detergent was used to resuspend the membranes. The sample was split in half and 20µl of *Bacillus thuringiensis* PIPLC (0.014 u/l, ICN-Flow) added to one half, whilst 20µl of H₂O was added to the other. The samples were then incubated with slow shaking at 37°C for 3 hours and then cooled to 0°C. The TX-114 concentration was then made up to 1% by the addition of a stock solution (11%) and the extraction carried out as before. All TX-114 used in these experiments was first precondensed according to the method of Bordier (Bordier, 1981).

Non-denaturing gel electrophoresis

GPI-anchored proteins and their amphiphilic counterparts are often indistinguishable on SDS-page despite predicted size differences, which is

probably a result of the anionic detergent binding to the lipid moiety providing a negative charge density more than enough to compensate for the size contribution of the lipid. However, some have been distinguished by their size heterogeneity on non-denaturing gels (Palfree and Hammerling, 1986; Hammelburger et al., 1987; Toutant et al., 1989), using such detergents as Triton X-100, Sodium deoxycholate and octylglucoside. These detergents should bind to the hydrophobic membrane-associated region of the protein and contribute only to the size of the solubilized molecule and not to its charge.

TX-100 gels:

15% polyacrylamide slab gels were prepared using a Tris-triton buffer (0.375M Tris.HCl pH 8.8, 1% TX-100) and a Tris-glycine upper reservoir buffer (0.025M Tris.HCl pH 8.8, 0.192M glycine) and Tris lower reservoir buffer (0.025M Tris.HCl pH 8.3). Samples were diluted 1:1 in loading buffer (40mM Tris.HCl pH 9.5, 30% glycerol, .002% bromophenol blue) and preheated to 37°C for 15 min prior to running at 150 volts for 45 min. No stacking gel was used.

Sodium deoxycholate gels:

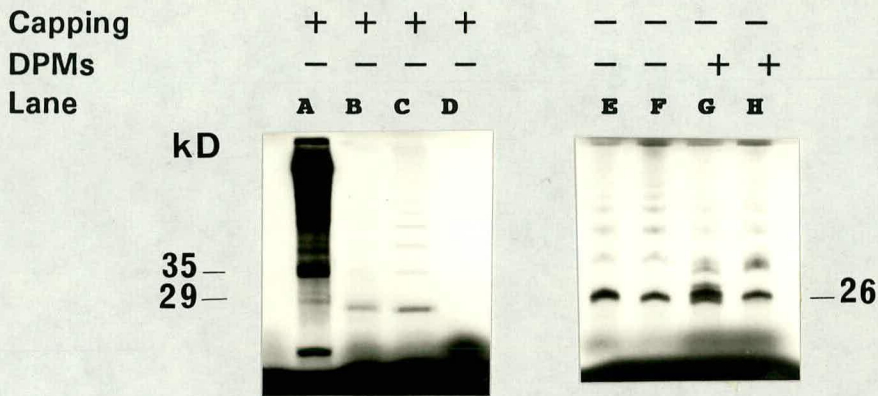
These were prepared essentially as above except 0.25% sodium deoxycholate was substituted for the TX-100.

Results

Size heterogeneity in *in vitro* translated PrP:

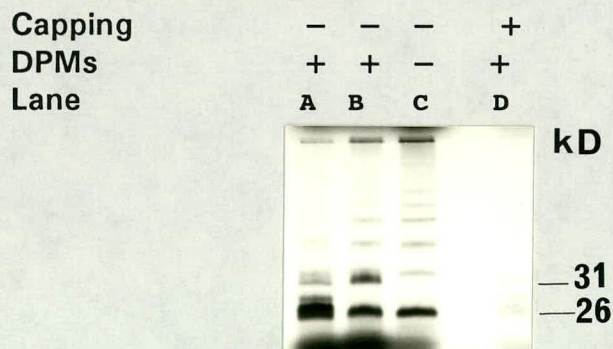
Both the wild type and mutant PrP give rise to a single band of approx. 26kD on SDS-page (Fig.11). No corresponding band is seen in the RNA negative control. The diffuse band at approx. 14-20kD seen in all reactions including the control corresponds to a globin molecule from the RRL (Promega). Faint bands of higher molecular weight at regular intervals up

Figure 11. Translation of wild-type and mutant mRNA (capped and uncapped) in RRL.



One fifth of a translation reaction from 10ng of mRNA with and without capping reagent and DPMs as indicated. Brome Mosaic Virus (Lane A), pSVPr251 (Lane B), pSVPr241 (Lane C), no RNA control (Lane D), pSVPr241 (Lane E), pSVPr251 (Lane F), pSVPr241 (Lane G), pSVPr251 (Lane H).

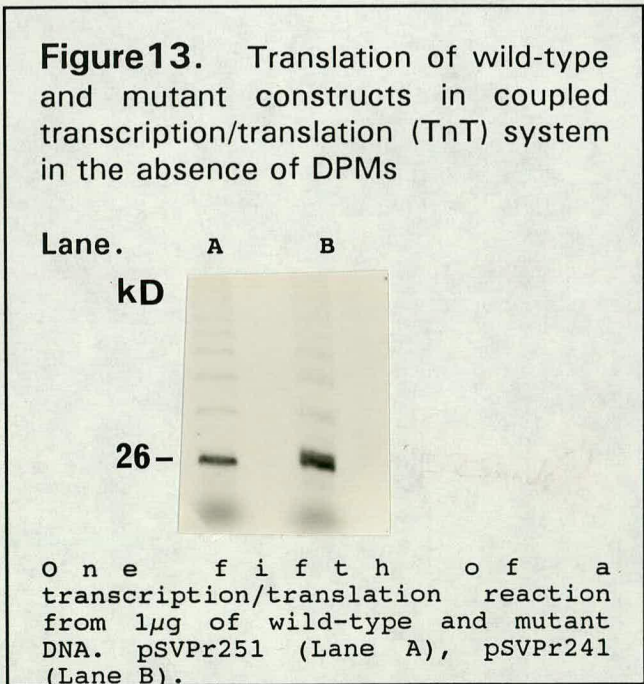
Figure 12. Translation of wild-type and mutant mRNA (capped and uncapped) in the presence and absence of DPMs.



One fifth of a translation reaction from 10ng of mRNA with and without capping reagent and DPMs as indicated. pSVPr241 (mutant) (Lane A), pSVPr251 (wild-type) (Lane B), pSVPr251 (Lane C), pSVPr241 (Lane D). DPM= dog pancreas membranes.

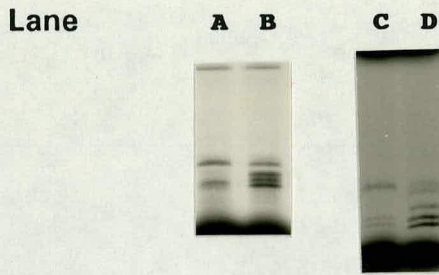
to the top of the gel were consistently observed when DPMs were omitted and also when uncapped RNA was used as a transcription substrate (Fig. 11, Fig. 12). These bands were also observed when the TNT system was used without membranes (Fig. 13) and were not observed in the RNA negative control lane (Fig. 11, Lane D). Similar bands were observed when a fusion protein containing the ninety amino-terminal amino acids of PrP fused to a luciferase reporter gene (Promega) was expressed in the RRL system but not when the luciferase reporter alone was expressed (M. Firoozan, pers. commun.) Discussions with the manufacturers suggested that these could possibly be an artefact of the system when used with PrP. Alternatively, they could suggest that the protein, when overexpressed

outside its natural environment, is incorrectly folded or does not interact with the proper chaperones and is allowed to aggregate. When DPMs were included wild type and mutant constructs gave rise to differing multiple band patterns (Fig. 14). The wild type protein typically had two major bands which corresponded to the unprocessed 26kD form

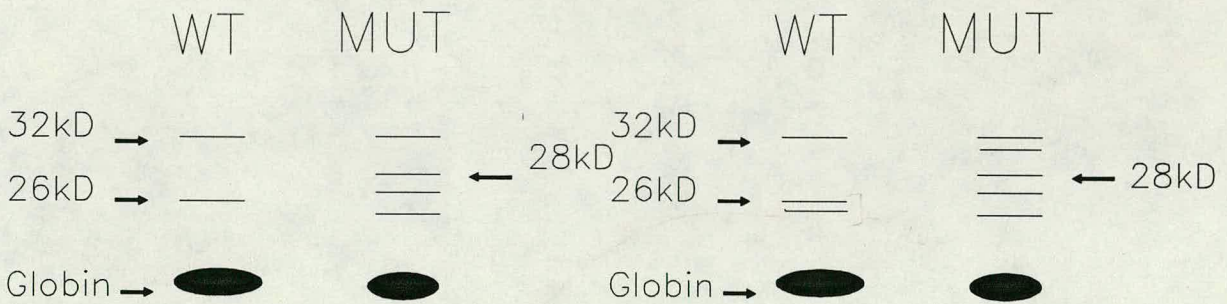


seen in the absence of DPMs and a band of approx. 33-35kD, which may exist as a doublet as two bands could be resolved on some gels. Similarly, in some reactions a second band is seen below the 26kD band. The larger bands are believed to correspond to the processed and N-glycosylated forms of the protein, with the doublet consisting of N-terminally cleaved, GPI-

Figure 14 Translation products of wild-type and mutant in the presence of DPMs.



Translation products produced with the uncoupled (Lanes A and B) and coupled (Lanes C and D) *in vitro* transcription/translation systems. pSVPr251 (Lanes A and C), pSVPr241 (Lanes B and D). See accompanying line diagrams.

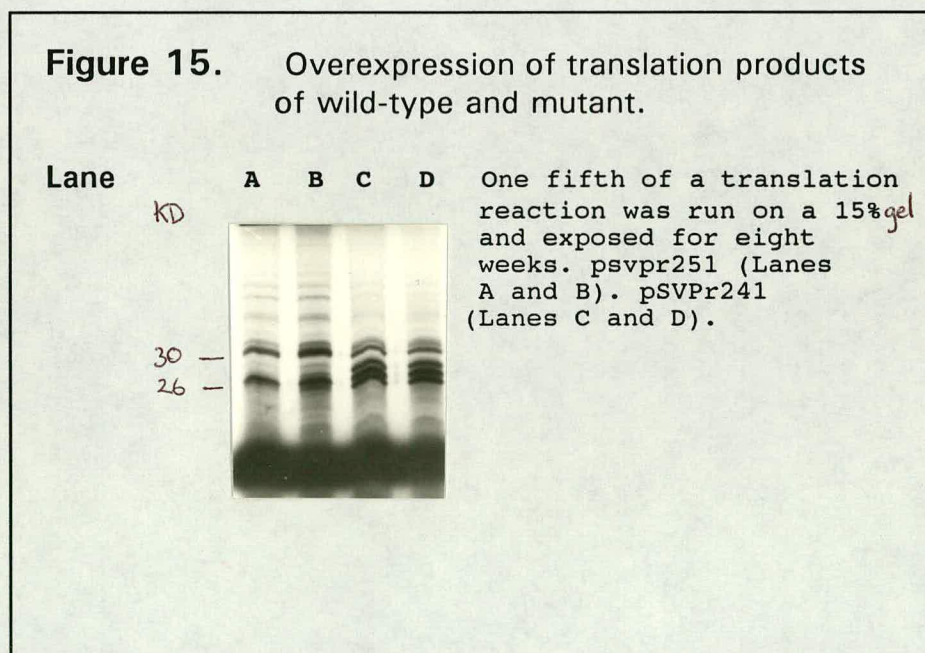


Line diagram of Fig. 14 A,B

Line diagram of Fig. 14 C,D

anchored proteins, with either one or two of the predicted N-glycosylation sites filled. The 26kD band probably represents the unprocessed form of the protein and the slightly smaller band possibly represents the N-terminally cleaved but as yet unglycosylated protein (Fig.14, Lanes A and C).

In the mutant a different pattern was observed (Fig.14, Lanes B and D). The unprocessed protein may be fractionally larger than that observed in the wild type but the difference is so small that it is difficult to resolve. Three other bands are observed, the largest of which corresponds to the processed, glycosylated form seen in the wild type. The negative charge of the lipid moiety in the GPI-anchored protein compensates for its larger size on SDS gels and consequently we would expect to be unable to differentiate between the fully glycosylated protein and the GPI-less protein by size using this system. Of the two other bands the smaller (24kD) may correspond to the N-terminally cleaved, unglycosylated form which is only sometimes observed in the wild type. The identity of the fourth band of intermediate size has not yet been conclusively assigned. Fig.15 is a very over exposed gel which clearly shows the major protein species.



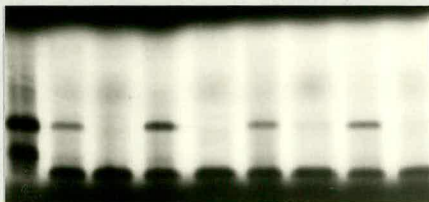
Further manipulations of these proteins were designed to show whether these predictions were correct or not.

Protease protection assays:

This experiment was designed to decipher which of the observed bands were translocated across the membrane, and consequently, would be protected from the action of exogenously added proteases and would have undergone co-translational processing, such as N-terminal signal cleavage. The disruption of the membrane by the addition of detergent should abolish this protection. The results showed that in the wild type the 26kD band disappears with PK treatment, whilst the 33-35kD bands are protected (Fig. 16). A similar result is observed when the treated proteins are immunoprecipitated with the PrP polyclonal antibody IB3 (Fig.17). This correlates with the idea that the 26 kD band represents the unprocessed translation product which has not passed through the membrane and has not had its N-terminal and C-terminal signal sequences cleaved. In the mutant (Fig18, LanesD,E,F, Fig.19,Lanes D-I) the second largest band disappears, which suggests that this is the corresponding unprocessed translation product, whilst the smaller molecular weight band which is protected could be an N-terminally cleaved band which has crossed the membrane, but has not been glycosylated. All the higher molecular weight bands are protected, which implies that they are all located on the interior of the membrane vesicle and have thus passed through the membrane and have undergone co-translational processing. The size (33-35kD) of the largest band in both mutant and wild type correlates to what we would

Figure 16. Proteinase K treatment of wild-type protein.

PK	-	+	+	+	+	+	+	+	+
DET	-	-	+	-	+	-	+	-	+
Lane	A	B	C	D	E	F	G	H	I



One fifth of each translation reaction was incubated with 1 μ l of Proteinase K (1mg/ml) at 0°C for 1 hour in the presence of 1% Triton X-100 as indicated. PK = proteinase K, DET = detergent. Untreated (Lane A). PK treatment only (Lanes B,D,F,H). PK plus detergent treatment (Lanes C,E,G,I).

Figure 17. Immunoprecipitation of Proteinase K treated wild-type protein.

Immunoptn.	-	+	+	+	+	+	+
PK	-	-	-	+	+	+	+
DET	-	-	-	-	+	-	+
Lane	A	B	C	D	E	F	G



Immunoprecipitation of wild-type protein with the poly-clonal anti-PrP antibody 1B3 after PK treatment in the presence or absence of detergent. Unprecipitated, untreated protein (Lane A), Untreated immunoprecipitated protein (Lanes B and C), PK treated protein in the absence of detergent (Lanes D and F), PK treated protein in the presence of 1% TX-100 (Lanes E and G).

Figure 18. Proteinase K treatment of wild-type and mutant protein.

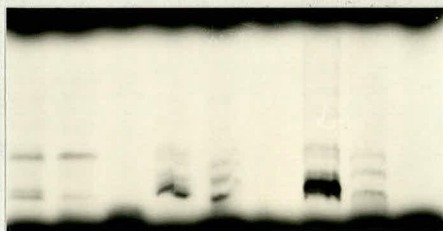
PK	-	+	+	-	+	+	-
DET	-	-	+	-	-	+	-
Lane	A	B	C	D	E	F	G



Translation products from the constructs pSVPr251 (wild-type) and pSVPr241 (mutant) were treated with Proteinase K, in the presence and absence of detergent as described in Fig.6 and the text. PK=proteinase K, DET= 1% TX-100. pSVPr251 (Lanes A,B,C), pSVPr241 (Lanes D,E,F), no RNA control (Lane G).

Figure 19. Proteinase K treatment of two different mutants.

PK	-	+	+	-	+	+	-	+	+
DET	-	-	+	-	-	+	-	-	+
Lane	A	B	C	D	E	F	G	H	I



Proteinase K treatment was carried out as described in previous figures and the text. pSVPr251 (Lanes A,B,C), pSVPr241 (Lanes D,E,F), pSVPr228 (Lanes G,H,I). + PK treatment (Lanes B,C,E,F,H,I). + 1% TX-100 (Lanes C,F,I) PK= proteinase K, DET= 1% TX-100.

expect for the glycosylated protein. The smaller molecular weight (28kD) band which is only seen in the mutant was thought at this stage to be a possible glycosylation intermediate.

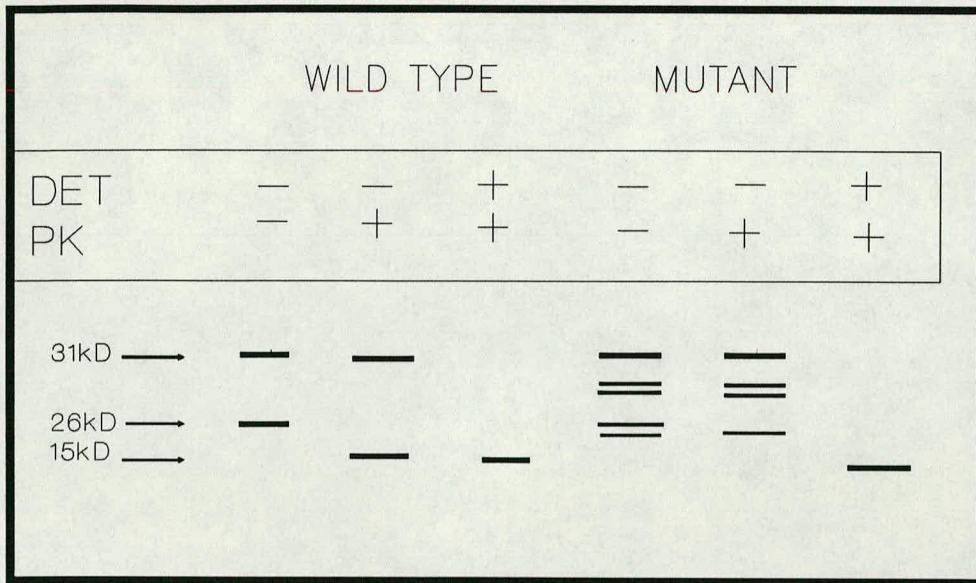
In the wild type PK treatment gave rise to a protease resistant band of approx. 10Kd which appears to be a degradation product (Figs.16 and 17) and which does not appear to be present in the mutant (Fig.18,19,20). With this exception addition of detergent abolished the resistance to degradation of all species, thus this mutation does not give rise to the production of protease resistant PrP in this system.

Immunoprecipitation:

Following immunoprecipitation of the wild type protein with the polyclonal antibody IB3, it was found that all the bands previously observed on SDS-PAGE were recognised by this antibody (Fig.17,20,21). The 10kD protease degradation product picked up in the PK experiments was also recognised by this antibody (Fig.17,20,21). When the mutant protein was immunoprecipitated a pattern of five bands was observed. The largest of these appears to correlate with the largest band observed without immunoprecipitation. The other four bands may be two sets of doublets, implying that one of the four bands previously observed does not immunoprecipitate with this antibody. Further evidence for this theory comes from PNGase experiments (see below and Fig.22), where, after immunoprecipitation, the undigested mutant protein has a pattern of only three bands, two of which, on better resolution could have been doublets. The identity of exactly which of the four original bands is not being immunoprecipitated has been difficult to assess.

Deglycosylation procedures:

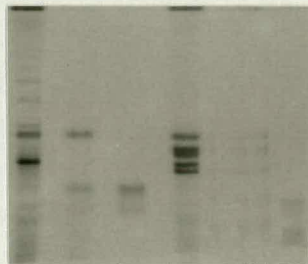
This experiment was designed to investigate whether the large molecular weight species observed were indeed the result of carbohydrate addition at



Line diagram of immunoprecipitated Proteinase K treated wild-type and mutant proteins. (see Fig.20)

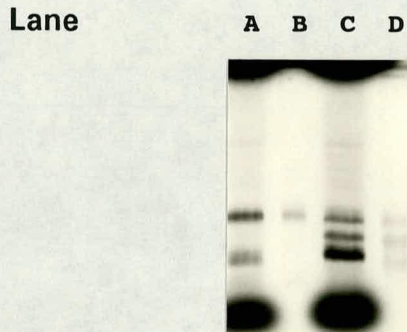
Figure 20. Immunoprecipitation of Proteinase K treated wild-type and mutant proteins.

PK	-	+	+	-	+	+
DET	-	-	+	-	-	+
Lane	A	B	C	D	E	F



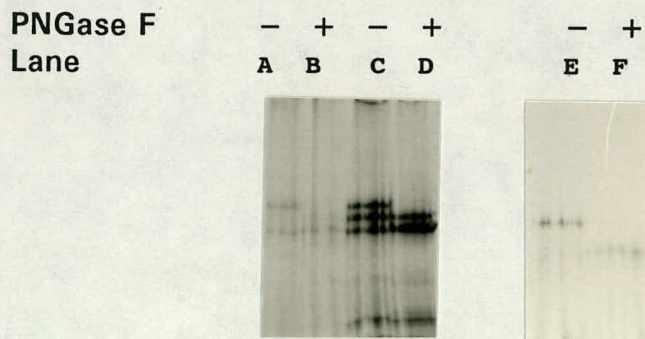
After each Proteinase K treatment the reaction was stopped with 2mM PMSF and each sample immunoprecipitated overnight with 1B3, a polyclonal anti-PrP antibody, before collection on Sepharose A beads and elution with 0.5% sds, 50mM NaCl. PK= Proteinase K, DET= 1% TX-100. pSVPr251 (Lanes A,B,C), pSVPr241 (Lanes D,E,F). + PK treatment (Lanes B,C,E,F), + TX-100 (Lanes C,F)

Figure 21. Immunoprecipitated versus non-immunoprecipitated wild-type and mutant proteins.



One fifth of a non-immunoprecipitated sample (5 μ l) was diluted in sample buffer and run (Lanes A and C) while one half of a translation reaction (10 μ l) was immunoprecipitated as described in the text (Lanes B and D). pSVPr251 (Lanes A and B), pSVPr228 (Lanes C and D).

Figure 22. Deglycosylation of wild-type and mutant proteins by PNGase F.



Proteins were first immunoprecipitated as described and then treated with 1 unit of PNGase F at 37°C overnight. pSVPr251 (Lanes A, B, E, F), pSVPr241 (Lanes C and D). PNGase F (Lanes B, D, F)

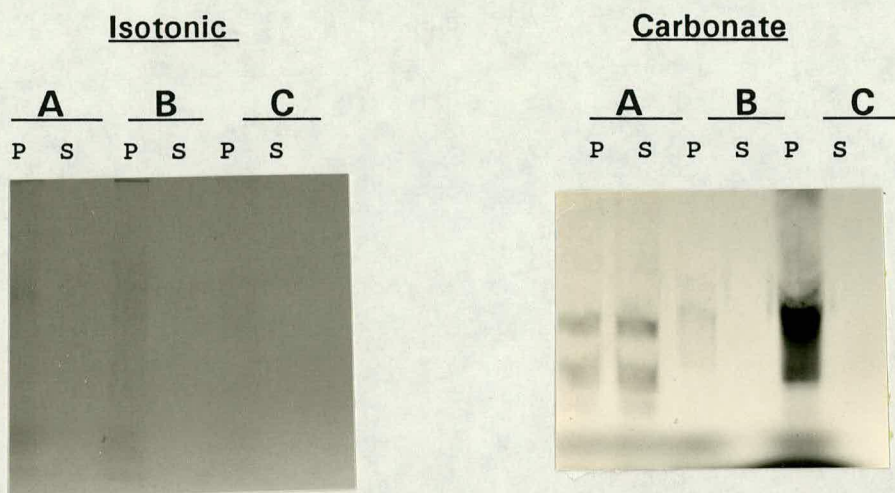
the predicted N-linked glycosylation sites. The enzyme used, PNGase F, specifically removes N-linked oligosaccharides by hydrolyzing the GlcNAc-Asn bond which attaches the carbohydrate to the protein. If the proteins are glycosylated then enzyme treatment should result in *accelerated movement in the gel*. Suitable reaction conditions for the action of this enzyme required that the protein was first immunoprecipitated. As noted above only three of the four bands in the mutant protein are immunoprecipitated by IB3. PNGase treatment of both mutant and wild type led to *acceleration in the gel* (Fig.22). In each case it was only the highest molecular weight band that was removed by action of the enzyme, implying that this is the only glycosylated species. Increasing the duration of incubation with the enzyme did not lead to the disappearance of the other bands. This leaves the origin of the intermediate band (28kD) in the mutant unidentified as it is probably not a glycosylation intermediate. As expected the running of the unprocessed band was not affected by this treatment. Purified ovalbumin exhibited *acceleration in the gel* after PNGase treatment, thus showing that the enzyme functions under the described conditions.

Sodium carbonate extraction:

Sodium carbonate extraction is known to strip peripherally associated proteins from the membrane, but to leave integral membrane proteins in the membrane fraction (Perara and Lingappa, 1985). It was not known before this experiment exactly how a GPI-anchored protein would behave in this system, but it was thought that it would remain associated with membrane fraction. In the first experiment the wild type protein remained in the membrane fraction in both cases when treated with either an isotonic buffer or the carbonate buffer, as did the 241 mutant, whereas the 228 mutant was associated with the membrane fraction in isotonic buffer and displayed a shift of approx. 50% into the supernatant fraction on carbonate extraction (Fig.23). This would imply that the wild type is not stripped by

this treatment but this result is put into doubt by the association of the

Figure 23. Sodium -Carbonate extraction of wild-type and mutant proteins.



One translation reaction was split in half and each half extracted with either an isotonic buffer or a Sodium Carbonate buffer pH 11.5 at 0°C for 30 min and then centrifuged at 50,000rpm for 1 hour. Pellets were resuspended in 1% Triton and sample buffer and supernatants precipitated with methanol.

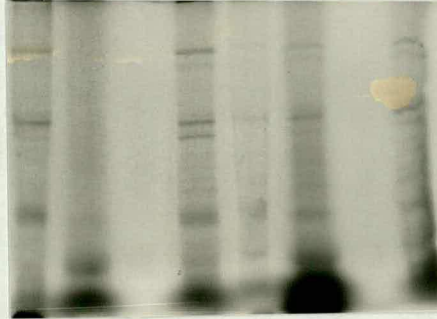
A= pSVPr251, B= pSVPr241, C= pSVPr228, P= pellet fraction, S= supernatant fraction.

mutant with the membranes in the isotonic buffer . We would not expect a secreted protein to associate with the membrane fraction . It was thought that the microsomal vesicles might be remaining intact, thus trapping the mutant protein in their lumen and causing to come down in the membrane fraction. Also, in this experiment a fresh translation reaction of mutant 228 was used, whereas the wild-type and mutant 241 translation reactions had been stored at -70°C before use and this may have affected their extractability in the carbonate buffer (Fig.23). Therefore a different experimental design was attempted. It is known that the stripping action of the carbonate buffer is largely a function of its alkali pH so an experiment

involving buffers of different pH coupled with sonication to ensure total

Figure 24. Extraction of wild-type and mutant proteins with alkaline buffer and sonication.

WT				MUT			
pH8		pH10		pH8		pH10	
P	S	P	S	P	S	P	S

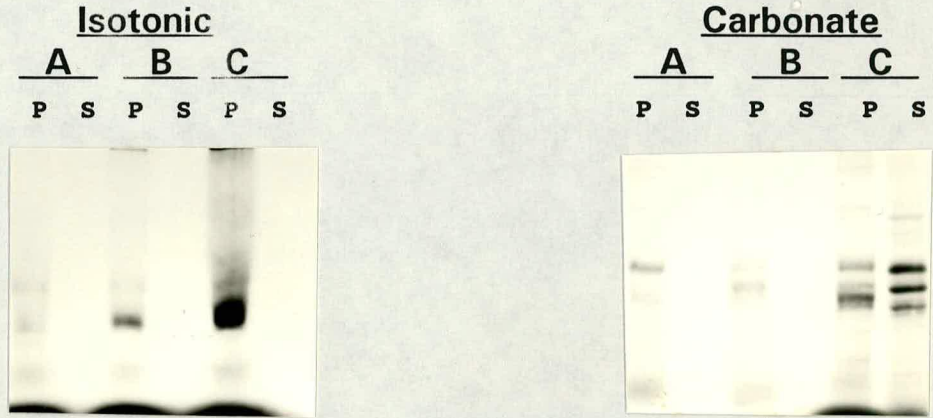


The product of one translation reaction (25 μ l) was diluted to 1ml in either a buffer of pH8 or pH10, and then sonicated with 3x10sec bursts. Samples were then centrifuged for 1 hour at 50,000rpm at 2°C and pellet and supernatant fractions collected and run on a gel. WT= pSVPr251 (wild-type), MUT= pSVPr241 (mutant)

disruption of the microsomal vesicles was attempted. In this experiment the wild type protein was found in the membrane fraction at pH 8 but shifted totally to the supernatant at pH 10 (Fig.24), whereas the mutant protein was found mostly in the supernatant at pH 8 and totally so at pH 10 (Fig.24). A subsequent experiment coupling sonication with the original isotonic and carbonate buffers led to a totally conflicting result, where both wild type and mutant were found in the membrane fraction in isotonic buffer and where only the wild type showed a shift to the supernatant on carbonate extraction (Fig.25). These results are confusing but imply that the wild type is stripped from the membrane by this treatment and thus does not exhibit the properties of an integral membrane protein. The situation with the mutant is less clear, as it behaves much like a secreted protein in

the second experiment but more like an integral membrane protein in the

Figure 25. Extraction of proteins with Sodium-Carbonate and sonication.



This experiment combined the carbonate buffer extraction with sonication. A= pSVPr251, B=pSVPr241, C= pSVPr228. P= pellet, S= supernatant.

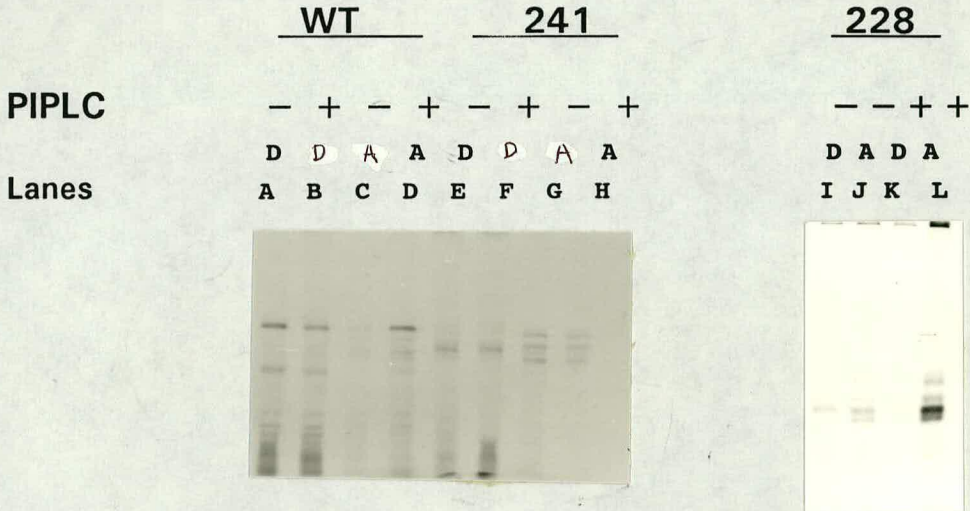
third set. One possible explanation is that the mutant protein is ^{secreted} but is clumped into aggregates under some conditions causing it to sediment with the membrane fraction. The GPI-anchored proteins GP-2 and THP have been shown to self-associate in an ion-dependent and pH-induced fashion *in vitro* after PIPLC cleavage (Fukuoka et al., 1992).

TX114 extraction and PIPLC treatment

PrP is a GPI-anchored protein which is susceptible to PIPLC treatment (Stahl et al., 1987). GPI-anchored proteins are known to separate into the detergent phase when extracted with the detergent TX114 (Bordier, 1981). On treatment with PIPLC they lose their hydrophobic glycolipid anchor and as a result will partition into the aqueous phase on TX114 extraction. Our results show that the wild type is found almost exclusively in the detergent

phase, but on PIPLC treatment shows a considerable shift into the aqueous

Figure 26. Treatment of wild-type and mutant proteins with PIPLC and subsequent extraction in TX114.



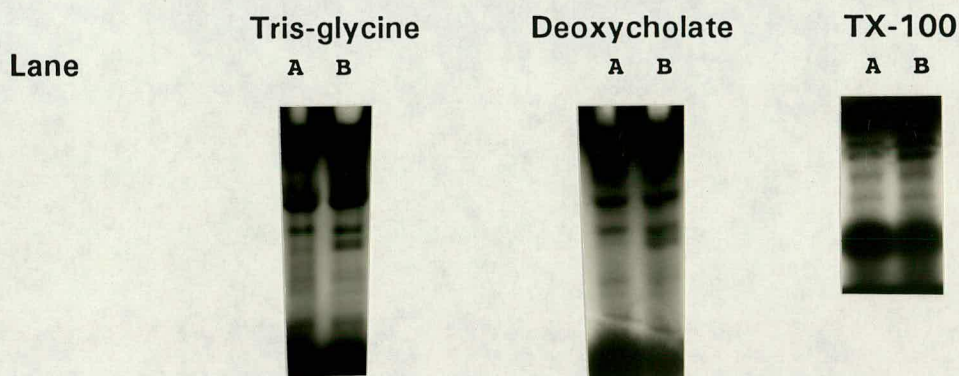
Two translation reactions were pooled and the membrane fraction sedimented as described in the text. These were extracted with a buffer containing 1% TX114, or 0.1% TX114 when PIPLC treatment was carried out. Samples were incubated with 0.014units of PIPLC for 3 hours at 37°C and the extraction buffer was then made up to 1% TX114 and the extraction continued as described in the text. WT= pSVPr251, 241=pSVPr241, 228= pSVPr228. pSVPr251 (Lanes A-D), pSVPr241 (Lanes E-H), PsvpR228 (Lanes I-L)

phase (Fig.26). This is consistent with its containing a GPI-anchor, with the residue in the detergent being protein from which the enzyme has not removed the glycolipid anchor. In contrast the bulk of the mutant is found in the aqueous phase and PIPLC treatment does not alter the pattern of its phase partitioning (Fig.26). This is consistent with the protein not having a GPI anchor. Interestingly, one of the four mutant bands seems to be poorly extracted into the aqueous phase. This is the second smallest band and the one that was previously postulated to be the unprocessed translation product. The corresponding band in the wild type also seems to partition poorly into the aqueous phase.

Non-denaturing gel electrophoresis:

Some workers have been able to use non-denaturing gels to differentiate between the GPI-anchored and hydrophilic forms of a protein, (Palfree and Hammerling, 1986; Hammelburger et al., 1987; Toutant et al., 1989). We tried to do so using a number of different gel formulations, which ranged from a basic Tris.-glycine gel to ones containing TX100 or sodium deoxycholate. However, these attempts were inconclusive as in each case most of the protein was trapped in the wells and it was difficult to say what those bands that were observed represented (Fig.27). It looks as if the highest molecular species, which should represent the fully processed protein, is migrating at the same rate in both wild type and mutant, which suggests that this method cannot be used to differentiate between them. Attempts to run PrP extracted from the brains of scrapie infected mice on similar gels were also unsuccessful (J.Hope, pers. commun.).

Figure 27. Non-denaturing gel electrophoresis of wild-type and mutant proteins.



Different types of detergent were used in an attempt to differentiate between the GPI-anchored and the hydrophilic forms of the protein on polyacrylamide gels. A= pSVPr251 (wild-type), B= pSVPr241 (mutant).

Conclusions

Examination of our PrP mutants in the RRL cell free system has shown that they differ from the wild type. They exhibit an extra band of 28kD which is translocated across the membrane but does not appear to be a glycosylation intermediate. All bands of the wild type protein were immunoprecipitable with the anti-PrP polyclonal IB3 . It is possible that one of the bands in the mutant is not. The availability of peptide specific antibodies, such as N- and C- terminal specific antibodies, would have been very useful in assigning definite origins to each of the observed protein species.

We have shown that the mutant proteins behave as expected for a hydrophilic form of the protein lacking a GPI-anchor as they are found in the aqueous phase of TX114 extractions and this phase distribution is not affected by the action of PIPLC, in contrast to the wild type which is found in the detergent phase and shifts to the aqueous on PIPLC treatment. Thus we conclude that similar to previously examined GPI-anchored proteins, inserting truncation mutations into the open reading frame of the PrP gene causes the loss of a C-terminal signal sequence for GPI-addition . Both the mutants that were examined, pSKPr241 and pSKPr228, which are truncated by thirteen and twenty-six amino acids respectively, gave rise to the same pattern of protein species. Thus we conclude that the removal of ten of the seventeen hydrophobic C-terminal amino acids is sufficient to prevent the addition of a GPI-anchor. The removal of a further thirteen amino acids , including the predicted attachment site of the GPI (Ser 231), does not give rise to any observable difference in the pattern of protein species observed. It was not possible to establish the presence or absence of a GPI-moiety on the recombinant proteins by separation on non-denaturing gels. In the absence of DPMs a regular series of higher molecular weight bands was observed (Figs. 12, 13, 14, 15) ranging in size from 35kD up to 150,000kD.

These bands are also seen to a lesser extent when DPMs are included, but uncapped RNA is used in the translation reaction (Fig.12,13,14). It is possible that these bands are an artefact of the overproduction of the protein when there is a limiting amount of membranes present and thus only a proportion of the nascent protein can be processed. The unprocessed, unglycosylated protein may have a greater tendency to aggregate. There is some evidence for this from experiments in which site-directed mutagenesis has been used to produce non-glycosylated PrP in scrapie infected cells (Taraboulos et al., 1990). These researchers found that PrP^{Sc}, the aggregated, protease resistant form of the protein, is produced more quickly in cells expressing the non-glycosylated form of the protein than in those containing the wild-type.

CHAPTER FIVE: Tissue culture analysis of mutants

Introduction

Tissue culture cells provide a much more realistic representation of the actual conditions that prevail in the whole organism than cell-free studies, without involving the time and high costs of animal work. Proteins expressed in tissue culture can be expected to be post-translationally modified in a similar manner to native proteins. It was predicted from the cell free work (Chp.4), that the wild-type PrP, having a GPI-anchor would be associated almost exclusively with the cells, whilst the mutant protein, having no GPI-anchor, would be secreted into the tissue culture medium. It was hoped that these constructs, under the influence of the viral SV40 promoter would overproduce the proteins which would then be easily detectable in amounts that could be used for further biochemical examination. It is necessary to be able to distinguish between the recombinant protein and any endogenous protein that may be present in the cell type utilised. In this case the use of a cell line of neural origin like neuroblastoma cells was ruled out as our recombinant wild-type PrP should directly resemble the endogenous protein. Therefore a cell line was chosen, CV1 cells (monkey kidney cells) that had previously been shown to express recombinant PrP and which did not express endogenous PrP by ELISA assays (Rogers et al., 1990).

General methods

Cells

CV1 cells, (an adherent monkey kidney cell line) and mouse neuroblastoma cells (N₂as) were obtained from ICN-Flow.

Media.

Initially, following the recommendations of the suppliers Minimum Essential Eagles medium (MEEM) supplemented with 10% fetal calf serum (FCS) and

certain nutrients as outlined below , was used as a growth medium. Subsequently, this was changed to Dulbeccos Modification of Eagles medium (DMEM) as this appears to be a better medium for efficeint transfection (pers.comm. K.Chapman).

MEEM was obtained from Flow-ICN as a 10x concentrate and diluted with tissue-culture grade H₂O as outlined below.

MEEM

10X Medium	50ml
100% Fetal calf serum	50ml
100x L-Glutamate	5ml
100x Non-essential amino acids	5ml
7.5% Sodium bicarbonate	16.5ml
H ₂ O	374.5ml

DMEM was obtained from Gibco-BRL as a 1x solution which was supplemented as outlined below.

DMEM

1X Medium	500ml
100% Fetal calf serum	50ml
100x non-essential amino acids	5ml

All media was supplemented with 1% pen/strep .

Maintenance of cells

Cells were grown to confluence and were initially divided 1/10 , but this ratio was reduced to 1/4 in later experiments in order to reduce stress on the cells during the transfection process. Cells were released from the surface of the flask by the action of a solution of trypsin (TVP) as outlined below.

TVP

Phosphate buffered saline (PBS) Ca and Mg free,	100ml
Chicken serum	1ml
2.5% Trypsin (ICN-Flow)	1ml
0.5M EDTA	200 μ l

1ml of TVP was added per each 25cm² of flask and this then incubated at 37°C for 3 min. At this stage the cells rounded up and detached from the bottom of the flask. The TVP was diluted out by the addition of at least an equal volume of medium and clumps of cells broken up by repeated pipetting up and down. Fresh medium was added and the cells incubated at 37°C and in 6% CO₂ in a Heraeus 6000 incubator.

Freezing cells

Frozen stocks of cells were prepared by detaching the cells from the flask as described above and collecting them by centrifugation at 3,000rpm for 3min. The cells were then resuspended in 10ml (per 75cm² flask) of freezing mix (see below) and the centrifugation step repeated. The cells were then resuspended in 5ml of the freezing mix and aliquoted into 0.5ml aliquots in Nunc screw top freezing vials. These were transferred into a polystyrene container and kept overnight at -70°C before transfer to liquid nitrogen where they could be stored indefinitely.

For thawing, vials were removed from liquid nitrogen and quickly thawed at 37°C. They were diluted in 5ml of medium and immediately collected by centrifugation, before being resuspended in 10ml of media and plated out in a 25cm² flask. This step had the result of removing the DMSO (Sigma) in the freezing mix, which is toxic to the cells when unfrozen.

Freezing mix

MEEM	6ml
DMSO	2ml
FCS	2ml

DNA for transfection

The DNA used for transfection was double banded on caesium chloride gradients (see Chp.2) and used in its supercoiled state, as nicked DNA and the presence of residual RNA and chromosomal DNA decreases transfection efficiency. Cells were transfected with 10 μ g DNA including the test DNA (1 μ g) and a control DNA (1 μ g) that is known to transfect efficiently. The control was chosen to have an activity which is easily monitored, in this case the chloramphenicol acetyl transferase (CAT) gene under the control of the Rous Sarcoma Virus (RSV) promoter. Thus the transfection efficiency was measured by the levels of CAT activity expressed by the cells. The amount of DNA was increased to 10 μ g using the vector pGEM.

As well as the pSV series of constructs two further constructs were used in later transient assay experiments. These contained the wild-type ORF and an ORF with a truncation mutation at codon 231 (IAH2 and IAH4a respectively) under the control of the human cytomegalovirus and had been shown to produce recombinant protein in tissue culture (A.Bennett pers. comm.) DNA stocks of these constructs were prepared as described above for the pSV series.

CAT assays

Chloramphenicol acetyl transferase (CAT) is an enzyme that transfers an acetyl (or but^yryl) group from acetyl (or butryl) coenzyme-A onto chloramphenicol. In the CAT assay expression of the gene was measured by the ability of extracts from cells into which the CAT gene had been transfected to transfer this acetyl group onto a radiolabelled chloramphenicol

molecule. Molecules with the extra group attached partitioned into the xylene phase when extracted in this solvent. The amount of CAT activity present was measured by measuring the radioactivity present in this phase compared to that obtained from extracts from cells into which no CAT DNA had been transfected.

The procedure followed for a 35mm dish is outlined below.

1) The supernatant was removed from the cells and the cells scraped off into 1ml of ice cold PBS.

2) Cells were collected by centrifugation and washed in a further 1ml of PBS and the centrifugation step was repeated.

3) Cells were lysed in 50 μ l of lysis buffer (10mM TrisHCl. pH 8, 2mM MgCl₂ , 0.5% Nonidet P-40) , heated for 7 min at 60°C and centrifuged at 13,000rpm for 10 min.

5) Mix A was prepared:-

TrisHCl pH 8	100 μ l
¹⁴ C Chloramphenicol	200 μ l
2.5mM But ^y ryl CoA	200 μ l
H ₂ O	500 μ l

6) 43 μ l of mix A was added to the 50 μ l of cell lysate and left at 37°C for 5 hours.

7) 200 μ l of xylene was added and the tube vortexed for 5 min.

8) 180 μ l of the xylene (top) layer was removed to a fresh tube and 100 μ l of TE added to it. This was vortexed briefly and then centrifuged for 3 min.

9) The top layer was taken off to a fresh tube and a further 100 μ l of TE added to it, after which the centrifugation was repeated.

10) The top layer was taken off and 2ml of scintillation fluid added to it. This was counted in a scintillation counter.

The heating step destroyed other enzyme activities and a negative control (untransfected or mock transfected cells) was always included in the assay for comparative purposes. Mock transfected cells were treated with the

transfection reagent but no DNA was added

Transfection

Many different protocols are now available for the transfer of exogenous DNA into the interior of mammalian cells (Sambrook et al., 1989). There are many different variables in the efficiency of these protocols, including different cell type, the stage of growth of the cell, the medium, and the state of the DNA used for the transfection. As great difficulty was experienced in successfully transfecting these cells a number of different protocols were attempted before a method that worked consistently was found.

Lipofection.

This is a method where a chemical analogue of the lipid bilayer is used to form micelles containing the DNA to be transfected, which are subsequently fused with the cell membrane, thus releasing the DNA into the cells. It has been shown to be 5-100x more efficient than Calcium Phosphate transfection depending on cell type (Felgner et al., 1987) and therefore was attempted first. Lipofection reagent is commercially available (Gibco-BRL, Lipofectin) and was used according to the manufacturers instructions, as described below for 35mm dishes.

- 1) Cells were seeded and grown to 40-60% confluency.
- 2) 10 μ g of DNA was mixed with 1ml of serum-free medium (SFM).(Mix A)
- 3) 10 μ l of Lipofectin reagent was mixed with 100 μ l of SFM.(Mix B)
- 4) Mixes A and B were combined and left at RT for 10-15 min.
- 5) Cells were washed x2 in SFM.
- 6) 800 μ l SFM was added to each tube ,mixed gently and added to the cells.
- 7) These were incubated at 37°C for 3-5 hours or longer.
- 8) 1ml of medium containing 20% FCS was added.
- 9) The incubation was continued for 48-72 hours and the cells assayed.

Prolonged exposure of the cells to Lipofection reagent in SFM caused cell death, so a balance between optimised transfection efficiency and maximum permissible cell death had to be achieved, usually by trial and error. Optimum conditions for CV1 cells allowed for 5 hours of incubation in the reagent.

Lipofection results

Lipofection was carried out as described above, and the cells were observed during the process. A certain amount of cell death occurred during the serum-free phase, presumably the result of disruption of the plasma membranes of some cells. However, after the 48-72 hour incubation period the cells had recovered and grown to full confluency. CAT assays performed on early Lipofection experiments gave very high levels of CAT activity- 50-100x that of an untransfected or mock transfected cell (Table1). These transfected cells were used to try and isolate the recombinant proteins (see below). After the initial couple of experiments it was not possible to isolate PrP from cells or supernatants of cells transfected with either the wild-type or mutant construct. Various different techniques were utilized, including TX114-extraction, direct methanol precipitation of cell supernatants with methanol, concentration of supernatants in Amicon concentrators, and pulse-chase labelling of proteins with ³⁵S-labelled methionine (see results below). It became clear that the protein was not being expressed in these cells, which prompted a reexamination of the CAT assay results for these transfections. It was noticed that there was a rapid drop off in the efficiency of transfection after the initial few experiments (Table 1), until the levels of CAT activity were only 3-10x that of the untransfected cells. Many variations in the basic protocol were tried to improve this efficiency. New reagents and new media made no improvement. Increasing the amount of Lipofectin from 10 μ l through to 50 μ l, and increasing or decreasing the amount of time that the

reagent was left on the cells did not help. New cells were brought out of freezing and also obtained from another laboratory and new stocks of DNA

Table 1:Lipofection results

Exp.no.	DNA	Counts	Times x background	Method/amount
1	none	160	1	Lipofection
1	rsvCAT	5721	35.7	Lipofection
2	none	83	1	Lipofection
2	pSVPr251(wt)	5896	71	Lipofection
2	pSVPr241	5627	67	Lipofection
3	none	249	1	Lipofection
3	pSVPr251	2152	8.7	Lipofection
3	pSVPr241	1830	7.3	Lipofection
4	none	133	1	Lipofection
4	pSVPr251	115	< 1	Lipofection
4	pSVPr241	349	2.6	Lipofection
5	rsvCAT	3509	?	Lipofection
5	pSVPr251	2431	?	Lipofection
5	pSVPr241	3660	?	Lipofection
6	none	991	1	Lipofection
6	rsvCAT	2590	2.6	Lipofection
6	pSVPr251	1573	1.5	Lipofection
6	pSVPr241	2317	2.3	Lipofection
6	pSVPr228	2686	2.7	Lipofection

Exp.no.	DNA	Counts	Times x background	Method/amount
7	none	88.4	1	Lipofection
7	rsvCAT	142.7	1.61	Lipofection
7	pSVPr241	88.8	1	Lipofection
8	none	295.77	1	Lipofection
8	rsvCAT	1812	6.14	Lipofection
8	pSVPr251	2110	7.1	Lipofection
8	pSVPr241	2578	8.7	Lipofection
9	none	182	1	Lipofection
9	rsvCAT	763	4.1	Lipofection
9	pSVPr251	227	1.2	Lipofection
9	pSVPr241	546	3	Lipofection
10	none	93	1	Lipofection
10	rsvCAT 10 μ g	576	6	Lipofection 20 μ l
10	rsvCAT 10 μ g	5800	62	Lipofection 25 μ l
10	rsvCAT 10 μ g	913	9.8	Lipofection 30 μ l
10	rsvCAT 10 μ g	5169	55	Lipofection 35 μ l
10	rsvCAT 10 μ g	9216	99	Lipofection 40 μ l
11	none	149	1	Lipofection
11	rsvCAT	13290	89	Lipofection
11	pSVPr251	8366	56	Lipofection

Exp.no.	DNA	Counts	Times x background	Method/amount
16	pSVPr251	647	1.5	Lipofection
16	pSVPr241	930	2.1	Lipofection

prepared. Using new cells led to an initial increase in efficiency, but this rapidly dropped off as before, and high levels of transfection efficiency could not be achieved consistently. As the only time that PrP had been isolated from the transfected cells was one of the initial experiments where the levels of CAT activity in the transfected cells was 67-70x that observed in the untransfected cells, it was concluded that a high level of transfection efficiency was necessary for expression at detectable levels with these constructs. As this method was unable to consistently produce high levels of transfection efficiency other methods were tried.

Electroporation

Electroporation is a method of introducing foreign DNA into cells by placing the cells and DNA in a specialized cuvette in a suitable buffer and then briefly passing a large electric current through them. This causes temporary pores to open in the plasma membranes of the cells and the DNA enters via these channels. The optimum conditions for electroporation vary greatly from cell-type to cell-type so it is helpful to have some previous knowledge of conditions that have been successfully utilised in the cell-type of interest. In these experiments conditions used for CV1 cells by other workers (Chu et al., 1987, Doncheva et al., 1991) were used as a starting point. A Bio-Rad Gene-Pulser plus capacitance extender were used throughout.

The variables involved in such an experiment include i) media in which the electroporation takes place, ii) voltage, iii) capacitance, iv) whether the cells are stored on ice before and/or after the pulse, v) the amount of DNA, vi)

the % confluency of the cells when harvested and vii) the number of cells per cuvette. All different combinations of these variables were used according to the general protocol described below.

- 1) Cells were grown to near confluency and harvested.
- 2) Cells were resuspended in the medium of choice and counted.
- 3) Cells were resuspended at a density of 5×10^6 cells in $400 \mu\text{l}$ of chosen buffer.
- 4) $10 \mu\text{g}$ (total) DNA was added to each cuvette (precooled on ice) and to this the $400 \mu\text{l}$ of cells was added. All were stored on ice for 2 min.
- 5) The electroporator was set to 250 volts and $960 \mu\text{F}$ capacitance.
- 6) The cuvette was placed in the electroporator and both buttons pressed until the digital display showed "high".
- 7) Samples were stored on ice for 10 min, then plated out into 60mm dish with an appropriate amount of medium and incubated for 48 hours.

Electroporation results

Although this method has been used to transfect CV1 cells (Chu et al., 1987) we were unable to duplicate these results. Every combination of parameters that was tried (Table 2) led to 80-100% cell death and therefore this approach was abandoned. The exact reasons why this technique was so lethal to the cells was not ascertained, but it may be possible that the culture conditions that were used were not optimum for cell survival using electroporation. As described below, new cell stocks grown in DMEM, split 1/4 instead of 1/10 and who had fresh media applied a couple of hours before transfection were successfully transfected by the Calcium Phosphate method, so it is possible that these changes would have influenced transfection efficiency with electroporation also.

Table 2: Electroporation results

EXP.No	No. Cells	Media	Volts	Capc.	Tc	on ice before	on ice after	CAT/% survival
1	1.4X10 ⁶	SFM	250	960	14.7	_____	5min	5 x
2	3X10 ⁶	SFM	250	960	12.5	_____	5min	10%
3	3x10 ⁶	SFM	250	960	12.5	_____	5min	10%
4	3x10 ⁶	SFM	250	960	12.5	_____	5min	10%
5	3x10 ⁶	SFM	250	960	12.5	_____	5min	10%
6	1.4x10 ⁶	SFM	300	960	11.9	_____	5min	all died
7	1.5x10 ⁶	PBS	300	960	11.6	_____	5min	all died
8	1.5x10 ⁶	HEPES	250	960	11.8	_____	5min	all died
9	2.5x10 ⁶	SFM	250	960	13.1	_____	5min	all died
10	2.5x10 ⁶	PBS	250	960	17.9	_____	5min	all died

EXP.No	No. Cells	Media	Volts	Capc.	Tc	on ice before	on ice after	CAT/% survival
11	2.5x10 ⁶	HEPES	250	960	12.0	_____	5min	all died
12	2x10 ⁶	SFM	250	960	14.2	_____	5min	all died
13	1x10 ⁶	SFM	250	960	13.9	_____	5min	all died
14	2.5x10 ⁶	SFM	200	960	21	10min	5min	all died
15	2.5x10 ⁶	SFM	250	500	11.3	10min	5min	all died
16	2.5x10 ⁶	SFM	300	960	15.1	10min	5min	all died
17	2.1x10 ⁶	PBS 0.3ml	250	960	14.7	10min	20min	all died
18	2.1x10 ⁶	PBS	250	960	11.8	10min	20min	all died
19	2.1x10 ⁶	SFM	250	960	12.9	10min	20min	all died
20	2.1x10 ⁶	SFM	200	960	13.2	10min	20min	all died

EXP.No	No. Cells	Media	Volts	Capc.	Tc	on ice before	on ice after	CAT/% survival
10	2.5x10 ⁶	PBS	250	960	17.9	_____	5min	all died
11	2.5x10 ⁶	HEPES	250	960	12.0	_____	5min	all died
12	2x10 ⁶	SFM	250	960	14.2	_____	5min	all died
13	1x10 ⁶	SFM	250	960	13.9	_____	5min	all died
14	2.5x10 ⁶	SFM	200	960	21	10min	5min	all died
15	2.5x10 ⁶	SFM	250	500	11.3	10min	5min	all died
16	2.5x10 ⁶	SFM	300	960	15.1	10min	5min	all died
17	2.1x10 ⁶	PBS 0.3ml	250	960	14.7	10min	20min	all died
18	2.1x10 ⁶	PBS	250	960	11.8	10min	20min	all died

EXP.No	No. Cells	Media	Volts	Capc.	Tc	on ice before	on ice after	CAT/% survival
19	2.1×10^6	SFM	250	960	12.9	10min	20min	all died
20	2.1×10^6	SFM	200	960	13.2	10min	20min	all died

Calcium Phosphate Precipitation

Due to the failure of the above two protocols to produce consistent results a third transfection method, that of Calcium Phosphate Precipitation was tried. A fresh supply of cells were obtained from another laboratory who were using this method, reagents purchased from Promega throughout and used according to the manufacturers instructions (Promega technical manual 1990) as outlined below.

1) 3×10^5 cells were plated out per 60mm dish and grow to 60-70% confluence.

2) The spent media was replaced with fresh media 2-3 hours before transfection.

3) Two tubes were prepared for each transfection as follows:

i) 2x HEPES-buffered saline (HBS)	300 μ l
ii) DNA	10 μ g
2M CaCl ₂	37 μ l
	H ₂ O to 300 μ l

4) (ii) was added to (i) dropwise whilst mixing thoroughly at RT.

This was left at RT for 30 min.

5) Samples were vortexed briefly then added dropwise to the plates and returned to the 37°C incubator for 4 hours.

6) The media containing the CaCl₂ was removed and washed x1 with ordinary media. Fresh media was added and incubated for a further 48-72 hours.

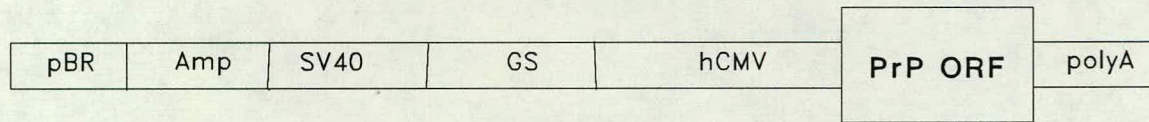
For this cell type it was not necessary to perform a glycerol shock after the four hour incubation and it was important to remove all the CaCl₂ mixture at this point, as leaving it on for longer proved to be very toxic to the cells.

Calcium Phosphate Precipitation results

Once the optimum conditions for these cells had been worked out this method was able to give reproducible results (Table 3, selected results).

With the constructs pSVPr251, pSVPr241 and pSVPr228 transfectants consistently had CAT activities 50-150x greater than those of untransfected or mock transfected cells. Levels of CAT activity for cells cotransfected with the constructs IAH2 and IAH4a (Fig.28) were, however, much lower, in the region of 2-10x background. This was found to be a consistent result, despite the fact that DNA stocks of all the constructs were prepared in exactly the same manner. This method obviously did put stress on the cells, as seen if the Calcium Phosphate mixture was left on the cells for too long, but they were able to recover completely in a short space of time once this mixture had been removed. The high levels of CAT activity in transfected cells (Table 3) showed that high levels of transfection efficiency were being obtained.

Figure 28. IA2/4a



pBR= pBR328 vector sequence
AMP= ampicillin resistance gene
SV40=SV40 early promoter
GS= glutamine synthetase gene
hCMV= human cytomegalovirus promoter-enhancer
polyA = SV40 polyadenylation signal
PrP ORF =PrP wild type or mutated open reading frame

Table 3: Calcium Phosphate results

Exp.no.	DNA	Counts	X background	Method
1	none	162	1	Calcium PO ₄
1	rsvCAT	13034	80	Calcium PO ₄
1	pSVPr251	12198	75	Calcium PO ₄
1	pSVPr241	4851	30	Calcium PO ₄
1	IAH4a	719	4	Calcium PO ₄
1	IAH2	0 all died	0	Calcium PO ₄
2	none	91.6	1	Calcium PO ₄
2	rsvCAT	11884	129.5	Calcium PO ₄
2	pSVPr251	8593	93	Calcium PO ₄
2	pSVPr241	17086	186	Calcium PO ₄
2	IAH2	168	1.8	Calcium PO ₄
2	IAH4a	679	7	Calcium PO ₄
3	none	say 100	1	Calcium PO ₄
3	pSVPr251	20,469	204	Calcium PO ₄
3	pSVPr241	18,395	183	Calcium PO ₄
3	IAH2	671	6.7	Calcium PO ₄
4	none	222	1	Calcium PO ₄
4	pSVPr251	23,452	105.6	Calcium PO ₄
4	pSVPr241	14,582	65	Calcium PO ₄

Exp.no.	DNA	Counts	X background	Method
4	IAH2	372	1.6	Calcium PO ₄
4	IAH4a	1484	6.6	Calcium PO ₄
5	none	429	1	Calcium PO ₄
5	pSVPr251	60163	140	Calcium PO ₄
5	pSVPr241	60935	142	Calcium PO ₄
5	IAH2	2399	5.5	Calcium PO ₄
5	IAH4a	9715	22	Calcium PO ₄

Isolation and analysis of protein from transfected cells and cell supernatants

A number of different strategies were utilized to isolate recombinant proteins from the cells and supernatants of transfected cells.

TX114 extraction

This was performed essentially as described in the previous chapter for cell-free translated proteins with adjustments of volume as described below:

From cells:

- 1) Cells were harvested and washed x2 in PBS.
- 2) Cells were resuspend in 1.5ml of extraction buffer (see chp.4)
- 3) Samples were sonicated for 30 sec. and left on ice for 30 min., centrifuged 10 min, 2000rpm ,at 0°C
- 5)Supernatants were centrifuged at 65,000 rpm in a Beckman TL100.2 rotor for 1 hour at 2°C.
- 6)The supernatants were overlaid onto 1.8ml of 6% sucrose buffer (see

chp.4). (N.B.the pellet from this step was stored and used in the float experiment described below)

7) This was incubated at 30°C for 10 min. and the phases separated by centrifugation.

8) Both detergent and aqueous phases were methanol precipitated.

From cell supernatants:

For extraction from cell supernatants the extraction buffer was made up to 2.8x normal strength and with a starting volume of 3ml of supernatant the procedure was carried out in the same manner as above, adjusting the volumes appropriately.

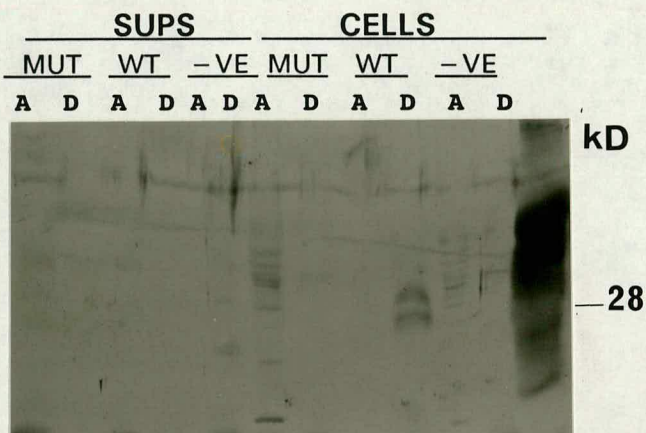
When TX114- extracted proteins were to be treated with the enzyme PIPLC the amount of detergent in the extraction buffer was reduced and the incubation carried out as described in chp.4.

TX114 results

In an initial experiment with TX114-extraction immunoreactive protein was seen in the detergent phase of cells transfected with the wild-type DNA but not in the aqueous phase (Fig.29). An equivalent protein was not seen in cells transfected with the mutant DNA (Fig.29). No proteins were seen in either supernatant fraction of this experiment (Fig.29), but this may have been due to the difficulty in resuspending the large pellet obtained from a starting volume of 3ml. It was thought that it would be necessary to use as much of the supernatant as possible in order to detect the recombinant protein.

To establish whether the TX-114 extraction could extract PrP from cells under our experimental conditions the extraction of PrP from N₂a cells was

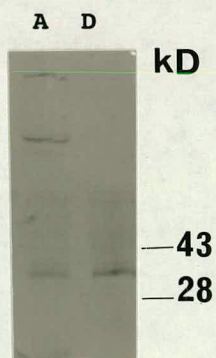
Figure 29. Anti-PrP immunoblot of TX114 extracted proteins from transfected CV1 cells and supernatants.



Lanes

One 35mm dish of cells was washed and harvested and resuspended in 1.5ml of TX114 extraction buffer and supernatants (1.5ml) were extracted with 2.8x extraction buffer and both samples centrifuged as described in the text. The supernatants of this centrifugation were overlaid onto 6% sucrose buffer and phase separation achieved by immersion in a 30°C water bath. Both the detergent (D) and the aqueous (A) phases were methanol precipitated. SUPS= supernatants, MUT= pSVPr241 (Lanes A,B,G,H), WT= pSVPr251 (Lanes B,C, I,J), -VE= mock-transfected cells (Lanes E,F, K,L).

Figure 30. Anti-PrP immunoblot of TX114 extracted protein from N₂a cells.



TX114 extraction was performed on a 35mm dish of N₂a cells as described in the text. D= detergent phase, A= aqueous phase. The gel was blotted with the polyclonal antibody 1B3; blotting with another polyclonal anti-PrP antibody 1A8 gave a similar result (data not shown).

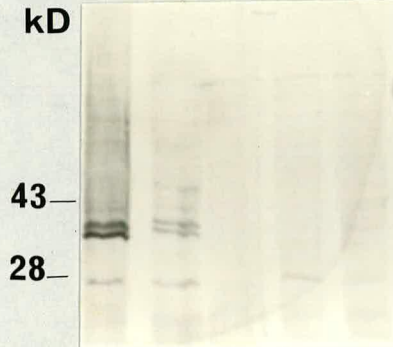
attempted. PrP is an endogenous protein in these cells and would be expected to be present at a lower level than a recombinant protein under the control of a viral promoter. A major immunoreactive band of 33kD was observed (Fig.30) mostly in the detergent phase as would be expected for wild-type PrP which implies that the TX114 extraction and blotting methods are sufficient for the detection of small amounts of the protein. A second round of extraction may have removed the remaining protein from the aqueous phase. Because of the problem of detecting recombinant protein in supernatants, different methods were tried (see below). As described in the transfection results, this failure to detect the recombinant protein led to a thorough examination of the transfection efficiency and no further protein extractions were attempted until a satisfactory system had been established. When TX114-extraction continued to give negative results after a good level of transfection efficiency had been established with the Calcium Phosphate Precipitation method it was undertaken to investigate the state of the RNA. Also the constructs IAH2 and IAH4a (Fig.28) which were known to express the recombinant proteins, were used to test if any of our protein extraction procedures were not working. We also acquired a stably transfected CHO cell line (CHOL761h) (Bebbington 1991) (by permission A.Bennett IAH, Compton) which stably expresses the same construct as IAH2, that is a truncation mutation at codon 231. This was used to compare the protein products with those of the transiently transfected CV1-cells.

Examination of the "float" fraction

In a number of cases it has been found that a proportion of a particular glycoprotein, often a large proportion of the total, is insoluble in TX114. This fraction of the protein can be extracted from the pellet in step 6) in the

Figure 31. Immunoblot of whole cell extracts

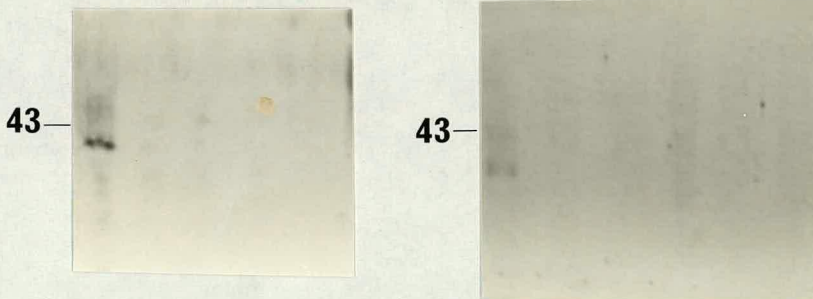
Lanes A B C D E



Cells from a 35mm dish were harvested and washed in PBS before lysis with 1% TX-100 and resuspension in FSB. IAH4a (Lane A), IAH2 (Lane B), pSVPr241 (Lane C), pSVPr251 (Lane B), mock transfected control (Lane E).

Figure 32. Methanol precipitation of supernatants from transfected cells

Lanes A B C D E A B C C D E



250 μ l aliquots of supernatant were precipitated with 4x methanol at -20°C, pelleted, dried and resuspended in running buffer. IAH4a (Lane A), IAH2 (Lane B), pSVPr241 (Lane C), pSVPr251 (Lane D), mock-transfected control (Lane E).

phase of the supernatant can be explained by the fact that only one TX-114 extraction was performed on this fraction. Many workers have found that multiple rounds of extraction are necessary to extract all the hydrophilic protein into the aqueous phase. The protein found in the supernatant shows a pattern of two doublets one of 24-26kDa and the other of 30-33kDa (Fig.34), and is present in much greater amounts than the protein found in the transiently transfected cells (Fig.35, compare Lanes D & A)

Other methods of protein isolation from supernatants of the pSV series of constructs

Firstly, incubating the cells in serum free media for the 48-72 hours post-transfection was tried as a means of reducing the amount of serum proteins present in the media. However, this approach was unsuccessful as the CAT assays were completely negative and nothing was seen on immunoblots indicating that the return to serum positive media after transfection with Lipofectin is an essential step for successful transfection. A Centricon concentrator was used according to the manufacturer's instructions to reduce the volume of the supernatant and run this directly on a gel, but no proteins were detected from any of these samples (not shown).

Radiolabelling proteins with ³⁵S methionine

For pulse-chase radiolabelling of nascent proteins with ³⁵S methionine 35mm dishes of cells were transfected as usual and grown for 48-72 hours and then treated as follows. This experiment was carried out using the pSV series of constructs.

- 1) The supernatant was removed and the cells washed x1 in complete media minus- methionine (Gibco-BRL).

- 2) 0.25ml of complete media minus methionine was added and incubated at 37°C for 1 hour.
 - 3) 120 μ Ci of ³⁵S-methionine (DuPont) was added and dishes incubated for 2 hours.
 - 4) This supernatant was harvested and stored for immunoprecipitation.
 - 5) The cells x3 were washed in PBS, 2ml of standard media added and the dishes incubated for a further 1 hour.
 - 6) This supernatant was harvested and immunoprecipitated .
 - 7) The cells were lysed and immunoprecipitated as described in Chp.2.
- Samples were loaded onto 15% polyacrylamide slab gels, run, dried and exposed as described in Chp.2

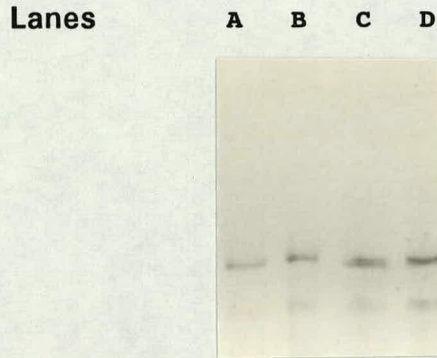
Results of ³⁵S labelling

Labelling newly translated proteins with ³⁵S-methionine and immunoprecipitation is a method whereby small amounts of a particular protein can be detected. This was carried out as described above and no detectable differences could be seen between either the wild-type and mutant or between either of these and the negative control cells (Fig.36). This was true for both cell and supernatant fractions (Fig36). This result implied that the recombinant proteins were not being expressed at all and at this point repetition of the TX114 experiment was attempted unsuccessfully and reexamination of transfection efficiency undertaken (as above).

Blotting with anti-CRD serum

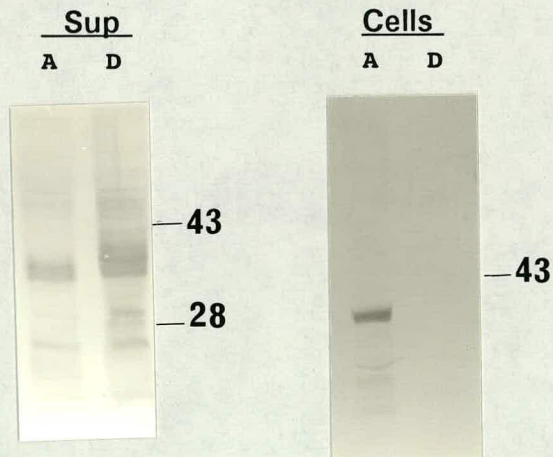
PrP contains a CRD epitope when it has been cleaved with PIPLC and in its

Figure 33 Methanol precipitation of supernatants from the stably transfected cell line CHOL761h.



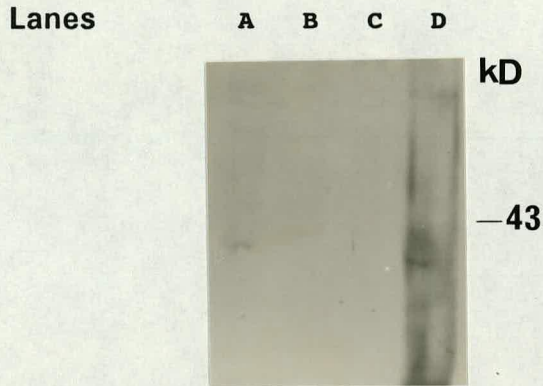
Aliquots of cell supernatant of increasing volume were precipitated with methanol, pelleted, dried and resuspended in running buffer. The gel was immunoblotted with antibody 1A8. Lane A 50 μ l, Lane B 100 μ l, Lane C 200 μ l, Lane D 250 μ l.

Figure 34. Immunoblot of TX114-extracted protein from CHOL761h cells and supernatant.



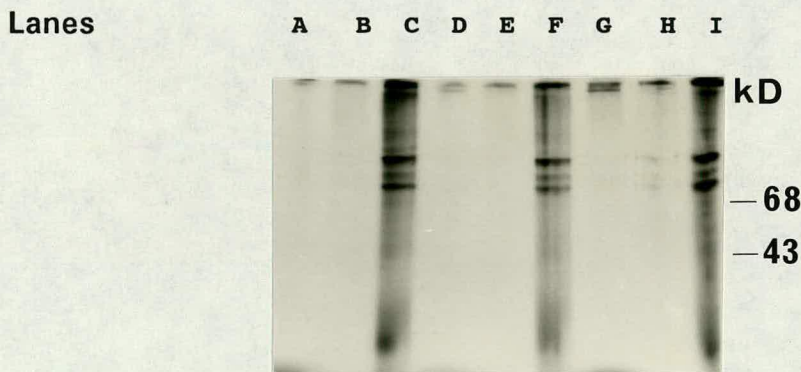
Cells were extracted with TX114 as described previously, supernatants were first methanol precipitated and then resuspended in TX114 extraction buffer, and subsequently treated as other samples. A= aqueous, D= detergent.

Figure 35. Comparison of methanol precipitated protein from cell supernatants of different transfectants.



150 μ l aliquots of supernatants from cells transiently transfected with the IAH constructs were compared to those taken from the supernatant of the stable cell line CHOL761h. Each was methanol precipitated and immunoblotted with the antibody 1A8. IAH4a (Lane A), IAH2 (Lane B,C), CHOL761h (Lane D).

Figure 36. Radiolabelling of proteins with 35 S-methionine and immunoprecipitation with the polyclonal antibody 1B3.

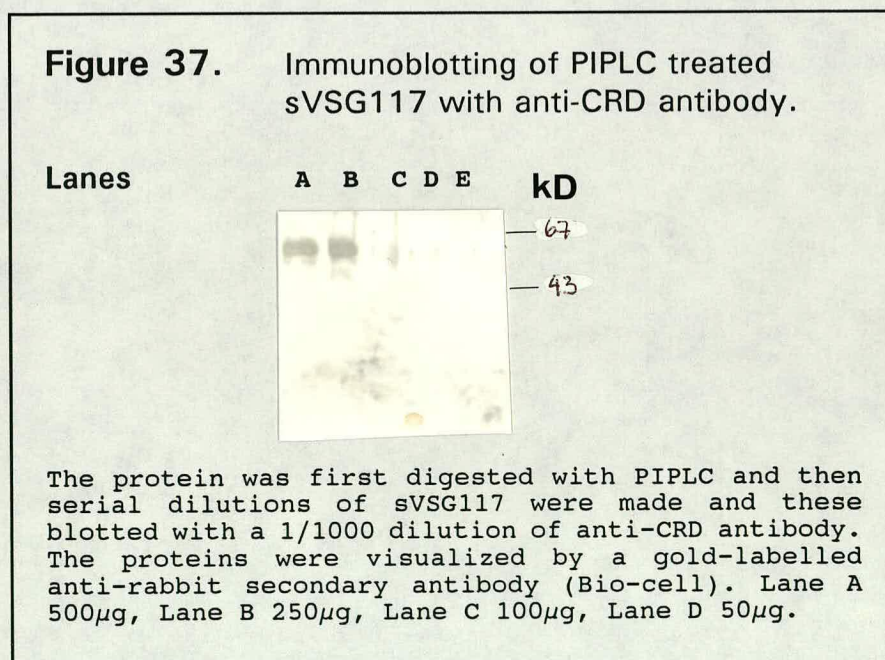


35mm dishes of cells were first washed with, and then incubated in, media minus methionine, after which 35 S-labelled methionine was added to each dish (120 μ C) and the dishes incubated at 37°C for one hour. At this time the supernatant was removed and stored on ice (1 hour sup) and replaced with standard media and incubated for a further two hours. Then this supernatant (3 hour sup) was removed and immunoprecipitated along with the one hour supernatant and the cells themselves. pSVPr241 (Lanes A,B,C), pSVPr251 (Lanes D,E,F), mock transfected control (Lanes G,H,I) 3 hour supernatant (Lanes A,D,G), 1 hour supernatant (Lanes B,E,H), cells (Lanes C, F I).

GPI anchored form should react with anti-CRD antibodies after such cleavage. A soluble or non GPI-anchored form should not react with such an antibody after PIPLC treatment. A whole-rabbit serum raised against an intact soluble form of a *T.brucei* VSG was obtained as a gift from M.Ferguson, along with some purified sVSG117 to act as a positive control. The antibody was used at a dilution of 1/1000 and visualized by a gold-labelled anti-rabbit 2 anti-body (Bio-cell) in the manner described in chp.2.

Anti-CRD immunoblot results

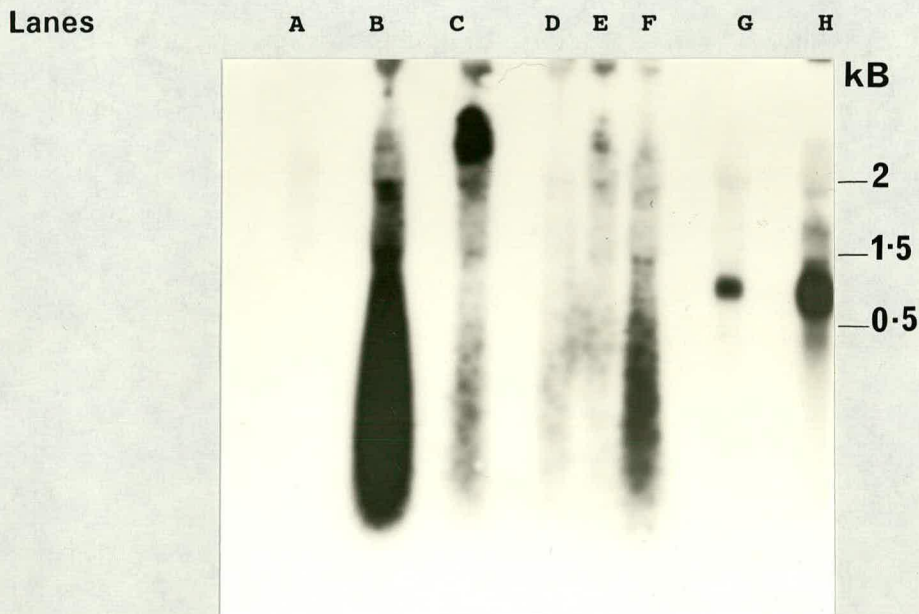
A titration experiment with our 2 antibody system showed that up to 250ng of sVSG117 protein per sample was necessary in order to get a positive signal (Fig.37) Negative results were obtained with all the transiently transfected constructs of PrP, both wild-type and mutant . This was possibly because the amounts of protein present in the samples were not very high before PIPLC treatment and thus the CRD epitope was undetectable in this system.



Total RNA extraction and Northern blots

Total RNA was extracted from cells by use of the reagent RNAzol, and blots and hybridizations carried out as described in chp.2.

Figure 38. Northern hybridization of total RNA from transiently transfected CV1 cells.



Total RNA was prepared from transfected cells using RNAzol, a one step preparative method, and separated on formaldehyde gels, before blotting onto Genescreen membrane. These were then hybridized with a double stranded probe prepared from PrP ORF cDNA. All samples on this gel were transfected and harvested at the same time with all the same reagents. Mock-transfected control(Lane A), pSVPr251 35mm dish (LaneB), pSVPr241 35mm dish (Lane C), pSVPr251 60mm dish (Lanes D,E), pSVPr241 60mm dish (Lane F), IAH2 (Lane G), IAH4a (LaneH).

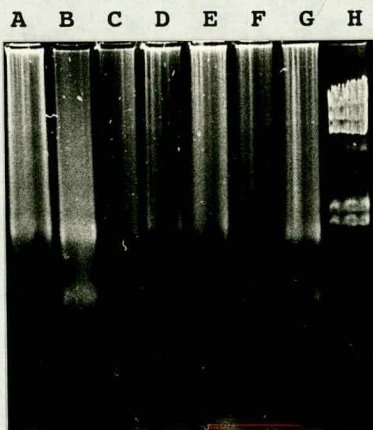
Northern hybridization results

RNA was extracted from CV1 cells transiently transfected with pSVPr251, pSVPr241, pSVPr228, IAH2 and IAH4a, blotted and hybridized with a probe made from the PrP ORF as described in Chp.2. Stringent washing steps at 65°C gave the pattern observed in Fig.38. RNA from the two IAH constructs existed as a single band of the predicted size (900bp) whilst RNA from the other constructs appeared on the autorad as a band and large smear. As all the transfections were carried out on the same batch of cells at the same time, and all RNA preparation and handling was done with the same reagents at the same time it is highly unlikely that the RNA from one set of constructs should be degraded by contaminating RNases and the other not. Also, visualization of ribosomal RNA for each sample on agarose gels before blotting (Fig.39) showed similar patterns for all the samples, with no obvious degradation in the pSV construct RNA. Therefore, it was concluded that the RNA from the pSV constructs was being degraded in the cells whilst the IAH RNA was not.

Cotransfection of RNA polymerase

Figure 39. Agarose electrophoresis of aliquots of total RNA prepared from transfected CV1 cells.

Lanes



One tenth volume of total RNA samples prepared from either 35mm or 60mm dishes was run on a 1% agarose gel and stained with ethidium bromide. pSVPr241 60mm (Lane A), pSVPr251 60mm (Lane B), IAH4a 35mm (Lane C), IAH2 35mm (Lane D), pSVPr241 35mm (Lane E), pSVPr251 35mm (Lane F), mock-transfected control 35mm (Lane G), λ HindIII size markers (Lane H).

From the results of the Northern analysis (Fig.38) it was concluded that the RNA expressed from the SV40 promoter of the pSV series of constructs was being degraded in the cells. As these constructs also contain a T7 promoter, from which the recombinant PrP genes were successfully expressed in the cell-free system (see Chp.4), it was decided to attempt a new technique that has been developed recently whereby T7 RNA polymerase is cotransfected with the construct (Gao and Huang 1993) and can drive expression of a foreign gene controlled by the T7 promoter. These workers found that expression of a reporter gene was transient, declining after 30 hours, consistent with the notion that T7 polymerase does not enter the nucleus and the transcription takes place in the cytoplasm of the transfected cells.

The previous workers used Lipofection to achieve this transfection, but bearing in mind the difficulties experienced using this system with our cells both Lipofection and Calcium Phosphate precipitation were used in these experiments. A Luciferase gene under the control of the T7 promoter (pPOLY(A)-luc(T7) , Promega Technical Bull. 126) was cotransfected along with the pSV series of constructs and purified T7 polymerase to act as a reporter gene. The Promega Luciferase assay system (Promega technical bulletin no.101) was used to monitor luciferase activity according to the manufacturers instructions. A general outline of the technique is given below.

- 1) The cells were transfected according to the usual protocol, including 1 μ g of the luciferase and test constructs along with 100units of purified T7 polymerase (Promega).
- 2) The cells were incubated for 30 hours at 37°C in 6% CO₂.
- 3) The cells were washed x2 with PBS.
- 4) Cells were overlaid with 150l of 1x Promega cell lysis buffer (25mM Tris-phosphate pH 7.8, 2mM DTT, 2mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton X-100) and left at RT for 10-20

min.

5) Cells were scraped off into an Eppendorf and pulsed in a microfuge to bring down the cell debris.

6) 100 μ l of Luciferase assay reagent (20mM Tricine, 1.07 mM [MgCO₃]4Mg[OH]2.5H₂O, 2.67mM MgSO₄, 0.1mM EDTA, 33.3mM DTT,, 270 μ l coenzyme A, 470 μ l luciferin, 530 μ l ATP) was added to 20 μ l aliquots of each sample and immediately read in a scintillation counter for 10-20 counts of 10 sec.

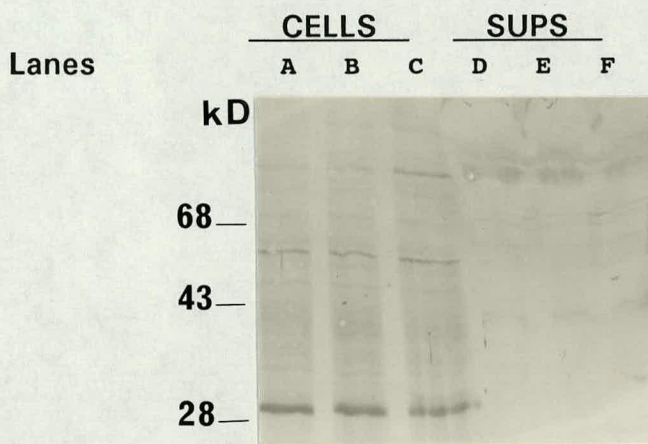
Co-transfection of RNA polymerase results

The reaction catalysed by firefly luciferase is the oxidation of beetle luciferin with concomitant production of a photon. When luciferase activity is present the light intensity of the reaction is constant for about 20 sec., then decays slowly, with a half-life of about 5 min. When no luciferase activity is present the background counts do not decay and remain fairly constant over the time period of the assay. In repeatable experiments, mock-transfected cells gave such a signal, with the number of counts remaining about the same over the 3-4 min of the assay.

Cells that had been cotransfected with the luciferase constructs and either the wild-type or mutant PrP pSV construct (pSVPr251 and pSVPr241 respectively) had much higher initial counts and these declined steadily over the course of the assay. This implies that the luciferase gene was being successfully transcribed from the T7 promoter by the action of the co-transfected T7 polymerase. It was hoped that this would mean that the recombinant PrP genes would also be transcribed from the upstream T7 promoter and that as in the cell-free system, this RNA would remain stable and be translated in the cell into protein. Therefore, aliquots of cell lysates and methanol precipitated aliquots of cell supernatants were examined for the presence of PrP protein by SDS-page and immunoblotting as previously described. Fig.40 shows that neither the wild-type or mutant cells or

supernatants contained any immunoreactive proteins that were not seen in the negative control. Time constraints prevented investigations as to whether stable specific PrP mRNA was produced and as no proteins were being produced this technique was not pursued further.

Figure 40. Expression of pSV constructs from the T7 promoter in tissue culture by cotransfection of T7 polymerase.



100 units of purified T7 polymerase was cotransfected into CV1 cells along with the constructs pSVPr251 and pSVPr241, both of which contain a PrP ORF behind a T7 promoter. A luciferase reporter plasmid containing a T7 promoter was also cotransfected. The cells were lysed and part used in a luciferase activity assay whilst the remaining cell lysate was examined for the presence or absence of PrP protein. Supernatants were methanol precipitated and cells and supernatants blotted with 1A8. Mock-transfected cells (Lanes A,D), pSVPr251 (Lanes B,E), pSVPr241 (Lanes C,F).

Conclusions

During the course of these experiments it was discovered that the transfection efficiency for these cells varied greatly from one technique to another. The lipofection technique initially gave very good results, with high levels of CAT activity being achieved, but these levels quickly deteriorated. The exact cause of this decline in transfection efficiency was never pinpointed as replacing the media, cells, reagents and DNA with new stocks and altering the conditions of the transfection reaction did not consistently give rise to better results. The conditions used mimicked those used by previous workers with these cells (Felgner et al., 1987) but it was concluded that the low levels of transfection generally observed would preclude detecting low levels of recombinant protein. Electroporation of these cells proved to be equally difficult, with all combinations of conditions leading to cell death. The cells were regularly checked for mycoplasma infection and appeared morphologically healthy, so the reason for their sensitivity in this system remains unknown.

A number of changes in the general maintenance of the cells were adopted when the switch to calcium phosphate precipitation transfection was made. Firstly, the media was changed from MEEM to DMEM, this having no effect on the growth of the cells but the latter was suggested to be a better media for transfections (K.Chapman pers.commun.). Secondly, the ratios at which cells were divided were altered from 1/10 to 1/4 when cells were to be transfected, and fresh media was applied to the cells 2-3 hours before the transfection. These measures were designed to avoid stressing the cells as much as possible and to allow them to recover after the transfection. Even so, some cell death did occur whilst the Calcium Phosphate solution was on the cells and the timing of this step was crucial as, if left on too long it was highly toxic to the cells. This method was very reproducible once the optimum parameters had been established. It is not known why one set of

constructs based on the pSVK3 vector (Fig.10) led to much higher levels of CAT activity than another based on the pEE14 vector, (Fig.28), (Bebbington, 1991), whilst the amounts of recombinant protein obtained from the former were so much lower than from the latter. The pEE14 vector contains the major immediate-early gene promoter-enhancer of human cytomegalovirus (hCMV) as well as the 5' untranslated region and the leader intron and a polyadenylation signal from SV40 . This is followed by a multi-cloning site , into which the PrP wild-type and mutated ORFs have been cloned (Fig.1).

It also contains a glutamine synthetase selectable marker under the control of the SV40 early promoter to enable the establishment of stable cell lines. From these experiments it would appear that these constructs give rise to a high level of expression of the recombinant proteins in these cells. The reason for the degradation of the pSV construct RNA is not understood, as these constructs gave rise to abundant recombinant protein in the cell free system (Chp. 4) from their T7 promoters. As all stocks of DNA were prepared in the same manner, the same CAT vector being used in each case and the fact that making of fresh stocks did not alter this pattern of CAT activity led to the conclusion that some intrinsic difference in the nature of the vectors was governing these properties.

When high levels of transfection were achieved PrP immunoreactive protein was observed in the detergent phase of TX114-extracted cells transfected with the wild-type construct pSVPr251, whilst none was visible in cells transfected with the mutant construct pSVPr241 . No PrP was precipitated by methanol from the supernatants of the wild-type transfected cells at any time whilst trace amounts were seen in those of the mutant-transfected cells, although this was not always reproducible (Fig. 32) The finding of the wild-type protein in the detergent phase of the cells (Fig.29, Lane J) is indicative of its being a GPI-anchored protein, whilst, as we would expect, little protein is found in the mutant-transfected cells. Most of the mutant

protein would be expected to be found in the supernatant, if indeed it has no GPI-anchor attached. The results of the Northern hybridization experiments suggest that the RNA is being degraded. If this happened only on some occasions or at a slow rate, then some protein could be translated before the RNA was degraded and this could account for the presence of immunoreactive protein in some experiments and its very low levels or absence in others.

The pSVK3 vector contains an SV40 small T antigen splice site and SV40 early polyadenylation site and is designed to be an expression vector, so the RNA should be capped and have a poly A tail and would be expected to be stable *in vitro*. The constructs also retain the original six bases 5' to the start codon, ATG, as these contain the Kozac consensus sequence for the correct initiation of translation (Kozac, 1983), so initiation of protein synthesis should proceed efficiently from the correct start codon.

From experiments using the constructs IAH2 and IAH4a we can see that the wild-type (IAH2) is contained mostly in the cells (Fig.31), although very small traces of it are found in the supernatant (Fig.32). Some endogenous PrP is shed from the surface of N₂a cells in tissue culture in a soluble form (Caughey et al., 1989; Borchelt et al., 1993), but this is a small proportion of the total. The mutant (IAH4a), however, is found predominantly in the supernatant (Fig.32), with some protein still found in the cells (Fig.31).

In the mutant protein from the stably transfected cell line CHOL761h protein found in the cells partitioned into the aqueous phase, whilst the vast majority of the protein was found in the supernatant (Fig.34). This implies that the mutant protein does not have a GPI-anchor. The pattern of banding of this protein implied that it is properly glycosylated and only differs from the wild-type by the absence of the GPI (Fig.33).

These results have outlined the difficulties of working with transient expression, when even using a strong promoter like that of the cytomegalovirus the recombinant protein is not always abundant or easily

detectable (IAH2). Even when using a cell line that has frequently been used previously the difficulties of achieving efficient transfection are evident. The presence of the mutant protein in the cells and supernatant does not have an obvious pathogenic effect on the cells, which appear morphologically normal.

From these results it can be concluded that the removal of the hydrophobic amino acids from the extreme C-terminus of the PrP protein removes the signal for GPI-addition, and these proteins are then subsequently secreted into the media of tissue culture cells.

Introduction

This project was designed to investigate whether disruption of the post-translational addition of a GPI-anchor to PrP could account for the observed differences between the normal and disease specific forms of the protein and also to investigate whether GPI addition has any significance for the normal function of the protein. This was investigated by engineering a murine PrP protein in *in vitro* systems from which the signal sequence for GPI-addition has been deleted. A full length protein has been produced in the same systems so that direct comparisons between the normal and mutated forms can be made. These *in vitro* studies have allowed detailed biochemical study of the mutated protein and have shown that GPI addition to PrP follows the same general rules that apply to other glypiated proteins. Removal of the C-terminal amino acids leads to the failure of GPI-addition and the subsequent secretion of the majority of the protein from the cell. The mutated protein appears to be glycosylated in a similar manner to the wild type protein, although the molecular weight of these glycosylated forms is lower than that observed for PrP extracted from mouse brain. This result is analagous^{to} other observations with recombinant PrP in cell free systems and tissue culture and is thought to result from the inability of these systems to add complex glycans (Caughey et al, 1989) Overexpression of the mutated PrP in Monkey Kidney cells (CV1) was not toxic for the cells and did not lead to any obvious morphological changes. The possible significance of these mutations for the disease process will be examined *in vivo* by the production of transgenic (Tg) mice carrying these mutations. The replacement of normal PrP gene with the mutated forms is being carried out using homologous recombination in embryonic stem cells in this laboratory (J.Manson).

Conversion of PrP^C to PrP^{Sc}

Anchorless forms of PrP.

Scrapie and other related transmissible encephalopathies lead to the accumulation in the brain and spleen of a disease specific form of a membrane protein, PrP. The disease specific form (PrP^{Sc}) differs in its physical and chemical properties from the normal form (PrP^C) although no covalent changes between the two have yet been discovered (Stahl et al., 1990). These differences are believed to be governed by a post-translational change. Both PrP^C and PrP^{Sc} have been shown to contain GPI anchors (Stahl et al., 1987). During the course of this work it became possible to purify larger quantities of the normal PrP protein and consequently a more detailed chemical analysis of it has been carried out (Stahl et al., 1993). These analyses have up until now been unable to detect any differences in the GPI-anchors of PrP^C and PrP^{Sc}. This, however, does not preclude the possibility that a small amount of an anchorless form of the protein could act as either a seed for amyloidogenesis or a catalyst for the conformational conversion of the normal protein to the disease specific form (Come et al., 1993). As the ratio of PrP^{Sc} molecules to infectivity is 1:10⁵ the aberrant molecule may escape detection (McKinley et al., 1983). Disease specific PrP extracted from hamster brains contains a significant fraction (~ 15%) that is truncated at its C-terminus and does not contain a GPI-anchor (Stahl et al., 1990). It is not known if this truncated protein has any significance in the formation of PrP^{Sc}. Also, at least three cases of CJD have been associated with a mutation in the PrP gene that would lead to a protein truncated at its C-terminus (Kitamoto et al., 1993). However, it has also been shown recently that a C-terminally truncated form of PrP is shed from the surface of primary cultures of uninfected hamster brains and also in homogenates of uninfected hamster brains, accounting for about 10% of the total PrP (Borchelt et al., 1993). Recombinant hamster

PrP overexpressed in CHO cells also gave rise to a shed form of PrP, again constituting approximately 10% of the total, indicating that it is not a shortage of GPI-precursor that gives rise to the shed form of PrP, but rather a cleavage event. Endogenous PIPLC activity has been ruled out as these shed forms do not react with anti-CRD antibodies. A truncated form of PrP which does not contain a CRD epitope has also been detected in the CSF of normal patients (Tagliavini et al., 1992). These results suggest that a non-anchored form of PrP may normally be produced *in vivo*.

Subcellular location of PrP^{Sc} synthesis

As the precise molecular nature of the changes between PrP^C and PrP^{Sc} are not known discovering where in the biosynthetic pathway this conversion takes place may give clues as to what happens. Evidence from work in tissue culture cells has shown that PrP^{Sc} is synthesised from a protease sensitive precursor that is almost certainly PrP^C (Caughey and Raymond, 1991). PrP^{Sc} synthesis does not occur if the cells are treated with Brefeldin A, an anti-fungal drug that causes the dissolution of the Golgi stacks (Taraboulos 1992). Thus, the endoplasmic reticulum alone is not sufficient for synthesis. Also, removal of PrP^C from the cell surface with PIPLC or Dipase prevents PrP^{Sc} synthesis (Caughey and Raymond, 1991, Borchelt et al., 1992). PrP^{Sc} has been found to accumulate in cytoplasmic vesicles identified as secondary lysosomes, but treatment with lysosomotropic amines does not prevent its synthesis (McKinley et al 1991, Caughey and Raymond, 1991, Taraboulos et al., 1992). From these lines of evidence it has been concluded that transit of the precursor to the cell surface is necessary and PrP^{Sc} probably occurs in an endosomal compartment before reaching the lysosome. PrP^C is rapidly degraded in the lysosome, whereas PrP^{Sc} undergoes only some N-terminal trimming and shows no further degradation, thus leading to its fatal accumulation.

The possibility does exist that the conversion might take place at the surface

of the cell, or that PIPLC or other enzymes enter the cell prior to digestion. The mechanisms whereby PrP^C or PrP^{Sc} are taken up by the cell are not known. PrP has been associated with non-clathrin coated invaginations called caveolae, as are other GPI-anchored proteins, and may be internalized through these patches. Alternatively, free flow endocytosis may be responsible. Recent work with the avian homologue of PrP, chPrP, has shown that this protein undergoes multiple rounds of recycling from the cell surface to an early endosomal compartment (Shyng et al., 1993) About 5% of the protein is cleaved near the N-terminus before it is returned to the cell surface and these truncated forms appear to have greater stability at the cell surface than the intact molecule and may be endocytosed less efficiently. From the above an endosomal location for the PrP^C to PrP^{Sc} conversion is envisaged.

Non-glycosylated PrP

Two different methods have been used to produce PrP molecules that are not glycosylated in *in vitro* systems. Firstly, treatment with tunicamycin, a drug which prevents N-linked glycosylation, and secondly, site directed mutagenesis of the consensus sites for addition of N-linked oligosaccharides (Taraboulos et al 1990, Caughey et al., 1989, Rogers et al., 1990). One group reported that none of the mutant protein was transported to the cell surface and another that the unglycosylated protein did reach the cell surface, but at a much slower rate than the fully glycosylated protein (Rogers et al., 1990, Taraboulos et al., 1990 Caughey et al., 1989) The first group found that despite this, in scrapie infected cells, a 19kD protease resistant form of PrP was produced. This raises the possibility of an alternative pathway for the cellular processing of the unglycosylated protein (Olden K. et al., 1982). The unglycosylated protein was also converted to PrP^{Sc} more rapidly than the fully glycosylated protein, leading to the speculation that it may be intrinsically less stable than the fully glycosylated

one (Taraboulos 1990).

C-terminally truncated PrP in Scrapie infected N₂a cells

Recently workers in another laboratory have produced C-terminally truncated PrP in scrapie infected N₂a cells (Rogers et al., 1993) They have shown that this truncation (from codon 231) gives rise to a protein in the cells that is approx. 2kD smaller than wild-type when both are treated with tunicamycin. This protein was not sensitive to degradation with 50% hydrofluoric acid (HF), which hydrolyzes phosphodiester bonds and should result in the removal of a GPI-anchor, whereas the wild-type exhibited a reduction of approximately 2kD. Even in the absence of tunicamycin the truncated protein was very poorly glycosylated compared to the wild-type. After Proteinase K treatment a small amount of protease resistant PrP was seen in these cells, suggesting that the protein without the GPI-anchor is still a substrate for conversion to PrP^{Sc}. These proteins have been engineered to contain an epitope recognised by anti-hamster PrP antibodies in order to recognise them against the endogenous mouse PrP of the cells in which they are expressed. This work does not refer to a secreted protein and whether such a protein was protease resistant or not.

Our results with similar mutants in CV1 cells do not give identical results to the above experiments. The bulk of the PrP synthesized in these cells is secreted (Fig.s 32,33,34,35) and appears as a broad band or doublet of approx.31-33kD. Exact sizing in this system has proved difficult as the PrP polyclonal antibody 1A8 used in these immunoblots tends to stain the markers also. The molecular weight of the observed proteins and the fact that they are secreted suggests that these proteins follow the normal biosynthetic pathway for PrP^C and are glycosylated during their transit to the cell surface (Caughey et al., 1989). The protein found in the supernatant of the stably transfected cell line CHOL761h lends particular support to this theory as the pattern of bands observed resembles that seen

for the wild-type as seen in cell free systems (Fig.14, Lanes A and C). This result needs to be confirmed by the treatment of these proteins with a deglycosylase, such as PNGase F and will be carried out on mutant protein isolated from transgenic mice. TX114 was carried out on these proteins (Fig.34) but the protein in the supernatant was not completely extracted into the aqueous phase as would be expected for a non-anchored protein, but this may prove to be simply a technical question of repeating the extraction more than once, as the quantity of protein in the supernatant fraction is large. Immunoreactive PrP was also found in the cells of both cells transiently transfected with the truncated protein (Fig.31, Lane A) and also in the stably transfected cells (Fig.34). In the transiently transfected cells this protein exactly resembles that seen in whole cell extracts from cells transfected with the wild-type construct (Fig.31). In the stable cell line CHOL761h, the PrP in the cell is found exclusively in the aqueous phase on TX114 extraction, suggesting that it indeed does not contain a GPI-anchor.

From the above results it can be concluded that in this system most of the mutated PrP follows the normal pathway of intracellular trafficking and is transported to the cell surface where it is not anchored due to its lack of a GPI anchor. Some PrP, which also does not contain a GPI anchor, is seen to remain in the cell. Whether this protein is glycosylated or not is not certain. This may represent protein that has not yet been secreted or indeed, may represent a minor alternative pathway of intracellular trafficking. If this is a minor pathway and the bulk of the mutant protein is secreted this may explain why Rogers et al. were only able to see very small amounts of PrP^{Sc} with their mutants in scrapie infected cells. Thus PrP^{Sc} can still be produced from an anchorless precursor, but such a mutant may be very inefficient at its production. This may be additional support for the idea that most PrP^{Sc} is produced from PrP^C after it has been endocytosed (Caughey et al.1991a,1991b, Borchelt 1990) from the cell surface.

These results also imply that for the majority of PrP the absence of the GPI anchor does not effect its intracellular targetting. GPI anchors have been established as markers for apical targetting in certain epithelial cell lines (Lisanti et al, 1991), but their role in targetting in neural cells may be much more complex (Dotti et al 1991, Faivre-Sarrailh 1993). The possibility exists that sub-populations of PrP exist that behave in different ways and it is one of these that gives rise to an alternative pathway of intracellular trafficking. The possibility that these possible sub-populations may be defined by differences in their GPI anchors is illustrated, for instance, by the existence of heterogeneity of release of GPI anchored proteins from different cell lines with PIPLC derived from either *Bacillus thuringiensis* or *S.aureus* (Low et al 1988). PrP GPI has been shown to have at least six different glycoforms (Stahl 1992), the significance of which is not yet known.

PrP topography in cell-free systems

Cell-free translation of recombinant PrP in two different systems has given rise to two different topographies for the protein (Hay et al., 1987a, 1987b). When the wheat germ extract (WG) system was used a predominantly transmembrane form was produced (Hay et al., 1987b), whilst the RRL system gave rise to a fully translocated protein (Hay et al., 1987a). These studies gave rise to the discovery of a stop transfer effector sequence in PrP and interaction with a cytosolic factor was implicated in effecting these differences (Lopez et al., 1990). Membrane interactions may have some significance for PrP and scrapie as infectivity has long been associated with membrane fractions.

In the cell free work undertaken here with the RRL system and C-terminally truncated PrP an extra band of ~28kD was identified (Fig.s 14 and 15) in both the mutants pSVPr241 and pSVPr228, but not in the wild-type pSVPr251. This does not appear to be a glycosylation intermediate as it is

unaffected by treatment with the deglycosylase PNGase F, whereas the larger 33kD band is completely degraded by this enzyme (Fig.22). Neither does it appear to be an integral membrane form of the protein, as it is fully protected from proteinase K digestion in the absence of detergent (Fig.11-20), implying that it is a fully translocated form. This band does not appear to be present in the truncation mutant IAH4a when expressed in CV1 cells (Fig.31), nor is it present in PrP extracted from the stable cell line CHOL761h (Fig.33 and 34). The pSV series of mutants did not produce enough protein in tissue culture to ascertain whether or not this band was present. Therefore it is not possible to state whether it is produced as a product of cell free translation or as a product of these particular constructs.

Conformational changes in PrP-the "protein only" hypothesis

Theories have arisen to explain the replication of the infectious agent in the absence of a nucleic acid which suggest that an incoming molecule of PrP^{Sc} is able to interact with existing PrP^C and change its conformation such that it acquires the properties of PrP^{Sc} (Prusiner, S., 1992, McKinley et al., 1991, Pan et al., 1993). Proteins that form amyloid have been shown to contain a high proportion of β -sheet conformation as opposed to α -helix (Kisilevsky 1987). Recent work with synthetic peptides corresponding to parts of the PrP protein has shown that some peptides predicted to have α -helix conformation actually adopt a β -sheet conformation *in vitro* (Gasset et al., 1992). As shown by electron microscopy and Congo Red staining these peptides spontaneously form amyloid and this leads to the suggestion that the conversion of PrP^C to PrP^{Sc} involves the changing of PrP α -helices to β -sheets (Pan et al., 1993).

The existence of scrapie strains are accounted for ⁱⁿ this model by the proposal that the incoming PrP^{Sc} molecule converts existing PrP^C into an exact replica of itself and that these molecules differ from each other in

some way other than primary sequence, for instance differential glycosylation (Endo et al., 1989, Prusiner, S., 1993) . Such variations might govern the interactions of the incoming PrP^{Sc} with only subsets of existing PrP^C molecules, thus explaining the influence of primary PrP sequence on incubation periods (Westaway et al., 1987) or target it to specific sets of neurons, thus explaining the diverse and reproducible patterns of pathology observed with different strains of scrapie (Bruce et al 1991).

It has been found that the expression of certain GPI anchored proteins is regulated between different types of neuron (Rosen et al 1992) . These workers found variations in the relative abundance and degree of glycosylation of GPI-anchored proteins including Thy-1, F3/F11 and the 120kD form of N-CAM on superior cervical ganglion neurons, dorsal root ganglion neurons and granule cell neurons. Whether GPI-anchored proteins are axonally or dendritically targetted also seems to be subject to regulation (Faivre-Sarrilha et al., 1992) Thus the possibility exists that differential expression or glycosylation of PrP on neuronal cell surfaces may give rise to subsets of cells that are more susceptible to infection, or to infection with a particular strain of scrapie. The finding that PrP from different sources has different solubilities with respect to extraction with the detergent TX114 is also interesting in this respect (see "Float" experiments Chp.5). In this case I was unable to detect any PrP in the insoluble pellet formed when CV1 cells were extracted with TX114, but in this laboratory other workers routinely find that up to 50% of the PrP from infected and uninfected mouse brain is found in this fraction (J.Hope, pers.commun.). This again suggests the existence of different forms of PrP in different cell types that may exhibit very different behaviours.

It is also possible that the infectious agent replicates by interfering with the folding process of normal PrP and interacts with the foldases and chaperones that control the correct configuration of the molecule, rather than having a direct interaction with PrP itself (Wills, P., 1992). Forms of

these diseases exist in humans (Will, R., 1993, Tateishi 1993, Gambetti 1993) and thirteen different mutations in the PrP gene have been found to segregate with human TE type diseases (CJD, GSS, FFI). It is thought that at least some of these act by lowering the activation energy required to convert PrP^C to PrP^{Sc} (Weissmann 1993). Association of PrP^C with PrP^{Sc} is seen to lower this necessary activation energy even further, whilst the spontaneous conversion of PrP^C to PrP^{Sc} -a very rare event- has the highest activation energy of all (Gadjusek 1988). The occurrence of spontaneous neurodegenerative disease in Tg mice bearing the codon 101 (equivalent to the human codon 102 leucine-proline substitution associated with GSS) has been put forward as evidence supporting the supposition that the abnormal PrP protein alone is sufficient to cause the disease (Hsiao et al., 1989).

Inhibitors of PrP^{Sc} formation

A number of different substances have been shown to inhibit the production of PrP^{Sc} both in cells and *in vivo* and also to prolong incubation periods in mice and hamsters (Caughey and Race, 1992, Caughey et al., 1993, Caughey and Raymond, 1993, Ladogana et al., 1992, Xi et al., 1992, Diringer et al. 1991, Farquhar, 1986). These prominently include the sulphate polyanions and the dye Congo Red, which has long been used to stain amyloid. The mechanism of inhibition is not fully understood but seems to work by preventing the formation of PrP^{Sc}, having no effect on preexisting PrP^{Sc}. A comparison of the inhibitory activities on PrP^{Sc} formation of these compounds in tissue culture correlated well with their prophylactic qualities *in vivo* (Caughey 1993), suggesting that it is indeed this ability to prevent PrP^{Sc} formation that gives them their anti-scrapie qualities. It is proposed that these substances are analogues of cellular sulfated glycosaminoglycans (GAGs) which may be involved in the formation of amyloid deposits and act by preventing PrP-GAG interactions (Caughey 1993).

The mechanism of action of Amphotericin B, which was shown to delay the accumulation of PrP^{Sc} without altering scrapie replication (Xi et al., 1992) is likely to have a different mechanism of action as it is not polyanionic in nature.

Application of such inhibitors to prevent amyloidogenesis and thus reduce clinical symptoms is being further investigated.

Normal function of PrP

The normal function of PrP is unknown, but the high level of conservation at both RNA and protein levels amongst mammalian species argues that it may be an important one.

The cell surface location and GPI anchor of PrP suggest a number of possible functions. It may act as a receptor and could be involved in receptor-mediated internalization of ligands. The finding that the avian homologue of PrP, chPrP, is constitutively recycled from the cell surface to an early endosomal compartment would support such a role (Shyng et al 1993). PrP's association with caveolae, along with other GPI anchored proteins might suggest a ligand internalizing role.

Due to its homology with chPrP, which was first isolated as a factor inducing acetylcholine receptors in chicken motoneurons, a similar role was proposed for mammalian PrP (Harris et al., 1992). However, this proposal has been negated by the fact that the regulation of cholinergic receptor expression in Tg mice, in which the PrP gene has been ablated (null mice, PrP^{0/0}) and no PrP protein is produced, is completely normal (Brenner et al., 1992)

Its cell surface location, GPI anchor and embryonic expression (Manson et al., 1992) also suggest a possible function as a cell-cell adhesion factor, similar to N-CAM and the cadherins. The transmembrane and GPI-anchored forms of N-CAM are generated by differential splicing of mRNA and it is not known how they differ in function. Such molecules are involved

in the development of the cellular architecture of the CNS.

PrP mRNA is expressed in many different cell and tissues. *In situ* hybridization (Manson et al 1992, Brown et al, 1990) and oligonucleotide primer extension and S1 mapping (Westaway et al 1987) have been used to show mRNA expression in neurons in the CNS, in the lungs, where it is associated with the alveolar lining and septal interstitial cells, muscle cells in heart, and to a lesser extent in the spleen, liver and kidney (Brown et al, 1990). It is possible that in these different cell and tissue types PrP adopts different functions. It has been suggested that PrP on the surface of normal human lymphocytes may act as an activation-released soluble signal for the activation of other subsets of lymphoid cells (Cashman et al., 1990). The presence of a GPI-anchor could facilitate such a release, although soluble forms of PrP found to date are not released by endogenous PIPLCs (Borchelt et al., 1993)

Experiments with PrP^{0/0} mice

Results with Tg null mice show that no phenotype is observed and the mice appear physically and behaviourally normal and are fully fertile (Bueler et al., 1992). This suggests that either the function of PrP is a non-essential one or else that this function can be taken over by another protein in the null mice. A third possibility is that PrP only becomes important under certain circumstances, for instance stress, and therefore this function has not yet been observed in the null mice.

Null mice (PrP^{0/0}) have been shown to be resistant to infection with scrapie when inoculated with infected mouse brain intracerebrally, where normal Tg littermates were susceptible to the disease within the expected incubation period (Bueler et al., 1993).

Heterozygotes (PrP^{0/+}) were shown to have incubation times much longer than would be expected for the model used. The introduction of Tg hamster genes into these mice rendered them susceptible to infected hamster

inoculum but not to mouse infected inoculum. These results implicate that the scrapie agent cannot replicate in the absence of the PrP gene product and that, as shown by the results with the heterozygotes, that the rate at which the disease progresses is proportional to the amount of PrP^C that is present. This conclusion is supported by the finding with conventional transgenic animals that with higher numbers of copies of the PrP transgene shorter incubation periods result (Prusiner 1990). The fact that the null mice do not contract scrapie and appear perfectly normal in the absence of the PrP protein implies that it is the accumulation of the abnormal protein rather than the disruption of the normal function of PrP that is the cause of pathogenicity.

Anchor-less PrP and its function

Expression of constructs coding for a truncated PrP protein in CV1 cells led to most of the protein being secreted from the cell instead of being attached to the cell membrane. This alteration ought to severely affect the normal function of the protein. However, no pathological effects were seen in tissue culture. This is consistent with the results described above with mice who produce no PrP protein which appear functionally normal. Experiments with Tg mice expressing these truncation mutations are underway. These mice will presumably secrete their PrP from the cell surface. These experiments should tell us if the secreted protein aggregates in the uninfected brain, and whether these aggregates, should they arise, cause disease. The fate of normally GPI-membrane anchored proteins in patients suffering from paroxysmal nocturnal hemoglobinuria (PNH), in which a specific defectⁱⁿ a gene coding for a GPI-biosynthetic precursor has been implicated (Takeda et al 1993), has not been examined in detail. Increased serum levels of a soluble form of PrP may render the individual more susceptible to the risk of developing a transmissible degenerative encephalopathy. The production of Tg animals carrying mutations in their PrP gene should answer many of

the remaining questions regarding the molecular mechanisms involved in the conversion of PrP^C to PrP^{Sc}.

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Appendix 1

Antibiotics

Name	Stock	working conc.	storage
Ampicillin	100mg/ml	100 μ g/ml	-20°C
Streptomycin	10mg/ml	100 μ g/ml	-20°C
Kanamycin	25mg/ml	70 μ g/ml	-20°C

Alkaline gel loading buffer

NaOH	300mN
EDTA	6mM
Ficoll	18%
Bromophenol green	0.15%
XCFF	0.25%

1 x Alkaline gel running buffer

50mN NaOH
1mM EDTA

Agarose gel loading buffer

Ficoll	1g
1 x TAE	8ml
0.5M EDTA	2ml
Bromophenol blue	0.025%
Xylene cyanol	0.05%

Bind-Silane

Ethanol	5ml
10% Acetic acid	170 μ l
Bind-Silane	17 μ l

Chromic acid

Add 200ml 70% Nitrous acid to 1 litre H₂O.

To this add 150g of potassium dichromate and make up to 1.5 litres.

50 X Denharts solution

Ficoll	0.5g
Polyvinylpyrrolidone	0.5g
Bovine serum albumin(BSA)	0.5g

H₂O to 50 ml

Store as 1ml aliquots at -20°C

DEPC treatment

Diethylpyrocarbonate (DEPC) was added to H₂O at a final concentration of 0.1%, left overnight in a fume hood and autoclaved before use.

Dialysis tubing

Cut tubing into suitable lengths.

Boil for 10 min in 1 litre 10% sodium carbonate.

Discard the buffer and boil x 3 in 1 litre H₂O.

Pour off H₂O and store in TE at 4°C.

Rinse with distilled H₂O before use.

EDTA

Weigh out enough EDTA to make 500ml of a 0.5M solution. Dissolve in approx. 200ml and pH to 8.0 with NaOH and make up to 500ml with H₂O.

Equilibrating phenol

Phenol was obtained from Gibco-BRL and stored at -20°C prior to equilibration.

It was defrosted by placing in a 65°C waterbath overnight.

The next day 0.1% 8-hydroxyquinalone was added, ie., 0.1g was added to 75ml of neat phenol and the volume made up to 100ml with 1M Tris.Hcl pH9. This was mixed and the phases allowed to separate. The aqueous layer was removed and the extraction with 1M Tris repeated x 2. Then the buffer was change to 0.1M Tris and repeated x 2. The pH of the aqueous layer should be 8.0.

Phenol was stored at 4°C. All manipulations were carried out in a fume hood.

Ficoll-dye EDTA (FDE)

0.2M EDTA 500µl

Ficoll type 400 0.3g

trace Bromophenol blue

H₂O to 1ml.

Formamide sample buffer

10x MOPS 100µl

Formamide(99%) 200µl

Formaldehyde(40%w/v) 120µl

Final sample buffer (FSB)

Glycerol 1.0ml

10% SDS 2.0ml

Bromophenol blue 2.0mg

Stacking gel buffer 1.25ml

H₂O to 10ml

Just prior to use add 1 μ l β -mercaptoethanol per 20 μ l of sample buffer.

Minimal agar

Add 6g Difco agar to 300ml H₂O , autoclave.

Cool to 60°C, add:	Spitzen salts	80ml
	20% glucose	4ml
	Vitamin B1 (2mg/ml)	0.1ml
		H ₂ O to 400ml

OLB Buffer

Mix solutions A, B and C in the ratios 100:250:150, aliquot and store at -20°C.

Solution A:-	Solution O	470 μ l	
	β -Mercaptoethanol	9 μ l	
	20mM dATP	12.5 μ l	
	20mM dTTP	12.5 μ l	
	20mM dGTP	12.5 μ l	store -20°C

Solution O:-	Tris	1.1g	
	MgCl ₂	0.25g	
		pH to 8 with HCl	
		H ₂ O to 10ml	

Solution B:-	Hepes	6.51g	
		pH to 6.6 with NaOH	
		H ₂ O to 12.5ml	

Solution C:-	pN ₆	Pharmacia	store -20°C
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PBST

100x Phosphate buffered saline tablets	5	
Tween 20	0.5g	
	H ₂ O to 500ml	

Polyacrylamide gels for sequencing

For an 8% gel:

40% Acrylamide solution	7ml
9M Urea	33ml
10% Ammonium persulphate	170 μ l
Temed	30 μ l

RF1

Rubidium chloride	12g	100mM
Mangenes chloride	9.9g	50mM
1M Pottassium acetate	30ml	30mM
Calcium chloride	1.5g	10mM
Glycerol	15.15ml	15%

H₂O to 100mlSterilize through a 0.22 μ m filterRF2

0.5M MOPS	20ml	10mM
Rubidium chloride	1.2g	10mM
Calcium chloride	11g	75mM
Glycerol	15.15ml	15%

H₂O to 100mlSterilize through a 0.22 μ m filterSDS-PAGE stacking buffer

Tris	6.06g
10% SDS	4 ml
	pH to 6.8 with HCl
	H ₂ O to 100ml

SDS-PAGE separating buffer

Tris	18.2g
10% SDS	4ml
	pH to 8.8 with HCl
	H ₂ O to 100ml

5X SDS-page running buffer

Tris	15g	
Glycine	72g	
SDS	5g	
	H ₂ O to 1 litre	store at 4°C

Semi-dry blotting buffer

Glycine	2.93g	
Tris	5.81g	
SDS	0.38g	
20% Methanol	200ml	
	H ₂ O to 1 litre.	store at 4°C

10 x SSC

NaCitrate	88.3g
NaCl	175.3g

H₂O to 1 litre.

20 x SSCP

NaCl	140.3g
NaCitrate	88.4g
NaH ₂ PO ₄ ·2H ₂ O	62.4g
NaOH to pH 7.4	
H ₂ O to 1 litre.	

20 x SSPE

NaCl	448g
H ₂ PO ₄ H ₂ O	55.2g
EDTA	14.8g
4N NaOH	90ml

H₂O to 2 litres.

50 X TAE

Tris	242g
Glacial acetic acid	57ml
0.5M EDTA	100ml

H₂O to 1 litre

10 X TBE

Tris	108g
Boric acid	55g
0.5 M EDTA	20ml

H₂O to 1 litre

TBST

1M Tris.Hcl pH 8.0	100μl
1M NaCl	1.5ml
Tween-20	5μl

H₂O to 10ml

TE

1M Tris.Hcl pH 8.0	10ml
0.5M EDTA	2ml

H₂O to 1 litre

Top agar

Tryptone	10g
Yeast extract	5g
NaCl	5g
Difco agar	7g

H₂O to 1 litre.

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