Analysis of Transient Gene Expression in Ovine Cells: A Role for the PrP Gene 3'UTR.

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Declaration.

I declare that this thesis has been composed entirely by myself and that the work presented herein is my own, except where otherwise stated. All experiments were designed by myself, in collaboration with my supervisors. No part of this thesis has been, or will be, submitted for any other degree, diploma or qualification.

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I am especially grateful to my family. The reasons are many and space limited, but thank you Mum, Dad and Lorna. For your support, love and for always having faith in me. I hope you are proud of what we have achieved. Finally, to the man who said "the only thing worse than doing a PhD is living with someone doing a PhD", thank you. To the good life$_x$

To Nana.

Abstract

Analyses of the PrP gene of several mammalian species have found that it exhibits a high degree of DNA sequence homology. However, exon III of the sheep gene is much larger than that found in other species due to insertions of ruminant-specific elements within the PrP 3'UTR. The ovine 3'UTR is also differentially processed to produce at least two mRNA transcripts (4.6 and 2.1kb) by alternative polyadenylation. Differential expression of the two transcripts is observed with respect to tissue specificity and level of transcription.

The primary objective of this study has been to examine the hypothesis that regions specific to the sheep 3'UTR act as an inhibitor. In a previous thesis, *in vitro* expression in mouse N2a cells using reporter gene constructs with the chloramphenicol acetyl transferase (CAT) gene linked to PrP sequences had suggested that there might be an inhibitor of translation in the PrP gene 3'UTR . Transient transfection methods were developed to generate optimal levels for *in vitro* expression of the CAT/PrP-3'UTR constructs in immortalised Cheviot fetal brain-derived cell lines from both scrapie resistant and susceptible genotypes and primary cell lines derived from cerebellum and liver tissues of the Icelandic sheep breed, *Ovis brachyura borealis pall.* Expression of a series of CAT/PrP-3'UTR vectors in cell lines derived from different PrP genotypes found that sequence between nucleotides 2000-2700 on the PrP 3'UTR may show a tendency to reduce protein expression in the cells derived from brain tissue of scrapie-resistant genotype and in a tissue specific manner.

Additional vectors were produced to express ovine PrP mRNAs, similar to the endogenous 4.6kb and 2.1 mRNAs transcripts, but altered to display a monoclonal epitope site (3F4) enable transiently expressed PrP protein within ovine cell cultures to be detected. Expression of the PrP constructs in mouse neuroblastoma (N2a) cells has shown that both constructs were viable. However, the immunodetection methods employed in this thesis could not distinguish between transiently expressed 3F4-labelled ovine PrP and endogenous PrP within sheep cell lines.

Results presented here confirm that the 3' UTR found in the 2.1kb mRNA is capable of supporting gene expression. A role for a specific sequence in the ovine PrP gene 3'UTR in controlling protein expression in brain-derived cell lines has been proposed. Also, the function of the regulatory sequence may be dependent on tissue origin. PrP^C is vital for the replication of the TSE agent. Controlling the amount of available PrP^C *in vivo* may influence susceptibility and development of TSE diseases.

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Abbreviations.

A	Adenine (nucleic acid sequence)
	Alanine (amino acid sequence
Aβ	Amyloid peptide, amyloid-β
ACm	Acetylated chloramphenicol
AD	Alzheimer's disease
AP	Amyloid plaque
APP	Amyloid precursor protein
ATP	Adenosine triphosphate
bp	base pairs
BSE	Bovine spongiform encephalopathy
β-gal	β-galactosidase
C	Cytosine (nucleic acid sequence)
	Cysteine (amino acid sequence)
CAT	Chloramphenicol acetyl transferase
cDNA	Complimentary deoxyribnucleic acid
CFI	Cleavage factor I
CJD	Creutzfeldt-Jakob disease. Prefix s: sporadic; v: variant;
Cm	Chloramphenicol.
CNS	Central nervous system
CoA	Coenzyme A
cpm	Counts per minute
CPSF	Cleavage/polyadenylation specificity factor
CstF	Cleavage stimulation factor
CWD	Chronic Wasting Disease
Da	Daltons
dNTP	Deoxynucleotide –5'-triphosphate
ddNTP	2'3'-dideoxynucleoside-5'-triphosphate
Dpl	*Prnd* gene product designated dopple
DTT	Dithiothreitol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSE	Downstream element
e-IF	Elongation initiation factor
EDTA	Ethylenediaminetetra-acetic acid

ENS	Enteric nervous system
ER	Endoplasmic reticulum
EV	Endocytotic vesicles
F	Phenylalanine
FCS	Foetal calf serum
FDC	Follicular dendritic cells
FFI	Fatal familial insomnia
FTIR	Fourier transform infrared spectroscopy
FSE	Feline Spongiform Encephalopathy
G	Guanine (nucleic acid sequence)
	Glycine (amino acid sequence)
G	Golgi appartus
GALT	Gut associated lymphoid tissue
GPI	Gylcoinositol phospholipid
GSS	Gerstmann-Straussler-Sheinker disease
H	Histidine
HIVv	Human immunodeficiency virus
HuPrP	Human PrP
I	Isoleucine
IAH	Institute for animal health
IMDM	Iscoves modified Dulbeccos medium
IS120.Cer	Icelandic sheep brain-derived cell line, genotype $AA_{136}RR_{154}RR_{171}$
IS120.Liv	Icelandic sheep brain-derived cell line, genotype $AA_{136}RR_{154}RR_{171}$
IS120.Kid	Icelandic sheep brain-derived cell line, genotype $AA_{136}RR_{154}RR_{171}$
kb	Kilobases
kDa	Kilodaltons
L	Leucine
LB	Luria-Bertani broth
LINE	Long interspersed nuclear elements
IL-6	Interluekan-6
LRS	Lymphoreticular system
M	Methionine
MoPrP	Mouse PrP
mRNA	Messanger RNA
MRP8	Migratory inhibitory factor-related protein

N	Adenosine, cytosine, guanine or thymmidine (nucleic sequence)
	Asparagine
	Nucleus
NGF	Nerve growth factor
NPU	Neuropathogenesis unit
nt	Nucleotide
ORF	Open reading frame
P	Proline
pA	Polyadenylation
PAB II	Poly A binding protein II
PAP	Poly A polymerase
pA80BR	Cheviot brain-derived cell line, genotype $AA_{136}RR_{154}QR_{171}$
pA80SK	Cheviot skin-derived cell line, genotype $AA_{136}RR_{154}QR_{171}$
PCR	Polymerase chain reaction
PDGF	Periodontal ligament growth factor
PrP	Protease resistant protein or prion
Prnd	Dopple gene
Prn-p	Mouse PrP gene
Prn-p $^{0/0}$	PrP null mice
PRNP	Human PrP gene
PrP^{C}	Cellular (host) PrP protein
PrP^{Sc}	Disease-specific PrP protein, proteinase resistant
Q	Glutamine
R	Arganine
RFLP	Restriction fragment length polymorphism
RCF	Relative centrifugal field, xg
RNA	Ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
S	Serine
SAF	Scrapie associated fibril
sA80BR	Cheviot brain-derived cell lines genotype $VV_{136}RR_{154}QQ_{171}$
SCID	Severe combined immunodeficent mice
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sem	Standard error mean

SHa	Syrian hamster
SINE	Short interspersed nuclear elements
SSBP/1	Sheep scrapie brain pool 1
ssDNA	Single stranded DNA
T	Thymidine (nucleic acid sequence)
	Threonine (amino acid sequence)
Tg	Transgenic
TME	Transmissible Mink Encephalopathy
TSE	Transmissible spongiform encephalopathy
USE	Upstream element
UTR	Untranslated region
V	Valine

Chapter 1 :Transmissible Spongiform Encephalopathies.

1.1 General Introduction.

Transmissible spongiform encephalopathies (TSEs), also known as prion diseases, are fatal degenerative disorders of the central nervous system (CNS) affecting both humans and animals. TSEs are characterised by disturbances in behaviour and movement, spongiform changes in the brain and the deposition of an abnormal isoform of a host-encoded protein (PrP) in the CNS and to a lesser degree in lymphoid tissue. There appears to be no immune response to infection and potential therapeutics investigated to date have only delayed the onset of disease in animal studies.

Transmission of TSEs to laboratory animals has highlighted the unusual properties of the pathological agent such as long incubation period and resistance to chemical and physical inactivation. The identity of the TSE agent has remained an enigma for more than 60 years due to the failure to isolate a particle which conforms to the criteria of TSE: transmissibility, replication, propagation of strain characteristics, neuronal loss, and accumulation of an abnormal host encoded protein. Suggested aetiologies for the TSE agent include that of a proteinaceous infectious particle (prion), a DNA/protein hybrid (virino) or an unconventional virus. The prion hypothesis suggests that a partially protease-resistant abnormal isoform (designated PrP^{Sc}) of a normal host encoded glycoprotein (PrP^{C}) (Bolton *et al*, 1982; McKinley *et al*, 1983; Oesch *et al*, 1985) is the major, or sole, component of the TSE infectious agent (Prusiner, 1982; Prusiner *et al*, 1982). It is proposed that the agent can replicate, without a nucleic acid (Griffith, 1967; Prusiner, 1982), by converting the normal host-encoded isoform to the disease isoform (Prusiner, 1991;Jarrett and Lansbury, 1993). According to the prion hypothesis, strain characteristics of TSEs are encoded for in the conformation of PrP^{Sc} (Caughey *et al.*, 1998; Safar *et al.*, 1998). Whether PrP^{Sc} is the infectious agent still has to be proven. The virino and virus theories both predict that the TSE agent has an independent informational

molecule encoding strain specific characteristics. The virino model suggests that the TSE agent-specific molecule, probably a nucleic acid (Dickinson and Outram, 1988), is protected by PrP. Formation of PrP^{Sc} may be due to the association of the virino nucleic acid with the host PrP^{C} protein. The virus model suggests that the agent-specific nucleic acid encodes strain-specific characteristics and the viral coat contains PrP^{Sc} (Rohwer and Gajdusek, 1980)

The normal PrP isoform, PrP^{C}, is detectable in many uninfected and TSE-infected tissues (Bendheim *et al.*, 1992). In contrast, PrP^{Sc} is only associated with TSE infection (Rubenstein *et al.*, 1987, Ikegami *et al.*, 1991). The PrP gene structure and organisation is known to be highly conserved between species suggesting that it may play a fundamental physiological role. However, the precise function of PrP is still unclear. To investigate the role of PrP, both in the cell and as a factor of TSE development, panels of transgenic mice have been created by several laboratories. Analysis of transgenic models has revealed the importance of host genetics (Race, 1990) for susceptibility to TSEs. Mice lacking the PrP gene (PrP null) develop normally and show no severe phenotype (Bueler *et al.*, 1992;Manson *et al.*, 1996). Furthermore, when inoculated with scrapie these PrP null mice appear to be resistant to disease (Bueler *et al.*, 1993; Sailer *et al.*, 1994). Conversely, animals over-expressing PrP are more susceptible to TSEs (Scott *et al.*, 1989; Prusiner, 1990; Bueler *et al.*, 1992). Expression of the PrP gene therefore seems to be vital to the development of TSEs.

This thesis deals primarily with the expression of the PrP gene within sheep, the natural host of scrapie, and attempts to correlate the importance of control mechanisms of PrP gene expression to disease susceptibility, transmission and development. The aim of this first chapter is to introduce the complexities of the TSE agent and the host PrP gene products.

Table 1.1 : TSE diseases of humans and animals

Natural Host	Disease	Occurrence
Sheep, Goats	Scrapie	Unknown[a]
Cattle	Bovine Spongiform Encephalopathy (BSE)	Iatrogenic[b]
Mule deer	Chronic Wasting Disease (CWD)	Unknown[c]
Mink	Transmissible Mink Encephalopathy (TME)	Unknown[d]
Cats (domestic, captive)	Feline Spongiform Encephalopathy (FSE)	Iatrogenic[e]
Human	Creutzfeldt-Jakob disease (CJD)	Sporadic[f]
		Inherited[g]
		Iatrogenic[h]
	New variant CJD (vCJD)	Iatrogenic[i]
	Gerstmann-Straussler-Scheinker Syndrome (GSS)	Inherited[j]
	Kuru	Iatrogenic[k]
	Fatal Familial Insomnia (FFI).	Inherited[l]

Note; [a](Cuille, J. and Chelle, P.L., 1936; Parry, 1983;Chelle, 1942; Dickinson, 1976), [b](Wells *et al*, 1987; Fraser *et al*, 1988), [c](Williams and Young, 1980), [d](Hartsough and Burgher, 1965), [e](Wyatt *et al*. 1990), [f](Creutzfeldt, 1920; Jakob, 1921), [g and h](review Prusiner, 1993), [i](Will *et al*, 1996; Chazot *et al*, 1996), [j](Gerstmann, 1928), [k](Gajdudesk and Zigas, 1957), [l](Lugaresi *et al*, 1986)

1.2 TSEs of Humans and Animals.

TSE diseases have been described in both animals and humans (Table 1.1). Characteristic pathology of TSEs is generally confined to the CNS, particularly the brain. Examination of TSE-infected brain reveals a neuropathology which can vary in localisation and severity depending on the infecting strain of TSE. Pathological markers for TSE infection commonly include accumulation and deposition of an amyloid protein (PrP^{Sc}) in specific brain areas, vacuolation (spongiform appearance) and astocytic gliosis(Beck *et al.*, 1969; Gibbs *et al.*, 1968; Bruce and Fraser, 1975; Chou *et al.*, 1980; Wisniewski *et al.*, 1981; Tateishi *et al.*, 1984). Spongiform lesions are thought to be the result of formation of intracellular vacuoles (Prusiner, 1993).

Scrapie, which occurs naturally in sheep and goats, has been recognised in Europe for more than 250 years and is the best studied of animal TSEs. Clinical signs of scrapie include trembling, loss of motor control, intense itching, and paralysis, death due to scrapie in sheep most frequently occurs between the ages of 2-5 years. Generally, neuropathological examination of a scrapie infected brain reveals widespread deposition of PrP^{Sc}, vascular degradation and gliosis (Wood *et al.*, 1997). Since early demonstrations of its transmissibility (Cuille and Chelle, 1936), scrapie has become the experimental model for TSEs in general and is usually studied in both sheep and laboratory rodents.

TSEs have been identified in other animal species based on transmission, clinical and neuropathological symptoms familiar to scrapie (Table 1.1). The appearance of Bovine spongiform encephalopathy (BSE), first identified in 1986, coincided with changes in rendering procedures and the feeding of cattle of meat and bone-meal in Britain (Bradley and Matthews, 1992). Although not proven, the BSE outbreak is considered to be the result of cattle being fed meat and bone-meal derived from ruminant sources contaminated with scrapie (Wilesmith, 1991). Since 1988, feeding ruminants with ruminant derived bone meal has been banned and as a result reported cases of BSE have fallen in number (Wilesmith, 1993). Neuropathologically, BSE in cattle shares similar features to scrapie in sheep: astrocytosis, vacuolation, neuronal

loss and PrP amyloid plaque formation (Wells and Wilesmith, 1995). However, significant differences in the pattern of neuronal loss within specific brain regions are observed following inoculation of inbred lines of mice with scrapie and BSE (Bruce, 1998). Although BSE is clearly a distinct strain of TSE, changes in the rendering process which occurred just prior to the appearance of BSE may have allowed the selection of a rare strain of TSE. TSEs have also been reported in domestic cats (FSE) and captive zoo animals (Wyatt *et al.*, 1990; Jeffrey and Wells, 1988; Kirkwood *et al.*, 1990) . These cases are thought to have arisen through the animals being fed BSE-contaminated-meat derived from bovine sources. Transmissible Mink Encephalopathy (TME), first observed in farmed mink in 1965 (Hartsough and Burger, 1965) was caused by an agent with similar physiochemical properties to that of the scrapie agent (Marsh et al., 1969; Marsh et al., 1979).

Human TSEs can be described as inherited (GSS, CJD, FFI), sporadic (CJD), infection (Kuru) or iatrogenic (CJD) (Table 1,1) . Most forms, like scrapie, are experimentally transmissible to various animal species(Gibbs *et al.*, 1969). The clinical and pathological features of the different human TSEs can vary greatly but most show degrees of dementia, ataxia, spongiform degeneration and amyloid deposits composed of PrP protein fragments (Prusiner, 1993). However, the age groups in which these TSEs occur can vary. Generally, the age of onset for CJD is 50-60 years, although cases in young patients have been identified (Brown, 1985). For GSS the age of onset can be 24-63 years (Prusiner, 1993) and FFI has presented in 40-60 year olds (Lugaresi *et al.*,1986: Reder *et al.*, 1995).

Although sporadic CJD (sCJD) occurs more frequently than other forms, it is still very rare (approximately 1 case per 10^6 per year world wide), and factors involved in the cause of sCJD are unknown. Approximately 15% of CJD cases and almost all GSS (occurrence rate of 1-2 per 10-100 million (Master *et al.*, 1979, Prusiner 1993)) cases occur in a familial context, both showing an autosomal dominant pattern of disease segregation (Masters *et al.*, 1979). Iatrogenic forms of human TSEs are thought to result from accidental inoculation, transplantation of TSE infected material. Kuru for example, a unique neurodegenerative disorder among the Fore

Tribe of Papua New Guinea, is thought to have been transmitted through ritual cannibalism (Gajdusek, 1977). However, since the practice of cannibalism ended in 1955, the numbers of recorded Kuru cases have fallen significantly, although cases are still occurring almost 40 years after the last known ritual. A new variant of CJD (nvCJD, now known as vCJD) was identified in the UK in young patients who had a unique, clinical and pathological phenotype (Will *et al.*, 1996; Chazot *et al.*, 1996). vCJD cases all shared early onset of disease, initial psychiatric disturbances, prolonged duration of illness and unusual pathology: all characteristics which had never been described in UK CJD cases before. Recently it was shown that a similar TSE agent is responsible for both BSE and vCJD (Collinge *et al*, 1996; Bruce *et al.*, 1997; Hill *et al.*, 1997) (section 1.5.2.1).

1.3 The TSE Infectious Agent.

Originally, the scrapie agent was believed to be an unconventional slow virus that had so far eluded detection but this is now thought unlikely. A similar difficulty in agent identification was seen with the hepatitis C virus (Choo *et al.*, 1989). Intensive immunological methods failed to identify specific viral antibodies and antigens in hepatitis cases arising from blood transfusion. It was not until 1989 that Choo *et al*, cloned an antigen associated specifically with a blood-borne non-A, non-B, hepatitis (NANBH) and so identified the hepatitis C virus.

A curious feature of the TSE agent is the persistence of infectivity after treatment with methods effective of viral inactivation. Infectivity is resistant to nuclease treatment and high doses of ultraviolet and ionising radiations (Alper *et al.*, 1967; Latarjet, 1970; Millson *et al*, 1976) but is susceptible to procedures that denature, modify, hydrolyse or degrade proteins such as protease K or trypsin (Bolten *et al.*, 1982; Mckinley *et al.*, 1983). Inactivation of TSEs and the repercussions of accidental transmission first became apparent during a large scale vaccination programme of sheep against louping-ill virus, when at least 10% of the sheep developed scrapie. The louping-ill vaccine, assumed to be contaminated with scrapie

had been treated with 0.35% formalin (Greig, 1950). In humans, transmission of CJD was recorded after instruments used on a suspect CJD patient for brain surgery were "sterilised" by ethanol and formaldehyde before being used on other patient who later developed CJD ((Bernoulli *et al*, 1977).

No single method has been found which completely inactivates TSEs e.g. autoclaving, detergents, acids, oxidising agents (review Taylor, 1999). For example after autoclaving, scrapie strain, 22A, and BSE strain, 301V, both show enhanced survival at 138°C compared with 134°C. The failure to decontaminate tissue or surfaces by these methods has led to the suggestion that the TSE agent is thermostable under conditions which appear to fix the protein component PrP^{Sc}, and hence protect the agent from inactivation. Only one method to date has been shown to successfully inactivate TSEs. A combination of autoclaving and exposure to sodium hydroxide can apparently inactivate CJD and scrapie infectivity (Taguchi *et al.*, 1991;Taylor *et al.*, 1997; Ernst and Race, 1993). However, this method does pose its own hazards, especially to the operator with regards to autoclaving, and contact, with sodium hydroxide. Future studies on this inactivation method may provide a solution to decontamination.

1.3.1 Association of PrP^{Sc} with TSEs.

Post-mortem investigation of scrapie and CJD infected brain tissue revealed the deposition of an amyloid protein in rods and scrapie associated fibrils (SAF), fibre-like structures 100-1000nm in length (Merz *et al.*, 1981) which are not found in healthy controls. These filaments, when purified, enriched scrapie infectivity from several hundred fold to several thousand fold (Bolton *et al.*, 1982; Prusiner, 1982a) and were found to consist largely of a single protein 27-30 kDa (PrP 27-30) which appeared to be derived from a larger protein by proteinase K digestion (Prusiner, 1982a). PrP 27-30 was uniquely associated with TSE infected hosts and designated PrP^{Sc} (PrP *Sc*rapie). The discovery of PrP^{Sc} allowed for the isolation of cDNAs from libraries constructed from scrapie infected hamsters and mice (Oesch *et al.*, 1985; Chesebro *et al.*, 1985; Basler *et al.*, 1986; Locht *et al.*, 1986) revealing that PrP^{Sc} was

the product of a conversion from host gene, designated PrP^C (PrP *C*ellular), and not virally encoded (Oesch *et al.*, 1985; Basler *et al.*, 1986). Furthermore, when proteolysis was avoided, a PrP protein with the larger relative mass of 33-35kDa was identified in both infected and normal brains. Therefore PrP^C was expressed in both TSE infected and uninfected animals whereas PrP^{Sc} was uniquely associated with TSE infection. Similarities between PrP^C and PrP^{Sc} included SDS-PAGE mobility and antigenicity (Oesch *et al.*, 1985; Barry and Prusiner, 1986), N-terminal signal peptide cleavage (Basler *et al.*, 1986; Hope *et al.*, 1986), glycosylation (Caughey *et al.*, 1988) an addition of a glycosylphophatidylinositol (GPI) anchor at the C-terminus (Stahl *et al.*, 1990). However, distinct biochemical and physical properties were found to differentiate between PrP^C and PrP^{Sc} (section 1.6.1) which lead to the suggestion that PrP^{Sc} was post-translationally derived from PrP^C (Borchelt *et al.*, 1990).

PrP^{Sc} appeared to accumulate in TSE-infected hosts in the absence of a detectable nucleic acid and opinion grew that perhaps the scrapie agent was fundamentally different from other infectious agents. One view was that the major or sole component of the TSE agent was PrP^{Sc} itself which was capable of self-replication (prion). Alternatively, an agent-specific nucleic acid may still exist and so the term virino was introduced to distinguish the agent from conventional viruses and viroids.

1.3.2 The Prion hypothesis.

A prion is defined as an infectious, disease-specific isoform of a cellular protein which is capable of replication and accumulates during the disease process (Prusiner, 1982). The prion hypothesis predicts that replication occurs through the post-translational conversion of PrP^C to PrP^{Sc} via direct interaction of the two proteins to generate more PrP^{Sc}. Passage of PrP^{Sc} from one host to another then constitutes "infection" by initiating PrP^{Sc} formation in a new host and the presence of PrP^C is fundamental to this theory. Two ideas for prion replication have been proposed: the nucleated (or seeded) polymerisation model (Jarrett and Lansbury, 1993) and the conformational model (Prusiner, 1991) (Figure 1.1).

The nucleated polymerisation models states that PrP^{C} is in equilibrium with a PrP^{Sc}-like monomer, which, under physiological conditions, is low in concentration. PrP^{Sc} only accumulates when the equilibrium shifts because of an incoming high concentration of PrP^{Sc} oligomers that form and stabilise new PrP^{Sc} (Jarrett and Lansbury, 1993). In contrast, the conformational model requires the direct interaction of stable PrP^{C} and PrP^{Sc} monomers (Prusiner, 1991) to form a heterodimer and catalyse the conversion of PrP^{C} to PrP^{Sc}. The resulting PrP^{Sc}:PrP^{Sc} homodimer then dissociates to capture further PrP^{C} monomers. The high-energy requirement for this reaction may be overcome by the assistance of chaperones and pre-existing PrP^{Sc} monomers. Consistent with the nucleation model, cell free studies have shown that the conversion of PrP^{C} to PrP^{Sc} requires the presence of particles containing oligomers of PrP^{Sc} much larger than monomers or dimers.

Supporters of the prion hypothesis argue that the apparently spontaneous conversion of PrP^{C} to PrP^{Sc}, as would have to occur with genetic human TSE disease, is the result of destabilisation of the PrP^{C} protein by associated specific point mutations. Indeed analogous disease associated PrP^{C} variants produced in cell culture displayed a number of biochemical characteristics of PrP^{Sc}, suggesting aberrant folding of the molecules *in vivo* (Huang *et al.*, 1994; Cohen *et al.*, 1994; Cappai *et al*, 1999). However, thermodynamic stability studies on recombinant murine PrP variants have shown that not all pathologically linked amino acid replacements destabilise the protein structure (Liemann, *et al.*, 1999).

Conversion of recombinant PrP^{C} to PrP^{Sc} has been demonstrated *in vitro* (Kocisko *et al.*, 1994; Caughey *et al.*, 1995) and characteristic biochemical properties (proteinase K resistance) of PrP^{Sc} from different strains of TME were also transmitted to PrP^{C} in the cell free system (Bessen *et al.*, 1995). These examples proved that information could be transmitted by non-nucleic acid molecules and would seem to be strong evidence in favour of the prion theory. However PrP^{Sc} synthesised *in vitro* has not yet been shown to be infectious and transmissible (Caughey *et al.*, 1995).

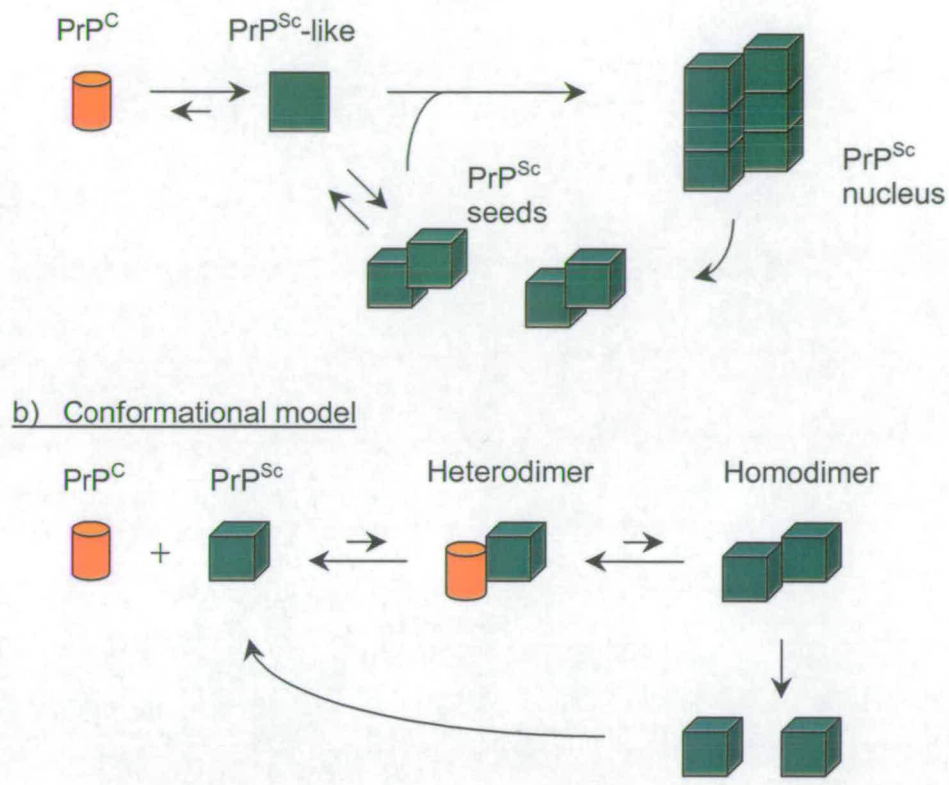


Figure 1.1: Proposed model for prion replication.

Self-replication of PrP^{Sc} has been proposed by two models. a) Nucleated polymerisation model (Jarrett and Lansbury, 1993) asserts that PrP^{C} (orange cylinder) conversion occurs when PrP^{Sc} (green cube) is at a critical concentration which favours incorporation of a PrP^{Sc}-like monomer into the PrP^{Sc} oligomer. b) Conformational model states that stable monomers of PrP^{C} and PrP^{Sc} interact directly to form PrP^{Sc} homodimer which can dissociate into active PrP^{Sc} monomers. (Prusiner, 1991).

1.3.3 The Virino hypothesis.

The virino theory suggests that PrP is a component of the infectious agent recruited into the infectious particle by an as yet unidentified host- independent informational molecule (Dickinson and Outram, 1988) which is expected to be an untranslated nucleic acid as nucleic acids are the only known biological molecules proven to be capable of carrying genetic information and propagating strain characteristics. TSEs have been exposed to nuclease treatment (Millson *et al.*, 1976), more extreme than would be required to inactivate most conventional micro-organisms. However rather than proving the absence of a nucleic acid, the property of resisting the effects of nucleic acid denaturants may simply indicate that the nucleic acid is either very small or is enveloped and thus protected by the PrP protein. It is hypothesised that the informational molecule of the TSE agent interacts with the host PrP^{C} protein (Farquhar *et al.*, 1998) and as a result of the conversion of PrP^{C} to PrP^{Sc} the nucleic acid is protected from degradation.

The virino hypothesis states that PrP^{C} exists in an equilibrium of ordered complexes that may act as a receptor or precursor for the infectious agent (Figure 1.2). The binding of the infectious agent to the PrP^{C} complex, and perhaps other molecules, would alter the three-dimensional structure of PrP^{C} such that it becomes PrP^{Sc} causing it to accumulate in and around cells as scrapie associated fibrils (SAF) (Hope, 1986). The calculated target size of the agent, determined by ionising radiation, is in line with the size of nucleic acid genomes in small viruses: estimated at $1.5x10^{6}$ Da (dsDNA) or $0.9x10^{6}$ Da (ssDNA) (Rohwer, 1991). For example the *porcine circovirus* virus has a ssDNA nucleic acid genome of $0.58x10^{6}$ Da (Tischer et al., 1982).

Attempts to identify TSE-specific nucleic acids have been largely unsuccessful, perhaps because they are too small, low in concentration or of an unusual structure. However, viral-like structures have been observed in TSE-affected brains (Diringer *et al.*, 1997). In another study, nucleic acids extracted from CJD brain tissue showed high infectivity levels and did not correlate to nucleic acids found in the mouse genome or uninfected brains (Dron and Manuelidis, 1996). The virino theory has yet to be disproved and is supported experimentally firstly by the existence of many strains

to be disproved and is supported experimentally firstly by the existence of many strains (difficult to reconcile with protein etiology, refer to section 1.4) and secondly by data which show that PrP^{Sc} and infectivity can be partially separated (refer to section 1.6.5).

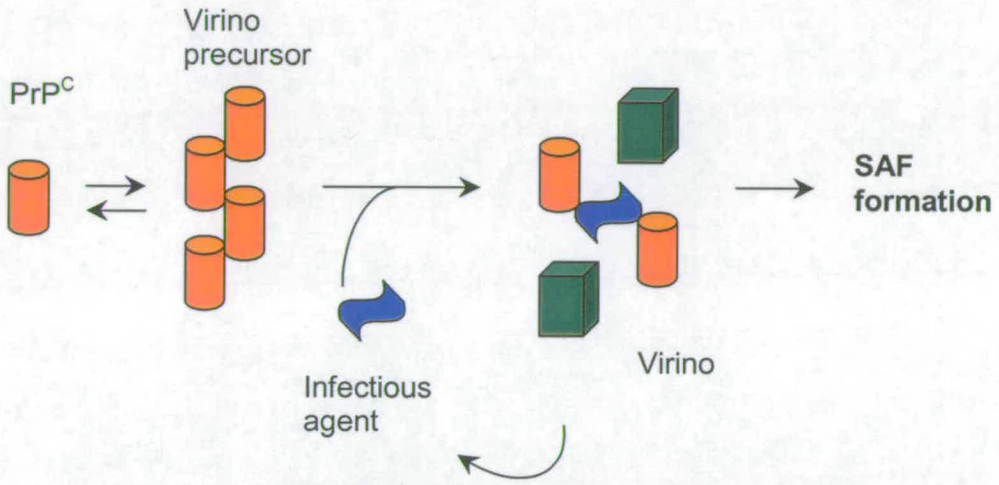


Figure 1.2: Proposed model for virino replication

The virino hypothesis states that PrP^{C} (orange cylinder) is in equilibrium as monomers and higher complexes (Hope, 1986). The TSE strain encoding molecule (blue ribbon) interacts with the complex and alters it properties, causing it to accumulate in the form of amyloid fibres which contain PrP^{Sc} (green cube).

1.3.4 Protein X

Protein X is an as yet unknown macromolecule which, according to supporters of the prion hypothesis may be involved in the conversion process of PrP^{C} to PrP^{Sc}. The concept of protein X was first described by Telling *et al.* (1995) when they observed that mice transgenic for a chimeric human-mouse PrP gene, Tg(MHu2M) mice, were highly susceptible to human TSEs (Telling *et al.*, 1995). Furthermore, expression of human PrP (HuPrP) on a null background (Tg(HuPrP)$Prnp^{0/0}$ mice) also resulted in mice susceptible to inoculation with human TSEs (Telling *et al.*, 1995). Previously it had been observed that Tg(HuPrP) mice were relatively resistant to inoculation with human TSEs (Telling *et al.*, 1994). The overall conclusions from these studies were that: 1) mouse PrP^{C} (MoPrP) inhibited conversion of $HuPrP^{C}$ to $HuPrP^{Sc}$; 2) a second mouse protein, designated protein X, was necessary for the conversion process, and; 3) mouse protein X binds to mouse PrP^{C} with higher affinity than it binds to $HuPrP^{C}$, thus inhibiting $HuPrP^{Sc}$ formation. Although several ligand binding studies have identified proteins capable of binding to PrP^{C} (Oesch et al., 1990; Kurschner and Morgan, 1995; Yehiely et al., 1997) their role , if any, in PrP^{C} conversion has not been examined.

Alternatively, subtle differences in the amino acid sequence between the host PrP^{C} and the infecting PrP^{Sc} protein may prevent prion replication by the host PrP^{C} protein "end blocking" the growing PrP^{Sc} polymer (Masel et al., 2000). According to the nucleated polymerisation model oligomers of PrP^{Sc} are the result of PrP^{Sc}-like monomers incorporated into the growing chain. Using mathematical modelling, Masel *et al.* (2000) proposed that the host PrP^{C} protein could bind to growing PrP^{Sc} oligomer and block the addition of further PrP^{Sc} molecules. Both protein X and the end-blocking theory were based on observations where there was only one amino acid difference between the host PrP^{C} and the infecting PrP^{Sc} protein (Priola et al., 1994; Kaneko et al., 1997; Zulianello et al., 2000). However, studies have shown that more than one point mutation difference existing between the host PrP^{C} and the infecting PrP^{Sc} protein does not protect the host from infection with TSEs from different species (Zulianello et al., 2000). To date both theories should be considered until further studies reveal the full pathway for PrP^{Sc} synthesis.

1.4 TSE Strains.

The major argument against the prion theory is the existence of multiple scrapie strains and this is often used as evidence for the other proposed etiologies. Many strains from naturally occurring scrapie cases in sheep and goats have been passaged through inbred mice. Each rodent-adapted scrapie strain has its own distinctive incubation period, clinical presentation, neuropathology, PrP^{Sc} deposition, and glycoform profile on Western blots (Fraser, 1976; Bruce *et al.*, 1991; Somerville, 1999). The incubation period and pathology is routinely used to identify the strain, or type, of TSE when passaged in inbred mouse lines. Furthermore, strain-specific neuropathology has lead to the development of a quantitative assay, lesion profiling (Fraser and Dickinson, 1968; Bruce *et al.*, 1991), which in contrast to the incubation period is not dose-dependent.

Another characteristic used to identify strains is the molecular heterogeneity of PrP^{Sc} which appears to retain a strain-characteristic glycosylation pattern when resolved on SDS-PAGE (Collinge *et al.*, 1996; Parchi *et al.*, 1996). This property of PrP^{Sc} led supporters of the prion hypothesis to argue that strain-specific properties of the TSE agent were enciphered within the attached carbohydrate moieties. However, the glycosylation pattern of PrP^{Sc} purified from different tissues, and cell types within a tissue, within a single host has been shown to differ (Hill *et al.*, 1999a; Rubenstein *et al.*, 1991; Somerville, 1999). This suggests that glycosylation is not dictated solely by the TSE strain but by co- and post- translational event and may be a result of the cell type of the species in which it occurs (Somerville, 1999).

1.4.1 Scrapie strain and the prion hypothesis.

In context of the prion theory, strain characteristics would have to be enciphered in the stable conformation of PrP^{Sc} and strain differences could therefore be explained by variations in the conformational state of the PrP^{Sc} protein (Bessen, 1994; Bessen 1995; Telling *et al.*, 1996; Caughey *et al.*, 1998; Safar *et al.*, 1998). PrP^{Sc} interacting directly with PrP^{C} would function as a template for replication of nascent PrP^{Sc} molecules and variations in incubation periods would be related to the

characteristic rate of conversion of PrP^C to PrP^{Sc} with the correct conformation for each strain. No high-resolution structures have yet been determined for PrP^{Sc} due to its tendency to form aggregates, which is incompatible with current NMR and X-ray diffraction techniques. Conformational studies using Fourier transform infrared (FTIR) spectroscopy have shown that PrP^{Sc} preparations from various hamster-adapted scrapie isolates have differences in the amount of β-sheet secondary structure (Caughey *et al.*, 1998). Additionally, a novel conformation-dependent immunoassay has claimed to characterise several hamster-adapted scrapie strains (Prusiner *et al.*, 1999). Neither system has yet been tested on strains of naturally occurring sheep scrapie where PrP^{Sc} levels can vary greatly between individual cases and different brain regions. It remains unclear what mechanisms enable the PrP protein alone to specify and retain biological TSE characteristics.

1.4.2 Scrapie strains and the virino theory

Supporters of the virino theory argue that the diversity of strains seems more plausibly explained by the existence of a nucleic acid which determines TSE strain characteristics rather than a single rogue protein. Western blot studies on PrP glycosylation patterns of TSE-infected laboratory rodents revealed that whereas the degree of glycosylation of PrP^{Sc} varied according to the TSE strain there was little effect on the glycosylation pattern of PrP^C (Somerville, 1999). These findings suggest that PrP^{Sc} is not the result of a conformational change in PrP^C . Instead PrP^{Sc} may be metabolised separately from PrP^C with the TSE agent directing PrP^{Sc} metabolism independently of the host PrP^C synthetic pathway.

1.4.3 Strain characteristics.

TSE strain characteristics after passage in hosts of different species have been used to examine possible links between TSEs. Transmission studies using eight unrelated BSE sources from cattle resulted in similar incubation periods and lesion profiles in mice suggesting that a single major strain of BSE was present in each source (Bruce, 1996). Other TSE sources i.e. cats, Nyala and Kudu also showed similar results supporting the suggestion that they too had been infected with the BSE strain (Bruce

et al., 1994; Fraser *et al.*, 1994) . Western blot analysis of PrP^{Sc} isolated from Cheviot sheep infected with BSE and experimental scrapie (CH1641) revealed striking glycoform similarity (Hope *et al*, 1999). However transmissions of natural scrapie from sheep to mice have given variable lesion profiles, with no individual source resembling BSE. This does not prove that BSE was not derived from scrapie or that BSE has not spread to sheep as TSE strain characteristics can change when passaged through a new species (Dickinson and Meikle, 1971).

The number of strains responsible for natural sheep scrapie is unknown and it may be that more than one strain is present in an affected flock at the same time. Limited strain typing studies on UK sheep scrapie isolates have so far revealed predominantly three scrapie strain types (Bruce, 1993) and a similar investigation of Icelandic scrapie showed a further three scrapie strains (Fraser, 1983). Knowledge gained from sheep PrP genotyping studies (section 1.7.2) has linked host genetics with susceptibility to different experimental scrapie isolates. This suggests that, in addition to scrapie strain and route of infection, host genetics plays a vital role in disease development. However, identification of high or low-risk PrP gene alleles can not totally explain the observed occurrence of natural scrapie in particular sheep breeds or PrP genotypes.

1.5 Scrapie Transmission.

1.5.1 Ovine transmission.

The natural route for scrapie infection is still unclear although there is evidence for acquired infection (lateral transmission) shown by healthy sheep and goats developing scrapie when housed in contact with scrapie sheep (Brotherston *et al.*, 1968; Haralambiev *et al*, 1973). In Iceland, scrapie management involved culling of scrapie affected flocks and establishing areas that remained scrapie free for 1–3 years after which farms were restocked with sheep from areas where scrapie had never been recorded. However, over the next 10 years, scrapie reoccurred on several of the farms suggesting either long term survival of the agent or an intermediate host (vector) (Palsson and Sigurdsson, 1959).

There is a clear possibility of maternal transmission of TSEs. Maternal transmission of BSE in cattle was thought to be one way of maintaining infection in BSE-affected herds at a low level with transmission occurring at a rate of 10% (Wilesmith *et al.*, 1997). Furthermore, BSE was observed not to be transmitted to embryos derived from BSE-infected goat donors (Foster *et al.*, 1999). The strongest evidence supporting maternal transmission of scrapie was recorded by Dickinson *et al* (1965) who observed that lambs born from infected ewes were seven times more likely to develop disease than lambs born to non-infected ewes. However, the precise route of maternal transmission is unknown. Several studies considering maternal transmission appear contradictory. Foote *et al.* (1993) showed that scrapie could not be transmitted via the embryo whereas studies at the NPU were not conclusive in that embryos donated from infected and non-infected ewes went on to develop scrapie (Foster *et al.*, 1992, 1996). The subsequent failure to detect infectivity in the uterus, ovary or mammary glands of clinically affected ewes supported the theory that maternal transmission did not occur. However recent studies have found evidence of infection in placenta. Therefore, one source of infection to newborn lambs may be at, or around, the time of birth. Such studies are being repeated in scrapie-free conditions in order to establish whether maternal transmission of scrapie really occurs (Hunter, personal communication).

1.5.2 Inter-species transmission of TSEs

Under normal conditions when scrapie is passaged between species, disease occurs only after a very long incubation period if at all and on subsequent serial sub-passages in the new species the incubation period shortens to a constant duration (Pattison and Jones, 1968; Dickinson, 1976; Kimberlin, 1979) and is known as the species barrier. The effect of the species barrier varies from apparent absolute resistance to increased incubation period dependant on: the species being infected, the host PrP gene, the species in which the TSE inoculum originated, route of infection and the strain of TSE. There are also situations where there appears to be no species barrier i.e. transmission of scrapie from sheep to goats (Greig, 1950; Brotherston *et al.*, 1968).

1.5.2.1 Transgenic analysis of the species barrier.

Experimental transmission of TSE from one host to a new host species is characterised by a prolonged incubation period (Table 1.2). This is known as the species barrier. For instance Chandler mouse scrapie can be transmitted to mice with incubation periods of approximately 120 days, while Syrian hamsters have an incubation period of up to 380 days with the same inoculum (Kimberlin and Walker, 1878; Kimberlin *et al.*, 1987). In contrast, hamster scrapie (263K) does not appear to transmit to mice despite a short incubation period of about 65 days when passaged in hamsters. To determine the controlling factors of the species barrier several lines of transgenic mice have been created (Scott *et al.*, 1989; Prusiner *et al.*, 1990). Transgenic (Tg) mice carrying the hamster PrP gene (Tg(SHa) mice) are susceptible to hamster scrapie but propagate neuropathology and PrP^{Sc} deposition characteristic of Syrian hamsters infected with hamster scrapie. (Scott 1989, Prusiner, 1990). Conversely Tg(SHa) mice inoculated with mouse scrapie show a prolonged incubation period compared to normal mice but similar neuropathology and PrP^{Sc} deposition (Scott *et al.*, 1989).

There is growing evidence that BSE has passed from cattle to humans causing an atypical new variant of CJD (vCJD). Recent emergence of vCJD was first recognised in young adults and showed a novel pathological phenotype (Will *et al*, 1996; Chazot *et al*, 1996). Lesion profile studies and glycoform analysis have reveal similarities between the BSE agent and vCJD (Collinge *et al*, 1996; Hill *et al.*, 1997; Bruce *et al.*, 1997). Transmission studies of BSE and vCJD have been completed in lines of Tg mice and would appear to support this theory (Telling *et al.*, 1994; Hill *et al.*, 1997;Scott *et al*, 1997). A recent study showed Tg mice expressing bovine PrP gene were highly susceptible to infection from primary passage BSE and vCJD and propagated incubation periods, neuropathology and PrP^{Sc} proteins which were indistinguishable by Western blotting analysis (Scott *et al*, 1999). It was generally assumed that humans would benefit from some degree of protection from the species barrier between cattle and humans however accumulating data suggests that the TSE agent responsible for BSE, and consequently vCJD, is highly adaptable at infecting new hosts.

Table 1.2:Demonstration of the species barrier to TSE transmission using transgenic models.

Scrapie source	Recipient	Mean incubation Period (days)	PrP^{Sc} type
Mouse[a]	hamster	378	Mouse
	Tg mouse (hamster PrP gene)[c]	175	Mouse
	Wild type mouse	120	Mouse
Hamster[b]	Hamster	65	Hamster
	Tg mouse (hamster PrP gene)[c]	75	Hamster
	Wild type mouse	>720	?

Note: [a](Kimberlin and Walker, 1978; Kimberlin *et al.*, 1987), [b](Scott *et al.*, 1989; Kimberlin *et al.*, 1989; Prusiner *et al.*, 1990), [c] Transgenic mice encoding at least one copy of the Syrian hamster PrP gene.

The transgenic studies have shown that neuropathology and synthesis of PrP^{Sc} is species specific and reflects the genetic origin of the inoculum. According to the prion hypothesis, TSE agent replication requires the interaction of the host PrP^{C} and the donor derived PrP^{Sc}, the species barrier effect is caused by differences in the primary sequence of the host PrP^{C} which reduced efficient PrP^{Sc} formation (Scott *et al.*, 1989; Prusiner *et al.*, 1990). In contrast, the virino hypothesis suggests that the species barrier is the result of agent adaptation to the new host. If PrP^{C} is the site for replication of the TSE agent differences between the host PrP^{C} primary sequence and the donor PrP^{C} primary sequence may prevent efficient interaction of the agent informational molecule (which encodes strain specific characteristics) and hence prolong incubation period (Dickinson and Meikle, 1971; Dickinson and Outram, 1979).

The species specificity observed in cell free conversion of radiolabelled recombinant PrP^{C} to PrP^{Sc} reflects the known species barriers in TSE transmission in animals (Kocisko *et al.*, 1994; Caughey *et al* 1995). In the cell free system PrP^{Sc} isolated

from scrapie-infected hamster brain tissue induced the conversion of hamster PrP^{C} to PrP^{Sc} but was unable to convert mouse PrP^{C} to PrP^{Sc} (Kocisko, 1995). This correlates with the fact that hamster scrapie strain 263K can infect hamsters but not mice.

1.6 Characterisation of the PrP Isoforms.

1.6.1 Biochemical and physical properties.

Comparative studies of PrP^{C} and PrP^{Sc} have highlighted many distinct features of the normal and disease-associated protein isoforms (summarised in Table 1.3). These properties are generally used to determine the type of PrP protein within tissues and the presence of PrP^{Sc} is assumed to be a marker for infectivity (section 1.6.4). PrP^{Sc} deposition has to date been detected mainly in the CNS, peripheral nervous system and (to a lesser extent) lymphoid tissue of scrapie affected sheep, mice and humans (Prusiner, 1993; Ikegami *et al.*, 1991;Prusiner, 1998)

1.6.2 Function of PrP^{C}.

Although widely expressed in a variety of different cell types, the function of PrP^{C} is still unclear, although the apparent high conservation observed across all species analysed to date suggests a key role for PrP in the metabolic process of the cell. This proposal is supported by housekeeping features of the PrP gene; wide spread expression, developmental regulation (section 1.9) and promoter structure (section 1.8). Its position on the cell surface of neurones has also suggested an involvement in synaptic function, cell-cell communication and/or adhesion (Manson *et al.*, 1992a). It was expected that mice deficient for the PrP gene (PrP null, *Prnp* $^{0/0}$) would show a phenotype distinct for the loss of PrP^{C} function, however the first null lines generated appeared to be normal (null line designated Zrch1, Bueler *et al.*,1992; null line designated Npu1, Manson *et al.*, 1994). Subsequently, it was found that these mice showed altered circadian activity (Tobler *et al.*, 1997) and electrophysiological (synaptic function) abnormalities (Collinge *et al.*, 1994). The

defects in synaptic function however, have never been reproduced and remain uncertain. The lack of phenotype due to PrP knockout may be the result of PrP^C being replaced by proteins with similar functions (Tremblay *et al.*, 1998, section 1.6.2.1).

Recently PrP^C was shown to bind copper ions (Cu^{2+}) through histidine residues within octarepeats near the N-terminus (figure 1.3, section 1.6.2.1). In PrP null mice, the activity of Cu/Zn superoxide dismutate (SOD, a major antioxidant enzyme) has been found to be substantially lower compared to wild type mice. These observations suggest that PrP may have a physiological function in controlling stress as a result of the generation of oxygen free radicals (Brown *et al.*, 1997; Stockel *et al.*, 1998; Viles *et al.*, 1999).

Table 1. 3: Summary of the biochemical and physical properties of PrP^C and PrP^{Sc}.

PrP^C	PrP^{Sc}
M_r 33-35kDa	M_r 33-35kDa[a]
Mature protein ~210 amino acids	Mature protein ~210 amino acids
Predominantly α helix structure	Predominantly β-sheet structure[b]
Completely sensitive to proteolysis	Protease resistant core, M_r 27-30kDa[a]
Soluble in detergent	Forms insoluble aggregates in detergent[a]
2 glycosylation sites	2 glycosylation sites[c]
Attached to cell surface by GPI anchor[d]	Primarily located within cells as fibrils or deposits
GPI anchor sensitive to cleavage by PIPLC	GPI anchor only sensitive to cleavage after denaturation [e]
Synthesis and degradation rapid (~6 hours)	Synthesis slow, degradation unknown [f]

[a](Meyer *et al.*, 1986), [b](Pan *et al.*, 1993), [c](Oesch *et al.*, 1985), [d](Stahl; *et al.*, 1990a), [e](Stahl; *et al.*, 1990b), [f](Caughey, 1993)

1.6.2.1 Prnd (Dopple) – the novel PrP-like gene

The lack of a severe phenotype for two *Prnp* null mice (*Prnp* $^{0/0}$) lines first gave rise to the suggestion that proteins might exist with functions that overlap with PrP^C. The development of two further mouse lines, Ngsk (Sakaguchi *et al.*, 19996) and Rcm0 (Moore, 1997), showed normal development but displayed late-onset ataxia. Furthermore, Ngsk Prnp $^{0/0}$ mice also showed loss of Purkinji cells (Sakaguchi *et al.*, 1996). Studies of the regions flanking the *Prnp* gene in mouse soon discovered a novel PrP-like gene, designated *Prnd* (Moore *et al.*, 1999). The *Prnd* gene contains an ORF encoding a protein named dopple (Dpl) which has 25% sequence identity to the C-terminal 2/3rd of PrP^C and is predicted to contain 3 α-helices and 2 disulphide bonds but lacks an octapeptide repeat domain found in PrP (section 1.6.4). *Prnd* homologues have been identified in rats, humans, sheep and cattle (Mead *et al.*, 2000).

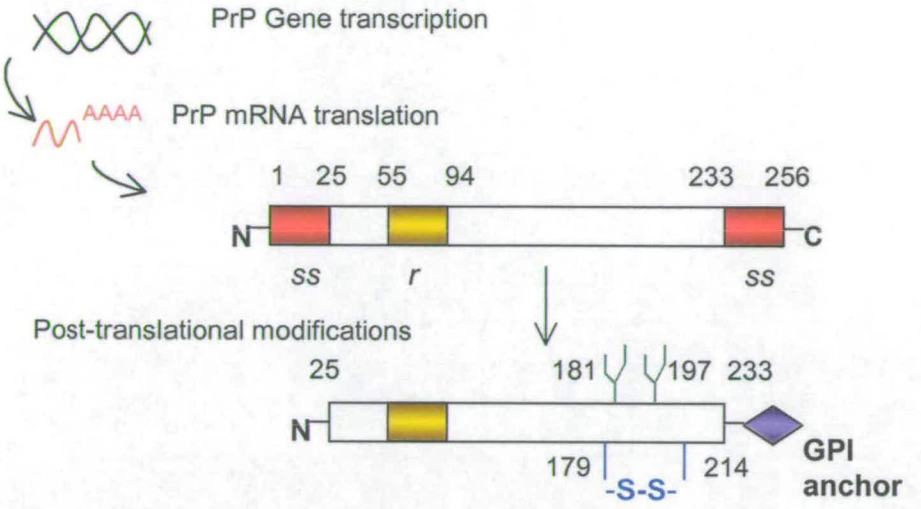
Inappropriate expression of *Prnd* may be linked to neurodegeneration. In normal mice, like PrP, Dpl mRNA is expressed during development but, unlike PrP does not seem to be abundant in the CNS. It was proposed by Moore *et al.* (1999) that loss of Purkinje cells in the Zrch1 and Rmo1 lines was due to long-range perturbations in gene expression as a result of cloning methods to create the $PrP^{0/0}$ mice. The expression pattern of Dpl mRNA in normal mice was also observed in the PrP null line Zrch1. In contrast, Dpl was up-regulated in the CNS of Ngsk and Rcm0 $Prnp^{0/0}$ mice both of which displayed signs of neurological illness and Purkinje cell loss (Moore *et al.*, 1999). Crossing Ngsk $Prnp^{0/0}$ mice with Tg mice over-expressing mouse PrP prevented neurodegeneration (Nishida *et al.*, 1999). These findings suggest that over expression of *Prnd* in the CNS is toxic to Purkinje cells, the Dpl and PrP may have similar biological functions but also that expression of *Prnp* and *Prnd* may be linked to neurodegeneration.

1.6.3 Synthesis of PrP^C.

Synthesis of PrP^C (figure1.3) appears to follow the secretory pathway (Caughey *et al.*, 1989). During biosynthesis the N-terminal signal sequence is cleaved (Basler *et al.*, 1986) and the C-terminal sequence replaced by a gylcosyl phosphatidylinositol (GPI) anchor (Stahl *et al.*, 1990). In most species, the mature PrP^C protein contains five glycine-rich octa/nona-peptide repeats and two hexapeptide repeats near the N-terminal region. The number of the octa/nona-repeats can vary between species with most having five but some, like bovine, can have five or six repeats (Goldmann *et al.*, 1991b) although an extra octarepeat in cattle does not seem to be associated with differences in BSE susceptibility. In goats, a short PrP allelic variant with only 3 octarepeats has been reported as causing no spontaneous pathological effect (Goldmann *et al.*, 1998). Within the PrP translation product, the N-terminal signal sequence targets the product to the endoplasmic reticulum where the C-terminal signal sequence promotes the addition of a GPI anchor (Oesch *et al.*, 1985). The modified protein is then transported to the golgi where glycosylation may occur at two sites, Asn181 and Asn 197. The C-terminal signal sequence is also thought to be involved in the targeting of PrP^C to caveolae-like domains on the cell membrane (Kaneko *et al.*, 1997). Deletion of the C-terminal signal sequence prevents attachment of PrP^C to the

cell surface and results in its secretion into the cell medium. Transport of PrP^C between the plasma membrane and endocytic vesicles has been observed in cell cultures and soluble forms of PrP^C have also been found in the medium of cultured cells and human cerebrospinal fluid (Tagliavini *et al.*, 1992; Shyng *et al.*, 1995). The metabolism of PrP^C is considered to be associated with non-acidic compartments bound by cholesterol-rich membranes (Taraboulos *et al.*, 1995) and has a half-life estimated at 3-6 hours in cultured cells (Caughey *et al.*, 1993). There is no evidence for PrP^{Sc} turn over in cells which may account for PrP^{Sc} accumulation *in vivo* (Caughey *et al.*, 1993). Once formed PrP^{Sc} accumulates in secondary lysosomes, on the cell surface or in the extracellular space.

a) Molecular events of PrP^C synthesis



b) Suggested Cellular Pathway in PrP synthesis

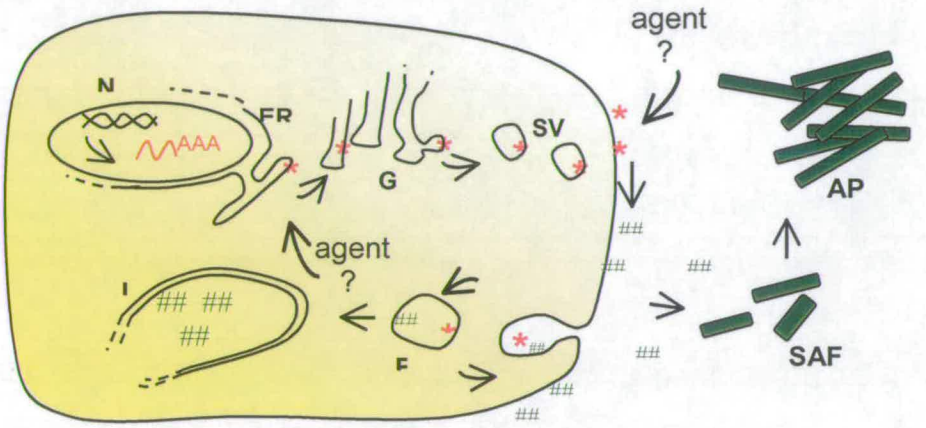


Figure 1.3: Proposed model for PrP^C and PrP^{Sc} synthesis

a) Maturation of the primary PrP translation product involves cleavage of signal sequences (*ss*) from the N and C terminal, addition of the GPI anchor, the formation of a disulphide bond (-s-s-) and glycosylation at one or two sites (residues 181 and 197). The octarepeat region is showed by *r* **b)** Synthesis of PrP^C (*)is considered to follow the secretory pathway: mature mRNA is transported from the nucleus (N) to be translated in the endoplasmic reticulm (ER) where it then moves to the golgi apparatus (G) in vesicles where it undergoes further modification. It is then transported to the cell membrane by secretory vesicles. Conversion of PrP^C to PrP^{Sc} (#) may occur at the cell surface or in endocytotic vesicles (EV) according to the prion hypothesis. Alternatively the agent may intercept the PrP^C synthetic pathway to create PrP^{Sc} independently. SAF; scrapie associated fibrils. AP: amyloid plaque.

1.6.3.1 Topology of PrP^C.

Biosynthetic studies of the PrP protein have suggested that two distinct forms are synthesised in the endoplasmic reticulum (ER): one that is fully translocated (secretory) and one which is transmembrane (Hay *et al.*, 1987;Yost *et al.*, 1990;). Digestion of the transmembrane form with proteases, in the absence of detergent, yields two fragments suggesting two topologies for the transmembrane PrP protein (Hay *et al.*, 1987; Lopez *et al.*, 1990). One topology with the COOH-terminal in the lumen (^{Ctm}PrP) the other with the NH2-terminal in the lumen (^{Ntm}PrP). Both transmembrane forms appeared to span the membrane over the same hydrophobic stretch of PrP (residues Ala_{113}-Ser_{135}) which has been designated the TM1 domain. Mutations, deletions and insertions into the TM1 and a second region, the stop transfer effector domain (STE, Lys_{104}-Met_{112}) can alter the amounts of each topological form of PrP (Yost *et al.*, 1990). The role of these topological forms *in vivo* was investigated in $PrP^{0/0}$ mice expressing transgenes with various mutation in the STE-TM1 domain. Those expressing the ^{Ctm}PrP form showed neurological signs of illness and neurological pathology similar to those in scrapie, but PrP^{Sc} was found to be absent (Hedge *et al.*, 1998). Mice expressing ^{Ntm}PrP transgene did not show any signs of illness. ^{Ctm}PrP was also found to accumulate in brain tissue from GSS affected patients who had a mutation of alanine to valine at codon 117 which is in the STE domain. The hypothesis proposed was that distinct topological forms of PrP^C may be involved in TSE disease pathogenesis.

1.6.4 Structural characteristics of PrP^C and PrP^{Sc}.

The PrP protein amino acid sequence is highly conserved between species with generally more than 90% amino acid identity (Schatzl *et al.*, 1997), and containing around 250 amino acids, the exact number depending on the species e.g. 256 in sheep, 254 in human and 253 in rodents. Both PrP^C and PrP^{Sc} are the product of the same host gene with no apparent TSE-associated differences in either the amounts of PrP mRNA or primary structure. Although the pattern of glyscosylation observed in PrP^C and PrP^{Sc} within a host has been shown to be distinct (Somerville,1999), pulse-chase metabolic labelling studies have shown that PrP^{Sc} may be derived from PrP^C

(Borchelt *et al.*, 1990; Caughey and Raymond, 1991), Therefore, the primary difference between the two isoforms may simply be conformational.

Nuclear Magnetic Resonance (NMR) studies on recombinant forms of mouse and hamster PrP^C have determined the N-terminal region (residues 23-120) of PrP^C to be flexible in solution while the C-terminal segment (residues 121-231) has a defined tertiary structure of three α-helices and a short two-stranded antiparallel β-sheet (Riek *et al.*, 1996; Riek *et al*, 1997; Donne *et al.*, 1997;James *et al.*, 1997). The C-terminal structure is independent of the flexible N-terminal tail as the structure is preserved in N-terminally truncated recombinant PrP forms.

The tertiary structure of PrP^{Sc} has not yet been determined because of the insolubility of this protein isoform. Limited X-ray diffraction studies have indicated that PrP^{Sc} is primarily in β-sheet conformation (Inouye and Kirschner, 1997). This finding has been confirmed using a synthetic polypeptide spanning amino acids 90 to 145 (Nguyen *et al.*, 1995) that was also found to form β-sheet complexes with PrP^C *in vitro*. Further studies using recombinant antibody Fab fragments against epitopes of PrP^C and PrP^{Sc} indicated that major conformational differences exist between the two isoforms in the region between amino acid residues 90 and 120 whereas the C-terminal structure may be the same in both (Peretz *et al.*, 1997). The protease resistance of PrP^{Sc} therefore may be conferred within the 90-120 segment of PrP^{Sc} as enzyme cleavage sites in this region are protected or hidden within the PrP^{Sc} structure.

1.6.5 PrP^{Sc} and the association with infectivity.

While protease resistance of PrP^{Sc} seems to be the marker for prion infectivity, a number of experiments appear to dissociate PrP^{Sc} from infectivity. Lasmezas *et al* (1997) reported that mice injected with BSE infected cattle brain homogenate showed symptoms of a neurological disease after an incubation period of one to two years, but biochemical analysis of the mouse brains did not detect PrP^{Sc} in more than 55% of cases (PrP^{Sc-}). Histological examination of the brain samples revealed neural

cell death in all mice, however, other classic changes associated with TSEs, such as neuronal vacuolation and astrocytosis, were limited to those mice with detectable PrP^{Sc}, (PrP^{Sc+}). On sub-passage, mice inoculated with PrP^{Sc+} mouse brain homogenate showed a shortened incubation period and PrP^{Sc} was detectable in all mice. During serial passage with homogenate from PrP^{Sc-} brains, inoculated mice showed classic TSE changes, had shorter incubation periods and PrP^{Sc} was readily detectable in brain extracts. The similarity of clinical disease seen in the sub-passages from PrP^{Sc-} and PrP^{Sc+} mice supports the idea that neuronal death is a major factor in TSE disease, and that the presence of lesions and gliosis is directly linked to the accumulation of PrP^{Sc}. Lasmezas *et al* (1997) raised the possibility that TSEs can be transmitted without detectable PrP^{Sc}, supporting the theory that an infectious entity in addition to PrP^{Sc} may transmit TSE. However the failure to detect PrP^{Sc} in the initial BSE-inoculated mice may have been reduced sensitivity of the anti-PrP antibodies to a low level of PrP^{Sc} present in the brain tissue.

More recently, two separate studies have shown that the protease resistance acquired by PrP in its conversion from PrP^{C} to PrP^{Sc} is not a sufficient marker for infectivity. It is known that, in contrast to normal mice, Tg mice expressing chimeric hamster-mouse PrP^{C} are susceptible to infection with hamster scrapie (section 1.5.2.1). Hill *et al.* (1999) expressed [^{35}S]-radiolabelled chimeric hamster-mouse PrP^{C} that acquired protease resistance following incubation with purified hamster scrapie in mouse erythroleukaemia cells. Transmission studies with the protease resistant chimeric PrP^{C} did not produce a scrapie-like disease in Swiss CD-1 mice. Additionally, Shaked *et al.* (1999) in the presence of dimethyl sulfoxide (DMSO) purified a fraction of protease resistant PrP^{Sc} that was resistant to high speed purification and aggregation. These soluble PrP27-30 molecules were able to aggregate on removal of DMSO but not into the characteristic rod structures of SAF. Furthermore when inoculated into hamster brains the purified PrP27-30 fraction proved to be 99% less infective compared to equivalent untreated PrP27-30 rods. These data argue that the conversion of PrP^{C} to protease resistant PrP^{Sc} alone is not responsible for the production of infectivity, and that protease resistance may not be a suitable test for the presence of the TSE agent.

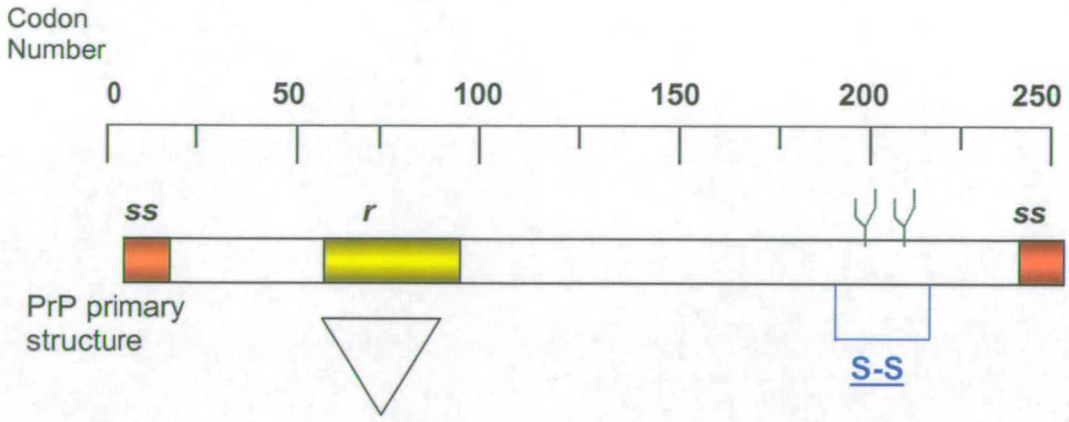
1.7 Host Genetics: PrP Gene Polymorphisms and Mutations

1.7.1 Human PrP gene polymorphisms and mutations.

To date, approximately 20 different *PRNP* point mutations or insertions of octapeptide repeats regions are known to segregate with inherited human TSE diseases (Prusiner, 1998). Some of these mutations are illustrated in Figure 1.4. The linkage between GSS and *PRNP* polymorphisms was realised with the discovery of a mutation, substituting proline to leucine at codon position 102 (Hsiao *et al.*, 1989), which has since been found in several GSS-affected families (Goldgaber *et al.*, 1989; Kretzschmar *et al.*, 1991). Three mutations have also been identified in patients with familial CJD (Goldgaber *et al.*, 1989; Owen *et al.*, 1989;Collinge *et al.*, 1989) and one for FFI . FFI and CJD are pathologically distinct from GSS. A silent mutation at codon 129 was found to modulate susceptibility to CJD(Palmer *et al.*, 1991) but also determine whether carriers of a pathological mutation at codon 178 will develop CJD or FFI (Medori *et al.*, 1992).

The mechanism by which these mutations cause TSE disease (if they do) is unclear. One explanation is that the mutations may cause the spontaneous conversion of PrP^{C} to PrP^{Sc} by destabilising the protein structure enabling a conformational change to PrP^{Sc}. Structural studies have shown that some of the *PRNP* mutations have the same stability as PrP^{C} suggesting that destabilisation of PrP^{C} does not lead to PrP^{Sc} formation (Huang *et al.*, 1994; Cohen *et al.*, 1994). Several lines of transgenic mice (Tg) have been created which appear to show spontaneous development of neurodegeneration (Westaway *et al.*, 1994a; Muramoto *et al.*, 1997; Hedge *et al.*, 1998; Shmerling *et al.*, 1998; Chiesa *et al.*, 1998). One line which over-expressed the mouse homologue (proline to leucine at position 101) of the human GSS-related proline to leucine mutation at 102 (Hsiao *et al.*, 1990; Telling *et al.*, 1996) showed signs of TSE pathology, PrP deposition and transmission of disease to similar Tg mice but not wild type mice (Hsiao *et al.*, 1994). However, PrP deposited in these mice was not proteinase K resistant (Telling *et al.*, 1996). A second mouse Tg line, developed to express the GSS mutation of 9 octapeptide repeat insertion into the PrP protein coding region, showed similar pathology to GSS and produced a PrP protein

which shared limited properties with PrP^{Sc} (Chiesa *et al.*, 1998). The possibility exists that over-expression of these transgenes may explain the observed phenotypes rather than expression of the mutation itself. For instance, following a gene targeting study to replace the wildtype mouse gene with a single copy of an allele with the proline to leucine mutation at position 102 no spontaneous degeneration was recorded (Moore *et al.*, 1995).



Human TSE	Insertions/ Deletions	Polymorphisms/ mutations
GSS	120bp-129M	**Pro102Leu**
	144bp-129M	Pro105Leu-129Met
	168bp-129V	**Ala117Val-129Val**
	192bp-129?	Tyr145STOP-129Met
	216bp-129M	Val180Ile-129Met
		Phe198Ser-129Val
		Gln217Arg-129Val
CJD	48bp-129?	**Asp178Asn-129Val**
	144bp=129M	**Glu200Lys-129Met**
		Val210Ile-129Met
		Met232Arg-129Met
FFI		**Asp178Asn-129Met**

Figure 1.4:Mutations and polymorphisms of the human PrP protein associated with human TSEs.

Several polymorphisms and mutations within the coding region or insertions in to region *r* (the octapeptide *repeat* region of the human PrP gene have been linked to TSE occurrence. Silent mutation at codon 129 (valine/ methionine) linked to susceptibility to CJD (129V/129M). Pathogenic mutations, shown in red, are known to be connected to TSE development in humans. *ss* denotes signal sequence., -S – S - , identifies the disulphide bond and the glycosylation sites shown as Y .Adapted from Liemann and Glockshuber (1998).

1.7.2 Ovine PrP gene polymorphisms.

Originally it was assumed that scrapie was a genetically inherited disease as disease appeared to segregate in the pattern of an autosomal recessive gene (Parry, 1962). However, controlled breeding experiments have shown that natural (Foster and Dickinson, 1988a) and experimental (Dickinson, 1974; Foster and Dickinson, 1988b) scrapie infections are dependant on a gene controlling scrapie susceptibility. To study scrapie susceptibility, a flock of South County Cheviots was founded in 1960 and selected into two lines depending on response to challenge with a source of experimental scrapie known as SSBP/1; positive line sheep succumbed to subcutaneous challenge whereas negative line sheep survived (Dickinson *et al.*, 1968 a+b). Since 1962 the flock has been closed to any outside breeding and is known as the Neuropathogenesis Unit (NPU) Cheviot flock. Offspring the result of crossing positive and negative line sheep were found to be susceptible to SSPB/1 but had a prolonged incubation period compared to positive line sheep (Dickinson and Outram, 1988). This finding suggested that a single autosomal gene, designated *Sip*, with two alleles was dominant for short incubation periods, was responsible for controlling scrapie incubation periods but the gene is now known to be much more complex than this. Restriction fragment length polymorphisms (RFLP) for the sheep PrP gene were found to be in accordance with the Sip genotype (Hunter *et al.*, 1989). This discovery raised the possibility that, as in the mouse (Dickinson *et al.*, 1968b; Carlson *et al.*, 1986; Hunter *et al.*, 1987), the PrP genotype has a major influence on scrapie susceptibility. From TSE transmission studies in mice the gene controlling scrapie incubation period, initially designated *Sinc*, was identified as the PrP gene (Moore *et al.*, 1998). Therefore the equivalent gene in sheep, know as *Sip*, is also likely to be the PrP gene.

In the PrP gene of sheep several polymorphisms have been detected (Goldmann *et al.*, 1990; Goldmann *et al.*, 1991a; La Planche *et al.*, 1993), three of which (PrP codons 136, 154, 171) are strongly linked to scrapie susceptibility differences (Figure 1.5).

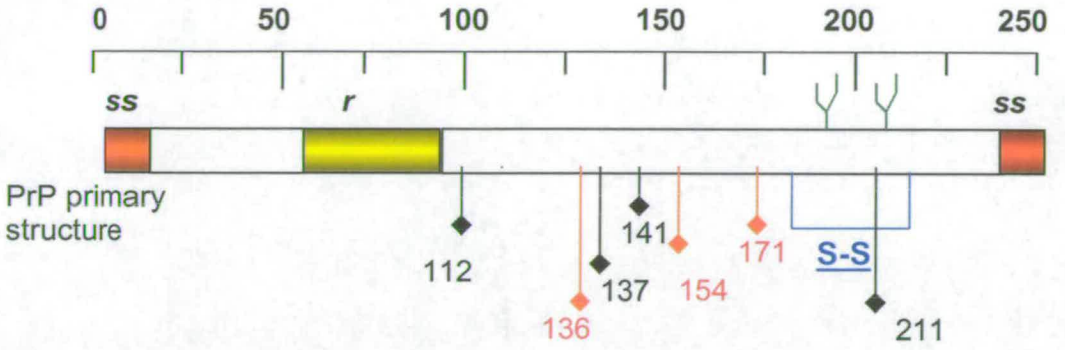


Figure 1.5: Polymorphisims of the ovine PrP protein.

Several polymorphisms within the ovine PrP gene have been linked to scrapie incidence. Variation at codons 136, 154 and 171 (red codons) have been linked to scrapie susceptibility. *ss* denotes signal sequence, *r* represents the repeat region, -S – S - identifies the disulphide bond and the glycosylation sites shown as Y

To date, with respect to scrapie susceptibility and the three codons discussed above, there are five known allelic variations of the PrP gene. These are;

1) $A_{136}R_{154}Q_{171}$
2) $A_{136}H_{154}Q_{171}$
3) $A_{136}R_{154}R_{171}$,
4) $V_{136}R_{154}Q_{171}$
5) $A_{136}R_{154}H_{171}$.

The PrP alleles and their frequencies within a particular breed of sheep are very specific with some alleles being more common in one breed compared to another (Table 1.4) (Dawson *et al.*, 1998, review). The combination of PrP alleles (genotypes) is also important. For example, heterozygote genotypes carrying $V_{136}R_{154}Q_{171}$ are at significant risk of scrapie incidence but not when paired with $A_{136}R_{154}R_{171}$. In some breeds $V_{136}R_{154}Q_{171}$ is rare, e.g. Suffolks where, although scrapie incidence is low, the allele $A_{136}R_{154}Q_{171}$ appears to confer highest risk of scrapie. (Hunter *et al.*, 1994; Westaway *et al.*, 1994). In most sheep breeds, homozygosity for alanine at codon 136, A_{136}, seems to offer resistance to scrapie development from both natural and experimental infection whereas valine at PrP codon 136, V_{136}, leads to disease with a short incubation period after challenge with SSBP/1 (Hunter *et al.*, 1996). To date only one PrP $AA_{136}RR_{154}RR_{171}$ sheep world

wide has been documented to be scrapie positive (Ikeda et al., 1995). Following subcutaneous (sc) challenge with SSBP/1, NPU Cheviots homozygous for valine (positive line sheep) at codon 136 develop scrapie after an incubation period of 167 days +/- 5, VA_{136} heterozygotes have an incubation period of 322 days +/- 10. AA_{136} homozygotes (negative line sheep) resist similar inoculation with SSBP/1. However, this is not the case when BSE or an alternative scrapie strain, i.e. CH1641, is the source of TSE agent (Goldmann *et al.*, 1994a; Goldmann *et al.*, 1994b). When challenged with BSE or CH1641 PrP codon 171 appears to be predominantly associated with scrapie incidence: glutamine homozygotes, QQ_{171}, succumb to intracerebral (ic) challenge, QR_{171} heterozygotes have a longer incubation period and RR_{171} homozygotes are relatively resistant within their lifespan. In addition to highlighting the role of PrP polymorphisms, these data may suggest that particular polymorphisms or genotypes are associated with susceptibility to different strains of scrapie.

Studies have shown that scrapie is not a spontaneous genetic disease in sheep. In a recent report scrapie-associated PrP genotypes were recorded in sheep from countries free of scrapie (Hunter *et al.*, 1997) where rigorous methods are taken to protect sheep from scrapie. This would suggest that although the PrP genotype is important to the progression of the disease, scrapie infection relies on the additional factor of an infectious agent. There is, however, a genetic component in the control of disease incidence as described above which adds more controversy to the debate over the identity of the scrapie agent. If the prion theory is correct, the polymorphisms seen in sheep PrP protein may favour spontaneous conversion of PrP^{C} to PrP^{Sc}. However this does not explain the obvious lateral transmission of scrapie between sheep nor why scrapie is not observed in countries where susceptible genotypes have been identified? If however the virus/ virino theories are correct, how does this explain the incidence of TSEs in humans without apparent infection, why are some sheep seemingly resistant to scrapie development? Although the PrP gene has been proven to influence scrapie susceptibility, little is understood about the control of gene expression or the interaction of the PrP protein with other gene products or indeed the infectious agent.

Table 1.4 : PrP alleles and their distribution in various sheep breeds. Scrapie susceptibility shown as high risk (red, bold) or low risk (blue bold). (Dawson *et al*, 1998)

Breed	**Predominant allele**	**Scrapie susceptibility**
Cotswold, Hampshire Down Soay Vendeen	$A_{136}R_{154}Q_{171}$ $A_{136}R_{154}R_{171}$	**$A_{136}R_{154}Q_{171}$/$A_{136}R_{154}Q_{171}$** **$A_{136}R_{154}R_{171}$/ $A_{136}R_{154}R_{171}$**
Suffolk	$A_{136}R_{154}Q_{171}$ $A_{136}R_{154}H_{171}$ $A_{136}R_{154}R_{171}$,	**$A_{136}R_{154}Q_{171}$ /$A_{136}R_{154}Q_{171}$**
Blue de Maine, Wensleydale Border Leicester, Poll Dorset,	$A_{136}R_{154}Q_{171}$ $A_{136}R_{154}R_{171}$ $V_{136}R_{154}Q_{171}$	**$A_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171}$** **$V_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171}$** **$A_{136}R_{154}R_{171}$/ $A_{136}R_{154}R_{171}$**
Charollais	$A_{136}R_{154}Q_{171}$ $A_{136}R_{154}R_{171}$ $V_{136}R_{154}Q_{171}$ $A_{136}R_{154}H_{171}$	**$A_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171}$** **$V_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171}$** **$A_{136}R_{154}R_{171}$/ $A_{136}R_{154}R_{171}$**
Bluefaced Leicester	$A_{136}R_{154}Q_{171}$ $A_{136}R_{154}R_{171}$ $A_{136}H_{154}Q_{171}$	**$A_{136}R_{154}Q_{171}$ /$A_{136}R_{154}Q_{171}$** $A_{136}R_{154}R_{171}$/ $A_{136}R_{154}R_{171}$
Cheviot, Dalesbred, Herdwick Scottish Blackface, Shetland Swaledale, Welsh Mountain	$A_{136}R_{154}Q_{171}$ $A_{136}R_{154}R_{171}$ $A_{136}H_{154}Q_{171}$ $V_{136}R_{154}Q_{171}$	**$V_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171}$** **$A_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171}$** $A_{136}R_{154}R_{171}$/ $A_{136}R_{154}R_{171}$
Texel, Lleyn	$A_{136}R_{154}Q_{171}$ $A_{136}R_{154}R_{171}$ $A_{136}H_{154}Q_{171}$ $V_{136}R_{154}Q_{171}$ $A_{136}R_{154}H_{171}$	**$A_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171}$** **$A_{136}R_{154}H_{171}$/ $V_{136}R_{154}Q_{171}$** **$V_{136}R_{154}Q_{171}$/ $V_{136}R_{154}Q_{171}$** $A_{136}R_{154}R_{171}$/ $A_{136}R_{154}R_{171}$

1.7.2.1 PrP 3'untranslated region polymorphisms.

Three restriction fragment length polymorphisms (RFLP) for restriction digestion with the enzyme *Eco*RI, have been described in the PrP 3'UTR. The RFLP are 6.8kb (e1), 5.2kb (e2) and 4.kb (e3), with e1 and e3 acting as differential markers for susceptibility (Goldmann *et al.*, 1990; Hunter *et al.*, 1991). The e1 RFLP (relatively rare in healthy controls) has been associated with increased susceptibility to experimental and natural scrapie cases whereas the e3 RFLP is associated with relative scrapie resistance in studies across different sheep breeds throughout the world (Hunter *et al.*, 1991; Hunter *et al.*, 1992; Maciulis *et al.*, 1992; Muramatsu *et al.*, 1992). Hunter *et al.* (1992) described the distribution of the alleles in 167 sheep of different breeds and cross breeds affected with natural scrapie as e1:73-75%, e2: 3-1% and e3: 24%. The e3 allele was associated mainly with resistant genotypes, whereas the e2 allele did not appear to segregate preferentially with either susceptible or resistant genotypes. A comparative study of the Suffolk e1 and e3 3'UTR highlighted several further differences between the two alleles (Cheung, 1996). Amongst six polymorphic sites (including the *Eco*RI site) there was one extra instability motif present on the e1 allele compared with the e3 allele. Although the e1 allele does not have 100% association with scrapie occurrence, it may confer increased scrapie susceptibility in the presence of other known polymorphisms or to infection with specific scrapie stains (Hunter *et al.*, 1991; Hunter *et al.*, 1992).

Disease-associated mutations of a 3'UTR are not unique. A polymorphism in the 3'UTR sequence of the stromal cell-derived factor (SDF)-1 gene of G to A (SDF1-3'A) has been associated with increasing susceptibility to human immunodeficiency virus (HIV) type 1 (van Rij *et al.*, 1998; John *et al.*, 2000). Patients homozygous for the G-A polymorphism (SDF1-3'A/3'A) display accelerated progression to AIDS but a prolonged disease process (Van Rij *et al.*, 1998). The SDF-1 gene encodes the chemokine receptor ligand which binds the T-tropic HIV-corecpetor CXCR4 and may be one route of infection for the virus. In support of this theory, the presence of the SDF1-3'A allele in infected mothers increases the likelihood of transmission of the HIV virus to an infant, regardless of the infants SDF1 genotype (John *et al.*, 2000).

1.8 PrP Gene Structure.

1.8.1 PrP gene structure.

The genomic structure of the PrP gene is well conserved amongst different species and is described as containing three exons and two introns (Figure 1.6). The isolation and comparison of cDNA and genomic DNA clones has shown that mouse, sheep and cattle PrP genes contain three exons (Westaway *et al.*, 1987; Puckett *et al.*, 1991;Westaway *et al.*, 1994; Horiuchi *et al.*, 1998; Lee *et al.*, 1998). Both exons I and II encode for 5' untranslated region (UTR, 140bp) whereas exon III encodes 5'UTR (10bp), the full open reading frame (ORF, approximately 760bp depending on species) and a 3'UTR which can vary from 1.2 to 3.3kb in length (section 1.8.4) (Goldmann *et al.*, 1990; Goldmann *et al.*, 1993; Westaway *et al.*, 1994; Inoue *et al.*, 1997; Horiuchi *et al.*, 1998). Sequence adjacent to, and 5' from, exons I and II has been associated with promoter and regulatory elements (Baybutt and Manson, 1997). Exon II is transcriptionally expressed in most species, yet human PrP mRNA appears to contain only exons I and III (Westaway *et al.*, 1994) despite strong homology for exon II in human genomic sequence when compared with other species (Lee *et al.*, 1997). The Syrian hamster (SHa) was once thought to express PrP mRNA containing only exons I and III until mRNAs containing exon II were observed in astrocytes from scrapie-infected hamster brain (Li and Bolton, 1997). The function of PrP exon II remains unclear and it may be that human exon II is expressed in mRNAs of certain cell types or tissues.

The length of the mRNA 5'untranslated (UTR) region is known to vary in different species and may be due to variable transcription start sites or alternative splicing generating different transcripts but which code an identical protein. For example, in mice the transcription start site has been shown to vary between 30 and 60bp upstream of the exon I/intron I boundary (Westaway *et al.*, 1987). Similarly in bovine tissue, two mRNAs with 5'UTRs that vary by 100 nucleotides are thought not to be the result of transcription originating from one start site but alternative splicing at the exon I/ intron I boundary (Horiuchi *et al.*, 1997). No alternative 5'UTR has been identified in sheep.

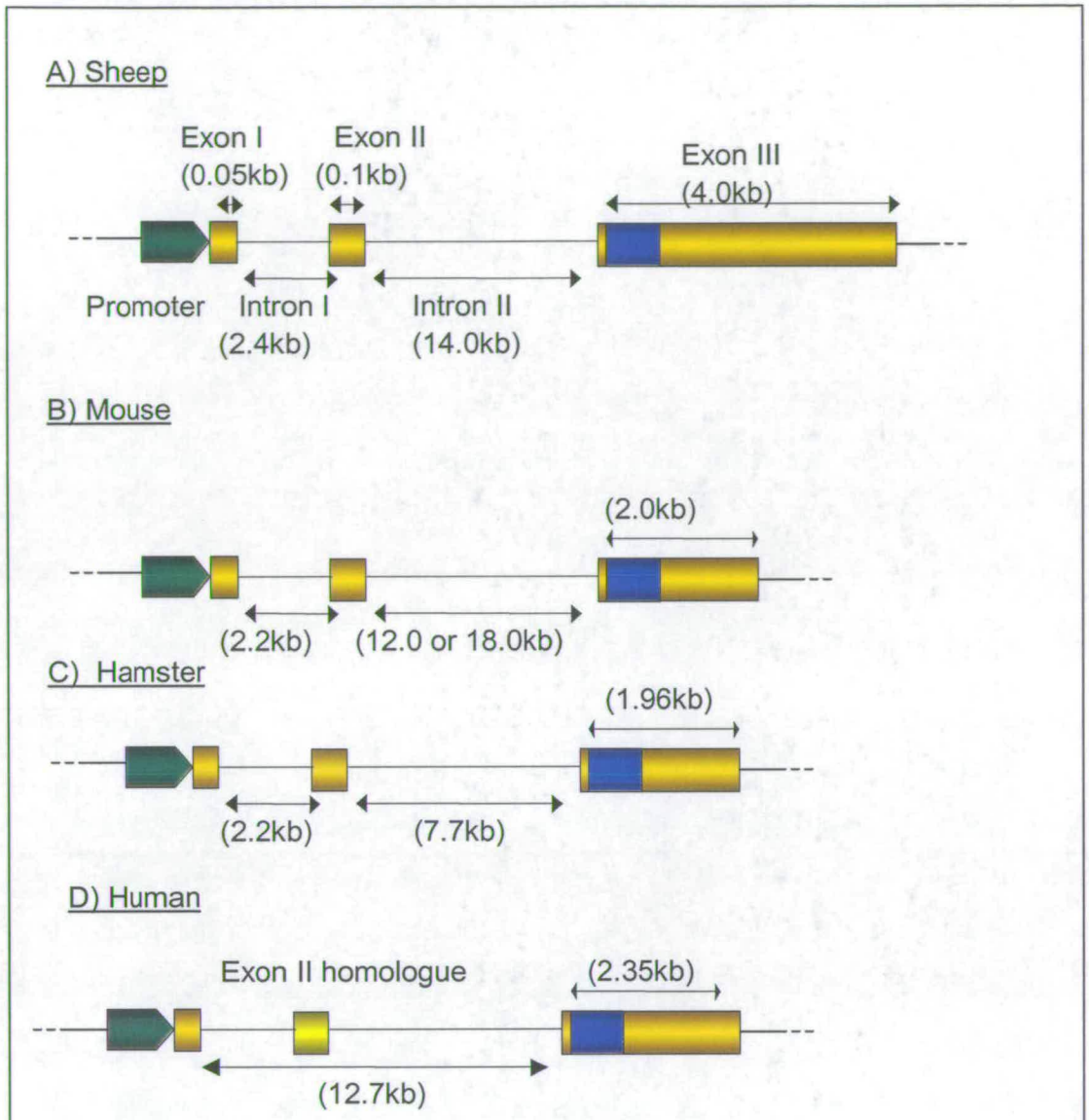


Figure 1.6 : PrP gene structure in different species

The PrP gene shows essentially conserved structure across different species. Compiled from Goldmann *et al.*, (1990); Li and Bolton (1997); Lee *et al.*, (1998)

1.8.2 PrP promoter region.

The promoter region of the PrP gene lacks typical regulatory elements known to control the precise initiation of transcription, for example TATA boxes. Instead, multiple copies of G-C rich repeats, similar to those present in house-keeping genes, have been found near the transcription start site in humans, ruminants and mouse PrP genes (Puckett *et al.*, 1991;Dynan *et al.*, 1986; Westaway *et al.*, 1994; Baybutt and Manson, 1997). These G-C rich motifs may function as recognition binding sites for the transcription factor SP1 (Mcknight and Tjian, 1986). Binding sites for other transcription factors have been identified in the PrP promoter region but the number and location of these factors appeared to be species dependant (Lee *et al.*, 1998). Recent analysis of the Cheviot and Suffolk sheep PrP promoter region at NPU discovered polymorphisms for an SP-1 consensus site. However, it is unknown whether this polymorphism is linked to scrapie susceptibility (i.e. other PrP polymorphisms) or sheep breed (O'Neill *et al*, in preparation).

1.8.3 PrP introns.

There is evidence that control of PrP gene expression may also extend to sequences within the intronic regions. Recent reporter analysis studies have shown that deletions within the mouse intron I can influence the level of gene expression due to the presence of two independent sequences capable of promoter activity and promoter suppressor activity (Baybutt and Manson, 1997). Deleting intron II from the PrP gene lead to undetectable levels of PrP expression in Purkinje cells of the cerebellum without affecting the level of PrP expressed in other brain areas (Fischer *et al.*, 1996), implying that intron II may contain sequences necessary for cell-specific PrP expression. The size of PrP intron I is typically 2 to 2.4kb. In humans, however this may be as great as 12.7kb, assuming the absence of a homologous exon II in the genomic sequence. Furthermore, the size of intron II is known to vary between, and within, species. In mice two variants of intron II have been observed as approximately 18kb (*Prn-p*^{a}) and 11kb (*Prn-p*^{b}) in length (Westaway *et al.*, 1994a). Originally the variation in length was interpreted as a 6kb deletion in the *Prn-p*^{b} intron 2, however it has now been reported that it results from an insertion of

a retroviral genome into the *Prn-p*^{a} allele, which has been identified as a typical LTR-gag-pol-env-LTR retroviral structure (Lee *et al.*, 1998).

1.8.4 The ovine PrP exon III.

As described in section 1.8.1 the structure of the PrP gene in well conserved, however the size of exon III varies considerably between ruminants and other species. Exon III of the sheep gene (like that of cattle and goats) is much larger than that found in other species (Figure 1.6): ruminants 4000 nucleotides (Goldmann *et al.*, 1990), rodents 2000 nucleotides (Chesebro *et al.*, 1985), and humans 2350 nucleotides (Puckett *et al.*, 1991). The difference lies within the 3'UTR region of the sheep PrP gene which has apparent insertions of ruminant-specific elements (Goldmann *et al.*, 1993; Lee *et al.*, 1998) including long and short interspersed repetitive sequences reminiscent of transposable elements (Figure 1.7). Sequence analysis of the PrP 3'UTR from several Suffolk and Cheviot sheep PrP alleles has shown that the 3'UTR is polymorphic, contains several potential polyadenylation sites and a variable number of ATTTA instability motifs (Goldmann et al., 1990; Cheung, 1996; Goldmann, 1999). Whether these regions play any role in PrP expression is unknown and is the subject of this thesis.

Despite the considerable differences in length of PrP 3'UTRs between species there is a high degree of conservation in specific regions of the 3'UTR (Goldmann *et al.*, 1990) (Figure 1.7). On the basis of cross-species sequnce compariosns, sheep, human and mouse PrP gene 3''TRs have been divided into sections A to G. Only the sheep 4.6kb mRNA 3'UTR encodes all seven regions, the human having A, B, C, E and G and the mouse having A, C, E and G. The sheep has two additional regions D and F which include SINE and LINE elements (Figure 1.7). SINE and LINEs, or Short and Long Interspersed Nuclear Elements, are repetitive DNA sequences found dispersed throughout the genome at high frequency. SINEs and LINEs are considered to have originated from transposable or retroviral elements which can replicate by reverse transcription (Fanning et al., 1987). The mechanism of insertion of transposable elements is unclear but is though to involve a processes such as sequence-specific insertion, S-phase insertion, ectopic excision and recombinational

editing (Wichman *et al.*, 1992). Unlike conventional single copy nuclear genes, the copy number of transposable elements can vary without apparent phenotypic effects. Transposable elements can also achieve horizontal transfer between species (Maruyama and Hartl, 1991: review Hartl *et al.*, 1997). SINEs and LINES can exist as active or inactive transposable elements, where inactive forms are likely to have arisen from the loss of sequence necessary for transposition (Engels, 1989; Garza *et al.*, 1991). Interestingly, inactive elements can become active in the presence of active elements which complement the defect in the inactive copies. However, often inactive forms do not appear to have obvious deletions to distinguish them from active copies (Moerman and Waterston, 1989; Hartl,, 1989).

Regarding the ovine PrP gene, the LINE element shows homology to a mariner DNA transposon relic from the *Mellifera* (honeybee) subfamily of mariner elements (Lee *et al.*, 1998). Both the inactive and active form of the mariner element have been identified in certain Dresophila species (Jacobson *et al.*, 1986; Hartl, 1989). The active mariner element is 1289 nt long and contains a single open reading frame encoding a putative protein of 345 amino acids (Jacobson *et al.*, 1986). The effect of the SINE or LINE elements on ovine PrP gene expression will be considered in this thesis.

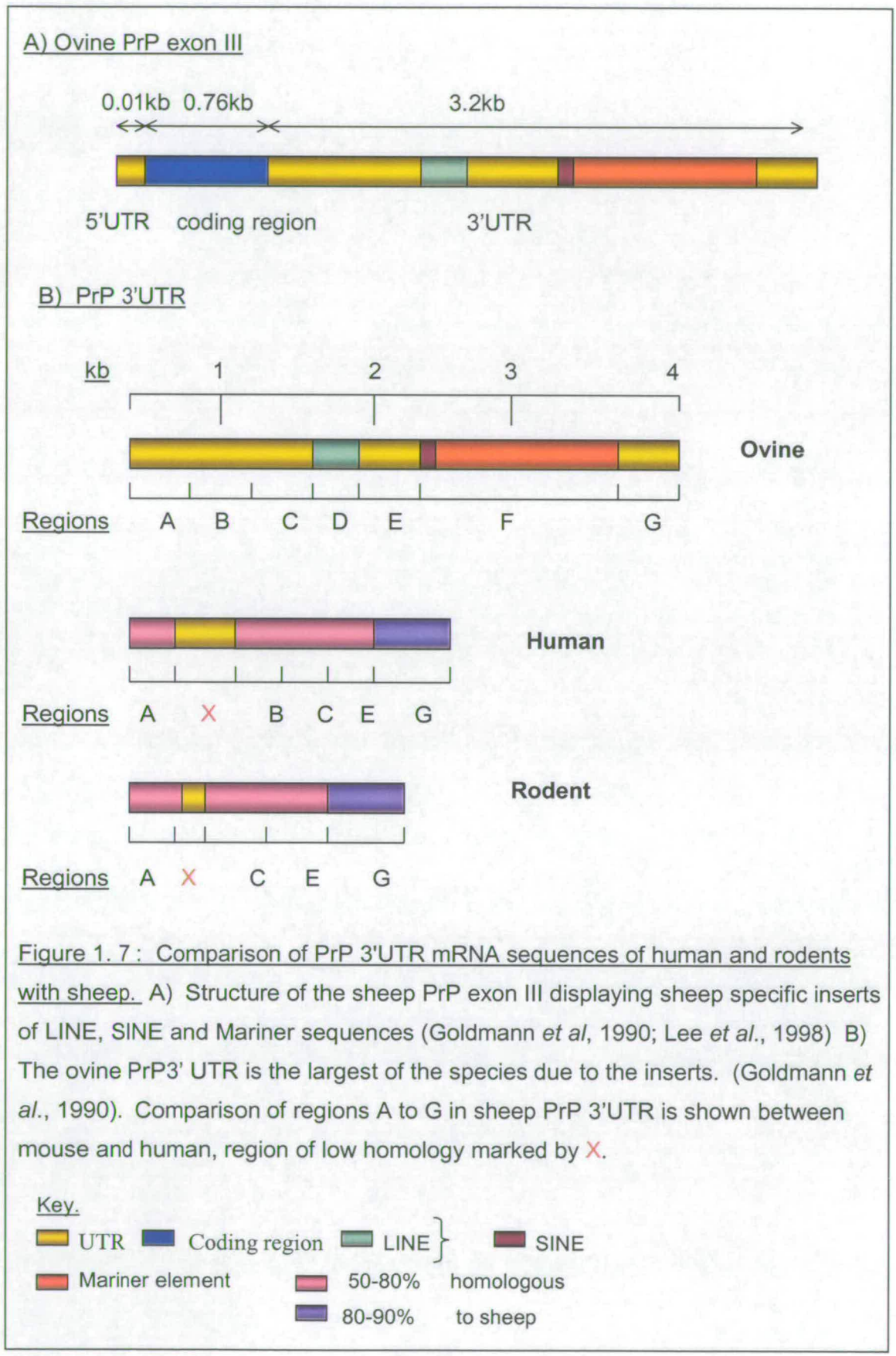


Figure 1. 7 : Comparison of PrP 3'UTR mRNA sequences of human and rodents with sheep. A) Structure of the sheep PrP exon III displaying sheep specific inserts of LINE, SINE and Mariner sequences (Goldmann *et al*, 1990; Lee *et al.*, 1998) B) The ovine PrP3' UTR is the largest of the species due to the inserts. (Goldmann *et al.*, 1990). Comparison of regions A to G in sheep PrP 3'UTR is shown between mouse and human, region of low homology marked by X.

1.9 PrP Gene Expression.

The genetic organisation of the PrP gene has been determined but the mechanisms controlling gene expression are still unclear, although it is known that the PrP gene is regulated both developmentally and in a cell-specific manner. As mentioned in section 1.5.2.1, expression of the PrP gene is vital for TSE development in that no PrP expression offers apparent resistance to infection but over-expression increases TSE susceptibility. Understanding the mechanisms controlling PrP expression may led to further understanding of susceptibility and scrapie development

1.9.1 PrP mRNA.

In sheep, PrP mRNA isolated from brain has an apparent length of 4.6kb, cattle and goats have a similar transcript, whereas rodents and humans express mRNAs of approximately 2.5kb (Goldmann *et al.*, 1990; Inoue *et al*, 1997; Horiuchi *et al.*, 1998, Goldmann *et al.*, 1999). Furthermore, the ovine PrP gene produces more than one mRNA transcript (Hunter *et al.*, 1994). In addition to a full length 4.6kb mRNA transcript, a second smaller mRNA species of 2.1kb can be detected and was first seen only in peripheral tissues. The 4.6 kb mRNA is found in all tissues, with the highest expression level in brain. The 2.1kb mRNA is found in all peripheral tissues at levels of up to 30% of total PrP mRNA, but only at very low levels in the brain (Goldmann *et al* 1999).The smaller additional mRNA is also found in goats and at very low levels in cattle, although no equivalent mRNA has been found in mice and human tissues (Hunter *et al.*, 1994; Goldmann *et al.*, 1999). The smaller sheep mRNA transcript is the result of usage of an alternative polyadenylation signal at an upstream site in the 3'UTR, at nucleotide position 1523 in the mRNA transcript (Goldmann *et al.*, 1999). The 2.1kb PrP mRNA lacks several features present in the 4.6kb mRNA, such as repetitive sequences, long and short interspersed elements (LINES/ SINES), a transposable element, instability motifs, and a highly conserved 3' region (Cheung, 1996; Lee *et al.*, 1997).

1.9.2 Tissue and cell type PrP expression.

Expression of PrP mRNA in many species, detected by Northern analysis, is typically highest in the brain with intermediate levels found in heart and lung, whereas liver and spleen have low or barely detectable levels (Oesch *et al.*, 1985; Caughey *et al.*, 1988; Goldmann *et al.*, 1999). In sheep peripheral tissues the 2.1kb mRNA contributes to a third of the total PrP transcripts but is barely detectable in the brain. In contrast, the 4.6kb mRNA is found in all tissues with the highest expression level in brain. Furthermore, the apparent levels of PrP protein vary between brain and other peripheral tissues (Horiuchi *et al*, 1995). Horiuchi *et al*, (1995), reported that PrP^C protein was abundant in neural and non-neural tissues such as spleen and lungs but the levels of detectable PrP^C protein in different tissues and individual sheep were not consistent. On comparison, the proportion of PrP mRNA to PrP^C varied between brain and other tissues. For example the amount of detectable PrP mRNA was expressed five times higher in the brain than in the kidney, however the amount of PrP^C detected in brain was forty fold more than the kidney. Horiuchi *et al* (1995), concluded that translation efficiency or the course of protein synthesis, including degradation, might differ between the brain and other tissues. An alternative explanation may be that in peripheral tissues the 2.1kb PrP mRNA could be favoured for translation over the 4.6kb mRNA (Goldmann *et al.*,1999)

The level of PrP^C expression varies among the different cell types found within rodent brain, the highest expression estimated to be in the cortex and striatum (Sales *et al.*, 1998), Purkinje cells of the cerebellum (DeArmond *et al.*, 1987), neurones of the septum, thalamus and caudate putaman (Manson *et al.*, 1992). In contrast, the caudate nucleus, neocortex and granular layer of the hippocampus show little or no PrP expression in hamster brain (Kretzschmar *et al.*, 1986; DeArmond *et al.*, 1992). Furthermore, brain cells which express detectable PrP mRNA may not show significant levels of PrP^C protein (Manson *et al.*, 1992). The differential expression pattern of PrP gene observed between tissues and within the brain displays tissue and cell-type transcriptional and translational control of the PrP gene.

1.9.3 Developmental regulation of PrP expression.

Developmental regulation of the PrP gene has been demonstrated in several species (McKinley *et al.*, 1987; Lazarini *et al.*, 1991; Manson *et al.*, 1992; Harris *et al.*, 1993; Hunter *et al.*, 1994;Moser *et al.*, 1995). In the developing mouse embryo, prenatal expression of PrP can be detected using *in situ hybridisation* by day 13.5 in brain, spinal cord and peripheral tissue (Manson *et al.*, 1992). Postnatally in the mouse brain, PrP expression was found to increase 4-fold from birth to day 20 to a level that was maintained throughout adult life (Lazarini, *et al.*, 1991).

In sheep, the 4.6kb and 2.1kb PrP mRNA transcripts can be detected throughout all developmental stages: foetus, lamb and adult (Goldmann *et al.*, 1999). Northern hybridisation signals for PrP mRNA are detectable at day 98, increasing 100-fold by day 134 and a further 2-fold in the lamb to a level maintained throughout adult life. The ratio of the 2.1kb transcript to the 4.6kb mRNA varies between tissue types during development with the highest level of the 2.1kb mRNA in the spleen and kidney, intermediate in heart and lowest in the brain.

1.9.4 Factors known to control PrP expression.

From *in vitro* and *in vivo* studies there is evidence that PrP mRNA expression may increase in the presence of specific cytokines (Satoh *et al.*, 1998) and growth/differentiation factors (Table 1.5) such as interluekan–6 (IL-6), nerve growth factor (NGF) (Mobley *et al.*, 1988; Lazarini *et al.*, 1994) and migratory inhibitory factor-related protein(MRP8) (Kniazeva *et al.*, 1997). In contrast, PrP expression is inhibited in human fibroblasts by the presence of periodontal ligament growth factor (PDGF). Cytokines and growth factors are known to stimulate cell growth and differentiation, however they may also function as a chemoattractant. The growth factor PDGF is a known chemoattractant it also functions to signal for proliferation, therefore a signal for mitogenesis may suppress PrP mRNA synthesis (Kniazeva *et al.*, 1997).

Table 1.5 : Factors known to be involved in regulating PrP expression

Cell Type	Factor	Influence
Cholinergic neurons	Nerve Growth Factor (NGF)	Increases choline acetyltransferase activity coincidentally with PrP mRNA (Mobley *et al.*, 98)
Human neuroblastoma (SK-N-SH)	Cytokines e.g. IL-1b, TNF-α IFN-γ	All increase PrP mRNA expression up to four fold (Satoh *et al.*, 1998) Decrease in PrP mRNA expression (Satoh *et al.*, 1998)
HL-60 cells (human premyloid)	Retinoic acid	Down regulation of cell surface PrP^C concentration (Dodelet *et al.*, 1998)
PC-12 cells	Interleukin-6 (IL-6) Nerve growth factor (NGF)	Increases the level of PrP mRNA expression (Lazarini, *et al.* 1994; Wion *et al.*, 1988)
Pancreatic endrocrine (beta cells)	Recombinant hGH Dexamethasone	Both factors induces beta cell maturation and increasing PrP mRNA levels (Atouf *et al.*, 1994)
Periodental ligament	Migration inhibitory factor-related protein (MRP8) Platelet-derived growth factor (PDGF)	Stimulates PrP mRNA expression Represses PrP mRNA expression (Kniazeva *et al.*, 1997)
Hepatic stellate cells (liver)	Cardon tetrachloride (CCl_4)*	Stimulation of PrP mRNA expression in response to oxidative stress (Ikeda *et al.*, 1998)

* Toxic chemical to liver cells, induces oxidative stress

1.9.5 PrP expression and TSE development.

Northern analysis has not revealed any consistent differences in the level of PrP gene expression between normal and scrapie-infected tissue (Caughey *et al.*, 1988). However, Northern analysis may not have detected subtle differences of less than 2-fold which may have a major effect on disease development. Overexpression of the PrP gene is known to decrease the incubation period (time of inoculation to development of disease) of transgenic mice when inoculated with experimental scrapie. Therefore, any increase in PrP expression may increase susceptibility to scrapie infection and speed-up the disease process. Interestingly, in uninfected hamster the level of PrP mRNA containing all three exons (section 18.1) was estimated to be 30-50% of the total PrP mRNA, this level increased 2.5 fold during scrapie infection (Li and Bolton, 1997). This may suggest that although the level of PrP expression may not alter during scrapie infection, control over gene expression might.

1.10 Role of the PrP 3'UTR in of Gene Expression.

1.10.1 General 3'UTR formation.

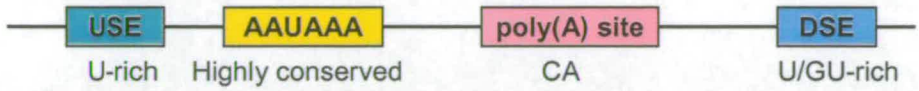
Correct formation of the mRNA 3'UTR is a key regulatory step in the expression of many eukaryotic genes. Defects in mRNA 3'UTR formation can significantly alter the growth, viability, development and health status of a cell. For example in humans, a loss of function of the EAAT2 glutamate transporter protein due to abnormal RNA polyadenylation leads to motor neurone degeneration (Lin *et al.*, 1998). In yeast cells, a failure to correctly modify the enzyme polyApolymerase (PAP) during the cell cycle is thought to slow down cell growth rate causing an accumulation of cells in the G_0-G_1 phase (Zhao and Manley, 1996).

The molecular mechanisms of 3'UTR formation and its interaction with other aspects of mRNA processing are slowly being understood. For instance, a functional polyadenylation signal is crucial for transcription termination by RNA polymerase II (Connelly and Manley, 1988; Proudfoot, 1989) and transport of the message from the

nucleus to the cytoplasm (Huang and Carmichael, 1996) which is also thought to involve an interaction with RNA polymerase (Long et al., 1995). In the cytoplasm the polyA tail mediates stability and translation efficiency of the message (Sachs et al., 1986; Ford et al., 1997; Preiss and Hentze, 1998; Wickens et al., 1997). Evidence exists therefore that the 3'UTR formation and regulation plays a vital role in normal cell growth and development. Furthermore, controlling the amount of available mRNA, or translation efficiency, directly affects the amount of protein synthesised.

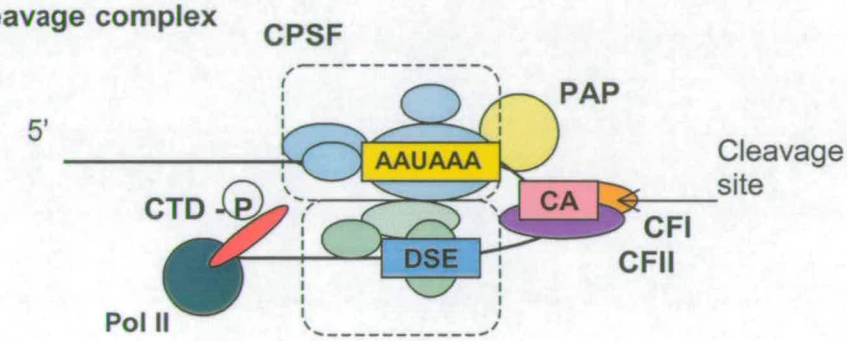
Three sequence elements are required for efficient 3'UTR formation in mammalian pre-mRNA (Figure 1.8). Firstly, a highly conserved polyadenylation signal, AAUAAA. This hexanucleotide, required for both cleavage and poly(A) addition (Manley, 1988;Wahle and Keller, 1992; Wickens, 1990), is found attached to 80% of all pre-mRNAs. Mutations within this sequence greatly reduces the efficiency of 3'UTR processing (Wickens and Stephenson 1984; Sheets *et al.*, 1990). Secondly, cleavage occurs at the poly(A) addition site, 11-23nt downstream of the hexanucleotide. In most genes the poly(A) site is defined by a CA dinucleotide. Finally, the presence of a GU- or U-rich element downstream (approximately 30 nucleotides, known as the downstream element, DSE) of the poly(A) site may affect the cleavage site position and the efficiency of cleavage (MacDonald et al.,1994; Gil and Proudfoot, 1987). Other sequence elements have been found to influence 3'UTR formation in both a positive and negative way. For example in viral pre-mRNAs, the presence of a U-rich upstream sequence element (USE) can greatly enhance poly(A) site recognition (Key et al.,, 1998; Carswell and Alwine, 1989). USEs have only been identified in a few eukaryotic genes e.g. complement factor C2 and lamin B2 (Brackenridge et al., 1997; Moreira et al., 1998). Many protein factors have also been identified as necessary for accurate cleavage and polyadenylation (refer to figure 1.8).

A) Mammalian mRNA sequence elements

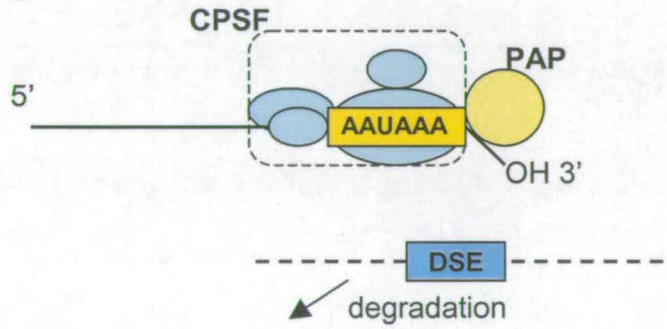


B) Model for cleavage and polyadenylation in mammalian cells

i) Cleavage complex



ii) Cleavage



iii) Poly(A) addition

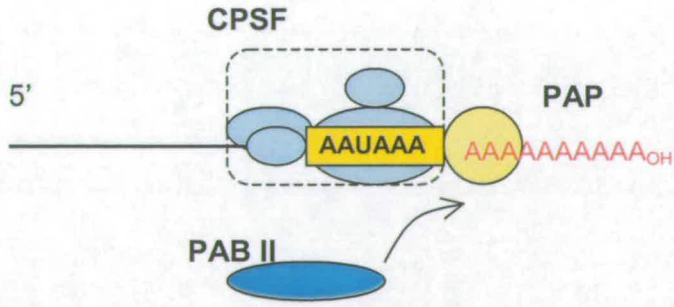


Figure 1.8: Schematic model for mammalian 3'end formation

Figure legend on page 48.

Figre 1.8 Schematic model for mammalian 3'end formation. The mammalian cleavage complex assembles through cooperative binding of Cleavage/Polyadenylation Specificity Factor (CPSF) at the AAUAAA site and Cleavage Stimulation Factor (CstF) at the Downstreamn Element (DSE, U- / GU-rich). Interactions between CPSF and CstF define the region for cleavage, a process assited by Cleavage Factor I (CFI). The arrangement of the cleavage complex requires CF II and Poly(A)Polymerase (PAP), but the arrangement of CFI and CF II are unclear. After cleavage, CPSF and PAP remain bound to the RNA. CSPF recruits PAP to the AAUAAA containing sequence and slow polymerisation of a poly(A) tail begins. Cooperative interactions between CPSF, PAP and Poly(A)-Binding protein II (PABII) promotes rapid poly(A) tail formation. When the tail reaches approximately 250 adenosine residues, elongation slows and poly(A) addition is terminated by a mechanism not yet understood (Figure adapted from Zhao *et al*, 1999; Lodish *et al*, 1995).

1.10.2 Control of cleavage and polyadenylation – tandem poly(A) sites.

Cleavage and polyadenylation can be regulated to vary the amount and type of mRNA a cell produces (Edwalds-Gilbert *et al.*, 1997). The majority of eukaryotic genes have only a single polyadenylation signal, however the existence of numerous poly(A) sites at the 3'end of an exon can give rise to several possible outcomes for 3'UTR formation (figure 1.9). Control over the efficiency and activity of the enzymes within the cleavage/ polyadenylation complexes (Figure 1.8) govern what 3'end will be produced. Within the scope of this thesis only the tandem terminal poly(A) sites will be discussed further, for reviews on other mRNA 3'end formation please refer to Edwalds-Gilbert *et al.* (1997) and Zhao et al. (1999).

The alternative usage of poly(A) sites has been shown to be both tissue and developmentally regulated. When presented with several polyadenylation signals within the 3'-terminal exon, the choice of poly(A) site can be based on the strength of the signal sequences (conservation of the AAUAAA site, presence of DSE, USE) and the addition of poly(A) stabilising factors (Zhao et al., 1999). It is possible that differential regulation of the *cis*-acting acting proteins of the cleavage/ polyadenylation complex at different poly(A) sites may give rise to alternative transcripts. Hence, producing transcripts of different translation efficiency and stability directly affects the amount of protein produced.

A recent observation of alternative usage of tandem poly(A) sites involved the eukaryotic initiation factor 2α (eIF-2α, involved in protein synthesis) where two mRNAs, 1.6 and 4.2kb, were transcribed at varying ratios in different tissues (Ernst et al., 1987; Cohen et al., 1990). *In vitro* translation studies showed that the 1.6kb mRNA was less stable, but more efficiently translated than the 4.2kb transcript. Activation of T cells increased the abundance of the 1.6kb mRNA 11.5-fold and the 4.2kb mRNA only 4-fold (Miyamoto et al., 1996). The activation of T cells initiates the cell cycle to proceed from G_0 to S phase which is associated with an increase in PAP activity and rate of polyadenylation (Miyamoto et al., 1996) indicating that polyadenylation may respond to a change in cellular environment. These results indicate that for eIF-2α, alternative usage of the poly(A)site for the 1.6kb mRNA

allows for increased efficiency of mRNA synthesis, translation and therefore increased protein levels. Additionally, in the testes a third poly(A) site is employed to produce a transcript of 1.7kb, suggesting that testes-specific polyadenylation factors may be recognising different elements within the eIF-2α pre-mRNA transcript (Miyamoto et al., 1996).

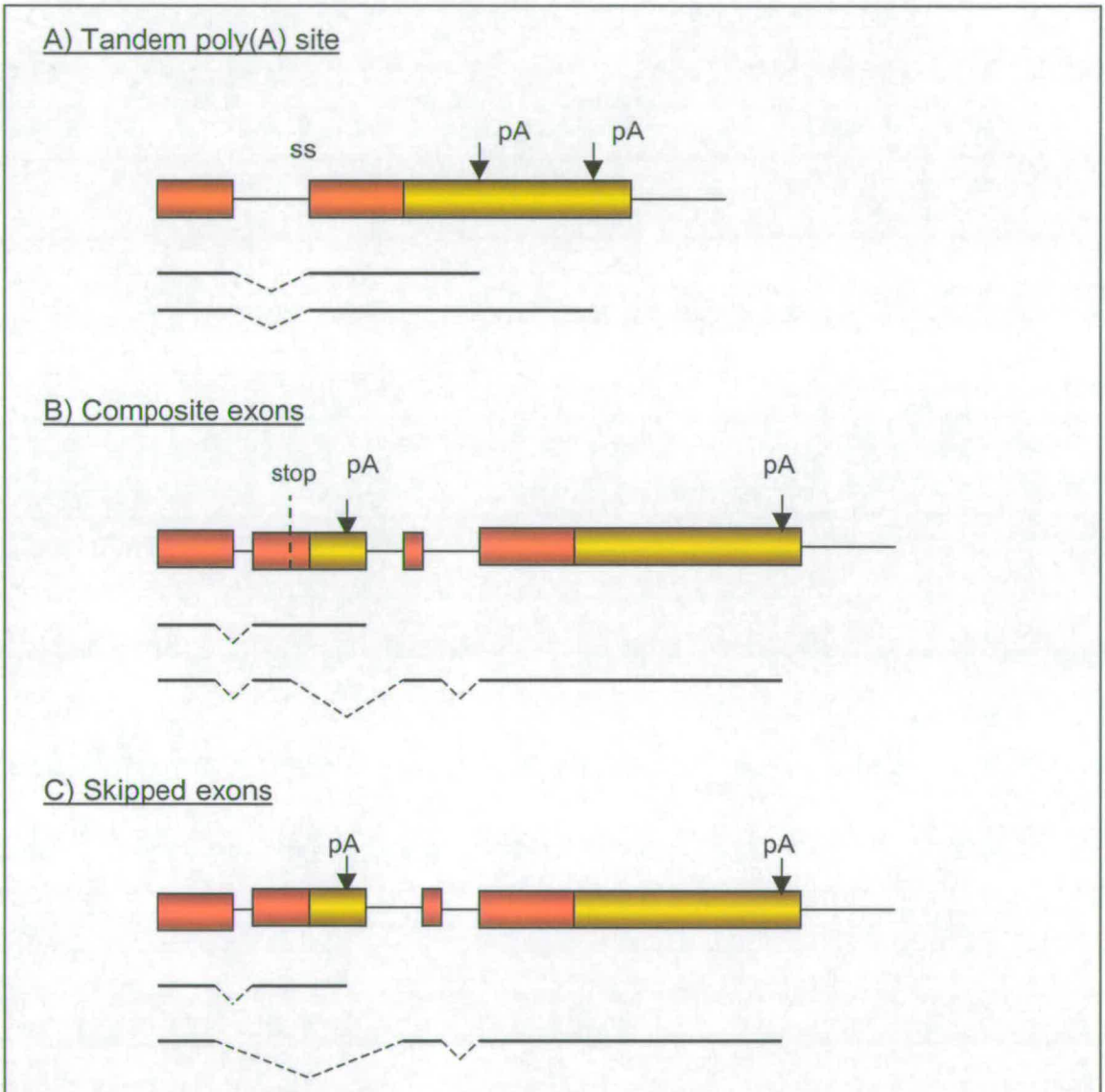


Figure 1.9 :Alternative polyadenylation choices. Types of alternative polyadenylation for 3'UTRs in mammalian cells. A) Tandem poly (A) site; choice of more than one p(A) signal in the last exon. B) Composite exons; choice of defining the end of an exon by alternative 5' splicing. C) skipped exons; more than one terminal exon (Zhao et al., 1999).

1.10.3 Differential expression of the ovine PrP gene.

Expression of the ovine PrP gene may regulate the production of two or more mRNA transcripts through the process of polyadenylation. Conserved polyadenylation signals at nucleotide positions 1253, 1523, 4038 and 4063 have been confirmed in both the Cheviot and Suffolk sheep PrP gene, potentially signalling for PrP mRNAs of 1.5 –4.5kb in length (Goldmann unpublished data; Goldmann *et al.*, 1999). In peripheral tissues of Cheviot sheep, PrP mRNAs of 2.1 kb and 4.6kb have been described, often at varying levels depending on the tissue (Horiuchi *et al.*, 1995; Goldmann *et al.*, 1999). For example, in spleen and uterus tissues the 2.1kb and 4.6b PrP mRNAs may both be expressed at high levels whereas in heart tissue the 2.1kb mRNA is expressed at a lower than the 4.6kb, as detected by Northern blot hybridisation (Goldmann *et al.*, 1999). However the highest level of PrP mRNA expression can be found in brain tissue predominantly from the 4.6kb mRNA, where the 2.1kb mRNA is present at very low levels (0.5-2% total PrP mRNA, Goldmann, 1999). The observed differential expression of the ovine PrP gene has been detected in both scrapie-resistant and scrapie-susceptible genotypes. Analysis by 3'RACE and SI nuclease protection assays of poly(A)- enriched mRNA from brain, kidney and spleen tissues confirmed that ovine PrP mRNA was likely to be processed at polyadenylation signals $AATAAA_{1523}$ and $ATTAAA_{4063}$. Importantly, long range RT-PCR and 5'-RACE analysis has shown that the 5'UTR sequence of the 2.1kb and 4.6kb mRNAs are identical (Westaway *et al.*, 1994) confirming that during transcription of the ovine PrP gene, two mRNAs are the product of alternative polyadenylation and not alternative splicing.

Post-transcriptional control of PrP mRNA may be directed through the 3'UTR. In the first instance, Denman *et al* (1991) showed that 30% of PrP mRNA was prevented from undergoing translation by the association of the 3'UTR with ribnucleoprotein particles in the cytoplasm. The sequestered mRNA shows the potential for increasing cellular PrP protein concentration in a short period. Also, *in vitro* expression of constructs with the chloramphenicol acetyl transferase (CAT) gene linked to various regions in the PrP 3'UTR was analysed by RT-PCR and CAT protein activity and showed that sequences within the 3'UTR were capable of

altering the levels of protein synthesis but not the level of mRNA (discussed in depth in chapter four). Taken together, these results suggest that the 3'UTR may be acting to control gene expression.

The ovine PrP gene structure is similar to that in other species i.e. mouse, bovine and hamster, for introns I and II, and exons II and I (section 1.8.1). However, the sheep 3'UTR region within exon III contains two ruminant specific regions, disease associated polymorphisms and two known polyadenylation sites which are both used in peripheral tissues (section 1.8.4). Considering these features of the sheep PrP gene 3'UTR, questions relating to the contribution of the untranslated region in controlling the level of normal PrP protein and scrapie susceptibility. As susceptibility to scrapie is associated to the level of PrP protein expression, a link between translational control of PrP expression and incidence of scrapie in sheep may also exist.

1.11 Aims and Objectives.

The aim of this thesis was to further investigate the role of the ovine PrP gene 3'UTR in its capacity to regulate gene expression in an ovine *in vitro* system. Previous work at the NPU (Cheung, 1996) had shown that a construct with the *in vivo* 4.6kb PrP gene 3'UTR was capable of repressing *in vitro* protein levels equivalent to a construct with the *in vivo* 2.6kb PrP 3'UTR. Deleting sequence within the 3'UTR led to an increase in protein levels but crucially, RT-PCR analysis showed no alteration in mRNA levels expressed from the constructs. This result would suggest that a mechanism exists for controlling the amount of protein synthesised from the PrP mRNA through sequence present in the mRNA. The ability to selectively control PrP expression in this way may also account for apparently low levels of PrP protein detectable in peripheral tissue compared to brain.

The hypothesis to test was whether the repressor element, associated with the PrP gene 3'UTR, functioned in ovine cells and, if so, could it differentially mediate levels of PrP protein in brain and peripheral tissue *in vitro*. Additionally, any association between the repressor element of the UTR with scrapie susceptibility (i.e. PrP genotype) was to be examined. To study the role of the PrP gene 3'UTR in controlling *in vitro* expression of the PrP protein, PrP constructs resembling the *in vivo* observed 2.1kb and 4.6kb PrP mRNAs were designed and mutated to express ovine PrP protein with the hamster 3F4 epitope. The altered ovine PrP protein should allow for analyse by immunological detection of expression from the constructs in sheep cell lines derived from different PrP genotypes and tissue origin. To achieve these aims it was essential to establish and optimise a reliable method for transfection of various ovine primary and immortal cell lines available at the NPU. During the time necessary for cloning the ovine PrP constructs and developing the transfection methods, confirmation of the CAT expression studies by Cheung (1996) in ovine cell lines were completed to further identify expression control regions within the ovine PrP gene 3'UTR.

2.1 General Chemicals, Solutions and Centrifugation.

The chemicals and enzymes used with in this study were purchased from Sigma (Dorest, UK), Gibco-BRL (Paisley, UK), DBH (DORSET, UK), Roche Diagnostics (East Sussex, UK), Qiagen Ltd. (West Sussex, UK) or Promega (Southampton, UK). All solutions were prepared with high quality chemicals, "e.g. Electran grade" (BDH, DORSET, UK) and when necessary were sterilised by autoclaving or filtering. Water used in the making of the solutions was obtained from a Millipore milli-QUF system and further autoclaved when required. Recipes for all solutions used in this thesis are listed in Appendix A. Unless otherwise stated, procedures for general methodology were adapted from Sambrook *et al*, 1989.

Centrifugation of solutions up to 30ml was performed in a Beckman J2-21 using rotor JA-14 at relative centrifugal fields (RCF) of 30,000 xg. For maximum centrifugal fields of 44,000 xg, 15ml solutions were centrifuged using the Beckman J2-21, JA-20 rotor. For RCFs up to 3,750 xg, 30ml volumes were centrifuged in a refrigerated GS-6R, rotor GHS 3.8 (Beckman Ltd, Buckinghamshire, UK). For ultra-centrifugation, maximum a RCF of 541,000 xg was achieved using an Optima TL Ultra-centrifugation 100,000 6R (Beckman Ltd, Buckinghamshire, UK) with rotor TLA 100.4. Micro-centrifugation for spins up to 13,400 xg in 0.5-1.5ml Eppendorf tubes was carried out in a bench top centrifuge (MSE Micro Centaur, Sanyo).

2.2 General Methods for DNA Cloning

2.2.1 Restriction enzymes.

Restriction enzymes recognise and cut at defined sites in DNA sequences depending on base composition and length. The number and size of fragments generated by a restriction enzyme depend on the frequency of occurrence of the restriction site in the DNA to be cut. DNA substrates generated by restriction digestion are either

analysed for cleavage patterns or used in subsequent experiments such as cloning, sequencing, labelling or hybridisation.

Single digestion or multiple digestion

Single restriction digests or multiple digestions were performed to a standard method; 16µl sterile water, 2µl restriction enzyme 10x buffer, 1µl DNA sample (0.2-1µg in water), and 1µl enzyme (2-10 units) or 0.5µl restriction enzyme 1 (10units/ml), plus 0.5µl restriction enzyme 2 (10units/ml) for multiple digestion. Components were mixed gently then centrifuged briefly at 13,400xg in a micro-centrifuge. The digestion reaction was then incubated at the appropriate temperature (usually 37°C) for one hour. The reaction was stopped by adding 4µl of bromophenol blue loading dye, and the results were visualised by agarose gel electrophoresis.

2.2.2 Modifying enzymes.

Modifying enzymes can be used to synthesis, degrade or modify portions of nucleic acids in a defined manner. Modifying enzymes are most commonly used in cloning experiments.

2.2.2.1 T4 DNA ligase.

T4 DNA ligase was used to catalyse the formation of a phosphodiester bond between adjacent 5'-phosphate and 3'-hydroxyl in cloning double stranded DNA molecules with cohesive or blunt ends. Using an appropriate vector:insert ratio the typical ligation reaction was as follows; 1µl T4 DNA ligase (Roche Diagnostics, East Sussex, UK), 1µl ligase 10x buffer and nuclease-free water to a final volume of 10µl. Components were mixed gently then centrifuged briefly at 13,400xg in a microcentrifuge. For the ligation of cohesive ends the ligation reaction was incubated at 14°C for 16 hours; for blunt ends the ligation reaction was incubated at 25°C for 16 hours.

2.2.2.2 T4 Polynucleotide Kinase (T4 PNK)

T4 PNK catalyses the transfer of the γ–phosphate from ATP to the 5'-terminus of polynucleotides or mononucleotides and was used in the radiolabelling of probes used in differential hybridisation experiments. To label an oligonucleotide probe the following method was used: 5μl [^{32}P]-ATP (10 mCi/ml) was added to 1μl oligonucleotide (25pmol), 2μl 10x kinase buffer, 1μl T4 PNK (Roche Diagnostics, East Sussex, UK) and nuclease free water to a final volume of 20μl. The reaction was mixed and incubated at 37°C for 1 hour in a lead-shielded vessel.

2.2.2.3 Shrimp Alkaline Phosphotase (SAP).

SAP catalyses the dephosphorylation of 5'phosphates from DNA and is used to dephosphorylate restricted cloning vectors to prevent religation.. For cohesive-end cloning, SAP (Roche Diagnostics, East Sussex, UK) was added directly to the restriction digestion after one hour incubation at 37°C (bromophenol blue loading dye was not added at this stage): 1μl 10x dephosphorylation buffer and 1μl SAP. The reaction was incubated at 37°C for a further 10 minutes. To dephosphorylate blunt-ended DNA fragments, SAP was added to the completed digestion and incubated for 60 minutes at 37°C. SAP was inactivated by 15 minutes at 65°C.

2.2.2.4 DNA polymerase I large fragment (Klenow).

Klenow enzyme is the large fragment of DNA polymerase I. The enzyme catalyses the addition of mononucleotides from deoxynucleosides-5'phosphates to the 3'-hydroxyl terminus of a template DNA and was used for 5'-overhang fill-in for cloning. Klenow also catalyses the removal of 3'- overhangs. To fill in 5'-overhang ends: 40mM of each dNTP and 1unit of Klenow per μg of DNA was added directly to the restriction buffer after one hour digestion incubated at 37°C. The reaction was incubated at 25°C for 10 minutes. For the removal of 3' -overhangs 1unit of Klenow (Roche Diagnostics, East Sussex, UK) per ug of DNA was added to the digest and incubated at 25°C for a further 20 minutes. Klenow is inactivated by heating at 75°C for 10 minutes.

2.2.3 Agarose gel electrophoresis.

The results of restriction digestion or PCR can be analysed using agarose gel electrophoresis following staining of the DNA with ethidium bromide and visualisation by UV light. For agarose gel electrophoresis the following method was used (Helling *et al*, 1974, Fischer *et al*, 1971). To prepare the gel, 1g Agarose was dissolved in 1xTBE buffer, and heated in a microwave for 4 minutes on medium high heat. The volume of the agarose mixture was made up to 100ml with dH_2O and ethidium bromide added to final concentration of 0.5μg/ml and the gel (14cm x 10cm x 1cm) poured and allowed to set for 2 hours. The DNA samples were prepared in $1/10^{th}$ volume of bromophenol blue loading dye, mixed, then loaded on to the gel. The gel was run at 100volts, 25mA until satisfactory separation of the DNA samples was achieved. The DNA was visualised under UV light.

2.2.3.1 Isolating DNA fragments from agarose gels.

To isolate bands from a gel, the gel was run as normal and DNA visualised under UV light and the band isolated using Qiagen Gel extraction kit (Qiagen Ltd., West Sussex, UK) according to the manufacture's protocol.

Following visualisation under UV light, the DNA band of interest was cut out from the agarose gel with a clean scalpel and placed in a centrifuge tube. The gel fragment (1 volume) was incubated in Buffer QG (3 volumes, where 100μg ~ 100μl) at 50°C for 10 minutes until the gel slice had completely dissolved, after which 1 volume iso-propanol was added to the sample and mixed. To bind DNA to the Qiaquick spin column, the agarose-DNA solution was spun through the column for 1 minute at 10,000 xg in bench centrifuge. The column was then washed with 0.75ml of Buffer PE and spun as before. The flow-through was discarded and the column spun dry for an additional 1minute at 10,000 xg. To elute the DNA 30μl of sterile water was added to the column and centrifuged for 1 minute at 12,000 xg in microcentrifuge. The DNA was then used directly or stored at –20°C for further use.

2.2.4 Modified Inoue method for transformation competent *E.coli* cells.

The following method was used to produce competent bacterial strains for efficient transformation. A starter culture, 5ml 2xYT, was inoculated with a XL-1 Blue colony and grown over night at 37°C in a shaking incubator, the same day 20-40 1.5ml Eppendorf tubes were prepared by pre-freezing at -70°C. Next day, a large scale inoculation (100-200ml) was prepared by diluting the starter culture 1:500 into 2XYT –10mM $MgSO_4$ which was incubated at 23°C, shaking (250rpm) to OD_{600}=0.6. The cells were collected by centrifugation using the Beckman J2-21, rotor JA-14, at 1380 xg in 50ml Corex tubes for 10 min, 4°C and the supernatant discarded. The cells were gently resuspended in ice-cold Buffer TB in 1/3rd the original culture volume and incubated on ice for 10 minutes. After repeating the centrifugation step, the cells were resuspended in ice-cold Buffer TB in 1/10th the original culture volume and transferred to a 15ml Corex centrifugation tube. DMSO was added to a final concentration of 7% and the cells incubated on ice for a further 10 minutes. Aliquots, 0.5mls were then dispensed in to the pre-frozen Eppendorf tubes and flash-frozen in liquid N_2 before storing at –70°C

2.2.5 Transformation of *E.coli* cells.

In order to transform *E.coli* cells the following protocol was used. To 25µl *E.coli* JM109 cells, 0.5µg of DNA was added and the mixture incubated on ice for 30 minutes, heat shocked at 42°C for 90 seconds and incubated on ice for 10 minutes as before. To the transformation mixture, 500µl LB broth (no ampicillin) was added and allowed to shake for 1 hour at 37°C. The transformed culture, 200µl, was streaked on to an ampicillin agarose plate (1µl/ml). Plates were incubated at 37°C over night, upside down. Single colonies were then selected and grown in 3ml of ampicillin agar broth overnight at 37°C, in a shaker. The following day plasmid DNA can be isolated. For storage of bacterial colonies on agarose plates, single colonies were picked to inoculate 3ml ampicillin LB broth which was incubated at 37°C with shaking for 16 hours. Fresh ampicillin agarose plates were then streaked with the culture and incubated at 37°C over night, upside down.

Colonies obtained from cloning experiments were screened by isolating small-scale DNA preparations (mini-preps) for restriction digestion and gel analysis or DNA sequencing reactions.

2.2.6 Small scale plasmid DNA preparation by alkaline lysis.

In order to prepare small scale amounts of plasmid DNA the following protocol was used (Birnboim and Doly, 1979, Ish-Horowicz,D. *et al*, 1981). An overnight culture (1.5ml) of transformed *E.coli* was spun for 15 seconds at 13,400 xg in a microcentrifuge. The supernatant was discarded and the pellet re-suspended in 100µl of TGE (25mM Tris-HCl, pH8.0, 10mM EDTA, 50mM glucose), then 200µl of 0.2M NaOH, 10% SDS solution was added to lyse the cells. The samples were mixed briefly and incubated on ice for 5 minutes. To remove the SDS and allow for renaturation of the plasmid DNA 150µl 3M potassium acetate solution was added, the sample was mixed and incubated on ice for 5 minutes then spun for 5 minutes, as before, to remove the precipitated protein and chromosomal DNA. The supernatant was transferred to a fresh Eppendorf tube and the plasmid DNA purified by ethanol precipitation.

2..2.7 Ethanol precipitation of plasmid DNA

To ethanol precipitate plasmid DNA, 2x volume 100% ethanol was added, mixed, and the sample incubated -20°C for 30 minutes then spun for 10 minutes at 13,400 xg. The pellet was resuspended in 30-50µl dH_2O or alternatively in RNaseA/ dH_2O mixture (10µg/ml) to digest residual RNA. For solutions up to 15ml, ethanol precipitation was preformed in Cortex tubes and centrifuged (Beckman J2-21, rotor JA-20) at 29,000 xg.

2.2.8 Large scale DNA preparation by alkaline lysis.

To obtain large scale amounts of plasmid DNA the following protocol was used (Birnboim and Doly, 1979, Ish-Horowicz,D. *et al*, 1981). A starter culture of 10ml ampicillin agar broth with transformed *E.coli* was prepared and incubated at 37°C for

8 hours with shaking, then added to 300ml ampicillin agar broth and incubated as before. Next day, the culture was split into 2x 250ml GSA bottles and centrifuged (Beckman, JA-14) for 10 minutes, 5520 xg at 4°C. The supernatant was discarded. The cells were resuspended in 25ml of TGE (25mM Tris-HCl, pH8.0, 10mM EDTA, 50mM glucose) and 5µl (10µg/ml) of lysozyme added and the cells left at room temperature for 10 minutes. To lyse the cells 60ml of a 0.2M NaOH, 10% SDS solution was added, mixed well and the bottles left on ice for 5 minutes. The lysate was neutralised by adding 30ml 3M potassium acetate, mixed, and the bottles left on ice for 15 minutes then spun as before to pellet the chromosomal DNA and cell debris.

The supernatants were pooled, filtered through gauze into a 500ml cylinder and 0.6 volume iso-propanol added then spun for 10 minutes in a fresh 250ml GSA bottle, 9820 xg at 4°C, as before. The supernatant was discarded and the pellet air dried for approximately one hour. The DNA pellet was resuspended in 4ml of TE buffer, pH 7.4,transferrd to an autoclaved Cortex tube and the volume made up to 12ml with sterile distilled water. The plasmid DNA was recovered by ethanol precipitation as previously described. The pellet was air dried then resuspended in 2ml TE buffer, pH 7.4.

To prepare a caesium chloride gradient in Beckman Quickseal tubes, 4g CsCl was added to a Corex tube and the DNA/ TE buffer mixture added, TE buffer was added to a final volume of 5ml. Once the caesium chloride had dissolved, 100µl ethidium bromide (5µg/ml) was added before preparing the DNA solution in a Beckman 5ml Quickseal tube. Ultra-centrifugation was carried out in the Optima TL Ultra-centrifugation 100,000 6R, rotor TLA-100.3 (Beckman, Ltd) for 16 hours, at 160,000 xg, 20°C. The plasmid band (lower) of the CsCl preparation was removed using a wide bore needle syringe and the volume made up to 5ml with autoclaved distilled water. The ethidium bromide was removed using 5ml of water saturated butan-1-ol and the DNA layer (top) extracted three times. Ethanol precipitation was used to recover the DNA followed by centrifugation at 8K for 10 minutes then the

pellet air dry and resuspended in distilled water or TE, pH 7.4. Alternatively the pellet was dissolved in RNaseA/ dH_2O mixture (10μg/ml) to digest residual RNA.

2.3 General Methods for DNA Mutagenesis and Sequencing.

Purified plasmid single stranded DNA (ssDNA) can be used in sequencing reactions and as a template upon which mutagenesis can be performed. Two methods were used to isolated ssDNA.

2.3.1 Preparation of single strand plasmid DNA (ssDNA) using M13K07 Helper Phage.

To prepare ssDNA for DNA sequencing reactions the following procedure was used. Ampicillin LB broth (3ml) was inoculated with transfected bacteria and 100μl of M13KO7 helper phage added, and incubated for 90 minutes in a shaking incubator at 37°C, and kanamycin added to a final concentration of 70μg/ml. The bacteria were incubated overnight at 37°C, with shaking. Approximately 1ml of an overnight culture was spun in microcentrifuge, 13,400xg for 60 seconds then 800μl of the supernatant transferred to fresh tube. A solution of PEG6000 and NaCl was added to a final concentration of 4% PEG, 500mM NaCl and left at room temperature for 30 minutes, then spun for 10 minutes as before. The pellet was dissolved in 200μl TE pH 7.4, mixed with 200μl phenol, incubated at 60°C for 5 minutes, then incubated on ice for 10 minutes. The tubes were spun for 10 minutes, 13000 xg, the DNA/buffer phase (top) removed to a fresh tube and mixed with 150μl phenol/chloroform(1:1). The extraction procedure was repeated and 10th volume 3M sodium acetate and 2x volume 100% ethanol was added to the top layer. This was stored at -20°C overnight, and the precipitate was pelleted by centrifugation for 10 minutes, 13000 xg. The pellet was air dried and dissolved in 15μl dH_2O for a DNA concentration of 0.5-1μg/μl.

2.3.2 Preparation of single stranded plasmid DNA by denaturation.

An alternative method to using helper phage in the production of single stranded DNA is to denature the double stranded DNA chemically. To one volume of dsDNA sample $1/10^{th}$ volume 2M sodium hydroxide and $1/10^{th}$ volume 2mM EDTA was added. The DNA solution was incubated at 37°C for thirty minutes then ethanol precipitated at –20°C for a further thirty minutes. The DNA pellet was washed with 70% ethanol then left to dry at room temperature for 15 minutes. Thereafter the pellet was dissolved in 20-30µl dH_2O for a DNA concentration of 0.5-1µg/µl.

2.3.3 Mutagenesis by single oligonucleotide hybridisation.

To create the hamster-specific anti-PrP epitope (designated 3F4, Kasack et al., 1987) in the sheep PrP protein coding region (Chapter Five, section 5.3) the following method was used (Gillam, *et al.*, 1997). To denature 10µg pPSHPrP, the following components were added: 10th volume 0.2M NaOH and 10th volume 0.25M EDTA. To phosphorylate 10µl (10pmol/ml) oligonucleotide primer the following mix was prepared: 1µl bacteriophage T4 polynucleotide kinase (10U/ml) in the presence of 1µl 100M DTT, 2µl 10mM ATP and 4µl distilled water, the mixture was incubated for 30 minutes at 37°C, placed at -70°C for 10 minutes then stored at -20°C. To 2µl of phosphorylated primer 1µl single stranded DNA template with 1µl 10x TM buffer, and 6µl water was added. The annealing reaction was incubated at 80°C and then cooled to room temperature for approximately 30 minutes. To the 10µl template-primer complex, 0.5µl Klenow fragment of DNA polymerase (5U/ml), 1µl T4 DNA ligase (10U/ml), 1µl 5mM ATP, 1µl 5mM dNTPs, 1µl 100mM DTT and 4µl water were added and mixed. The reaction was incubated for 12-20 hours at 12°C. After extension/ligation, *E.coli* cells were transfected and colonies screened for positive clones by restriction enzyme digest or hybridisation. For summary of oligonucleotides used see Table 2.1 and Appendix A.

2.3.3.1 Screening for successful mutagenesis- restriction enzyme digestion.

To screen for positive colonies restriction enzyme digestion was used. Individual colonies were grown in 3ml ampicillin agar broth for 16 hours and plasmid DNA

obtained by alkaline lysis. Ethanol precipitated DNA was digested with *Hind*III and *Xba*I as described in Appendix A. The DNA fragments were separated and visualised by agarose gel electrophoresis.

2.3.3.2 Differential colony hybridisation.

An alternative method to screen for positive colonies is differential colony hybridisation (Grunstein *et al.*, 1975). Colonies were lifted onto duplicate nitrocellulose membranes and the membranes washed in denaturing buffer for two minutes, then soaked in neutralising buffer for five minutes and debris removed in washing buffer. The DNA was fixed to the membranes by UV irradiation. The membranes were incubated with oligonucleotides, 20pmol/ml, homologous to the mutated and wild-type sequence, with 2µl 10x kinase buffer, 1µl T4 polynucleotide kinase, 5µl ^{32}P ATP (370MBq/ml) and sterile water to final volume of 20µl for 1-1.5 hours at 37°C. The radiolabelled probes were precipitated with 5µl *E.coli* tRNA(5mg), 20µl 4M ammonium acetate and 120µl 100% ethanol, placed at -20°C for one hour, then spun for 15 minutes at 13,400 xg, and the pellet dissolved in 100µl sterile water. The radiolabelled probe was added to the pre-hybridisation mix and washed over the membranes for four hours at 37°C. The filters were washed twice with 2xSSPE / 0.05% SDS at room temperature for ten minutes, followed by one or two more stringent washes at higher temperature. After exposure to Kodak XAR-5 film at -70°C for six hours or over night with a Lightning Plus intensifier screen, positive colonies were identified if only present on the filters hybridised with the mutated oligomer probe and not the on those hybridised with the wild type oligomer.

2.3.4 DNA Sequencing: Chain-termination sequencing of DNA.

Using ssDNA as a template, the chain termination sequencing reaction involves the synthesis of a DNA strand (initiated by an oligonucleotide primer) by DNA polymerase. In four separate reactions, one for each deoxynucleotide (dNTP), a nucleotide analogue is added which can not support chain elongation (a dideoxynucylotide,ddNTP). This results in a population of chains of various lengths. A radioactive nucleotide is also included in the synthesis so that the chains may be

visualised by autoradiography after separation by electrophoresis. Sequencing of ssDNA was completed using the Sequenase Version 2.0 DNA Sequencing Kit (Amersham Life Sciences, Buckinghamsire, UK) according to the manufacturer's protocol. Oligonucleotides used as primers for DNA polymerase are described in Table 2.1.

2.3.4.1 Sequencing reaction

To anneal the oligonucleotide primer to the ssDNA the following method was used. In a 0.5ml centrifuge tube 1µl primer (1pmol/reaction) was added to 7µl DNA (5-7µg) and 2µl Reaction Buffer. The annealing reaction was incubated at 65°C for 2 minutes in a hot water bath which was then allowed to cool to below 35°C over 30 minutes to complete the annealing process. To label the template-primer complex the following was added to the completed annealing reaction on ice: 1µl 0.1MDTT, 2µl diluter labelling mix (1:5 dGTP labelling mix), 0.5µl [^{35}S] –dATP , 1µl Mn buffer and 2µl diluted Sequenase Version 2.0. The labelling reaction was mixed and incubated at room temperature for 2-5 minutes.

For the chain termination reaction 2.5µl of each ddNTP (A, C, G and T) was aliquoted into a capped centrifuge tube and warmed to 37°C for at least 1 minute. Labelling reaction, 3.5µl was added to each of the four prepared ddNTPs, and incubation at 37°C continued for 3-5 minutes. After which 4ml Stop solution was added to the Termination reaction, mixed and the samples stored on ice until required. Prior to loading onto the prepare polyacrylamide gel, the samples were heated to 75-80°C for 2 minutes, then 2-3µl loaded on the gel immediately.

2.3.4.2 Denaturing gel electrophoresis for sequencing.

The result of sequencing reactions can be resolved by polyacrylamide gel electrophoresis. The gel is prepared as follows. For a 7% polyacrylamide gel, 5.7ml 40% acrylamide/bis-acrylamide solution was mixed gently at 35°C with 17.5g urea, 4ml 10x TBE buffer, 10ml distilled water and 80µl 10% APS. Once the urea had completely dissolved the volume of the polyacrylamide gel solution was adjusted to 40ml with distilled water and 30µl undiluted TEMED added. The gel was then

poured (34cm x 17cm x 0.4mm) and allowed to polymerise for 2 hours at room temperature.

Before loading the samples, the gel was pre-run for 15 minutes at 1800V, 45 W to warm the gel. Conditions used to run the sequencing reaction were 1800V, 35W for a required time. After which the gel was soaked in a solution of 5% acetic acid, 15% methanol for 15 minutes to remove the urea, covered with plastic-wrap and dried in a gel drier for 1hour at 80°C. The gel was then exposed to Kodak BioMax MR-1 film (emulsion side in direct contact with gel) in a Lightning Plus Intensifier screen. In general, overnight to 36 hour exposures were sufficient to develop autoradiography.

2.3.5 Polymerase Chain Reaction (PCR).

Polymerase Chain Reaction (PCR) was used to amplify DNA sequences of interest. For PCR the concentration of the primers should be approximately 20pmol/l, and the final concentration 200mM of dNTPs. The following standard reaction was used; 1µl Taq Poymerase (Roche Diagnostics, East Sussex, UK), 0.4µl 25mM dNTP, 5µl 10x buffer (Roche Diagnostics, East Sussex, UK), 1µl oligo 1 (20pmol), 1µl oligo 2 (20pmol), 3µl DNA (10µg) and sterile water to 50µl. Controls were set up to check primers i.e positive control PCR reactions with both primers and a template known to work, and a negative control with no DNA template. Standard PCRs were run on a Biometra Personal Cycler (Biorad Laboratories Ltd, Hertfordshire, UK), 35 cycles of: step 1; 95°C for 1 minute, 15 seconds: step 2; 62°C for 1 minute, 15 seconds: step 3; 72°C 1 minute, 15 seconds.

2.3.6 Reverse Transcription –PCR.

Reverse transcription –PCR (RT-PCR) was used to synthesis cDNA from total RNA extracts prepared from mouse and ovine cell lines using The RNAzol™ B method (AMS Biotechnology, Oxon,UK). Media was removed, and the cells washed twice in PBS before 1ml RNAzol™ B was added to each 6-well plate at 4°C. To disrupt the cells the plate was rocked gently and the RNA solublised by pipetting. The RNA

solution was transferred to a fresh Eppendorf and 0.1ml chloroform added, the solution mixed then incubated on ice for 15 minutes. After centrifugation at 13,400 xg for 15 minutes the upper aqueous layer was removed to a fresh tube and the RNA precipitated by adding an equal volume iso-propanol and incubating on ice for a further 15 minutes. The RNA pellet was washed with 75% ethanol and stored in 75% ethanol at -20^{O}C until required.

RT-PCR using Superscript II was carried out according to manufacturer's protocol Gibco-BRL (Paisley, UK). Total RNA, 1-5μg, and 1μl oligo dT (500μg/ml) were added to 12ml sterile water and heated to 70°C for 10 minutes then chilled on ice briefly. The following components were added to the total RNA and mixed gently: 4μl 5x First Strand buffer, 2μl 0.1M DTT and 1μl 10mM dNTP. This was incubated at 42°C for two minutes then 1μl of Superscript II added and mixed gently by pipetting, the reaction was incubated for a further 50 minutes at 42°C. The reverse transcriptase reaction was stopped by heating to 70°C for 15 minutes. cDNA can now be used for PCR as described in section 2.3.5.

Table 2.1:Summary of oligonucleotides used during PCR and mutagenesis.

Name	Oligonucleotide sequence	Target
A023[N]	CTGACAGCCGCAGAGCTGAGAG	PrP promoter, 5' sequence
A025[N]	CTCATTCCCTAATCTTCA	PrP promoter, 5' sequence
A045[N]	CCGTGCAGAGGAGGAGCTG	PrP promoter, 3' sequence
13741[N]	GAGGCCTGAGGTGGATAGCGGTTGC	PrP ORF, nt 217 3' sequence
SalPM[c]	GCAGGTGTCGACTAATATCC	5' end of ovine PrP promoter region with SalI site added (underlined).
SstPM[c]	GGTCTGCAGTTTAAAGAGCTCCGCGGCTATT	3' end of ovine PrP promoter region with SstI site added (underlined)
SwilD[c]	CCTGCCACATGCTTC	Ovine PrP ORF, nt 337-351, anti-sense strand
E439[m]	GCTCCAGCTGCAGCAGCTCCTGCCATATGC	3F4 epitope sequence/ *Nde*I site in ovine PrP ORF, nt 337-351, anti-sense strand (mutation underlined)
H3F4[c]	CCTGCCATATGCTTC	3F4 epitope sequence/ *Nde*I site in ovine PrP ORF, anti-sense strand (mutation underlined)
E337[c]	GAAGCATATGGCAGG	Introduces 3F4 epitope sequence/ *Nde*I site in ovine PrP ORF, nt 337-351, sense strand (mutation underlined).
P1[N]*	CCGATACCCGGGACAGGGCAG	Ovine PrP ORF, nt 188-208, sense strand

Note:
Oligonucleotides synthesised by [c]Cruachem Ltd.(Glasgow, UK) or MWG-Biotech Ltd (Milton Keynes, UK) or [N] available at NPU. * Designed by Dr Gerard O'Neill.

2.4 General Methods for Tissue Culture.

2.4.1 Preparation of new plates/flasks.

All plastic wear was purchase from Costar (Buckinghamshire, UK). The cells were grown in 75cm^2 flasks which were treated as follows. The flasks (Costar Ltd, Buckinghamshire, UK) were incubated with 0.1% gelatin at room temperature for 2 hours in the hood and rinsed with sterile phosphate buffered saline (PBS,Gibco-BRL, Paisley, UK) prior to use.

2.4.2 Handling cells to be passaged.

To passage the cells the following protocol was observed. The culture medium was removed and the cells rinsed with PBS then 3ml trypsin/ versene solution was added to the 75cm^2 flask (refer to Appendix A for all solutions). The flask was rocked gently and placed in the incubator for a few minutes and when all the cells were off the flask they were collected in a 15ml centrifuge tube. The flask was rinsed with 2ml sterile PBS which was added to the collected cells. The cells were spun at 2000 xg for 5 minutes, and the pelleted cells resuspended in 2ml of complete culture medium. The resuspended cells (0.2ml) were added to a new flask and placed in the incubator at 33°C with 5% CO_2. The remaining cells were stored at -20°C.

2.4.3 Cells for frozen storage.

Cells for storage were prepared by the following protocol. After collection and pelleting the cells, the supernatant was decanted to remove all excess trypsin/ versene solution The pellet was resuspended in 3ml freezing down medium, and 1ml of resuspended cells pipetted into cryovials. The vials were wrapped in paper towel and placed directly into -70°C overnight in an upright position, then the cells were placed in liquid N_2 until required.

2.4.4 Removing cells from frozen storage.

To remove cells from storage for re-growth the following method was used. The vials were removed from liquid N_2 and thawed rapidly in a warm water bath, the outside of the vial was sprayed with 70% Methanol to clean it. The cells were added to flasks prepared with 0.1% gelatin, 10ml of culture medium added slowly, and the cells incubated at 33°C. Next day the medium was replaced with fresh medium thus removing the dimethyl sulphoxide (DMSO; Sigma, Dorset, UK).

2.4.5 Culturing ovine cell lines.

The NPU sheep-derived cell lines were grown at 33°C in 5% CO_2. A change in morphology was observed when the cells were grown at 37°C which may be due to the presence of vectors chosen to produce stable cell lines (Hew John, Moredun Institute, Edinburgh, personal communication). The primary cells were grown at 37°C in 5% CO_2. The cells were fed every three to four days with complete media (Appendix A) as required. The tops of the flasks were unscrewed loose while in the incubator.

2.4.6 Culturing Neuroblastoma cells (N2a)

Neuroblastoma cells (N2a) were available at the NPU. The cell requirements for N2a cells are different from the sheep cells in that the N2a cells are kept at 37°C instead of 33°C as for the ovine cells. Plates and media were prepared as for ovine cells, N2a cells are grown in the presence of complete culture medium. The cells were fed every three to four days with complete media (Appendix A) as required. The tops of the flasks were unscrewed loose while in the incubator.

2.4.7 Immunostaining for ovine brain-derived cell type identification.

For classification of brain cell lines specific neuronal antibodies (Table 3.1) were used to test for several neuronal markers and were detected by fluorescein isothiocyanate (FITC) labelled secondary antibodies.

2.4.7.1 Fixing brain-derived cell lines for immunostaining.

The cells were passaged (Section 2.4.2) as usual and a 500µl aliquot added to a 60mm culture dish containing a 10-well glass slide which had been treated with gelatin as described for the preparation of plates and flasks (Section 24.1). Complete media, 5ml, was added to the dish and the cells were incubated at 33°C for 16 hours. Before fixing, the cells were washed twice in warm PBS for 5 minutes, shaking. The cultures were fixed with methanol/acetone solution (1:1) for 5-10 minutes at 4°C. The cells were then washed with PBS as before and then stored at 4°C for up to 1 week prior to immunostaining.

2.4.7.2 FITC-immunostaining of *in vitro* ovine brain-derived cultured cells.

Using a wax pen (PAP pen supplied by Agar Scientific Ltd, UK, product number L4197), circles were drawn around the inner rim of each well on the glass slide. The cells were washed three times with warm PBS (37°C) for 5 minutes then incubated with 30µl FITC Blocking solution (refer to Appendix A) for 15 minutes at room temperature. The primary antibody, pre-diluted in 5% Blocking solution (if necessary), was added as 25µl aliquots to the wells (Table 3.1). This was incubated at room temperature for 1 hour, taking care to ensure the solution remained within the PAP circle. Control wells were incubated with 5% Blocking solution only. The cells were then washed as before with warm PBS. The secondary antibody, 25µl (Table 3.1; FITC-conjugated Affinipure goat anti-mouse, Jacksons ImmunoResearch Lab. Inc, Luton, UK) was prepared in 5% Blocking solution according to the supplier's protocol and incubated on the cells in the dark for 1 hour while gently shaking at room temperature. The cells were then washed as before with warm PBS in the dark and the slide mounted with a water-based mounting solution. The stained slides were then observed using fluorescent microscopy (Nikon eclipse E800) and results recorded on film.

2.5 Methods for Transient Transfection.

2.5.1 Preparation of DNA for transfection.

Quality of DNA for transfection is crucial as contaminating protein, RNA and chemicals can adversely affect transfection efficiency. DNA for transfection was purified using CsCl gradients (2.2.8) or endotoxin-free kits (Qiagen Ltd., West Sussex, UK). For transfection a OD_{260}:OD_{280} ratio at or about 1.8 was obtained with the final DNA concentration approximately 1mg/ml.

2.5.2 Control vectors.

The control vector pCAT3-Promoter (Promega, Southampton, UK) was used to optimise transfection methods by analysing reporter activity in cell lysates. To control for transfection efficiency or cell lysate recovery from transfection experiments the vector pSV-β-Galactosidase (Promega, Southampton, UK) was used. β-galactosidase (β-gal) is a commonly used reporter molecule for co-transfection. It is assumed that both reporter vectors are transfected with the same efficiency in a single transfection reaction but that different cell populations may be transfected at varying efficiencies. Measuring the activity of β-gal allows cell extracts to be measured for transfection efficiency and lysates adjusted accordingly for further CAT assays.

2.5.3 Calcium phosphate precipitation.

To transfect ovine and N2a cells by calcium phosphate precipitation the following protocol was observed (Gorman,1982). Cells were passaged (Section 2.4.2) at 50-100% confluent and resuspended in 2ml complete culture medium, of which 0.2ml was seeded into a 6-well plate pre-treated with 0.1% gelatin (Section 2.4.1) and incubated overnight at 33°C. For transfection, 5μg of plasmid DNA was mixed with 250μl 2xHBS (Appendix A) and 220μl sterile water. To precipitate the DNA, 31μl of 2M calcium chloride was added slowly and the mixture incubated at room temperature for 30 minutes. To each well, 160μl of the calcium phosphate/ DNA

precipitate was added and the cells incubated at 37°C overnight. Next day the cell medium was replaced and cells harvested 24 hours later.

2.5.4 Electroporation of brain-derived cell lines.

For electroporation of stable ovine brain cells lines the method described by Li, 1997, was used (Li *et al.*, 1997). On the day before electroporation the cells were passaged and seeded into 75cm^2 culture flasks and the medium replaced approximately 1 hour before harvesting. The cells were trypsinized and resuspended in 5ml of complete culture medium, an aliqout could be removed at this stage for cell counting. The cells were collected by centrifugation and resuspended in 0.8ml 1xHBS and transferred to an electroporation cuvette in preparation for electroporation. A DNA solution was prepared for electroporation by dissolving a pellet containing 5µg of plasmid DNA in 80µl 1xHBS, this was added to the cell suspension. The cells were electroporated (Biorad Gene Pulser, Biorad Laboratories Ltd, Hertfordshire, UK) (Table 3.3) then allowed to rest at room temperature for 10 minutes before being plated onto a 75cm^2 flask pre-treated with gelatin (Section 2.4.1) and returned to 33°C incubator. The cells were harvested 48 hours after electroporation.

2.5.5 Liposome-mediated transfection.

2.5.5.1 Liposome Reagent Dosper.

Transfection using Dosper was carried out according to manufacturer's protocol (Roche Diagnostics, East Sussex, UK). The day before transfection the cells were passaged (as for calcium phosphate) into 6-well plates at 33°C. On the day of transfection the cells were 60-80% confluent for transfection. Different liposome/ DNA mixtures were prepared to determine the optimal proportions for transfection. For example; 6µl Dosper/ 1.5µg DNA. For this 1.5µg DNA was diluted in HBS to a final volume of 50µl (solution A) and 6µl Dosper was diluted with HBS to a final volume of 50µl (solution B). Solutions A and B were mixed together gently by pipetting and incubated at room temperature for 15 minutes to allow the Dosper/DNA complex to form. Without removing culture medium, the Dosper/DNA

solution was added drop wise to the cells, mixing gently by rocking the culture dish. After 6 hours incubation the transfection medium was replaced with 2ml fresh complete medium and the cells incubated at 37°C. The cells were harvested to determine the level of reporter gene activity 48 hours after starting transfection.

2.5.5.2 Liposome Reagents Tfx™-20 and Tfx™-50.

Transfection using Tfx™-20 and Tfx™-50 was carried out following the manufacturer's protocol (Promega, Southhampton, UK). The cells were prepared in a 6-well plate at 33°C as in calcium phosphate transfection method. The cells were 60-80% confluent for transfection. Before use, the liposome reagents were dissolved in 400µl nuclease-free water and stored at –20°C. On the day of transfection the reagent was thawed on ice and different ratios of liposome/ DNA mixtures were prepared to determine the optimal proportions for transfection. For example: 2:1, 3µl reagent per µg DNA; 3:1, 4.5µl per µg DNA; 4:1, 6.0µl per µg DNA.

The Tfx™ reagent/ DNA solution was incubated at room temperature for 10-15 minutes to allow the Tfx/DNA complex to form, after which 1ml of serum-free Iscoves Dulbeccos Modified Medium (Gibco-BRL, Paisley, UK) was added to the Tfx™ reagent/ DNA mixture. The cells were washed twice with PBS before 1ml Tfx™-50/ DNA mixture was added to each well. After 1-2 hours incubation at 37°C, the transfection medium was replaced with 2ml of fresh complete culture medium and the cells incubated at 37°C. The cells were harvested to determine the level of reporter gene activity 48hours after starting the transfection.

2.5.6 Dendrimer Technology: Superfect Transfection Reagent.

Transfection using Superfect was carried out according to he manufacturer's protocol (Qiagen Ltd., West Sussex, UK). The cells were passaged 24 hours prior to transfection into 6-well plates, seeding 2-8x10^5 cells per well and incubated at 33°C. The cells were 60-80% confluent on the day of transfection. Plasmid DNA was prepared by diluting 2µg of DNA in TE, pH7.4, to a concentration of 0.1µg/µl and

added to 100µl cell growth medium containing no serum or antibiotics. To the plasmid DNA solution, 9µl of Superfect transfection reagent was added. To allow the DNA-dendrimer complex to form, the sample was mixed by vortexing for 10 seconds then left at room temperature for 10–15 minutes.

While the complex was forming the growth medium on the cells was removed and the cells washed twice with 1ml PBS. To the reaction tube containing the transfection complexes 2ml complete culture media was added, mixed by pipetting then added immediately to the cells. The cells were then incubated at 37°C in 5% CO_2 for 2-3 hours, after which the medium containing any remaining complexes was removed from the cells. The cells were washed twice with 1ml PBS and 2ml complete culture media added to the cells and incubated at 33°C, 5% CO_2. After 48 hours, the cells were harvested and evaluated in the appropriate way.

2.5.7 Effectene Transfection Reagent.

Effectene reagent was used according to the manufacturer's protocol (Qiagen Ltd., West Sussex, UK). The cells were 60-80% confluent for transfection. Different ratios of Effectene/ DNA-Enhancer mixtures were prepared to determine the optimal proportions for transfection (Table 2.2). A solution was prepared containing 0.5µg DNA, with Enhancer and Enhancer buffer to a final volume of 150µl. Effectene reagent was added to the DNA-Enhancer mixture as required, mixed and incubated at room temperature for 5 to 10 minutes. While the Effectene complexes formed, the growth medium was removed from the cells to be transfected, the cells washed with PBS and 1ml fresh culture medium overlaid on to the cells. For transfection, 0.5ml complete culture media was added to the transfection reaction, mixed by pipetting and immediately applied drop wise to the cells. The plate was gently rocked to ensure uniform distribution. After 16 hours incubation with the complexes at 37°Cand 5% CO_2 , the transfection medium was replaced with 2ml fresh complete culture medium and the cells incubated as before. The cells were harvested to determine the level of reporter gene 48 hours after transfection.

Table 2.2: Pippetting scheme for optimising transfection with Effectene reagent (Qiagen Ltd, West Sussex, UK) 0.5μg pCAT3-Promoter to primary cell lines in 6-well plates.

Component	Ratio of DNA to Effectene Reagent		
	1:10	1:25	1:50
DNA (μg)	0.5	0.5	0.5
Enhancer buffer (Buffer EC, μl)	100.0	100.0	100.0
Enhancer (μl)	3.2	3.2	3.2
Effectene (μl)	4.0	10.0	20.0

2.5.8 Cell extract preparation.

2.5.8.1 Freeze/thaw method

The transfected cells were harvested by freeze/thawing for CAT assay in the following way. The medium was removed and the cells washed in 1x PBS before 1ml TENS buffer (Appendix A) was added directly to the cells and left at room temperature for ten minutes. Using a cell scraper, the cells were collected and transferred to a micro-centrifuge tube and centrifuged at 13,400 xg for 1 minute. The pellet was dissolved in 100μl 0.25M Tris-HCl, pH 8.0 and subjected to three freeze-thaw cycles, vortexing after each cycle. Cell debris was removed by centrifugation as before and the supernatant transferred into a fresh tube to be assayed directly or stored at –70°C.

2.5.8.2 Reporter Cell Lysis buffer.

The transfected cells were harvested lysis by Reporter Cell Lysis Buffer (RCLB; Promega, Southampton, UK) in the following way. The medium was removed and the cells washed twice in 1xPBS. Sufficient volume of 1X RCLB was added to cover the cells (400μl for 60mm dish) and the dish rocked gently at room temperature for 15 minutes. All areas of the plate were scraped to ensure all visible cell debris was collected, and using a pipette the cell lysate was transferred to an 1.5ml Eppendorf tube and incubated on ice briefly. The cell debris was removed by

centrifugation at full speed in a microcentrifuge for two minutes. The cell lysate (supernatant) was transferred to a fresh tube and used directly or stored at –70°C.

2.5.9 Chloramphenicol Acetyltransferase (CAT) enzyme assay.

To assay for CAT activity the standard method used is as follows (Seed, *et al.*, 1988). To 100µl cell extract, 3µl [^{14}C]chloramphenicol (0.05mCi/ml), 5µl n-butyryl Coenzyme A (5mg/ml) (Promega, Southampton, UK) and sterile water to 125µl was added. The reaction was incubated at 37°C for one hour. To carry out both liquid scintillation (LSC) and thin layer chromatography (TLC) assays the sample was divided in two 75µl aliquots. To terminate the reaction: for LSC 300µl mixed xylenes was added; for TLC 500µl ethyl acetate was added. From LSC the amount of acetylated products were measured on a scintillation counter. From TLC the acetylated products were either isolated for LSC or the measured directly by densometric reading using a Kodak Imagine Station 440 and 1D image analysis software (section 2.5.12).

2.5.10 Liquid Scintillation Count (LSC) assay.

The sample in xylene was vortexed for 30 seconds, spun in a microcentrifuge for three minutes at 13,400 xg and the upper layer removed to a fresh tube. Fresh 0.25mM Tris-HCl, pH 8.0, 100µl, was added and the spin repeated, extracting the upper layer again. A known volume, or 200µl, was added to a scintillation vial with 2ml scintillation fluid and the counts per minute (cpm) measured.

2.5.11 Thin Layer Chromatography (TLC) assay.

The sample in ethyl acetate was vortexed for 1 minute, spun in microcentrifuge at 13,400 xg for three minutes, the top layer was transferred to a fresh tube and dried under vacuum. The residue was dissolved in 10µl ethyl acetate and spotted on to a silica gel plate. The plate was run in a pre-equilibrated tank with chloroform:methanol (97:3) for 30 minutes then allowed to dry at room temperature.

The plate was covered with plastic wrap and exposed to Kodak XAR-5 film overnight with a Lightning Plus Intensifier screen.

2.5.12 Densometric reading of autoradiographs.

Following TLC and development of autoradiographs, it was possible to measure directly the density of the acetylated products using a Kodak Image Station and processing the results using the Kodak 1D Image Analysis software. The measurement recorded is the "net" figure that is calculated by the sum intensity of the pixels within a selected area minus the background.

2.5.13 β-Galactosidase enzyme assay.

To assay for the level of transient β-gal activity the method used was the β-Galactosidase enzyme assay System (Promega, Southampton, UK). Cell extract was prepared by the freeze thaw method (Section 2.5.8.1) and 50μl cell extract added to 100μl of Assay 2x Buffer and mixed vigorously. The β-gal assay was incubated at 37°C for 30 minutes or until faint yellow colour had developed. The reaction was stopped by adding 500μl of 1M $NaCO_3$ and the colour reaction measured by reading the absorbance at 420nm.

2.6 General Methods for Protein Analysis.

2.6.1 PrP extraction from adherent cells using Triton X-114

For transient expression assays, cell extracts were prepared 48 hours after transfection. Cells extracts from non-transfected cells were also prepared to measure endogenous levels of cellular protein levels. Approximately $2x10^7$ cells were collected from100mm petri dishes for PrP protein extraction by harvesting the cells in TENS buffer (Section 2.5.8.2, Appendix A). Using a cell scraper the cells were collected and transferred to a microcentrifuge tube and spun at top speed for 1 minute. The supernatant was removed and the cells stored dry at –20°C. Prior to extraction all rotors and tubes used were pre-cooled to 4°C. Also, the TL100 and the

GS-6R centrifuges were cooled to 2°C and 0°C respectively at least 30 minutes before use. Both centrifuges have temperature controls allowing the rotors (TLA-100.3) to be cooled to at least 0°C.

The cells were resuspended in 2ml Extraction buffer (Appendix A), 2µl of 10mM PMSF protease inhibitor was added and the cells sonicated for 30 seconds at power level 8, tune 3 (Micro-ultrasonic cell disrupter, Konics). The extract was transferred to a pre-cooled 15ml screw top tube and incubated on ice for 30 minutes. After centrifugation for 10 minutes at 900 xg, 0°C in the GS-6R the supernatant was removed using a pasteur pipette and transferred to a thick walled TLA-100.3 tube. The supernatant (SP_1) was left to cool on ice for approximately 10 minutes then spun in the ultracentrifuge for 1 hour at 164,000 xg, 2°C. The pellet was discarded and the supernatant (SP_2) overlaid onto 3ml Sucrose buffer (Appendix A) then incubated at 30°C for 15 minutes.

The GS-6R rotor was pre-warmed to 30°C for the next stage of extraction. SP_2 was spun in the GS-6R for 10 minutes at 380 xg at 30°C. With a pasteur pipette, 2.5ml was removed from the aqueous layer (SD_1) and cooled on ice. The remaining sucrose bed was removed and discarded, the detergent layer(D_1) was transferred to a screw-top Eppendorf tube and stored at 4°C. To SD_1, 220µl Triton X-114 was added and vortexed then incubated on ice for 1 hour. SD1 was overlaid onto 3ml Sucrose buffer, incubated at 30°C for 15 minutes then spun as before in the GS-6R for 10 minutes at 380 xg, 30°C. With a pasteur pipette, 2ml was removed from the aqueous layer (SD_2), the remaining sucrose bed discarded and the detergent layer (D_2) was transferred to a screw top Eppendorf tube and stored at 4°C. D_1 and D_2 were pooled then split into two aliquots. The protein was then precipitated by chloroform/methanol extraction and stored at –20°C in methanol.

2.6.2 Chloroform/methanol extraction

This method was used to precipitate protein from cell extracts following Triton X-114 treatment. For a 200µl extract: 800 µl methanol, 400 µl chloroform and 600 µl

H_2O was added, vortexed and spun in a micro-centrifuge for 10 minutes at 2000rpm. The aqueous layer was removed and 800µl methanol added, the sample was mixed and centrifuged as before. The pellet was stored in methanol at –20°C until required for SDS-PAGE and Western blotting (Section 2.6.4).

2.6.3 Immunoprecipitation

For immunoprecipitation the following method was adapted from Firestone and Winguth (1990) and used to detect transient and endogenous PrP^C expression (Chapter 5, Section 5.5). Cell extracts were prepared from approximately $2x10^7$ cells, 48 hours after transfection using Reporter Cell Lysis Buffer (RCLB, Section 2.5.6.2). Cell extract from non-transfected cells was also prepared to measure endogenous levels of PrP^C protein levels. Cell lysate was spun briefly at 2,000 xg and the supernatant transferred to a fresh tube. The primary antibody (Table 2.3) was diluted in TBST (2x volume of the supernatant, Appendix A) and added to the lysate supernatant and incubated for 16 hours at 4°C while gently mixed. To precipitate the PrP immunocomplex, 100µl protein-A Sepharose solution (10% in TBST) was added to the lysate solution and incubated for a further hour at room temperature, gently mixing. The sepharose beads were collected by centrifugation at 3,000rpm, 10 minutes, and washed in 100µl 5x TBST. This procedure was repeated three times. After the third wash the beads were resuspended in 50µl 1x sample buffer to elute the bound PrP complex which was then denatured at 90°C for 10 minutes and processed by SDS-PAGE and Western blotting (section 2.6.4)

2.6.4 Chemiluminescence blotting.

To detect *in vitro* PrP^C protein, Western blots were carried out as follows (Towbin, *et al.*, 1979). All equipment and solutions described in Appendix A. After methanol/chloroform precipitation the protein pellet collected by micro-centrifugation for 10 minutes at 13,400 xg. The pellet was dissolved in 30µl 1x sample buffer and incubated at 90°C for 10 minutes before being loaded onto a 12% acrylamide gradient gel. The gel was run at 200V, 75mA for 1 hour.

In preparation for protein transfer onto a PVDF membrane, 2x 6 pre-cut pieces of 1F filter paper (6cm x 8.5cm) were soaked in Transfer buffer for 10 minutes. A sheet of pre-cut PVDF membrane (6cm x 8.5cm) was soaked in methanol for 5-10 seconds, washed in water for 1 minute then soaked in Transfer buffer for five minutes. For blotting the following was assembled on the semi-dry blotter: 6 pieces of filter paper, PVDF membrane, gel and 6 pieces of filter paper. A current of 2mA/sq cm was applied for 1 hour.

After blotting, the PVDF membrane was washed with methanol for five seconds and left to dry at room temperature for 15 minutes. The membrane was re-wet with methanol, rinsed with sterile water then washed twice with TBS before incubating with 1% Western Blocking Reagent Solution (Roche Diagnostics, East Sussex, UK: Appendix A) at room temperature for one hour while rocking gently. The primary antibody was prepared in 0.5% Western Blocking Reagent Solution (Table 2.3) and incubated with the membrane for 2 hours at room temperature while being gently rocked. The membrane was washed twice for 10 minutes with 20ml TBST, then washed twice for 10 minutes with 0.5% blocking solution. The secondary antibody was prepared in 0.5% blocking buffer (Table 2.4) and added to the membrane and incubated at room temperature for 30 minutes while being gently rocked. The membrane was then washed for 15 minutes with 20ml TBST, this was repeated a further three times before rinsing the membrane with water for 90 seconds. Pre-mixed detection reagent (Appendix A) was added to cover the membrane and left to incubate for 60 seconds, the blot was wrapped in transparent film and exposed to Kodak XAR-5 film for 30 seconds, 3 minutes and 10 minutes.

2.6.5 Primary antibodies

To date, no antibody can reliably distinguish between PrP^{C} and PrP^{Sc}. Furthermore most antibodies will cross-react with PrP protein purified from two or more species as a result of the highly conserved PrP gene. For the purposes of this investigation the antibodies chosen for PrP protein analysis are shown in Table 2.2. As discussed in Chapter One the polyclonal antibody 1B3 recognises PrP purified from many species and was used as a control for PrP expression between the mouse and sheep cell lines. However 1B3 also cross-reacts with several other cellular protein s and can give high background during Western blotting. Recently the monoclonal antibody 6H4 became commercially available (Prionics, Zurich). 6H4 shows specificity to PrP from several species and was used as a control between the mouse and sheep cell lines. The monoclonal antibody FH11 had been shown to be ruminant specific and was chosen to detect ovine PrP expression from the transiently expressed constructs in the mouse N2a cell line. To detect transiently expressed ovine PrP from the plasmids pNPU3F4.2 and pNPU3F4.4 in the sheep cell lines the monoclonal antibody 3F4 was used.

Table 2.3: Primary antibodies used for immunodetection of ovine and mouse PrP.

Antibody	PrP motif (amino acids)	Specificity	Conditions for use (dilution)	Supplier
1B3 Rabbit polyclonal	14-36; 83-102; 119-139; 188-212	Mouse, Sheep, Goat	10µl in 12ml 0.5% blocking solution, incubate room temperature for 2 hours, shaking (1:1200)	C. Farquarson, NPU, Edinburgh
FH11 Mouse monoclonal	PQGGG (51-55 hu)	Goat, Sheep	5µl in 25ml 0.5% blocking solution, incubate at room temperature for 2 hours, shaking (1:5000)	C. Birkett, IAH Compton
3F4 Mouse monoclonal	KHMA (109-112 hu)	Hamster	1µl in 12ml 0.5% blocking solution, incubate at room temperature for 16 hours, shaking (1:12000)	R.Kascsak, MRDD, New York (Kascsak *et al.*, 1987)
6H4 Mouse monoclonal	DYEDRYYRE (aa 144-152 hu)	Mouse, Sheep, Goat	5µl in 15ml 0.5% blocking solution, incubate at room temperature for 16 hours shaking (1:3000),	Prionics AG, Zurich (Korth *et al.*, 1997)

2.6.6 Secondary antibodies

Table 2.4: Peroxidase-conjugated secondary antibodies used for detection PrP after Chemiluminescence blotting. Supplied by Jacksons ImmunoResearch Laboratories Inc., Luton, UK.

Antibody	**Primary antibodies**	**Conditions for use**
Goat-anti-rabbit-POD	1B3	1µl in 25ml 0.5% blocking solution, incubate at room temperature for 30 minutes, shaking
Rabbit-anti-mouse-POD	FH11 3F4 6H4	5µl in 12ml 0.5% blocking solution, incubate at room temperature for 30 minutes, shaking

Chapter 3 : Development of transient transfection systems for *in vitro* expression studies in Ovine derived cell lines.

3.1 Introduction

The use of cell culture in molecular biology is an important tool for elucidating the functions of genes and their products. The advantage of using insect or mammalian cell lines, as opposed to bacterial cultures or cell free systems, is the presence of cellular machinery for correct post-translational modification. It can be expected then, that proteins expressed *in vitro* using mammalian cell culture will be modified in a similar manner to native proteins *in vivo*.

The process of introducing nucleic acids into cells by non-viral methods is known as transfection. Two approaches exist for expression of transfected genes: stable, long-term transfection and transient expression (Southern and Berg, 1982; Gorman *et al.*, 1982). Stable transfection involves the integration of the reporter gene into the host chromosome to produce cell lines that constitutively express the gene of interest and uses a selection process to screen against non-transfected cells. The alternative method of transient transfection was used for the work described in this thesis. This method involves cloning a reporter gene into an expression vector (plasmid) which is introduced into cultured cells in high copy number where it exists independently in the cell and appears to degrade slowly. The efficiency of transient transfection is dependent on the number of cells which take up the transfected DNA. The products from reporter genes following transient transfection can be analysed 48-72 hours post transfection.

Several methods exist for the development of stable or transient transfection, based on the utilisation of different physical and chemical principles; calcium phosphate precipitation (Graham and van der Eb, 1973), electroporation (Wong and Neumann, 1982), liposome-mediated transfection (Fraley *et al.*, 1980; Felgner *et al.*, 1987), and dendrimer-mediated transfection (Tang *et al.*, 1996). The parameters for transfecting

cells by these methods vary considerably for each cell type and therefore need to be optimised for efficient transfection.

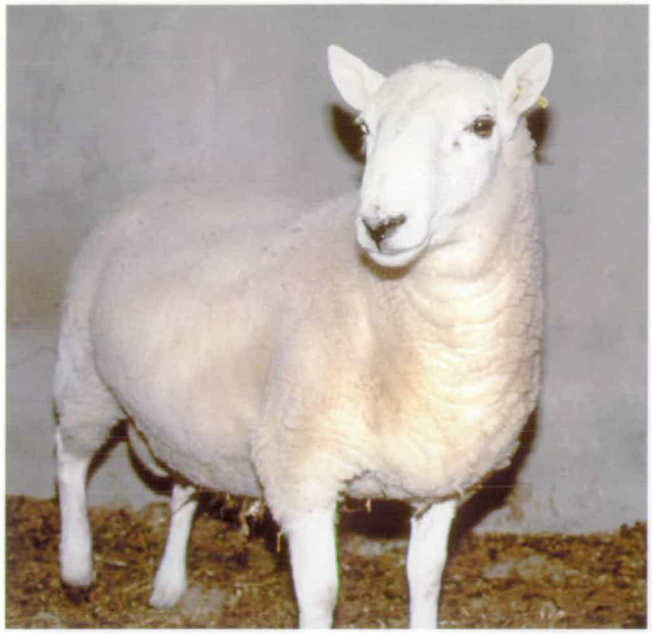
Previous *in vitro* studies involving the sheep PrP 3'UTR (discussed in Chapter Four) were undertaken in mouse neuroblastoma cells (N2a) (Cheung, 1996; Goldmann *et al.*, 1999). It is feasible that the mouse-derived cell line does not have the same transcription/ translation factors necessary for control of an ovine gene as would be found in sheep cells. The aim of this Chapter is to outline the methods tested for efficient transient transfection of various ovine cell lines. As sheep cells lines are not commonly used, with only limited lines available commercially, it was necessary to obtain suitable ovine cells lines. With this in mind, stable sheep cell lines were obtained from Dr Huw John, Moredun, derived from NPU Cheviot sheep (Section 3.2) and primary cell lines (Section 3.3) were created by Dr Gerard O'Neill, NPU, from an Icelandic sheep breed. It was also important to identify the cell types present in the brain cell lines, as it is known that the level of PrP expression in different brain areas can vary. This may have implications for the reporter gene activity as discussed in Section 3.4.

3.2 Generation of Stable Ovine Cell Lines for Transient Transfection Studies.

Immortilised cell lines were derived from brain and skin from $VV_{136}RR_{141}QQ_{171}$ (scrapie susceptible) and $AA_{136}RR_{141}QR_{171}$ (scrapie resistant) NPU Cheviot sheep by Dr Huw John, Moredun Institute, Edinburgh. The cell lines were kindly given to the NPU for the studies in this thesis. The method used to create stable cell lines from NPU cheviot sheep (Figure 3.A) by Dr Huw John, Moredun Institute, Edinburgh was modified from published methods (John *et al*, 1994; John, 1994). Foetuses from these sheep were removed by caesarean Section at 80 days into gestation and tissue cells extracted from neural (brain, spinal cord) and peripheral tissues (spleen, lungs, skin). Briefly, the extracted tissue was washed and treated with a 0.25% trypsin solution and the tissue digested for 10 minutes at 37°C. The supernatant was discarded and trypsin digestion repeated three times. The separated cells were recovered by sedimentation and resuspended in Iscove's modified Dulbecco's Medium (IMDM, Gibco), 10% foetal calf serum, (FCS, Gibco). The Moloney murine leukaemia virus (MoMLV) based vector, pZIPNeo, containing the marker neomycin was transfected to generate continuous proliferation in cultured cells. Monolayer cells ($1x10^6$) for retroviral transfection were cultured at 37°C overnight then exposed to retroviral-containing medium for 24 hours. Successfully transfected cells were selected by treating the cultures with medium containing the antibiotic neomycin, G418, 2mg/ml.

The established cell lines were sustained at 33°C instead of 37°C as it was observed that at the higher temperature they underwent morphological changes (John, personal communication). It was unclear whether this was a result of the vector used for stable transformation of the cells. The brain cell lines (Figures 3.2-3.3) derived from each genotype (sA80BR from $VV_{136}RR_{141}QQ_{171}$; pA80BR from $AA_{136}RR_{141}QR_{171}$) grew successfully at the NPU and were used in transient expression studies. The skin cells (Figure 3.4) derived from $AA_{136}RR_{141}QR_{171}$ NPU Cheviot sheep also grew well. Unfortunately the skin cell line derived from sheep of susceptible genotype suffered several fungal infections, failed to grow at a satisfactory rate, and was discounted within this study.

A

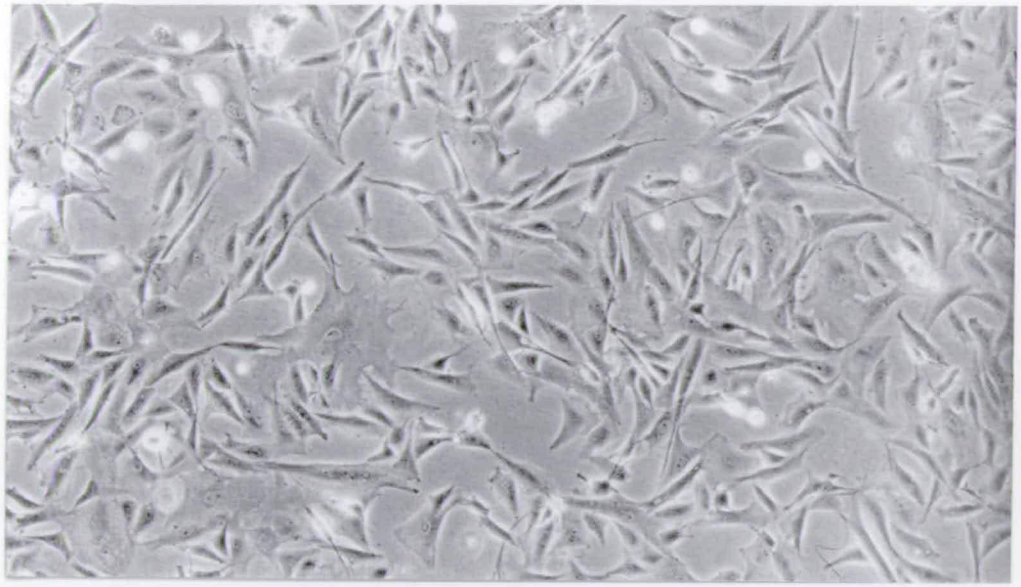


B

Figure 3.1: Sheep breeds used in preparation of cell lines.

(A) Example of Cheviot sheep breed as used by Dr Huw John to create the stable cell lines sA80BR and pA80BR as described in Section 3.2. (b) Example of the Icelandic breed, *Ovis branchura borealis pall*, used by Dr Gerard O'Neill to establish primary cell lines from various tissues as described in Section 3.3.

A



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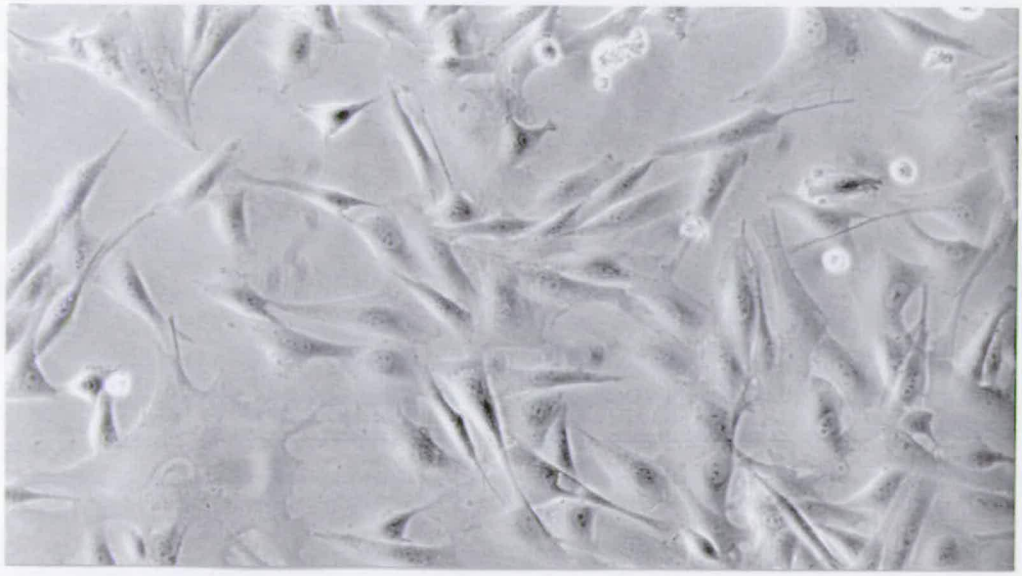
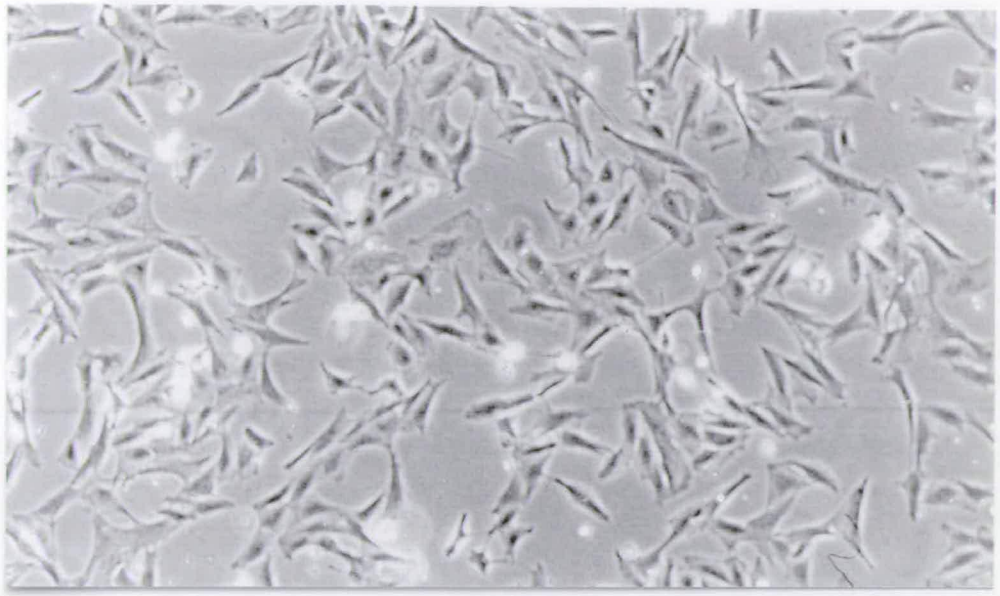


Figure 3.2: NPU Cheviot sheep brain-derived cell line sA80BR

Brain cell line, sA80BR, derived from a scrapie susceptible Cheviot sheep ($VV_{136}RR_{154}QQ_{171}$), passage number 13. (A) x25 magnification (B) x50 magnification.

A



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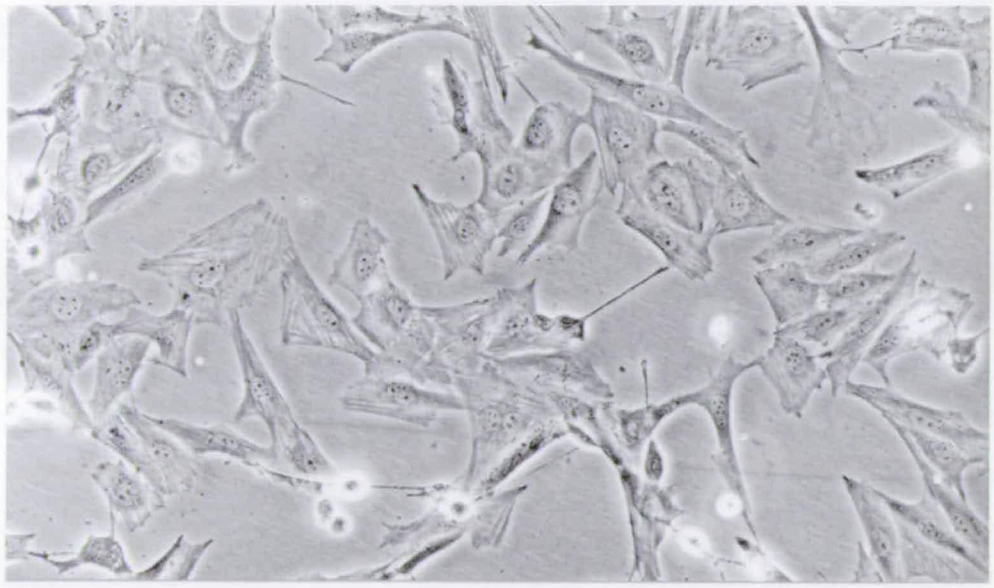
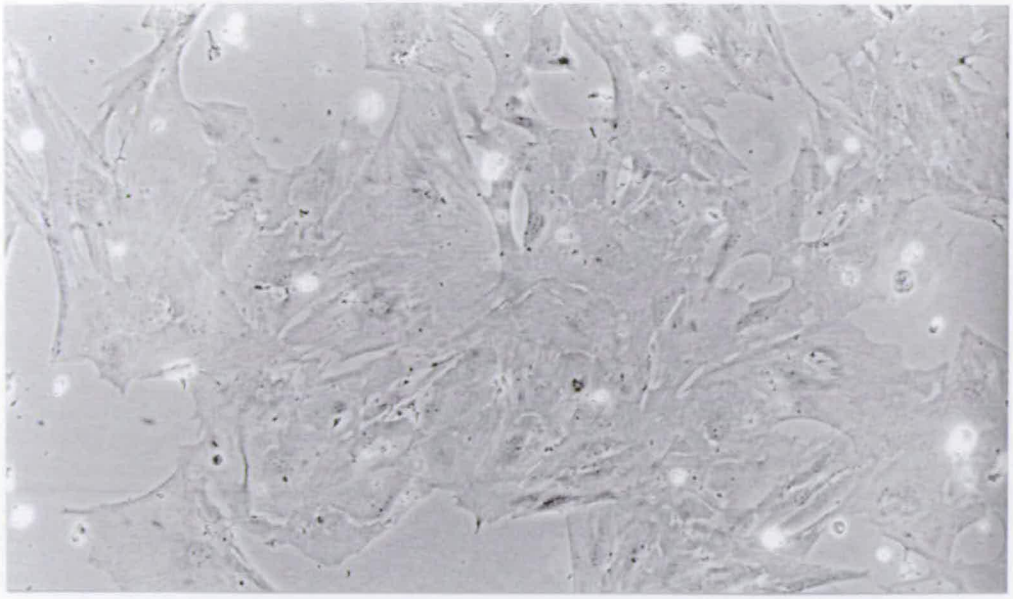


Figure 3.3: NPU Cheviot sheep brain-derived cell line pA80BR

Brain cell line, pA80BR, derived from a scrapie resistant Cheviot sheep ($AA_{136}RR_{154}QR_{171}$), passage number 10. (A) x25 magnification (B) x50 magnification.

A



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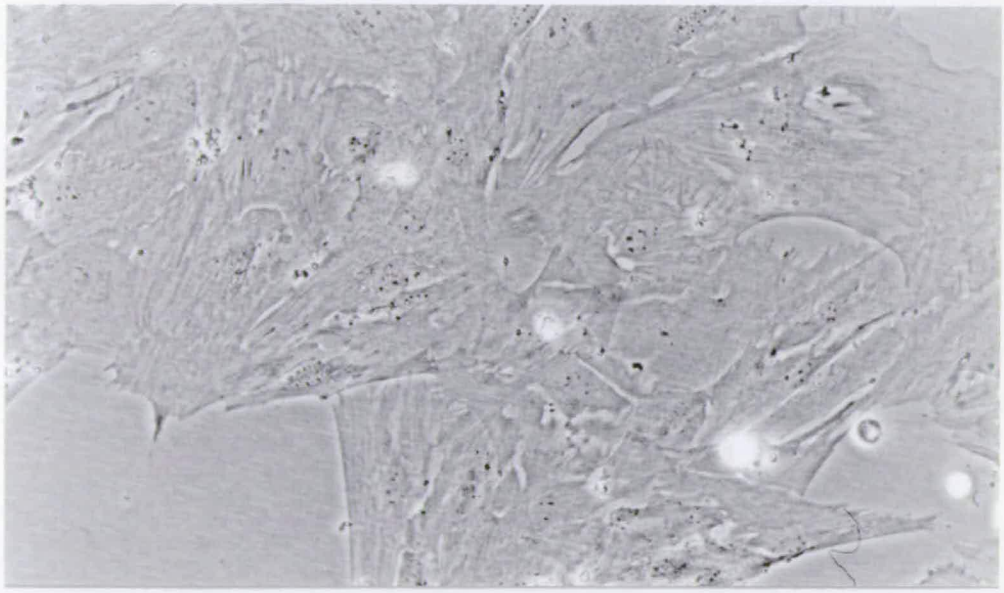


Figure 3.4: NPU Cheviot sheep skin-derived cell line pA80SK

Skin cell line, pA80SK, derived from a scrapie resistant Cheviot sheep ($AA_{136}RR_{154}QR_{171}$),passage number 3. (a) x25 magnification (b) x50 magnification.

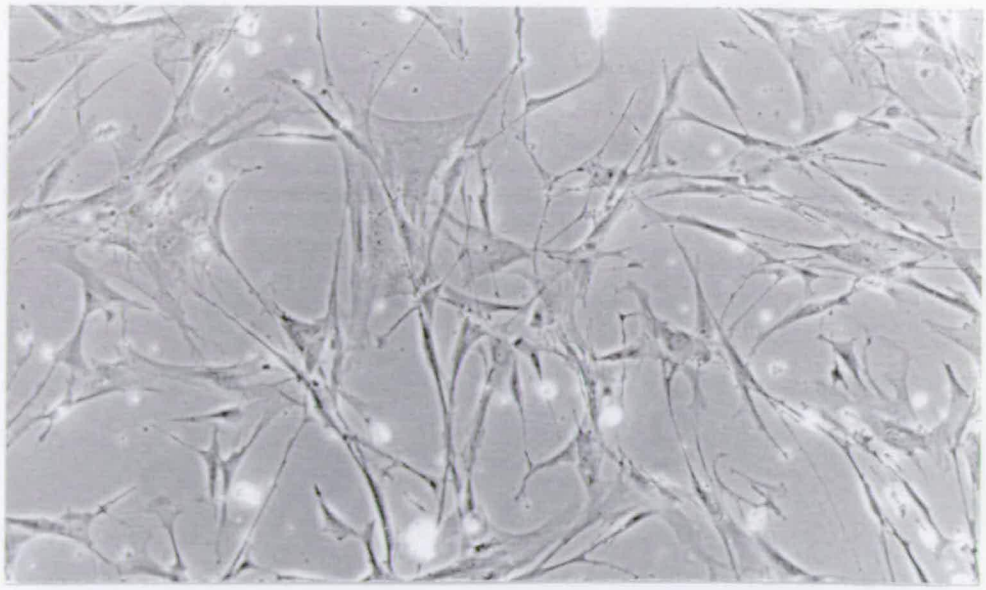
3.3 Generation of Primary Ovine Cell lines for Transient Transfection Studies

Primary cell lines were derived from a male sheep of the Icelandic breed (Figure 3.1B), *Ovis brachyura borealis pall*, by Dr Gerard O'Neill by standard cell culture methods (Freshney, 1987). The animal was 1.5 years of age and had a PrP genotype of $AA_{136}RR_{154}QQ_{171}$ which is scrapie susceptible in the Icelandic sheep breed. Tissue was recovered, in $2cm^2$ segments, from brain and other tissues and macerated into smaller fragments with scalpel blades. The tissue fragments were treated with a 0.25% trypsin solution and the tissue digested for 30 minutes at 37°C. The digested tissue was allowed to settle, the lysate was removed and the cell debris pelleted. Fresh trypsin solution was added to the cell debris and the process repeated three times. The cell lysate and debris fractions were re-suspended in 10ml Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 10% foetal calf serum (FCS, Gibco).

Aliquots, 2mls, of the cell lysates were added to pre-gelatinised (0.1%) $25cm^2$ flasks and incubated at 37°C, 5% CO_2. Fresh medium was applied to the cells after 16 hours. When explants were observed to be growing from the cell colonies, the flasks were agitated to dislodge the growths and the cells washed in fresh DMEM containing 10% FCS. The primary cell cultures were passaged when they reached confluency or frozen for storage.

Cells lines created by Dr O'Neill from cerebellum tissue (IS120.Cer), liver tissue (IS120.Cer) and kidney tissue (IS120.Kid) were intended for use within this present study (Figures 3.5-3.7). Both IS120.Cer and IS120.Liv grew well at NPU as adherent cultures with distinct cellular morphology. Unfortunately, the cell line IS120.Kid grew at a slow rate, unsuitable for transfection analysis and so were not used in the transient expression studies, but were used for PrP analysis in Chapter Five. General cell requirements and recipes for the solutions used in tissue culture are found in Chapter Two and Appendix A.

A



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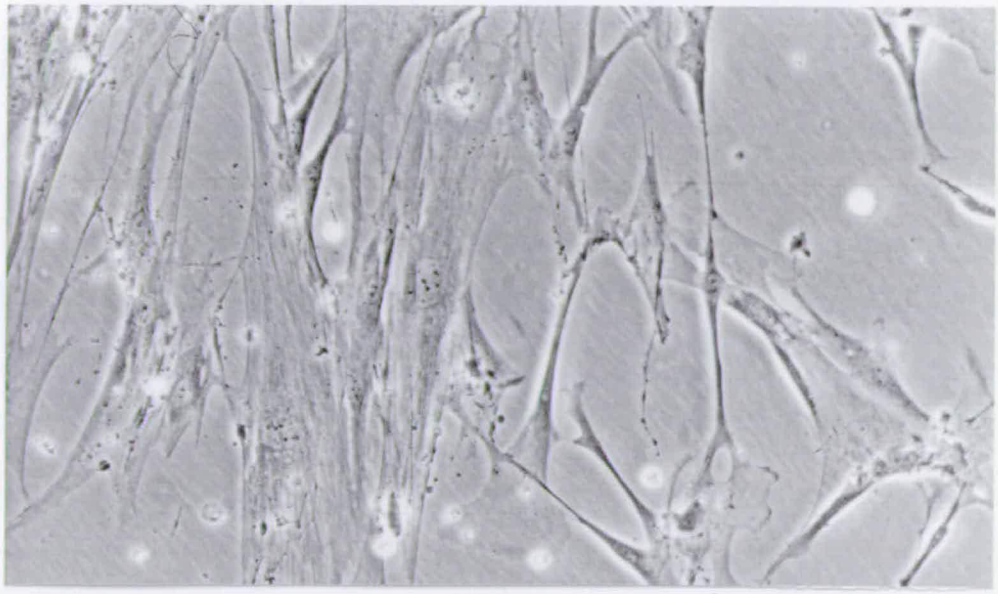
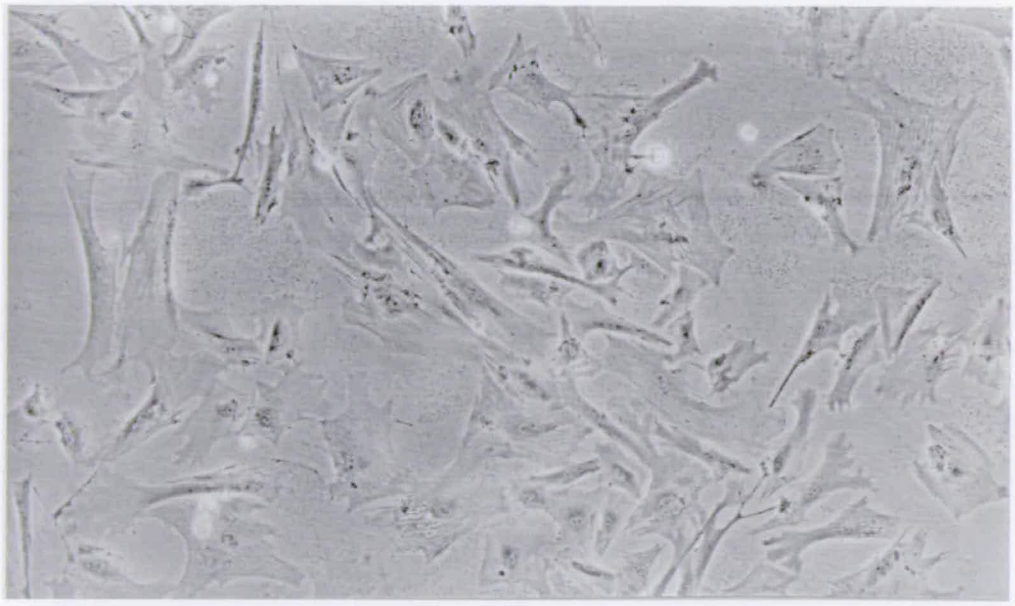


Figure 3.5: Icelandic sheep brain-derived cell line IS120.Cer

Cell line, IS120.Cer, was derived from the cerebellum of an Icelandic sheep breed, *Ovis brachyura borealis pall,* scrapie susceptible genotype ($AA_{136}RR_{154}QQ_{171}$), passage number 6. (A) x25 magnification (B) x50 magnification.

A



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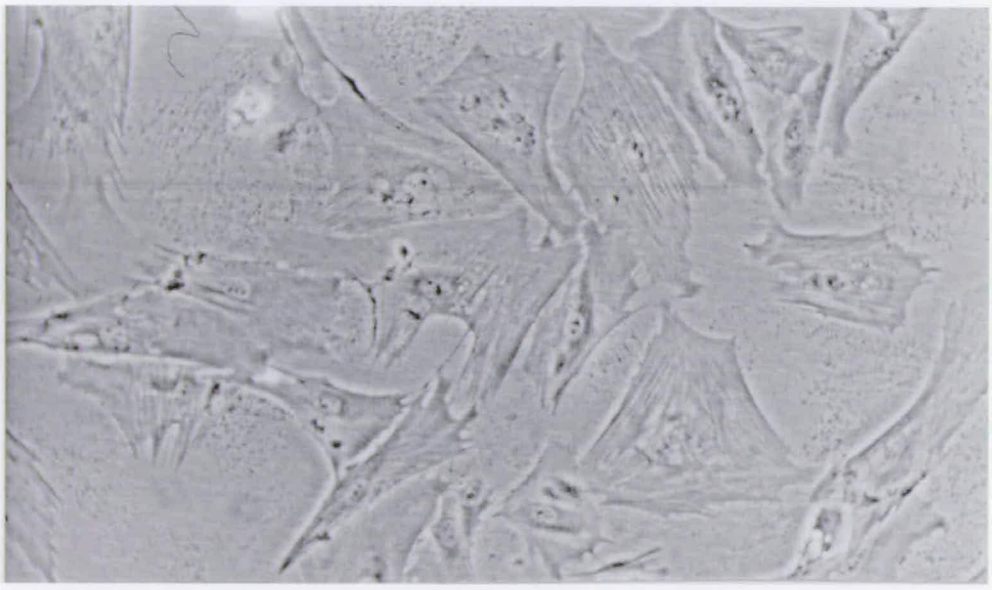

Figure 3.6: Icelandic sheep liver-derived cell line IS120.Liv.

Liver cell line, IS120.Liv, derived from liver tissue of an Icelandic sheep breed, *Ovis brachyura borealis pall,* PrP genotype $AA_{136}RR_{154}QQ_{171}$, passage number 10. (A) x25 magnification (B) x50 magnification.

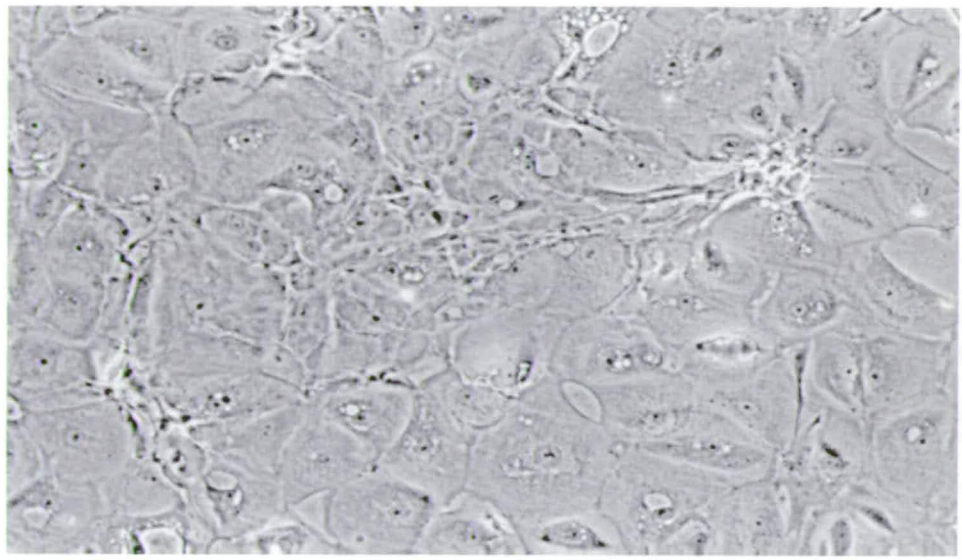

Figure 3.7: Icelandic sheep kidney-derived cell line Liv: IS120.Kid

Kidney cell line, IS120.Kid, derived from kidney tissue of the Icelandic breed, *Ovis brachyura borealis pall* (scrapie susceptible genotype), passage number 6. (a) x25 magnification.

3.4 Classification of Ovine Brain-derived Cell Lines.

The PrP gene has been shown to be expressed *in vivo* in brain fractions (Rubenstein *et al.*, 1986; Kascsak*et al.*, 1986; Manson *et al.*, 1992a; Harris *et al.*, 1993), a wide variety of non-neuronal tissues (Rubenstein *et al.*, 1986 Bendheim *et al.*, 1992; ; Horiuchi *et al.*, 1995; Fournier *et al.*, 1998; Goldmann *et al.*, 1999) and *in vitro* by numerous cell lines of different origin (Wion *et al.*, 1988; Satoh *et al.*, 1998; Dodelet *et al.*, 1998; Lazarini, *et al.* 1994; Atouf *et al.*, 1994; Kniazeva *et al.*, 1997; Ikeda *et al.*, 1998). In sheep it is known that the level of PrP mRNA expression varies between tissues with the highest level detectable in the brain (Goldmann et al., 1999).

Within the brain, although neuronal PrP mRNA expression has been documented by *in situ* hybridisation, PrP mRNA is also readily detectable in astrocytes and oligodendrocytes (Raeber *et al.*, 1997; Moser *et al.*, 1995). In the developing hamster brain the level of PrP mRNA expression in astrocytes is initially comparable to that of the neurones, increasing two-fold during postnatal development (Moser *et al.*, 1995). A significant proportion of brain PrP^C is the result of glial cell expression. It has been shown that the level of PrP mRNA expressed within individual brain regions and cell types can vary. Regional differences in the expression level of PrP mRNA seen through the developing hamster brain persists within the adult (Mobley *et al.*, 1988). Furthermore, the constitutive expression of PrP mRNA observed in numerous human neuronal cell lines alters in specific cell lines in response to cytokine treatments (Satoh *et al*,1998), which suggests cell type specific regulatory mechanisms.

It was necessary to establish the cell types present in the brain cell cultures used in this study as this may have implications for the level of transient expression from the PrP constructs (discussed in Chapter Five).

3.4.1 Experimental approach: FITC-immunostaining of *in vitro* ovine brain-derived cultured cells.

Several specific neuronal antibodies were used for classification of cell types present in the sheep brain cell lines. The brain cell lines were prepared for immunostaining by fixing with methanol: acetone (1:1) and bound antigens were detected by fluorescein isothiocyanate (FITC) labelled secondary antibodies in the method described briefly below and in detail in Section 2.4.7.2. Classification of the brain cell line sA80BR was repeated twice, results shown in Figure 3.8. FITC-staining of the cell lines pA80BR (results not shown) and IS120.Cer (Figure 3.9) was completed once.

Pre-fixed and blocked cells were incubated with the primary antibody (Table 3.1), pre-diluted in 5% Blocking solution at room temperature for 1 hour. Control cells were incubated with 5% Blocking solution only. The secondary antibody, labelled with fluorescein isothiocyanate (FITC), was diluted according to the suppliers' protocol (Jacksons, UK) in 5% Blocking solution (Table 3.1) and incubated on the cells in the dark for 1 hour, gently shaking at room temperature. The cells were then washed with warm PBS (37°C) in the dark and the slide mounted with Aquamount solution (BDH,UK). The stained slides were observed using fluorescent microscopy and photographed using a Nikon Eclipse E800 (Figures 3.8 and 3.9).

Table 3-1 : Antibodies used to classify cells present in ovine brain derived cell cultures. Secondary antibodies are FITC-conjugated Affinipure goat anti-mouse/ rabbit, (Jacksons ImmunoResearch Lab. Inc, Luton, UK).

Name and Specificity		Detect	Source	Dilution
1°	Anti-glial fibrillary acidic protein (GFAP, Sigma G-3893) Monoclonal	Astrocytes Glial cells	Mouse	1/600
	Neurofilament 200 (N200, Sigma N-0142) Monoclonal	Neuronal cells	Mouse	1/500
	Anti-fibronectin (Sigma F-3648) Polyclonal	Fibroblasts	Rabbit	1/100
2°	Goat anti-mouse FITC	Mouse IgG	Goat	1/50
	Goat anti-rabbit FITC	Rabbit IgG	Goat	1/30

3.4.2 Results: Immunohistochemical staining of ovine brain-derived cell lines using fluorescein isothiocyanate (FITC) labelled antibodies.

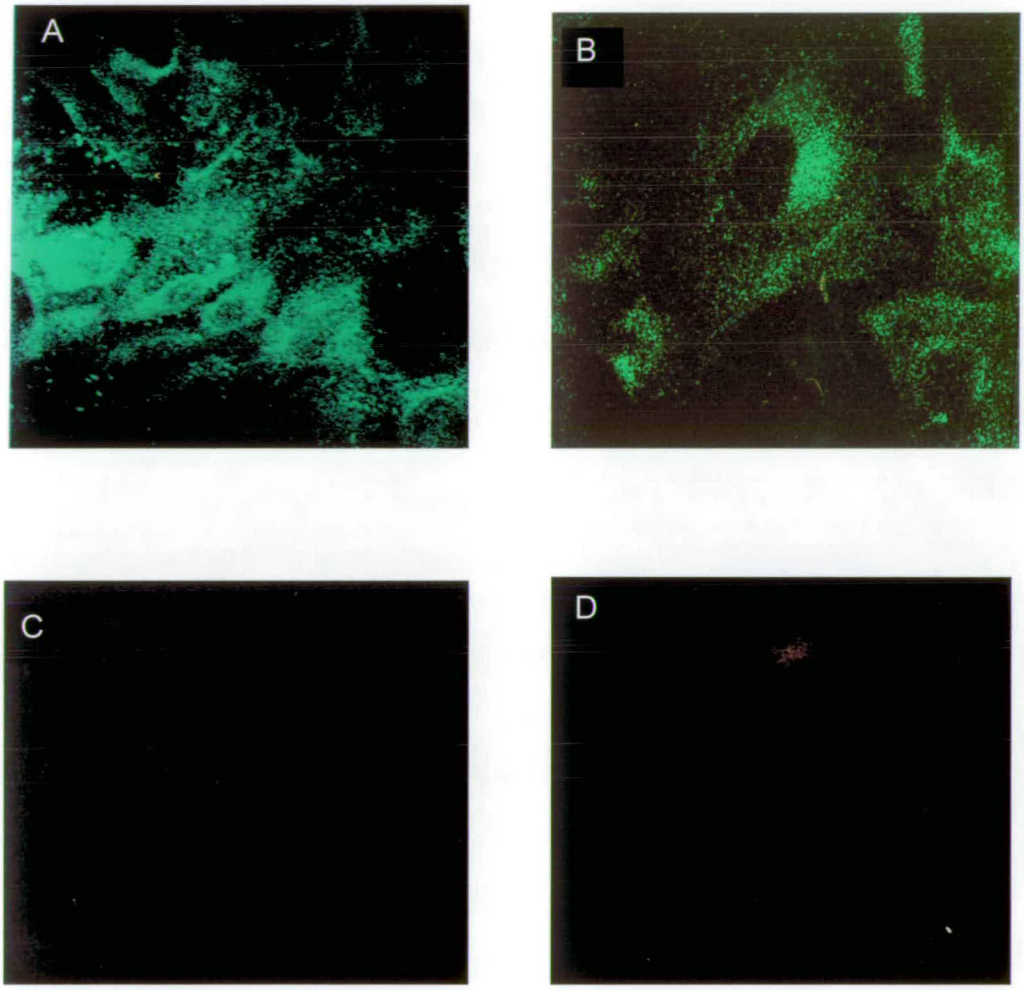

Figure 3.8 : Classification of cell types present in Cheviot brain sA80BR cell line.

Immunohistochemical staining of cell line sA80BR using fluorescein isothiocyanate (FITC) labelled secondary antibodies. (A) Positive staining of anti- N200 captured at x20 magnification, (B) Positive labelling of anti-GFAP captured at x40 magnification, (C) Negative labelling for anti-fibronectin, (D) Negative control using goat anti-mouse FITC.

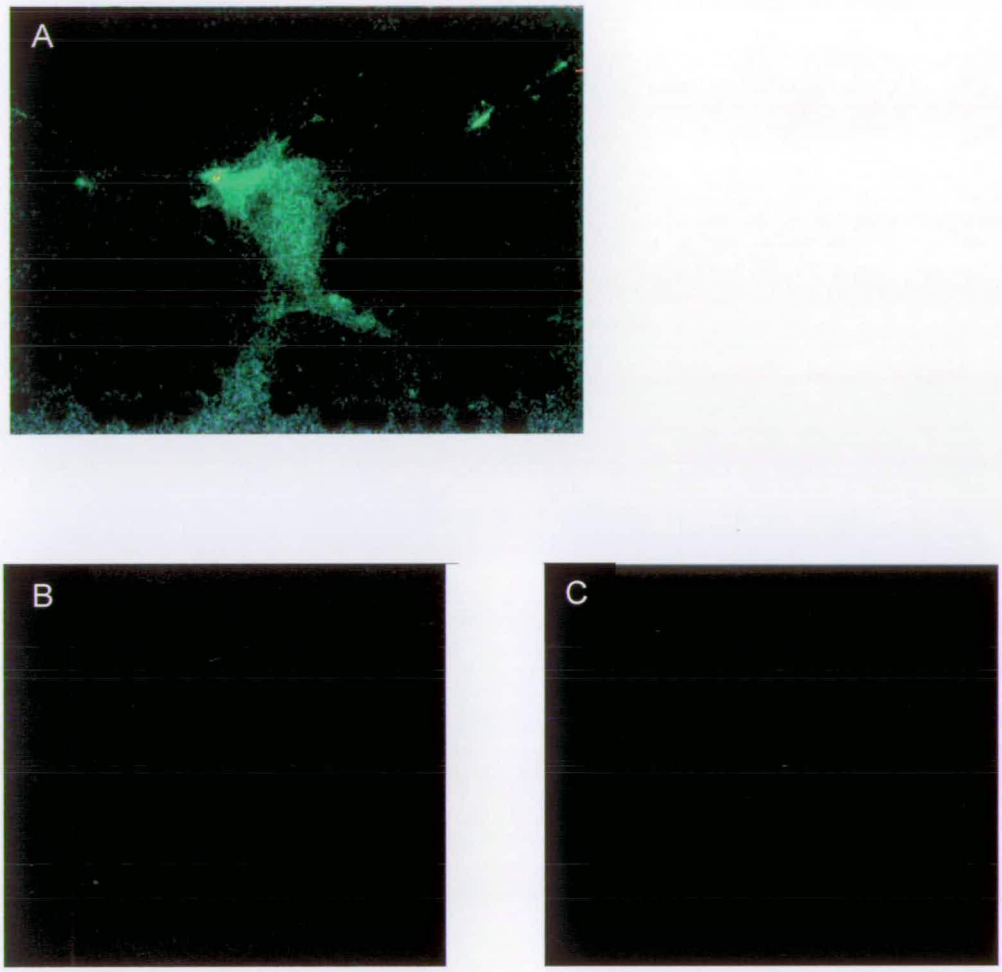

Figure 3.9: Classification of cell types present in Icelandic breed cell line, IS120.Cer

Immunohistochemical staining of primary Cerebral cell line using fluorescein isothiocyanate (FITC) labelled secondary antibodies. (A) Positive staining of anti-N200 captured at x40 magnification, (B) Negative staining of anti-GFAP, (C) Negative control using goat anti-mouse FITC.

The Cheviot ovine brain cell line sA80BR stained positive for anti-N200 and anti-GFAP (Figure 3.8, Table 3.1), suggesting the presence of neuronal and glial derived cells. Staining for anti-N200 was strong, abundant and easily observed at low magnification (x20 magnification). In contrast, staining for anti- GFAP was weaker, less abundant and only visible at a higher magnification (x40). The ovine brain cell line sA80BR did not stain with anti-fibronectin suggesting the absence of fibroblasts within the cell culture. The Cheviot brain cell lines pA80BR also stained positive for anti-N200 and anti-GFAP and negative with anti-fibronectin. The same pattern of staining was observed in pA80BR as the sA80BR cell line, again suggesting a heterogeneous culture of neuronal and glial derived cells (results not shown).

The primary brain cell line IS120.Cer prepared from *Ovis brachyura borealis pall* stained positive for anti-N200 (Figure 3.9), indicating the presence of cells derived from neuronal tissue within the cell culture. Staining for anti-N200 was strong and abundant, defining the nucleus of the cells well. The cells did not stain with anti-GFAP or anti-fibronectin suggesting the absence of astrocytes and fibroblasts within the culture.

All the brain-derived cell lines tested by FITC were positive for the presence of neuronal cells.

3.5 Transient Transfection of Ovine Cell Lines.

An ideal method for transfection should have the following properties: efficient in reporter gene transfer; low toxicity for the cells; and reproducibility. Many different methods exist, each has its advantages and limitations, and a method that works for one cell type may not work for another. Furthermore, different lines of culture cells can vary greatly in their ability to assimilate and express exogenously added DNA. Therefore choosing the method for transfection for a new cell line can be trial and error to begin with.

In particular, it is believed that because of their highly differentiated state, neuronal cell lines are relatively resistant to conventional transfection methods. For this reason it was decided to concentrate to begin with on finding an efficient method for transfection for sheep brain cells.

3.5.1 Experimental approach.

The procedures used for handling cell lines are described fully in Chapter Two. Briefly, all DNA was prepared to ensure it was endotoxin free and cell culture work was carried out in an area which could be UV treated to eradicate microbial contaminants. The methods tested to establish transient expression in sheep brain cells (Section 2.5) included calcium phosphate precipitation, electroporation, lipofection and dendrimer technology. The control vector pCAT3-Promoter (Promega, UK, Appendix A) which expresses chloramphenicol acetyl transferase (CAT), was used to optimise transfection methods by analysing CAT activity in cell lysates. The vector pSV-β-Galactosidase, which expresses β-Galactosidase, was used to control for transfection efficiency or cell lysate recovery from transfection experiments.

3.5.2 Results.

3.5.2.1 Calcium phosphate precipitation.

The method of calcium phosphate precipitation for transfection was one of the first to be described (Graham and van der Eb, 1973). Transfection mediated by calcium phosphate involves mixing DNA directly with a calcium chloride solution and phosphate buffer to form a fine precipitate that covers the cultured cells. The mechanism is not fully understood but it is thought that precipitated DNA enters the cytoplasm of the cell by endocytosis and is transferred to the nucleus. The factors that influence efficiency of calcium phosphate transfection are primarily the amount of DNA in the precipitate and the length of time the precipitate is left on the cells. A calcium phosphate optimisation scheme is shown in Table 3.2, full details of the protocol followed are described in Chapter Two (Section 2.5.3) and full data in Appendix B 1.0, Table B 1.1.

Calcium phosphate is widely used for transient transfection and has been used in this laboratory for recent reporter gene studies on other cell lines (Baybutt and Manson 1997; Cheung, 1996). The cells, sA80BR, were harvested 48 hours post-transfection with the vector pCAT3-Promoter and a CAT assay was performed, the results of which were analysed by thin layer chromatography (TLC). A typical autoradiograph developed from TLC following calcium phosphate transfection is shown in Figure 3.10. Using the developed autoradiogragh, it was possible to isolate the acetylated products from the silica plate and the level of CAT activity determined by measuring [^{14}C] by liquid scintillation counting (LSC, Section 2.5.10). Relative CAT activity was determined (Table 3.2.) by comparing the CAT activity of the reporter construct to a positive CAT control reaction (100%). The positive CAT control was 1 unit of purified CAT enzyme (Promega, UK). The calcium phosphate transfection method was repeated three times (twice in duplicate) and found ineffective for transient transfection of the pCAT3-Promoter plasmid to ovine brain cell cultures. Little cell death was observed irrespective of amount of DNA or time of exposure to precipitate.

Table 3.2: Optimisation and results of transfection of sA80BR with pCAT3-Promoter (Promega) following calcium phosphate precipitation (refer also to Figure 3.10).

DNA (μg)	Exposure to precipitate (hr)	Positive CAT (cpm)	Cell CAT (cpm)	Relative CAT activity (%)
10	6	n/d	0.0	n/d
10[a]	16	36130.82	0.0	0.00
5[a]	16	36130.82	10.93	0.03
5[b]	16	22744.3	345.72	1.5
5[b]	16	22744.3	294.61	1.2

Note. Positive CAT was the measured acetlyated product (100%) when 1 unit of purified chloramphenicol transferase was incubated with acetyl Coenzyme A (5mg/ml) and [^{14}C]chloramphenicol (0.05mCi/ml). Cell CAT activity was the measured acetylated product isolated by TLC from a CAT assay on the sA80BR cell lysate after calcium phosphate precipitation of pCAT3-Promoter (Promega, UK). [a] signifies that the experiments were completed at the same time and [b] denotes a duplicated transfection.

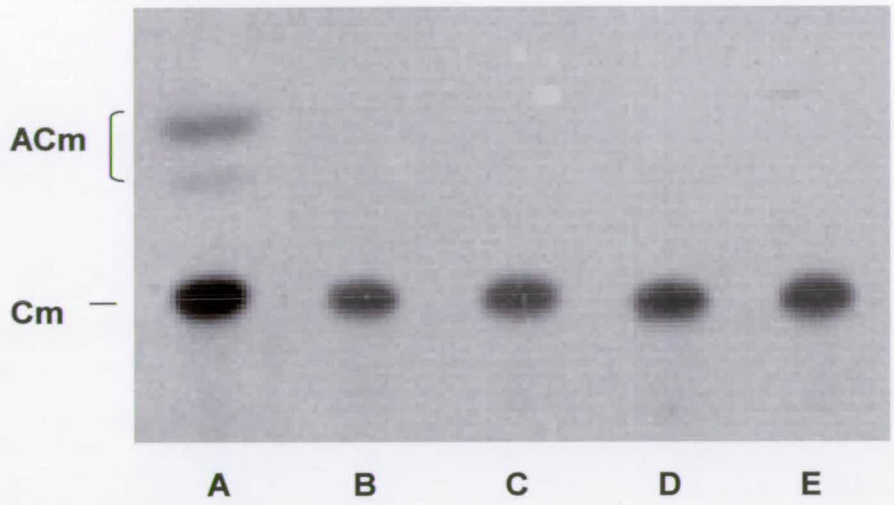

Figure 3.10 :Transfection of ovine brain cell line sA80BR using the calcium phosphate method. (A) Positive control, 1 unit of purified CAT enzyme; (B,C) repeat assays from non-transfected cells; (D,E) cell lysate assays from cells transfected with 5μg pCAT3-Promoter (Promega). ACm: acetylated chloramphenicol, Cm: chloramphenicol.

3.5.2.2 Electroporation of brain-derived cell lines.

The physical method of electroporation is based upon perturbation of the cell membrane by an electrical pulse, forming pores that allow the uptake of nucleic acids into the cell (Shigekawa, *et al.*, 1989). Consequently, the concentration of DNA is less likely to be a factor determining transfection than are the duration and strength of pulse for each cell type. A balance must be found between conditions that kill cells and conditions that allow efficient transfer of DNA.

The vector pCAT5 (Appendix A) was used for testing this method of transfection as it was shown to have higher expression *in vitro* than pCAT3-Promoter and may have enhanced the likelihood of detectable CAT expression (Baybutt, personal communication and Table 3.8). The method used for electroporation is fully described in Chapter Two, Section 2.5.4, and full data presented in Appendix B. Cells were harvested 48 hours post-transfection and a CAT assay performed, the results of which were analysed by TLC. Relative CAT activity was determined by TLC and autoradiography (Figure 3.11, Table 3.3.). The amount of acetylated substrate produced from the transfected cell lysates was compared to a positive CAT control reaction (100%). Full data can be found in Appendix B 1.0, Table B 1.2-1.3.

Significant cell death was suffered under most conditions, particularly when the parameters 5μg DNA, 800V and 300μF were used. Cell death under other conditions was approximately 40-60%. Electroporation was not effective in transient transfection of the pCAT5 plasmid (Table 3.3). Altering the conditions for electroporation did not affect the efficiency of transfection.

Table 3.3: Result of transfection of pCAT5 into sA80BR cells following electroporation under different conditions. Determined by TLC and autoradiography (Figure 3.11)

Cell passage Number	DNA (μg)	Voltage (V)	Capacitance (μF)	Cell Death (%)	Positive CAT (cpm)	Cell CAT (cpm)	Relative CAT activity (%)
5	5	800	300	100	100	0	0.0
	25	800	300	80-50		0	0.0
	50	800	300	50-40		0	0.0
6	20	200	960	70	100	0	0.0
	20	250	960	60		0	0.0
	20	300	960	60		0	0.0

Note. Positive CAT was the measured acetlyated product (100%) when 1 unit of purified chloramphenicol transferase was incubated with acetyl Coenzyme A (5mg/ml) and [^{14}C]chloramphenicol (0.05mCi/ml). Cell CAT activity was the measured acetylated product anylised by TLC and LSC following CAT assay on the sA80BR cell lysate after electroporation of pCAT5.

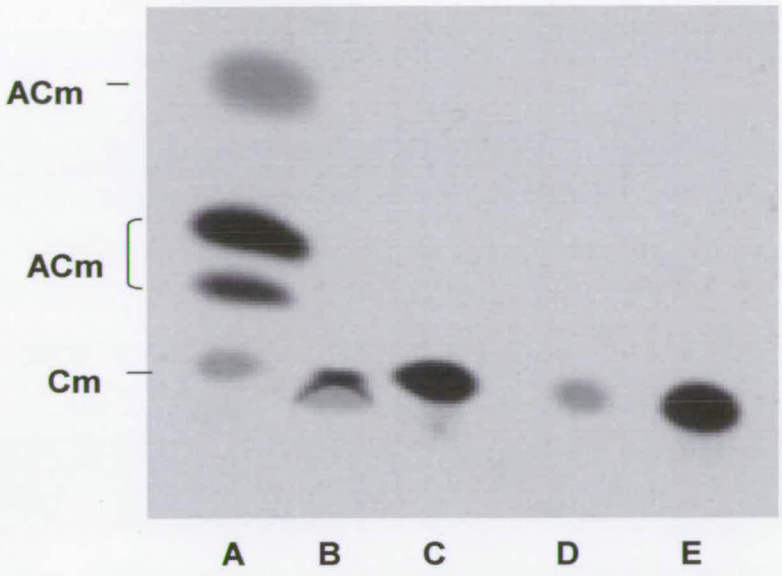

Figure 3.11:Electroporation was ineffective in transfection of ovine brain cell line sA80BR with the reporter vector pCAT5. (A) Positive CAT reaction, 1unit of purified CAT enzyme (Promega) (B) Negative CAT reaction (C) Electroporation of 20μg pCAT5, 200V, 960μF (D) Electroporation of 20μg pCAT5, 250V, 960μF (E) Electroporation of 20μg pCAT5, 300V, 960μF . ACm: acetylated chloramphenicol, Cm: chloramphenicol.

3.5.2.3 Liposome-mediated transfection.

The use of synthetic cationic lipids for transfection offers advantages of high efficiency gene transfer usually to cells which are resistant to calcium phosphate transfection (Fraley *et al.*, 1980; Felgner *et al.*, 1987). Lipofection involves encapsulating DNA or RNA with liposomes. The cationic head of the lipid associates with the negatively charge phosphates on the nucleic acid. The nucleic acid/lipid complex then fuses, or associates, with the overall negatively charged cell membrane and results in the internalisation of the nucleic acid into the cell. Following endocytosis the complex appears in endosomes then later in the nucleus.

Commercial cationic lipids tested on the stable ovine brain cell lines were Tfx^{TM}-50 and Tfx^{TM}-20 reagent (Promega, UK), Dosper (Roche Diagnostics, UK) and Effectene (Qiagen Ltd, UK). These reagents are preparations of polycationic lipids, each unique in the cationic lipid supplied. Tfx^{TM}-20 and Tfx^{TM}-50 contain the same cationic lipid but different molar concentration of a fusogenic lipid. It is vital to develop optimal transfection conditions as factors such as cell line, clonal variety within the cell line, culture conditions and the DNA being transfected can influence transfection efficiency. The following parameters are also known to alter transfection efficiency: the charge ratio of lipid reagent to DNA, transfection time, and the absence or presence of serum. The charge ratio of lipid to DNA is vital for transfection as a net neutral or positive charge is required to ensure successful micelle formation. In general increasing the amount of available lipids improves transfection efficiency but at high levels lipids can be toxic to cells, also the length of time cells are exposed to a liposome reagent may be toxic. The high concentration of proteins present in serum may prevent formation of micelles essential for DNA transfection. However, the absence of serum from cells for a considerable time is stressful to the cells and may result in poor DNA uptake or cell death. A full description of each of the methods followed for liposome-mediated transfection can be found in Chapter Two (Section 2.5.5) and data listed in Appendix B.

A) Liposome transfection: Dosper (Roche Diagnostics)

Liposome transfection using the Dosper reagent (1μg/μl, Roche Diagnostics) was carried out as recommended by the manufacturer's protocol (Section 2.5.5.1). For optimisation of transfection (Table 3.4) of the pCAT3-Promoter plasmid (Promega, Appendix A) various ratios of Dosper (μg/ml) and DNA (μg/ml) were tested to give different charge ratios of Dosper reagent to DNA. Full data can be found in Appendix B. 1.0, Tables B 1.4-1.5. The Dosper reagent did not appear to be toxic to the cells however transfection of the ovine cell line, sA80BR, was attempted for a total of three times, each being negative (Table 3.4, Figure 3.12). As this was a new method to the laboratory, it was decided to test the procedure in mouse N2a cells, a cell line which has been transfected before with pCAT3-Promoter by calcium phosphate. This was carried out with the assistance of Dr Gerard O'Neill, but the results were also negative (Table 3.5).

The cells were harvested 48 hours post-transfection and a CAT assay performed, the results being analysed by LSC and confirmed as before by TLC (Figure 3.12). The relative CAT activity from the transfected cells was calculated as a percentage of CAT activity (100%) determined from the positive CAT control.

Transfection reagent, Dosper was not effective in transfecting the ovine brain derived cell line sA80BR.

Table 3.4: Results from two separate transfections of sA80BR with 3μg pCAT3-Promoter (Promega) following lipofection using Dosper reagent (Figure 3.12).

Passage number	Dosper Reagent (μl)	Charge ratio (Dosper:DNA)	Positive CAT(cpm)	Cell CAT (cpm)	Relative CAT activity (%)
11	0	0	3763.11	69.55	1.85
	2	1:1		0.0	0.0
	4	2.5:1		0.0	0.0
	6	4:1		72.54	1.93
13	0	0	92427.94	249.37	0.27
	2	1:1		94.74	0.1
	4	2.5:1		17.89	0.02
	6	4:1		10728	1.17

Table 3.5: Result of transfection of murine N2a cell line (passage number 189) with pCAT3-Promoter (Promega) following lipofection using Dosper reagent.

DNA (μg)	Dosper Reagent (μl)	Charge ratio (Dopser:DNA)	Positive CAT (cpm)	Cell CAT (cpm)	Relative CAT activity (%)
1	2	4:1	108213.89	2444.38	2.24
1.5	2	2.5:1		6.07	0.01
2	2	2:1		0.0	0.0
3	2	1.3:1		365.64	0.33
3	0	0		0.0	0.0
0	2	0		16.99	0.01

Note. Positive CAT was the measured acetlyated product (100%) when 1 unit of purified chloramphenicol transferase was incubated with acetyl Coenzyme A (5mg/ml) and [^{14}C]chloramphenicol (0.05mCi/ml). Cell CAT activity shown in table 3.4 refers to measured acetylated product anaylsed by TLC and LSC on the sA80BR cell lysate after lipofection of pCAT3-Promtoer with Dosper reagent (Roche Diagnostics, UK). Table 3.5 shows Cell CAT activity measured from N2a cell lysates after Dosper transfection of pCAT3-Protmoer (Promega, UK).

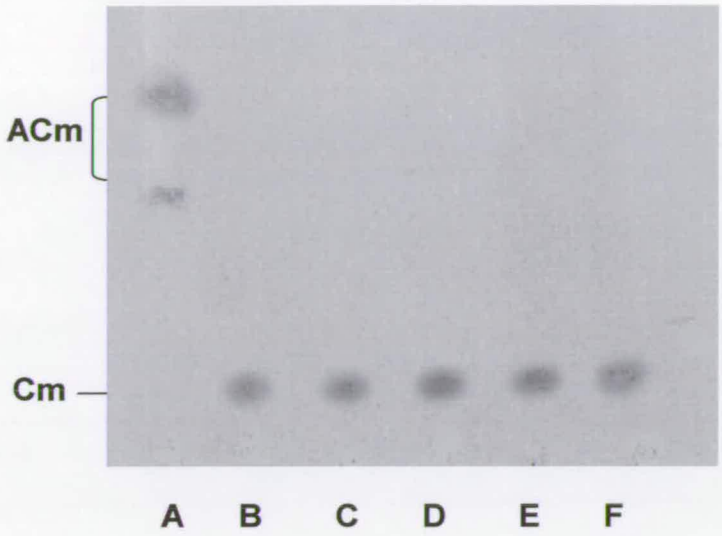

Figure 3.12: Transfection of the ovine brain cell line sA80BR with pCAT3-promoter plasmid by the liposome reagent Dosper. Altering the ratio of DNA to lipsome reagent did not increase transfection efficiency. (A) Positive CAT reaction, 1unit for purified CAT enzyme (Promega) (B) Negative CAT reaction (C) Liposome transfection with 0μl of Dosper reagent (D) Liposome transfection with 2μl of Dosper reagent (E) Liposome transfection with 4μl of Dosper reagent (F) Liposome transfection with 6μl of Dosper reagent. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

B) Liposome transfection: Tfx50^{Tm} (Promega)

Liposome transfection of sA80BR with the plasmid pCAT3-Promoter (Promega) using the Tfx50^{Tm} reagent (Promega) was examined according to the manufacturer's protocol. The Tfx50^{Tm} reagent was resuspended in 400µl nuclease-free water to give a final concentration of 1.75µg/µl. Various concentrations of DNA and transfection reagent were tested to alter the charge ratio between the DNA and lipid components of the transfection reaction (Table 3.6 and 3.7). Several parameters known to effect the efficiency of Tfx 50^{Tm} reagent transfection were tested: y the ratio of DNA to Tfx50^{Tm} reagent and presence or absence of serum (Table 3.7). As described in Chapter Two (Section 2.5) cells were harvested 48 hours post-transfection and a CAT assay performed, and results analysed by TLC (Figure 3.13) and LSC. The relative CAT activity (Table 3.7) from Tfx50^{Tm} transfected cells was calculated as a percentage of CAT activity determined from the positive CAT control (100%).

The conditions for transfection with Tfx50^{Tm} were favoured in the absence of serum (Figure 3.13b, lanes 3-5), with a charge ratio of 2:1. CAT activity was low in these studies probably because of the amount of DNA used (see Figures 3.13 and 3.14) Optimum transfection of pCAT3-Promoter (Promega) into sA80BR gave 33% conversion of chloramphenicol to acetyl chloramphenicol when compared to the positive control with a charge ratio of 2:1 and increasing concentration of DNA (Figure 3.14, lanes D-F, Table 3.7, rows 10-12). Cell death was observed under conditions where the liposome reagent Tfx50^{TM} was in contact with the cells from more than two hours, when high concentrations of DNA were used and when high DNA: Tfx50^{Tm} ratios were tested. Liposome transfection using Tfx50^{TM} was tested on sA80BR under different experimental conditions, full data can be found in Appendix B. 1.0, Tables B 1.6-1.8.

Liposome transfection using Tfx50^{Tm} was further tested on mouse N2a cells as a control for transfection efficiency (Table 3.8). Tfx50^{Tm} was a powerful transfection reagent on N2a cells when used at the same conditions as those optimal for the sheep cell line sA80BR (Figure 3.15).

Table 3.6: Charge ratio of Tfx^{Tm} :DNA with reference to the volume of Tfx^{Tm} in each reaction.

Charge ratio (Tfx^{TM}:DNA)	Tfx^{TM} (ml) reagent per μg DNA
2:1	3.0
3:1	4.5
4:1	6.0

Table 3.7: Result of three separate transfections of sA80BR with pCAT3-Promoter (Promega) following lipofection using Tfx50^{Tm} according to various conditions.

Cell passage Number	**DNA (μg)**	**Charge ratio (Tfx50^{TM}:DNA)**	**Positive CAT (cpm)**	**Cell CAT (cpm)**	**Relative CAT activity (%)**
16	3	2:1	108638.48	900.67	0.83
	3	3:1		1779.91	1.64
	3	4:1		110.81	0.1
17	3	2:1	107282.88	283.1	0.26
	3	3:1		113.35	0.11
	3	4:1		531.85	0.5
	3	2:1*	107282.88	7553.87	7.04
	3	3:1*		4851.03	4.52
	3	4:1*		1556.45	1.45
18	3	2:1*	94205.49	2108.88	1.71
	6	2:1*		5584.09	5.4
	10	2:1*		15947.73	33.33
	3	3:1*		1586.23	1.15
	6	3:1*		2232.76	1.84
	10	3:1*		1122.0	1.85

*absence of serum

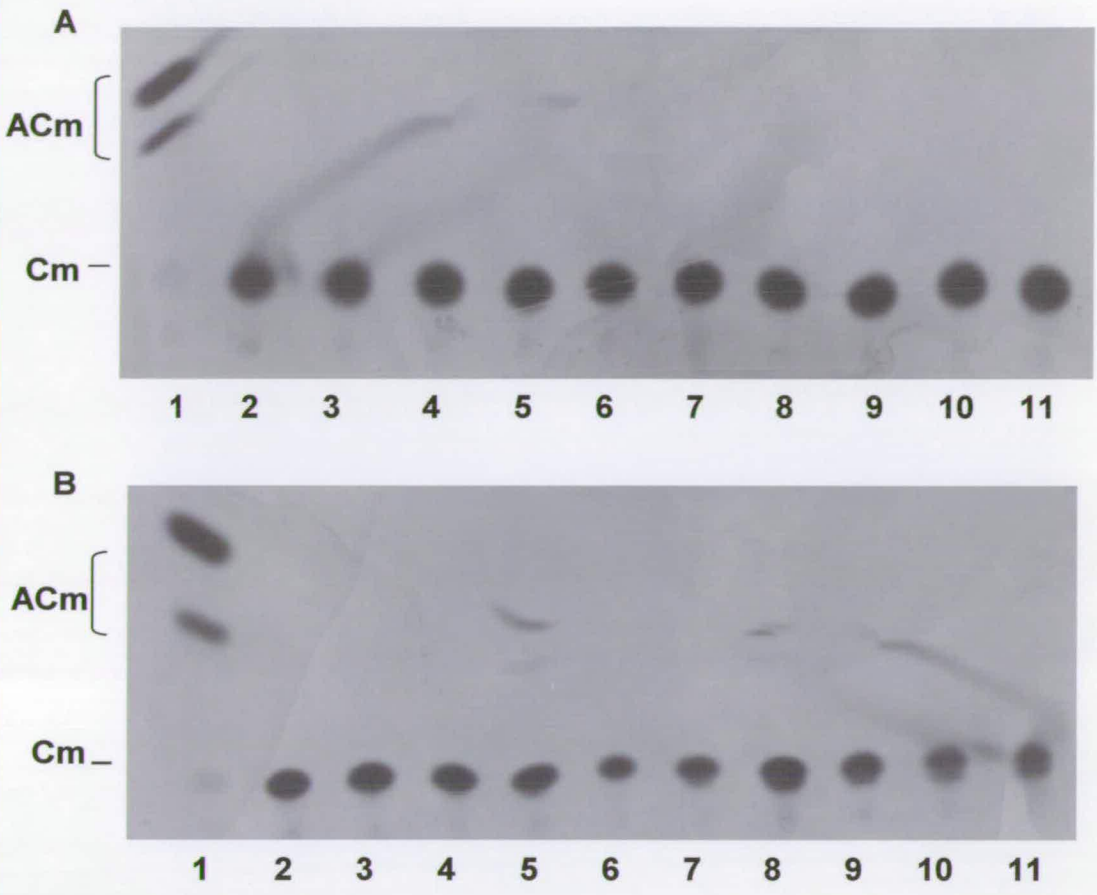

Figure 3.13:Transfection of sA80BR with pCAT3-Promoter using Tfx50^{Tm} reagent. (A) in the presence of serum (B) in the absence of serum. On both plates lanes; 1, 1 unit purified CAT: 2, negative control. To optimise for the ratio of DNA to Tfx50^{Tm} reagent various conditions were used: lanes 3-5, Tfx50^{Tm}: DNA ratio of 2:1: lanes 6-8, Tfx50^{Tm}: DNA ratio of 3:1; lanes 9-11, Tfx50^{Tm}: DNA ratio of 4:1. To optimise for the amount of DNA available for the transfection reaction various concentrations were tested: 3,6,9, 0.5μg pCAT3-Promoter: 4, 7,10, 1μg pCAT3-Promoter: 5,8,11 3μg pCAT3-Promoter. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

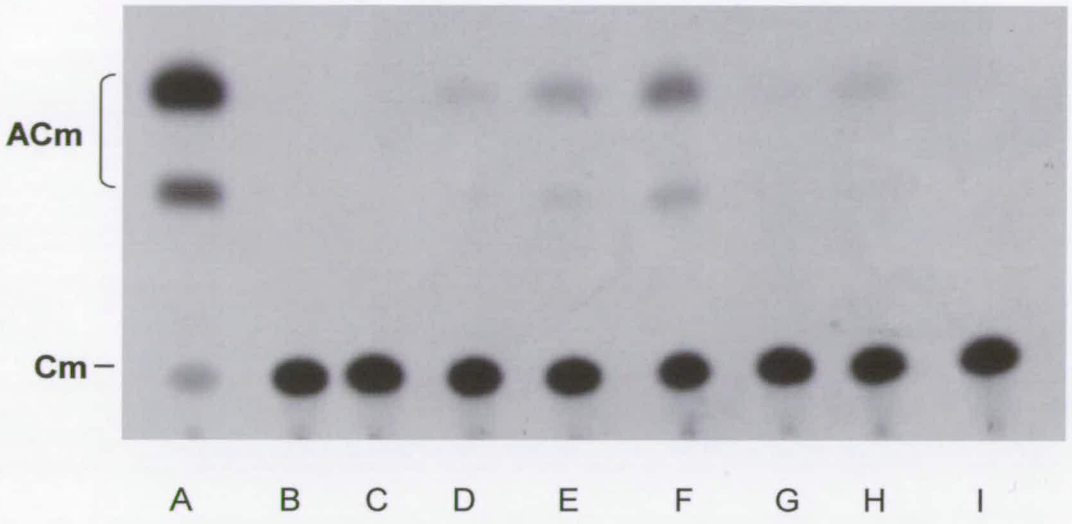

Figure 3.14: Transfection of sA80BR cells with pCAT3-Promoter and increasing amounts of DNA at ratios of 2:1 (D-F) and 3:1 (G-I) with Tfx50^{Tm} reagent. Lane A, positive CAT control,1 unit purified CAT (Promega): B, negative CAT control: C; non transfected cells: D and G; 3μg pCAT3-Promoter: E and H; 6μg pCAT3-Promoter: F and I; 10μg pCAT3-Promoter. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

Table 3.8: Result of transfection of mouse N2a cell line following lipofection using Tfx50^{Tm} according to the various conditions used.

Vector	DNA (μg)	Charge ratio (Tfx50^{Tm}:DNA)	Positive CAT (cpm)	Cell CAT (cpm)	Relative CAT activity (%)
pCAT3-Promoter	1.0	2:1	108017.1	36242.53	33.55
	3.0	2:1		67536.27	62.52
pCAT5	1.0	2:1		78157.07	72.36
	3.0	2:1		92845.96	85.50

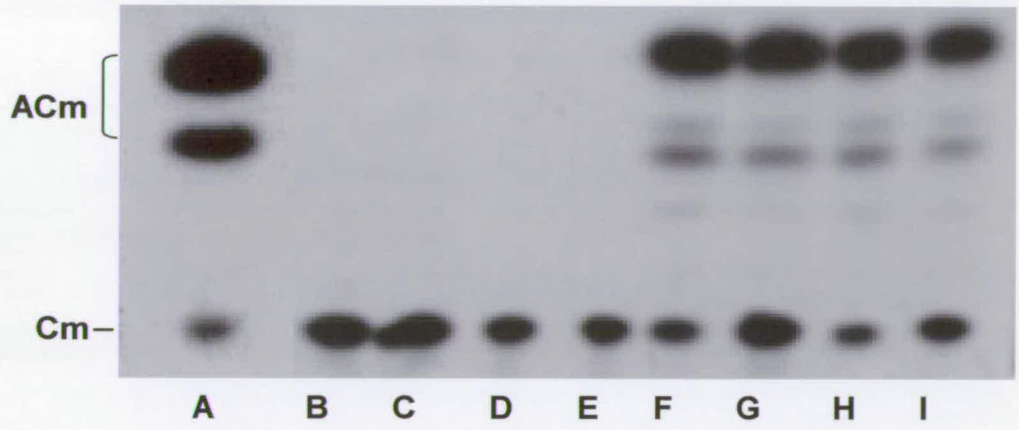

Figure 3.15:Transfection of N2a cells with pCAT3-Promoter reporter gene using Tfx50^{Tm} reagent. Ratio of DNA to Tfx50^{Tm} used was 3:1 Lane A, positive CAT control, 1unit purified CAT (Promega): B and C; non transfected cells: D and E; 3μg pCATBasic: F and G; 3μg pCAT3-Promoter: H and I; 3μg pCAT5. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

C) Liposome transfection: Tfx20^{Tm} (Promega)

Liposome transfection was examined according to the manufacturer's protocol. The reagent Tfx20^{Tm} was dissolved in 400µl nuclease-free water to a final concentration of 4µg/µl. As with Tfx50^{Tm} several parameters known to effect the efficiency of Tfx 20^{Tm} reagent transfection were tested: primarily the ratio of DNA to Tfx50^{Tm} reagent and presence or absence of serum. Various DNA and liposome reagent concentrations (Table 3.6) were tested to alter the net charge ratio. Cells were grown in 24-well plates for transfection analysis and harvested 48 hours post-transfection. A CAT assay was performed, the result of which was analysed by TLC and the amount of acetylated chloramphenicol determined using densitrometry (Kodak Image Station, Section 2.5.12) as opposed to LSC. The advantage to using this method of analysis is that it allows the CAT assays to be read directly and avoids handling of radioactive products. The relative CAT activity (Table 3.9) from Tfx20^{Tm} transfected cells was calculated as a percentage of CAT activity determined from the positive CAT control in the normal manner.

Cell death was observed using Tfx20^{Tm} when the reagent was exposed to the cells for more than 4 hours. Optimum transfection (Table 3.9) of 1µg pCAT3-Promoter (Promega) into sheep sA80BR cell line gave 49% conversion of chloramphenicol to acetyl chloramphenicol when compared to the positive control using Tfx20^{Tm} (Promega) reagent at a ratio of 3:1 to the pCAT3-Promoter DNA in the absence of serum (Figure 3.16). Transfection of pCAT3-Promoter was attempted several times, full data can be found in Appendix B. 1.0, Tables B 1.9. However, this method proved to be unreliable as it was not possible to repeat the transfection to the same degree of success as shown in Table 3.9.

Table 3.9: Result of one transfection, carried out in triplicate, of sA80BR with pCAT3-Promoter (Promega) following lipofection using Tfx20^{Tm} according to various conditions, in the absence of serum.

Passage Number	DNA (μg)	Charge ratio (Tfx20^{TM}:DNA)	Positive CAT (net intensity, 10^5)	Cell CAT (net intensity, 10^5)	Relative CAT activity (%)
5	0.25	2:1	5.22	0.47	9.02
	0.5	2:1		0.36	6.91
	0.75	2:1		1.07	20.6
	1.0	2:1		1.76	33.81
5	0.25	3:1		1.31	25.08
	0.5	3:1		1.41	27.02
	0.75	3:1		1.46	28.06
	1.0	3:1		2.56	49.01
5	0.25	4:1		0.67	12.82
	0.5	4:1		1.25	23.91
	0.75	4:1		0.02	0.39
	1.0	4:1		1.21	23.18

Note. Acetylated products from CAT assays were isolated by TLC and measured by densitometry (refer to Section 2.5). The density of staining was measured with regards to the background and the net intensity eg 5222458.16 which was abbreviated to $5.22x10^5$. Positive CAT was the measured acetylated product (100%) when 1 unit of purified chloramphenicol transferase was incubated with acetyl Coenzyme A (5mg/ml) and [^{14}C] chloramphenicol (0.05mCi/ml). Cell CAT activity was the measured acetylated product isolated following a CAT assay and TLC on the sA80BR cell lysate after lipofection of pCAT3-Promoter with Tfx20^{Tm} (Promega).

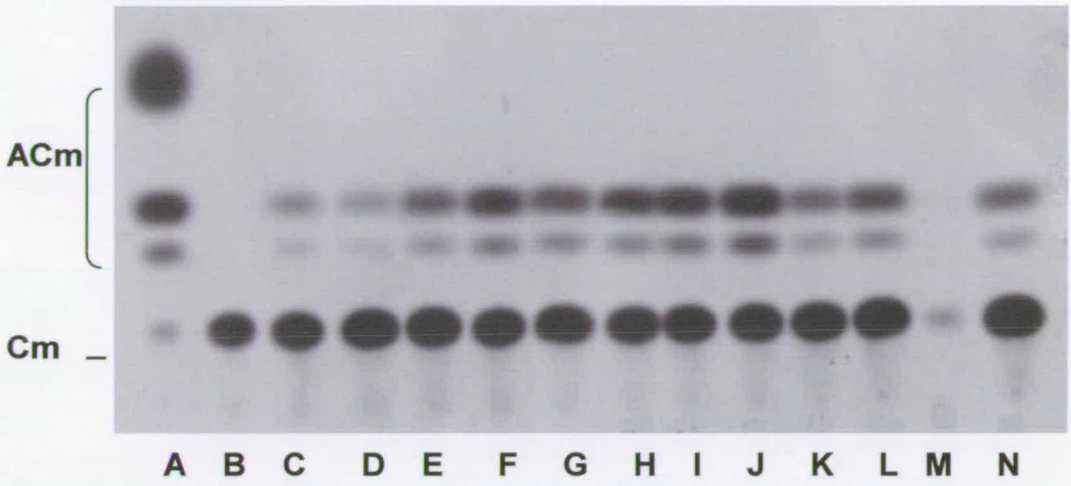

Figure 3.16:Transfection of sA80BR cell line with pCAT3-promoter using Tfx20^{Tm} reagent. Lane A; positive control, 1unit purified CAT; lane B; negative control; lanes C-F; Tfx20^{Tm} :DNA ratio of 2:1; lanes G-J: Tfx20^{Tm} :DNA ratio of 3:1; lanes K-N: Tfx20^{Tm}:DNA ratio of 4:1. The amount of DNA in each transfection reaction was also controlled as; lanes C, G, K: 0.25μg; lanes D, H, L: 0.5μg; lanes E, I, M; 0.75μg; lanes F, J, N; 1.0μg. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

D) Liposome transfection: Effectene reagent.

The Effectene reagent is a lipid based reagent which has been proven to efficiently transfect a wide variety of cell types, including primary cell lines. In contrast to other liposome reagents, the first step in Effectene transfection condenses the DNA into a compact structure that allows the Effectene reagent to form regular, uniform micelle structures around the condensed DNA. These micelles interact with the cell membrane and the DNA is transported into the cells as previously described. Effectene has also been designed for use in the presence of serum, which reduces the stress on the cells. Due to time constraints Effectene was the only transfection reagent to be tested on the primary cell lines derived from the Icelandic sheep breed.

Transfection using the Effectene reagent was carried out according to the manufacturer's protocol for primary cells (Chapter Two, Section 2.5.7). As with other liposome based reagents it was necessary to optimise the charge ratio between the lipid component and DNA for each cell line (Table 2.2, Section 2.5.7). Cells were harvested 48 hours post-transfection and a CAT assay performed, the result of which was analysed by TLC and the amount of acetylated chloramphenicol determined using densitometry (Kodak Image Station, 2.5.12). In contrast to previously tested liposome-based reagents, no significant cell death was observed as the cells were exposed to Effectene during the transfection reaction. Furthermore, Effectene transfection gave consistent levels of detectable CAT activity and considerably less DNA was used for each transfection. For full details on Effectene transfection levels refer to Appendix B 1.0, Tables 1.11-1.14.

Transfection of 1μg pCAT3-promoter into primary liver cell line, IS120.Liv, was optimal when an Effectene:DNA ratio of 25:1 was used (Figure 3.17, Table 3.10). Under these conditions the relative CAT activity was 103 and 123% when compared to the positive control (Table 3.10). Effectene reagent was extremely effective at transiently transfecting ovine primary liver cells.

Table 3.10: Result of one transfection, carried out in triplicate, of IS120.Liv, grown in 6-well plates, with pCAT3-Promoter (Promega) following lipofection using Effectene according to various conditions.

Passage Number	DNA (μg)	Charge ratio (Effectene:DNA)	Positive CAT (net intensity, 10^5)	Cell CAT (net intensity, 10^5)	Relative CAT activity (%)
6	0.2	10:1	4.01	0.0	0.0
	0.4	10:1		2.25	56.17
	1.0	10:1		1.09	27.29
6	0.2	25:1	4.01	1.27	31.64
	0.4	25:1		4.3	107.33
	1.0	25:1		4.16	103.89
6	0.2	50:1	4.01	0.39	9.75
	0.4	50:1		2.04	51.12
	1.0	50:1		1.36	34.13

Note. Acetylated products from CAT assays were isolated by TLC and measured by densitometry (refer to Section 2.5). The density of staining was measured with regards to the background and the net intensity eg 4007509 abbreviated to 4.01×10^5. Positive CAT was the measured acetylated product (100%) when 1 unit of purified chloramphenicol transferase was incubated with acetyl Coenzyme A (5mg/ml) and [^{14}C] chloramphenicol (0.05mCi/ml). Cell CAT activity was the measured acetylated product isolated following a CAT assay and TLC on the sA80BR cell lysate after lipofection of pCAT3-Promoter with Effectene (Qiagen).

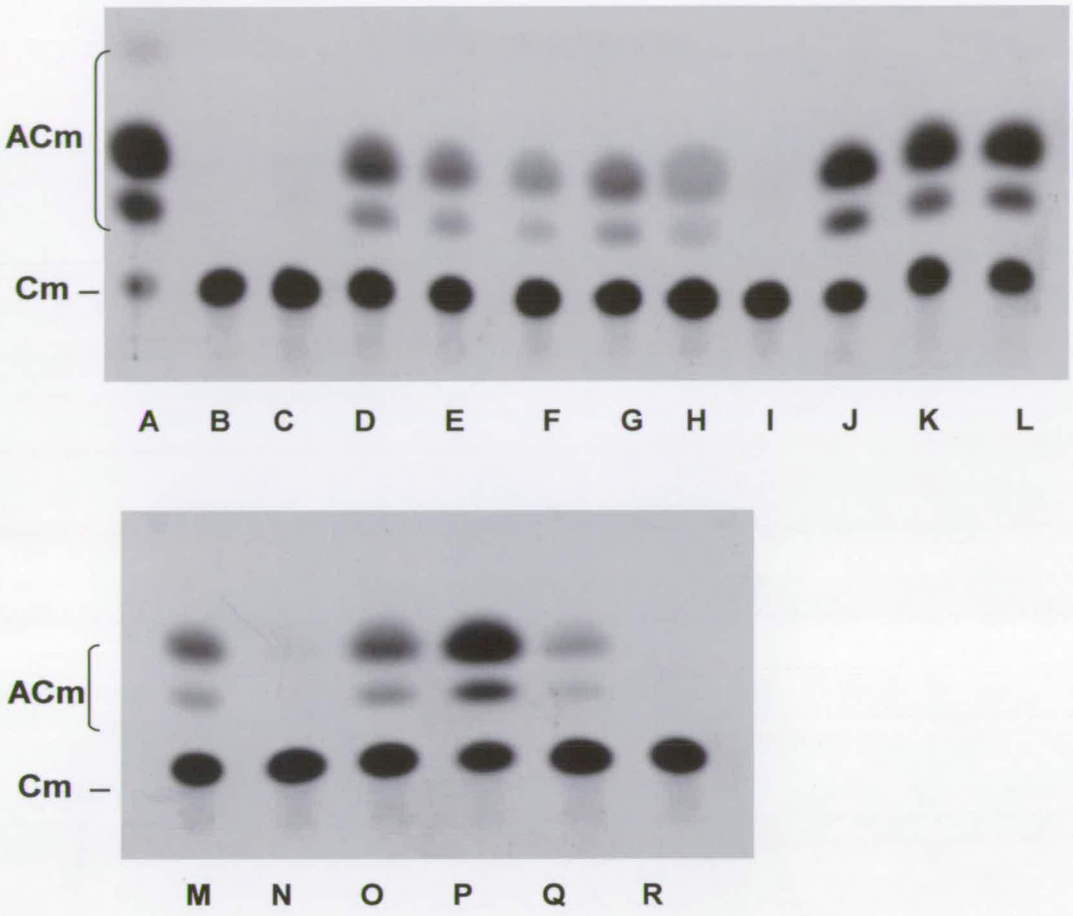

Figure 3.17: Transfection of IS120.Liv cell cultures with pCAT3-promtoer by Effectene reagent. Varying ratios of Effectene to DNA were tested. Lane A; positive control, 1 unit purified CAT; lane B; negative control; lanes C-F: Effectene: DNA ratio of 10:1; lanes G-L: Effectene : DNA ratio of 25:1; lanes M-R: Effectene:DNA ratio of 50:1. Amount of DNA tested was; 0.2μg (C, G, H, M, N); 0.4μg (D, I, J, O,P); 1.0μg (E, F, K, L, Q, R) ACm: acetylated chloramphenicol, Cm: chloramphenicol.

The efficiency of Effectene in the transient transfection of primary ovine cerebral cells, IS120.Cer, was also observed (Figure 3.18). Conditions for optimal transfection of 0.5μg pCAT3-Promoter into the cerebral cell line grown in 24-well plate were found to be an Effectene:DNA ratio of 10:1 (Table 3.11). Due to time limitations it was only possible to perform this experiment once, in duplicate.

Table 3-11: Result of three separate transfections, carried out in duplicate, of IS120.Cer cell line, grown in 24-well plates, with pCAT3-Promoter (Promega) following lipofection using Effectene according to various conditions.

Cell passage Number	DNA (μg)	Charge ratio (Effectene: DNA)	CAT activity (net intensity, 10^5)	
5	0.1	10:1	0.0	0.0
	0.2	10:1	10.23	0.38
	0.5	10:1	13.41	1.72
5	0.1	25:1	0.0	0.0
	0.2	25:1	0.0	7.42
	0.5	25:1	2.73	0.0
5	0.1	50:1	0.0	0.0
	0.2	50:1	0.0	0.0
	0.5	50:1	0.0	0.0

Note. Acetylated products from CAT assays were isolated by TLC and measured by densitometry (refer to Section 2.5). The density of staining was measured with regards to the background and the net intensity eg 1023404 which was abbreviated to $10.23x10^5$. Cell CAT activity was the measured acetylated product isolated following a CAT assay and TLC on the IS120.Cer cell lysate after lipofection of pCAT3-Promoter with Effectene (Qiagen).

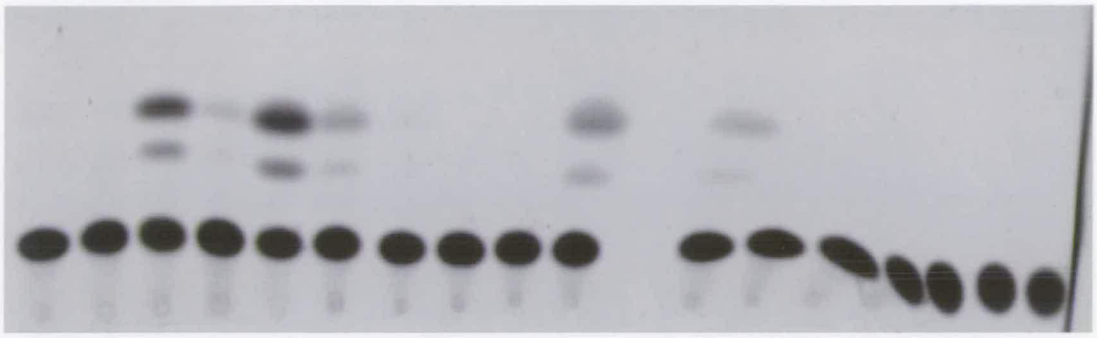

Figure 3.18:Transfection of pCAT3-Promoter into IS120.Cer cell line using Effectene. Varying ratios of Effectene to DNA were tested in duplicate. A positive and negative control was not included on this plate. Lanes A-F: Effectene: DNA ratio of 10:1; lanes G-K: Effectene:DNA ratio of 25:1; lanes L-Q: Effectene: DNA ratio of 50:1. Concentrations of DNA used were: A-B, G-H,L-M with 0.1μg; C-D, I-J N-O with 0.2μg; E-F, K, P-Q with 0.5μg. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

3.5.2.4 Dendrimer technology: Superfect Transfection Reagent.

Superfect Transfection Reagent is a specifically designed polycation, which at the molecular level is spherical in shape with branches radiating from a central core and terminating with charged amino groups (Tang *et al.*, 1996; Tang and Szoka, 1997). It is believed that the Superfect Reagent assembles the DNA into compact structures similar to histones, and having a net positive charge the DNA-Superfect complex associates with negatively charged receptors on the cell membrane facilitating the entry of DNA into the cell. Once inside the cell, Superfect is thought to protect the DNA during transport to the nucleus. Superfect Reagent has been shown to efficiently transfect a variety of different cells lines and primary cells lines.

As with liposome based transfection reagents, the amount of available dendrimer reagent will effect the formation of transfection complexes. For efficient transfection, the overall net charge of Superfect-DNA complex should be slightly positive to promote the interaction with negatively charged groups (e.g. sialylated glycoproteins) on the cell surface. Various schemes were used to optimise the charge ratio of Superfect reagent to DNA (Table 3.13).

Table 3.12: Charge ratio of Superfect: DNA with reference to the volume of Superfect in each reaction for cell growing in 6-well plates.

DNA (μg)	**Charge ratio (Superfect:DNA)**		
	3:1	**6:1**	**15:1**
1	3	6	15
2	6	12	30
3	9	18	45

Transfection of sA80BR cell line with pCAT5 and pCAT3-Promoter using Superfect reagent was as described in Section 2.5.6. Cells were harvested 48 hours post-transfection and a CAT assay performed, the results of which were analysed by TLC and the amount of acetylated chloramphenicol determined by LSC. The relative CAT activity (Figure 3.19, Table 3.13) from Superfect transfected cells was calculated as a percentage of CAT activity determined from the positive CAT control in the normal manner. Full data is presented in Appendix B 1.0, Table 1.10. Transfection of the vectors pCAT5 and pCAT3-Promoter into sA80BR cell line was optimal when a charge ratio of 3:1 (Superfect: DNA) was used to transfect 5μg of reporter vector (Table 3.13).

Table 3.13: Result of transfection of sA80BR with pCAT3-Promoter (Promega) and pCAT5 (Appendix A) using Superfect reagent according to various conditions.

Passage Number	DNA (μg)	Charge ratio (Superfect: DNA)	Positive CAT (net intensity, 10^5)	Cell CAT (net intensity,10^5)	Relative CAT activity (%)
9	2[a]	15:1	30.93	0.0	0.00
	5[a]	6:1		11.11	35.90
	10[a]	3:1		36.01	116.43
10	5[b]	3:1	19.62	22.75	115.91
	5[a]	3:1		24.85	126.60
11	1[b]	3:1	127.79	17.07	13.36
	2[b]	3:1		50.03	39.15

Note. [a] denotes transfection with the vector pCAT5
[b] denotes transfection with the vector pCAT3-Promtoer
Acetylated products from CAT assays were isolated by TLC and measured by densitometry (Section 2.5). The density of staining was estimated with regards to the background and gives the value as net intensity eg 3093140 which was abbreviated to $30.9x10^5$. Positive CAT was the measured acetylated product (100%) when 1 unit of purified chloramphenicol transferase was incubated with acetyl Coenzyme A (5mg/ml) and [^{14}C] chloramphenicol (0.05mCi/ml). Cell CAT activity was the measured acetylated product isolated following a CAT assay and TLC on the sA80BR cell lysate after transfection of pCAT3-Promoter with Superfect (Qiagen Ltd, UK).

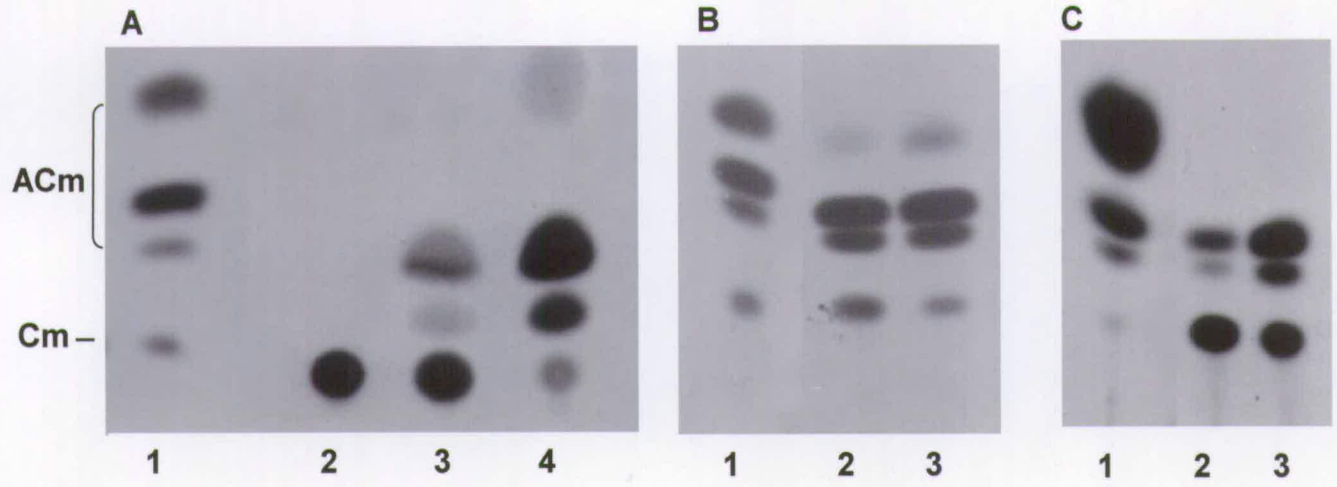

Figure 3.19: Transfection of sA80BR cell line with pCAT5 and pCAT3-Promoter (Promega) with Superfect reagent (Qiagen).

Optimisation of transfection of sheep sA80BR brain cell line with pCAT5 (Appendix C) and pCAT3-Promoter (Promega) using Superfect reagent (Qiagen). (A): lane 1: positive control, 1unit CAT; lane 2; 2μg pCAT5, charge ratio of Superfect : DNA of 15:1: lane 3; 5μg pCAT5, charge ratio of 6:1: lane 4; 10μg of pCAT5, charge ratio of 3:1. (B) lane 1: positive control CAT: lane 2; 5μg pCAT3-Promoter, charge ratio of 3:1: lane 3: 5μg pCAT5, charge ratio of 3:1. (C) lane 1: positive control CAT; lane 2: 1μg pCAT3-Promoter, charge ratio of 3:1; lane 3: 2μg pCAT3-Promoter, charge ratio 3:1. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

3.6 Discussion.

3.6.1 Classification of cell types present in brain-derived cell cultures.

The ovine cell lines intended for transfection experiments were stable (sA80BR, pA80BR and pS80SK) and primary cell lines (IS120.Cer and IS120.Liv) as described in Sections 3.2 and 3.3. The morphology of the cells present in the stable brain cultures was not typically neuronal or astrocyte like (Figures 3.2 and 3.3). This is probably an artefact of the retroviral transformation method used to create the stable cell lines. Although one advantage of stable cell lines is their immortality, however it is known that the introduction of an oncogenic gene can alter the phenotype and/or the growth pattern of the transformed cell (Rous, 1970; Bishop, 1985). For example, the mouse mammary tumour virus (a RNA tumour virus/ retrovirus) carries an oncogenic gene within its genome which causes uncharacteristic growth patterns and appearance (neoplastic transformation) of infected mammary gland cells (Butel et al., 1977). The morphology of cells present in the primary brain culture IS120.Cer (Figure 3.5) was more typical of neurones with long, thin processes extending from the main cell body. These cells also appeared to grow as a network of colonies connected by the cellular extensions.

Prior to screening transfection methods, the use of fluorescent immuno-cytochemistry (F-ITC) was used to identify the cell types present in the brain cell cultures. The stable brain cells, sA80BR and pA80BR, were a heterogeneous culture of neuronal and glial cells (Figure 3.8). The primary brain cell line derived from the cerebellum of the Icelandic breed, *Ovis brachyura borealis pall,* IS120.Cer appeared to be a homogenous culture of cells derived from neuronal tissue (Figure 3 9). All brain cell lines were grown in the presence of NGF. When NGF was removed from the stable cell lines, sA80BR and pA80BR, the growth rate slowed down but did not arrest, nor was there any change in morphology. The lack of requirement for NGF may be an effect of stable transformation and heterogeneous culture. In contrast, when NGF was removed from the primary cerebellum cell line, IS120.Cer, cell growth was severely inhibited, the cells

appeared to shrivel and the network of cell processes was withdrawn towards colonies which had formed. To maintain uniformity between the brain cell cultures for future comparison, the presence of NGF was maintained throughout this study.

3.6.2 Optimisation of transient transfection of ovine cell lines.

The methods investigated are discussed below and compared in figure 3.20. Various methods were tested in the attempt to find a reliable transfection method for the stable Cheviot brain cell lines. As both sA80BR and pA80BR appeared to be similar morphologically and showed the same staining patterns to anti-N200 and anti-GFAP, it was assumed that they would also react in the same manner the various transfection reagents.

3.6.2.1 Calcium phosphate.

Transfection using the method of calcium phosphate precipitation has been proven by others to be effective for producing stable and transient expressing cells. Crucial to transfection using this method is the formation of a DNA precipitate which is dependent on the pH of the phosphate buffer (2xHEBS). The optimal pH range for transfection is extremely narrow: typically between 7.05 and 7.12 (Graham, *et al.*,1973) and during storage the pH of the solution may change. Furthermore the pH of the medium may alter to become too acidic reducing transfection efficiency by causing protein precipitation, noted by a colour change in the cell medium from red to orange. On occasion, even when conditions would appear to be optimal (i.e. DNA concentration, solution pH, and cell density) transfection by calcium phosphate simply does not work.

Calcium phosphate precipitation was ineffective at transfecting sheep brain cell line sA80BR with pCAT3-Promoter. Both the phosphate buffer and calcium solution were freshly made and the pH checked to be pH 7.05. On every transfection attempt, a DNA

precipitate was observed to have formed confirming that the pH of the phosphate buffer was within the optimal range. Furthermore no cell death or colour change in the medium was apparent suggesting the cell medium pH was suitable for transfection by calcium phosphate. Calcium phosphate was therefore not used in further studies.

3.6.2.2 Electroporation.

The use of high voltage electric shocks for transfection of fibroblasts was first described by Wong and Neuman (Wong and Neumann, 1982; Neumann *et al.*, 1982) and later proven to be effective for other cell types known to be resistant to calcium phosphate precipitation (e.g. lymphocytes, Potter *et al.*, 1984). Disadvantages of electroporation include the need for specialised equipment, almost five-fold more cells and DNA than the calcium phosphate method and the high rate of cell death. The critical parameters for electroporation are maximum voltage of the shock and the duration of the current pulse (capacitance) which must be optimised for each cell type. The electroporation buffer may also affect transfection efficiency. Low-resistance buffers (high salt, e.g. HeBS) may require high voltages and sensitive cells may prefer electroporation in tissue culture medium though it has been shown that magnesium and calcium ions in the medium lower electroporation efficiency (Neumann *et al.*, 1982). It is known, however, that some cell lines will transfect poorly under standard conditions.

Under the conditions used (Table 3.3) electroporation was ineffective for transient transfection of the CAT reporter gene, pCAT5, into the sheep cell line sA80BR. There was no difference in the efficiency of transfection when either PBS or HeBS was used as the electroporation buffer. Electroporation of cells resuspended in culture medium was not tested. Significant cell death was recorded with all conditions tested. Electroporation of sA80BR cell line may have been possible using further combinations of high voltage/ low capacitance or low voltage/ high capacitance however the procedure was lengthy and expensive in term of resources used. A decision was therefore taken to discontinue testing this method of transfection.

3.6.2.3 Lipofection.

The commercially available Dosper (Roche Diagnostics, UK), Tfx20^{Tm} and Tfx50^{Tm} (Promega, UK) and Effectene (Qiagen, UK) liposome-based transfection reagents were tested for their ability to transfect pCAT3-Promoter into sheep brain cell line sA80BR (Section 3.5.2.3). Dopser reagent did not efficiently transfect sheep sA80BR or mouse N2a cell lines with pCAT3-promoter. The active lipid in Dosper is 1,3-Di-Oleoyloxy-2-(6-Carboxy-spermyl)-propylamid which may have been unable to form functional micelles with the pCAT3-Promter or the micelles which were formed were not efficiently absorbed by the cells. Alternatively proteins present in the cell medium may have been competing with the DNA for the positively charged chain on the lipids and thus reducing transfection efficiency. Liposome reagents Tfx50^{Tm} and Tfx20^{Tm} (Promega, UK) showed limited efficiency for the transfection of pCAT3-Promoter to ovine brain cell line sA80BR in the absence of serum and were toxic to the cells, 3-30% and 10-50% respectively (Figure 3.20). However the Tfx reagents (a mixture of the synthetic lipid N,N,N',N'-tetramethly-N,N'-bis(2-hydroxyethyl)-2,3-di(oleoyloxy)-1,4-butanediammonium iodide and DOPE) had poor reproducibility between transfection experiments. This may be primarily due to the fact that the shape and size of the micelles which form in each reaction will vary slightly and so alter the rate and efficiency of DNA uptake into the cell.

In contrast to other liposome based reagents, Effectene (Qiagene, UK) uses a DNA enhancer to improve transfection efficiency. The enhancer functions to condense the transfected DNA into a compact structure with which the lipids freely interact to form regular micelles. Thus eliminating the possibility of size, charge and shape irregularities between transfection reaction. Effectene reagent was efficient in transfecting primary liver (IS120.Liv) and brain cell lines (Is120.Cer) derived from the Icelandic sheep (Tables 3.10 and 3.11, Figure 3.20). Effectene reagent was also not toxic to the cells in the presence of serum or antibiotic, and significantly less DNA was required to transfect with cell lines to achieve high levels of CAT expression.

With all liposome based transfection reagents the health of the cells is vital to transfection. A reduced transfection efficiency was observed with cells of high passage number. Cell lines of low passage number were therefore used in later transient expression studies.

3.6.2.4 Dendrimer technology.

Superfect transfection reagent (Qiagen, UK) has only been commercially available since 1997 but is similar to Effectene in that it functions to condense the transfected DNA into regular sized vessels which are easily and efficiently adsorbed into the cell. As with liposmes the charge ration of the polycation dendrimer and DNA must be optimised for efficient transfection (Tang *et al.*, 1996; Tang and Szoka, 1997). However the advantages of Superfect over liposome reagents include: the presence of serum actually increases transfection efficiency, antibiotics do not effect Superfect function, and reproducibility is good if cell density at time of transfection is consistent. Superfect reagent was efficient in transfecting pCAT3-Promoter into sheep brain cell line sA80BR with 40-80% efficiency (Section 3.5.2.4 and Figure 3.20). Optimal transfection was observed with increased DNA amount (5μg) and a charge ratio of 3:1 (Table 3.14). During use of Superfect reagent, no cell death was observed. Superfect reagent was proven to be the most efficient transfection reagent for ovine brain cell line sA80BR and as such was used for further transient expression studies of the CAT-PrP 3'UTR vectors (Chapter Four) and PrP mini-gene vectors (Chapter Five).

A comparison of all the method used to develop a transfection method for the immortal sheep cell lines is shown in Figure 3.20.

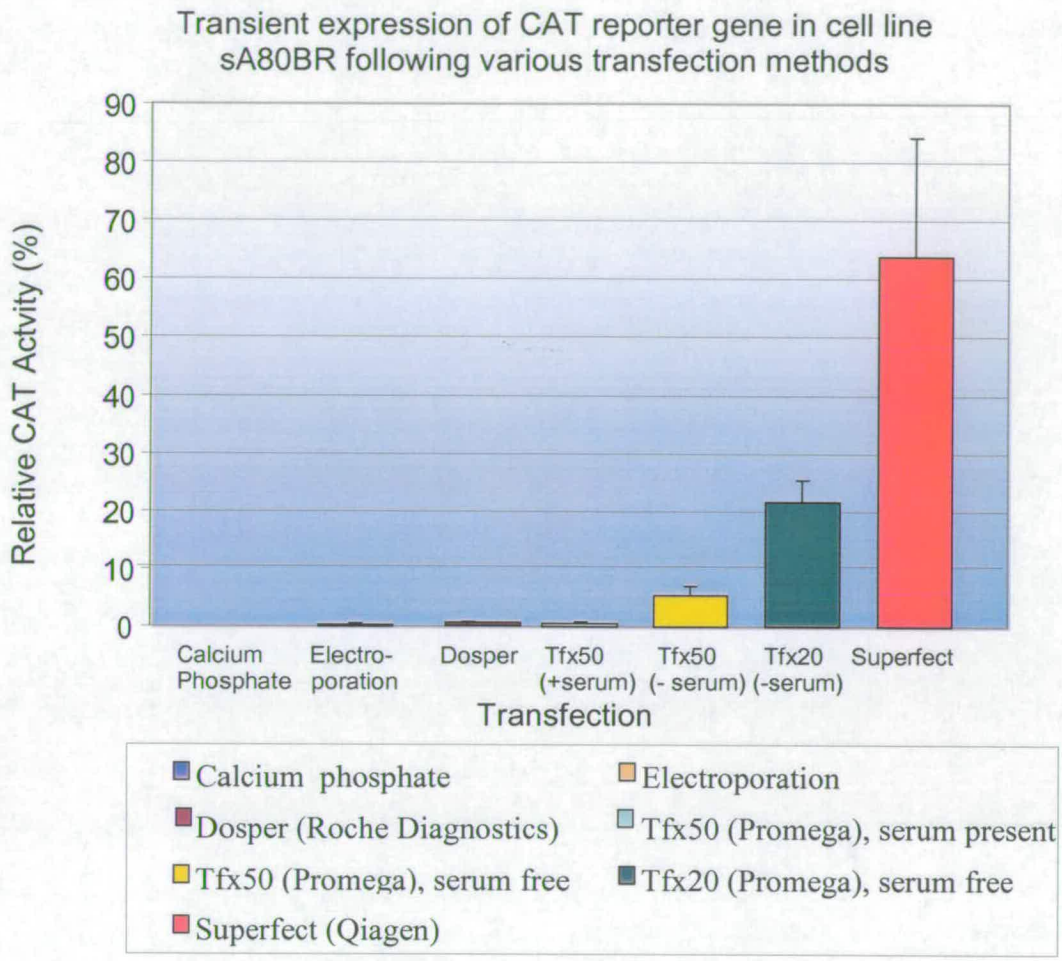

Figure 3.20: Summary of transfection efficiencies of CAT reporter gene by various methods in cell line sA80BR. Ovine brain cell lines sA80BR was optimally transfected with pCAT3-promoter by Superfect transfection reagent (Qiagen Ltd, UK). See Appendix B for full data. SEM shown. High variability in Superfect CAT activity is due to low level of expression when low amounts of DNA were used as part of the optimisation screening.

Chapter 4 : Transient Expression of CAT Reporter Genes in Various Ovine Cell Lines and the Function of the PrP 3'UTR.

4.1 Introduction.

The term gene expression commonly refers to the entire process of DNA transcription, RNA processing and mRNA translation into protein. Regulation at any stage in this process could lead to differential gene expression. Control over gene expression allows for a cellular response to external stimuli or the execution of developmental pathways, ensuring the correct genes are activated at the correct time (spatial and temporal control). Maturation of 5' capped mRNA requires two primary steps: 1) cleavage and polyadenylation at the 3' end and 2) ligation of exons (splicing). In eukaryotes, RNA processing can control gene expression by on- off regulation, exon splicing, or alternative cleavage and polyadenylation at polyA sites (Chapter one, Section 1.10; Zhao *et al.*, 1999). Such alternative processing pathways are often regulated in a cell type-specific manner.

Post-transcriptional control of gene expression is mostly mediated through the untranslated regions. Secondary structures and protein binding motifs present in the 5' region have been shown to influence translation initiation and efficiency (Dever, 1999). Initially the 3'UTR was considered to function only to control polyadenylation of the maturing mRNA for export into the cytoplasm for translation, with the length of the poly (A) tail determining the stability of the mRNA. In the absence of efficient polyadenylation, the pre-mRNA molecule will be degraded before translation. For example, autoregulation of the snRNP protein U1A prevents wasteful synthesis of the U1A protein when the U1 snRNP splicing machinery is not required (Gunderson *et al.*, 1994). However, investigations have shown that further sequences and structures within the 3'UTR are also capable of effecting stability, location and translation efficiency of mRNA and hence controlling gene expression (Chapter 1, Section 1.10).

The stability of an mRNA is a function of its synthesis and the rate of degradation (review Ross, 1995). The stability of an mRNA also determines how tightly the rate of synthesis of the encoded protein can be controlled. Many short-lived mRNA transcripts contain multiple, often overlapping AUUUA sequences within their 3'UTRs (Shaw and Kamen, 1986; Fan *et al.*, 1997). The mechanism by which these sequences function is not yet understood. In several cases, the rate of degradation is also regulated by RNA-protein interactions in the 3'UTR. Expression of the transferrin receptor is regulated by the intracellular iron concentration through iron-response element (IRE) located within the 3'UTR: a drop in iron concentration stabilises Tfr mRNA and promotes synthesis of the Tfr protein (Roa *et al.*, 1986).

In mammalian cells translational control via the 5' UTR is well documented however little is known of the function of the 3'UTR during translation. From studies in yeast, an interaction between the poly(A) tail and a cytoplasmic poly(A) binding protein (PABP) may influence translation efficiency (Sachs *et al.*, 1986).

4.1.1 Protein levels mediated by the ovine PrP 3'UTR.

As discussed in Chapter 1 (Section 1.10), controlling the process of cleavage/ polyadenylation may regulate the amount of protein synthesised. One method frequently used to determine DNA sequences vital to gene expression involves constructing reporter vectors with deletions in the flanking region of interest i.e. promoter regions, 5' or 3' UTRs or introns. Assaying for *in vitro* expression of the reporter gene determines if inserted DNA sequences stimulate or inhibit expression of the reporter vector compared to a control expressing a bacterial gene. Using this method of analysis the ovine PrP 3'UTR was the subject for a previous Ph.D. project at the NPU (Cheung, 1996). The PrP 3'UTR fragment used for the CAT analysis work was originally cloned from a Suffolk sheep with the PrP allele $A_{136}R_{154}Q_{171}$ (Goldmann *et al.*, 1991a). Previous analysis of the cloned PrP 3'UTR by RFLP has shown it to it to be from an e1 allele. Deletions were made in the PrP 3'UTR (Figure 4.1) inserted downstream of the reporter gene chloramphenicol transferase (CAT) and used in a series of transient transfection experiments in a mouse N2a cell line (Cheung, 1996). As the only differences between the constructs lay in the 3'UTR, the assumption was made that the level of measured CAT activity was a direct result of the influence of PrP 3'UTR sequence inserted into the reporter vector. If the level of CAT activity decreases when a region of 3'UTR has been removed, it can be considered that the deleted sequence was essential for expression. Conversely, if the CAT activity rises the deleted sequence may have an inhibitory effect.

In the previous work by Cheung (1996) the CAT constructs included two which resembled the *in vivo* PrP mRNA 3'UTR sequences (Hunter *et al.*, 1994; Goldmann *et al.*, 1999) with a UTR of 3.2 kb (as found on 4.6 PrP mRNA) and 0.7kb (the 2.1kb PrP mRNA). *In vitro* expression in mouse N2a cells of CAT/PrP-3'UTR vectors showed that sequences within the 3'UTR had an inhibitory effect on levels of CAT protein produced (Cheung, 1996; Goldmann *et al.*, 1999). Interestingly the constructs pEYR and pD17 which resemble the *in vivo* PrP transcripts (mRNAs 4.6kb and 2.1kb respectively) produced amongst the lowest CAT activities. Mutating the sequence $ATTAAA_{1523}$ to $TATAAA_{1523}$ in both pEYR and pD17 caused a 60% decrease in detectable CAT activity suggesting very low *in vitro*

expression of CAT in the presence of the full length PrP 3'UTR. Further studies by Cheung (1996) comparing the amount of RNA transcribed *in vitro* (cell free system and N2a cells) from the constructs pEYR, pD36(Figure 4.1), and the CAT control (pCAT3-Promoter, Promega Ltd, UK) judged by semi-quantitative RT-PCR analysis, showed no significant difference in the level of mRNA produced from the vectors over the 48 hour transfection period. In contrast, the level of CAT activity detected for constructs pEYR, pD36 and the CAT control was 12.4%, 44.9% and 100% respectively. Taken together, Cheung's results suggested that the 3'UTR may be acting to control PrP gene expression.

The aim of this chapter was to investigate the role of the ovine PrP 3' UTR in controlling expression of the CAT gene with regards to sheep tissue specificity and PrP genotype. The chimeric CAT-PrP 3'UTR vectors were transiently expressed in transformed Cheviot foetal brain cell lines with PrP genotypes $VV_{136}RR_{154}QQ_{171}$ (susceptible) and $AA_{136}RR_{154}RQ_{171}$ (resistant). The level of expression from the CAT-PrP 3'UTR vectors was also assayed in cerebellum and liver cell line from the Icelandic sheep breed, *Ovis brachyura borealis pall* , which had a susceptible genotype of $AA_{136}RR_{154}QQ_{171}$.

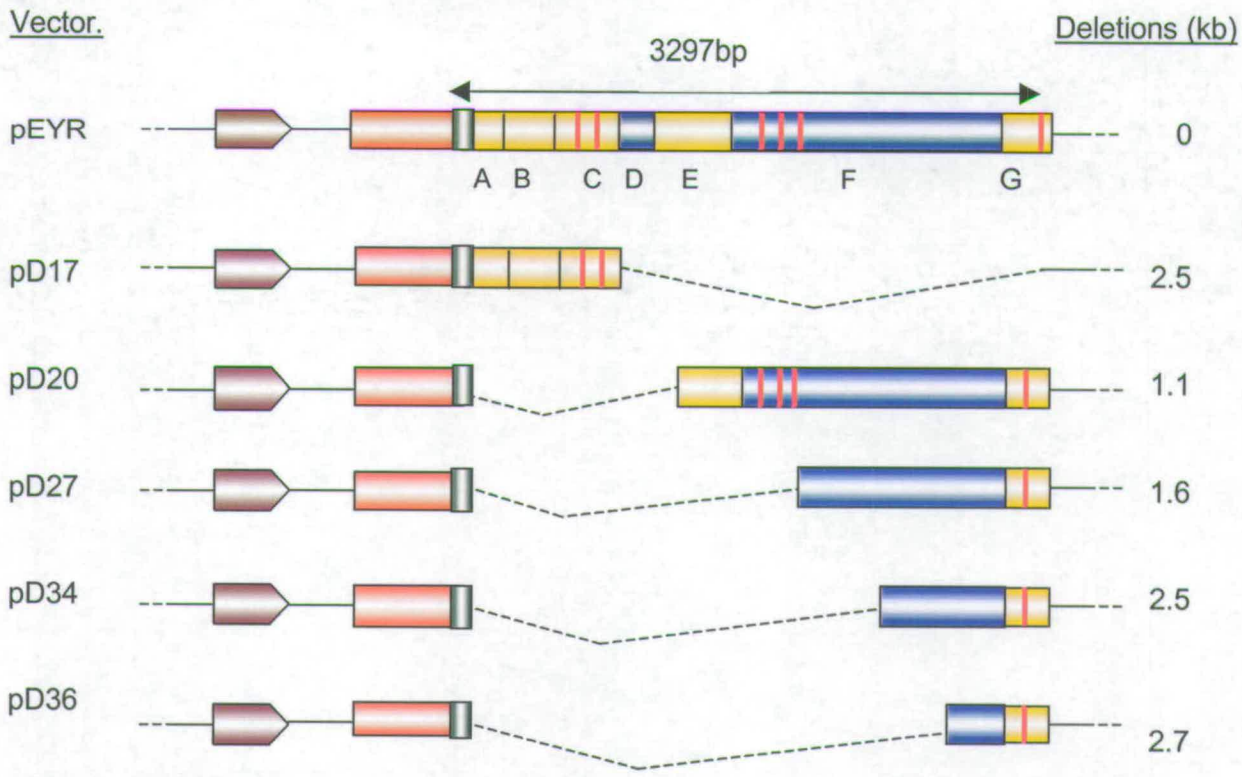

Figure 4.1: Chimeric CAT/PrP-3'UTR constructs. A series of CAT reporter vectors were created by Cheung (1996) containing the sheep PrP 3'UTR cloned down stream of the CAT ORF. Deletions were made within the 3'UTR region and CAT expression studied in mouse N2a cells (Cheung, 1996, Goldmann, 1999). These vectors have now been transiently expressed in sheep cell lines from various tissue origins and PrP genotypes.

Key. SV40promoter region | CAT coding region | Ovine PrP 3'UTR | Ovine PrP coding region | Ovine specific PrP 3'UTR sequence | Conserved polyadenylation signal | Deletion

Table 4.1: Structural properties of the UTR in chimeric CAT/PrP-3'UTR vectors.

Vector	PrP 3'UTR length (kb)	PrP 3'UTR region	Instability motifs	Conserved polyadenylation signals
pEYR	3.2	A-G	5	5
pD17	0.7	A-C	0	1
pD20	2.1	E-G	4	4
pD27	1.4	F-G	3	1
pD34	1.1	f-G	3	1
pD36	0.5	f-G	2	1
PCAT3-Promoter	0.9	n/a	*	1

Note. Refer to Figure 4.1 and Cheung (1996) Goldmann *et al.*(1999)
*No ATTTA consensus sequence

4.2 PrP 3'UTR: Translational Control of the CAT Reporter Gene in Ovine Brain-derived Cell Lines of Susceptible and Resistant PrP Genotype.

4.2.1 Experimental approach.

The aim of this section was to investigate whether or not sequences within the PrP 3'UTR displayed control over expression of the CAT gene in brain cell lines derived from NPU Cheviot sheep of scrapie susceptible (cell line sA80BR, $VV_{136}RR_{154}QQ_{171}$) and resistant (pA80BR, $AA_{136}RR_{145}QR_{171}$) PrP genotype. Chimeric CAT/ PrP -3'UTR plasmids (Cheung, 1996, Figure 4.1) were transiently transfected by Superfect reagent (Qiagen) into the brain cell lines using the method discussed in Chapter 3. For each experiment approximately $2x10^7$ cell were recovered from a single well of a 6-well plate. Transfection efficiency was controlled for by co-transfecting with pSV-β-galactosidase and the relative CAT activity recorded from each expression vector was proportioned to the level of CAT activity from pCAT3-Promoter (Promega) which was assumed to be 100%. The results of the CAT assays were analysed by TLC and the amount of acetylated product was measured by LSC directly from the TLC plate or by densometric reading of the autoradiograph using a Kodak Image Station (Chapter 2, Section 2.5.12). Throughout the study, the cells appeared to remain healthy and no change in growth pattern was observed. A full record of data obtained can be found in Appendix B, Sections B 2.1-2.2. Typical autoradiographic films developed for each of the cell lines are shown in Figure 4.2A and 4.2B.

4.2.2 Results and analysis. Comparative transient expression studies of chimeric CAT-PrP 3'UTR in brain cell lines sA80BR and pA80BR.

4.2.2.1 Results.

The collected results from the five assays performed in cell line sA80BR are shown in Figure 4.3A and the seven assays performed in cell line pA80BR in Figure 4.3B. The data presented in Table 4.2 and 4.3 shows results of statistical analysis completed on the transformed CAT measurements recorded for both cell lines.

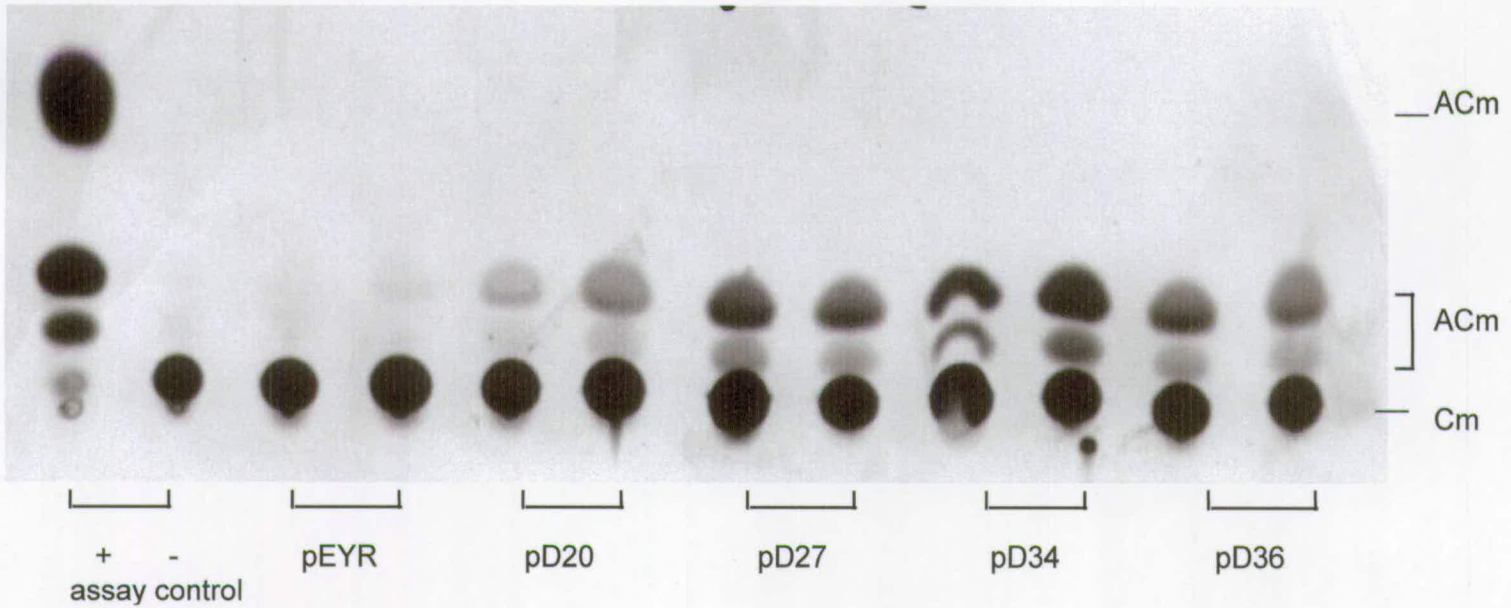

Figure 4.2 A:Transient expression of chimeric CAT-PrP 3'UTR plasmids in cell line sA80BR.
Products from CAT transfection experiments were separated by TLC and the acetylated product was isolated and quantified by scintillation counting. The assay positive control contained1 unit of purified CAT enzyme (Promega), negative control contained 0.25 M Tris and [^{14}C]chloramphenicol only. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

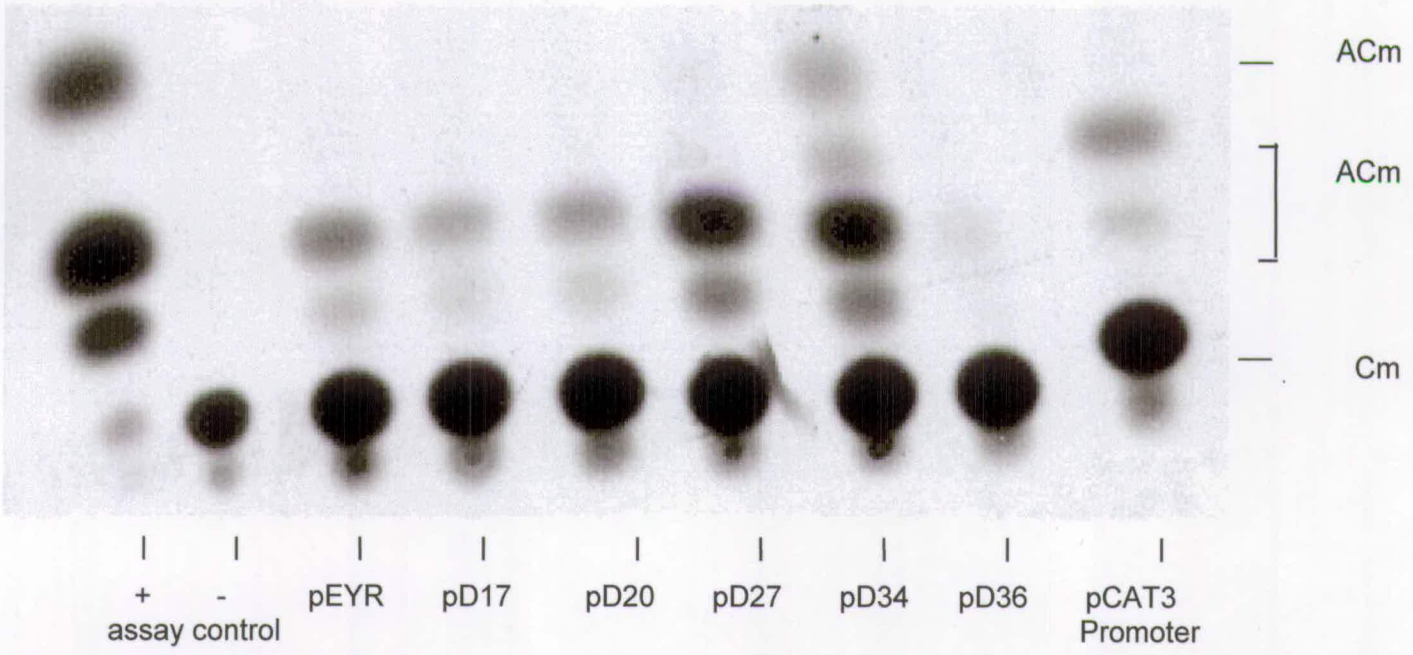

Figure 4.2 B:Transient expression of chimeric CAT-PrP 3'UTR plasmids in cell line pA80BR.

Products from CAT transfection experiments were separated by TLC (section 2) and the acetylated product was isolated and quantified by scintillation counting. The assay positive control contained 1 unit of purified CAT enzyme (Promega), negative control contained 0.25 M Tris and [^{14}C]chloramphenicol only ACm: acetylated chloramphenicol, Cm: chloramphenicol.

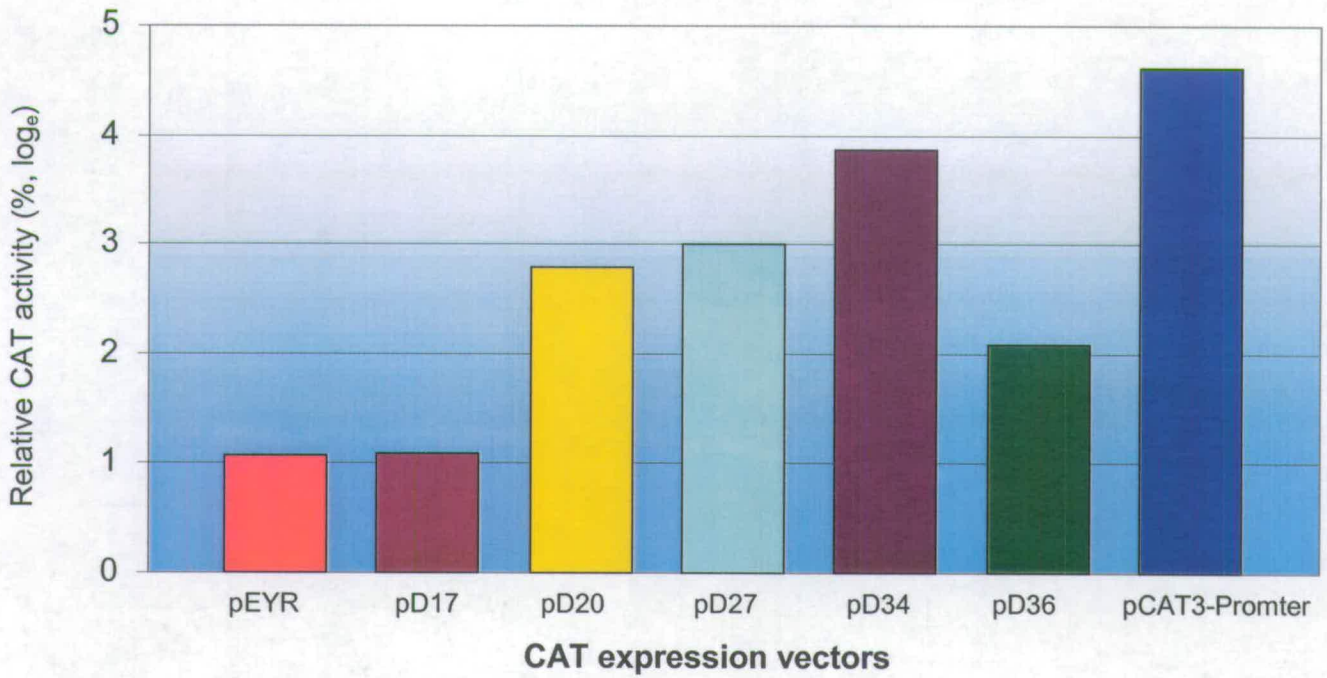

Figure 4.3 A: Relative CAT activity (%) measured in cell lines sA80BR.

TransientCAT expression of chimeric CAT-PrP 3'UTR plasmids in ovine cell lines derived from Cheviot brain cell line sA80BR (PrP genotype $VV_{136}RR_{154}QQ_{171}$). The amount of acetylated chloramphenicol product was measured for each CAT/PrP-3'UTR vector and calculated as a percentage of the vector pCAT3-Promoter (100%) for each transfection experiments. The results were plotted on the $\log_e$ scale as the level of detectable CAT activity from separate transfection experiments varied in magnitude.

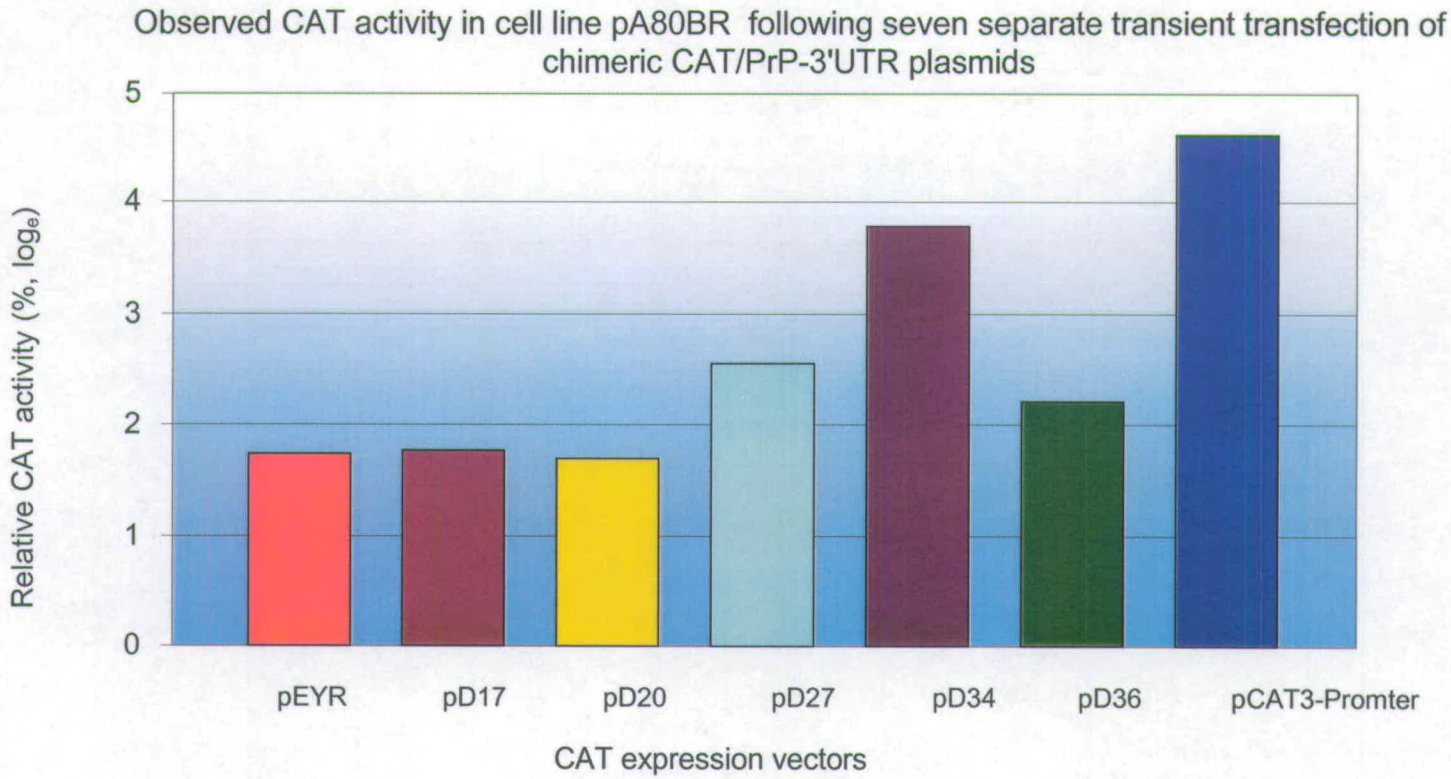

Figure 4.3 B: Relative CAT activity (%) measured in cell lines pA80BR.

Transient CAT expression of chimeric CAT-PrP 3'UTR plasmids in ovine cell lines derived from Cheviot brain cell line pA80BR (PrP genotype $AA_{136}RR_{154}QR_{171}$). The amount of acetylated chloramphenicol product was measured for each CAT/PrP- 3'UTR vector and calculated as a percentage of the vector pCAT3-Promoter (100%) for each transfection experiments. The results were plotted on the $\log_e$ scale as the level of detectable CAT activity from separate transfection experiments varied in magnitude.

Table 4.2 Statistical analysis of expression from CAT/PrP-3'UTR constructs in cell lines sA80BR and pA80BR.

Constructs for comparison	Cell line	
	sA80BR (p =≤)	pA80BR (p=≤)
p17 with pEYR	0.42	0.97
p 20 with pEYR	0.14	0.96
p27 with pEYR	0.1	0.37
p34 with pEYR	0.02	0.05
p36 with pEYR	0.52	0.55
pEYR with pD17	0.99	0.97
pD17 with pD20	0.1	0.95
pD20 with pD27	0.63	0.41
pD27 with pD34	0.03	0.21
pD34 with pD36	0.15	0.08

Note. In rows 1-5, the mean CAT activity recorded for each construct was compared against the CAT activity from pEYR (resembling in vivo 4.6kb PrP mRNA 3'UTR). In rows 6-10 the significance of the change in CAT expression as sequential deletions were introduced into the 3'UTR is considered. Analysis was completed by a t-test on the transformed data, please refer to Appendix B, Section B 2.2.1 . For sA80BR and pA80BR the degrees of freedom were 8 and 11 respectively.

Table 4.3 Comparison of expression from CAT/PrP-3'UTR constructs in cell lines sA80BR and pA80BR.

Constructs for comparison	Between cell lines sA80BR and pA80BR (p=≤)
PEYR	0.57
pD17	0.52
pD20	0.26
pD27	0.54
pD34	0.93
pD36	0.91
PEYR with pD17	0.54
pD17 with pD20	0.83
pD20 with pD27	0.36
pD27 with pD34	0.74
pD34 with pD36	0.98

Note. In rows 1-5, the mean CAT activity recorded for each construct was compared against equivalent expression in each cell line. In rows 6-10 the significance of the change in CAT expression as sequential deletions were introduced into the 3'UTR is considered. Analysis was completed by a t-test on the transformed data, please refer to Appendix B, Section B 2.2.1. The degrees of freedom were 10.

4.2.2.2 Analysis.

Independent transient transfections of ovine brain cell lines derived from scrapie resistant and scrapie susceptible PrP genotypes were completed with the chimeric CAT/PrP-3'UTR constructs (Figure 4.1). In both cell lines, the CAT activity recorded between experiments varied by an order of magnitude. To allow for statistical analysis, the value for CAT activity (% CAT) was transformed on to the $\log_e$ scale (Snedecor and Cochran, 1967). Before Students t-test was completed on the transformed data, the level of variance between experiments was examined for significance using Analysis of Variants (ANOVA) (full explanation and working in Appendix B, Section B 2.5). In summary, ANOVA confirmed that variance did occur between transfection experiments but, importantly, that this variance was not significant in terms of *in vitro* expression of the plasmids. This means that although there was a great deal of variation in the overall magnitudes of the standardised CAT results between experiments, the pattern of relative differences in expression from the series of plasmids was consistent across all experiments. Further analysis was carried out using a t-test on the probability that the 3'UTR sequence present in the CAT/PrP-3 UTR constructs influenced the level of detectable CAT activity (Table 4.3).

As observed in mouse N2a cell line (Cheung, 1996; Goldmann *et al.*, 1999), *in vitro* expression of constructs pEYR and pD17 (resembling 3'UTRs of in vivo PrP mRNA) was lower than the other plasmids. In both cell lines, an increase in the level of detectable CAT activity was observed as deletions were introduced into the PrP 3'UTR sequence in the 5' to 3' direction. For the sA80BR cell line this increase was observed to begin when sequence upstream of 3'UTR nucleotide (nt) position 2000 had been deleted (construct pD20) and continued to increase, peaking at a deletion up to nt 3400 (pD34) (Figure 4.2A). Although the difference in mean CAT expression between pEYR and pD20 was not significant (Table 4.2, $p=\leqslant 0.14$), the figures do support a tendency for increased expression from constructs pD20 to pD34 (Table 4.2, rows 1-6). For the pA80BR cell line, an increase in CAT activity was observed after deletion of sequence upstream of nt position 2700 (pD27, Figure 4.2B). However, this increase in detectable CAT activity was not significantly

different to the wild type construct pEYR (Table 4.2). As observed in sA80BR, CAT activity peaked in pA80BR with construct pD34 ($p = 0.05$). A direct comparison of expression from the CAT/PrP-3'UTR constructs in the two cell lines reveals no significant difference (Table 4.3).

An alternative explanation for the observed difference in CAT gene expression between the sA80BR and pA80BR cell line is the efficiency of the transformation process. As described in Chapter Three, Section 3.2, the Cheviot cell lines were immortalised by transformation with the Moloney murine leukaemia virus. The nature of retroviruses allows integration of the viral genome into the host genome by illegitimate recombination where the viral genome has no sequence homology with the insert site. Consequently the two cell lines sA80BR and pA80BR will be transformed differently which may give rise to differences in efficient gene expression and control. However, similarities in CAT expression were observed between the stable and primary cell lines as described in Section 4.3 which argues against any effect on gene expression in the Cheviot cell lines which may be attributed to the transformation process.

4.3 Translational Control of the CAT Reporter Gene Constructs in Ovine Neuronal and Peripheral Cell Lines .

4.3.1 Experimental approach.

The aim of this Section was to evaluate the role of sequences within the sheep PrP 3'UTR in controlling transient expression of CAT in ovine cell lines derived from neuronal and peripheral tissues. At the start of this study, the intention was to compare CAT expression in the stable brain cell lines to similar transfections in skin derived cell lines. However, as discussed in Chapter Three, several problems were encountered in the growing and transfection of the skin cells and they were discounted from this particular study. As an alternative, the primary cell lines (Chapter Three) derived by Dr Gerard O'Neill from cerebellum (designated IS120.Cer) and liver (designated IS120.Liv) tissues from the Icelandic sheep

(genotype $AA_{136}RR_{154}QQ_{171}$ which is scrapie susceptible in this breed) were used. Chimeric CAT/ PrP -3'UTR plasmids (Cheung, 1996, Figure 4.1) were transiently transfected by Effectene reagent (Qiagen) into the primary cell lines using the method discussed in Chapter Three and Chapter Two, Section 2.5. For each experiment approximately $2x10^7$ cell were recovered from a single well of a 6-well plate. As before, transfection efficiency was controlled for by co-transfecting with pSV-β-galactosidase and the relative CAT activity recorded from each expression vector standardised against the level of CAT activity from pCAT3-Promoter (Promega) which was assumed to be 100%. The results of the CAT assays were as previously described (Chapter Two, Section 2.5.12). Throughout the study, the cells appeared to remain healthy and no change in growth pattern was observed. A full record of data obtained can be found in Appendix B, Sections B2.3 and 2.4. Typical autoradiographic films developed for each of the cell lines are shown in Figures 4.4A and 4.4B.

4.3.2 Results and analysis. Comparative transient expression studies of chimeric CAT-PrP 3'UTR constructs in cell lines IS120.Cer and IS120.Liv..

4.3.2.1 Results.

The collected results from the six assays in cell line IS120.Cer are in Figure 4.5A and the five assays in cell line IS120.Liv in Figure 4.5B . The data presented in Tables 4.4 and 4.5 shows results of statistical analysis completed on the transformed CAT measurements recorded for both cell lines.

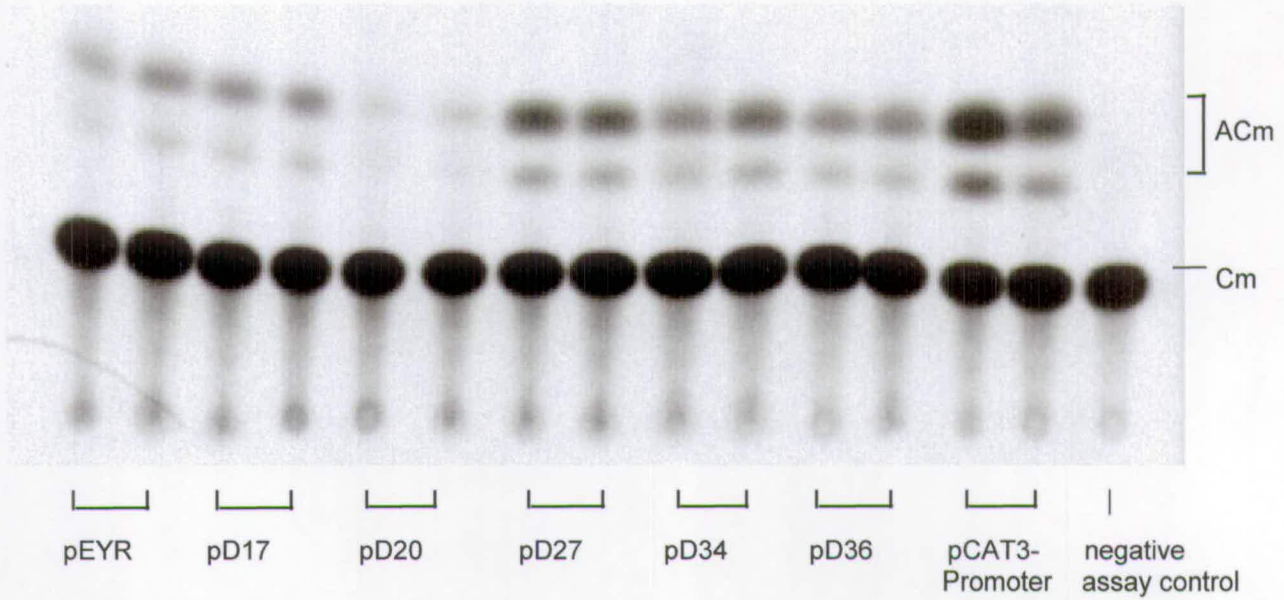

Figure 4.4A: Transient expression of chimeric CAT-PrP 3'UTR plasmids in cell line IS120.Cer. Products from CAT transfection experiments were separated by TLC and the acetylated product isolated and quantified by scintillation counting. Typical autorad from one separate transfection is shown. The assay positive control contained 1 unit of purified CAT enzyme (Promega), negative control contained 0.25 M Tris HCl and [^{14}C]chloramphenicol only; ACm: acetylated chloramphenicol, Cm: chloramphenicol.

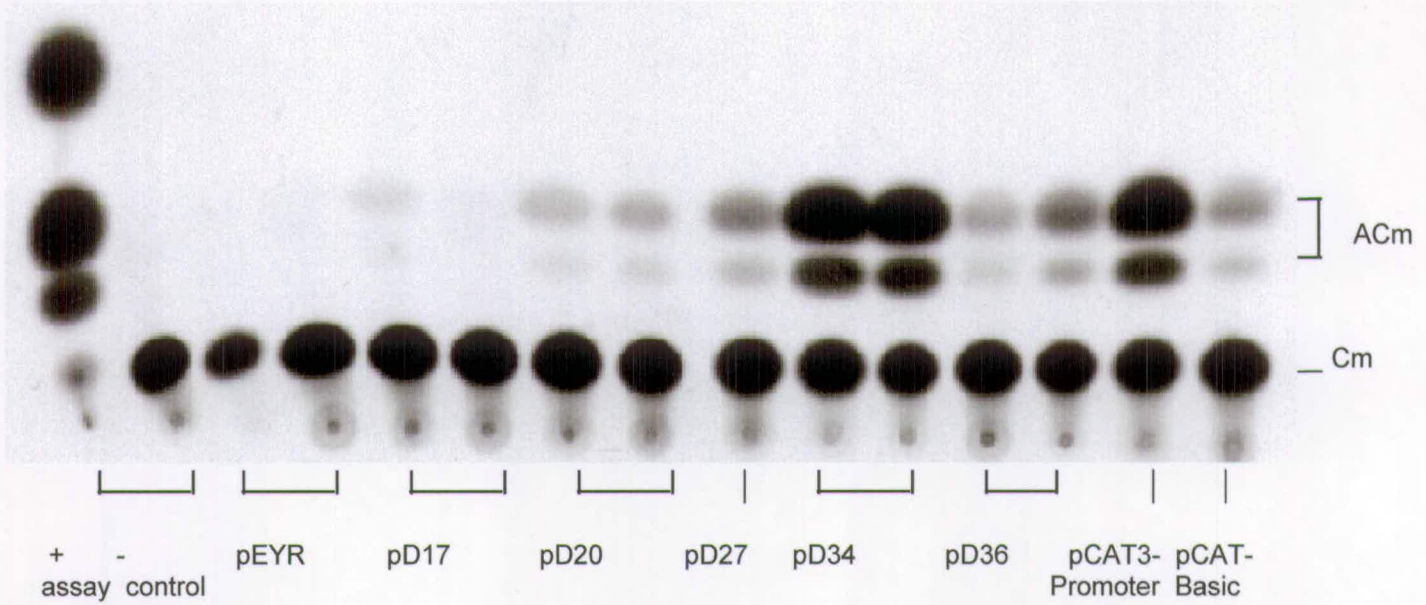

Figure 4.4 B: Transient expression of chimeric CAT-PrP 3'UTR plasmids in cell line IS120.Liv. Products from CAT transfection experiments were separated by TLC (section 2) and the acetylated product was isolated and quantified by scintillation counting. Typical autorad from one transfection is shown. The assay positive control contained 1 unit of purified CAT enzyme (Promega), negative control contained 0.25 M Tris HCl and [^{14}C] chloramphenicol only. ACm: acetylated chloramphenicol, Cm: chloramphenicol.

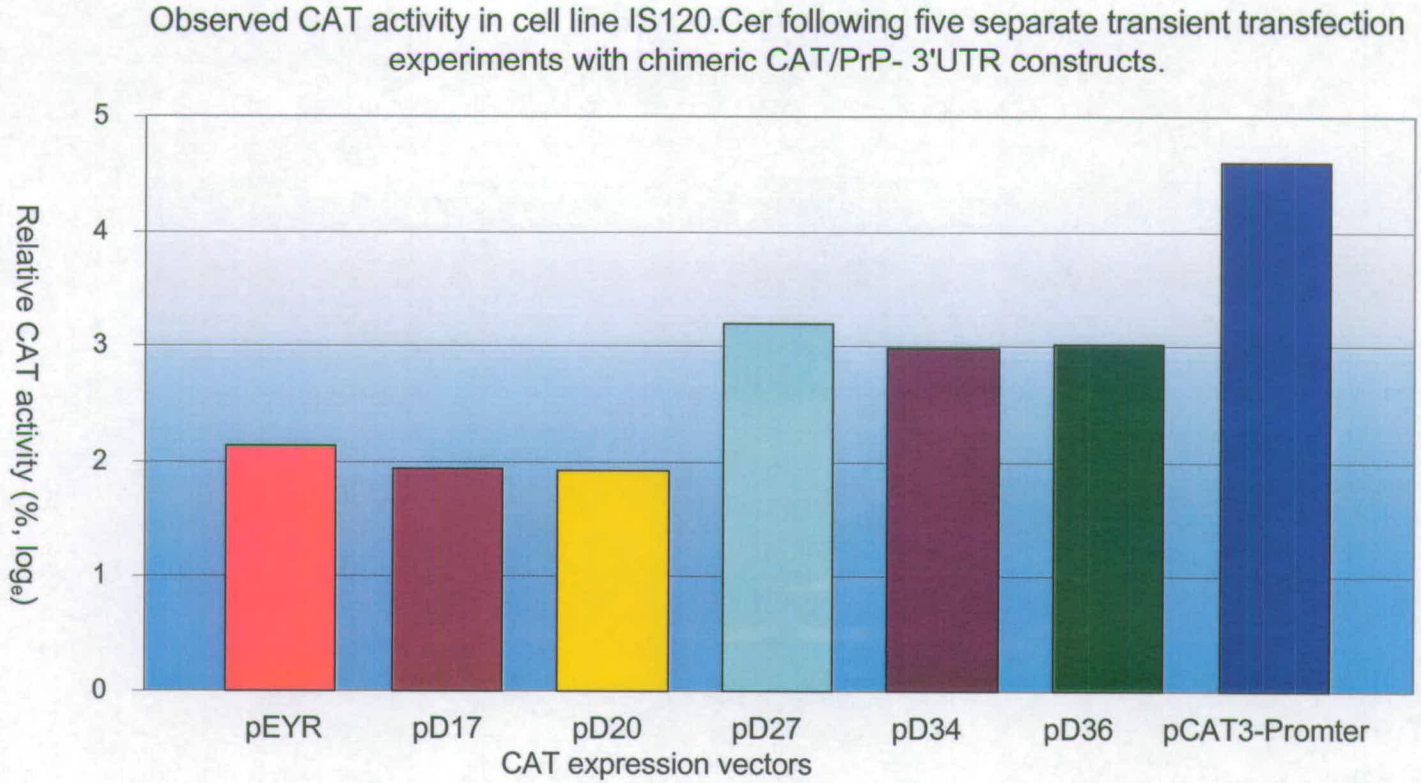

Figure 4.5 A : Relative CAT activity (%) measured in cell line IS120.Cer Transient expression of chimeric CAT-PrP 3'UTR plasmids in ovine cell lines derived from cerebellum tissue (cell line IS120.Cer) from the Icelandic sheep breed *Ovis brachyura borealis pall* (genotype $AA_{136}RR_{154}QQ_{171}$.) Relative CAT activity was calculated by the amount of acetylated chloramphenicol produced in each CAT assay with regard to pCAT3-Promoter (100%) and plotted on the $\log_e$ scale.

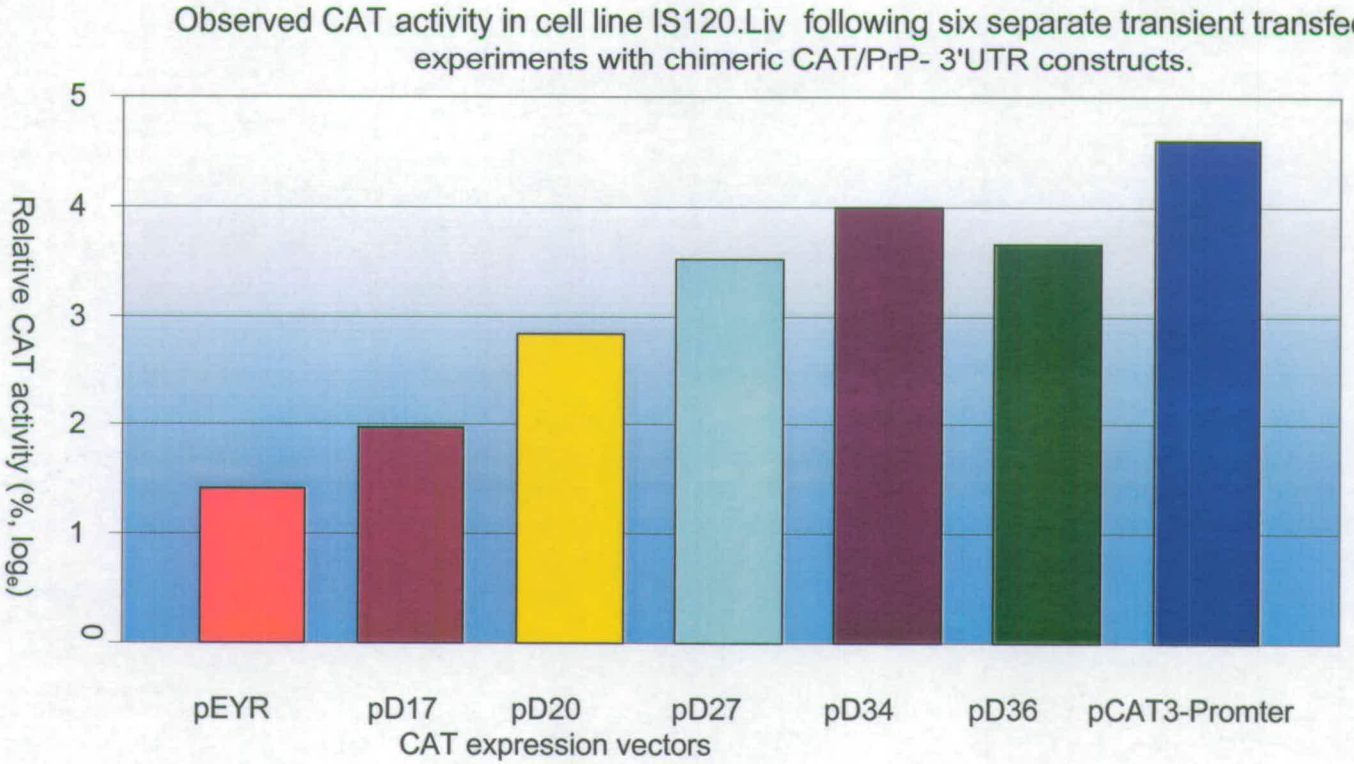

Figure 4.5 B : Relative CAT activity (%) measured in cell line IS120.Liv . Transient expression of chimeric CAT-PrP 3'UTR plasmids in ovine cell lines derived from liver tissue (cell line IS120.Liv) from the Icelandic sheep breed *Ovis brachyura borealis pall* (genotype $AA_{136}RR_{154}QQ_{171}$.) Relative CAT activity was calculated by the amount of acetylated chloramphenicol produced in each CAT assay with regard to pCAT3-Promoter (100%) and plotted on the $\log_e$ scale.

Table 4.4: Statistical analysis of expression from CAT/PrP-3'UTR constructs in cell lines IS120.Cer and IS120.Liv.

Constructs for comparison	Cell line	
	IS120.Cer (p=≤)	IS120.Liv(p=≤)
p17 with pEYR	0.35.	0.73
p 20 with pEYR	0.53	0.3
p27 with pEYR	0.12	0.19
p34 with pEYR	0.06	0.09
p36 with pEYR	0.05	0.12
PEYR with pD17	0.73	0.73
pD17 with pD20	0.96	0.3
pD20 with pD27	0.02	0.36
pD27 with pD34	0.7	0.53
pD34 with pD36	0.9	0.12

Note. In rows 1-5, the mean CAT activity recorded for each construct was compared against the CAT activity from pEYR (resembling in vivo 4.6kb PrP mRNA 3'UTR). In rows 6-10 the significance of the change in CAT expression as sequential deletions were introduced into the 3'UTR is considered. Analysis was completed by a t-test on the transformed data, please refer to Appendix B, Table B 2.4.14.6. For IS120.Cer and IS120.Liv the degrees of freedom were 5 and 4 respectively.

Table 4.5: Comparison of expression from CAT/PrP-3'UTR constructs in cell lines IS120.Cer and IS120.Liv.

Constructs for comparison	Between cell lines IS120.Cer and IS120.Liv(p=≤)
pEYR	0.52
pD17	0.98
pD 20	0.08
pD27	0.83
pD34	0.14
pD36	0.23
PEYR with pD17	0.75
pD17 with pD20	0.55
pD20 with pD27	0.55
pD27 with pD34	0.56
PD34 with pD36	0.18

Note. In rows 1-5, the mean CAT activity recorded for each construct was compared against equivalent expression in each cell line. In rows 6-10 the significance of the change in CAT expression as sequential deletions were introduced into the 3'UTR is considered. Analysis was completed by a t-test on the transformed data, please refer to Appendix B, Table B 2.4.6 The degree of freedom were 9.

4.3.2.2 Analysis.

Independent transient transfections with the chimeric CAT/PrP- 3'UTR constructs were completed in primary cell lines derived from brain and liver tissue from the Icelandic sheep to investigate the role of the 3'UTR in gene expression and tissue specificity. As described in Section 4.2.2, the CAT data tended to vary in magnitude between experiments. For statistical analysis on the calculated relative CAT activty (%) was transformed onto the $\log_e$ scale and ANOVA completed (Appendix B, Section 2.5). ANOVA showed that again transfection efficiency did not effect the expression of the transfected plasmids and that the pattern of variation between the plasmids themselves was consistent and significant. Further analysis on the standardised data was completed using t-test (Tables 4.4 and 4.5).

In both cell lines expression from the constructs resembling the *in vivo* PrP mRNA 3'UTRs (pEYR and pD17) was lower than the remaining constructs, with the exception of pD20 in the cerebellum cell line (Figure 4.5A). As before, the level of detectable CAT activity from the constructs the 3'UTR deletions increased when compared to pEYR. For IS120.Cer this increase began sharply when sequence upstream of 3'UTR nucleotide (nt) position 2700 had been deleted (construct pD27), and remained high with deletions up to nt 3600 (constructs pD34 and pD36, $p=$ ≤ 0.06 and 0.05 respectively, Table 4.4, Figure 4.5A). Furthermore in IS120.Cer, the largest and most significant increase in CAT activity was detected between construct pD20 and pD27 ($p=\leq 0.02$, Table 4.4). For IS120.Liv a steady increase in CAT activity was observed during sequential deletion of the 3'UTR, peaking with a deletion upto nt position 3400 (construct pD34, Figure 4.5B). Expression of the CAT/PrP-3'UTR constructs in IS120.Liv did not reveal significant CAT from any one construct on comparison with pEYR. A direct comparison of expression from the CAT/PrP-3'UTR constructs in the two cell lines did show a tendency for the level of CAT expression from pD20 to be lower in IS120.Cer compared with IS120.Liv, although this was not highly significant ($p=\leq 0.08$, Table 4.5).

4.4 Comparison of Transient Expression Chimeric CAT-PrP 3'UTR Plasmids in Mouse and Sheep Derived Cell Lines.

4.4.1 Introduction

To identify a sheep specific regulatory region within the PrP 3'UTR which may influence gene expression a comparison was made between the published CAT/PrP-3'UTR data in the mouse N2a cell line (Goldmann *et al.*, 1999) and the sheep tissue-derived cell lines. In all experiments, expression of CAT from the experimental plasmids was standardised against the level of CAT from the control plasmid pCAT3-Promoter (Promega Ltd, UK), which was assumed to be 100% on each occasion, and the relative CAT activity calculated. For the purpose of this comparison, the previously published CAT data in mouse N2a cell was standardised on the $\log_e$ scale and the mean CAT activities from each cell line plotted. A comparison between the mean CAT activity in all cell lines was completed (Figure 4.6, Table 4.6). Also considered was a comparison of CAT/PrP-3'UTR constructs expressed in the stable and primary brain cell lines (Table 4.7).

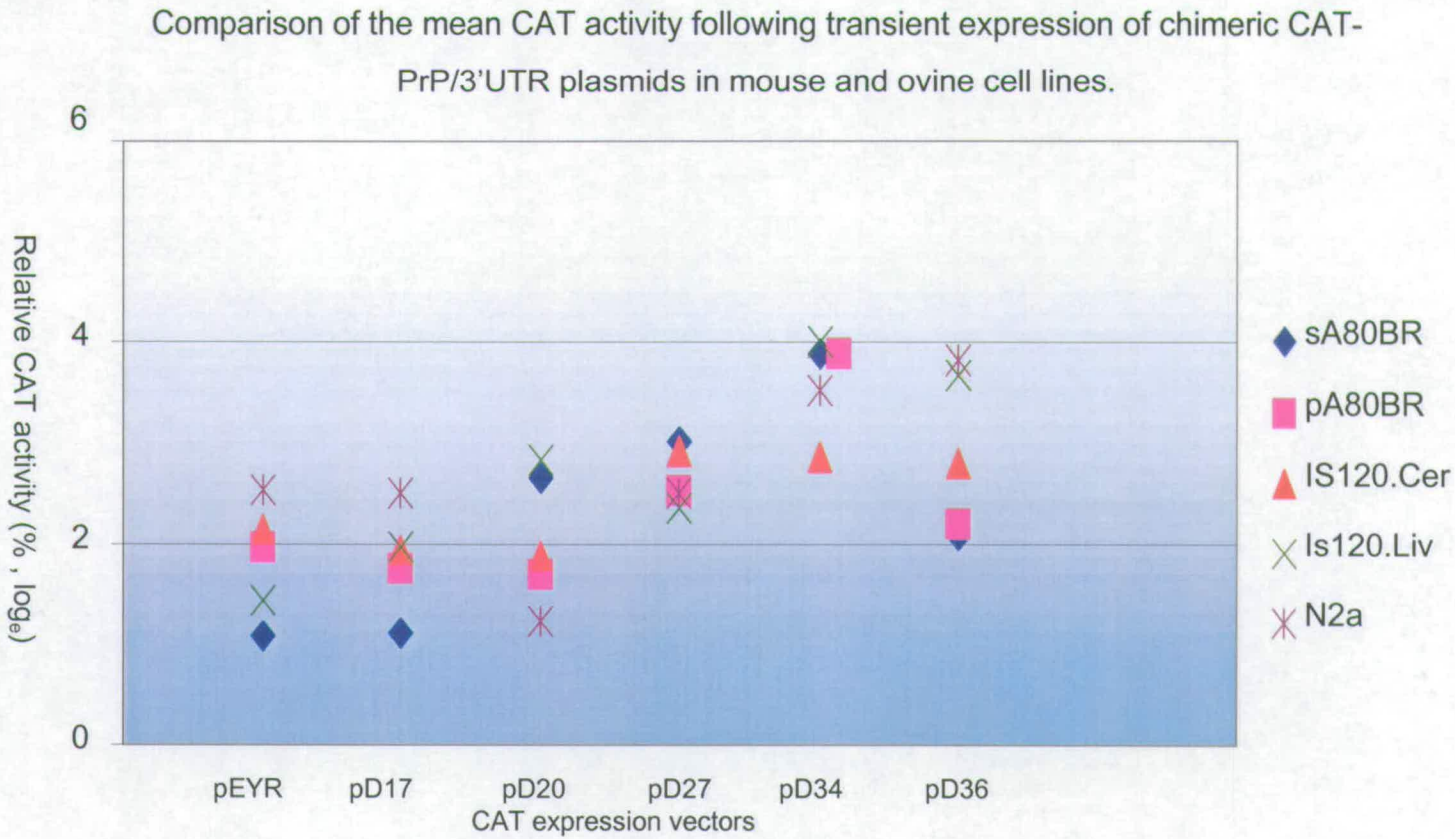

Figure 4.6. A comparative study of the mean transient CAT expression levels from chimeric CAT-PrP/3'UTR plasmids in ovine immortal (sA80BR and pA80BR) and primary (IS120.Cer and IS120.Liv) cell lines with the published data from a mouse N2a cell line (Goldmann *et al* , 1999)

Table 4.6 Statistical analysis of expression from CAT/PrP-3'UTR constructs in cell lines sheep cell lines compared with mouse N2a cell line (Goldmann et al., 1999).

Constructs expressed in N2a cells	Comparative Expression in cell line (p≤)			
	sA80BR	pA80BR	IS120.Cer	IS120.Liv
pEYR	0.31	0.47	0.72	0.49
pD17	0.25	0.47	0.57	0.65
pD20	0.006	0.51	0.13	0.009
pD27	0.67	0.97	0.54	0.41
pD34	0.81	0.83	0.64	0.75
pD36	0.33	0.29	0.62	0.93

Note. The significance of the change in CAT expression in the sheep cell lines compared to the mouse N2a cell lines as sequential deletions were introduced into the 3'UTR is considered. Analysis was completed by a t-test on the transformed data summarised in Appendix B, Tables 2.2.7 and 2.4.6. Mouse data presented in Goldmann et al., 1999 and provided by Dr Gerard O'Neill for analysis.

Table 4.7 Statistical analysis of expression from CAT/PrP-3'UTR constructs in stable Cheviot brain cell lines sA80BR and pA80BR compared with the primary cerebellum cell line, IS120.Cer.

Constructs expressed in IS120.Cer cells	Comparative Expression in cell line (p≤)	
	sA80BR	pA80BR
pEYR	0.31	0.58
pD17	0.36	0.84
pD 20	0.01	0.79
pD27	0.77	0.41
pD34	0.008	0.27
pD36	0.016	0.16

Note. The significance of the change in CAT expression in the stable cheviot-brain cell lines compared to the primary sheep-cerebellum cell line as sequential deletions were introduced into the 3'UTR is considered. Analysis was completed by a t-test on the transformed data summarised in Appendix B, Tables 2.2.7 and 2.4.6.

4.4.2 Results.

4.4.2.1 Effect of tissue origin.

The standardised mean CAT activity measured from transient expression of plasmids pEYR (*in vivo* 4.6kb PrP mRNA 3'UTR, Figure 4.1) and pD17 (in vivo 2.1kb PrP mRNA 3'UTR) in mouse N2a cell was not statistically different on comparison with IS120.Liv, IS120.Cer and pA80BR cell lines. There was a tendency for expression of pEYR and pD17 to be lower in CAT activity in sA80BR cell line compared to N2a (Figure 4.6). However, due to the large standard variation observed for pEYR and pD17 expression in sA80BR this difference in not significant (Table 4.6). No significant difference in transient CAT expression was observed from plasmids with PrP- 3'UTR sequence in either the pA80BR or the IS120.Cer cell line compared with the N2a cell lines. In contrast, the mean value of CAT measured from construct pD20 was significant higher in both sA80BR and IS120.Liv compared with equivalent expression in N2a cells (p=≤0.006 and 0.009 respectively, Table 4.6).

4.4.2.2 Effect of stable or primary cell lines.

There was no significant difference in expression from any of the constructs between cell line IS120.Cer and cell line pA80BR (Table 4.7). However, deleting sequence upstream of nt positions 2000 (pD20) and 3400 (pD34) lead to significantly higher CAT expression in sA80BR cell lines compared with IS120.Cer (p=≤0.01 and 0.008 respectively Figure 4.6 and Table 4.7). In contrast a deletion upto nt position 3600 resulted in significantly lower CAT expression in sA80BR compared with IS120.Cer (p=≤0.016, Figure 4.6 and Table 4.7).

4.6 Discussion.

4.6.1 Effect of the Ovine PrP genotype on *in vitro* CAT-PrP 3'UTR Expression

Transient transfection experiments show that the amount of detectable CAT protein can be controlled by presence of particular 3'UTR sequence of the ovine PrP gene. Interestingly there was no significant difference in CAT expression from CAT/PrP-3'UTR constructs which resembled the in vivo PrP mRNAs (pEYR and pD17, Figures 4.1, Tables 4.3, 4.5, 4.6 and 4.7). In general, an increase in CAT activity was observed as deletions were introduced in the 5' to 3' direction (Figures 4.3 and 4.5). This may be suggestive of increasing efficiency of transcription/ translation as the transcripts become shorter, allowing for quicker processing and loss of instability motifs. However, a comparison of the CAT activity detected with regards to the length of UTR sequence shows this is not the case. CAT expression from pEYR (UTR length= 1.9kb) and pD17 (UTR length = 0.7kb) are the same level in each of the cell lines, also pD36 (UTR length= 0.9kb) is not significantly higher than the longest length.

An alternative explanation is the deletion of sequence that is capable of repressing/ inhibiting expression in the full-length transcript. Data presented here shows that the presence of sequence between nt positions 2000-27000 of the ovine PrP 3'UTR can significantly repress transient CAT expression in sheep derived cell lines. Over and above general trend of increased expression, as deletions are introduced into the 3'UTR sequence in the 5' to 3' direction a significant increase in protein expression was observed when sequence 2000-27000 was deleted in cell lines sA80BR (scrapie susceptible Cheviot brain-derived) and IS120.Liv (scrapie susceptible Icelandic liver-derived). This response was not observed in either the pA80BR (scrapie resistant Cheviot brain-derived) or IS120.Cer (scrapie susceptible Icelandic brain-derived) cell lines.

The association between genotype and scrapie susceptibility is well documented with the $VV_{136}RR_{154}QQ_{171}$ genotype linked to highest susceptibility in all breeds and $AA_{136}RR_{154}RR_{171}$ the lowest. (Section 1.x). The presence of V at codon 136 shows increased susceptibility with A136 may offer resistance. When V_{136} is rare in a breed, scrapie predominantly occurs in carriers of the A_{136} allele. This is the case with the Icelandic sheep breed. If the function of the putative repressor element at PrP 3'UTR sequence 2000-2700 was to act on susceptible genotypes alone it might be expected that the same pattern of transient CAT expression would have been observed in the cell lines of the same scrapie status. However both sA80BR and IS120.Cer are derived from brain tissue from scrapie-susceptible sheep but show significantly different CAT protein expression patterns from three of the CAT constructs (Table 4.7). Instead cell lines pA80BR and IS120.Cer, which have similar PrP genotypes ($AA_{136}RR_{154}QR_{171}$ and $AA_{136}RR_{154}QQ_{171}$ respectively), show similar CAT expression profiles. This observation argues for an alternative hypothesis that an additional factor may also be involved in influencing scrapie susceptibility.

This thesis proposes that a repressor element functioning through the ovine PrP 3'UTR at nt sequence 2000-27000 may be involved in controlling gene expression and that loss of this control may increase scrapie susceptibility. It is proposed that the sequence contained with in the nt 2000-2700 may reduce protein levels to greater effect in sheep with genotypes normally associated with resistance. The putative repressor region could function alone as a structural motif or recognition factor for nucleic acid binding proteins but does not appear to function efficiently in ovine brain tissue containing at least one $A_{136}R_{154}Q_{171}$ allele. Alternatively, this region may interact with sequences at the 5' or 3' region of the PrP gene. It is interesting to note that this repressor region is 5' of the polymorphic *Eco*RI site (nucleotide position 3440), loosely associated with scrapie incidence (Hunter *et al.*, 1991). These issues will be the subjects of more discussion in Chapter Six.

4.6.2 Effect of peripheral or neuronal tissue origin on gene expression.

In the previous section, a putative repressor was described within the 3'UTR, nucleotide position 2030-2700, which may function to increase scrapie susceptibility with regards to gene expression in brain-derived cell lines. Although this result may lead to further study in scrapie susceptibility it fails to address whether the 3'UTR shows any tissue specific control over gene expression besides alternative polyadenylation. Results presented here again confirm that sequences within the 3'UTR are capable of influencing *in vitro* protein levels but are not responsible for tissue specific expression. The level of PrP expression within liver tissue has been difficult to determine. To date, in liver tissue PrP protein has not been reported (Horiuchi *et al.*, 1995) and PrP mRNA has only been shown at very low levels in normal liver (Goldmann *et al.*, 1999) but has been shown to increase in activated hepatic cells (Ikeda *et al.*, 1998). If the 3'UTR was responsible for controlling selective tissue expression it may have been expected that CAT protein levels would have been drastically different, or negligible, in the liver cell line compared with the cerebellum cell line. However there was no significant difference in the overall mean level of CAT protein expression, and only marginal differences in expression of some of the plasmids.

CAT protein levels detected in the cerebellum cell line from the plasmid containing sequence deleted upstream from nt position 2000 (pD20) were significantly lower when compared to expression from the construct with sequence upstream from nt 2700 (pD27). Expression from pD20 also had the tendency to be lower in the brain cell line compared with the liver cell line. Deleting sequence inclusive of nts 2000-2700 did not significantly alter expression in the liver cell line. These observations would suggest that the putative repressor region was not functional in ovine liver cells. In general, protein levels were higher in the liver cell line compared to the cerebellum cell line, in contrast to the *in vivo* pattern of expression of PrP. This may indicate that brain tissue is more efficient in controlling gene expression.

In context with the results presented for PrP genotype and susceptibility, the increase in CAT expression as a result of deletions between nucleotides 2000 and 2700 may

be due to the presence of a repressor element which is not functional in peripheral tissue of sheep. Whether this statement can be applied generally to sheep peripheral tissues or to tissues from scrapie susceptible sheep is unknown, as only one peripheral tissue has been tested within this study. The issues raised in this Chapter will be discussed in full in Chapter Six.

Chapter 5 : The Ovine PrP 3'UTR: Differential Control of PrP Gene Expression in Ovine Brain and Peripheral Tissue?

5.1 Introduction.

Although the genetic structure of the ovine PrP gene has been determined (Chapter 1, Section 1.8), factors controlling the expression of the gene are still unclear and may be vital to our understanding of scrapie development. Hunter *et al.* (1994) reported that PrP gene expression was not exclusive to the CNS when they demonstrated that PrP mRNA could be detected in neural and non-neural tissues of developing sheep and mice. It was also found that in sheep peripheral tissues in addition to the previously reported 4.6kb mRNA (Goldmann *et al.*, 1990), a second mRNA species of 2kb was present (Hunter *et al.*, 1994; Horiuchi *et al.*, 1995). The 4.6kb mRNA is found in all tissues, with the highest expression level in brain. The 2.1kb mRNA is found in peripheral tissues at levels of up to 30% of total PrP mRNA, but only at very low levels in the brain (Horiuchi *et al.*, 1995; Goldmann *et al.*, 1999). The smaller mRNA is also found in goats at comparative levels to sheep but only at very low levels in cattle (Goldmann *et al.*, 1999). The sheep 2.1kb mRNA is the result of an alternative polyadenylation signal at an upstream site in the 3'UTR at position 1522 (Goldmann *et al.*, 1990). The 4.6kb transcript is polyadenylated (poly(A)) at position 4046. The truncated PrP mRNA lacks several features present in the 4.6kb mRNA, such as repetitive sequences, instability motifs and a highly conserved 3'region (for more detail refer to Chapter 1, Section 1.8.4). At present there is no data regarding the stability of either the 4.6kb or 2.1kb PrP mRNA. Further more, semi-quantitative RT-PCR on constructs encoding the CAT reporter gene and PrP 3'UTR sequence resembling the *in vivo* 4.6kb and 2.1kb mRNAs showed no difference in stability or translatability associated with the 3'UTR (Cheung, 1996).

Horiuchi *et al.* (1995) confirmed the findings of Hunter *et al.* (1994) and also reported that PrP^C was detectable in neural and non-neural tissues such as spleen and lungs. Comparing PrP mRNA and PrP^C protein levels in sheep, the proportional relationship of PrP mRNA and PrP^C varied between the brain and other tissues (Horiuchi *et al.*,

1995). For example: PrP mRNA was found at levels 5 x higher in brain than kidney but the amount of PrP^C detected in brain was 40 x more than kidney. The presence of the second PrP transcript in sheep suggests the possibility of a complex control mechanism in gene expression where two different PrP mRNAs could result in differential expression or cellular location of the PrP protein. As a result of differential expression processes such as translational efficiency and/or the course of PrP synthesis, including degradation, may differ between brain and other tissues. Although it is not known what contribution each sheep mRNA makes to the overall level of detectable PrP^C, Goldmann *et al*, 1999, proposed that the 2.1kb PrP mRNA may be the predominantly expressed mRNA in peripheral tissues (Goldmann *et al.*, 1999).

The aim of this Chapter is to confirm whether both *in vivo* 3'UTRs are capable of supporting PrP protein synthesis and estimate the contribution each sheep PrP mRNA makes to PrP protein levels (Chapter Four, this thesis; see also Cheung, 1996; Goldmann *et al.*, 1999). As with the chimeric CAT studies, tissue specificity of PrP expression and any associations with PrP alleles linked to resistance or susceptibility to scrapie was to be studied in sheep cell lines of known PrP genotype and tissue origin. The cell liens described in Chapter Three were used in this study. Three ovine PrP constructs were created encoding the ovine PrP promoter region (500bp upstream of transcription start site), the ovine PrP ORF, and various lengths of the PrP gene 3'UTR. The constructs were designed to produce transcripts representing the full length mRNA (4.6kb), the short mRNA species (2.1kb) or an intermediate mRNA species, formed by alternative processing at potential poly(A) sites (conserved sites at nucleotide(nt) positions 2221,2284,2668) downstream from the 1522 site (Goldmann *et al.*, 1990). To detect transient PrP protein expression from these constructs, the cloned sheep PrP open reading frame (ORF) was manipulated to encode the hamster PrP specific 3F4 epitope in the amino acid sequence. These vectors were transiently transfected into ovine brain cell lines and any difference in expression was observed by Western analysis with the 3F4 anti-PrP antibody.

5.2 Designing Constructs for Transient PrP Expression.

5.2.1 Introduction

To allow study of differential expression of PrP *in vitro* due to alternative polyadenylation, constructs were designed to encode identical 5' and coding sequences but different lengths of ovine PrP 3'UTRs. Work completed at the NPU has shown that a 550bp region upstream from the Cheviot PrP transcription start site is capable of driving expression of CAT reporter gene *in vitro* (O'Neill, in preparation). The mouse PrP promoter has also been shown to be a strong driver of *in vitro* CAT expression (Baybutt and Manson, 1997). The constructs created for transient expression of ovine PrP were cloned to contain the Cheviot sheep promoter/ exon I region and a Suffolk sheep exon III, PrP allele $A_{136}R_{154}Q_{171}$ and e1. The cloned regions of the Cheviot PrP gene used in the new constructs were available at the NPU, full graphical representation found in Appendix A, Figures A.1-A.10.

The plasmid pNPU7PM carries the full length PrP exon III, and is expected to generate transcripts equivalent to the two in vivo PrP mRNAs by processing at known active poly(A) signals (nt 1522 and 4046; Goldmann *et al.*, 1990; Cheung, 1996; Goldmann *et al.*, 1999). Transcripts may also be produced from other conserved poly(A) signals between the two known functional sites (nt 2221, 2281, 2668). Construct pNPU2PM differs in the 3' region to pNPU7PM as it contains a truncated exon III which will allow transcription of one mRNA species only using the poly(A) site at 1522. Construct pNPU3PM contains an exon III of intermediate length encoding consensus polyadenylation signals at sites 1522, 2221, 2281 and 2668, of which the signal at 1522 is already known to be active. There is the possibility that any of the remaining signals could be used to form intermediate length mRNAs. At the start of the cloning project the levels of endogenous sheep PrP^C expression in the sheep cell lines were unknown, as the cell lines were not available. The original expression constructs, which would also express sheep PrP^C, later had to be redesigned to contain sequence encoding the hamster 3F4 epitope which allows for detection of protein produced by the reporter gene against a background of sheep PrP^C by means of using the anti-hamster PrP antibody 3F4 (Kascsak *et al.*, 1987; Section 5.3).

5.2.2 Construct pNPU7PM.

To study protein levels in relation to the full length PrP gene 3'UTR construct pNPU7PM was designed (Figure 5.1). The full length Suffolk PrP exon III, PrP allele $A_{136}R_{154}Q_{171}$ and e1, has been previously cloned and published (Goldmann *et al* 1990, accession number M31313.em_om). The fragment was available at NPU (construct p71, Appendix A, Figure A.3). The Cheviot promoter/exon I region was also available at the NPU, plasmid pNPU110-1, cloned from an NPU Cheviot sheep, PrP genotype $AA_{136}RR_{154}QQ_{171}$ (Goldmann, personal communication, Appendix A, Figure A.4).

To facilitate cloning of the promoter/exon I fragment upstream of exon III, restriction sites were created by PCR into the 5' and 3' ends of the promoter/exon I fragment generated from clone pNPU110-1 (Appendix A, Figure A.4) as no suitable cloning sites were present. Restriction digestion sites *Sal*I and *Sst*I were introduced by PCR to flanking regions of the promoter/exon I region using primers SstPM and SalPM (Table 2.1, Chapter 2 for full details). The 550 base pair fragment produced by PCR was sequenced in full before further cloning to ensure no mutations had been introduced (Section 5.2.5). Using restriction sites *Sal*I and *Sst*I, the promoter fragment was cloned upstream of PrP exon III in p71 to create vector pNPU7PM (Figure 5.1). The structure of the final construct was confirmed by restriction mapping and PCR with primers available at NPU (A025 and A045, Chapter Two, Table 2.1) which target sequence 3' of the promoter and 5' of the PrP open reading frame respectively.

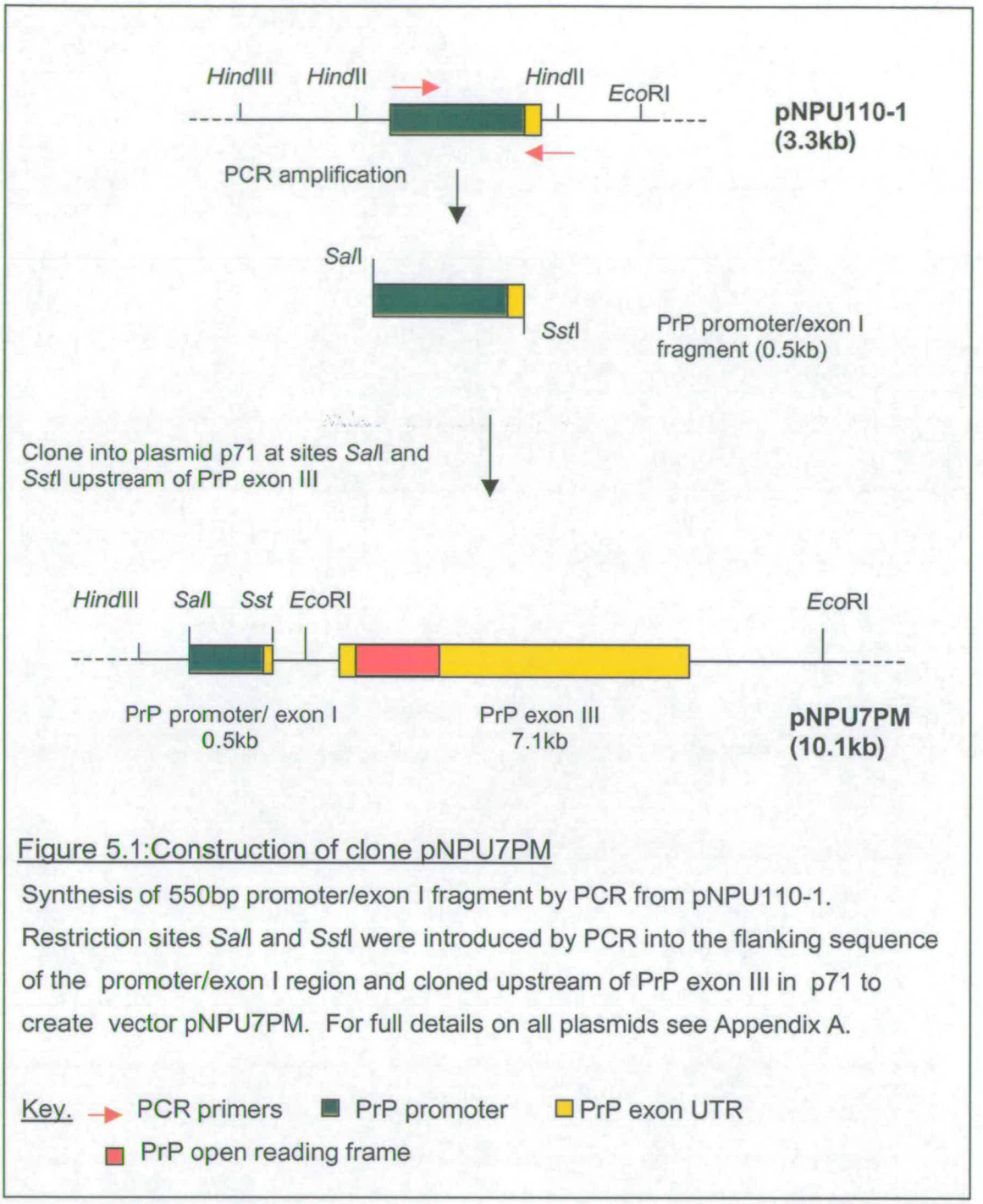

Figure 5.1:Construction of clone pNPU7PM

Synthesis of 550bp promoter/exon I fragment by PCR from pNPU110-1. Restriction sites *Sal*I and *Sst*I were introduced by PCR into the flanking sequence of the promoter/exon I region and cloned upstream of PrP exon III in p71 to create vector pNPU7PM. For full details on all plasmids see Appendix A.

5.2.3 Construct pNPU3PM.

It is not yet known whether polyadenylation signals other than those at 1552 and 4046 present in the 3'UTR are actively selected to produce alternative PrP mRNA transcripts *in vivo* at low levels. Construct pNPU3PM was therefore designed to display the conserved polyadenylation signals upstream from the signal at 4046bp, encoding mRNAs of intermediate lengths. The Cheviot sheep PrP promoter/exon I from pNPU110-1 was cloned into pBluescript (Stratagene, UK, Appendix A, Figure A.6) then into pGEM-7Zf(+) (Promega, UK) by ApaI – XbaI digestion to form pGEM.promoter (Appendix A, Figure A.7). The two step cloning strategy of the promoter region was necessary due to the high number of repeated restriction sites within the 3'UTR sequence. It was not possible to produce pNPU3PM by truncating pNPU7PM for the same reason. Instead, restriction digestion of p71 by *Eco*RI and *Cla*I produced a PrP exon III fragment of 3.6kb which was cloned into pGEM-7Zf(+) to create pGem3kb (Appendix A, Figure A.8). The promoter/exon I fragment was cloned upstream of the truncated PrP exon III in pGem3kb by *Xho*I digestion to create pNPU3PM (Figure 5.2). The structure of the final construct was confirmed by restriction mapping as well as PCR targeted to the promoter region and the 5'region of the PrP open reading frame.

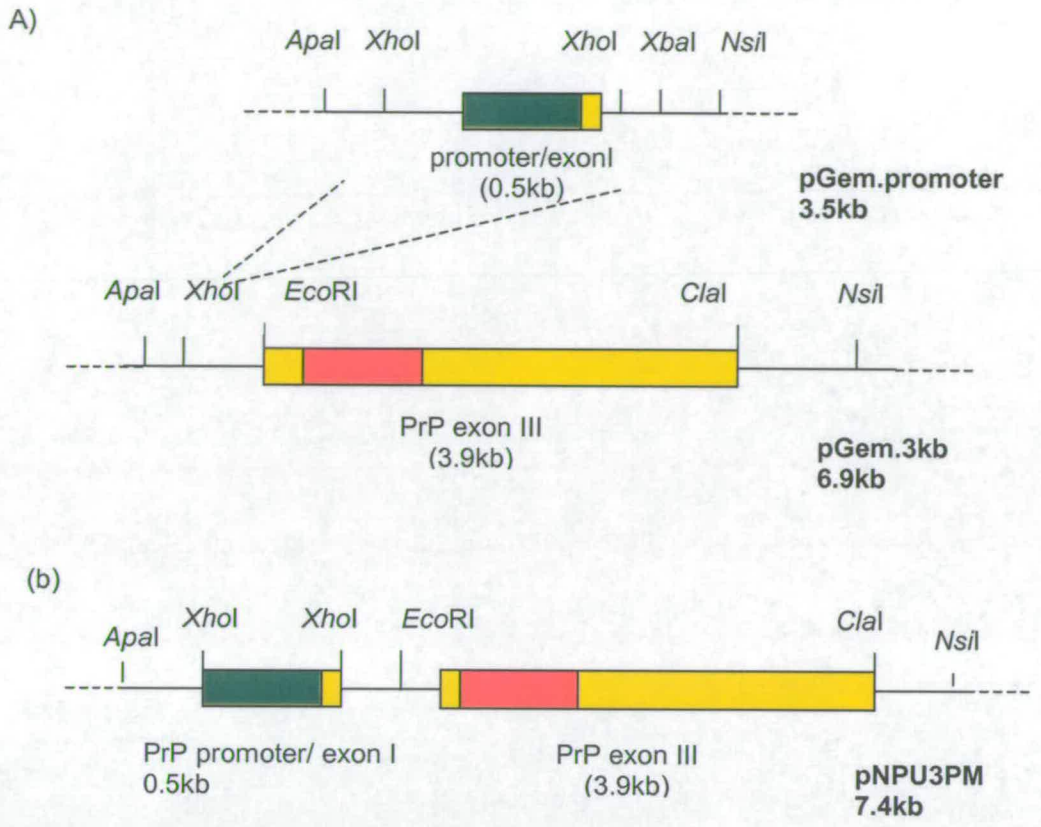

Figure 5.2: Construction of clone pNPU3PM.

A) Restriction digestion and purification of *Xho*I PrP promotor/ exon I fragment from pGem-promoter. B) Cloning of the PrP promoter/ exon I fragment upstream of the PrP modified exon III in pGem.3kb at site *Xho*I. For full details on all plasmids see Appendix A.

Key. PrP promoter PrP exon UTR PrP open reading frame

5.2.4 Construct pNPU2PM

To study protein expression *in vitro* in association with the 2.1kb PrP mRNA 3'UTR the vector pNPU2PM was designed to contain the ovine promoter, exon I and a truncated exon III with consensus polyadenylation signal nt 1522. A 1.5kb fragment was removed from pNPU3PM exon III (Section 5.2.3) by *Kpn*I-*Nsi*I digestion which, after gel purification, followed by *Pst*I digestion. The resulting 0.4kb *Kpn*I-*Pst*I fragment was again gel purified and inserted into the 5.9kb fragment produced by *Kpn*I-*Nsi*I digestion of pNPU3PM. The restriction digestion ends of *Pst*I and *Nsi*I are complimentary and were religated successfully to reform a circular plasmid and create pNPU2PM (Figure 5.3). The structure of the plasmid was confirmed by restriction mapping.

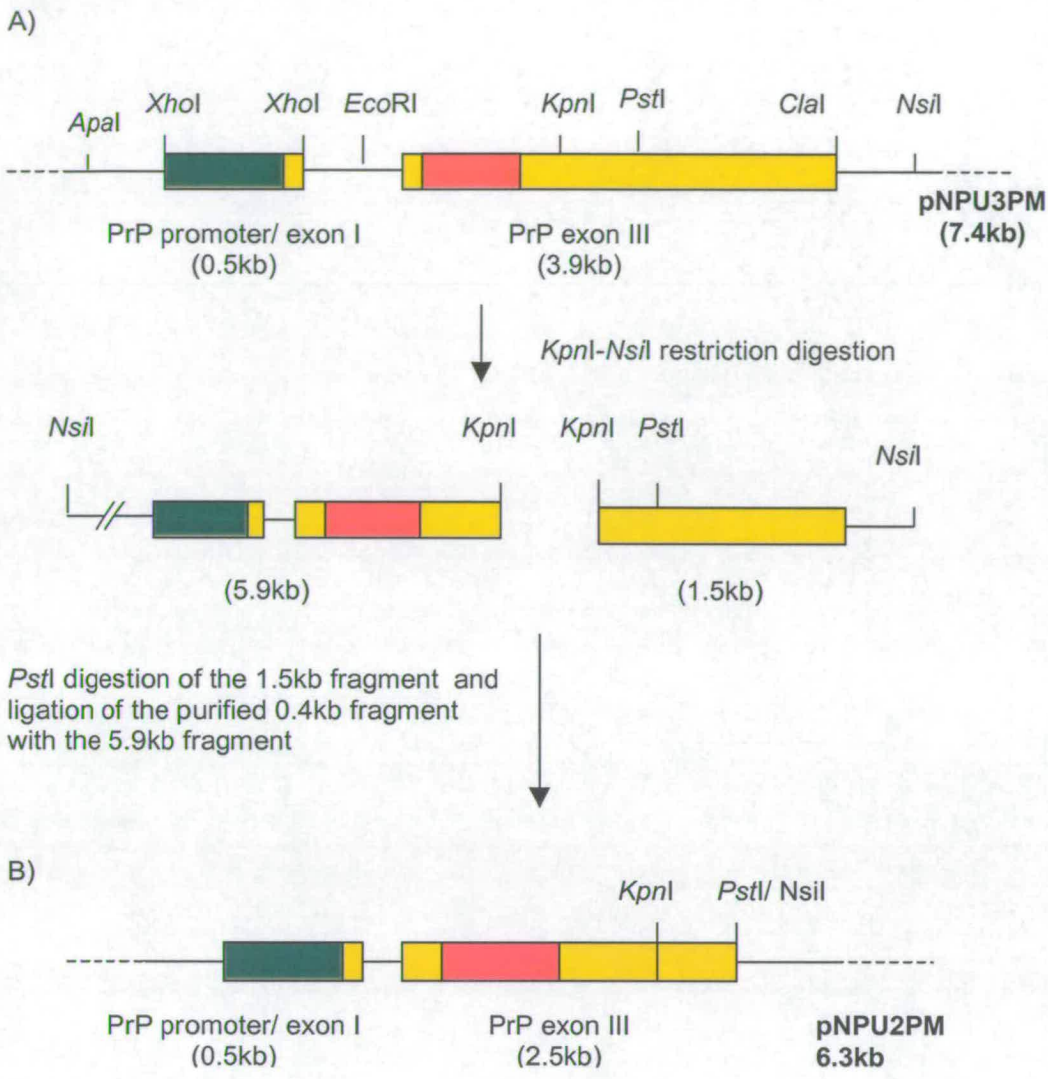

Figure 5.3:Construction of clone pNPU2PM.

A) *Kpn*I/*Nsi*I digestion produces a 5.9 kb and 1.5kb fragment, which were isolated, the 1.5kb fragment was further manipulated by *Pst*I digestion and a 0.4kb fragment purified. B) Ligation of 0.4kb *Kpn*I/*Pst*I fragment to *Nsi*I/ *Kpn*I 5.9kb PrP fragment possible due to complementary ligation of sites *Pst*I and *Nsi*I. For full details on all plasmids see Appendix A.

Key.- PrP promoter PrP exon UTR PrP open reading frame

5.2.5 *In vitro* expression of RNA from PrP constructs.

5.2.5.1 Introduction

Efficient gene expression requires recognised transcription promoter/ enhancer sequences, a transcription start site, correct splicing, and transcriptional termination signals (Lodish *et al.* 1995). Assuming that the full length PrP exon 3 contains the intact 3' acceptor site of splicing and 3' polyadenylation signals it was necessary to confirm that, following cloning, the ovine PrP constructs contained the consensus sequences for transcription initiation and splicing and so were capable *in vitro* expression.

5.2.5.2 Experimental approach.

Confirmation that transcription initiation and 5' donor splice sites were present was carried out by partial sequencing of the promoter region cloned into plasmids pNPU7PM and pNPU2PM (Section 5.5.5.3). Transcriptional activity from pNPU7PM and pNPU2PM was investigated by transiently expressing the constructs in mouse N2a cells as a protocol had not been developed for the ovine cell lines at this time. Expression from the constructs was determined by reverse transcription-polymerase chain reaction (RT-PCR, Section 5.2.5.4). Full details of the sequencing and RT-PCR protocols can be found in Chapter 2, Sections 2.3. As construct pNPU2PM was cloned from manipulating exon III of pNPU3PM, constructs pNPU2PM and pPU3PM had the same 5' PrP sequence. Therefore, *in vitro* expression detected from pNPU2PM was assumed to also represent transcriptional activity possible from pNPU3PM.

5.2.5.3 Sequencing promoter region for 5' donor site

Sequencing of cloned ovine promoter/ exon I region was completed as described in Section 2.3.4. Using different nucleotide primers targeted to the 5' and 3' regions of the promoter region, both strands of the promoter region were sequenced. The sequence data gathered was incomplete, perhaps due to difficulties encountered in attempting to sequence a region with a high GC content. However, a conserved 5' donor sequence was detected which suggests that splicing at the promoter/ exon I region should occur (Figure 5.4).

```
PrP plasmids;      1    ...gccc ccgcagctcc tcctctgcac ggcgactcac
                          |||| |||||||||| |||||||||| ||||||||||
   pNPU110-1;    391 ctccccgccc ccgcagctcc tcctctgcac ggcgactcac


PrP plasmids;     35 cagccctagt tgccagtcgc nnacngnngc agagnngaga
                     |||||||||| ||||||||||   || |  || ||||  ||||
    pNPU110-1;   431 cagccctagt tgccagtcgc tgacagccgc agagctgaga

PrP plasmids;     75 gcgncttctc tcccagaggc aggtaaa...
                     ||| |||||| |||||||||| |||||||
   pNPU110-1;    451 gcgtcttctc tcccagaggc aggtaaatag
                                          ↑ splice site
```

Figure 5.4: Sequence of ovine PrP promoter/ exon I region cloned into plasmids pNPU7PM and pNPU2PM. Consensus 5' splice site shown in bold text, splice site indicated by arrow.

5.2.5.4 *In vitro* detection of PrP mRNA expressed from PrP constructs.

Following transient transfection of mouse N2a cell lines with plasmid pNPU7PM and pNPU2PM mRNA was isolated and reverse transcribed. The protocol used is described in full in Section 2.3.6. Briefly, total RNA was precipitated following lysis of the cells using RNAzol. The same amount of total RNA was used for each RT-PCR reaction. PCR amplification of the purified mRNA molecules was completed using primers which were available at the NPU: A023 (targeting exon I region) and 13741 (targeting 5' region of exon III). The product of PCR on the reverse transcribed mRNA templates with primers A023 and 13741 (available at NPU, Appendix A) was predicted to be 250 bases in length if splicing occurred. If splicing did not occur a PCR product of approximately 750 bases would be detected as the cloned 3' sequence of intron II (approximately 500 bases) would not be spliced out. Furthermore, cross reaction of the primers with N2a cell endogenous PrP mRNA should lead to a PCR product of approximately 350 bases.

The result of one RT-PCR reaction is shown (Figure 5.5). Control parameters included within the experiment were RT-PCR and PCR on non-transfected N2a cells (lanes 4 and 7) and as a positive control for primers A023 and 13741, PCR was carried out on an ovine cDNA (RL110) supplied by Wilfred Goldmann (lane 3). PCR of the ovine cDNA clone should give a product of 350 bases as exon II will be present in the transcript. PCR-only was completed on total RNA extract from the transfected N2a cells to determine the level of remaining PrP constructs after transfection (lanes 5 and 6). RT-PCR products of 750 bases and 250 bases were detected following transient expression of the PrP constructs in N2a cells (lanes 8 and 9). The product at 250 bases is equivalent to successfully spliced mRNA transcribed from plasmids pNPU7PM and pNPU2PM. The product at 750 bases may reflect the presence of non-spliced mRNA or remaining plasmids template from the transfection reaction as determined in lanes 5 and 6. No PCR or RT-PCR products were detected in the negative controls, also PCR on the ovine cDNA produced a band of approximately 350 bases as expected. Therefore PrP constructs pNPU7PM and pNPU2PM have been shown by RT-PCR to be actively transcribed in a mouse N2a cell line.

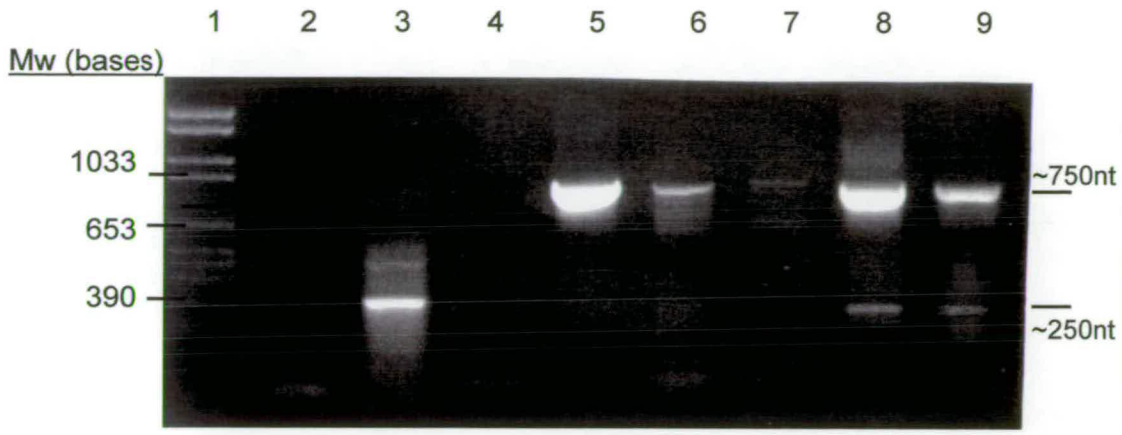

Figure 5.5:cDNA analysis of PrP constructs pNPU2PM and pNPU7PM in N2a cell line. Cell were harvested 49 hours post-transfection and total RNA extracted. Equivalent amounts of RNA were used for each RT-PCR reaction. Lane 1; 100bp ladder: lane 2;PCR negative control: lane 3; PCR positive control, ovine cDNA: lane 4; PCR of total RNA extracted from non transfected N2a cells: lane 5; PCR of total RNA extracted from N2a cells transfected with pNPU2PM: lane 6; PCR of total RNA extracted from N2a cells transfected with pNPU7PM: lane 7; RT-PCR on total RNA extracted from non-transfected N2a cells: lane 8; RT-PCR of total RNA extracted from N2a cells transfected with pNPU2PM: lane 9; RT-PCR of total RNA extracted from N2a cells transfected with pNPU7PM.

5.3 Creating the Hamster 3F4 Epitope by Site Directed Mutagenesis.

5.3.1 Introduction and approach.

To study expression of the PrP protein in ovine cell lines an assay system had to be developed to distinguish transiently expressed PrP^C from endogenous PrP^C. At the start of this study, the monoclonal antibody 3F4 was known to recognise specifically hamster PrP while not cross-reacting with sheep PrP at the sensitivity required for Western blot analysis (Kascsak *et al.*, 1987). It follows then that introduction of the 3F4 epitope into the sheep PrP amino acid sequence by mutation of the PrP nucleotide sequence should allow for the detection of transiently expressed PrP in sheep cell lines.

Mutations can be introduced into known cloned gene sequences by various methods that involve cleavage, degradation or synthesis of DNA. One advantage of this method is the precision with which the mutation can be introduced. The DNA template for oligonucleotide-mediated mutagenesis is normally ssDNA. When designing primers for single nucleotide mutagenesis several features must be considered;

1 Oligonucleotide primer must be generated complimentary to the correct strand of plasmid template for replication with DNA polymerase.

2 Length of the oligonucleotides must be sufficient to allow promoter annealing to the target sequence (approximately 17-19 nucleotides) with the desired mutation in the middle of the sequence.

3 Target DNA sequence should contain little sequence capable of forming complex secondary structure i.e. palindromic sequences.

4 Oligonucleotide should be unlikely to bind to regions on the DNA template other than the target sequence.

Alignment of the sheep and hamster PrP amino acid sequence (Figure 5.6) across the 3F4-recognition site shows that the sequences differ at one codon only. The sheep gene encodes for valine at position 115 as opposed to methionine in the hamster equivalent codon 112. Mutating the sheep coding region at nucleotide position 424 from G to A would convert codon 115 from valine to methionine, thus creating the potential 3F4 recognition site. This mutation also creates the restriction site *Nde*I within the PrP mutated ORF which can be used for screening during the cloning procedure.

A) Comparison of the hamster and ovine 3F4 epitope amino acid (aa) sequence:

Hamster: ^{104}K P K T N M K H M A G A A A^{117}

Ovine: ^{107}K P K T N M K H V A G A A A^{120}

B) Primer design for ovine PrP site directed mutagenesis.

Ovine aa sequence: ^{114}H V A G A A A A G A^{123}

Ovine nt sequence: 5′ ^{411}CAT GTG GCA GGA GCT GCT GCA GCT GGA GCA^{440} 3'

Mutagenic primer: 3' ^{29}GTA TAC CGT CCT CGA CGA CGT CGA CCT CG^{1} 5′
(designated E439)

Mutated .aa sequence: ^{107}K P K T N M K H M A G A A A^{120}

Figure 5.6: Mutating the sheep PrP nucleotide sequence to create the hamster 3F4 epitope in the amino acid sequence. A) Comparison of the hamster 3F4 epitope with the equivalent ovine amino acid (aa) sequence. Ovine sequence deviates from the hamster sequence at one codon only: codon 115 (red). B) Method used to design a reverse primer (E439) for mutagenesis of the ovine PrP nucleotide (nt) sequence spanning 411-439 to convert the sheep PrP codon 115 from valine to methionine thus creating the 3F4 recognition site.

5.3.2 Experimental Approach

Two variations on the oligonucleotide- mediated mutagenesis method were used in the attempt to introduce the single nucleotide substitution in the sheep ORF necessary to create the hamster 3F4 epitope. The first used a single mutagenic primer to induce synthesis of a DNA template, which would be amplified in a bacterial system. The second method was PCR based, introducing the mutation via a set of primers designed to amplify a precise region of the ORF, with one of the primers containing the mutation.

For both methods, primers were designed using the published Suffolk PrP exon III sequence (accession number M31313.em_om, Goldmann *et al* 1990). Full details of the primers sequences used can be found in Section 2.3, Table 2.1. Site directed mutagenesis was attempted using the clone pPSHPrP which was available at the NPU and contains only the PrP open reading frame and so increasing the likelihood of correct annealing of the primer to the target sequence (see Appendix A, Figures A.11-A.14).

5.3.2.1 Single-primer Oligonucleotide- mediated mutagenesis.

The method used for single primer oligonucleotide mutagenesis is full described in Section 2.3.3. Briefly, the oligonucleotide H3F4 was designed and used in mutagenic experiments with pPSHPrP. After annealing the phosphorylated mutagenic primer (10pmol/ml) to single stranded DNA (1μg) of pPSHPrP, the primer extension was allowed to occur over 15 hours at the appropriate temperature. DNA synthesis was initiated at the 3' terminus of the mutagenic primer and extended by DNA polymerase along ssDNA template to form a heterodulex. Once DNA synthesis was complete, XL-Blue *E.coli* cells were transfected and plated onto ampicillin agar plates. Colonies were screened for positive clones by differential hybridisation (refer to Section 2.3.3.2). Briefly, for screening large numbers of colonies, duplicate colony filters were produced by pressing nitro-cellulose membranes onto the experimental ampicillin plates. The filters were then treated to lyse cells and the DNA fixed to the membranes by UV irradiation. After four hours hybridisation, at 37°C, with radio-labelled oligonucleotides H3F4 (mutated PrP

sequence; Chapter Two, Table 2.1) or E377 (normal PrP sequence; Chapter Two, Table 2.1) the filters were washed twice at room temperature for 10 minutes, followed by a further 10 minutes at 47°C to remove any unbound probe then exposed to autoradiography film.

Colonies that appeared positive by differential hybridisation were further analysed by restriction digestion. Digestion of pPSHPrP with restriction enzymes *Hind*III, *Eco*RI and *Nde*I gives a defined pattern (Appendix A). The plasmid, pPSHPrP has a pUC 10/11 backbone with the PrP coding region inserted in the *Bam*HI site, digestion with *Eco*RI and *Hind*III cuts out the 0.8Kb PrP ORF fragment. Successful site directed mutagenesis at nucleotide 414 within the ORF would create a novel *Nde*I site, and is detected by digestion with *Eco*RI, *Hind*III and *Nde*I. The 0.8Kb *EcoRI/ Hind*III fragment should be digested with *Nde*I producing additional fragments of 0.35kb and 0.4kb.

5.3.2.2 Results – single primer mutagenesis.

Single primer mediated mutagenesis was repeated several times and any colonies that seemed potentially positive for the required mutant plasmid were assayed. Restriction fragment length polymorphism for the presence of *Nde*I was not detected in any colonies screened (results not shown). Most transformed cells appeared to contain only unmutated plasmids. Control digests were carried out to ensure that all enzymes used were active. It was therefore concluded that single-primer mediated mutagenesis had been unsuccessful at introducing the desired mutation into the sheep PrP ORF.

5.3.2.3 PCR –mediated mutagenesis.

Oligonucleotides specific to the sheep PrP ORF which were used to amplify a region approximately 250 bases in length (Chapter 2, Table 2.1). The first primer, p1, was available at the NPU and was homologous to the sense strand of the ORF from position 118-225, which includes a *Xma*I restriction site. The other primer, E439, was designed to be homologous to the anti-sense strand except at position 414 where it encoded a T instead of a C (Figures 5.6B, 5.7). Following PCR, the amplified fragment was digested with *Xma*I and *Pst*I, gel purified and ligated into pBluescript (Stratagene, UK) to create the construct pBSK-ORF.3F4. Potentially positive colonies for the mutated sequence were screened by restriction digestion with *Nde*I. Digestion with *Xba*I will linearize the transformed pBSK-ORF.3F4 (3.5kB), successful substitution at nucleotide 414 would have created a *Nde*I site therefore double digestion with *Xba*I/ *Nde*I was expected to produce fragments of 0.25kb.

5.3.2.4 Results – PCR mediated mutagenesis.

In contrast to the single-primer mediated mutagensis method, following ligation and transformation, PCR-mediate mutagenesis did not produce high numbers of colonies for screening, it was therefore possible to screen all colonies efficiently by restriction digestion with *Nde*I and *Xba*I. After three repeated attempts at PCR mediated mutagensis, a positive clone was identified by restriction digestion, the result was confirmed further by PCR (result not shown). Full sequencing (Figure 5.7) of the mutated PrP ORF ensured that no further mutations had been introduced into the fragment by PCR other than the intended mutation at nucleotide position 414. This plasmid is known as pPSH.3F4.

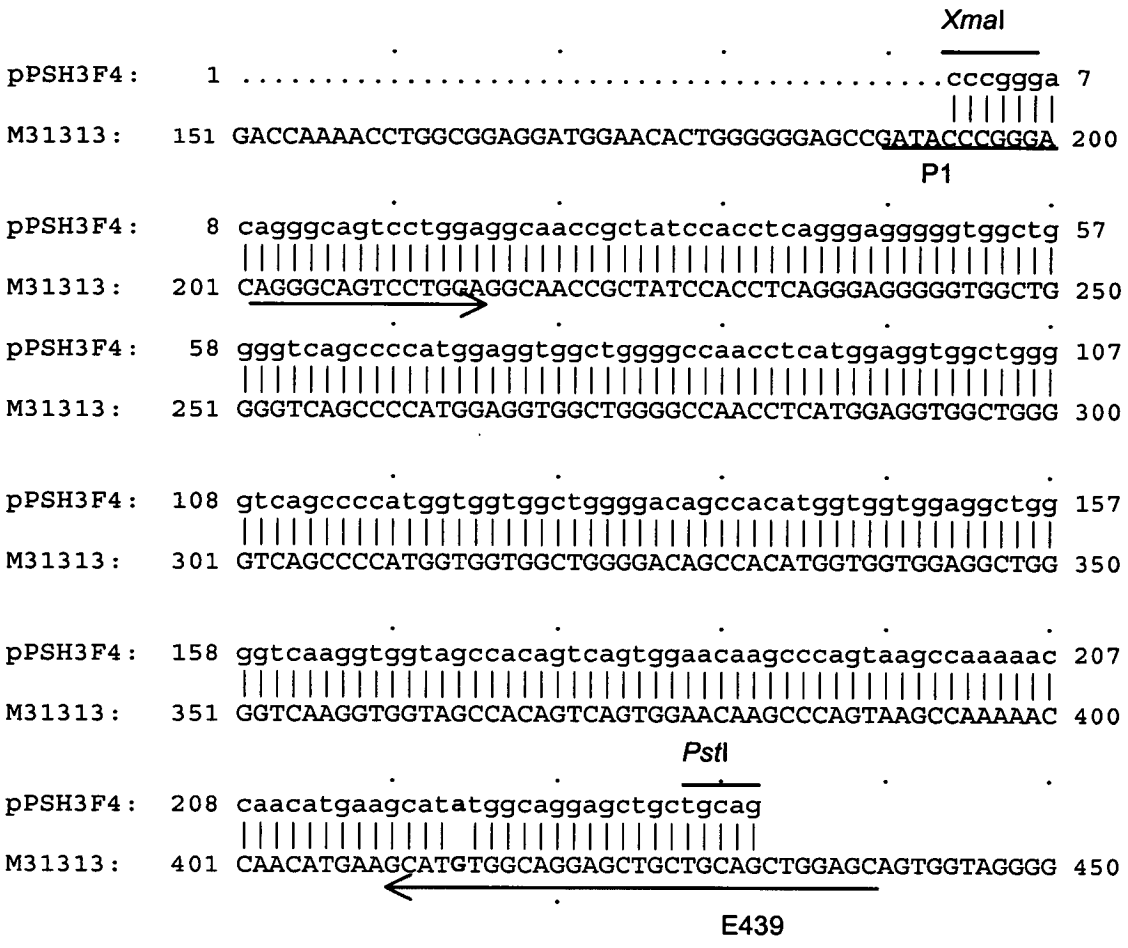

Figure 5.7: PCR mediated mutagenesis of the ovine PrP open reading. The plasmid pPSHPrP was used in mutagenesis studies to modify the ovine PrP open reading frame to encode the hamster 3F4 epitope. The clone pPSH3F4 was successfully created by PCR mutagenesis. Comparison of pPSH3F4 sequence (upper line) to the published Suffolk PrP open reading frame (accession number M31313.em_om, lower line) shows the mutation from G to A at the equivalent pPSH3F4 nucleotide position. Oligonucleotides represented by dark blue arrows.

5.3.3 Conclusions.

Site directed mutagenesis was successful in creating the hamster 3F4 epitope nucleotide sequence within the sheep ORF using the PCR mediated method rather than the single-primer mutagenesis method. The single primer method was chosen first primarily on the basis of theory – priming ssDNA synthesis from a mutagenic oligonucleotide should have conserved the mutation during dsDNA formation. Secondly the planned screening method of hybridisation and restriction digestion should have been efficient and should have reduced the background of non-mutated clones. However the method was time consuming and did not produce any positive clones. Several reasons may explain the failure of the single primer mediated mutagenesis. For example: incomplete polymerisation, primer displacement by DNA polymerase, and host mismatch-repair in favour of the original sequence. Many new methods now described use two or more primers for mutagenesis. The presence of the other primers allow for selection or prevent displacement of the mutagenic primer. In contrast the PCR-mediated mutagenesis was successful and produced a positive clone quickly. This method was convenient and efficient in producing the desired mutagenesis. Sequencing was completed to ensure that no further mutations were introduced by PCR.

5.4 Insertion of 3F4 Fragment into Clones pNPU7PM and pNPU2PM.

The mutated sheep nucleotide sequence in clone pPSH.3F4 (Section 5.3) was inserted into clone pNPU7PM and pNPU2PM to allow for detection of transiently expressed sheep PrP in sheep cell lines against a background of endogenous sheep PrP protein using 3F4 anti-PrP antibody. Due to time restrictions, a decision was taken to concentrate on transient expression of PrP from plasmids pNPU2PM and pNPU7PM as these constructs resemble the known ovine endogenous *in vivo* PrP mRNAs. Construct pNPU3PM was therefore not modified further by the insertion of the 3F4 sequence.

5.4.1 Experimental approach and results.

Briefly, the 3F4 epitope was created by PCR-mediated site directed mutagenesis in plasmid pPSHPrP to create the clone pPSH3F4 (Section 5.3). By restriction digestion with enzymes *Dra*III and *Hinc*II a 0.3kb fragment containing the mutated sheep sequence was isolated from pPSH3F4 and cloned into the construct pNPU2PM (Figure 5.8). Correct orientation and insertion of the fragment into pNPU2PM was confirmed by restriction mapping and partial sequencing. The presence of the 3F4 epitope was confirmed by restriction digestion with NdeI (Figure 5.10a, lane 3). The resulting plasmid, pNPU2.3F4, contains the PrP promoter/exon I, a truncated exon III to position 1929, and encodes for the 3F4 epitope in the amino acid sequence. Following conformation of the structure of plasmid pNPU2PM.3F4 it was possible to clone the 3F4 epitope into plasmid pNPU7PM to create pNPU7PM.3F4 (Figure 5.9). Restriction digestion of PNPU2.3F4 with *Bam*HI and *Xba*I lead to the purification a 1.5kb fragment carrying the PrP ORF mutated to encode the 3F4 epitope. The 1.5kb fragment was inserted into pNPU7PM using *Bam*HI and *Xba*I and confirmed by partial sequencing and restriction mapping. Again the presence of the 3F4 epitope was confirmed by digestion with NdeI (Figure 5.10B). Purification of the BamHI/Xba I site from pNPU7.3F4 and further digestion with NdeI produced a fragment of 1kb (Figure 5.10B, lane 5)

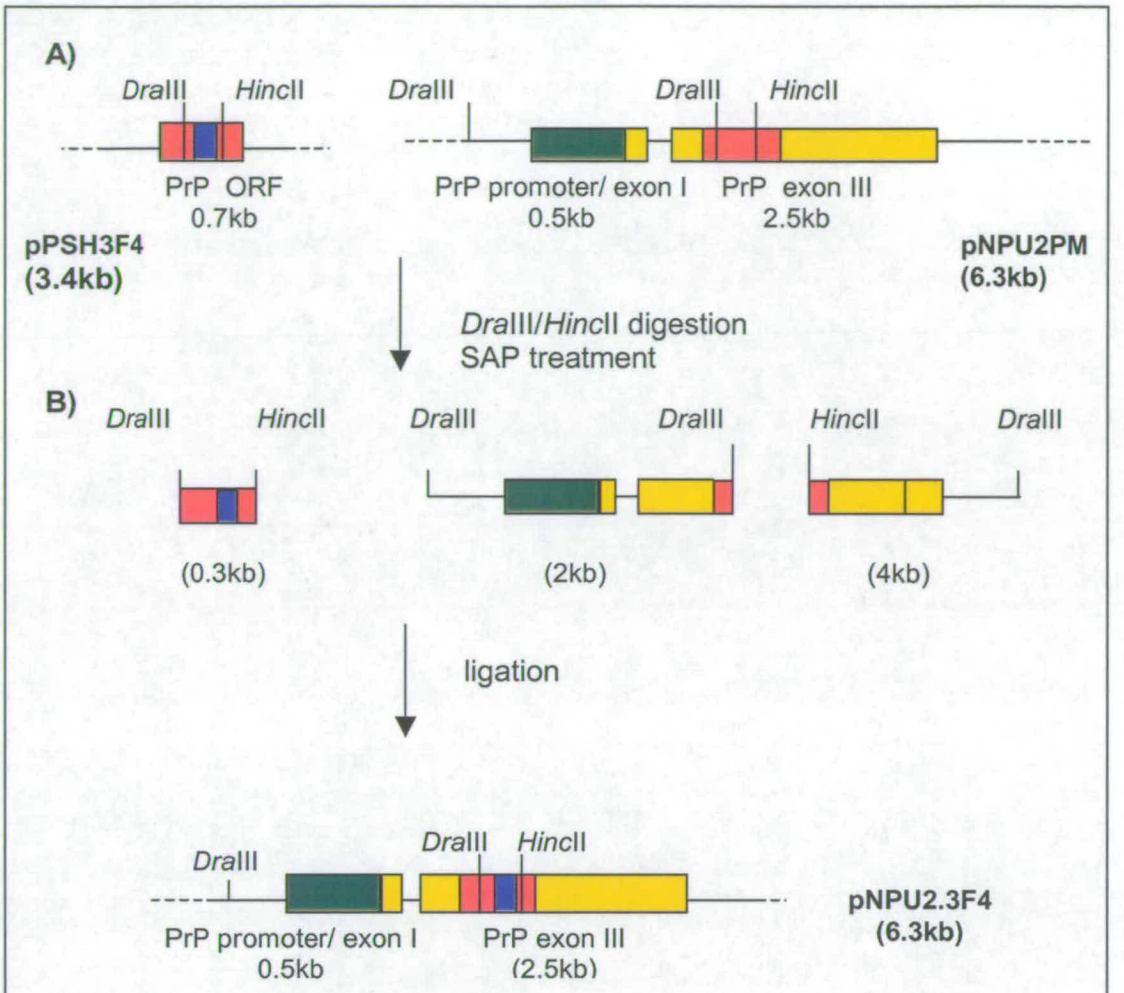

Figure 5.8: Cloning of pNPU2.3F4.

A) Restriction digestion with DraIII/ HincII and shrimp alkaline phosphatase (SAP) treatment of plasmids pNPU2PM and pPSH3F4. B) Fragments 2kb and 4kb were purified from pNPU2PM digestion and set up in a three way ligation with the 0.3kb fragment from pPSH3F4 digestion. The 0.3kb fragment carries the PrP coding region encoding the 3F4 epitope.

Key. PrP promoter PrP exon UTR PrP open reading frame 3F4 epitope

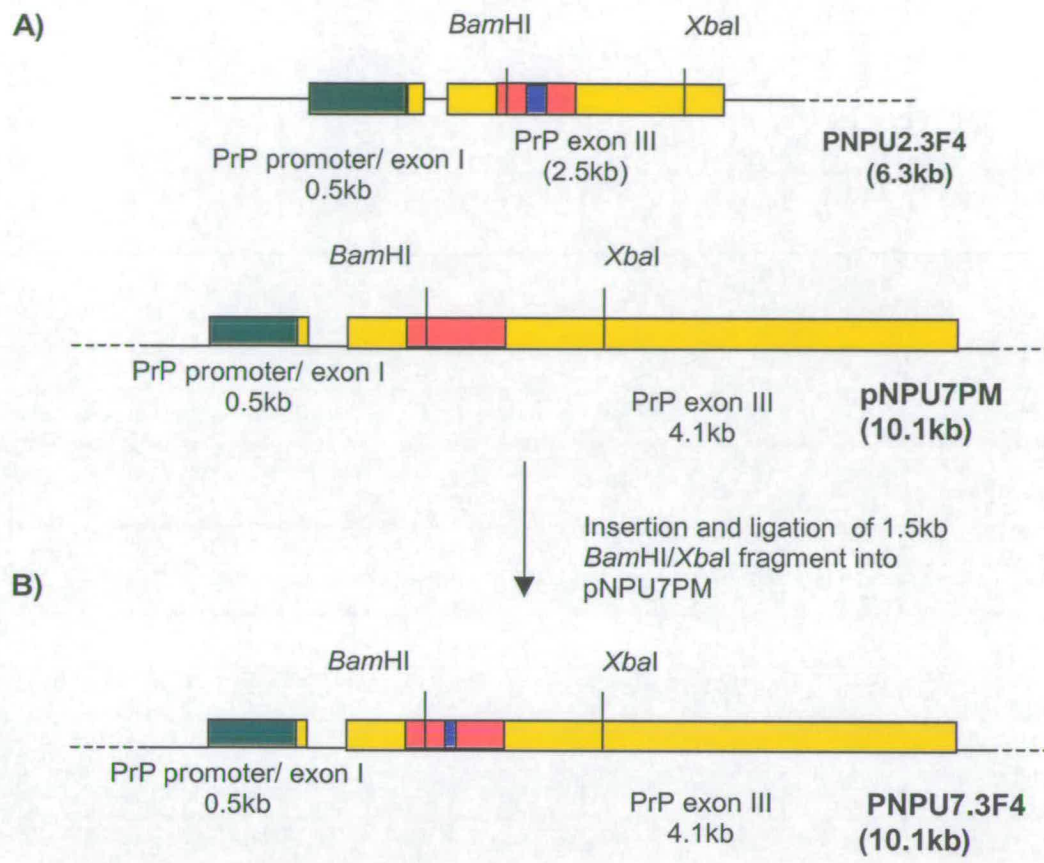

Figure 5.9: Cloning of pNPU7.3F4

A) Restriction digestion with *Bam*HI/ *Xba*I of plasmids pNPU3F4.2 and pNPU7PM. B) The 1.5kb fragment from pNPU3F4.2 digestion, carrying the 3F4 epitope, was ligated into the 8.6kb fragment of pNPU7PM after *Bam*HI/*Xba*I digestion to form pNPU3F4.4

Key. PrP promoter PrP exon UTR PrP open reading frame 3F4 epitope

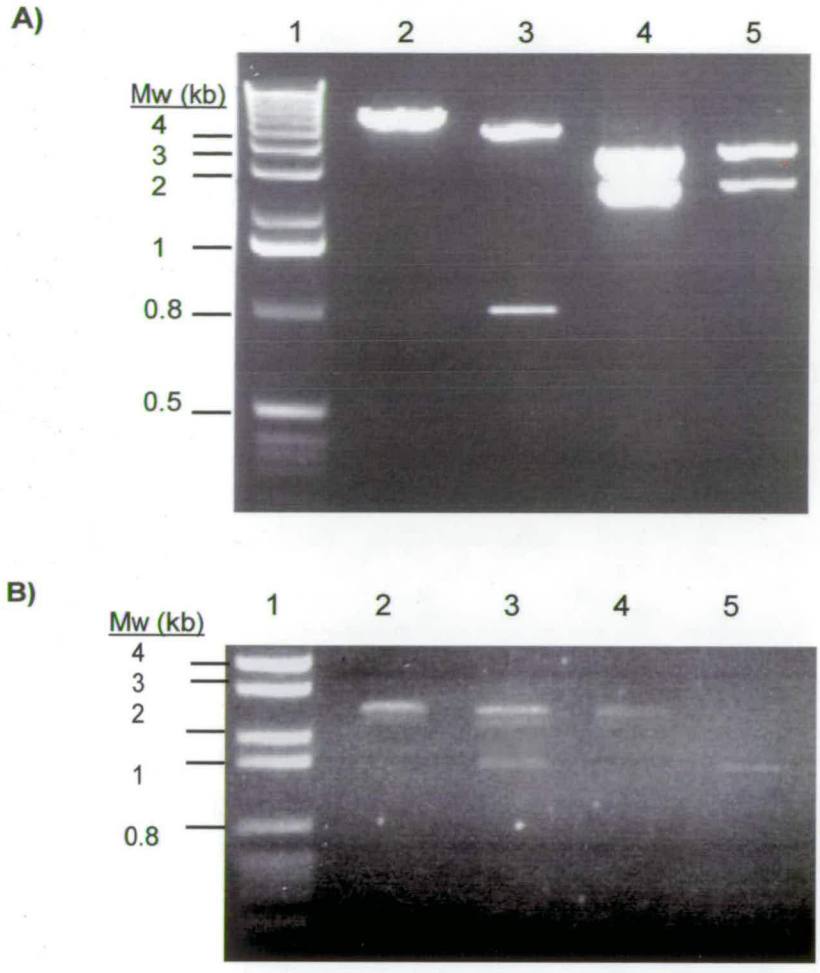

Figure 5.10: Screening pNPU2.3F4 and pNPU7.3F4 clones for mutagenic *Nde*I site. A)Restriction digestion of pNPU2.3F4 with *Nde*I to confirm presence of mutated ovine sequnce. Digestion of pNPU2.3F4 with *Nde*I should produced a 0.8kb fragment if the mutagenesis is successful. Lane 1; 1Kb markers: lane 2; *Nde*I restriction digest of pNPU2PM; lane 3; *Nde*I restriction digest of pNPU2.3F4: lane 4; *Bam*HI /*Sca*I restriction digest of pNPU2PM: lane 5; *Bam*HI/*Sca*I restriction digest of pNPU2.3F4. B) Restriction digestion of pNPU7.3F4 to confrim the presence of mutated ovine PrP sequnce. Purification and digestion of the *Bam*HI/ *Xba*I fragment from pNPU2.3F4 and pNPU7.3F4should produce a 1.1kb fragment if mutagensis has been successful. Lane I; markers VI: lane 2; *Nde*I resitriction digest of *Bam*HI/*Xba*I fragment of pNPU7PM; lane 3; *Nde*I restriction digest of *Bam*HI/*Xba*I fragment of pNPU2.3F4: lane 4; *Nde*I digestion of *Bam*HI /*Xba*I fragment of pNPU7.3F4 clone7: lane 5; *Nde*I digestion of *Bam*HI/*Xba*I fragment from pNPU7.3F4 clone 8.

5.5 Immunological Detection of Transiently Expressed Ovine PrP^C Protein in Mouse and Ovine Cell Lines

5.5.1 Introduction

In vitro expression is frequently used for the study of protein structure and function, protein–protein interactions, antibody production and mutagenesis. It can also be employed to assess the levels of protein synthesis as a result of enhancer/ repressor elements controlling gene expression at the level of transcription or translation. Analyse of a protein sample often involves purification which can be dependent on the biochemical properties of the protein i.e. solubility or size. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has become the most popular method for analysis and isolation of small amounts of protein. Successful SDS-PAGE allows for individual gel bands to be visualised and recovered, or transferred onto a membrane, for further analysis. The most commonly used methods for visualising bands after gel electrophoresis are Coomassie blue and silver staining which often require stringent conditions making proteins difficult to manipulate further. Coomassie and silver stains do not differentiate between proteins within a sample, staining the majority of proteins present within the isolated fraction. For more specific analysis of particular proteins immunological detection is essential.

Immunological detection of proteins is possible using either monoclonal or polyclonal antibodies. Antibodies are widely used to detect antigens in a variety of methods; immunodetection can be classified as either solution based (enzyme-linked immunosorbent assay (ELISA), immunoprecipitation) or sample-immobilisation based (i.e. Western, dot blotting). In general the method for immunodetection is determined by the sensitivity and specificity of the antibodies available, nature of sample or tissue, and ease of purification/ extraction of target protein. Always of importance are the conditions for stringency in antibody-antigen detection as changes in the environment (i.e. temperature, salt concentration, and presence of detergents) may cause non-specific binding or limited antibody-antigen binding. Furthermore, antibodies may recognise epitopes that may be denatured or buried during processes

such as SDS-PAGE or Western blotting and so antibody-binding sensitivity may be reduced.

Developing a reliable method for PrP^C protein extraction was vital for the study of endogenous and transient PrP^C expression studies. Methods for PrP^{Sc} isolation are well established (first described by Hope *et al.*, 1986), however this is not the case with PrP^C. PrP^C differs in physical properties to PrP^{Sc} which means different isolation methods must be used to isolate PrP^C. Firstly PrP^C is detergent soluble (e.g. Sarkosyl) but detergents can make samples viscous and difficult to analyse by SDS-PAGE. Secondly, PrP^C is not as physically stable as PrP^{Sc} and may be degraded by the presence of strong denaturing agents or heat treatment. Further, as PrP^C is proteinase K sensitive, steps must be taken to prevent proteinase contamination. PrP protein has a distinctive band pattern on SDS-PAGE, it has a molecular weight of 33-35kDa (Meyer *et al.*, 1986) and separates into three bands due to the glycosylation status (un-, mono-, di- glycosylated)(Oesch *et al*, 1985; Barry and Prusiner, 1986). The development of a suitable method for PrP extraction and detection was greatly assisted by the advice and guidance of Angela Chong, Neuropathogenesis Unit, Edinburgh.

As discussed in Section 1.9 the level of PrP^C protein detected in different cell types and tissues can vary considerably, with the highest level of PrP^C protein recorded in the brain levels (Oesch *et al.*, 1985; Caughey *et al.*, 1988; Manson *et al.*, 1992a and b; Sales *et al.*, 1998; Goldmann *et al.*, 1999). Within the brain PrP expression is thought to be largely associated with neurones and astrocytes (Kretzschmart *et al.*, 1986; Manson *et al.*, 1992a; Harris *et al.*, 1993; Moser *et al.*, 1995 Raeber *et al.*, 1997). It was necessary to determine the level of endogenous PrP^C expression, if any, within the ovine cell lines as this may have implications for the detection level of transiently expressed PrP^C from the PrP constructs. For instance, although the immortalised brain cell lines (sA80BR and pA80BR) tested positively for the presence of neurones and glial cells (Chapter 3) they may be altered intrinsically so that they express low, or high, levels of PrP^C. Also, one may expect PrP^C expression from the primary cerebellum cells, IS120.Cer (positive for neuronal cells, Chapter 3) as PrP expression in neurones is

well established (Kretzschmart *et al.*, 1986; Manson *et al.*, 1992a; Harris *et al.*, 1993). The reported level of PrP expression in peripheral tissues can vary greatly (Horiuchi *et al.*, 1995; Goldmann *et al.*, 1999). In sheep, PrP mRNA expression has been shown to be relatively high in the kidney despite the apparent absence of detectable PrP^C (Horiuchi *et al.*, 1995). In contrast, heart tissue shows high level of both PrP mRNA and PrP^C protein (Horiuchi *et al.*, 1995; Goldmann *et al.*, 1999).

The aim of this Section was to develop an immunological detection method for transiently expressed ovine PrP^C in brain and peripheral tissue-derived cell lines. Transient expression the PrP plasmids described in Section 5.4, may provide information relevant to the understanding of events leading to differential expression of the PrP protein and whether the factors that govern the level of PrP protein synthesis are found in the 3'UTR of the PrP gene.

5.5.2 Experimental Approach.

At the start of this study the level of PrP^C protein expression in the cell lines was unknown and so the unmodified PrP plasmids (pNPU2PM, pNPU3PM, pNPU7PM) were transfected in to the sheep cells in the hope of being able to detect increased levels of PrP expression as a result of transient expression. Most emphasis however, was placed on the transient expression of the PrP constructs encoding the 3F4 epitope (pNPU2.3F4 and pNPU7.3F4). The constructs were also expressed in mouse N2a cells as a control for extraction and immunological detection methods.

N2a and ovine cell lines (sA80BR, pA80BR, pA80SK, IS120.Cer and IS120.Liv) were transfected with the ovine PrP constructs (Sections 2.5 and 3.5). Successful transfection was confirmed by co-transfection and colorimetric assay of β-galactosidase (β-gal). For approximately $6x10^7$ cell, β-gal assays completed on the recovered cell lysates allowed for differential transfection efficiency between experiments. Three methods were tested for efficient protein extraction from the cell lines: chloroform/methanol precipitation; Triton X114 detergent extraction; immunoprecipitation. The level of purified PrP^C isolated was estimated by Western blot analysis. A full description of each method can be found in Sections 2.6.

Chloroform/ methanol extraction was the simplest of the methods involving lysis of the cells by shearing in the presence of SDS then precipitation in chloroform/methanol - a quick, crude protein preparation. Triton X114 detergent extraction is a laborious, precise process, temperature dependent, requiring several washes of a sucrose gradient with Triton X114 solution to extract all protein. Extraction by Triton X114 can produce a sample collection that can be pooled and concentrated by methanol precipitation. Immunoprecipitation involves lysing cells in a cell lysis buffer, the antibody is applied overnight and isolated the next day by the addition of Sepharose A beads. Sepharose beads are covalently modified with isolated protein A molecules that occur naturally on the cell walls of *Staphylococcus aureus* and bind to the Fc region of IgG or IgM molecules. The strategy behind immunoprecipitation is the interaction of the antigen with the antibody and then the addition of excess Sepharose A containing protein A to bind all appropriate antibodies. The immunoabsorbed antigen is purified from the crude extract by low-speed centrifugation. Immunoprecipitation can be more sensitive than other methods if the antigen is in low concentration or sensitive to degradation.

Both SDS-PAGE and Western blotting were completed after each extraction to determine the presence of PrP (Section 2.6.4). The antibodies used for Western analysis and immunoprecipitation are outlined in Table 2.3. Antibodies FH11 and 3F4 were kindly supplied by Chris Birkett, IAH, Compton and Richard Kascsak, MRDD, New York, respectively. The secondary antibody was a peroxidase-conjugated affinipure IgG complex. Purified PrP^{Sc} samples were kindly provided by Angela Chong and Kasia Sobotnicki as positive controls for the SDS-PAGE and Western blotting procedures (full description of sample preparation and use in Section 2.6). Positive controls were prepared from scrapie-affected mice or hamster brains available at the NPU. At the start of preparation a known weight of brain tissue (grams) was used for PrP^{Sc} extraction. The purified sample is referred to as the amount of protein (gram Equivalent (gE)) extracted from a known weight of brain (Chong, personal communication). Within this thesis both proteinase K treated and non-treated sample were used. For SDS-PAGE, 25µl of a control sample, dissolved in 100µl 1x sample buffer (Chapter 2, Section 2.6) was loaded on to a gel. Serum controls for the

antibodies available at the NPU were completed prior to this study (Angela Chong, personal communication: for 3F4 and 6H4 please refer to Kascsak *et al.*, 1987 and Korth *et al.*, 1997 respectively).

5.5.4 Results.

5.5.4.1 Chloroform/methanol extraction.

Chloroform/ methanol extraction of mouse N2a cells transfected with plasmids pNPU2PM, pNPU3PM and pNPU7PM was repeated eight times. The ruminant-specific antibody FH11 was used for immunological detection of transiently expressed ovine PrP^C. A typical Western bot is shown in Figure 5.11A. Despite several attempts, transiently expressed ovine PrP was not detected from any of the PrP constructs in the mouse N2a cell line (Figure 5.11A, samples 1-3). Successful extraction of endogenous N2a PrP^C by chloroform methanol extraction was demonstrated by immunoblotting with the polyclonal antibody 1B3 (Figure 5.11B. samples 1-3). Furthermore, the level of PrP^C extracted from N2a cells transfected with pNPU7PM does appear to be higher compared to the cells transfected with either pNPU2PM or pNPU3PM (Figure 5.11B, compare sample 3 to 1 and 2). However, this result was never repeated and no internal control was probed for, i.e α-actin, on the original blot which would have confirmed increased transient expression from pNPU7PM compared with the other PrP constructs.

Similar transfections and extractions were carried out on the ovine cell lines sA80BR, pA80BR and pA80SK. A typical immunoblot result is shown in Figure 5.12. Chloroform/ methanol extraction was repeated four times on the transfected sheep cell lines and ovine PrP expression analysed by Western blotting coupled with antibodies 1B3 and FH11. At the time of this study the monoclonal antibody 3F4 was not available at NPU and so the expectation at this stage was not to distinguish between endogenous and transiently expressed PrP^C but to detect an increase in PrP^C levels in the transfected cells compared to non-transfected cells. However, chloroform/methanol extraction was not efficient in extracting detectable levels of PrP^C from non-transfected or transfected ovine cells (Figure 5.12).

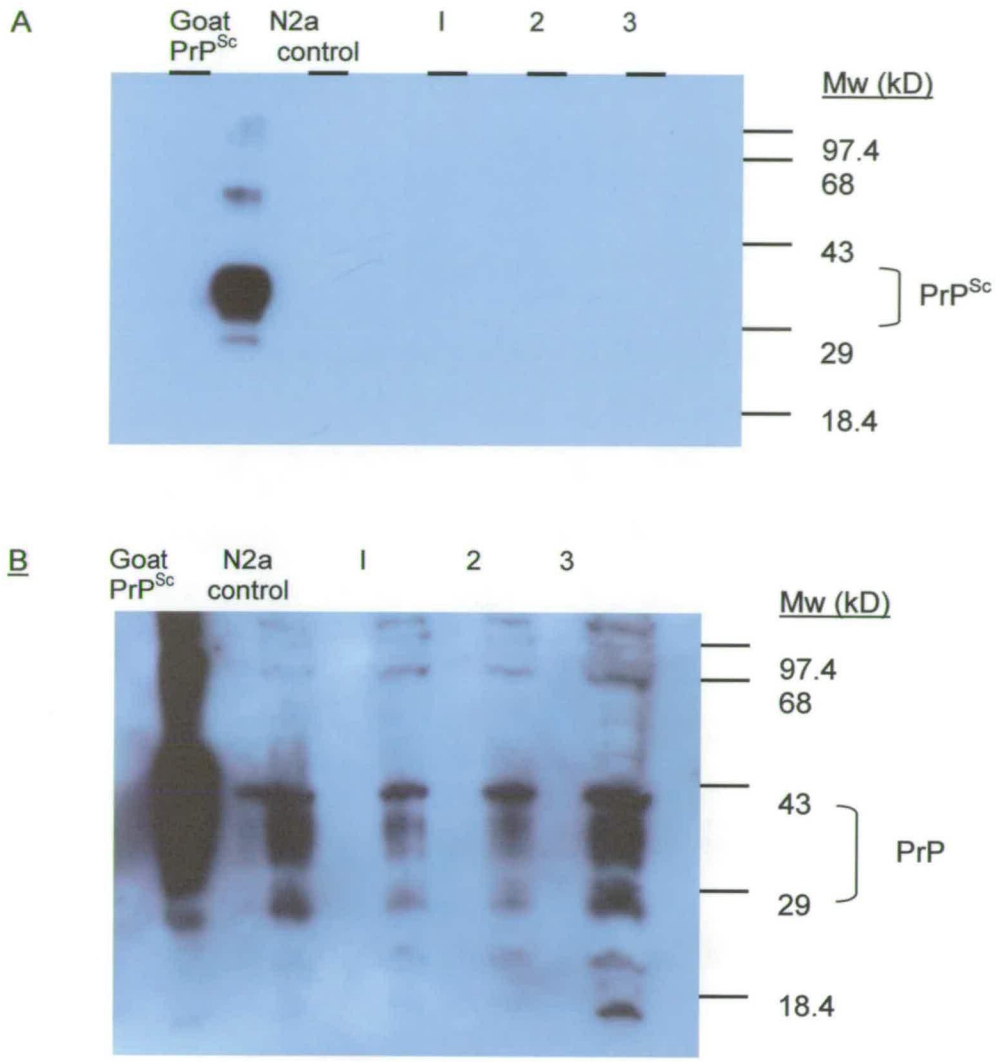

Figure 5.11: Chloroform methanol extraction of endogenous and transiently expressed PrP^C from a mouse N2a cell line. Following extraction, the precipitate was dissolved in 1x sample buffer for SDS-PAGE and then transferred on to a charged nylon membrane for Western blotting. Antibody control was purified goat PrP^{Sc} ,0.002 gE; N2a control is non-transfected cells processed for extraction; sample 1, N2a cells transfected with pNPU2PM ; sample2, N2a cells transfected with pNPU3PM; sample 3, N2a cells transfected with pNPU7PM. A) Immunodetection with the ovine specific antibody FH11. B) Immunodetection with the polyclonal antibody 1B3. Detection of lower molecular weight bands may indicate degradation of PrP and higher molecular weight bands may be PrP dimers or trimers. Alternatively extra bands may be due to cross reactivity of the 1B2 antibody (Chong, personal communication0.

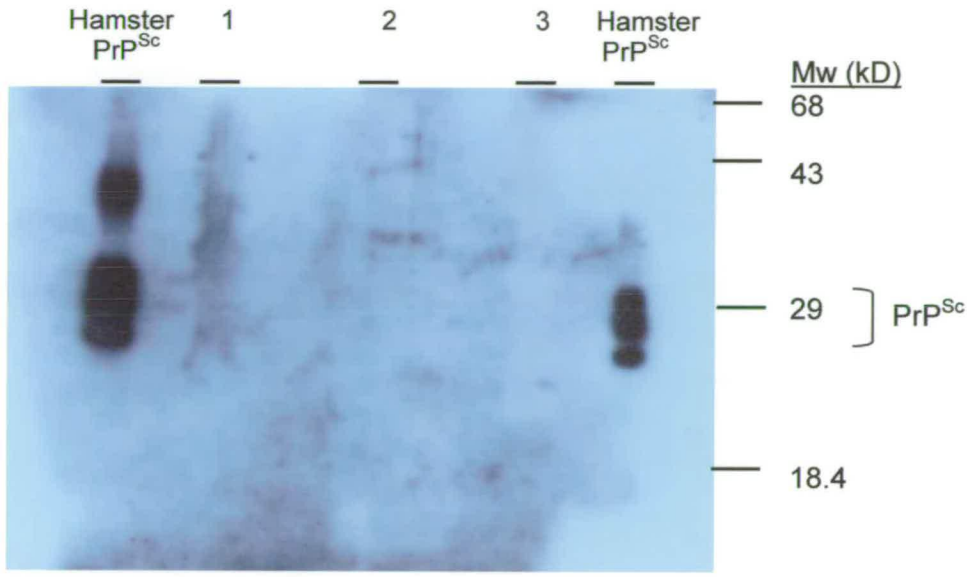

Figure 5.12: Chloroform/methanol extraction of endogenous ovine PrP from cell lines sA80BR, pA80BR and pA80SK. From chloroform/ methanol extraction, the protein precipitate was dissolved in 1x sample buffer and loaded for SDS-PAGE. The antibody positive control was 0.002gE (left) and 0.0002gE (right) of mouse PrP^{Sc} which had been proteinase K treated. Following western blotting, the polyclonal antibody 1B3 was used to detect PrP^{C} from sA80BR (sample 1), pA80BR (sample 2) and pA80SK (sample 3).

5.5.4.2 Detergent extraction.

During development of Triton X-114 extraction from mouse and ovine cells, the antibodies 3F4 and 6H4 became available at NPU and were used in conjunction with 1B3 and FH11 in an attempt to detect endogenous and transiently expressed PrP^C. Detergent extraction using Triton X-114 was completed a total of eighteen times on N2a cells transiently transfected with the PrP constructs. Figure 5.13 shows a blot with one half probed with the polyclonal antibody 1B3 and the monoclonal antibody 3F4. The right-hand side of the blot is a typical result using 1B3, confirming protein extraction. Immunodetection with the hamster specific antibody 3F4 on the left-hand side of the blot shows in lane 2 a protein of approximately 27 kDa that is equivalent to truncated, un-glycosylated form of PrP^C protein. Using Triton X-114 extraction it was not possible to detect transiently expressed ovine PrP in N2a cells using the ruminant specific monoclonal antibody FH11 (Figure 5.14)

Purification of endogenous and transiently expressed ovine PrP by Triton X-114 was completed a total of nineteen times followed by immunodetection with the antibodies 1B3, FH11, 3F4 and 6H4. In the majority of instances the level of PrP protein purified was not sufficient to be detected at the level of Western blotting using these antibodies. The immunoblot shown in Figure 5.15 is typical of control and transfected ovine cell lines after detergent extraction, SDS-PAGE purification and Western blotting with 1B3. On one occasion, ovine PrP was detected by the antibody FH11 in sA80BR cells which had been transfected with the plasmid pNPU7PM (Figure 5.15B, lane 7), PrP^C detected had an apparent molecular weight of 31kDa which would be approximate to a un-glycosylated PrP protein. In Figure 5.15 no endogenous PrP^C was detected, imunoblotting similar samples with hamster anti-PrP 3F4 antibody failed to confirm this result (Figure 5.16). Using detergent extraction it was not possible to detect transiently expressed PrP with the hamster specific antibody 3F4 (Figure 5.16).

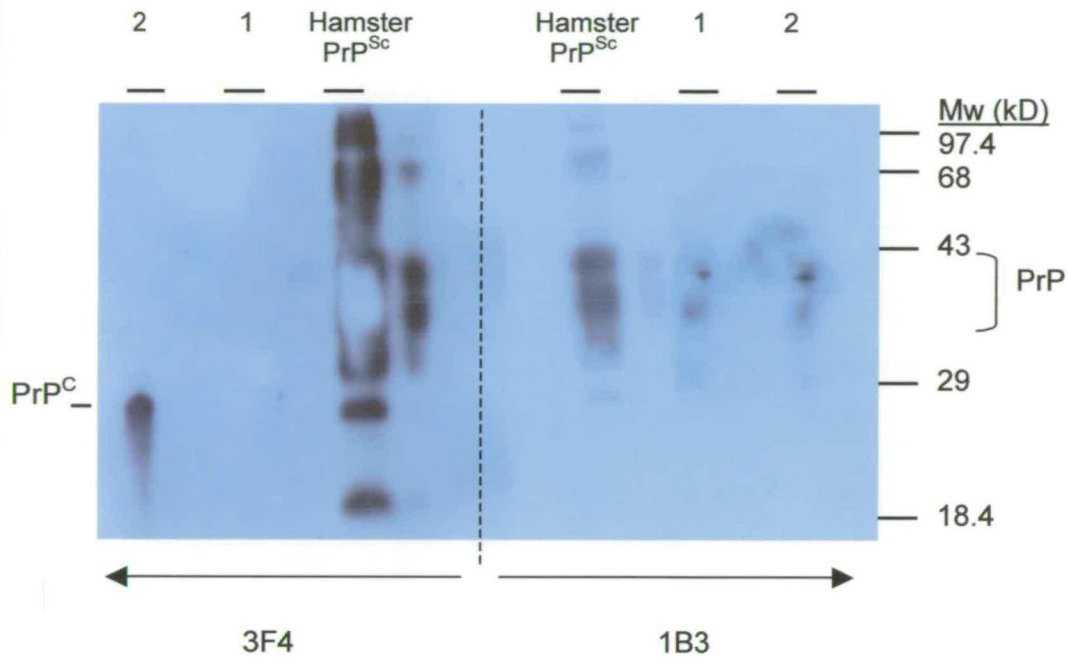

Figure 5.13: Detergent extraction and detection of transiently expressed PrP^C from N2a cells with 3F4. . Cell lysate was recovered from control N2a cells (sample 1) and N2a cells transfected with the construct pNPU2.3F4 (sample 2) and PrP^C purified by Triton X-114 extraction. The precipitate was dissolved in 1x sample buffer and processed by SDS-PAGE, transferred onto PVDF membrane and membrane split into two equal halves; one half was probed with 1B3, the other with 3F4. The positive antibody control was a purified hamster PrP^{Sc} (0.05gE), non proteinase K treated.

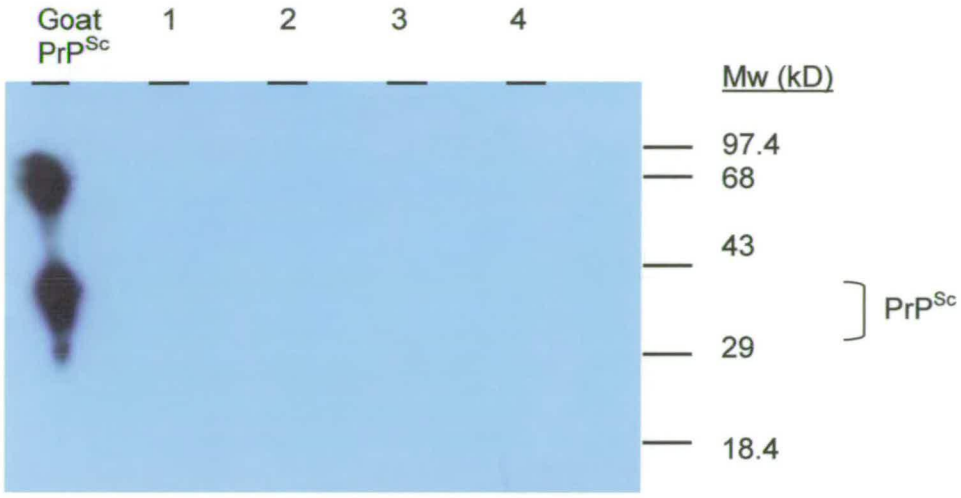

Figure 5.14:Detergent extraction of ovine PrP^C from N2a cells and detection with antibody FH11 Cell lysate was recovered from control N2a cells (sample 1) and N2a cells transfected with the constructs pNPU2PM (sample 2), pNPU3PM (sample 3), and pNPU7PM (sample 4) and protein purified by Triton X-114 extraction. The precipitate was dissolved in 1x sample buffer and processed by SDS-PAGE as shown above and then transferred onto a PVDF membrane (western blotted). The ruminant specific antibody FH11 was used to probe the membrane . The positive antibody control was a purified goat PrP^{Sc} (0.025gE) non proteinase K treated.

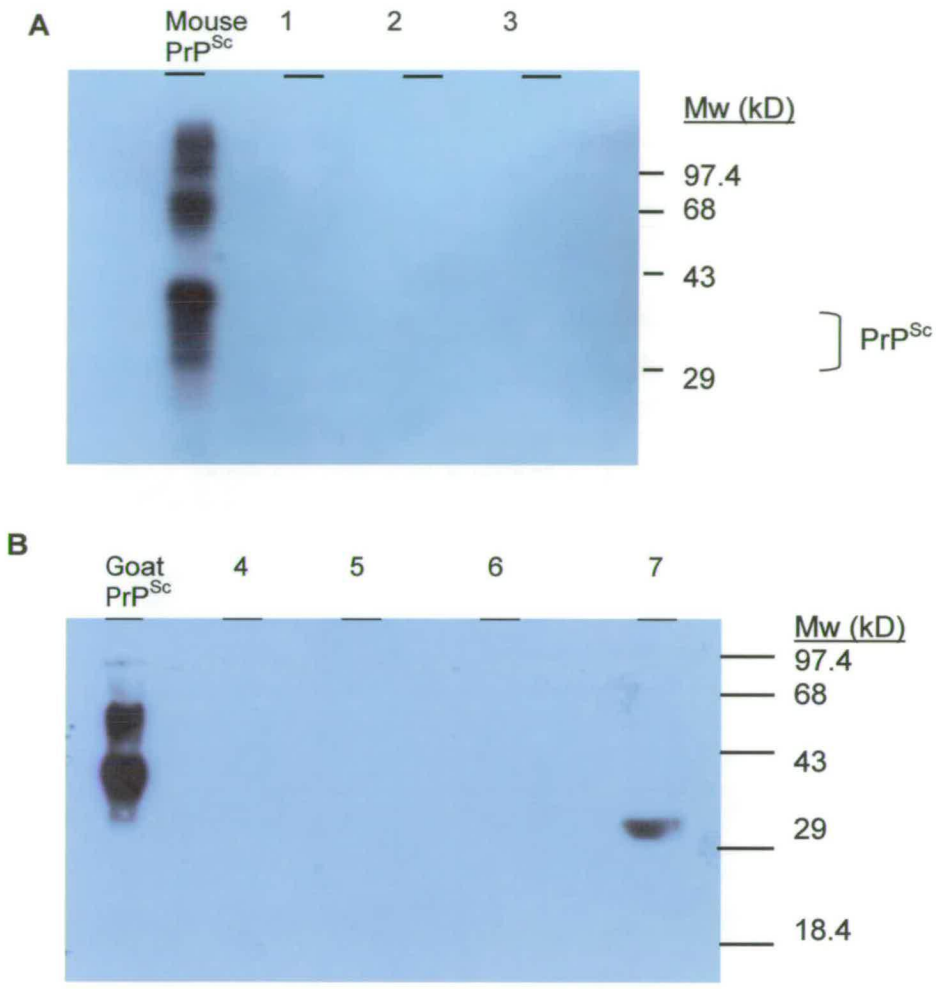

Figure 5.15: Detection of PrP^C protein from ovine cell lines following detergent extraction. Cell lysate was recovered from control ovine sA80BR cells and ovine cells transfected with the PrP constructs and protein purified by Triton X-114 extraction. The precipitate was dissolved in 1x sample buffer and processed by SDS-PAGE and western blotted. The positive antibody control was a purified mouse (A) and goat (B) PrP^{Sc} (0.1gE) non proteinase K treated. A) Immunoblotted with antibody 1B3. Sample1: non-transfected sA80BR cells; sample 2: sA80BR cells transfected with pNPU2PM; sample 3: sA80BR cells transfected with pNPU7PM. B) Immunoblotted with antibody FH11. Sample 4: non-transfected sA80BR cells; sample 5: sA80BR cells transfected with pNPU2PM; sample 6: sA80BR cells transfected with pNPU3PM; sample 7; sA80BR cells transfected with pNPU7PM.

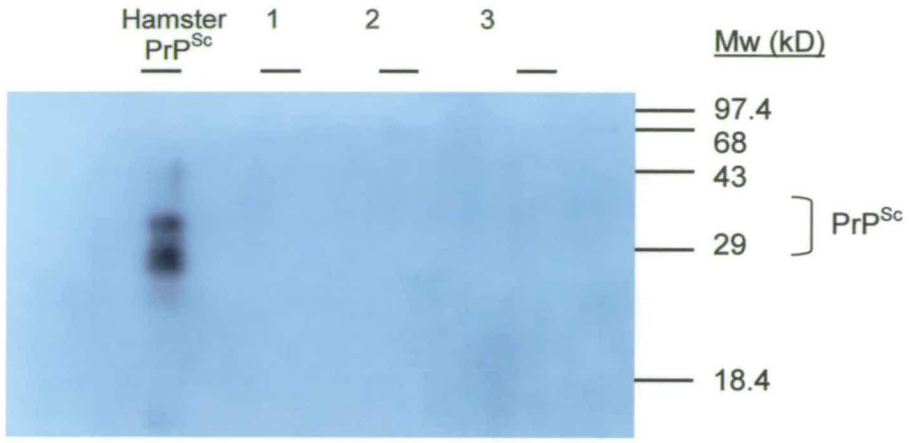

Figure 5.16:Triton X-114 extraction of transiently expressed PrP^C in sA80BR with the antibody 3F4. Cell lysate was recovered from control ovine sA80BR cells and ovine cells transfected with the PrP constructs pNPU2.3F4 and pNPU7.3F4. Protein was purified by Triton X-100 extraction. The precipitate was dissolved in 1x sample buffer and processed by SDS-PAGE and Western blotted with the hamster anti-PrP 3F4. The positive antibody control was a purified hamster PrP^{Sc} (0.05gE) non proteinase K treated. Sample1: non-transfected sA80BR cells; sample 2: sA80BR cells transfected with pNPU2.3F4; sample 3: sA80BR cells transfected with pNPU7.3F4.

5.5.4.3 Immunoprecipitation.

Protein purification by immunoprecipitation was carried out on five separate occasions for N2a cells and four times for ovine cell lines populations which had been transfected with the PrP constructs encoding the 3F4 epitope. After harvesting of the cell, the cell lysate was incubated for sixteen hours with antibodies 3F4 or 6H4. SDS-PAGE and Western blotting with 1B3 to detect endogenous and transiently expressed PrP^C protein followed precipitation of the immunocomplex. Figures 5.17 and 5.18 show typical blots from PrP^C protein purified by immunoprecipitation from both N2a and ovine cell lines.

As with chloroform/methanol extraction (Section 5.5.4.1) cross reactivity of the anti-PrP antibody 1B3 was observed. This does make interpretation of the blots more difficult. Immunoprecipitation with complete culture medium was negative (data not shown) where as immunoprecipitation with all cell-extract preparations showed positive for PrP. The blots in Figures 5.17 and 5.18 show PrP^C extracted from N2a cells transfected with constructs pNPU2.3F4 and pNPU7.3F4. From Figure 5.17 all of PrP^C has been precipitated with the Sepharose beads and does not remain in the supernatant. PrP^C was detected in both non-transfected and transfected N2a cells despite using hamster anti-PrP 3F4 monoclonal antibody. Expression of the PrP constructs was also examined in ovine cell lines, Figure 5.18 shows PrP^C extraction from sA80BR. Despite detection of higher molecular weight proteins, extraction of PrP^C protein also from both cell lines using immunoprecipitation was successful and on most occasions all three glycoforms of PrP^C were visible (i.e un-, mono- and di-gylcosylated). It is apparent that although immunoprecipitation was capable of extracting PrP^C from cell lysate preparations it was not possible to distinguish transiently expressed PrP^C from endogenous PrP^C, despite the transient PrP constructs encoding the hamster 3F4 epitope.

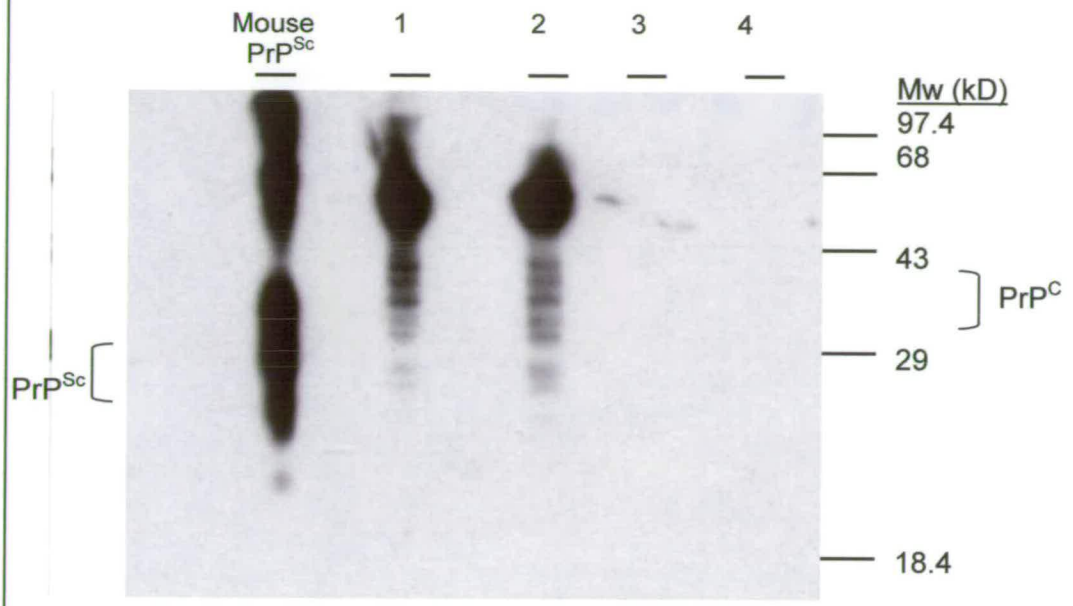

Figure 5.17: Immunoprecipitate of PrP^C protein from N2a cells with 3F4. Cell lysate was recovered from N2a cells transfected with the constructs pNPU2.3F4 and pNPU7.3F4 for immunoprecipitation overnight with the hamster specific antibody 3F4. The precipitate was collected and protein eluted and processed by SDS-PAGE and Western blotting with the polyclonal antibody 1B3. The supernatant collected from the precipitate was methanol precipitated and also processed. The antibody control for Western blotting was mouse PrP^{Sc}, (0.05gE) proteinase K treated. Sample 1, immunoprecipitate from mouse cells transfected with pNPU2.3F4: sample 2; immunoprecipitate from mouse cells transfected with pNPU7.3F4: sample 3; supernatant from sample 1: sample 4; supernatant from sample 2

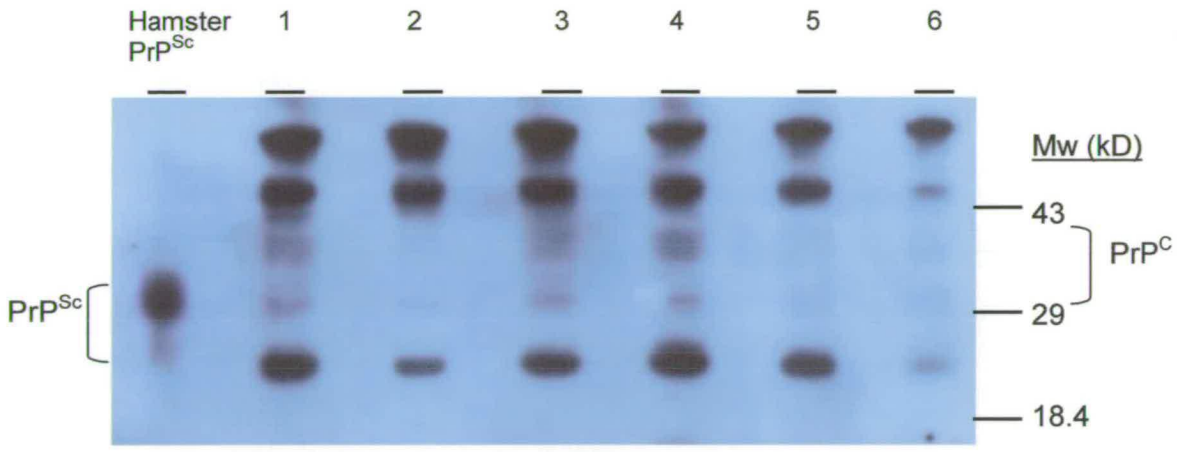

Figure 5.18: Immunoprecipitation of PrP^C protein from N2a and sA80BR cell lines transfected with 3F4 encoding PrP constructs. Cell lysate was recovered from N2a and sA80BR cell lines from both controls and lines transfected with the constructs pNPU2.3F4 and pNPU7.3F4 for immunoprecipitation overnight with the hamster specific antibody 3F4. The precipitate was collected and protein eluted and processed by SDS-PAGE and Western blotting with the polyclonal antibody 1B3. The antibody control for Western blotting was hamster PrP^{Sc} (0.05BE) non proteinase K treated. Sample 1, immunoprecipitate from non-transfected mouse cells; sample 2, immunoprecipitate from N2a cells transfected with pNPU2.3F4, sample 3, immunoprecipitate from N2a cells transfected with pNPU7.3F4; sample 4, immunoprecipitate from non-transfected sA80BR cells; sample 5, immunoprecipitate from sA80BR cells transfected with pNPU2.3F4, sample 6, immunoprecipitate from sA80BR cells transfected with pNPU7.3F4.

5.6 Discussion

The ovine PrP gene 3'UTR has been shown to mediate *in vitro* protein levels. *In vivo* expression from the PrP gene is not fully understood. Hunter *et al.* (1994) described two PrP mRNAs transcribed mRNAs (2.1kb and 4.6kb), these transcripts are known to be identical in 5' sequence differing only in the length of the 3'UTR as a result of alternative polyadenylation (Goldmann *et al.*, 1990). It is still not known whether the 2.1kb mRNA is a transcript for PrP protrein. As discussed in Chapter One (Section 1.9) the level of PrP mRNA, the ratio of the two transcripts and the amount of detectable PrP protein varies greatly between tissues (Horiuchi *et al.*, 1995; Goldmann *et al.*, 1999). In an attempt to study the relationship between PrP^C levels and the *in vivo* ovine PrP transcripts, PrP constructs were designed with 3'UTRs as those described for the 4.6kb and 2.1 kb mRNAs.

Expression from the constructs was to be driven by a region 500bp upstream of the transcription start site on the Cheviot PrP gene. This region has been shown previously to initiate transcription of a CAT reporter gene in ovine cell lines (O'Neill *et al*, in preparation). The promoter region, which also includes exon I and the splice donor site, was cloned upstream of the Suffolk e1 PrP exon III for all constructs used in this study. Restriction digestion of the PrP exon III 3'UTR at defined sites created UTRs of varying lengths with known polyadenylation sites. Transient expression of the PrP constructs concentrated mainly on the plasmids pNPU2PM, pNPU23F4, pNPU7PM and pNPU7.3F4. Constructs pNPU2PM and pNPU2.3F4 are truncated at nucleotide (nt) 1929 and so only encoded one active pA site (nt 1523). Constructs pNPU7PM and pNPU7.3F4 contain the full length PrP gene 3'UTR and therefore encodes conserved pA sites at 1522, 2221, 2281, 2668 and 4046. RT-PCR analysis showed constructs pNPU2PM and pNPU7PM were actively transcribed in a mouse N2a cell line. The use of cell free translation assays may have confirmed protein synthesis form the PrP construct.

Immunological detection of PrP^C proved difficult if not impossible. Although PrP^C was easily extracted and detectable from the N2a cell line, endogenous PrP^C from the ovine cell lines was more difficult to detect and the methods of PrP^C extraction

employed in this thesis had varying results. Protein extraction from N2a cells was successful using all methods described. Endogenous protein from ovine cell lines was only reliably determined by immunoprecipitation. Using N2a cells as a control for transfection and extraction, transient expression from the ovine PrP constructs was not detected by FH11, regardless of the extraction method. FH11 also failed to detect endogenous and transient PrP^C expression in the ovine cells. Following Triton X-114 extraction, a 31-32kDa protein was detectable by FH11 in sA80BR cells which had been transfected with pNPU7PM (Figure 5.15). The antibody FH11 recognises an epitope in the PrP amino acid sequence (aa sequence 51-55; Somerville, personal communication) which is upstream of the proteinase K site in the N-terminus region. The protein detected in Figure 5.15 may indicate that the PrP^C synthesised *in vitro* by sA80BR cells is un-glycosylated. It is not possible to state clearly that the PrP^C detected (Figure 5.15) was not endogenous, however from the same set of experiments no endogenous PrP^C was detected. Similar experiments in N2a cells using the monoclonal antibody 3F4 (Figure 5.13) showed detergent extraction of a protein with molecular weight 29kDa, suggesting a PrP^C protein that is both un-glycosylated and N-terminally degraded. Weighed with the failure of FH11 to detect transient and endogenous PrP^C expression and the detection of a truncated protein, the cell lines may not be synthesising/ modifying mature PrP^C correctly. Alternatively the PrP^C may have been degraded by proteinases despite action taken to prevent protein degradation i.e. extraction carried out on ice and in the presence of proteinase inhibitors.

Mutating the cloned ovine PrP ORF gene sequence in constructs pNPU2PM and pNPU7PM to encode the hamster 3F4 epitope hsould have allowed for the detection of transiently expressed PrP^C on a background of endogenous ovine PrP^C. Prior to the start of this study the hamster anti-PrP 3F4 antibody was recognised as not cross-reacting with the sheep PrP protein (Kascsak *et al.*, 1987). Probing for PrP^C with 3F4 following detergent extraction of N2a cells successfully detected transient expression from PrP construct pNPU2.3F4 (Figure 5.13). However, despite several attempts PrP^C expression from constructs pNPU2.3F4 and pNPU7.3F4 was never detected in ovine cell lines following Triton X-114 extraction. Immunoprecipitation

with the antibody 3F4 was developed for extraction of transiently expressed PrP^C from constructs pNPU2.3F4 and pNPU7.3F4 from both N2a and ovine cell lines. Results from immunoprecipitation were difficult to interpret but appeared to show precipitation of endogenous and transiently expressed PrP^C from both the mouse and sheep cell lines (Figure 5.17 and 5.18). Due to the absence of a cell lysate negative control for the PrP 3F4 antibody it is difficult to say with confidence that PrP has been detected. However the pattern of protein detected between molecular weights30-36kD is characteristic for PrP^C as is the pattern of three distinct bands corresponding to the gylcosylation status (un-, mono- and di- glycosylated). Furthermore, from published data available at the start of this study, 3F4 was understood not to cross react with endogenous ovine PrP. From Figure 5.18 protein bands characteristic of PrP were detected in the non-transfected ovien cell line (Figure 5.18, lane4), indicating that ovine PrP^C was detectable by immunoprecipitation. In support of this idea, a recent report (Baron *et al.*, 1999) has shown that *in vitro* expression of sheep PrP fusion proteins was detectable by Western blotting using the hamster monoclonal antibody 3F4.

Despite detection of expression, at both the RNA and protein level, from the PrP construct in N2a cells, detection of transient expression within ovine cell lines was not achieved. There are several reasons for this failure. Firstly, the original paper describing anti-hamster PrP antibody (Kascsak *et al.*, 1987) used purified PrP^{Sc} from mice and hamsters brains infected with hamster scrapie strain (263k). At the start of this study the anti-hamster PrP antibody was thought to detect only hamster and human PrP while not detecting sheep, rat, cattle and rabbit PrP (Kascsak *et al.*, 1987). It was only recently that 3F4 has been described as cross reacting with the sheep PrP protein (Baron *et al.*, 1999). Secondly, it has also been shown that the level of recoverable PrP^{Sc} from different brain-tissue samples can vary depending on the method of protein extraction and the specific PrP antibody (Madec *et al.*, 1998). Furthermore, within the same study the choice of anti-PrP antibody also lead to variable results. Madec *et al*, (1998) compared two widely used methods for extraction of PrP^{Sc} based on detergent methods (Sarkosyl) and showed that the methods varied on sensitivity, depending on the starting levels of PrP^{Sc} within the

tissue (Madec *et al.*, 1998). Also when coupled with a ruminant specific PrP monoclonal antibody the methods were again distinguishable in their efficiency to detect PrP^{Sc}. Madec *et al*, 1999, speculate that such variation in extraction efficiency may be affected, for example, by the presence of lipid in the tissues. It may be expected then that extraction methods that worked for N2a cells did not work for the ovine cell lines as the ovine cells had different endogenous PrP^{C} levels and contained different cellular concentrations of molecules such as lipids. Optimising an extraction method solely for ovine cells may have led to detection of endogenous and transiently expressed PrP^{C}.

Also, it would appear that PrP^{C} synthesised from the PrP constructs was truncated and unmodified. Incorrect expression of the PrP^{C} construct may be a due to the absence of exon II or full length sequence of introns I and II. As discussed in Chapter one (Section 1.8.3) the mouse intron I has been shown capable of promoter activity and promoter suppresser activity (Baybutt and Manson., 1997). The absence of intron II from the PrP gene lead to undetectable levels of PrP expression in Purkinje cells of the cerebellum without affecting the level of PrP expressed in other brain areas (Fischer *et al.*, 1996), implying that intron II may contain sequences necessary for cell-specific PrP expression. Lacking full PrP gene sequence, *in vitro* expression from the PrP constructs may have lead to reduced (or abnormal) protein synthesis. The synthesis of a truncated PrP^{C} protein would account for why the ruminant specific antibody FH11 was unable to detect transiently expressed PrP.

Transient expression of PrP^{C} protein from the ovine PrP constructs in mouse N2a cell lines has shown that the PrP gene 3'UTR associated with the 2.1kb transcript is capable of supporting translation. However, this was not confirmed in ovine cell lines. Work presented within this Chapter highlights the need for more research into PrP extraction methods. There is a concern that alternative extraction methods used in different laboratories may give rise to ambiguous reporting of the presence of PrP protein in various tissue samples. Finally due to the conserved nature of the PrP gene it is difficult to reliably obtain species specific antibodies to the PrP protein. Antibodies that recognise defined host-derived PrP proteins may be forced to cross-

react with PrP from other species if conditions for extraction do not denature the PrP protein sufficiently or do not allow for stringent protein purification. The development of improved extraction methods for PrP^C and sensitive PrP antibodies may give more accurate results for future PrP^C analysis.

Chapter 6 : Final Discussion and Conclusions. Implications for Scrapie Susceptibility.

6.1 Results Summary and Discussion.

Prior to the start of this study, *in vitro* expression experiments with the chloramphenicol acetyl transferase (CAT) gene linked to the PrP 3'UTR had revealed an inhibitor effect on translatability of the CAT reporter gene in a mouse neuroblastoma cell (N2a) (Cheung, 1996; Goldmann *et al.*, 1999). The PrP 3'UTR fragment used for the CAT analysis work was originally cloned from a Suffolk sheep, with PrP allele e1 and $A_{136}R_{154}Q_{171}$ (Goldmann *et al.*, 1991a). The CAT-PrP-3'UTR constructs available at the NPU were used to further study the role of the 3'UTR in determining protein levels in ovine cell lines derived from different tissues and PrP genotype origins. The cell lines used in this study were derived from Cheviot brain tissue, PrP genotypes $VV_{136}RR_{154}QQ_{171}$ (scrapie susceptible) and $AA_{136}RR_{154}QR_{171}$ (scrapie resistant), and cerebellum and liver from an Icelandic sheep breed, PrP genotype $AA_{136}RR_{154}QQ_{171}$ (scrapie susceptible). As observed in mouse N2a cells, the PrP 3'UTR was capable of altering the detectable level of CAT protein in brain-derived sheep cells.

In all ovine cell lines tested in this study, no significant difference was observed in the level of transiently expressed CAT protein between CAT/PrP-3'UTR constructs pEYR and pD17. Constructs pEYR and pD17 were designed to resemble in vivo PrP mRNAs 4.6kb and 2.1kb respectively. From the lack of indifferent control of protein synthesis from constructs pEYR and pD17, the conclusion may be that tissue origin and PrP genotype does not affect gene expression. However there was a significant difference in the observed response of the cell lines transiently transfected with CAT constructs containing deletions within the 3'UTR sequence (summarised in Figure 6.1). CAT activity from a construct with sequence deleted upstream from PrP 3'UTR nt position 2000 was significantly up-regulated compared to pEYR and pD17 in cell lines sA80BR (Cheviot $VV_{136}RR_{154}QQ_{171}$ brain cell line) and IS120.Liv (Icelandic

sheep $AA_{136}RR_{154}QQ_{171}$ liver cell line). This response was not observed in the pA80BR (Cheviot $AA_{136}RR_{154}QR_{171}$ brain cell lines) and IS120.Cer (Icelandic sheep breed cerebellum $AA_{136}RR_{154}QQ_{171}$ cell line) (Figure 6.1, Tables 4.2, 4.5 and 4.7). Instead, in cell lines pA80BR and IS120.Cer, the level of expression from pEYR, pD17 and pD20 increased gradually following deletions of sequences upstream from nt position 27000. Interestingly CAT/PrP-3'UTR constructs produced a similar expression pattern in the Icelandic ($AA_{136}RR_{154}QQ_{171}$) liver cell line as the Cheviot ($VV_{136}RR_{154}QQ_{171}$) brain cell (Section 4.4, Figure 4.6), both genotypes in these breeds are known to be scrapie susceptible.

Results presented in this thesis confirm the inhibitory effect of the PrP gene 3'UTR. It is further proposed that the sequence 2000-2700 (region E-part F) of the 3'UTR functions as a repressor of PrP gene expression in brain cells of sheep encoding PrP genotypes normally associated with scrapie resistance. The repressor element is proposed to be an RNA-binding protein acting through 3' UTR sequence 2000-2700 (regions E-part F, Figure 4.1).

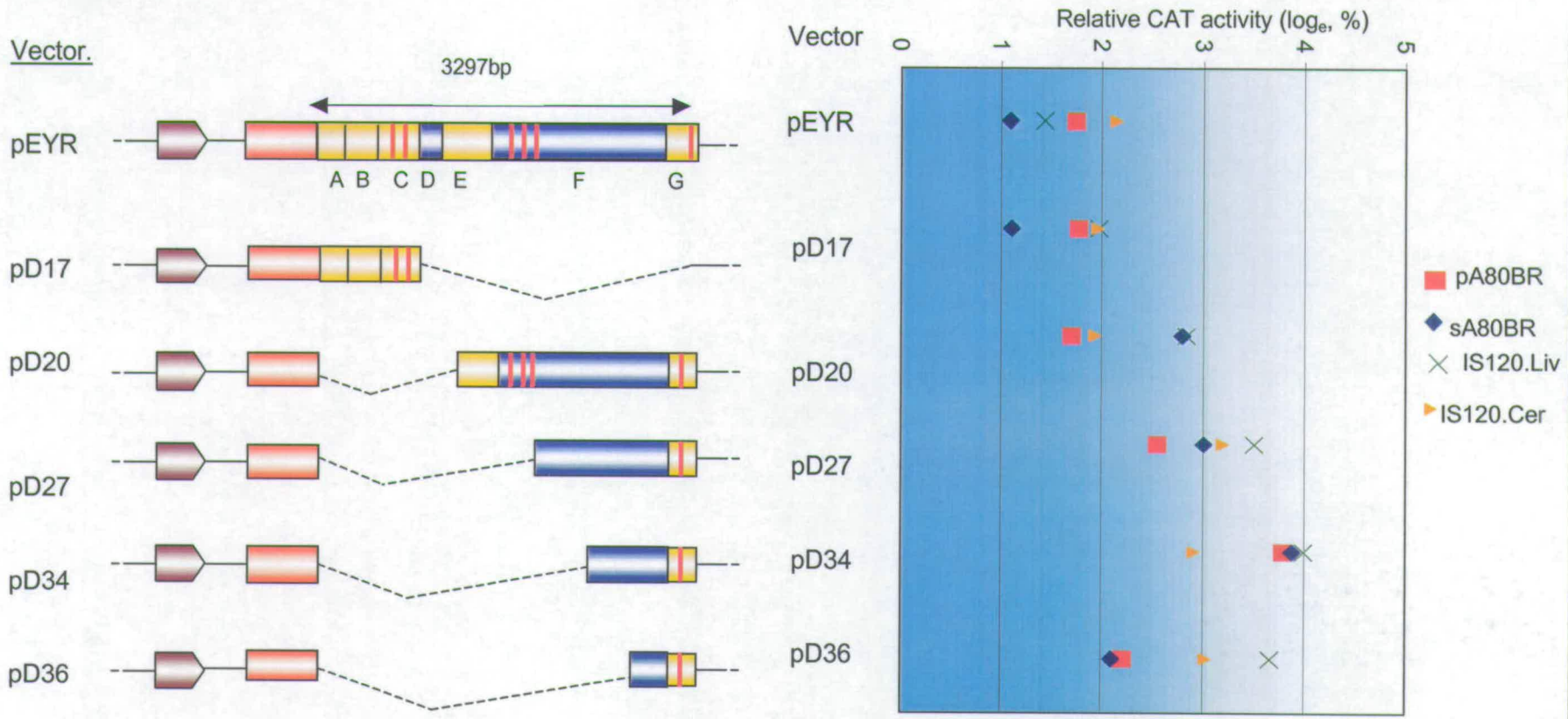

Figure 6.1. Summary diagram of transient CAT/PrP-3'UTR constructs in ovine derived cell lines (Chapter Four)

Several points for discussion for PrP gene expression arise from the above observations. Firstly, the 2.1 kb and the 4.6kb PrP 3'UTRs are capable of supporting the same level of protein synthesis regardless of PrP genotype, this observation suggests that sequence shared by both 3'UTRs can mediate steady state expression. The 2.1kb mRNA may be responsible for constitutive expression in peripheral tissues. Furthermore in $VV_{136}RR_{154}QQ_{171}$ brain cells, the 4.6kb mRNA may have potential for increasing protein levels on the removal of a repressor element. Factors binding to the repressor element of the 4.6kb PrP mRNA may reduce protein levels from the 4.6kb mRNA by increasing the rate of mRNA degradation or inhibiting translation.

Over expression of the PrP gene is known to increase susceptibility of transgenic mice to infection with TSEs (Scott *et al*,, 1989; Prusiner *et al.*, 1990), therefore loss of control, or up-regulation, of PrP^C protein expression would also increase scrapie susceptibility. Natural scrapie transmission must occur through a peripheral route. Expression of the PrP gene in ruminants is known to differ between brain and peripheral tissues through differential transcription of PrP mRNA species (Hunter *et al.*, 1994; Goldmann *et al.*, 1999). According to the prion hypothesis, increasing the amount of PrP^C protein would increase the amount of template for conversion of PrP^C to PrP^{Sc} (Jarret and Lansbury, 1993; Prusiner, 1993). On the other hand, the virino hypothesis, the increased concentration of PrP^C would increase the number of replication sites of the scrapie agent. The function of peripheral tissue in scrapie is slowly being understood: perhaps in sheep susceptible to scrapie abnormal expression of PrP protein in peripheral tissues is induced by the scrapie agent to promote agent replication. Such an argument is proposed in this thesis where loss of control of gene expression through a repressor element in the PrP 3'UTR may lead in adherent expression of PrP which in turn allows for increased potential for PrP^{Sc} formation.

The creation of PrP null mice has shown that expression of the PrP gene is vital for TSE development (Bueler *et al.*, 1993; Sailer *et al.*, 1994). In mice, TSE disease incubation period is also determined by the level of PrP gene expression (Scott *et al.*,

1989; Prusiner, 1990; Bueler *et al.*, 1992). To date there is little evidence that sheep of different breeds or genotypes show significantly different PrP gene expression levels which can account for increased scrapie susceptibility in known populations. For example, tissue distribution of PrP^C protein, determined by western blotting was similar in Suffolk sheep, PrP genotype $AA_{136}RR_{154}QR_{171}$ and Blue-de-Dorset, PrP genotype $AA_{136}RR_{154}QQ_{171}$ but were found to be different in a second Suffolk sheep of genotype $AA_{136}RR_{154}QR_{171}$ (Horiuchi et al., 1995). Furthermore the observed PrP^C glycosylation pattern between sheep and different sheep tissues varied, the authors speculated that heterogeneity might arise from cell type specific PrP^C expression or the influence of PrP genotype. Considering the later point, published sequence data from different breeds acknowledges several point mutations within the PrP gene 3'UTR between different sheep breeds and between sheep of the same breed (Goldmann et al., 1990; Westaway et al., 1994; Lee et al., 1998). The existence of polymorphic sites in the 3'UTR may allow for differential regulation of the PrP mRNA and therefore regulation of PrP protein through translation of the PrP transcripts.

6.2 PrP Genotype and Scrapie Susceptibility.

PrP genotype strongly influences susceptibility of sheep to scrapie. In 1962, Parry concluded that natural scrapie was a genetic disease, caused by an autosomal recessive gene but which was also transmissible (Parry, 1962). The discovery of maternal and lateral transmission (Brotherston *et al.*, 1968; Dickinson *et al.*, 1965; Dickinson and Outram, 1988) and sheep with scrapie susceptible genotypes in scrapie-free countries (Hunter *et al.*, 1997) indicates that scrapie is an infectious disease. The current view is that host genetics are important for natural scrapie incidence but that it is not purely a genetic disease (Hunter *et al.*, 1995; Foster *et al.*, 1996b).

Analysis of natural and experimental scrapie in the NPU South Country Cheviot sheep flock (section 1.7, Table 1.4) has made a considerable contribution to the understanding of scrapie susceptibility. With this flock it was demonstrated that a

major gene, now designated PrP, controlled scrapie susceptibility (Dickinson and Outram, 1988; Hunter *et al.*, 1987; Hunter *et al.*, 1989; Hunter *et al.*, 1991; Hunter *et al.*, 1996). Polymorphic variants of the PrP gene have been extensively characterised and associated with incidence of scrapie in sheep (Goldmann *et al.*, 1991a;Hunter *et al.*, 1994), goats (Goldmann *et al.*, 1996) and humans (Collinge *et al.*, 1991). To date, seven polymorphic amino acid codons have been described in sheep with three known to be vital in determining scrapie susceptibility, these are codons 136, 154 and 171 (Goldmann *et al.*, 1990; Goldmann *et al.*, 1991; Laplance *et al.*, 1993; Bosser *et al.*, 1996; see also Chapter 1, section 1.7).

In NPU Cheviots, sheep homozygous for valine at codon 136 (V_{136}) have a short incubation period after challenge with experimental scrapie (SSBP/1), whereas sheep heterozygous at codon 136 with alanine (A_{136}) have longer incubation periods (Goldmann *et al.*, 1990). Alanine homozygotes, AA_{136}, are linked with scrapie resistance. Different TSE strains respond to different PrP polymorphisms. For example, Cheviots homozygous for glutamine at codon 171 (QQ_{171}) are susceptible to experimental challenge with BSE or scrapie strain CH1641 where as arginine at codon 171 (R_{171}) leads to longer incubation periods (Goldmann *et al.*, 1994). A polymorphism for histidine at codon 154 (H_{154}) has been associated with resistance to scrapie infection in NPU Cheviots (Hunter *et al.*, 1996). PrP polymorphisms at codons 136 and 171 have been associated with natural scrapie infection in throughout the world and in various sheep breeds (Laplanch *et al.*, 1993; Belt *et al.*, 1995; Ikeda *et al.*, 1996).

Intensive PrP genotyping studies have revealed five allelic variations associated with scrapie incidence, the frequency and distribution of the allelic variants differ from breed to breed (section 1.7, Table 1.4; Dawson *et al*, 1998). For example, the PrP allele $V_{136}R_{154}Q_{171}$ is rare in Suffolk sheep but not in Texels or Cheviots and in contrast $A_{136}R_{154}H_{171}$ allele is common in Texels but not in other breeds. In breeds which encode V_{136} alleles (valine breeds), e.g. Cheviots, Swaledales and Shetlands, the allele $V_{136}R_{154}Q_{171}$ shows the strongest association with scrapie susceptibility, sheep homozygous for this allele almost always develop scrapie in affected flocks.

PrP allele $A_{136}R_{154}Q_{171}$ is often associated with scrapie susceptibility, especially in breeds where the $V_{136}R_{154}Q_{171}$ allele is rare i.e. Suffolk and Icelandic sheep breed. In valine breeds the $A_{136}R_{154}Q_{171}$ allele tends to show a lower or incomplete penetrance to scrapie infection, as shown by longer survival times or relative resistance compared to $V_{136}R_{154}Q_{171}$ encoding sheep (Goldmann *et al.*, 1991a; Clouscard *et al.*, 1995). Also, in valine breeds combining the $V_{136}R_{154}Q_{171}$ allele with $A_{136}H_{154}Q_{171}$ or $A_{136}R_{154}R_{171}$ offers relative resistance to scrapie infection (Laplanche *et al.*, 1993; Hunter *et al.*, 1996).

There are on occasion however, incidences of scrapie or resistance to scrapie, which defy the well-defined polymorphisms discussed above. For instance, sheep homozygous for PrP allele $V_{136}R_{154}Q_{171}$ have been found, apparently scrapie free, at an age well past life expectance of a sheep with such a susceptible genotype (Hunter *et al.*, 1997). In contrast sheep with the $V_{136}R_{154}Q_{171}$ allele coupled to $A_{136}R_{154}R_{171}$ allele, normally linked to relative resistance, were found to be affected by scrapie (Hunter *et al.*, 1997). Additionally in non-valine breeds (Suffolk) sheep homozygous for allele $A_{136}R_{154}Q_{171}$ do not always develop scrapie whereas scrapie did occur occasionally in sheep with genotype $AA_{136}RR_{154}QR_{171}$ (Hunter *et al.*, 1997). To date on one case of scrapie has been identified in $AA_{136}RR_{154}RR_{171}$ throughout the world (Ikeda et al., 1995)

Due to the observed variation in PrP allelic variants in scrapie-affected sheep, factors other than known PrP gene polymorphisms must control susceptibility. Results presented within this thesis show detectable differences in *in vitro* protein levels between CAT/PrP-3'UTR constructs transiently expressed in brain cell lines derived from sheep with different PrP genotypes and scrapie susceptibilities. Brain cell lines used in this thesis were derived from a Cheviot $VV_{136}RR_{154}QQ_{171}$ (scrapie susceptible), Cheviot $AA_{136}RR_{154}QQ_{171}$ (scrapie resistant) and an Icelandic sheep breed, $AA_{136}RR_{154}QQ_{171}$ (scrapie susceptible). The PrP gene 3'UTRs associated with the 2.1 kb or 4.6kb PrP mRNA did not result in varying amount of protein. However, constructs containing 3'UTR regions E-G (pD20, figure 4.1) did function differently in the brain cell lines with genotypes $VV_{136}RR_{154}QQ_{171}$ compared with

$AA_{136}RR_{154}QQ_{171}/AA_{136}RR_{154}QR_{171}$. Brain cells derived from sheep with at least one copy of the $A_{136}R_{154}Q_{171}$ allele appeared capable of repressing expression from constructs containing sequence 2000-2700 (i.e. pEYR and pD20) equivalent to the construct with UTR resembling the 2.1kb PrP mRNA (pD17). In contrast, $VV_{136}RR_{154}QQ_{171}$ brain cell line appeared incapable of repressing expression from pD20 (Figure 4.3)

The association between the speculative repressor quality of PrP 3'UTR and PrP genotype does not apply to scrapie susceptibility. The Cheviot brain cell line $VV_{136}RR_{154}QQ_{171}$ is known to be scrapie susceptible where as the Cheviot $AA_{136}RR_{154}QR_{171}$ brain cell line is resistant to natural scrapie. The Icelandic sheep breed in contrast has a genotype of $AA_{136}RR_{154}QQ_{171}$, a genotype normally associated with resistance to natural scrapie in Cheviots, but scrapie susceptible in Icelandic sheep (Thorgeirsdottir *et al.*, 1999). So despite sharing similar PrP genotypes and response to the CAT/PrP-3'UTRs constructs, the Icelandic cerebellum and the $AA_{136}RR_{154}QR_{171}$ Cheviot brain cells are derived from sheep of different scrapie susceptibilities. PrP allele disease linkage is well established, therefore other factors must function to increase, or decrease, the susceptibility of a particular sheep, and breed to scrapie. Several reasons may be given for this: control of PrP expression may differ depending on the host genotype; susceptibility may also be determined by further, as yet unknown polymorphisms in the PrP untranslated region or other genes may have a role in TSE infection; different scrapie strains may have different affinities for different PrP alleles.

The implications here are that breed differences may allow for differential control of PrP gene expression during scrapie infection i.e. variation in genes or gene products other than PrP influence scrapie susceptibility. PrP polymorphisms are strongly linked to incidence of scrapie however other factors may act co-ordinately with PrP to influence scrapie susceptibility. These genes may be involved in controlling expression of the PrP gene. It is feasible that expression of the PrP gene may , in part, be controlled by sequences within the 3'UTR as proposed within this thesis. Suppression of gene expression, either at the level of transcription or translation, will

reduce the amount of available PrP protein which has been strongly linked with a role in facilitating infection of TSEs.

6.3 Brain and Peripheral Expression of the PrP Gene.

The discovery of selective polyadenylation of the PrP gene in brain and peripheral tissue first led to the idea that the function of the PrP 3'UTR is far more complex than previously believed (Hunter *et al.*, 1994). The 4.6kb transcript is preferentially produced in sheep brain tissue, and both mRNA species have also been shown to be present at low level in bovine tissue (Goldmann e t al.,1999). The level of PrP mRNA in peripheral organs is lower than in the brain (kidney PrP mRNA approximately seven times less than brain) despite the presence of two mRNAs. However the level of PrP protein is significantly higher in the brain than in peripheral organs (more than 40 times the level of the kidney). Although no significant difference in PrP mRNA level has been detected between scrapie-free and scrapie-infected sheep, an increase in the PrP protein is consistently observed. This is assumed to be due to accumulation of PrP^{Sc} which is not degraded by the cells. An increase in protein levels that are not associated with an increase in mRNA levels may also be the result of increased stability or translatability of the mRNA. Therefore, translational control of the PrP gene may be vital for maintaining protein levels. Sequence present within the 3'UTR were shown to regulate *in vitro* protein synthesis (Cheung, 1996; Goldmann *et al.*, 1999; this thesis.)

Understanding the molecular events of differential PrP gene expression may lead to an understanding of scrapie susceptibility. The protein associated with TSEs, PrP^{Sc}, has been found in peripheral and neural tissues and expression of the PrP gene is known to vary in different tissues. Relating this to the disease, although the conditions for the conversion of PrP^{C} to PrP^{Sc} are still unknown, the existence of PrP^{C} in many tissues suggests that the scrapie agent may potentially be able to replicate in a variety of tissues. Natural scrapie must be transmitted via a peripheral rather than the intracerebral route so the differential expression of PrP in peripheral tissues is of particular interest (section 6.4).

To address whether the 3'UTR also shows tissue specific control over gene expression, *in vitro* expression studies of the CAT/PrP-3'UTR constructs were completed in ovine cells derived from cerebellum and liver tissues of the Icelandic sheep breed *Ovis brachyura borealis pall* (genotype $AA_{136}RR_{154}QQ_{171}$). In general, CAT protein levels were higher in the liver cells compared to the cerebellum cells (Figure 4.7A and B), in contrast to the *in vivo* pattern of expression of PrP (Horiuchi *et al.*, 1995). This may indicate that brain tissue, the site of TSE pathology, is more efficient in controlling gene expression. As observed in the brain cell lines, there was no significant difference in the overall mean level of CAT protein expression, with the majority of the constructs displaying a characteristic expression pattern. However, expression from the construct with a 1.1kb deletion at the 5' end of the 3'UTR (containing regions E-G, pD20) was significantly higher in the liver derived cell line compared to the cerebellum derived cell line (Compare figures 4.5A and 4.5B). A further deletion of 0.7kb appeared to remove repression of protein expression in the cerebellum cell line. Interestingly, pD20 shows the same expression pattern in $VV_{136}RR_{154}QQ_{171}$ Cheviot brain cells as in $AA_{136}RR_{154}QQ_{171}$ Icelandic sheep liver cells (Figure 4.6, in that the observed repressor quality regions E-part F are not functional. Several reasons may explain this observation. Firstly PrP mRNA expression liver tissue is normally low with PrP protein detection limited (Horiuchi *et al.*, 1995; Goldmann *et al.*, 1999; Hunter personal communication). Therefore expression of CAT/PrP-3'UTR constructs may not be representative of control exerted by the PrP 3'UTR in peripheral tissues. Or perhaps repression from the 4.6kb mRNA 3'UTR is not necessary in peripheral tissue under normal conditions i.e. scrapie free, as the 2.6kb mRNA is constitutively expressed. Alternatively the repressor may only be functioning in brain derived cells with the $A_{136}R_{154}Q_{171}$ PrP allele. On consideration, the second account may be correct as the same region , nucleotides 2000-2700 also repressed expression in mouse N2a cells. Although the PrP genotype of the N2a cells is not known at this time, PrP protein expression is detectable in N2a cells.

In the brain, neurones were originally thought to be the site of agent replication and pathology as PrP mRNA was primarily located with neurones (Kretzschmart *et al.*, 1986; Manson *et al.*, 1992a; Harris *et al.*, 1993) suggesting that neurones were the site of synthesis for PrP^{Sc} which was then transported axonally to the white matter.

Support for this theory came from the study of scrapie development in transgenic mice expressing hamster PrP under the control of the neurone specific enolase (NSE) promoter (Race *et al.*, 1995). Expression of PrP in neurones was only sufficient to sustain infection when the transgenic mice were inoculated intracerebrally with hamster derived scrapie isolate. However, it has been observed that PrP^{Sc} deposition in astrocytes precedes neuronal loss (Diedrich *et al*, 1991) indicating that astrocytes may be a primary target for the scrapie agent (Diedrich *et al.*, 1991) and are subsequently primary producers of PrP^{Sc} which in turn leads to neuronal loss (Muller *et al.*, 1993). Alternatively, astrocytes can be mopping up PrP^{Sc} produced and exported by neurones. Recent studies have shown that a considerable amount of PrP mRNA within the brain is accounted to astrocyte expression (Moser *et al.*, 1995). Furthermore, PrP null mice transgenic for the hamster PrP gene under the control of the glial fibrillary acidic protein (GFAP, astrocyte specific) promoter were susceptible to intracerebral inoculation with hamster scrapie (Raeber *et al.*, 1997). These mice showed typical scrapie pathology with spongiform changes, astrocytosis in the neocortex and hippocampus and accumulation of PrP^{Sc}. The neurone and astrocyte specific expression studies described above highlight the relationship between neurones and astrocytes wherein astrocytes function to maintain the normal status of neurones and may indicate a co-operative relationship for astrocytes and neurone in TSE development. Loss of normal neuronal or astrocyte function during scrapie development may affect a common pathway and lead to dysfunction in both cell types.

The cell type involved in the replication of the scrapie agent *in vivo* has not yet been identified, however evidence is growing in favour of follicular dendritic cells in the spleen functioning as sites of propagation for the agent (McBride *et al.*, 1992, see also section 6.4). Strict control of PrP expression may be vital in tissues known to

express PrP at relatively high levels or those considered to be necessary for trafficking the scrapie agent to the CNS. Conversely the scrapie agent (or specific scrapie strains) may target particular PrP genotypes and tissues in which the PrP synthetic pathway can be modified to promote its replication.

6.4 Transmission of Scrapie

Natural transmission of scrapie is assumed to be via the oral route with the alimentary tract as a possible route of entry for the infectious agent . Ingestion of TSE-infected material is considered the major route of infection for TSE agents responsible for Kuru, vCJD, scrapie and BSE. Attempts to track the route of infection during scrapie development in mice and sheep have used two methods. Firstly immunoblotting and immunohistological detection of PrP^{Sc} are widely used as a marker for infectivity due to the observation that PrP^{Sc} is never found in the absence of TSE infection. However it has been shown that infectivity can be detected without PrP^{Sc} deposition so sensitivity of detection by the latter method may be limited. Mouse bioassay, although more time consuming can also be used to produce a lesion profile (section 1.4) and give further information on TSE strains.

Hadlow *et al*, 1980, 1982, used a mouse bioassay to show infectivity was detectable, and persistent, in lymphiod tissues of naturally infected sheep months, or years, before becoming detectable in brain (Hadlow *et al.*, 1982). This observation was confirmed by immunohistological detection of PrP^{Sc} in spleen, retropharyngeal lymph nodes, mesenteric lymph nodes and palatine tonsils of naturally infected, clinical positive scrapie sheep (van Keulen *et al.*, 1996). A similar PrP^{Sc} detection pattern was described recently during experimental transmission of BSE to lemurs (Bons *et al.*, 1999). These data suggested that during oral exposure, the TSE agent crosses the intestinal barrier via cells in the lumen of the digestive tract (including the tonsils). The cell populations involved in this transport are unknown but could involve M cells (which have been shown to be responsible for the uptake of bacteria, Trier, 1991). Alternatively, lymphocytes present in the tissues may also be involved and transport the agent through the lymphoreticular system (LRS) to the lymph nodes and spleen. Within the spleen

cells thought to be vital for scrapie replication are follicular dendritic cells (FDC) (McBride *et al.*, 1992). Severe combined immunodeficent (SCID) mice, which lack B and T lymphocytes and mature FDCs, are resistant to peripheral (intraperitoneal) challenge with scrapie and are unable to replicate the agent in their spleens (Fraser *et al.*, 1996; Lasmezas *et al.*, 1996; O'Rourke *et al.*, 1994). Furthermore, transgenic models with immune systems manipulated to produce lymphocytes but not mature FDCs were resistant to infection with scrapie strain ME7 (Brown *et al.*, 1999). Therefore the LRS, or specifically the gut associated lymphoid tissue (GALT), and the spleen may play a vital role in scrapie agent replication and spread of disease from peripheral tissues to the brain (CNS).

Several studies have indicated a second route for the spread of the disease in a host. Kimberlin *et al.* (1989b) demonstrated that splenectomy did not prevent mice developing scrapie when challenged intragastrically with mouse-adapted scrapie strain 139A. In hamsters, infection with scrapie strain 263K shows little involvement of the spleen in scrapie replication early in pathogenesis following oral infection (Beekes at el., 1996). More recently PrP^{Sc} deposition was recorded in myentric and submucosal plexuses of the gut wall, stomach, caudal oesophagus and enteric nerves of sheep with clinical signs of natural scrapie (van Keulen *et al.*, 1999). This was confirmed by Beekes and McBride (2000) where infection was detected early in pathogenesis in various GALT tissues and ENS ganglia which suggests that the enteric nervous system may be an alternative route. Once located in the ENS or GALT the agent is presumed to spread to the thoracic spinal cord or dorsal motor nucleus of medulla oblongata (van Keulen *et al* 1999; Beekes and McBride, 2000) via nerve cell pathways.

The route of infection and replication in the spleen would appear to be strain specific and host dependent. For example in hamsters, infection with scrapie strain 263K does not involve replication in the spleen (Kimberlin *et al.*, 1989b). Similarly, the spleen does not seem vital to BSE infection in cows, however infectivity has been reported in sheep experimentally infected with BSE (Foster *et al.*, 1996a). Also van Keulen *et al*, 1999, reported significantly more peripheral deposition of PrP^{Sc} in the ENS of sheep with highest genetically determined scrapie susceptibility compared with those with a PrP genotype associated with scrapie resistance (van Keulen, *et al.*, 1999).

The role of PrP gene expression in TSE susceptibility and development is slowly being understood. Fundamentally, factors known to increase TSE susceptibility are defined mutations and polymorphisms in the PrP coding region and, as determined by studies in transgenic mice, overexpression of the PrP gene. Scrapie is not a spontaneous genetic disease (Hunter *et al.*, 1997), therefore an infectious agent must be involved in disease development. Following oral contamination the agent may begin replication within tissues, or cell types, in which it can interact with the normal PrP^C synthetic pathway. Differential control, or expression, of the PrP gene between tissues may dictate the route of infection taken by the scrapie agent.

6.5 Controlling PrP^C Expression.

Production of an active protein from a gene requires multiple regulated steps, many of these steps involve some aspect of RNA processing. Disease caused by mutations that affect RNA processing are relatively rare compared to mutations that disrupt protein function. The vast majority of RNA processing faults linked to disease are due to the loss of function of a single gene as a result of mutations in elements required for pre-mRNA splicing. However, a few diseases are caused by alternations in transacting factors required for RNA processing. One interesting example of such a process was observed in expression of amyloid precursor protein (APP) mRNA (Amara *et al.*, 1999). Similar to TSEs, Alzheimer's disease (AD) is associated with deposition of an amyloid peptide (amyloid-β, Aβ) in the CNS and neurodegeneration (Ishiura *et al.*, 1991; Lendon *et al.*, 1997). Aβ formation is thought to be linked to abnormally processed, or high levels, of APPs as a result of over expression of APP mRNA (Johnson *et al.*, 1990; Ishiura *et al.*, 1991;Hardy, 1997). *In vitro*, APP mRNA levels were observed to increase in astrocytes in response to TGF-β_1 (Gray and Patel, 1993). Consequently, levels of transforming growth factor (TGF-β_1) were found to be increased in AD patients, to associate with lesions (Peress and Perille, 1995) and to promoter amyloid deposition in transgenic mice (Wyss-Coray *et al.*, 1997). Therefore TGF-β_1 appeared to be a candidate for increasing APP mRNA levels and promoting

AD. TGF-β_1 is a known transcriptional regulator (Massague, 1990), however TGF-β_1 has also been shown to regulate genes involved in mRNA stability (Amara *et al.*, 1996).

In vitro analysis of APP mRNA expression in human astrocytes in response to TGF-β_1 confirmed the results of Gray and Patel, 1993, but also showed that in the presence of TGF-β_1, APP mRNA stability increased five-fold (Amara *et al.*, 1999). As 3'UTRs are known to function in controlling mRNA stability the authors examined any possible role of APP mRNA 3'UTR in TGF-β_1 induced stabilisation and discovered that the APP mRNA 3'UTR conferred stability on the CAT reporter gene product (Amara *et al.*, 1999). Band shift and UV cross-linking assays revealed that sequence in the 3'UTR interacted with a cytoplasmic protein to form a 68kDa RNA-protein complex in response to TGF-β_1 treatment. Formation of the RNA-protein complex led to increased stability of the CAT mRNA. Deletion of the TGF-β_1 response element (region of UTR the TGF-β_1- responsive protein bound to) from the 3'UTR of APP mRNA prevented stabilisation of CAT mRNA in the presence of TGF-β_1. Amara *et al*, 1999, proposed that TGF-β_1 may increase and stabilise APP mRNA expression indirectly by regulating a responsive protein and its interaction with the 3'UTR sequence.

There is little knowledge on the PrP regulatory pathway and the role of PrP^C in peripheral and its contribution to scrapie agent infection and replication. Peripheral tissues are a potential source for scrapie replication and subsequent transport to the brain (section 6.4), understanding the mechanism of PrP expression is vital for elucidating the function of PrP^C in TSEs. From *in vitro* and *in vivo* studies there is evidence that the level of PrP mRNA expression may increase in the presence of specific cytokines and growth/differential factors such as interlukin –6 (IL-6), nerve growth factor (NGF), migratory inhibitory factor-related protein(MRP8) (Mobley *et al.*, 1988; Wion *et al.*, 1988; Lazarini *et al.*, 1994; Kniazeva *et al.*, 1997). In contrast, PrP expression is inhibited in human fibroblasts by the presence of platelet derived growth factor (PDGF). Therefore, known transacting factors (regulatory proteins) have already been shown to influence the level of detectable PrP expression. The majority of the transacting factors are transcription activators or regulators (Table 4.6). However as

discussed for Alzheimer disease (AD, Section6.5), transacting factors such as cytokines can also regulate at the post-transcriptional level.

Work presented within this thesis confirms that the ovine PrP gene 3'UTR contains a sequence capable of mediating PrP protein levels and proposes that sequence 2000-2700 represses expression from the 4.6kb mRNA. This speculative repressor sequence may be in either secondary structure or specific sequence (*cis*-acting) for the binding of regulatory factors (nuclear or cytoplasmic). Alteration of the PrP metabolic pathway by the agent may be indirectly through interaction with transcriptional/ translational machinery or directly with the pre-mRNA or mRNA (Figure 6.2). Cheung (1996) observed no change in mRNA levels but increased translatability (protein levels) from various CAT/PrP-3'UTR constructs. This would suggest that different PrP mRNAs show either enhanced stability or translation efficiency. If the scrapie agent were capable of modulating PrP metabolism increasing stability/translation of PrP mRNA would increase PrP protein levels, which in turn would enhance agent replication. The agent may bind directly to PrP mRNA or affect *trans*-acting factors that bind to the RNA. Genotype and scrapie susceptibility differences observed between, and within, flocks may be explained by efficiency of the scrapie agent to interact with PrP synthesis in sheep with different genetic backgrounds.

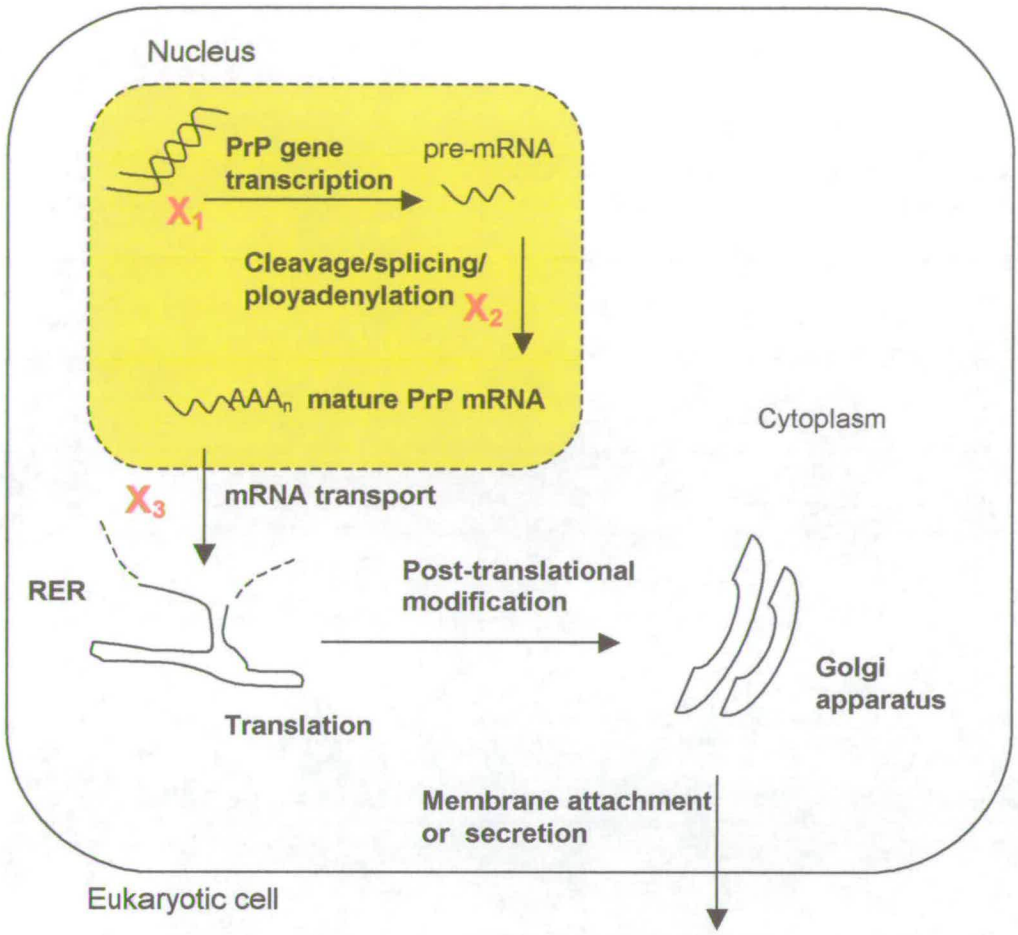

Figure 6.2 Potential sites for control of PrP gene expression.

Within this thesis, control of *in vitro* gene expression has been examined in association with the PrP gene 3'UTR. A putative repressor of expression has been proposed within the 3'UTR which functions preferentially in brain cells with at least one ARQ allele. Sites for repression include: X_1; down regulation of transcription, repression of transcription termination: X_2; interaction with *trans*-acting factors preventing efficient processing; differential regulation of polyA degradation; X_3; association with cytoplasm *trans*-acting factors which prevent translation or stabilise mRNA; down regulation of translation efficiency. RER: rough endoplasmic reticulum.

6.5 Final Thoughts and Future Work.

Expression of the PrP gene is vital for the development and spread of TSE disease.. Although scrapie in sheep and goats is known not to be spontaneous genetic disease, polymorphisms have been linked to increased susceptibility. However, how can a sheep of susceptible genotype remain apparently healthy when housed with an infected flock, or a sheep with resistant genotype develop scrapie? One explanation for selective development of scrapie in sheep of particular PrP genotype and breed is proposed in this thesis: differential control of PrP gene expression. Factors other than PrP genotype frequency may also influence scrapie susceptibility. A putative repressor –binding site in the PrP gene 3'UTR has been identified in the 4.6kb PrP mRNA 3'UTR sequence which acts specifically in brain cells derived from sheep with at least one $A_{136}R_{154}Q_{171}$ allele. However the repressor element does not seem to be associate strictly with scrapie resistance as the regulatory function was operational in $A_{136}R_{154}Q_{171}$ brain-derived cells from known scrapie resistant and susceptible sheep. Perhaps highly susceptible breeds and genotypes already have reduced control over PrP gene expression which can be exploited by the scrapie agent. During the process of replication, the agent may interfere with the pathway of PrP synthesis by increasing stability of the 4.6kb mRNA, leading to increased levels of the 4.6kb mRNA and may produce PrP^{Sc} as a by-product.

It is hypothesised that:

1. Scrapie susceptibility, although strongly linked to PrP gene polymorphisms, may also linked to control of PrP gene expression
2. In sheep with high-risk scrapie associated alleles the scrapie agent may be able to increase the level of PrP gene expression at the post-transcriptional level.
3. PrP expression may be controlled through transacting factors at a 3'UTR cis-element (specific sequence).
4. PrP gene expression is controlled in cell/tissue specific manner that may dictate the route of scrapie spread to the CNS.

Work for the future should be aimed at confirming the regulatory role of the 3'UTR in gene expression. Transient expression of the CAT/PrP-3'UTR constructs in cells derived from various non-neuronal tissues thought to participate in scrapie infection

(distal ileum, spleen, lymph nodes) would clarify whether or not the repressor was specific to the brain or not. It would be interesting to see if the CAT/PrP –3'UTR constructs showed the same expression in tissues known to express PrP in peripheral tissue i.e spleen or lung. The spleen would be of particular interest as this is one of the few non-neural tissues in which natural scrapie infectivity can be detected. Also, expression in follicular dendritic cells, one of the assumed sites for scrapie replication outside the CNS may, lead to further understanding of PrP expression and the disease process. Furthermore, wider *in vitro* expression analysis in cell lines derived from sheep of other PrP genotypes would confirm the association of $A_{136}R_{154}Q_{171}$ allele with reduced expressional control and indicate that other factors (based on genetic differences) may control PrP gene expression. If cells from PrP expressing peripheral tissues derived $AA_{136}RR_{154}QQ_{171}$ or $AA_{136}RR_{154}QR_{171}$ sheep also showed repression of the CAT/PrP-3'UTR vectors over sequence 2000-2700 this would further imply that control of PrP gene expression is vital for maintaining scrapie resistance. Transient expression of the PrP gene should be possible in the future with development of more specific antibodies and extraction methods. Using this approach a clearer understand may be reached on specific control of the PrP gene.

Studies should concentrate on potential binding of transacting factors to the PrP gene 3'UTR. This could be achieved by DNA footprinting (Galas and Schmitz, 1978), DNA mobility shift assays or gel retardation (Fried and Crothers, 1981; Garer and Revzin, 1981) and methylation interference assays (Sienbenlist and Gilbert, 1980). Confirmation of transacting factors by a combination of these methods could lead to purification and characterisation of the regulatory molecules, and then hopefully identification. Furthermore, the efficiency of *in vitro* expression could be monitored as cells response to chemical or physical treatments (growth factors, heat stress) potentially able to control gene expression through cell signalling pathways.

If one function of the 3'UTR is regulation of PrP gene expression via sequence at nucleotides 2000-2700 what about the 2.1kb PrP mRNA? The 2.1kb transcript is processed upstream of this putative repressor region so can we assume that this mRNA is controlled in the same way? No major difference has been recorded in expression of

the PrP transcripts between scrapie free and infected animals. From *in vivo* observations, it would appear that expression of the 2.1kb mRNA and the ratio of two PrP transcripts in particular tissues is further controlled through regions not included in this study.

Natural scrapie must be transmitted through the peripheral route, so understanding the differential expression of PrP gene is of particular interest. It cannot yet be speculated whether this putative repressor sequences functions as a structural motif or recognition factor for nucleic acid binding proteins. Further more, this region may indeed interact with sequences at the 5' region of the PrP gene, a matter not addressed within this thesis. It is interesting to note that this repressor region is immediately 5' of the polymorphic *Eco*RI site loosely associated with scrapie incidence (Hunter *et al.*, 1991). However, it is feasible to imagine that expression the PrP gene may be, in part, controlled by sequences within the 3'UTR. Suppression of gene expression, either at the level of transcription or translation, will reduce the amount of available PrP protein which has been strongly linked with a role in facilitating infection of TSEs. Consequently a loss of repressor function may lead to aberrant PrP expression. PrP gene expression is vital for scrapie development, therefore variation in gene expression control may alter the levels of PrP protein in tissue and hence scrapie agent infection, replication and ultimately pathology.

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Appendix A: Solutions, Reagents and Plasmids

General solutions.

Agar Broth.
20g of LB broth base to 1Lt of dH_2O.
Autoclave as for agar plates.
Add ampicillin to final concentration 1mg/ml.

Agar Plates.
32g of LB agar
make up to 1Lt with dH_2O
Autoclave for 15mins at 121°C

Agar can be poured immediately or stored for use. To melt, re-heat in microwave at low for 30 minutes, shake regularly. Leave for one hour to cool, then pour.

1% Agarose Gels.
1g agarose
100ml 1x TBE buffer
4µl Ethidium Bromide

Alkaline lysis solution 1.
50mM Glucose
2.5mM Tris Cl (pH 8.0)
2ml 0.5M EDTA stock
Prepare in 100ml aliquots, autoclave and store at 4°C. Glucose added after autoclaving.

Alkaline lysis solution 2.
7ml dH_2O
2ml 4M NaOH (final conc 20mM)
1ml 10%SDS

Alkaline lysis solution 3.
60ml 5M Potassium acetate (final conc 3M)
11.5ml Glacial acetic acid
28.5ml dH_2O

Ampicillin plate.
As agar plates; when the agar is ready to pour add ampicillin to final concentration of 1μg / ml.

Ethidium Bromide. CAUTION
0.25g Ethidium Bromide
100ml dH_2O
or 1x 10mg tablet to 1ml dH_2O. Make up to 2ml.

Loading buffer (agar gels)
0.25% bromophenol blue (0.025g)
50% glycerol + dH_2O

TB.
0.6g 10mM PIPES
2.18g 55mM $MnCl_2$
0.44g 15mM $CaCl_2$
3.73g 250mM KCL

TBE (10x).
108g Tris
55g Boric acid
40ml 0.5M EDTA pH 8.0
Make up to 1L

Tris-HCl (pH7.5)
0.1M Tris-HCl , pH7.4
pH to 7.4 with HCl
Distilled water to 100ml

2XYT Medium
10g LB Broth
5g Bacto-tryptone

Add dH_2O to 500ml, autoclave to sterilise. oH to 7.05.
Filter sterilise and store at 4°C (there is a brown precipitate autoclave instead)

General Tissue Culture solutions

Fetal Calf Serum (FCS).
500ml heat inactivated FCS aliquoted into 10x50ml, stored at -20°C. FCS heat inactivated at 56°C for 45 mins. Immediately before use centrifuge at 2000rpm for 10 minutes.

FCS supplied by Gibco-BRL .

Complete Culture Medium.
500ml of IMDM (Gibco)
50ml of centrifuged, heat inactivated FCS
Penicillin/Streptomycin to final conc. 100 units/ml
Add 5ml Pen/Strep to 500ml medium

Complete Culture Medium with Nerve Growth Factor(NGF).
100ml of complete culture medium
NGF to final conc. of 10ng/ml (Sigma N-0153)

Freezing Medium.
30ml of complete culture medium
1.5ml heat inactivated FCS (centrifuged)
5ml of DMSO
aliquot into 8x6ml and tore at -20°C

Trypsin/ Versene.
4ml 25%Trypsin
16ml 0.02% Versene

0.1% Gelatin.
0.4g gelatin (Biorad gelatin electrophoretic grade)
400ml dH_2O
autoclaved for 15 minutes at 121°C, store at 4°C

TENS.
40mM Tris-HCL pH 7.5
1mM EDTA pH 8.0
150mM NaCl

FITC Blocking buffer

0.2g BSA
0.745g Tris pH 7.4
To 100ml H_2O

General solutions and requirements for protein extraction

Protein Standards.
Prestained proein molecular weight Standards (Gibco BRL, 26041-020), 14,300-200,000 molecular weight range, lyophilized

Running conditions - 200V, 150mA (2 gels) for 1 hour.

Apparatus - Mini protean II Electrophoresis cell (Biorad)
- Semi-dry transfer cell (Biorad)

12% Acrylamide Resolving gel
10.02ml Acryl/bis mix
1.285g Tris
3.0g Sucrose
0.77ml 1M HCl
8.75ml TEMED
H_2O to 25ml
Store at 4°C. To pour gel, 7ml 12% acrylamide mix + 120µl APS

3.5% Acrylamide Stacking gel
5.82ml Acryl/bis mix
0.327g Tris
2.65ml 0.5M H_2SO_4
55ml TEMED
H_2O to 50ml
Store at 4°C. To pour gel, 3.8ml 3.5% acrylamide mix + 60µl APS. Apply on top of 12% gel.

10% Ammonium Persulphate (APS)
0.1g in 1 ml water
NB - make fresh daily

5x Sample Buffer (SB)
000.81g Tris
1.25g SDS
0.667ml 1M H_2SO_4
1.25ml β-mercaptoethanol

Lower Reservior Buffer
28.22g Tris
16.9ml 1M HCl
Distilled water to 500ml.
Note- make fresh on day of use.

Upper Reservoir Buffer (20x)
24.8g Boric acdi
49.6g Tris
10.0g SDS
Distilled water to 500ml.
For running: 10ml 20x stock to 190 ml distilled water for 2 gels.

Solutions for Chemiluminescence blotting.

Semi-dry transfer buffer.
2.93g glycine
5.81g Tris
0.37g SDS
200ml methanol
H_2O to 1L
Store at 4°C

TBS.
6.05g Tris base
8.76g NaCl
H_2O to 800ml, pH 7.5 with HCl - top up to 1L with water

TBST
For one blot: 0.25ml TWEEN (Roche) in 250ml TBS

1% Blocking solution.
Per blot: 1ml Blocking agent (Roche) in 100ml TBS

0.5% Blocking agent
Per Blot: 5ml Blocking agent in 100ml TBS

Detection solution.
Pre-warm solution A for 30 minutes at 25°C
Per blot: 1.5ml solution A, 15ml solution B. Incubate at room temperature for 30 minutes.

BM Chemiluminescence Blotting Substrate (POD) Cat no: 1 500 694 (Roche Diagnostics,UK)

Solutions for Triton X-114 extraction.

Extraction Buffer.
For 50ml;
5ml 0.1M Tris-HCl, pH 7.4 (10mM)
0.438g NaCl (150mM)
4.385ml Triton X-114 (1%)
note – Triton X-114 must be pre-condensed. Approximate concentration of Triton X-114 is 11%. Store at 4°C

Sucrose Buffer
For 50ml;
5ml 0.1M Tris-HCl, pH7.4 (10mM)
3g Sucrose (6%)
0.43g NaCl (150mM)
H_2O to 50 ml
Store at 4°C

Plasmids.

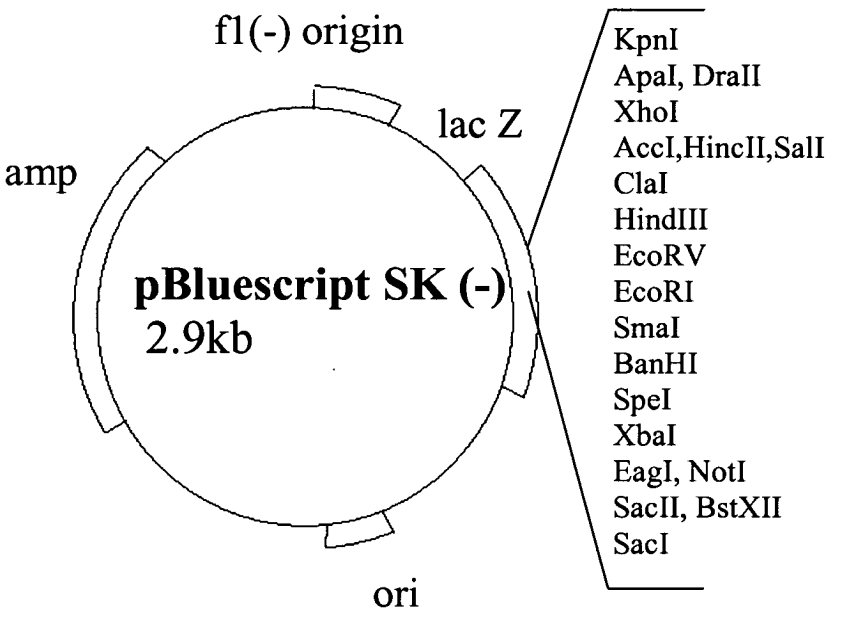

Figure A. 1 Cloning vector pBluescript SK II (-)

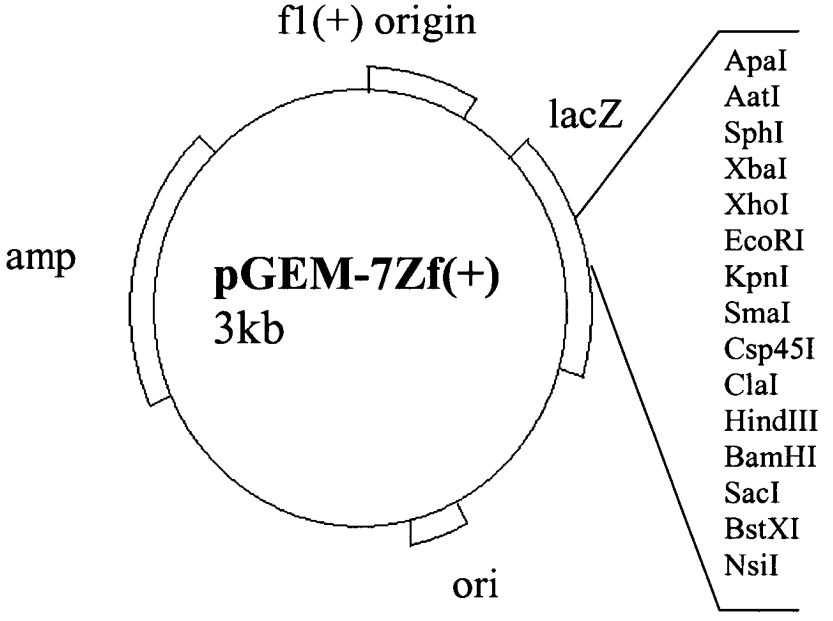

Figure A. 2 Cloning vector pGEM-7Zf (+)

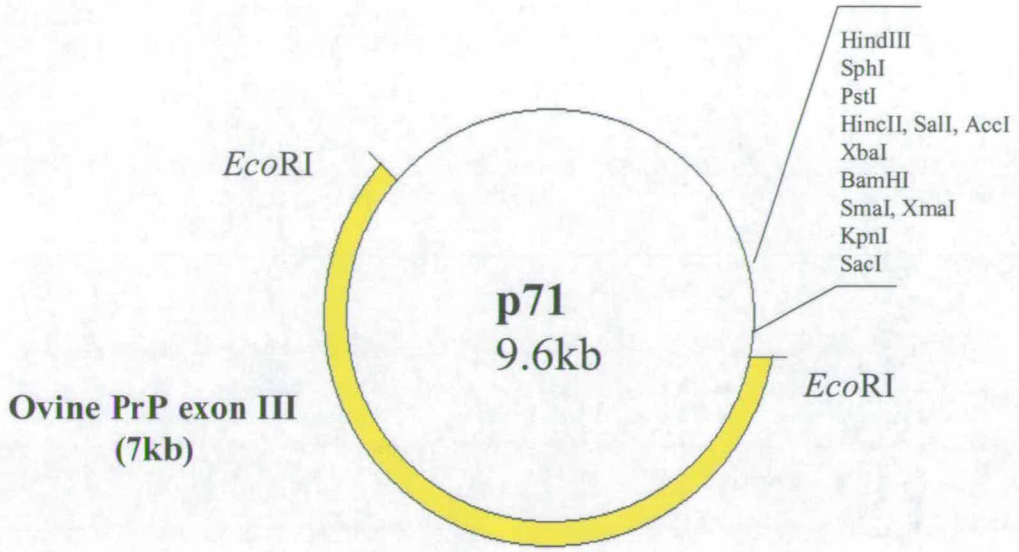

Figure A. 3 p71 pUC18 derived (Goldmann et al, 1990)

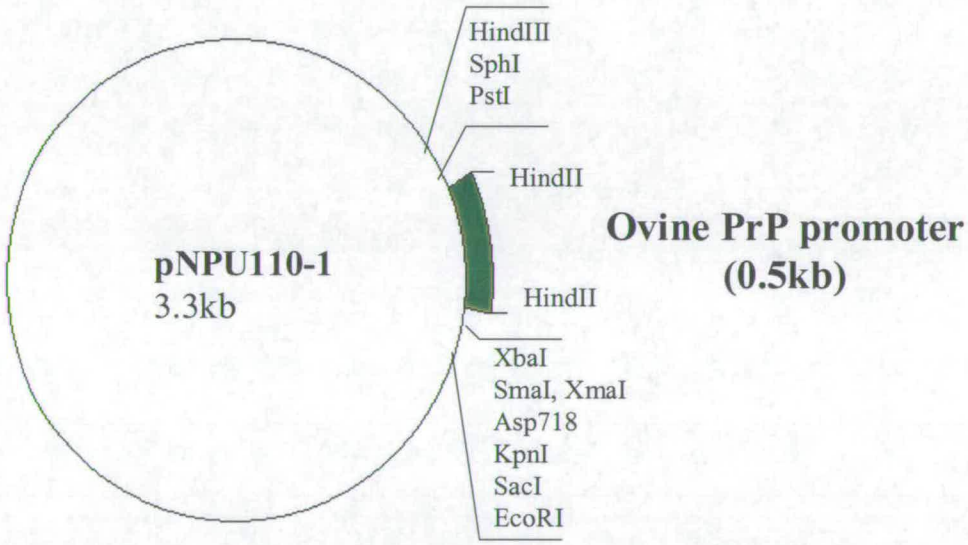

Figure A. 4 pNPU 110-1. PT7/T3a-18 derived. Wilfred Goldmann

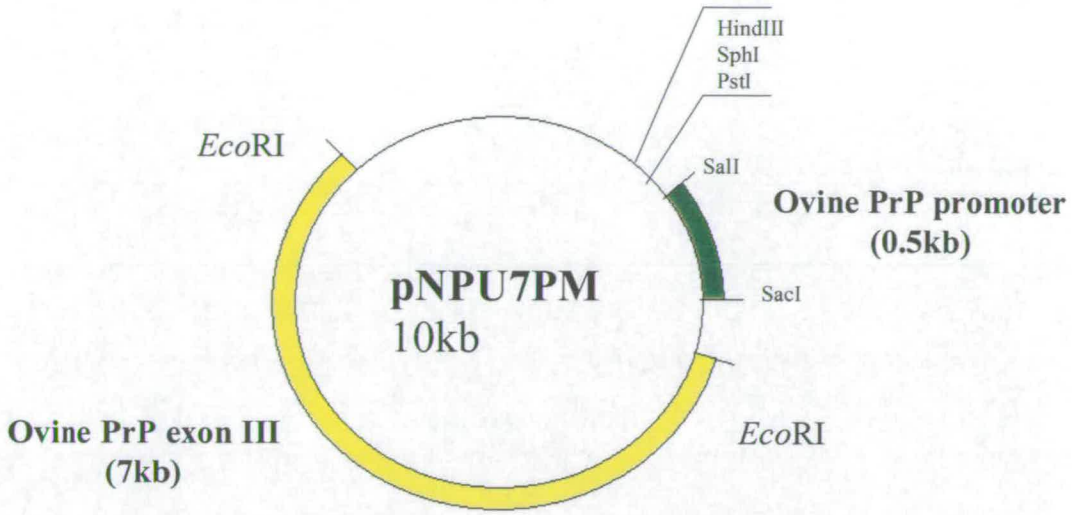

Figure A. 5 pNPU7PM

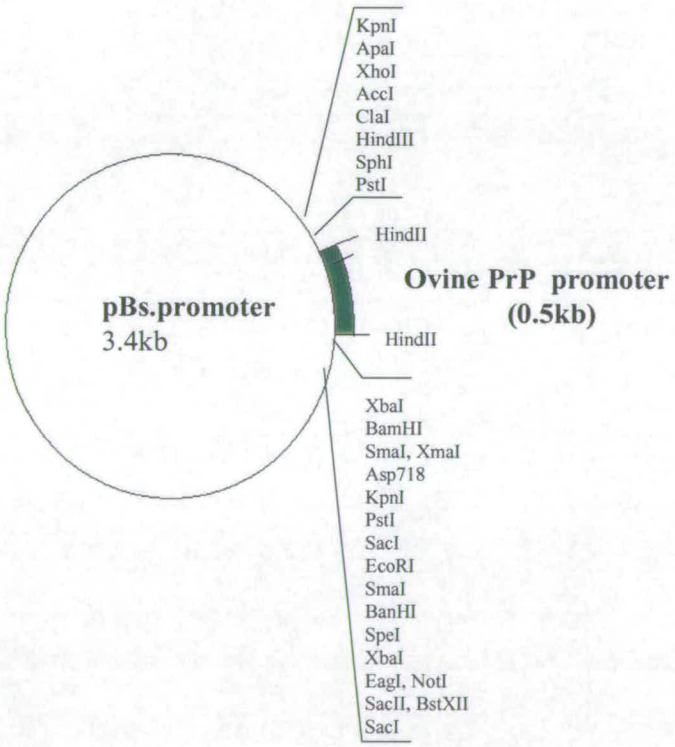

Figure A. 6 pBs.promoter. Cloned from pNPU 110-1 by HindIII-EcoRI digestion into pBluescript SK(-)

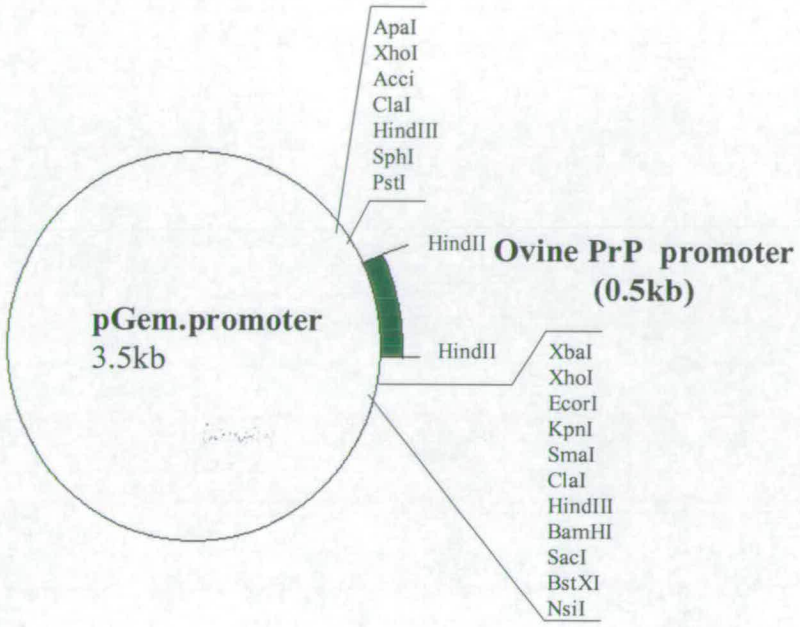

Figure A. 7 pGEM.promoter. Cloning by ApaI-XbaI digestion of pBs.promoterinto pGEM-7Zf(+)

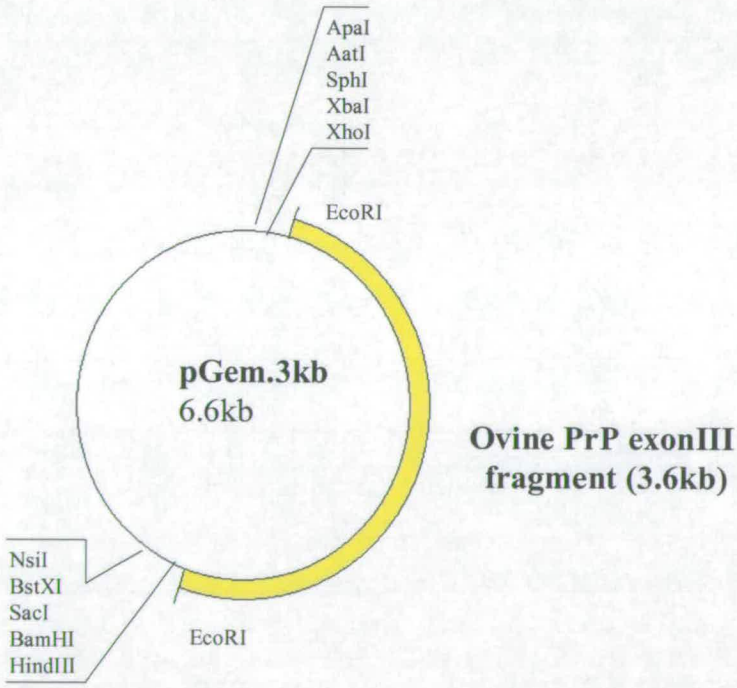

Figure A. 8 pGem.3kb Cloning of 3.6kb EcoRI-ClaI digestion of p71 into pGEM-7Zf(+)

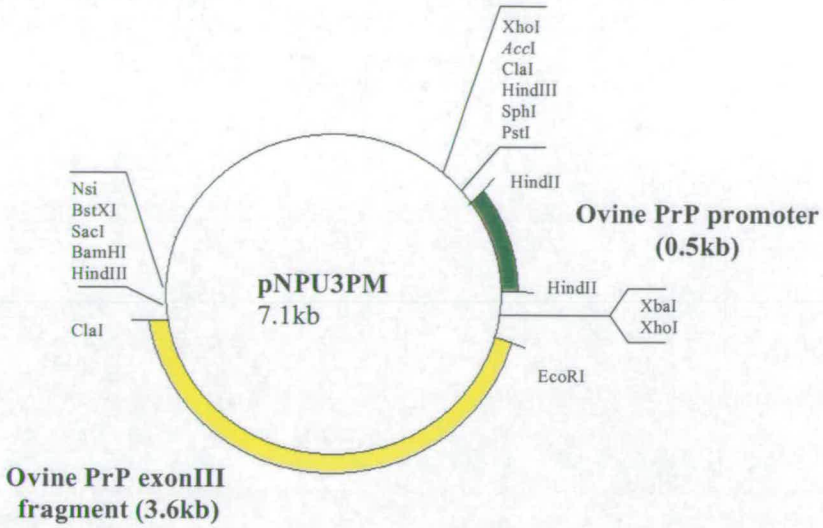

Figure A. 9pNPU3PM. Insertion of (XhoI)ApaI-XhoI digestion from pGem.Promoter into pGem.3kb to form vector with truncated exonIII

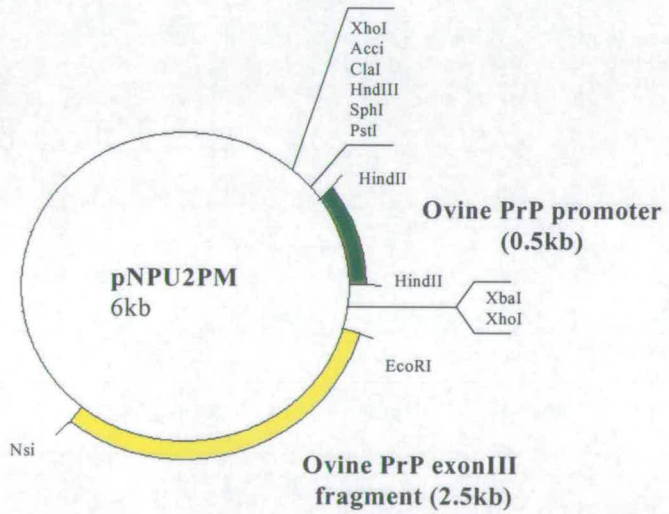

Figure A. 10 pNPU2PM. Created by removing 1.5kb Nsi-Kpn digestion from pNPU3PM and re-insertion of 0.4kb Kpn-Pst digestion .(Pst and Nsi are complimentary sites)

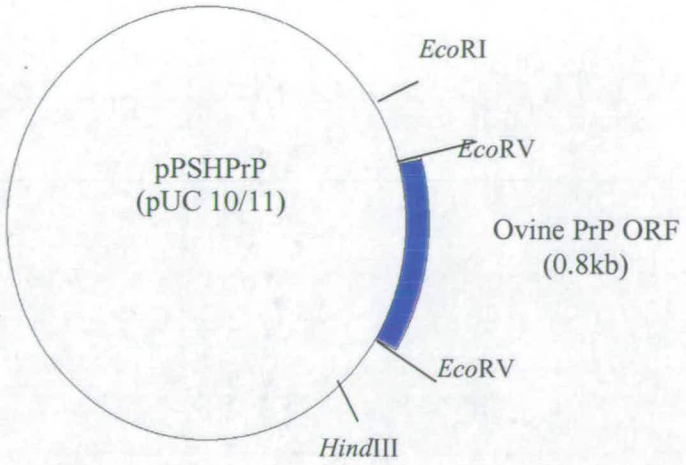

Figure A. 11 pPSHPrP Designed and supplied by Herbert Baybutt, NPU

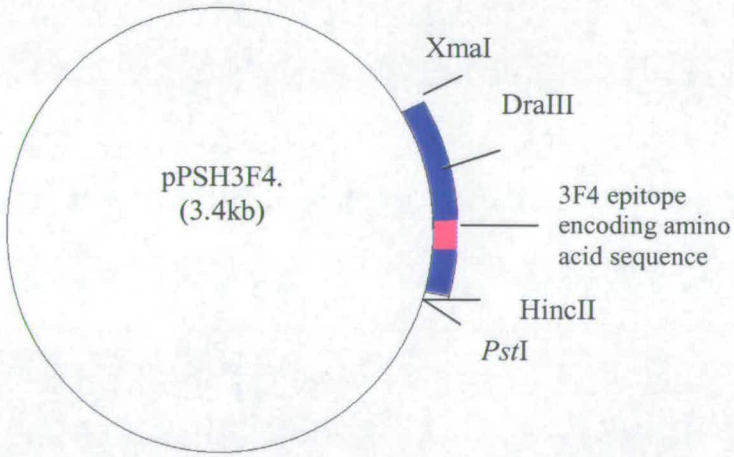

Figure A. 12 Construct pPSH3F4. Manipulation of the ovine ORF by PCR-mediated site directed mutagenesis introduced the hamster coding sequences for the amino acid epitope 3F4.. The manipulated ovine fragment (0.7kb) was cloned into clone pPSH3F4 (pBluescript backbone) using sites *Pst*I and *Xma*I.

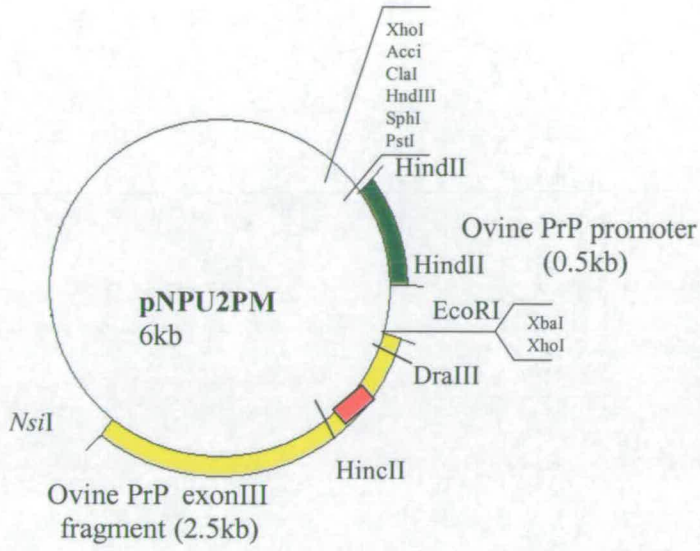

Figure A. 13 Manipulated ovine sequence was cloned from pPSH3F4 in to pNPU2.3F4 using sites *Dra*III and *Hinc*II

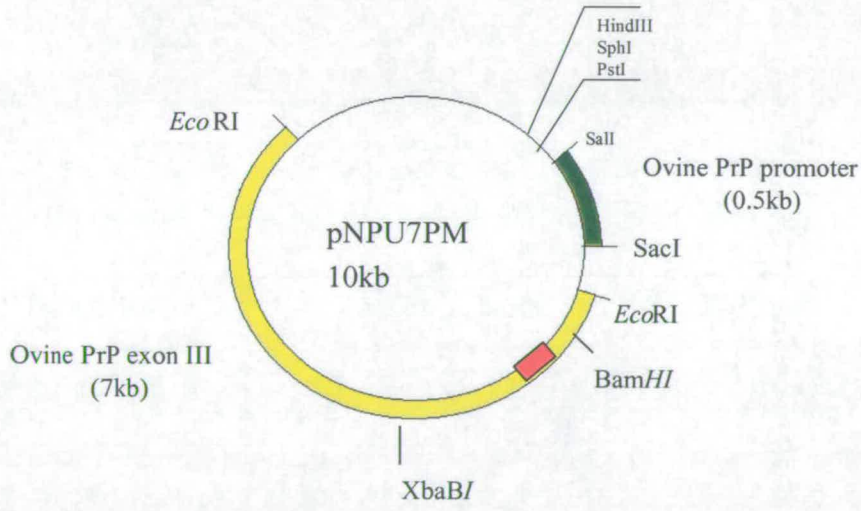

Figure A. 14 Restriction digestion with *Bam*HI/ *Xba*I of plasmids pNPU2.3F4 and pNPU7PM produced a 1.5kb fragment, encoding the nucleotide sequence for the 3F4 epitope, this was inserted into clone pNPU4.3F4.

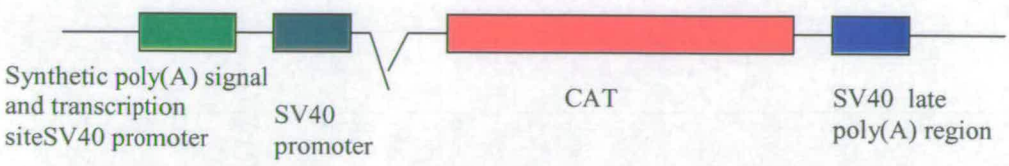

Figure A. 15 pCAT3-Promoter vector (Promega, Southhamptom UK).

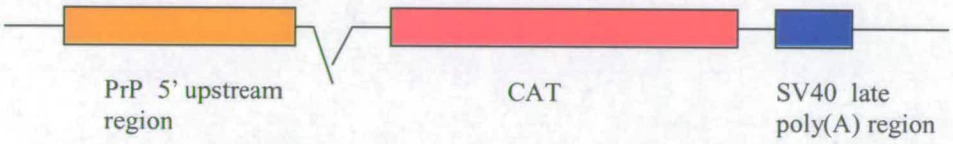

Figure A. 16 Construct pCAT5 was designed and supplied by Dr Herbert Baybutt and contains 1.5kb insert of sequence 5' to the mouse PrP start site cloned upstream of the CAT coding region

Appendix B

B 1.0 Optimisation of transfection protocol for ovine brain-derived cell line sA80BR.

Transfection methods tested are described in Chapter Two, Section 2.5. . The control vector pCAT3-Promoter (Promega, UK, appendix A) which expresses chloramphenicol acetyl transferase (CAT), was used to optimise transfection methods by analysing CAT activity in cell lysates. The vector pSV-β-Galactosidase, which expresses β-Galactosidase, was used to control for transfection efficiency or cell lysate recovery from transfection experiments. The cells, sA80BR, were harvested 48 hours post-transfection with the vector pCAT3-Promoter and a CAT assay was performed, the results of which were analysed by thin layer chromatography (TLC). Using the autorad developed from TLC, it was possible to isolate the acetylated products from the silica plate and the level of CAT activity determined by measuring [^{14}C] by liquid scintillation counting (LSC, section 2.5.10). Relative CAT activity was determined by comparing the CAT activity of the reporter construct to a positive CAT control reaction (assumed to be 100% for each experiment). The positive CAT control was 1 unit of purified CAT enzyme (Promega, UK). The negative control was cell lysate from non-CAT transfected cells

Table B 1.1 Transient transfection sA80BR by calcium phosphate.

Experiment Details	1	2	3[a]	4	5[a]
Cell Passage Number	9	13	13	14	14
[DNA] ug	10	10	5	5	5
Time (hours)	6	16	16	16	16
LSC (cpm)	815.00	42.70	58.59	622.84[b]	571.73[b]
Positive control	n/d	36178.48		22744.32	22744.32
Negative control	n/d	47.66		277.12	277.12
Relative CAT %	0	0	0	0	0

[a] Experiment 3 was a duplicate of 2 and experiment 5 a duplicate of 4
[b] Results negative by TLC

Table B 1.2 Transfection of brain sA80BR cell line by Electroporation.

Experiment Details	1	2	3a	4a
Cell Passage No.	12	5	5	5
DNA (ug)	50	5	25	50
Voltage, V	800	800	800	800
Capacitance	300	300	300	300
LSC (cpm)	1781.89	0[b]	0[b]	0[b]
Positive control	76458.28	54183.2		
Negative control	461.9	291.3		
Relative CAT %	1.74	0	0	0

[a] Experiment 3 and 4 were duplicates of 2
[b] Results negative by TLC

Table B 1.3 Transfection of brain sA80BR cell line by Electroporation.

Experiment Details	5	6a	7a
Cell Passage No.	6	6	6
DNA (ug)	20	20	20
Voltage, V	200	250	300
Capacitance	960	960	960
LSC (cpm)	0*	0*	0*
Positive control	34864.7		
Negative control	189.4		
Relative CAT %	0	0	0

[a] Experiment 6 and 7 were a duplicate of 5
[b] Results negative by TLC

Table B 1.4 Transfection of brain sA80BR cell line by Dosper reagent (Roche Diagnostics East Sussex, UK)

Experiment Details	1	2[a]	3[a]	4[a]
Cell Passage No.	11			
DNA (μg)	3	3	3	3
Dosper (μl)	0	2	4	6
LSC (cpm)	482.76[b]	351.61[b]	312.88[b]	485.75[b]
Positive control	4176.32			
Negative control	413.21			
Relative CAT %	1.85	0	0	1.93

[a]Experiments 2-4 were duplicates of 1
[b]Results negative by TLC

Table B 1.5 Transfection of brain sA80BR cell line by Dosper reagent (continued)

Experiment Details	5	6[a]	7[a]	8[a]
Cell Passage No.	13	13	13	13
DNA (μg)	3	3	3	3
Dosper (μl)	0	2	4	6
LSC (cpm)	655.63	501.64	424.15	1483.54
Positive control	92427.94			
Negative control	406.26			
Relative CAT %	0.27	0.1	0	1.17

[a]Experiments 6-7 were duplicates of 5

Table B 1.6 Transfection of sA80BR cell line by Tfx50 (Promega, Southampton, UK)

Experiment Details	1	2	3	1	2	3
Cell line	sA80BR			pA80BR		
[DNA] ug	3	3	3	3	3	3
Tfx50 ratio	2:1	3:1	4:1	2:1	3:1	4:1
LSC (cpm)	1534.74	2413.98	744.88	10566.09	8275.89	1162.57
Positive control	109272.6					
Negative control	634.07			748.84		
Relative CAT %	0.83	1.64	0.1	9.05	6.94	0.38

Note: sA80BR, cell passage number 16. pA80BR, cell passage number 10

Table B.17 Transfection of sA80BR cell line by Tfx50; serum free

Experiment Details		1	2	3	1	2	3
Cell Passage No					17		
Medium		serum	serum	serum	serum free	serum free	serum free
Tfx50 ratio		2:1	3:1	4:1	2:1	3:1	4:1
LSC (cpm) for 0.5		360.56	471.8	523.25	343.67	191.87	481.72
DNA (μg);	1	307.91	395.63	464.89	395.65	226.49	520.29
	3	605.95	436.2	854.7	7876.72	5173.91	1879.3
Positive control		107605.7					
Negative control		322.85					
Relative CAT %		0.04	0.14	0.19	0.02	0.	0.15
for;	0.5μg						
	1μg	0	0.07	0.13	0.07	0	0.18
	3μg	0.26	0.11	0.5	7.04	4.52	1.45

Table B 1.8 Transfection of sA80BR cell line by Tfx50 : serum free.

Experiment Details		1		2		3	
Cell Passage No		18		19		20	
DNA (ug)		3	3	2.5	2.5	1	1
		6	6	5	5	2.5	2.5
		10	10	10	10	4.5	4.5
Tfx50 ratio		2:1	3:1	1:1	2:1	2:1	2:1
LSC (cpm)	3μg	2108.88	1586.23	544.05	428.11	1103.84	814.62
	6μg	5584.09	2232.76	391.68	581	4097.65	1883.18
	10μg	15947.73	1122	663.57	3439.87	6207.42	8236.12
Positive control		94705.14		80840.94		71577.68	
Negative control		499.65		297.98		540.38	
Relative	3μg	1.71	1.15	0.31	0.16	0.79	0.39
CAT %	6μg	5.4	1.84	0.12	0.35	5.01	1.89
	10μg	33.3	1.85	1.28	8.17	16.72	22.5

Table B 1.9 Transfection of sA80BR cell line by Tfx20 (Promega Southampton, UK)

Experiment Details		1	2 [a]	3[a]	4	5	6[a]
Cell Passage No		4			6	7	
Tfx20 ratio		2:1	3:1	4:1	2:1	3:1	4:1
CAT (net intensity) for DNA (μg)	0.25	471123.1	1309975	669431.69	0	0	0
	0.5	360933.1	1411100	1248617.8	0	0	0
	0.75	1075654	1465413	20131.39	0	0	0
	1.0	1765868	2559327	1210601.4	0	0	0
Positive control		5222458					
Relative CAT %	0.25	9.02	25.08	12.82	0	0	0
	0.5	6.91	27.02	23.91	0	0	0
	0.75	20.6	28.06	0.39	0	0	0
		33.81	49.01	23.18	0	0	0

[a] Experiments 2 and 3 were duplicates of 1 and 6 was a duplicate of 5

Table B 1.10 Transfection of sA80BR cell line by Superfect (Qiagen West Sussex, UK)

Experiment Detail	1			2		3	
Cell Passage No	9			10		11	
Superfect (μl)/ μg DNA	3	6	15	3	3	1	2
Vector	pCATpromoter			PSV2	pCAT5	pCATpromoter	
CAT (net intensity)	3601188	1110619	0	2275107	2484897	1707032	5002714
Positive control	3093140			1962786		12778997	
Relative CAT %	116.43	35.9	0	115.91	126.6	13.58	39.15

Table B 1.11 Transfection of Ovine liver cells with Effectene

Date of Experiment		1	
Effectene (μl): DNA 1μg/μl)		5:1	25:1
CAT (net intensity) for DNA (μg)	0.2	0	0
	0.4	181978.2	131910
	1	833161.1	479150.4

Note; Cell passage number was 5

Table B 1.12 Transfection of Ovine liver cells with Effectene

Experiment Details	2[a]	3 [a]	4[a]
Effectene (ml): DNA 1mg/ml)	25:1	50:1	10:1
CAT (net intensity)	2298265	29017726	42403465
Positive control	45959099		
Relative CAT %	5	63.14	92.26

[a] Experiments 3 and 3 were duplicates of 2
Cell passage number for 2-4 was 4

Table B 1.13 Transfection of Ovine liver cells with Effectene (continued)

Experiment Details		1	2	3	4	5	6
Effectene (ml): DNA 1mg/ml)		10:1	10:1	25:1	25:1	50:1	50:1
CAT (net)	0.2	0	N/a	1267876	745141.4	0	390912.4
ForDNA (μg)	0.4	2251224	N/a	43013453	0	5661907	2048627
	1.0	1093490	728756.4	4163298	4964755	0	1367386
Positive control		4007509					
Relative CAT %		0	0	31.6	18.59	0	9.75
		56.17	0	107.33	0	141.28	51.12
		27.29	18.18	103.8	123.89	0	34.12

Note: Passage number was 6.

Table B 1.14 Transfection of Ovine Cerebellum cells with Effectene (Qiagen)

Experiment Details		1[a]	2[a]	3[a]	4[a]	5[a]	6[a]
Effectene (μl): DNA 1μg/μl)		10:1		25:1		50:1	
CAT (net)	0.1	0	0	0	0	0	0
for DNA (μg)	0.2	1023404	38819.91	0	741990.1	0	0
	0.5	1341039	172436.4	0	273313.9	0	0

Note : [a] Experiment 2 was duplicate of 1, 4 a duplicate of 3 and 6 and duplicate of 5.

B 2.0 Raw and Standardised CAT Data.

Following transient transfection of the various cell lines as drawn out in section 2.5, cell lysate was recovered and a CAT assay completed. The enzyme CAT catalyses the transfer of acetyl groups to acetyl choline, in this case the substrate is radio-labelled [^{14}C]chloramphenicol. To ensure equivalent recover of cell lysate independent on transfection efficiency, the cells were co-transfected with the plasmid β-galactosidase and the appropriate β-galactosidase assay carried out. The acetylated products were separated by thin layer chromatograph, and visualised by autoradiography, or by centrifugation by solubility in solvent (ethyl acetate).

After purification from unconverted substrate, the amount of radio-labelled product was quantified by liquid scintillation counting or by densometirc reading of the autoradiograph using the Kodac system. From scintillation counting the amount of 14C labelled product was measured as counts per minute (cpm). From densometirc quantification the amount of 14C product was calculated by measuring the density (Net intensity) of the acetylated product detected by autoradiograph. The net intensity was measured by a Kodak Image Station and processed using the Kodak 1D Image Analysis software (section 2.5). As a positive control for CAT activity, the cell were transfected with the plasmid pCAT3-Promoter (Promega), the amount of CAT activity from this plasmid was assumed to be 100% on each transfection and the chimeric CAT- PrP 3'UTRs were standardised against this value.

B 2.1 CAT activity recorded following transient transfection of chimeric CAT-PrP 3'UTR plasmids in cell line sA80BR

Table B 2.1.1 Experiment 1

Plasmid	Intensity (cpm)	Relative CAT%
PEYR	0	0
PD17	220.44	3.32
PD20	673.51	10.15
pD27	1757.96	26.50
pD34	2371.33	35.74
pD36	2058.43	31.03
pCAT3-Promoter	6634.08	100

Note: Cell passage number 17.

Table B 2.1.2 duplicate of Experiment 1

Plasmid	Intensity (cpm)	Relative CAT%
pEYR	57.59	0.87
pD17	0	0
pD20	3192.2	20.67
pD27	1346.4	8.72
pD34	4847.7	31.38
pD36	1425.77	9.23
pCAT3-Promoter	15447.4	100.00

Table B 2.1.3 Experiment 2

Plasmid	Intensity (cpm)	Relative CAT%
pEYR	101.75	12.64
pD17	77.02	9.57
pD20	120.54	14.97
pD27	149.23	18.54
pD34	536.01	66.58
pD36	285.72	35.49
pCAT3-Promoter	805.12	100

Note; Cell passage number 18

Table B 2.1.4 Experiment 3

Plasmid	Intensity (cpm)	Relative CAT%
pEYR	224.56	26.21
pD17	117.69	13.74
pD20	372.57	43.49
pD27	321.91	37.58
pD34	741.17	86.52
pD36	288.13	33.63
pCAT3-Promoter	856.69	100.00

Note; Cell passage number 4

Table B 2.1.5 Experiment 4

Plasmid	Intensity (cpm)	Relative CAT%
pEYR	264.08	6.87
pD17	194.84	5.07
pD20	320.46	8.34
pD27	784.46	20.40
pD34	1499.10	38.99
pD36	0	0
pCAT3-Promoter	3844.62	100

Note; Cell passage number 5

B.2.2 CAT activity recorded following transient transfection of chimeric CAT-PrP 3'UTR plasmids in cell line pA80BR

Table B 2.2.1 Experiment 1

Plasmid	Net Intensity	Relative CAT (%)
pEYR	N/A	N/A
pD17	23891.28	25.53
pD20	4486.75	4.79
pD27	N/A	N/A
pD34	86991.47	92.95
pD36	89598.09	95.73
pCAT3-Promoter	93592.05	100.00

Note ;Cell passage number 6

Table B 2.2.2 Experiment 2

Plasmid	Net Intensity	Relative CAT (%)
pEYR	182905.67	73.44
pD17	117674.85	47.25
pD20	148627.28	59.68
pD27	522107.44	209.63
pD34	659221.01	264.68
pD36	18817.08	7.56
pCAT3-Promoter	249061.13	100.00

Note; Cell passage number 7

Table B 2.2.3 Experiment 3

Plasmid	Net Intensity	Relative CAT (%)
pEYR	3942.81	2.41
pD17	2932.98	1.80
pD17	20858.55	12.77
pD20	28566.89	17.49
pD20	40480.69	24.78
pD27	7695.28	4.71
pD34	345771.91	211.66
pD34	367143.25	224.75
pD36	5408.90	3.31
pD36	35153.57	21.52
pCAT3-Promoter	163359.34	100.00

Note ;Cell passage number 10

Table B 2.2.4 Experiment 4

Plasmid	Net Intensity	Relative CAT (%)
pEYR	547266.5	16.28
pD17	146087	4.35
pD20	152586	4.54
pD27	325563.3	9.69
pD34	113979.7	3.39
pD36	166444.9	4.95
pCAT3-Promoter	3362523	100

Note; Cell passage number 11

Table B 2.2.5 Experiment 5

Plasmid	Net Intensity	Relative CAT (%)
pEYR	17675.97	2.93
pD17	2222.69	0.37
pD20	9802.06	1.62
pD27	30553.70	5.06
pD34	71699.00	11.86
pD36	12636.38	2.09
pCAT3-Promoter	583866.75	100.00

Note: Cell passage number 11

Table B 2.2.6 Experiment 6

Plasmid	Net Intensity	Relative CAT (%)
pEYR	15575.28	2.02
pEYR	13201.65	1.71
pD17	29188.72	3.79
pD20	454.75	0.06
pD27	29260.68	3.80
pD34	53264.00	6.92
pD36	68402.02	8.88
PCAT3-Promoter	769940.13	100.00

Note; Cell passage number 12

Table B 2.2.7 Comparison of sA80BR and pA80BR relative CAT activity $\log_e$ data.

Cell line	Vector	Collated % CAT activity							Mean	st.dev
sA80 BR	pEYR	-2.30	-0.03	2.54	3.27	1.94			1.08	2.26
	pD17	1.23	-2.30	2.27	2.63	1.64			1.09	1.97
	pD20	2.34	**3.03**	2.71	3.77	2.13			2.80	0.65
	pD27	3.28	2.18	2.93	3.63	3.02			3.01	0.60
	pD34	3.58	3.45	4.20	4.46	3.67			3.87	0.44
	pD36	3.44	2.23	3.57	3.52	-2.30			2.09	2.52
pA80BR	pEYR	0.75	4.30	0.92	0.00	2.79	1.11	0.59	1.74	1.51
	pD17	3.24	3.86	0.64	2.55	1.49	-0.78	1.36	1.77	1.59
	pD20	1.59	4.09	2.87	3.21	1.53	0.54	-1.90	1.70	1.99
	pD27		5.35	3.11	1.57	2.27	1.65	1.36	2.55	1.51
	pD34	4.53	5.58	5.36	5.42	1.25	2.48	1.95	3.79	1.84
	pD36	4.56	2.03	1.22	3.07	1.61	0.78	2.19	2.21	1.27

B.2.3 CAT activity recorded following transient transfection of chimeric CAT-PrP 3'UTR plasmids in cell line IS120.Cer

Table 2.3.1 Experiment 1

Plasmid	Intensity (cpm)	Relative CAT (%)
pEYR	472.82	5.26
pD17	513.55	5.71
pD20	593.04	6.59
pD27	586.09	6.52
pD34	2583.35	28.72
pD36	1756.54	19.53
PCAT3-promoter	845.8	9.40

Table 2.3.2 Experiment 2 (duplicate of 1)

Plasmid	Intensity (cpm)	Relative CAT (%)
pEYR	310.57	3.45
pD17	421.36	4.68
pD20	707.28	7.86
pD27	1024.84	11.39
pD34	911.11	10.13
pD36	8995.92	100.00
PCAT3-promoter	845.8	9.40

Note : Cell passage number 10, 3rd August, 1999

Table 2.3.3 Experiment 3

Plasmid	Intensity (cpm)	Relative CAT (%)
pEYR	86787.96875	19.7454362
pD17	107231.1953	24.40
pD20	32432.16797	7.38
pD27	281823.25	64.12
pD34	118816.8984	27.03
pD36	146027.7813	33.22
PCAT3-promoter	548012	124.68

Table 2.3.4 Experiment 4 (duplicate of 3)

Plasmid	Intensity (cpm)	Relative CAT (%)
pEYR	143812.75	32.72
pD17	123573.4219	28.11
pD20	38378.27734	8.73
pD27	238240.8438	54.20
pD34	212682.9531	48.39
pD36	148989.2813	33.90
PCAT3-promoter	331056.625	75.32

Note : Cell passage number 7, 3rd December, 1999.

Table 2.3.5 Experiment 5

Plasmid	Intensity (cpm)	Relative CAT (%)
pEYR	41931.85547	7.85
pD17	14404.44922	2.70
pD20	19829.95313	3.71
pD27	45802.77344	8.58
pD34	50073.15234	9.38
pD36	99413.63281	18.62
PCAT3-promoter	282509.25	52.92

Table 2.3.6 Experiment 6 (duplicate of 5)

Plasmid	Intensity (cpm)	Relative CAT (%)
pEYR	19697.68945	3.69
pD17	11226.77344	2.10
pD20	40888.75	7.66
pD27	226975.9531	42.52
pD34	79789.0625	14.95
pD36	95266.94531	17.85
PCAT3-promoter	785177	147.08

Note: Cell passage number 8

B 2.4 CAT activity recorded following transient transfection of chimeric CAT-PrP 3'UTR plasmids in cell line IS120.Liv

Table B 2.4.1 Experiment 1.

Plasmids	Net Intensity	Relative CAT (%)
PEYR	0.0	0.00
PD17	30313.4	3.44
PD20	74007.9	8.39
PD27	139327	15.80
PD34	616813	69.97
PD36	75743.2	8.59
PCAT3-Promoter	881595	100.00

Table B 2.4.2 Experiment 2

Plasmids	Net Intensity	Relative CAT (%)
pEYR	929.288	0.11
PD17	1511.5	0.17
PD20	81152.5	9.21
PD27	0	0
PD34	692904	78.60
PD36	184862	20.97

Note ; for both experiment 1 and 2 cell passage number was 11

Table B 2.4.3 Experiment 3

Plasmids	Intensity (cpm)	Relative CAT (%)
pEYR	576.68	86.63
PD17	456.97	68.64
PD20	445.11	66.86
PD27	382.79	57.50
PD34	2087.16	313.52
PD36	380.81	57.20
PCAT3-Promoter	665.73	100.00

Note: Cell passage number 11, 12-08-1999

Table B 2.4.4 Experiment 4

Plasmids	Net Intensity	Relative CAT(%)
pEYR	51798.16	25.68
PD17	46360.69	22.98
PD20	74095.64	36.73
PD27	25138.03	12.46
PD34	67181.86	33.30
PD36	164531.4	81.56
PCAT3-Promoter	201738.5	100.00

Table B 2.4.5 Experiment 5 (duplicate of 4)

Plasmids	Net Intensity	Relative CAT (%)
pEYR	52858.46	26.20
PD17	25038.26	12.41
PD20	14559.36	7.22
PD27	220116.6	109.11
PD34	16512.9	8.19
PD36	211291.2	104.74

Note; for both experiment 4 and 5 cell passage number 12

Table B 2.4.6 Comparison of IS120.Cer and IS120.Liv relative CAT activity log_e data.

Cell line	Vector	Collated % CAT activity						Mean	st.dev
IS120.Cer	pEYR	1.68	1.27	2.07	1.33	2.99	3.49	2.14	0.91
	pD17	1.76	1.56	1.03	0.79	3.20	3.34	1.95	0.94
	pD20	1.90	2.08	1.34	2.05	2.01	2.18	1.93	0.31
	pD27	1.89		2.16	3.75	4.16	3.99	3.19	1.13
	pD34	2.44	3.36	2.25	2.71	3.30	3.88	2.99	0.50
	pD36	2.33	2.98	2.93	2.89	3.51	3.53	3.03	0.42
IS120.Liv	pEYR	-2.30	-1.56	3.26	3.27	4.46		1.43	2.67
	pD17	1.26	-1.31	3.14	2.53	4.23		1.97	2.12
	pD20	2.14	2.23	3.61	1.99	4.20		2.83	1.00
	pD27	2.77	0.00	2.53	4.69	4.05		3.51	1.03
	pD34	4.25	4.37	3.51	2.12	5.75		4.00	1.33
	pD36	2.16	3.05	4.40	4.65	4.05		3.66	1.04

B 2.5 Analysis of variants.

Despite co-transfection to ensure consistent transfection efficiency, variation was observed in the overall level of CAT activity detected from independent transfection experiments. However, although the level of CAT measured may have varied between separate experiments the pattern of CAT expression from the individuals plasmids remained constant. It was necessary to determine if the observed variance between CAT values was significant. Analysis of variance (ANOVA) on the logged data was completed to look for significant differences (*F*) between the effect of the vectors overall and transfection efficiencies between each experiment. The purpose of analysis of variance (ANOVA) is to test for significant differences between population means. The *source of variation* in these experiments are the CAT-PrP/3'UTR plasmids and the efficiency of transfection. *Residual* variation estimates the with-in group variability, a factor that can not readily be accounted for in the current design. *Sum of squares* of a measurement of the amount of variation due to each source. *Degrees of freedom* are appropriate to the various sum of squares and reflect the number of populations (individual transfection experiments or plasmids). *Mean of squares* is the variance estimates obtained by dividing each sum of square by its appropriate degrees of freedom. The *variance ratio* is a comparison between the variance estimates and is used to determine the importance of the different sources of variation.

The overall effect of plasmids was found borderline significant in all cell lines (tables 1-4). This means that there are differences in the overall magnitudes of the standardised CAT results between samples, which may be a result of variable transfection efficiencies. However, the estimated variation when considering the both the overall plasmid effect and transfection effect together (interaction) was found significant. This indicates that although the overall level of CAT activity detected for individual transfection experiments may have varied, the differences in standardised CAT measurements between plasmids are reasonably constant across all samples.

Table B 2.5.1 Analysis of Variants (ANOVA) using CAT activity (log_e) obtained from transient transfection studies of cell line sA80BR.

Source of variation	Degrees of freedom	Sum of squares	Mean of squares	Variance ratio (F)	*F* probability
Plasmids	5	24.44	4.89	5.37	$0.100 > p > 0.050$
Transfections	3	21.53	7.18	7.89	$0.050 > p > 0.025$
Interaction	15	45.97	3.06	3.37	$p > 0.100$
Residual	4	3.64	0.91		
Total	30	95.4			

Table B 2.5.2 Analysis of Variants (ANOVA) using CAT activity (log_e) obtained from transient transfection studies of cell line pA80BR.

Source of variation	Degrees of freedom	Sum of Squares	Mean of Squares	Variance ratio (F)	*F* probability
Plasmids	5	30.21	6.04	5.69	$0.050 > p > 0.025$
Transfections	5	43.52	8.70	8.20	$0.025 > p > 0.010$
Interaction	25	73.89	2.96	2.78	$p > 0.100$
Residual	6	6.37	1.06		
Total	42	154.15			

Table B 2.5.3 Analysis of Variants (ANOVA) using CAT activity (log_e) obtained from transient transfection studies of cell line IS120.Cer.

Source of variation	Degrees of freedom	Sum of squares	Mean of squares	Variance ratio (F)	*F* probability
Plasmids	5	17.6	3.52	4.58	$0.010 > p > 0.005$
Transfections	2	7.52	3.76	4.89	$0.025 > p > 0.01$
Interaction	10	11.82	1.18	1.54	$p > 0.100$
Residual	18	13.84	0.77		
Total	36	50.79			

Table B 2.5.4 Analysis of Variants (ANOVA) using CAT activity (log_e) obtained from transient transfection studies of cell line IS120.Liv.

Source of variation	Degrees of freedom	Sum of Squares	Mean of Squares	Variance ratio (F)	probability
Plasmids	5	24.5	4.91	2.7	$0.1 > p > 0.05$
Transfections	2	48.9	24.4	8.213.50	$p > 0.010$
Interaction	10	31.9	3.19	2.781.8	$0.2 > p > 0.15$
Residual	12	21.7	1.81		
Total	30	127			