The 3' Untranslated Region of the PrP Gene

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Declaration

I hereby certify that this thesis has been composed entirely by myself, that the work presented is my own and that the work of others has been duly acknowledged.

Foo Cheung

Acknowledgements

"Do not follow, where the path may lead
    go instead where there is no path
        and leave a trail............."

The 3' Untranslated Region of the Sheep PrP Gene

Abstract

Transmissible spongiform encephalopathies (TSEs) form a family of fatal neurodegenerative diseases which affect both man and animals. TSEs are associated with spongiform change in the central nervous system (Masters and Gajdusek, 1982; Bastian, 1991) and aggregation of a host protein PrP, the expression of which is central to the understanding of susceptibility to TSEs (Review: Prusiner 1994). The PrP protein is found in two isoforms: the normal cellular form, designated as PrP^C, and the abnormal form, PrP^{Sc}, found only in diseased animals (Meyer et al., 1986; Oesch et al., 1985). PrP^{Sc} accumulates in amyloid plaques, discrete areas or intracellular deposits (Lantos, 1991). TSEs are transmissible by experimental and accidental inoculation with infected tissue and there is strong evidence for genetic control of disease incidence. The use of transgenic models in recent years has shown that there is a relationship between PrP expression level and disease progression. Lower-than-normal expression leads to extended incubation period and clinical phase (Beuler, 1993; Manson et al., 1994). In contrast, overexpression shortens incubation period and generates novel neurological syndromes associated with degeneration of skeletal muscle, peripheral nerves and the CNS (Westaway et al., 1994b). Natural differences in expression of PrP^C resulting from a variety of mutations within the promoter, enhancer, intron or non-coding sequences could therefore play a part in determining whether a host is either susceptible or resistant to disease.

This project was designed to investigate the potential of the sheep PrP gene 3'-untranslated region (UTR) to control expression of a reporter gene in an *in vitro* system

and in neuroblastoma (N2a) cells of mouse origin. Interest in this region of the PrP gene arose results from sheep PrP genetic studies (Hunter et al., 1991) and PrP mRNA (Hunter et al., 1994b) and protein studies (Horiuchi et al., 1995). In this project sequence analysis of the PrP gene 3' UTR region (e1 allele) showed that it was polymorphic with respect to the naturally occurring e1 and e3 allele and contained A,T-rich motifs and potential alternative polyadenylation signals. The 3'UTR of PrP was found to reduce translatability of a linked chloramphenicol acetyl transferase (CAT) reporter gene by some 2-fold in N2a cells, although this effect was not detectable in a cell-free system. Deletion of 2.8kb of the 3.2kb 3'UTR relieved the inhibitory effect *in vivo*. Further constructs were made by unidirectional deletions in the 3' UTR and the results indicated that the above inhibitory effect is probably caused by sequences within a 1kb central portion of the 3'UTR. However, the alternative possibility that it reflects an unspecific effect, correlated merely with the length of the 3'UTR tail on the mRNA, has not been excluded. Additional experiments showed by deletion and site directed mutagensis that an alternative polyadenylation signal ATTAAAdiscovered at position 1522 can be used in 3' end processing of RNA in N2a cells. These findings may have implications both for the expression of the PrP gene in the natural host of scrapie and for development and progression of disease. However, these implications would have to be explored in the natural host, not least because both PrP transcription and transmission of the disease differ in important ways between mouse and sheep.

Two main approaches were used in this project

In the first of these, equimolar concentrations of *in vitro* transcribed RNA differing in the length and orientation of the 3'UTR of sheep PrP were *in vitro* translated. Results suggest that the 3' UTR had no effect upon the levels of methionine [35S] labelled CAT

protein, as measured by scanning autoradiogram of SDS gels with a densitometer.

In vivo studies were carried out in neuroblastoma cell lines (N2a). Transient transfection of N2a cells with chimaeric CAT constructs possessing a 2794bp deletion in the 3' UTR of the PrP showed a significant increase in the translatability of CAT transcripts. When compared with constructs possessing the full length 3'UTR or a 1.2 kb deletion derivative. Additional *in vivo* studies showed that an alternative polyadenylation signal, 2 Kb from the etablished site was sufficient for 3' end processing. To connect such findings with disease requires an understanding of the expression of PrP in the natural host, the sheep. Natural transmission between animals, including maternal transmission, occurs in sheep but not in mice. Moreover it is known that the expression pattern is different in sheep from that in mice as there are two peripheral mRNAs only in the former. This is likely to be relevant for transmission of natural scrapie, which must be transmitted via a peripheral route, in contrast to the intracerebral injection- used in mouse experiments.

Abbreviations

A	Adenine
ACE	Adenylation cytoplasmic element
Amp	Ampicillin
Beta gal	Beta-galactosidase
bp	Base pair
BSA	Bovine serum albumin
BSE	Bovine spongiform encephalopathy
BHV	Bovine Herpes Virus
^{14}C	Carbon-14
C	Cytosine
CAT	Chloramphenicol acetyl transferase
Ci	Curie (s)
CIP	Calf intestinal phosphatase
CJD	Creutzfeldt-Jakob Disease
CoA	Coenzyme A
CNS	Central nervous system
CPE	Cytoplasmic Polyadenylation Element
CPM	Counts per minute
ddNTP	Dideoxynucleoside 5' triphosphate
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside 5'-triphosphate
DMEM	Dulbeccos modified Eagles medium
DMSO	Dimethylsulphoxide
DTT	Dithiothreithol

EDTA	Ethylenediaminetetra-acetic acid disodium
eIFs	Elongation initiation factor
FCS	Foetal calf serum
g	Relative centrifugal field
G	Guanine
GPI	Glycoinositol phospholipid
Hr	Hour (s)
Hsps	Heat Shock Proteins
IVTS	*In Vitro* Translation System
Kb	Kilobase
kDa	Kilodalton
lacZ	Beta-galactosidase gene
LMP	Low melting point
Min	Minute (s)
mRNA	Messenger RNA
mRNP	Ribonucleoprotein particles
NMR	Nuclear Magnetic Resonance
NPU	Neuropathogenesis Unit
OD	Optical density
ONPG	o-nitrophenyl-beta-D-galactopyranoside
ORF	Open reading frame
PAP	Polyadenylation polymerase
^{32}P	Phosphorus-32
Poly (A)	Polyadenylation
PCR	Polymerase chain reaction
PrP	PrP protein
PrP^{SC}	Protease K resistant PrP associated with infectivity
PrP^{C}	Cellular form of PrP

RNA	Ribonucleic acid
RPM	Revolutions per min
R/T	Room temperature
RT-PCR	Reverse transcription-PCR
^{35}S	Sulphur-35
SDS	Sodium dodecyl sulphate
Sec	seconds (S)
SEM	Standard error of the mean
SDS PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
T	Thymine
TAR	Trans activator region
tat	Trans activator protein
TE	Tris-EDTA
TSEs	Transmissible Spongiform Encephalopathies
TLC	Thin Layer Chromatography
UTR	Untranslated region
UV	Ultraviolet

(1.1) Introduction

Scrapie is a fatal, transmissible neurodegenerative disease of sheep which occurs naturally. It was the first known and is the best studied of the group of transmissible neurodegenerative diseases often described as the transmissible spongiform encephalopathies (TSEs). Examples of TSEs of animals and humans are shown in Table 1. TSEs are unusual because they may arise spontaneously in the population without any apparent cause, the so called sporadic forms (Brown, 1988; Hsiao & Prusiner, 1990; Owen et al., 1990), or they can be familial, tightly linked to mutations (Hsiao et al., 1989). In either case the disease can be transmitted by inoculation to laboratory animals. TSEs have gained increased media attention and have become the focus of public and economic interest because of the occurrence of bovine spongiform encephalopathy (BSE), the so called "mad-cow disease", and the possibility of transmission of the disease from animals to humans (Collinge and Rossor 1996; Will et al., 1996). Initial terminology of scrapie was based on descriptive terms that had arisen in relation to common clinical features. The naturally occurring disease was described in England as "rubbers" or "the gogles", in France as "la tremblance" (the tremble), in Germany as "Gnubberkrankheit" (itching disease) and "Traberkrankheit" (trotting disease). Scrapie was a Scottish term and was the first TSE described, although its infectious nature was not recognised until 1938 (Cuille and Chelle, 1938). Scrapie has been endemic in the United Kingdom for at least 200 years and the average age of sheep at appearance of scrapie symptoms is 3.5 years, with most animals affected between 2.5 and 4.5 years (Stamp, 1962; Dickinson et al., 1965).

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Disease	Natural host
Kuru	Humans
Creutzfeldt-Jacob disease	Humans
Gerstmann- Straussler-Scheinker syndrome	Humans
Fatal familial insomnia	Humans
Scrapie	Sheep and goats
Transmissible mink encephalopathy	Mink
Chronic wasting disease	Mule deer and elk
Bovine spongiform encephalopathy	Cattle
Feline spongiform encephalopathy	Cats
Exotic ungulate encephalopathy	Nyala and greater kudu

Table 1. Examples of transmissible spongiform encephalopathies and their natural hosts

(1.2) Aetiology

The aetiology of sheep scrapie has been debated for many years. Parry (1962) concluded that natural scrapie is an autosomal recessive genetic disorder which is also experimentally transmissible. Other have suggested that natural scrapie is the result of an infection with susceptibility and resistance controlled genetically by the host (Dickinson et al., 1965, 1974; Brotherston et al., 1968).

Oral/ alimentary transmission has been suggested as a route for the natural spread of scrapie in sheep (Hadlow et al., 1982), but the source of infection is unclear. Oral/ alimentary transmission has also been proposed as the route of infection of other TSEs such as BSE (Wilesmith et al., 1988), transmissible mink encephalopathy (Hartsough and Burger, 1965) and Kuru (Gadjusek, 1965). Little is known about the transmission of natural scrapie between sheep, although it is believed that vertical transmission from ewe to lamb does occur (Kimberlin, 1990; Schreuder, 1994). However the data that supports maternal transmission in sheep, date from a time when the genetics of disease susceptibility was poorly understood and the work needs to be repeated (Ridley and Baker 1995; Will 1995). The current view is that scrapie is an infectious disease where host genetic factors play a central role (Dickinson & Outram, 1988; Hunter, 1996). Recent data suggest that the scrapie agent can replicate in mites and that mites may represent a self-sustaining reservoir vector for scrapie agents (Wisniewski et al., 1996). Nematode larvae have also been postulated to play a part in transmission (Clouscard et al., 1995).

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(1.3) Clinical Signs and Diagnosis

Scrapie is not easy to study in the field: its appearance can not be predicted, there is no *in vitro* detection system for the infectious agent or preclinical, non-invasive diagnostic test. The term scrapie was used to describe the scratching behaviour often seen in infected sheep. Other indicators include loss of co-ordination and altered gait (Stamp, 1967). Diagnosis of scrapie in sheep has been primarily based on the histopathologic finding of neuronal and neuropil vacuolation in the brains of clinically affected sheep. However diagnostic problems arise, because vacuolation can be minimal or absent in suspected cases of scrapie or, conversely, when neuronal vacuolation is detected in healthy animals (Fraser, 1976; Zlotnik and Rennie 1958; Zlotnik, 1958). More reliable diagnostic tests are now based upon the presence of an abnormal protein designated PrP^{Sc} in diseased animals (Van Keuen et al., 1995).

(1.4) Biochemistry of the PrP Protein

(1.4.1) PrP^C

Central to all TSEs is a protein called PrP. In non-infected animals PrP protein is designated as PrP^C and is a 33-35 kDa host encoded membrane glycoprotein (Oesch et al., 1985; Stahl et al., 1987). The sheep PrP protein is 256 amino acids long and contains five tandem repeats of an eight amino acid motif (Pro-His-Gly-Gly-Gly-Trp-Gly-Gln) between residues

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54 and 95. Maturation of the PrP precursor protein involves a number of modifications of the primary translation product. Maturation of the hamster PrP protein is shown in Figure 1. An N-terminal signal sequence of 22 amino acids is cleaved off the primary translation product, glycosylation occurs at two asparagine residues, numbers 181 and 197, 23 C-terminal amino acids are removed while a glycosyl phosphatidylinositol (GPI) residue is attached to a serine residue at codon 231 and a disulphide bond occurs between cysteine 179 and cysteine 214.
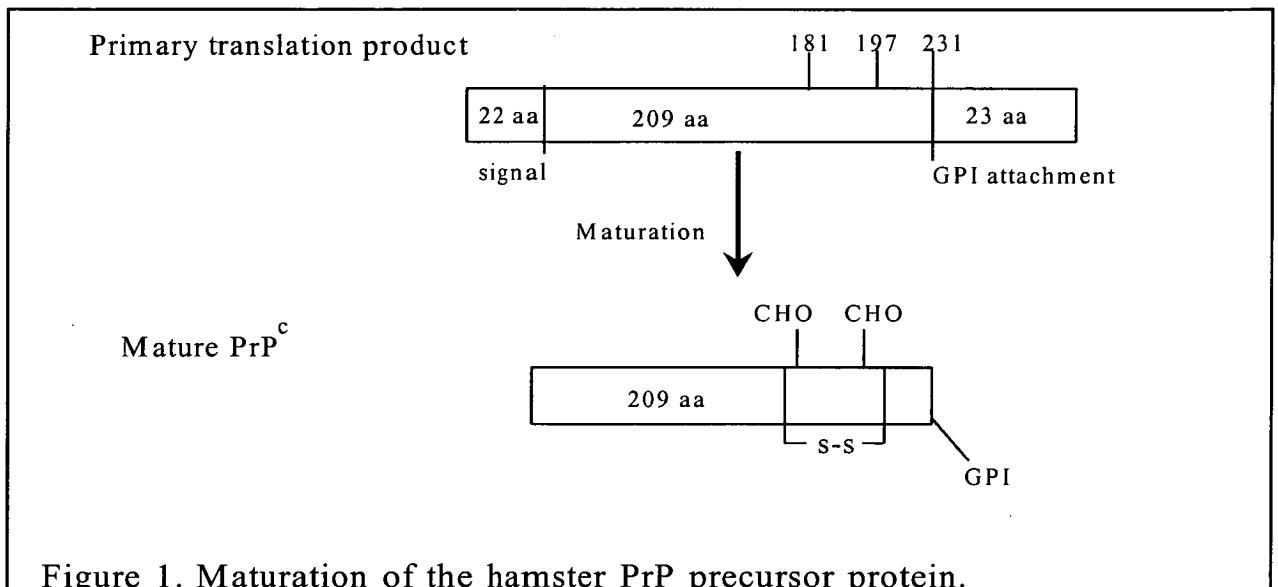
Figure 1. Maturation of the hamster PrP precursor protein.

Molecular modelling has been used to try to predict the structure of the normal protein based on its amino acid sequence (Cohen et al., 1995). Although calculations imply that the protein folds into a compact structure having four helices at its core (Cohen et al., 1995), NMR studies of recombinant PrP protein residues 121-231 (PrP 121-231) have shown the potential for 3 alpha helices and a 2 stranded anti-parallel beta sheet (Riek et al., 1996) in this region of the molecule.

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(1.4.2) PrP^{Sc}

An isoform of PrP^C designated as PrP^{Sc} accumulates in the central nervous system and

lymphoid tissues of sheep during the development of scrapie (Rubenstein et al., 1987;

Ikegami et al., 1991). Conversion of PrP^C to PrP^{Sc} occurs after exposure of the host to

scrapie agent. PrP^{Sc} is a biochemical marker for scrapie and related diseases, and it can also

be used as a marker for infectivity. PrP^{Sc} can be differentiated from PrP^C biochemically,

firstly by sedimentation after detergent treatment and secondly by its partial resistance to

proteases. Protease K digestion completely digests PrP^C but with PrP^{Sc} the resistant core

PrP27-30 remains after digestion. The N-terminus of PrP 27-30 is frayed and ranges from

residues 73-90 (Figure 2.) (McKinley et al., 1983; Meyer et al., 1986; Hope et al., 1986,

1988a, 1988b).

Figure 2. Operational definition of PrP^{Sc}. PrP^{Sc} is operationally defined as containing a protease K resistant core while PrP^C is completely sensitive to digestion.
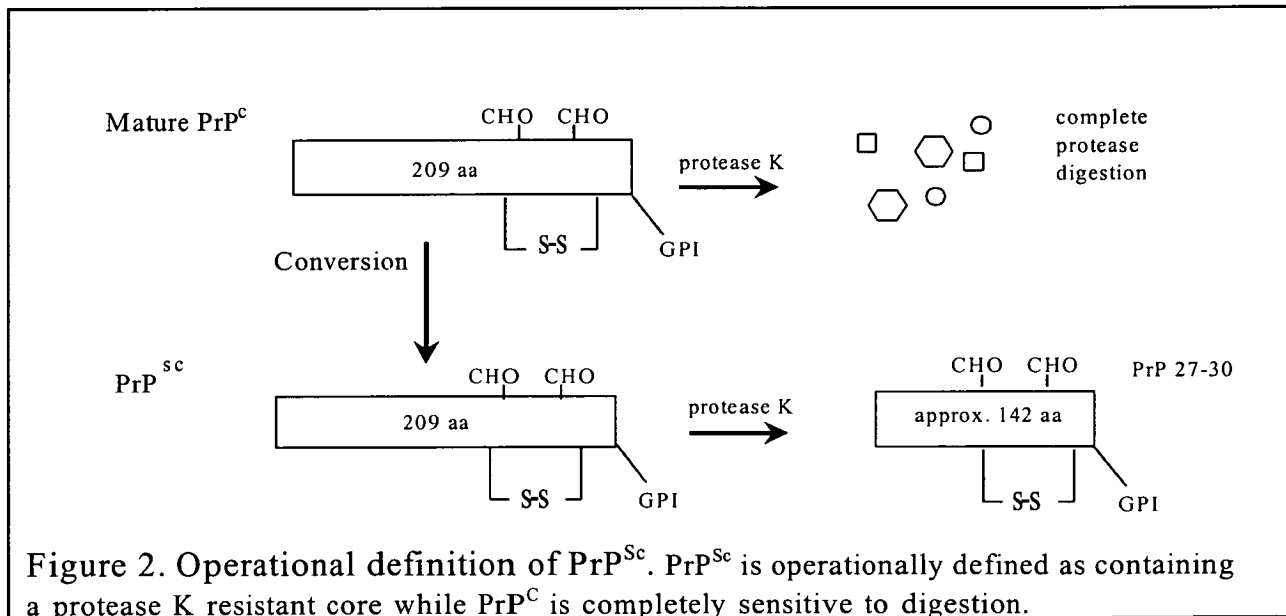
PrP^{Sc} has been detected in the brain by immunohistochemistry (DeArmond et al., 1992; Manson et al., 1992), and in extracts of brain by electron microscopy as fibrils (Merz et al., 1981) or amorphous aggregates (Manuelidis and Manuelidis, 1986) or by SDS-PAGE immunoblotting (Diringer et al., 1988; Rubenstein et al., 1986; Kascsak et al., 1987; Brown et al., 1986). Infectivity co-purifies, to some degree at least, with PrP^{Sc} during subcellular fractionation and several groups have reported a stoichiometry of about 100, 000 molecules of PrP^{Sc} per infectious particle using rodent models of these diseases (Diringer et al., 1988; Prusiner, 1991; Scott et al., 1991; Bolton and Bendheim, 1991).

(1.4.3)   Models for the Conversion of PrP^{C} to PrP^{Sc}

Models for the conversion of PrP^{C} to PrP^{Sc} and the nature of the infectious agent (Section 1.5.2) are the cause of intense debate and should be discussed separately. However, the central issue revolves around the idea that PrP^{Sc} is itself the infectious agent (protein-only model) generated either from a mutant PrP gene or converted from endogenous PrP^{C} by some sort of template process seeded by PrP^{Sc} from an exogenous source. These models do not include a requirement for nucleic acid but its involvement in the conversion of PrP^{C} to PrP^{Sc}, replication and infectivity cannot be excluded (Section 1.5.2), and so others believe that the involvement of PrP^{Sc} in infectivity is not so fundamental and that other factors are involved. The ability of PrP^{Sc} protein alone to be both infectious and self replicating has not been experimentally proven although various attempts have been made to confirm this.

There is general agreement that PrP^C and PrP^{Sc} differ primarily by conformation and several theories exist as to how the PrP protein comes to be misfolded and form aggregates. Conversion of PrP^C to PrP^{Sc} is thought to be due to a post translational event. Circular dichroism studies indicate that there is a higher number of beta sheets and reduced number of alpha helices in the disease specific protein (PrP^{Sc}) when compared with PrP^C (Gasset *et al.*, 1992, 1993). NMR on the other hand studies have shown that PrP^C contains a two-stranded anti-parallel beta sheet and three alpha helices (Riek et al., 1996). The experimental demonstration of a beta sheet in PrP^c is in contrast with theoretical model building predictions of an all helical structure of PrP^C (Cohen et al., 1995; Pan et al., 1993) and may be important for the initiation of the transition from PrP^C to PrP^{Sc}.

Using a simplified model, there may be various stages in transition that PrP protein undergoes: the normal cellular PrP^c to the scrapie isoform (PrP^{Sc}) and polymerisation then to scrapie amyloid fibres which accumulate extracellularly in the brain (Gajdusek, 1988; Prusiner, 1992).

The seeded nucleation model holds that PrP^{Sc} formation is a kinetic event dependent primarily on seeded nucleation, much like protein crystallization (Come et al., 1993, Jarret & Lansbury, 1993). When a crystal is put into supersaturated solution, that crystal can act as the seed for dissolved molecules to join and increase the size of the crystal. In the nucleation model PrP^C is postulated to unfold slightly, forming PrP^U and exposing a hydrophobic stretch of amino acids to the solvent. PrP^U dimerizes and a dimer has been isolated from mouse neuroblastoma cells expressing hamster PrP (Priola et al., 1995).

Dimers appeared to be covalently linked and stable to several strongly denaturing reagents including boiling in SDS. Interestingly, the dimer showed properties of both PrP^C and PrP^{Sc} in that it was protease sensitive, yet also tended to form aggregates. It was suggested that the dimer molecule may be an intermediate state in PrP^{Sc} formation (Priola et al., 1995). Furthermore, in a cell free system the protease sensitive dimer is capable of being converted to the protease resistant form in the presence of PrP^{Sc} purified from scrapie-infected hamster brains (Kocisko et al., 1994).

According to the seeded nucleation model described in Figure 3, dimer aggregation offers a means to the formation of an ordered structure which could act as a seed, or be acted upon by a seed of PrP^{Sc} (Priola et al., 1995). In the protein-only model for infection, the seed would be introduced from outside after which rapid aggregation of endogenous PrP^u would begin. Sporadic TSEs, on the other hand, would depend on spontaneous seed formation from endogenous PrP^U dimers. Equilibrium between PrP^C and PrP^U could easily depend on the cellular environment and be influenced by, for example, pH (Brown et al., 1991).

Figure 3 Seeded Nucleation Model. Hypothetical model for the conversion of PrP^c to PrP^{Sc}.
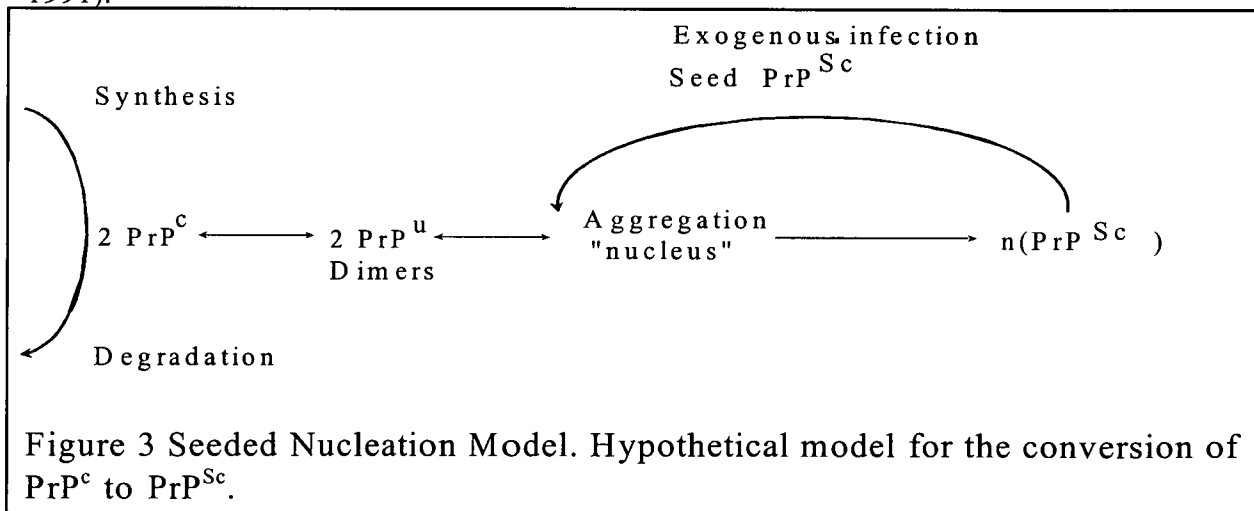
Other investigators suggest that the PrP protein can exist in a meta stable state capable of collapse to either of two more permanent states (Prusiner and DeArmond 1995). In this model PrP^C assumes a partially unfolded intermediate called PrP*, which can then become PrP^{Sc} or be degraded, or resume the PrP^C form (Figure 4). In addition, PrP^{Sc} would be able to serve as a template for the conversion of PrP* into PrP^{Sc}. The slow step is considered to be formation of PrP*. Under normal conditions the concentration of PrP* is low and formation of PrP^{Sc} is not significant. However, at some threshold level there would be enough PrP^{Sc} present to form a complex with any PrP* that forms, thus converting it to PrP^{Sc}. Infection with PrP^{Sc} would act as a template to promote the conversion of PrP* into PrP^{Sc}. Sporadic disorders would arise through events which cause an increase in the frequency of PrP*, and subsequently PrP^{Sc}, such as a mutation in the PrP gene ora rare chance interaction with a co-factor.

Figure 4. A conformational model for conversion of PrP^c to PrP^{Sc}.
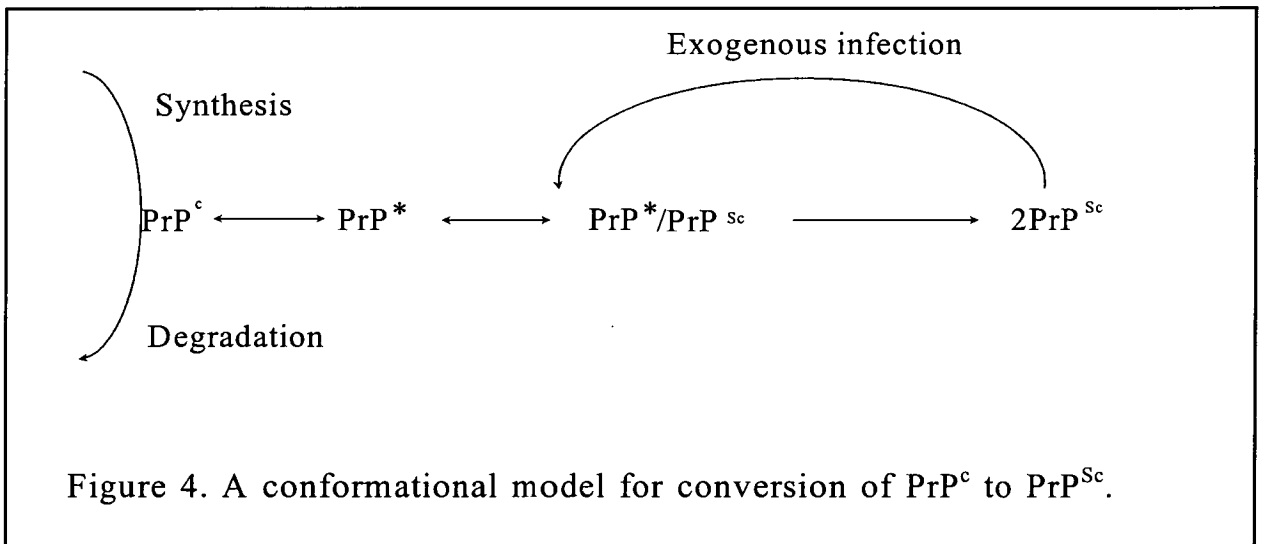
(1.4.4) Protein X and Heat Shock Proteins

Studies of the effect of scrapie on the distribution and expression of various heat shock proteins (hsps) in scrapie-infected cells revealed differences in comparison with healthy cells. While the majority of chaperones (proteins involved in folding and unfolding of proteins) are constitutively expressed and found in abundance in cells under normal conditions (Gething & Sambrook, 1992), some of the heat shock proteins are highly induced under cell stress. Hsp73 is constitutively expressed and is usually found in the cytoplasm. Exposure of neuroblastoma cells to stress results in the translocation of both hsp72 and hsp73 into the nucleus of the cell. In contrast to normal neuroblastoma cells, scrapie-infected cells exposed to stress inducing conditions, failed to translocate hsp73 into the nucleus and it is speculated that hsp73 may form a tight complex with PrP^{Sc} (Tatzelt et al., 1995). The hsp70 proteins (hsp 73 and hsp 72) have been described as undergoing a host of interactions with proteins, binding both folded and unfolded proteins (Ziegelhoffer et al., 1996) and these interactions may be important in PrP^{Sc} formation. Recent reports using the 2 hybrid system have shown that the alpha helical domains of PrP^{C} interact specifically with hsp 60 (Edenhofer et al., 1996).

Results of the CD studies suggest that PrP^{C} contains approximately 42% alpha helix and virtually no beta sheet, but PrP^{Sc} contains 30% alpha helix and 43% beta sheet (Pan et al., 1993). Despite the recent NMR studies of recombinant PrP which suggest more beta sheet may be present in PrP^{C}, the CD studies are still current and argue that the conversion of

alpha helices into beta sheets underlies the formation of PrP^{Sc}. Transgenic studies have

suggested that beta sheet of PrP^{Sc} binds to alpha helices of PrP^C while the PrP^C/PrP^{Sc}

complex binds to a macromolecule (provisionally designated protein X) through residues

of PrP^C and it is also possible that PrP^{Sc} is protein X. The formation of PrP^{Sc} would be a

specific, tightly regulated process mediated by protein X which might act as a molecular

chaperone in the refolding of PrP^C into PrP^{Sc} (Telling et al., 1995). A model has been put

forward that describes an infectious dimer of PrP^{Sc} which binds to an as yet unidentified

Protein X (Figure 5), forming a heterotrimeric complex that would bind with PrP^C to form

a heterotetramer. The heterotetramer would facilitate conversion of PrP^C to PrP^{Sc} while

protein X dissociates. The homotrimeric PrP^{Sc} complex could then act as a template to

facilitate the formation of another PrP^{Sc} molecule from PrP^c by binding another PrP^C

molecule. Protein X could then facilitate the dissociation of the PrP^{Sc} homotetramer into two

infectious PrP^{Sc} homodimers and form the heterotrimeric PrP^{Sc}/X complex, thus completing

the replication cycle.

Heterotrimeric complex   + PrP^c Heterotetramer complex

(2 PrP^{Sc} + Protein X) $\longrightarrow$ (2 PrP^{Sc} + Protein X + PrP^c)

2 PrP^{Sc}

Dissociation   Protein X

4 PrP^{Sc} $\longleftarrow$ 3 PrP^{Sc} $\longleftarrow$ PrP^c

Homotetramer complex   Homotrimer complex
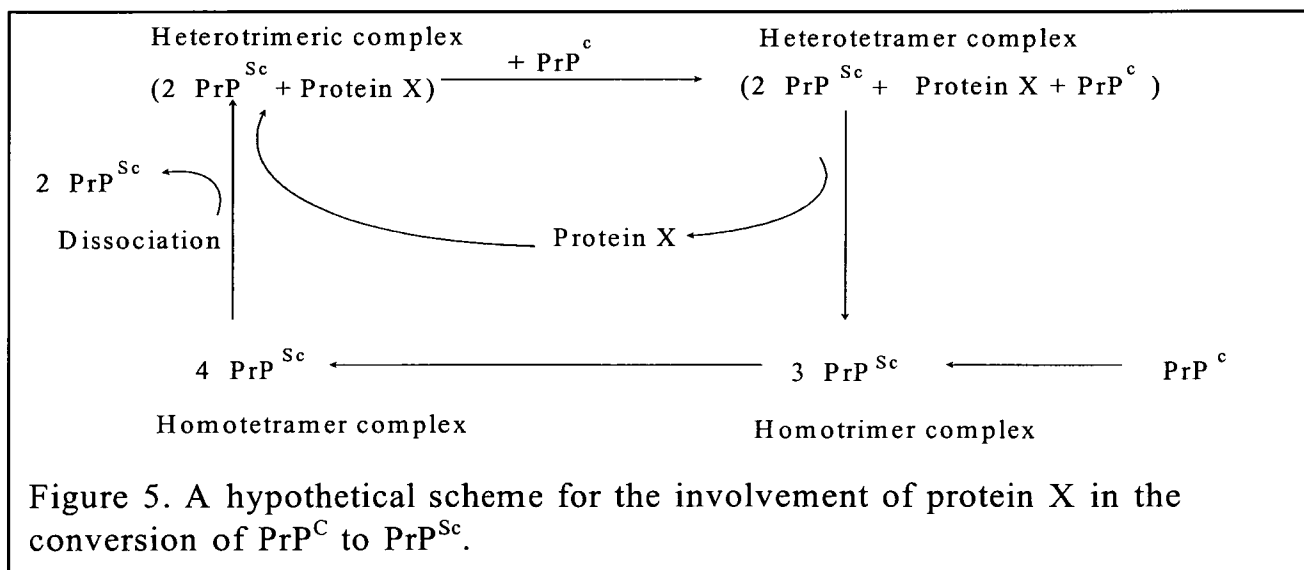
Figure 5. A hypothetical scheme for the involvement of protein X in the conversion of PrP^C to PrP^{Sc}.

The conversion of a protease sensitive to a resistant species of PrP protein can be performed *in vitro* by mixing PrP^C and PrP^{Sc} (Kocisko et. al., 1994). This should lead to a more factually based model for PrP conversion. These results have been extended to include *in vitro* formation of different types of scrapie-like proteins starting from a common PrP precursor (Kocisko et al., 1994). All of these reactions were dependent on the use of guanidinium to unfold the template PrP^{Sc} protein slightly, but not enough to diminish its associated infectivity. Production of mammalian PrP^{Sc} from PrP^C *in vitro* is very inefficient, for instance a system in which the ratio of PrP^C:PrP^{Sc} (both from the same species) was 1:50 yielded only trace amounts of PrP^{Sc} *de novo* (Kocisko et. al., 1994) and new infectivity produced would be much too small to detect by bioassay against background levels. The long time course for *in vitro* formation of protease resistant PrP combined with the inefficiency of the reaction and the requirement for slightly unfolding the template PrP^{Sc} molecule, suggest a requirement for conditions in the cell that are optimal for this process. A co-factor such as a chaperone may also be required. Perhaps if a complete cell-free system were set up, for instance one which contained purified protein X or had a purified heat shock protein added to the system, a more efficient *in vitro* conversion might be achieved. Similarly, if PrP^C to PrP^{Sc} conformation changes occur in the endocytotic pathway as suggested by Mayer et al., 1986, then *in vitro* experiments with extracts of endosome-lysosomes might increase the efficiency of the process.

(1.4.5) The Importance of Post-translational Modifications

After discovery that PrP^C and PrP^{Sc} were encoded by the same gene (Oesch et al., 1985) a search began for a post-translational modification that could give rise to the difference between the two isoforms. However, both isoforms are modified covalently in a very similar fashion. The PrP protein undergoes several post translational modifications before either being degraded as PrP^C or transformed into PrP^{Sc} and accumulated.

A signal sequence present near the C-terminus targets PrP protein synthesis to the endoplasmic reticulum (ER), where the sequence is cleaved leaving Ser at codon 231 as a site for attachment of a glycosyl-phosphatidylinositol (GPI) group. Addition of GPI serves to anchor to the cell surface and GPI is present on both PrP^C and PrP 27-30 (the protease resistant core of PrP^{Sc}). However synthesis of a truncated PrP lacking the GPI anchor attachment signal still allowing in the production of PrP^{Sc} *in vivo* thus eliminating GPI as a necessary factor for PrP^{Sc} production (Rogers et al., 1993). The truncated PrP^c protein could not be detected which is thought to be due to the rapid digestion of PrP^c protein as, lacking the GPI attachment site, it may be secreted.

PrP protein also undergoes glycosylation in the golgi. There are two consensus sites for asparagine linked glycosylation near the C-terminus of the PrP protein. Examination of the various forms of the PrP protein revealed that PrP^C, PrP^{Sc}, and PrP 27-30 are all glycosylated (Endo et al., 1989). While a myriad of different oligosaccharide structures are possible on the PrP protein (Endo et al., 1989), it is doubtful that these play a role in the

transformation of PrP^C into PrP^{Sc}, since a scrapie-infected cell line which can support PrP^{Sc} production, still does so in the presence of agents which block N-linked glycosylation (Taraboulos et al., 1990). The same conclusions have been reached by other methods, for instance by use of site directed mutagenesis to eliminate the sites of N-linked glycosylation (Caughey et al., 1995). PrP^{Sc} is still produced in the absence of sites for N-linked glycosylation suggesting that glycosylation is not an essential element in the transformation of PrP^C into PrP^{Sc}. However, in the *in vivo* situation glycosylation may be important for stability and transport to the cell surface (Petersen et al., 1996).

(1.4.6) Neurotoxicity of PrP Peptides, PrP^{Sc} and PrP^{Sc} in Brain Grafts

The abnormal form of the PrP protein (PrP^{Sc}) and a synthetic peptide of PrP (residues 106-126) are cytotoxic *in vitro* (Forloni et al., 1993; Brown et al., 1994; Selvaggini et al., 1993). The peptide PrP 106-126 has a beta-sheet secondary structure and forms amyloid-like fibrils (Tagliavini et al., 1994). The cytoxicity of the PrP peptide (PrP 106-126) was found to be dependent upon its state of aggregation, on continued cellular expression of PrP^C (Hope et al., 1996) and possibly also on the presence of microglial cells (Brown et al., 1996). These findings suggest that the region corresponding to residues 106-126 of the human PrP is important for neurotoxicity, and that cerebral accumulations of peptides including this domain may be responsible for the neuropathological changes that occur in PrP-related encephalopathies, such as GSS and CJD (Chen et al., 1995).

To determine whether scrapie pathology in mice comes about by neurotoxicity of PrP^{Sc}, acute depletion of PrP^{C}, or some other mechanism, neural tissue overexpressing PrP^{C} was grafted into the brains of scrapie-resistant mice lacking a PrP gene (PrP-null mice), and inoculated with brain homogenates from scrapie infected animals (Aguzzi et al., 1996). Infected grafts developed all the pathological changes normally associated with scrapie in mice (spongiosis, gliosis and neuronal death), and contained high amounts of PrP^{Sc} and infectivity, while neighbouring cells remained unaffected. The life span of the infected mice was not reduced, and no neurological symptoms developed. Surprisingly, graft-borne PrP^{Sc} migrated from the transplants in amounts sufficient to induce the formation of plaque-like deposits of PrP^{Sc} in the host brain outside the graft region. However even 16 months after infection, no pathological changes were seen in the PrP-null tissue immediately adjacent to the grafts and to such deposits. Therefore, in this case, availability of endogenous PrP^{C} to the infectious agent, rather than deposition of PrP^{Sc}, correlated directly with scrapie neurotoxicity *in vivo*.

(1.5.1) Infectious Process

Events in the infectious process such as the site of entry and primary multiplication of the agent, and the mechanism by which the agent is transported to the CNS remain obscure. Research has shown that the agent will pass along peripheral nerves and hence will travel in this way from a site of absorption to the brain (Farquhar et al., 1994).

Hadlow et al., (1979) found high infectivity titres in the brain, the spinal cord and the

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peripheral nervous system of scrapie affected sheep. Infectivity was also demonstrated at lower levels in ileum, colon, spleen, lymph node, tonsil, sciatic nerve and thymus (in order of decreasing concentrations). Despite relatively low titres, scrapie infectivity can be detected in non-neuronal tissues (Hadlow et al., 1982), and PrP^{Sc} is also found in non-neuronal tissues of scrapie infected sheep (Ikegami et al., 1991).

(1.5.2) The Infectious Agent

The nature of the agent causing TSE has generated intense discussion for more than a quarter of a century. There are three hypotheses for the nature of the infectious agent:

(1) The virus model; this model suggests that a nucleic acid encodes for pathogenic strain information and for the viral coat protein.

(2) The prion model or protein-only model: this model suggests that PrP^{Sc} alone is the infectious agent and does not require a nucleic acid molecule.

(3) The virino model; this model suggests that the pathogenic strain information is encoded by an agent-specific molecule (probably a nucleic acid) which is protected by a host encoded protein.

Virus-like structures have been shown by electron microscopy to be present in fractions from scrapie hamster brain, however their involvement in TSEs remains to be established (Diringer et al., 1994). It has been speculated that an information molecule (nucleic acid)

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is involved in infectivity and/or the conversion of PrP^c to PrP^{Sc}, although no candidate nucleic acid has yet been found (Sommerville, 1991).

Perhaps the strongest case for the presence of an information molecule associated with the infectious agent comes from the finding of multiple strains or distinct isolates of the infectious agent defined by specific incubation times, distribution of vacuolar lesions and patterns of PrP^{Sc} accumulation (Dickinson et al., 1968; Fraser and Dickinson 1973; Hecker et al., 1992).

There is no shortage of data that supports the view that the abnormally modified host-encoded cellular protein PrP^{SC} is a major and necessary component of the infectious agent (Review: Prusiner, 1995). Recent data supporting the prion hypothesis include observations that PrP^C will convert into protease resistant forms when incubated with preexisting PrP^{Sc} in a cell-free system (Kocisko et al., 1994, 1995; Bessen et al. 1995; Caughey et al., 1995). However, it is not known whether new scrapie infectivity is generated upon conversion of PrP^C to these protease resistant forms (Section 1.4.4).

The unresolved issue is whether nucleic acid is an essential component of the infectious agent. Biophysical and chemical studies have led to the hypothesis that the agent's infectivity is not susceptible to insults that would be expected to modify or hydrolyse polynucleotides, while treatment with protein denaturants reduces infectivity. (Milson et al., 1976; Prusiner et al., 1981; Diener et al., 1982; McKinley et al., 1983; Bellinger-Kawahara et al., 1987).

(1.5.3) Strain Variation in Models

There are clear results indicating that, like conventional microorganisms, the infectious scrapie particle exhibits strain differences. Isolates show different incubation times, neuropathology, ease of transmission to new species (Kimberlin and Walker, 1978), clinical manifestations and susceptibility to thermal inactivation (Dickinson and Taylor, 1978). Scrapie isolates have been transmitted to mice (Dickinson and Meikle, 1971; Bruce et al., 1991), sheep (Foster and Dickinson, 1988) and hamsters (Kimberlin and Walker, 1978; Kimberlin et al., 1989), while CJD isolates have been transmitted to mice (Mori et al., 1989; Kitamoto et al., 1990) and transmissible mink encephalopathy to hamsters (Kimberlin et al., 1986). There are currently about 20 phenotypically distinct strains isolated in mice by serially passaging scrapie or BSE from a wide range of sheep, goat and cattle sources (Bruce, 1993).

Mouse models provide a convenient system to analyse strain variation. The *Sinc* gene has two alleles (s7 and p7) in mice and is the most important gene controlling incubation time from inoculation to clinical disease (Dickinson et al., 1968). Good evidence suggests that the *Sinc* gene encodes the PrP protein, for example the s7 allele is linked to the PrP variant with leucine at codon 108 and threonine at codon 189 while the p7 allele has PrP with phenylalanine at codon 108 and valine at 189 (Westaway et al., 1987; Hunter et al., 1992). Transgenic studies also support the congruency of *Sinc* and PrP (Westaway et al., 1991).

Primary transmissions of TSE strains from natural hosts to mice result in very prolonged incubation periods, and, and in some cases, no disease at all. This is referred to as the "species barrier". However, subsequent passage through mice to mice of a single *Sinc* genotype results in a precise characteristic neuropathology and incubation period (Bruce et al., 1991, Bruce, 1993; Fraser and Dickinson, 1968; Kimberlin et al., 1989).

Recent transmission experiments from several unrelated sources of BSE from cattle to inbred mice gave strikingly similar pathology and incubation periods. Interestingly, transmissions from other new TSE sources (cats, kudu and nyala) gave similar results to BSE from cattle suggesting a similar source of infection. However, these results differed in incubation periods and pathology from transmission experiments when scrapie of sheep was injected into inbred mice (Fraser et al., 1992; Bruce et al., 1994). The modification and/ or selection of a particular strain might have resulted from the passage through cattle of scrapie affected tissue.

(1.5.4) PrP conformation and strain variation

Because efforts to find nucleic acids associated with infectivity have been unrewarding, the prion, or protein only hypothesis offers another explanation of strain variation. One possibility is that PrP proteins can adopt multiple conformations. Folding in one way might convert PrP^c to PrP^{Sc} highly efficiently, giving rise to short incubation times. Folding in another way might cause the conversion to work less efficiently. Similarly, one "conformer" might be attracted to neuronal populations in one part of the brain,

whereas another might be attracted to neurons elsewhere, thus producing different symptoms. Considering that PrP can fold in at least two ways, it would not be surprising to find it can collapse into other structures as well (Prusiner and DeArmond 1995).

(1.6) Molecular Biology of PrP Expression

(1.6.1) PrP Gene Structure

Location of the human PrP gene to the short arm of chromosome 20 (Liao et al., 1986; Sparkles et al., 1986; Puckett et al., 1991) and that of mice to the homologous region of chromosome 2 (Sparkles et al., 1986) implies that the gene was likely to be present before speciation of mammals. The PrP open reading frame (ORF) consists of a single exon in all known mammalian and avian PrP genes. Mouse (Westaway et al., 1994b) and sheep (Westaway et al., 1994a) PrP genes encompass 3 exons and 2 introns whereas the hamster and human PrP genes encompass only 2 exons and 1 intron (Puckett et al., 1991: Oesch et al., 1985). In all known PrP genes, the ORF and the 3' untranslated region (3'UTR) are contained within one exon, the farthest one downstream from the promoter in each case. The PrP ORFs are similar in length (about 760bp) (Goldmann et al., 1991) while the length of the 3' UTR is more variable. The additional exons code for the 5' UTR. (Figure 6).
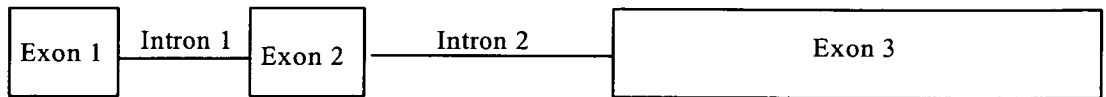
Figure 6. Schematic diagram of sheep and human PrP gene structure. Note that sheep have 3 exons and 2 introns while humans have 2 exons and 1 intron.

(1.6.2) Genetic Linkage of PrP and Susceptibility

Host genes control the incubation period of scrapie from infection to clinical disease and death. In mice, the major gene controlling the incubation period is *Sinc* (scrapie *inc*ubation period) which has two alleles, s7 and p7 (Dickinson et al., 1968). Studies of genetic linkage (Westway et al.,1989; Hunter et al., 1992) and transgenic mice (Hsaio et al., 1990; Prusiner et al., 1990) have suggested that the PrP gene is the gene *Sinc* in mice which controls the length of the incubation period of scrapie, but this is not yet conclusive (Westway et al., 1991). All $Sinc^{S7}$ mouse strains tested encode PrP protein with leucine at codon 108 and threonine at codon 189, whereas in all $Sinc^{P7}$ mouse strains the PrP protein has phenylalanine at codon 108 and valine at codon 189. The sheep homologue of *Sinc* is called *Sip* and also has two alleles , sA and pA (Dickinson and Outram, 1988). *Sip* is defined as the gene which controls the incubation period in NPU Cheviots following experimental inoculation with a scrapie isolate named SSBP/1.

Polymorphisms of the PrP gene are so tightly linked to different incubation times that it is thought to be the same gene as *Sip*.

(1.6.3) Polymorphisms In the Sheep PrP Open reading frame

Several polymorphisms have been described in the PrP gene of sheep several (Figure 7) (Goldmann et al., 1990, 1991; Laplanche et al., 1993; Belt et al., 1995; Iketa et al., 1995).

Figure 7. Schematic diagram showing the known polymorphisms in the sheep PrP open reading frame.
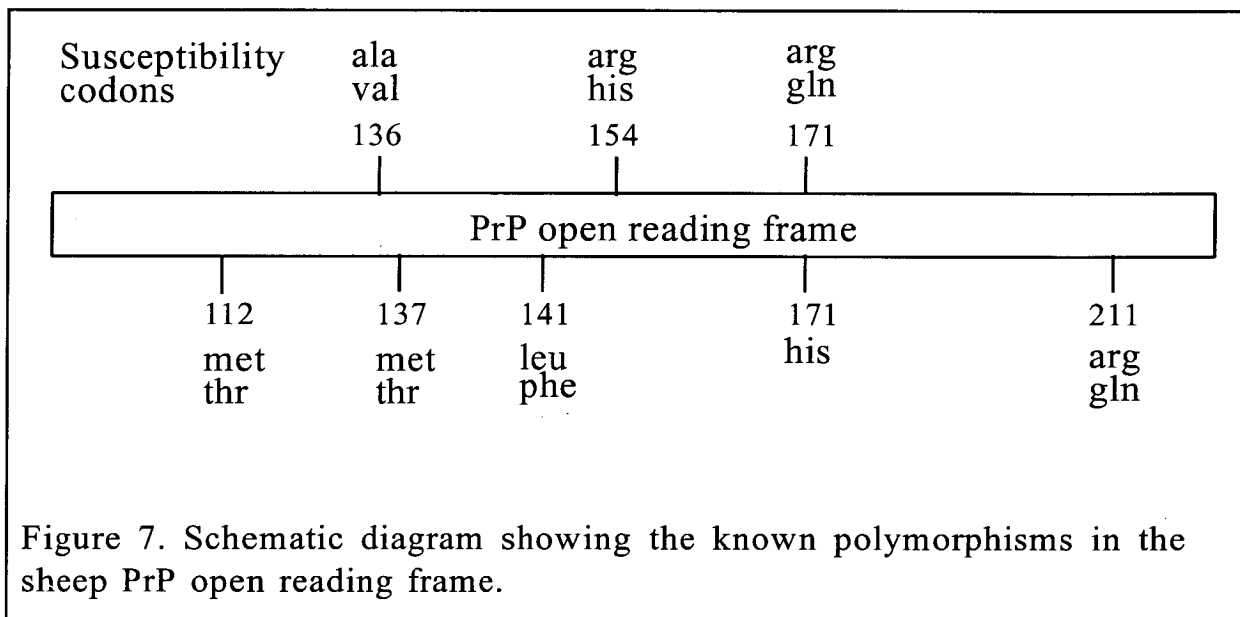
(1.6.4) Experimental Scrapie

Studies in which sheep have been injected with brain homogenate from scrapie infected sheep have shown that several factors determine whether the inoculated sheep develops scrapie: PrP genotype, route of inoculation, scrapie source and the breed (Review: Hunter, 1996). In NPU Cheviot sheep a range of experimental scrapie experiments have been carried out to assess whether there is any linkage of PrP ORF polymorphisms with susceptibility to scrapie (Figure 8). It was found that upon subcutaneous injection with a scrapie isolate SSBP/1, sheep that encode valine at codon 136 (V_{136}) in the PrP ORF on both alleles had short incubation periods while heterozygotes, sheep encoding valine at codon 136 on one allele and alanine on the other (VA_{136}), had longer incubation periods. In contrast alanine homozygotes (AA_{136}) were resistant to experimental scrapie (Goldmann et al., 1994).
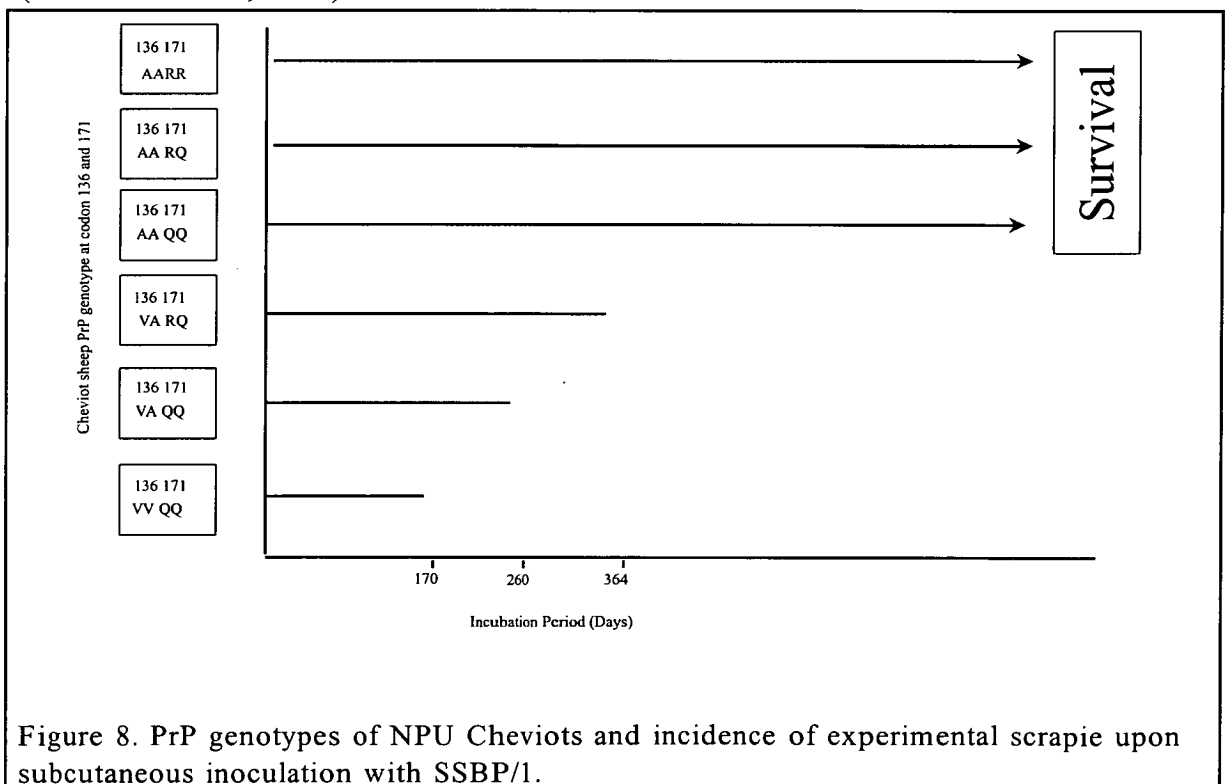
Figure 8. PrP genotypes of NPU Cheviots and incidence of experimental scrapie upon subcutaneous inoculation with SSBP/1.

However, when another scrapie isolate CH1641 or a BSE isolate was intracerebrally injected into Cheviot sheep an association of disease incidence was found not with codon 136 but primarily with codon 171 (figure 9). Animals homozygous for glutamine at codon 171 (QQ_{171}) had short incubation periods while those having one allele with arginine (RQ_{171}) have much longer incubation periods (Goldmann et al., 1994).

Figure 9. PrP genotypes of NPU Cheviots and incidence of experimental scrapie upon intracerebral inoculation with BSE.
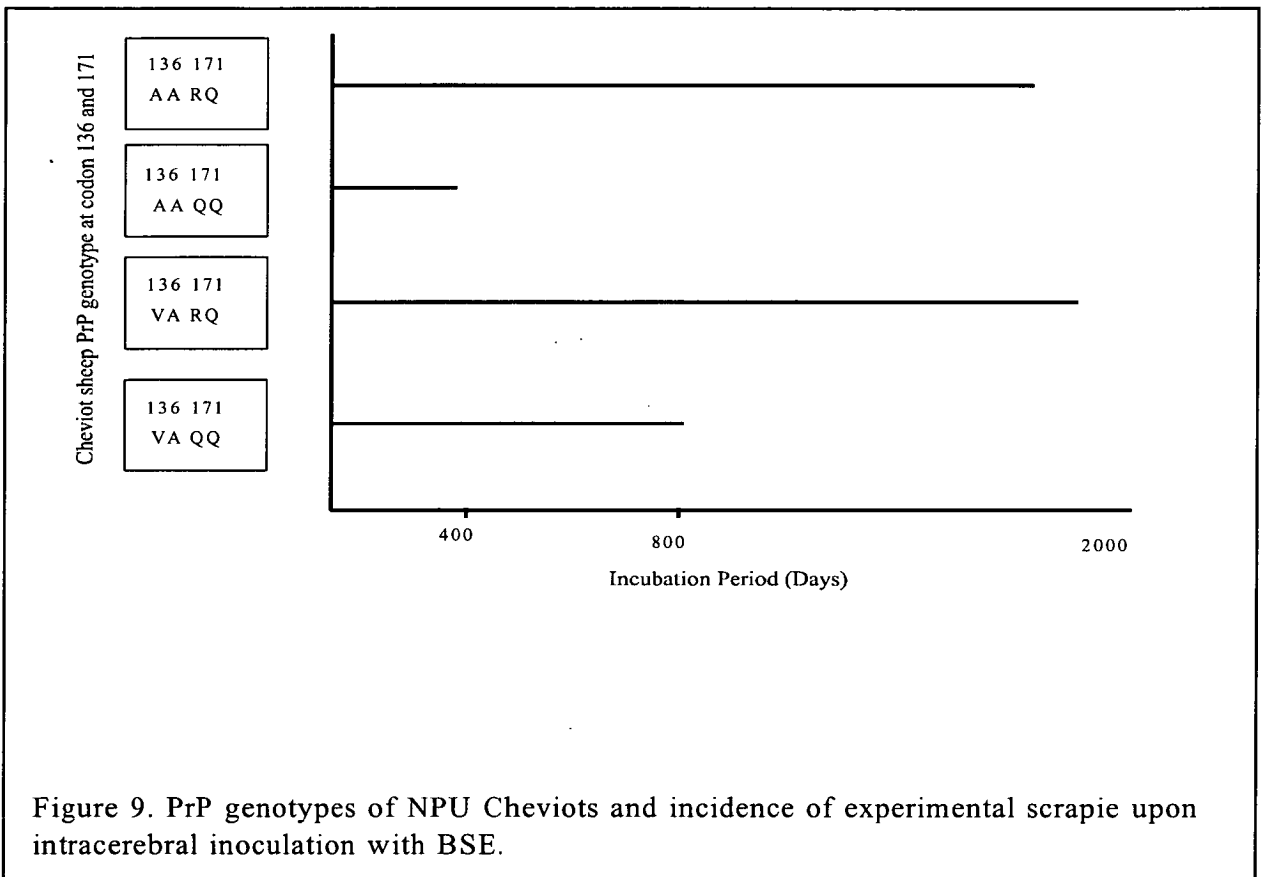
(1.6.5) Natural Scrapie

With natural scrapie the source, route, time and dose of infection are unknown and all could affect incidence and age-at-death of scrapie. Nevertheless there is strong evidence in sheep of the importance of PrP genotype in controlling disease incidence, including the polymorphisms at codon 136 (Goldmann et al., 1991; Maciulis et al., 1992; Hunter et al., 1993; Laplanche et al., 1993; Hunter et al., 1994a; Belt et al., 1995; Clouscard et al., 1995; Ikeda et al., 1995) and codons 154 and 171 (Laplanche et al., 1993; Goldmann et al., 1994; Westaway et al., 1994a; Belt et al., 1995; Hunter et al., 1996).

For example, in NPU Cheviot sheep, the genotype $VV_{136}RR_{154}QQ_{171}$ succumbs to natural scrapie at 700-900 days of age. Heterozygotes, $VA_{136}RR_{154}QQ_{171}$ also develop natural scrapie but live longer (1100-1200 days). Animals which are VA_{136} will survive if also heterozygous at either codon 154 (HR_{154}) or codon 171 (RQ_{171}) despite these genotypes being susceptible to experimental challenge with SSBP/1 (Hunter et al., 1996). All AA_{136} genotypes are apparently resistant in this outbreak of natural scrapie. Not all sheep breeds, or outbreaks, exhibit precisely the same PrP genetics, however.

In two separate studies of natural scrapie in Romanovs (Laplanche et al., 1993; Clouscard et al., 1995) an association of the V_{136} PrP allele of the PrP gene was found with incidence of disease (Laplanche et al., 1993) while in another the association with disease was with glutamine at codon 171 on both alleles of the PrP gene (Clouscard et al., 1995). In Suffolk sheep V_{136} is a rare allele (Hunter et al., 1996) and in studies of Suffolk sheep in the US, and Japan it was found that sheep affected with natural scrapie were all QQ_{171} (Westaway et al., 1994a; Ikeda et al., 1995).

(1.6.6) Polymorphisms in the 3'UTR of the Sheep PrP gene

An *Eco* RI restriction fragment e1 (6.8 kb) of the sheep PrP gene (Figure 10) has been associated with the Sip sA allele and its relative susceptibility phenotype in NPU Cheviot (Hunter et al., 1989, 1991) and USA Cheviot sheep (Maciulis et al., 1992) following injection with SSBP/1 scrapie. Swaledale sheep affected with natural scrapie also have high frequencies of e1 (Hunter et al., 1993). In a study of 160 naturally infected sheep of 32 breeds and cross breeds from the U.K. PrP-e1 was found to be present in up to 92% of the animals (Hunter et al., 1992). PrP-e1 is relatively rare in healthy age-matched controls which have higher frequencies of a shorter corresponding fragment, e3 (4.4 kb). A similar high frequency of e1 in scrapie sheep was also found in Suffolk sheep in Japan (Muramatsu et al., 1992). The polymorphic *Eco* RI site which gives rise to e1 and e3 lies in the 3' untranslated region (UTR) of the PrP gene and its persistent association with scrapie incidence has given rise to the idea that the 3'UTR may play some role in disease incidence through control of expression of the gene. However, the association between e1 and disease could well be due to the linked missense mutation affecting codon 171, while not dismissing the possible correlation with 3' UTR polymorphisms.
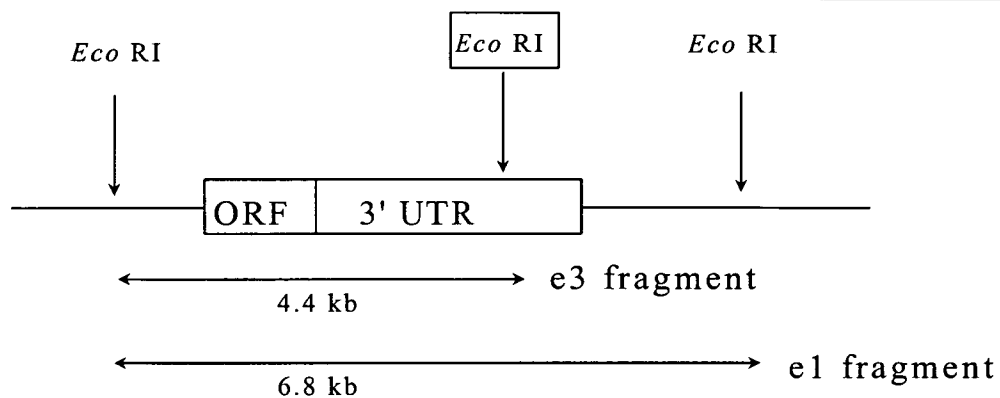
Figure 10. Schematic representation of the *Eco* RI restriction sites in the PrP gene. Polymorphic site is boxed and give rise to fragments e1 and e3.

(1.6.7) Transgenic Studies

A wealth of new information has been gained from transgenic mouse studies with and without endogenous PrP. Analysis of transgenic models in recent years has shown that there is a relationship between PrP expression level and disease progression. Lower-than-normal expression leads to extended incubation period and clinical phase (Beuler, 1993; Manson et al., 1994). In contrast, overexpression shortens incubation period or generates novel neurological syndromes associated with degeneration of skeletal muscle, peripheral nerves and the CNS (Westaway et al., 1994b).

Healthy transgenic mice with both copies of the PrP gene inactivated by homologous recombination (Bueler et al., 1992) cannot so far be infected with scrapie (Bueler et al.,1993; Prusiner et al.,1993). In hemizygotes the presence of one copy of the PrP gene resulted in susceptibility to scrapie but with longer incubation periods compared with wild type mice (Bueler et al.,1993). Consequently, PrP gene expression appears essential

for disease and neuropathology.

Although the normal function of PrP remains unknown, it has been proposed that PrP protein is required for normal synaptic transmission following the observation that long term potentiation (LTP) is weakened in PrP-null mice. Stimulus protocols which induce LTP in wild type and hemizygous mice induce only short lasting potentiation (STP) in PrP-null mice (Collinge et al., 1994). Other hypotheses suggest the involvement of PrP in circadian rhythms (Tobler et al., 1996) and in the long-term survival of Purkinje neurons (Saaguchi et al., 1996).

Earlier experiments involved the use of transgenic mice expressing high copy numbers of the hamster PrP gene in addition to the endogenous mouse PrP gene (Prusiner et al., 1990; Scott et al., 1989). By inoculation of these mice with hamster scrapie it was found that the incubation time was inversely proportional to the level of hamster PrP transcripts and hamster PrP^C concentration in transgenic mouse brains. At the same time, a direct correlation was found between the incubation time and the dose of hamster scrapie inoculated. Scrapie titre at the time of clinical illness was independent of the incubation time suggesting that the normal "species barrier", which prevents hamster scrapie affecting mice, is overcome by giving mice the hamster PrP gene. The PrP genes of Syrian hamsters and mice encode proteins differing at 16 positions, so these residues are candidates for the control of the species barrier and can now be analysed.

Proline at position 102 is found in all mammalian PrP genes sequenced so far while a missense mutation leading to a proline to leucine substitution at this position is linked to GSS in affected families (Hsiao et al., 1989). Transgenic mice expressing an

equivalent mutation at position 101 (position 102 in humans 101 in mice) , displayed

spontaneous neurological disease with pathology indistinguishable from murine scrapie

(Hsiao et al., 1990). It was therefore suggested that the primary lesion in GSS is the

proline to leucine change at codon 102.

An alternative explanation of the response of these transgenic mice relates to the multiple

copies of the transgene inserted into the mouse genome. Transgenic mice carrying

multiple copies of the hamster PrP gene have hamster scrapie incubation periods inversely

related to the number of transgene copies (Prusiner et al., 1990). The codon 102 (proline

to leucine) transgenic mice also carrying many copies of the transgene could simply have

been rendered exquisitely susceptible to a contaminating scrapie agent. It is also known

that multiple copies of normal PrP genes can result in illness (muscle myopathy) in

transgenic mice (Westaway et al., 1994b).

Transgenic mice expressing the different sheep PrP proteins are currently being made

(Hunter et al, in progress). The use of gene targetting to replace the endogenous mouse

PrP gene coding region with that of sheep should avoid the problems experienced with

multi-copy PrP transgenes. However the associations of particular PrP amino acid variants

with disease remain simply strong correlations without any real understanding of the

biochemical (if any) of these single amino acid changes. In a completely different

approach, investigations are being carried out into whether the PrP amino acid

polymorphisms, which contribute to differences in survival times (*in vivo*), influence the

in vitro conversion kinetics of PrP^C into PrP^{Sc} (Bosers et al., 1996).

(1.6.8) Introns

The large intron of PrP genes varies from 10-14 kb in size depending on the species (Basler et al., 1986; Bueler et al., 1992; Westaway et al., 1994a). Transgenic mice transcribing a PrP gene without an intron failed to express PrP protein and it has been speculated that the intron may have a significant role in PrP expression (Scott et al., 1989). However, another study has shown that a PrP mini-gene without an intron can express PrP protein (Race et al., 1995). Since introns are required for expression in other systems the PrP intron may be in some cases exerting a general rather than a specific effect on PrP protein expression (Brinster et al., 1988; Buchman and Berg, 1988). A relationship between splicing and 3' processing has been suggested (Robberson et al., 1990) and may account for the inability to detect PrP mRNA in transgenic mice lacking an intron (Fisher et al., 1996).

(1.6.9) Promoter Analysis

The expression of many eukaryotic protein-coding genes is controlled at the level of transcription. In these cases, the amount of mRNA in the cell is determined principally by the rate of initiation of transcription from the promoter which gives rise to the message. Over 40 different protein subunits regulate transcription by RNA polymerase II at specific promoters. The core subunits of eukaryotic RNA polmerase II are able to catalyze RNA synthesis, but are not capable of gene specific transcription . Instead, a

host of accessory or general transcription factors work in concert with RNA polymerase II to bring about promoter recognition and accurate transcription initiation (for reviews see Zawel and Reinberg, 1995; Tjian et al., 1996). The most prominent core promoter element is the TATA box, typically located upstream (-25 to -30) of the transcription initiation sites of many eukaryotic genes. PrP genes in animals and human lack a TATA box in the 5'-flanking region upstream of the transcriptional start sites (Basler et al., 1986; Westaway et al., 1994a; Puckett et al., 1991; Onodera et al., 1996). Functional transcriptional regulatory elements in the rat PrP gene were precisely localised by a series of 5' deletion mutants. Deletion analysis showed that an inverted CCAAT and adjoining Sp-1 binding sequences may be involved in the control of in transcription of the rat PrP gene (Onodera et al., 1996). These sequences have been shown to play a important role in numerous viral and eukaryotic genes (Chodosh et al., 1988; Briggs et al., 1986).

The sheep PrP gene shares four motifs over 250 nucleotides in the promoter region with Syrian hamster and human PrP genes (Basler et al., 1986; Puckett et al., 1991). These motifs may correspond to binding sites for functional transcription factors. The sheep promoter sequence diverges between these motifs and the mRNA start sites. It includes an AP-2-like consensus sequence, whereas the Sp1 and AP-1 sites present in the rodent and human genes are absent (Westaway et al., 1994). These differences might account for the difference in PrP mRNA levels between rodents and sheep. The PrP mRNA in mice and hamster spleens was found to be less than 1% of that in the brain (Caughey et al., 1988), while PrP mRNA in the sheep spleen was estimated to be about 10% of

that in the brain (Horiuchi et al., 1995).

(1.6.10) Transcription Start Sites

Human PrP gene transcription exhibits a single start site resulting in a homogeneous population of PrP mRNA with 136 nucleotides of 5' leader sequence in brain tissue (Puckett et al., 1991). In contrast, the hamster gene has multiple start sites within a 330bp promoter region producing a heterogenous mRNA population with 5' leader sequences of 150-300 nucleotides in brain tissue (Basler et al., 1986; Westaway et al., 1987). Similar multiple start sites have been shown for rat PrP transcripts (Onadera et al., 1996).

(1.6.11) Transcriptional regulation

There is no evidence of gross changes in the PrP mRNA levels (by Northern analysis) during disease development. However, subtle changes may occur. Potential transcription regulation can be seen during embryonic development and may account for differences between mRNA levels in the brain and peripheral tissues. Northern analysis in mice has shown low levels of PrP mRNA in the kidney, heart, lung and spleen, while high levels have been found in the brain, specifically within neuronal cells (Oesch et al., 1985; Manson et al., 1992 and Brown et al., 1990). Similarly, expression in the sheep is also widespread with the highest levels of mRNA found in the brain (Hunter et al 1993b;

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Iketa et al 1995) and is detectable in developing lambs before birth (Hunter et al 1993b). Rat PrP mRNA levels increase threefold during embryonic development from embryonic day 20 to adult day 70 (Lieberburg, 1987). Similar changes in PrP expression during development have been shown in mice (Manson et al., 1991).

Treatment of PC12 (rat) cells with nerve growth factor (NGF) has been shown to increase the levels of PrP mRNA by 9 fold without affecting of mRNA stability (Mobley et al., 1988).

(1.6.12) The Sheep PrP Promoter

It has been shown that minor sequence differences in promoter regions can lead to important phenotypic differences *in vivo*. For instance susceptibility to cerebral malaria is associated with a point mutation in the promoter of TNF-alpha (McGuire et al., 1994). The promoter and exon I sequences of mouse and sheep PrP genes exhibit surprisingly low overall homology (Westaway et al., 1994a) and have quite different putative transcription factor binding sites and sequence motifs. Existing transgenic mice harbouring several copies of the sheep PrP gene (Westaway et al., 1994b) have proved that the basic elements of transcription initiation are recognized in mouse, but the regulation of these multicopy transgenes has not been investigated. The replacement of the 5' gene flanking region of mouse PrP with the homologous region of the sheep PrP gene is an experiment which is now being carried out (Hunter et al., in progress), so that mouse PrP protein expression should be controlled from a sheep PrP promoter. The

resulting expression pattern of PrP mRNA and the distribution of PrP protein within the CNS and peripheral tissues will then be studied.

(1.6.13) The 3' UTR

The 3'untranslated region (UTR) is often the largest part of an mRNA and is defined as the region after the stop codon and before the site of polyadenylation. In the last several years this region has turned out to be central to post-transcriptional regulation of some mRNAs. RNA processing, export from the nucleus, mRNA stability, and intracellular localization and translation can be all regulated by the 3' UTR (Jansen et al., 1995). Gene expression is often regulated at the level of transcription but changes in the level of protein do not always correlate with the transcriptional rate, suggesting the operation of post transcriptional control mechanisms. The majority of studies on post-transcriptional control of mRNA expression have involved early embryos because maternally inherited mRNA and not DNA transcription is pivotal (Review: Macdonald and Smibert 1996). The first few hours of embryonic development, in most organisms, are marked by an absence of transcription. Thus the fully grown oocyte, in order to furnish itself with the necessary proteins and enzymes required during early embryogenesis (and particularly during early cell division), accumulates stockpiles of both proteins and mRNA. During meiotic maturation, or after fertilisation, gene expression is regulated by mobilising these protein and message stores. The 3' UTR has been shown in different cases to be "sufficient" and "necessary" for mRNA localisation,

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mRNA stability and translational regulation, both dependent on and independent of polyadenylation. Many eukaryotic mRNAs contain regulatory elements within the 3' UTR and interact with specific cytoplasmic proteins that modulate stability or translational competence .

(1.6.14) Post Transcriptional Control of PrP mRNA Expression During Disease

Several studies have suggested that PrP expression would be regulated at the post-transcriptional level and have analysed PrP expression during disease, *in vitro* and during normal conditions.

Normal PrP (PrP^C) is a protease-sensitive cell surface glycoprotein anchored in the membrane by a glycoinositol phospholipid (Stahl *et al.*, 1987). During the course of disease PrP^{Sc} accumulates in and around cells of the brain as protease-resistant deposits. Accumulation of PrP^{Sc} cannot be explained by differences in PrP mRNA stability or the rate of transcription in mice (Pfeifer et al., 1993) and may result from changes in post-transcriptional control.

In rat brain, 30% of PrP mRNA is thought to be sequestered in ribonucleoprotein particles (mRNPs) and is therefore potentially subject to translational regulation by attenuated initiation (Denman et al., 1991). Release of sequestered mRNA could result in the higher levels of PrP protein found in scrapie affected rat brains (Figure 11). A

35|s

model has been put forward to explain the inhibition of translation initiation by phosphorylation and binding of a protein to a specific 3' UTR motif independent upon polyadenylation (Figure 11). The model suggests that binding of the specific protein to the 3 'UTR acts as a nucleation signal for other proteins to bind to the mRNA, wrapping it up in a particle (mRNP) in which the RNA is inaccessible to the translational machinery. Possible candidates for proteins involved are the so-called Y-box proteins (Sommerville and Ladomery 1996).
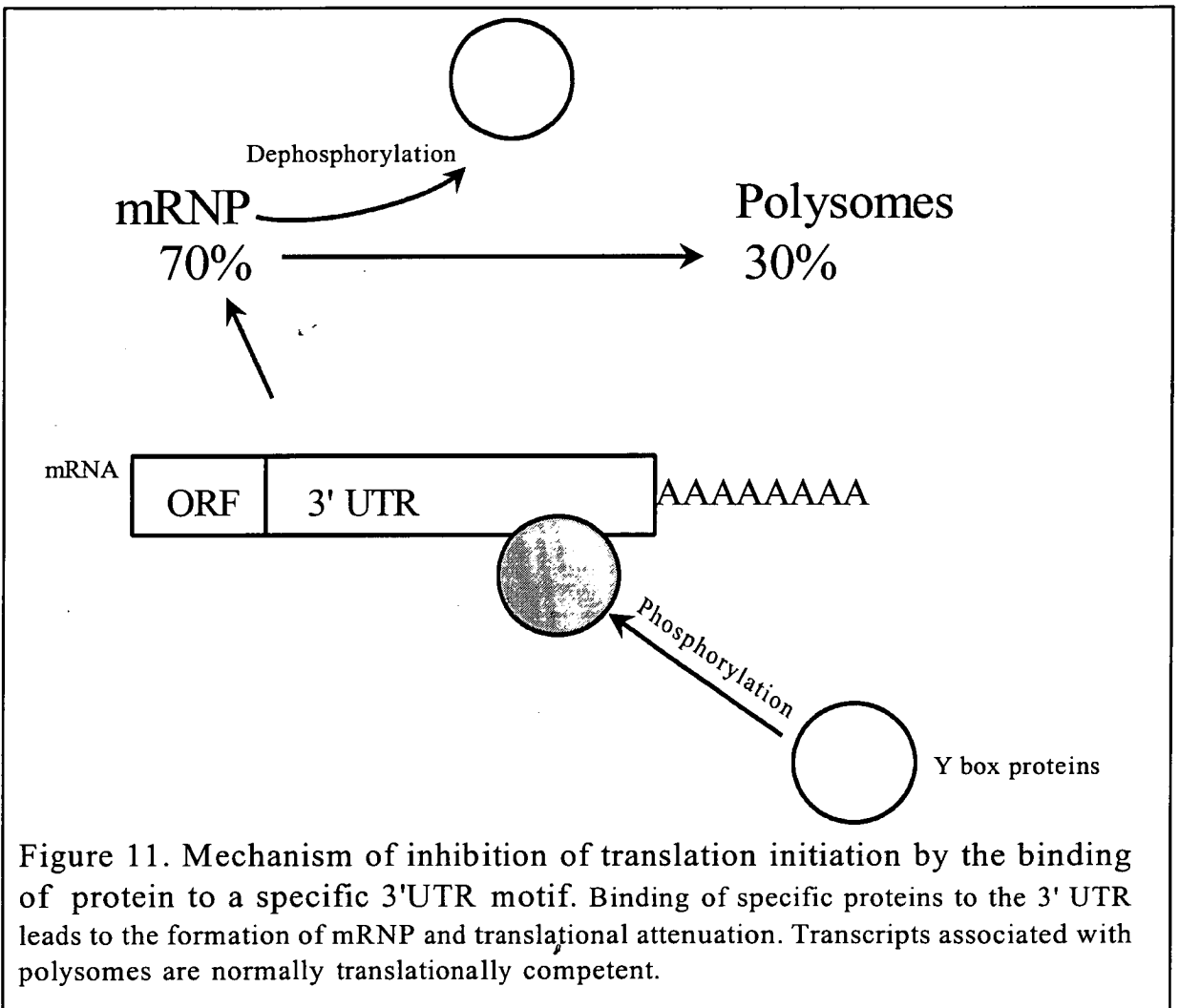
Figure 11. Mechanism of inhibition of translation initiation by the binding of protein to a specific 3'UTR motif. Binding of specific proteins to the 3' UTR leads to the formation of mRNP and translational attenuation. Transcripts associated with polysomes are normally translationally competent.

(1.6.15) PrP expression in cell cultures

A variety of cell types have been tested for their ability to produce PrP transcripts, and

synthesize PrP protein using PrP genes inserted into expression vectors. Neuroblastoma

cells and various fibroblastic cell lines have PrP mRNA levels similar to those of mouse

and hamster brain but the levels of PrP^C are less than 10% of those found in the brain

(Scott et al., 1988). Fibroblasts or HJC glioma cells transfected with expression vectors

containing PrP cDNA failed to increase significantly either the PrP mRNA or protein

levels. Removal of most of the sequences flanking the PrP ORF and insertion into the

SV40-derived expression vector, led to increased protein production in monkey COS-7

cells. It is not known whether the improved expression of PrP using the SV40 vector

system is a consequence of removing most of the flanking sequences from the PrP ORF

or simply the result of a more appropriate host cell background for expression of PrP

(Scott et al., 1988).

In a similar vein, using a fragment of PrP cDNA cloned from scrapie-infected mouse

brain (Chesebro et al., 1985; Locht et al., 1986), a plasmid expression vector was

constructed containing the metallothionine promoter and an SV40 polyadenylation site.

Upon transfection into mouse C127 epithelial/ fibroblast cells (Caughey et al., 1988b),

Northern analysis showed that PrP mRNAs encoded by both the plasmid and the

endogenous cellular PrP gene were expressed, with the former being less abundant.

Despite the relatively low levels of plasmid-encoded PrP mRNA, metabolic labelling and

PrP immunopreciptation experiments showed that the plasmid encoded mRNA was more efficiently translated than the endogenous PrP mRNA, suggesting that, if the scrapie brain and C127 PrP mRNAs are identical initially, removal or replacement of portions of the original noncoding regions with the metallothionine promoter at the 5' end and/ or the SV40 sequences at the 3' end might enhance their translational activity. One model has been put forward that explains in simple terms how the binding of specific proteins to cis-acting 3' UTR signals might be central to a mechanism controlling translation that involves polyadenylation of transcripts. The model assumes that binding of specific proteins to the cis-acting 3' UTR signals, such as the cytoplasmic polyadenylation elements (CPE) (McGrew and Richter, 1990) or adenylation control elements (ACE) (Bachvarova, 1992) found in vertebrate oocyte mRNAs, is central to the mechanism of control of translation involving polyadenylation. As shown in Figure 12 the trans-acting protein may be a negative repressor (or positive activator) binding to the 3' UTR to block (or activate) polyadenylation. Modification of the protein by phosphorylation/ dephosphorylation, might diminish (or enhance) its affinity for the RNA and hence activate (or inactivate) polyadenylation and translation.

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┌─────────────────────────────────────────────────────────────────────┐
│  ┌──────┬─────────────────────────────┐                             │
│  │ ORF  │ 3'UTR            CPE         │ AAAAAAAAAA                  │
│  └──────┴─────────────────────────────┘                             │
│                              ▲                                        │
│                              │  Cytoplasmic adenylation               │
│                              │  and translational activation          │
│                              │                                        │
│  ┌──────┬─────────────────────────────┐                             │
│  │ ORF  │ 3'UTR          CPE          │ AA                          │
│  └──────┴──────────────┓              ┘                             │
│                    (shaded ellipse)                                   │
│                                  Phosphorylation                      │
│                                         ◯                            │
└─────────────────────────────────────────────────────────────────────┘
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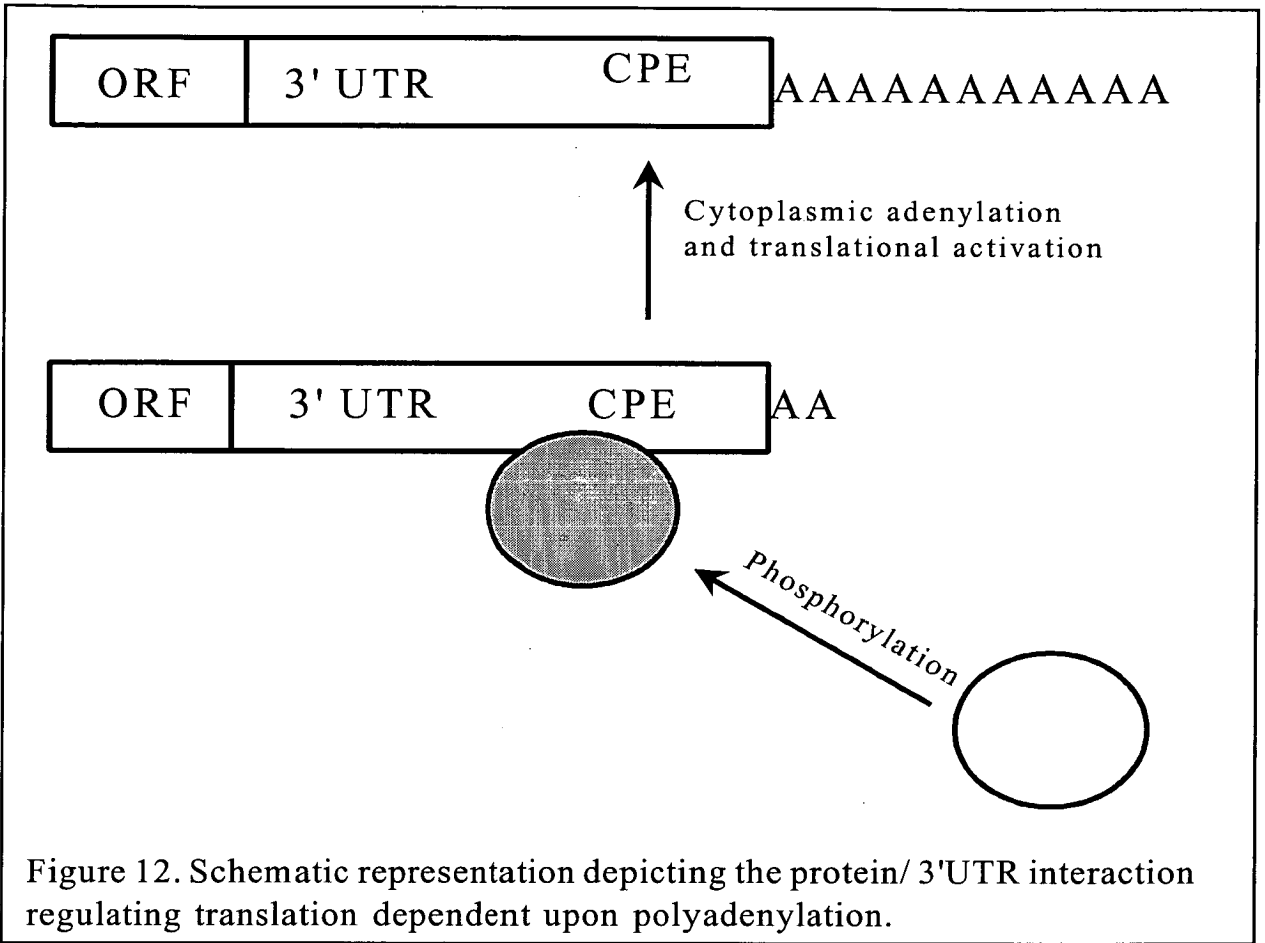
Figure 12. Schematic representation depicting the protein/ 3'UTR interaction regulating translation dependent upon polyadenylation.

(1.6.16) Protein/ 3'UTR interaction regulating translation and polyadenylation.

All eukaryotic cellular mRNA transcripts, with the exception of histone messengers, are provided with a poly (A) tail in the nucleus. After entering the cytoplasm, the poly (A) tail is progressively shortened as the mRNA ages. Very unstable mRNA species, such as those encoding the myc and fos proto-oncogenes, are deadenylated far more rapidly than the average mRNA (Review: Bachvarova, 1992). A wealth of evidence indicates that, besides to having a stabilizing influence on mRNA, long poly (A) tails are stimulatory for translation, both *in vitro* and *in vivo* (Wickens, 1992).

In those cases where translational activation is dependent on polyadenylation, it seems to be the dynamic process of extension of the poly A tail that regulates translation. A 3' UTR element AAUAAA is required for polyadenylation in addition to a second determinant which in *Xenopus* oocytes is UUUUUAU (Mc Grew et al., 1989; Fox et al., 1989) and in mouse oocytes, AUUUUAAU (Salles et al., 1992). Changes in the length of the poly (A) tail have been shown to be central in most cytoplasmic post-transcriptional control of gene expression. In the amphibian *Xenopus laevis*, experimental data have shown the importance of the poly (A) tail in translational initiation. Firstly, poly (A) mRNAs are recruited efficiently and effectively into polysomes for translation upon injection into oocytes when comparisons are made with non-polyadenylated mRNAs (Galili et al., 1988). Secondly recruitment of poly (A) mRNA into polysomes is coupled to elongation of the poly (A) tail during maturation of the oocyte into a fertilizable egg. Correspondingly in early embryos, deadenylation is coupled to mRNA release from the polysomes (Hyman and Wormington, 1988 ; McGrew et al., 1989 ; Paris and Philippe, 1990). Storage of cytoplasmic mRNA and activation during maturation of the oocyte have also been shown in mice (Vassalli et al., 1989) and Drosophila (Wharton and Struhl, 1991).

Other models have described how the 3' poly (A) tail and the 5'-terminal cap may interact to control translational activation (Kuge and Richter, 1995; Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). These models are reinforced by a number of studies showing that: (i) poly (A) and the cap act in concert to stimulate translation (Gallie, 1991); (ii) cap ribose methylation of mRNA occurs at a time of sea urchin development that is coincident with polyadenylation and translational activation (Caldwell and Emerson, 1985); and (iii) vaccinia

virus poly (A) polymerase (PAP) forms a heteroduplex with cap-specific 2'-O-methyltransferase (Schnierle et al., 1992).

(1.6.17) Sheep PrP transcription and translation

Comparisons of PrP mRNA and PrP^C levels have been made in sheep tissues. It was shown that the brain expressed PrP mRNA at a level about fivefold higher than in the kidney. On the other hand the brain contained at least fortyfold more PrP^C than the kidney. Consequently, in sheep there is potentially an 8 fold increase in the translational efficiency of PrP in brain realtive to kidney (Horiuchi et al., 1995). Another hypothesis is that the mRNA in brain is more stable than that in the other somatic tissues.

(1.6.18) mRNA Stability

Post-transcriptional control of gene expression can be achieved by changing the stability of mRNA. The stability of an mRNA influences gene expression by affecting the steady state level of the mRNA, and the rate at which the mRNA disappears following transcriptional repression and accumulates following transcriptional induction.

There are at least 4 determinants on an RNA molecule that determine stability: AU (AUUUA) rich regions, poly (A) tracts, stems and loops and coding region determinants. mRNA binding proteins are thought to be involved in the decay process by interacting with these

determinants.

mRNAs whose 3' UTRs contain AU-rich segments or AU-plus U rich segments are generally unstable. A complex formed between the poly (A) tract and poly(A)-binding-protein is thought to protect most or all of the mRNAs of mammalian cells from rapid degradation. Located within the 3' UTR or near the 3'-terminus of some mRNAs [eg. the mRNAs encoding the transferrin receptor (Swenson and Walden 1994)] are stem-loop elements, which are hypothesized to function as binding sites for regulatory proteins. Figure 13(b) outlines the transferrin receptor mRNA, whose 3' UTR contains several stem-loop regions (iron-response elements). These bind an iron regulatory protein which protects the mRNA from endonucleolytic attack. A complicated and large stem-loop structure within the 3' UTR of the insulin-like growth factor II includes an RNase cleavage site (Scheper et al., 1996). Portions of the coding regions of *fos* (Wellington et al., 1993) and *myc* (Yielding et al., 1996) mRNAs appear to be protein-binding sites, while beta-tubulin mRNAs function by an unknown process regulated in cis- through translation of the mRNA, and in trans- through the concentration of tubulin monomers in the cytoplasm (Ross, 1996).
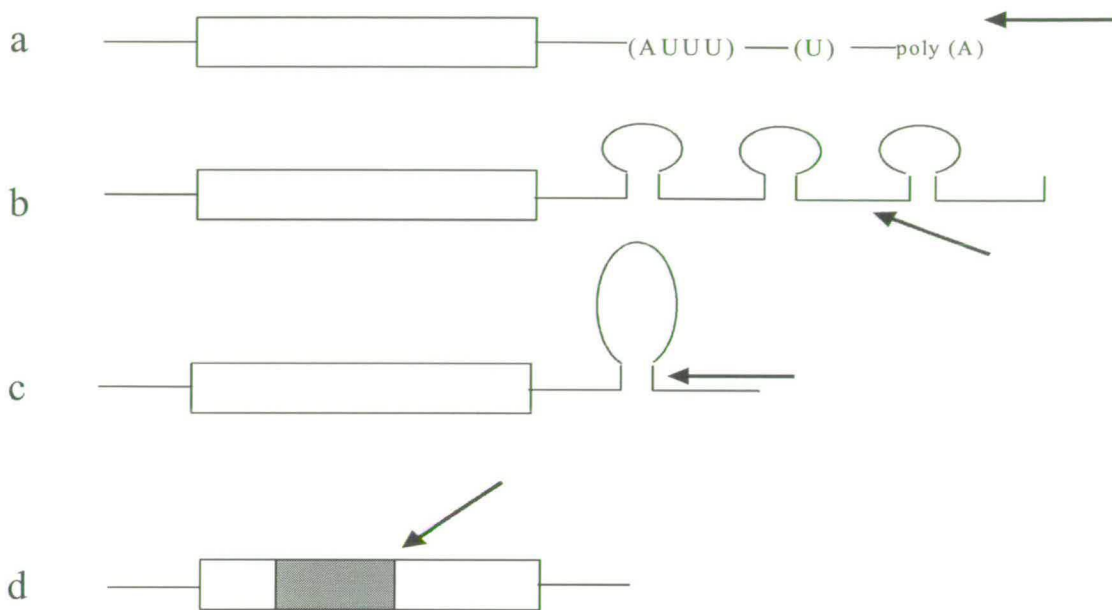
Figure 13. Sequences or structures in mRNAs that affect half-lives. Rectangles and lines denote coding and untranslated regions, respectively.
(a) AU- and U-rich regions, and the poly(A) tract, (b) Stem-loop elements as protein-binding sites, (c) Stem-loop regions as cleavage sites and (d) mRNA stability determinants in coding regions.

3'UTR-sequences that are AU rich have been shown to be associated with instability in many short lived mRNAs, including those coding for lymphokines and proto-oncogenes (Caput et al., 1986) and deletion within this region resulted in an increase in stability in these normally short lived mRNAs (Raymond et al., 1989; Wilson and Treisman 1988; Peppel et al., 1991). Conversely insertion of AU-rich sequences into the UTR of genes with normal stability leads to decreased mRNA stability (Shaw and Kamen, 1986; Raymond et al., 1989; Vakalopoulou et al., 1991; Shyu et al., 1989). It has been proposed that Au-A, a 34 kDa protein with specific

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binding capacity for AUUUA multimers, is part of a complex that is a substrate of nucleocytoplasmic transport or/and cytoplasmic metabolism. Exchanges of other RNA binding proteins for Au-A on AUUUA sequences in the cytoplasm could regulate mRNA degradation (Katz et al ., 1994). A recent study suggests that the pentamer AUUUA is not in itself sufficient for determining instability. Instead, the nonamer UUAUUUAUU is the minimal AU-rich motif that effectively destabilizes mRNA (Zubiaga et al., 1995). Not many studies have been made on the stability of PrP mRNA. However it has been shown that the stabilities of PrP mRNA in uninfected and scrapie-infected N2a cells do not significantly differ (Pfeifer et al., 1993) and this is in accord with studies that show that during infection, PrP mRNA levels do not differ markedly from levels found in non-infected hamsters and mice (Oesch et al., 1985, Chesebro et al., 1985; Robakis et al., 1986; Caughey et al., 1987).

(1.6.19) The 3' UTR of sheep PrP

Scrapie is a natural disease of sheep but occurs in mice only following experimental challenge. Moreover the pattern of PrP mRNA expression is different in sheep and mice (Hunter et al., 1994b). Study of PrP gene expression in sheep may therefore provide information more relevant to the understanding of natural scrapie. Analysis of recent publications (Review: Vassalli and Stutz, 1995) has shown that the 3' UTR of a number of genes is a repository for elements involved in controlling gene expresion at the post-transcriptional level.

The sheep exon containing the open reading frame coding for PrP is about 4 kb in size

(Goldmann et al., 1990) which is about twice as large as the equivalent exon in humans (2.35 kb) (Liao et al., 1986; Puckett et al., 1991) and rodents (2 kb) (Oesch et al., 1985; Basler et al., 1986; Locht et al., 1986; Westaway et al., 1987). Since the open reading frame coding for PrP protein is about 760bp in all genes so far studied, it is the 3'UTR that accounts for the increase in mRNA length.

In sheep brain there is a single PrP mRNA of 4.6 kb, whereas in peripheral tissues (eg, spleen, kidney) the 4.6 kb mRNA is accompanied by a smaller (2.1 kb) PrP mRNA (Hunter et al., 1994b; Horiuchi et al., 1995). When this PhD project was starting oligonucleotide hybridisation studies had suggested that the difference between the PrP transcripts was in the length of the 3' UTR (Figure 14). The 2.1 kb mRNA was therefore thought to lack several features present in the 4.6 kb transcript including a highly conserved region at the 3' end of the UTR (Goldmann et al., 1990) and therefore might be subject to quite different expression controls.
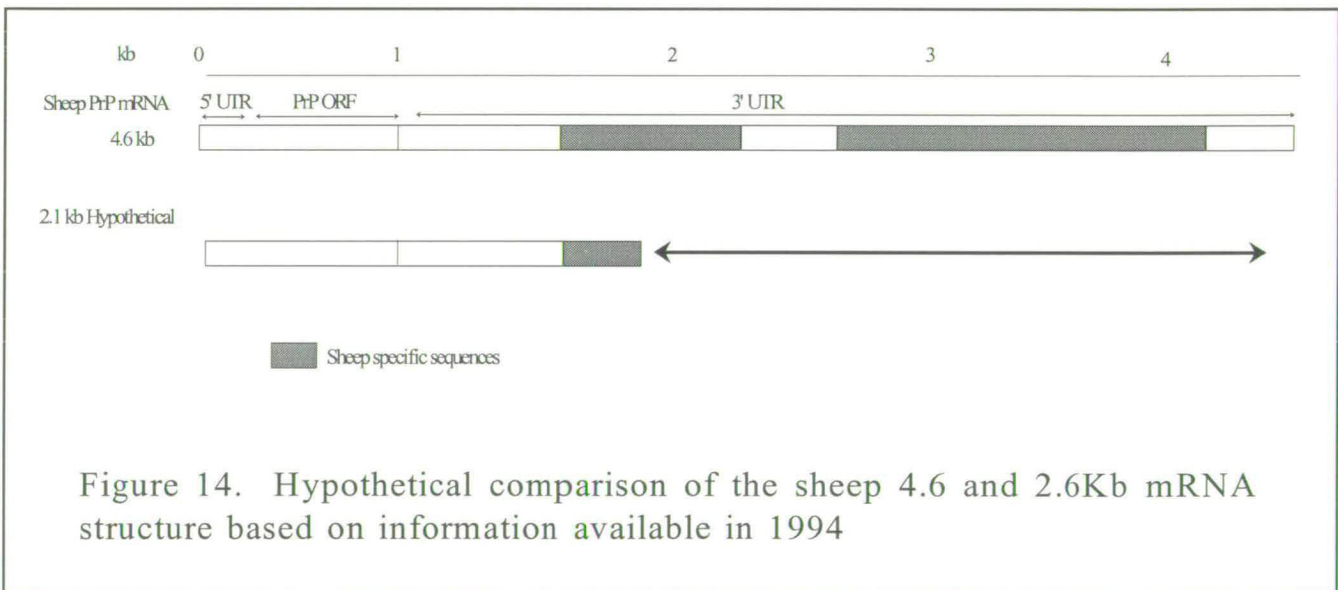
Figure 14. Hypothetical comparison of the sheep 4.6 and 2.6Kb mRNA structure based on information available in 1994

The differences between the two transcripts could therefore play a part in the spread of the infectious particle from the peripheral to the central nervous system. Thus an analysis of the 3' UTR may lead to a better understanding of the disease process. Interestingly, the mRNA for the Alzheimer's disease amyloid protein precursor also has two forms produced by alternative polyadenylation. The longer mRNA is translated more efficiently than the shorter one, and the evidence indicates that the region between the two polyadenylation sites is stimulatory for translation (Sauvage et al., 1992).

In transgenic studies PrP levels have been shown to be central to the timing of disease (Bueler et al., 1995; Manson et al., 1995). Thus polymorphisms in the sheep PrP 3' UTR might affect protein levels and the time of onset at, or susceptibility to scrapie (Hunter et al., unpublished).

Aims of this thesis

The aims of the work described in this thesis are as follows:

(1)   Sequence analysis of the 3' UTR of the sheep PrP gene.

(2)   Investigation of the potential of PrP gene 3' UTR sequences to control expression of a reportergene. There were two main approaches: use of

(a)   a Cell free system.

(b)   aTissue culture system.

In both systems, subclones of the sheep PrP 3'UTR were inserted 3' of the chloramphenicol acetyl transferase (CAT) gene and the effects on CAT synthesis were studied.

(3) Testing whether the region approximately 2kb upstream from the established polyadenylation site was sufficient to mediate 3' end processing.

Chapter 2   MATERIALS AND METHODS

MATERIALS

(2.1) Chemicals and enzymes

All chemicals and enzymes were purchased from Sigma, Gibco-BRL, Boehringer

Mannheim, Promega or Pharmacia and stored at the appropriate temperature and conditions

according to the manufacturer's instructions. Solutions were made in accordance to recipes

in the appendix of this thesis. Where possible they all were autoclaved before use, then

stored in appropriate aliquots and conditions. Water was routinely obtained from a Millipore

milli-QUF system and then subsequently autoclaved.

(2.2) Strains of *E.coli* used in this study

Strain	Genotype
CMK 603	*thr, leu, thi, supE, recBC,* T1^R, T5^R, hsdR, (lac-pro)del. F'lacl^9, *lacZΔM15 pro+*
DH5α	*F'/endA1,hsdR17(rk-mk+), supE44, thi-1, recA1, gyrA(NalR), relA1, Δ(lacZYA-argF)U169, (phi80dlac(lacZΔM15)*

Strains CMK 603 and DH5α were used for routine transformations.

GENERAL METHODS

(2.3) Phenol Extraction

Proteins were removed from DNA solutions by carrying out phenol extractions. An equal volume of (1:1) phenol/chloroform was added to the nucleic solution to be purified. The mixture was vortexed vigourously for 1 minute. Microcentrifugation (13000 rpm) was then carried out for 5 mins. The upper aqueous layer was removed and transferred to a fresh tube.

(2.4) Ethanol Precipitation

Salts were removed from DNA solutions by ethanol preciptation. To the nucleic acid sample were added 1/10 volume of 3M sodium acetate and 2 volumes of 100% ethanol. The mixture was vortexed for 30 seconds. The nucleic acid was then pelleted in a microcentrifuge (13000 rpm) for 10 minutes and washed in 70 % ethanol. The pellet was allowed to air dry and finally dissolved in TE buffer or water.

(2.5) Determination of nucleic acid concentration and purity

Quantitation of DNA was carried out by spectrophotometric measurement of the amount of ultraviolet irradiation absorbed by the nucleotide bases. Absorbance readings were taken at wavelengths of 260nm and 280nm. The reading at 260nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1.0 corresponds to approximately

50 µg/ml for double stranded DNA, 40 ug/ml for single stranded DNA and RNA, and 33 µg/ml for synthetic DNA oligonucleotides (Sambrook et al., 1989). The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have an OD_{260}/OD_{280} value of 1.8 to 2. Pure preparations of RNA have an OD_{260}/OD_{280} value of more than 2. Solutions with OD_{260}/OD_{280} significantly lower than the values given were re-purified by phenol extraction and ethanol precipitation.

METHODS USED IN CLONING

Plasmid cloning often involves the following steps: preparation, isolation and ligation of the vector and insert DNA, transformation of *E. coli* with the ligated products, screening of transformants by restriction analysis and partial sequencing.

(2.6) Restriction Enzyme Digests

Restriction endonuclease digestions were performed in the buffers supplied with the enzyme and according to the manufacturer's instructions. Typically, reactions were carried out at specified temperatures for 1 hour in a total volume of 20 µl containing 1-2 µg DNA and 5 units of enzyme. The DNA was then analysed by agarose gel electrophoresis to ensure that the restriction digestion had gone to completion.

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(2.7) Blunt-ending DNA fragments

For cloning experiments, it was sometimes necessary to fill 5' overhangs or remove 3' overhangs generated by restriction endonuclease to produce blunt ends as described in Sambrook et al., (1989).

(1) Filling in 5' overhangs

To fill 5' overhangs, reactions were carried out for 15 min in the buffer supplied with the enzyme, at room temperature, in a mix containing the following: 1µl of 0.5mM each dNTP, 20 µl of sterile water in which was dissolved 2 µg of DNA to be modified, and 2 units of DNA polymerase I (Klenow fragment). The reaction was stopped by heating to $75^{o}C$ for 10 min.

(2) Removal of 3' overhangs

To remove 3' overhangs, reactions were carried out for 15 mins in the buffer supplied with the enzyme, at $11^{0}C$, in a mix containing the following: 1µl of 0.5 mM dNTP , 20 µl of water in which was dissolved 2 µg DNA and 10 units of T4 DNA polymerase. The reaction was stopped by heating to $75^{o}C$ for 10 min.

(2.8) Separation of DNA fragments on agarose gels

Agarose gel electrophoresis as described by Sambrook et al., (1989) was employed to check the progression of a restriction enzyme digestion, to quickly determine the yield and purity of a DNA solution or PCR reaction, and to fractionate DNA molecules, for subsequent elution from the gel.

(1) Preparation and running of a 1% agarose gel

The gel cassette and chamber were rinsed in sterile water. For a 100 ml gel, 1 g agarose was melted in 100 ml 1X TAE. The solution was cooled to $65^{o}C$, 1 ml 10 mg/ml ethidium bromide was added while stirring, and the gel was cast. TAE buffer (1X) was used as running buffer. Separation was carried out at 60 mA and continued until the bromophenol blue front had migrated 75 % the length of the gel.

(2)Preparation of DNA samples

To the DNA sample was added one tenth volume of 10x sample buffer (50 % glycerol, 1 mM EDTA, pH8.0, 0.25 % bromophenol blue, 0.25 % xylene cyanol), and the mixture was loaded on to the previously prepared agarose gel.

(2.10) Isolation of DNA fragments from agarose gels

Three techniques were commonly used and were equally efficient for the recovery of restriction fragments from agarose gels for ligation reactions:

(1) Sephaglas, (2) Low melting temperature agarose, (3) Centrifugation through chromatography paper

(1) Sephaglas (Pharmacia)

The Sephaglas kit (Pharmacia) was used to isolate DNA fragments from agarose gels. Stored DNA bands were excised from agarose gels with a sterile razor blade under UV illumination and weighed. One volume of gel solubilizer was added to the gel slice in a microfuge tube and left for 10 minutes at 60°C and then 5 µl of Sephaglas were added per µg of DNA isolated (estimated based upon the known amount loaded) and again left for 10 minutes at room temperature. The Sephaglas with bound DNA was pelleted by centrifugation in a microfuge (13000 rpm) for 1 minute. The supernatant was discarded and the pellet washed in 500 µl ethanol. The washing in ethanol was repeated 3 times. Finally the DNA was eluted in 20 µl water.

(2) Low melting temperature agarose

DNA fragments were eluted from low- melting temperature agarose gels (Sambrook et al., 1989). The band of interest was excised with a sterile razor blade, placed in a

microcentrifuge tube, frozen at -70°C for 10 seconds, and then melted at 60°C. Then, an equal volume of TE-saturated phenol was added to the melted gel slice, shaken for 30 seconds, the mixture again frozen and thawed. After the second thawing, the tube was centrifuged (13000 rpm) and the aqueous layer removed to a new tube.

(3) Centrifugation through chromatography paper

DNA bands were excised from agarose gels after electrophoresis and sliced into smaller pieces with a sterile razor blade before centrifuging through blotting paper (Weichenham, 1991) as follows. Two 1.5 ml microfuge tubes with their lids removed were placed one inside the other. The upper tube was punctured at the base with a 19 gauge needle. A 2 cm square of Whatman 3MM paper was wrapped round the inside of the upper tube forming a cup-like structure. The gel pieces containing the DNA fragments were placed in this cup and centrifuged at 13000 X g for 1 min, thus forcing the DNA into the lower tube, along with the electrophoresis buffer. The DNA was purified by phenol-chloroform extraction and ethanol precipitation. The DNA pellet was dissolved in TE and the purity was determined by spectrophotometry.

(2.11) Ligations

Ligations were carried out according to the instructions and in the buffer supplied by the ligase manufacturer at $4^{O}C$ for 6 hours in sterile microfuge tubes containing 100 ng vector DNA, 100 ng insert DNA, and 1 Weiss unit T4 DNA ligase in a total volume of 20 µl.

(2.12) Preparation of competent cells

Two techniques were commonly used and were equally efficient for the preparation of frozen competent cells:

(1) Calcium chloride method

(2) Rubidium chloride method

Transformation efficiencies usually exceeded $1x10^{6}$ transformants $µg^{-1}$ of DNA.

(1) Calcium chloride method

According to the method of Cohen et al., (1972), a single colony of *E.coli* was cultured over night in 50 ml LB medium at $37^{O}C$ with moderate shaking. Of the resulting culture, 4 ml were used to inoculate 400 ml LB medium which wete incubated at $37^{O}C$ with vigorous shaking to an OD 600nm of approximately 0.35. The next step involved three successive centrfugations (each 3000 rpm for 7 min) and after each round of centrifugation the cells were resuspended in 50, 20 and 2 ml respectively of 0.1 M $CaCl_2$. The final 2 ml

of now ompetent cells were dispensed into prechilled, sterile microfuge tubes and flash frozen immediately and then stored at -70°C.

(2) Rubidium chloride method

According to the method of Sambrook et al., (1989), a single colony of *E.coli* was cultured overnight in 50 ml LB medium at 37°C with moderate shaking. 1ml of the resulting culture was used to inoculate 100 ml Psi broth which was incubated at 37°C with vigourous shaking to an OD 600nm of approximately 0.35. The culture was then left on ice for 15 minutes before the cells were pelleted in a 50 ml Costar tube at 3000 X g for 10 mins. The supernatant was discarded and the pellet resuspended in 40 ml of TfbI (30 mM rubidium chloride, 100 mM calcium chloride, 10 mM manganese chloride, 50 mM glycerol, adjusted to pH 5.8 with dilute acetic acid). The cells were pelleted as before, and resuspended in 4 ml of TfbII (MOPS 0.21g, 10 mM calcium chloride, 75 mM rubidium chloride, 10 mM glycerol, adjusted to pH 6.5 with dilute NaOH) and left on ice for 15 min. Aliquots (0.5 ml) of cells were dispensed into microfuge tubes, flash frozen in liquid nitrogen and stored at -70°C

(2.13) Transformation

Transformation was carried out on ice for 30 mins by the addition of 10-20 ng of ligated DNA products to competent cells (thawed on ice). The cell/DNA mixture was heated to 42°C for 2 minutes in a water bath and cooled on ice for 1 minute. 1 ml of LB was then

added followed by incubation at 37°C with gentle shaking for 1 hour to allow the expression of plasmid-encoded drug resistance. Finally, 300 µl of the transformed cells were plated out onto LB plates supplemented with 100 µg/ml ampicillin and incubated at 37°C for 16 hours.

IDENTIFICATION OF POSITIVE CLONES

(2.14) Colony blotting + hybridisation

In order to detect positive clones, a colony blotting method was used based on the procedure of Grunstein and Hogness (1975). A circular membrane (Hybond-N) was laid on top of agar plates containing the bacterial colonies to be screened. The membrane was removed with sterile forceps and soaked colony side up first on denaturing solution (0.2M NaOH, 1% SDS), left for 6 mins, and secondly soaked in neutralising solution (2.55M Potassium acetate pH 4.8) for 3 mins. The membrane was then washed in 2X SSC and left to dry on 3MM paper. Membranes were then fixed by UV irradiation to 0.4 J/cm^{-2}.

The membrane was prehybridised in 30ml of oligonucleotide prehybridisation solution (15 ml 6X SSC, 0.1% SDS, 1X Denhardts, 5 mg boiled sheared herring sperm DNA and 33 ml sterile water) at 65°C overnight in a box in a shaking water bath. The relevant probe was then added to 10 ml of fresh prehybridisation solution and the membrane sealed with it into a clean polythene bag. Hybridisation took place overnight at 42°C. Washing of membranes was in 0.1 x SSC, 0.1% SDS for 30 mins at 42°C.

(2.15) Mini-prep of plasmid DNA: alkaline lysis method

To identify bacterial colonies containing the plasmid of interest, only small amounts of plasmid DNA are required (3 μg). This method is based upon that of Birnboim and Doly (1979): Transformants were picked from a LB agar plate and grown overnight at 37°C in 5 ml LB supplemented with 100 μg/ml ampicillin. The bacterial cells were pelleted in a microcentrifuge (13000 rpm) for 20 seconds. The supernatant was discarded and the pellet resuspended in 100 μl of a solution containing 25 mM Tris-HCl, pH8, 10 mM EDTA and 50mM glucose. To lyse the cells 200 μl of a solution containing 0.2M NaOH, 1% SDS were then added, mixed and left for 10 min at room temperature. To neutralise the lysate and renature plasmid DNA, 150 μl 3 M potassium acetate were added and mixed. The cell debris and chromosomal DNA were pelleted in a microcentrifuge (13000 rpm) and the supernatant was transferred into a fresh tube. The plasmid DNA was ethanol precipitated and dissolved in TE buffer and restriction analysis was performed to identify the plasmid of interest.

(2.16) Large scale preparation of plasmid DNA

Large scale preparation of plasmid DNA was required for DNA sequencing, transfections, *in vitro* transcription, probe preparation and cloning. This method is based upon procedures of Birnboim and Doly (1979).

E.coli cells containing the plasmid of interest were cultured overnight at 37°C, with vigorous shaking, in 500 ml of LB supplemented with 100μg/ml ampicillin. The bacteria were pelleted by centrifugation at 5000 rpm for 15 minutes in a Beckman JA-14 rotor and then resuspended in 18 ml of a solution containing 25 mM Tris-HCl, pH8, 10 mM EDTA and 50 mM glucose. To lyse the cells, 40 ml of a solution containing 0.2 M NaOH, 1% SDS were added, mixed and left for 10 min at room temperature. The lysate was neutralised and the plasmid DNA renatured by adding 30 ml of 3 M potassium acetate. The cell debris and most chromosomal DNA were then pelleted by centrifugation at 5000 rpm for 15 minutes. The supernantant was transferred into Falcon tubes and DNA recovered by ethanol precipitation, centrifuged for 10 min at 3000 rpm in a GH 3.7 rotor and dissolved in 8 ml TE buffer. To this DNA solution, 8.8 g of CsCl and 0.8 ml ethidium bromide (10 mg/ml) were added and dissolved. The nucleic acid solution was transfered into a Beckman quick-seal tube and sealed. Centrifugation was carried out at 58000 rpm for 16 hours in a NVT65 near-vertical rotor.

The lower band containing the supercoiled plasmid DNA was removed with a 19 gauge needle attached to a syringe and sequentially extracted with an equal volume of butanol

until the pink colour was gone. The nucleic acid solution was finally ethanol precipitated according to section 2.4.

(2.17) Double strand DNA sequencing

Dideoxy sequencing of double stranded DNA was carried out using the Sequenase Version 2.0 system (U.S.B/ Amersham). Specific oligonucleotides designed and used as primer are shown in Section 2.18.

Alkaline denaturation of 3 μg of plasmid DNA was carried out by the addition of 0.1 volume of 2 M NaOH and 2 mM EDTA. The solutions was then neutralised with 2 μl 3 M sodium acetate. The denatured plasmid DNA was finally ethanol precipitated. 1 pmol of the primer and 2 μl Sequenase reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) were added to the DNA template to a final volume of 10 μl, before being heated for 5 mins at 65°C. The reaction was then allowed to cool to 37°C before placing on ice.

At room temperature the following were added to the reaction and left for 2 mins: 1μl 0.1 M DTT, 2 μl 1:15 diluted labelling mix (7.5 μM dGTP, 7.5 μM dCTP and 7.5 μM dTTP), 0.5 μl $(\alpha^{35}S)$ dATP (10 Ci per μl) and 2 μl 1:6 diluted Sequenase. Aliquots of 3.5 μl were mixed with the appropriate termination mix at 37°C and left at that temperature for 5 mins. Reactions were terminated by the addition of 4 μl of stop solution (95 % formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF). Samples were heated to 75°C before loading onto a prewarmed and pre-run 6% polyacrylamide gel.

(2.18a) Synthetic oligonucleotides used as primers for sequencing the 3'
PrP UTR

Primer	position	sequence
BOO8	824	TTCTCATAGTAGGATAGGGGC
A30	976	TTACAGCACTGGGAAATG
W7	1023	GTTCAGGGTAATCGAG
BO11	1242	ACACAGACGTCTCTTGATATTTCC
AO29	1348	AATACCCGTCTCCTGTAAA
5261	1603	CCCTACACTCTCAACCTGACATTTCTT
AO46	1934	CGTCAGGTACCACAGCGTCTCAG
W6	2346	TCTGTGATAAGACATGA
W10	2588	AGTGGGCAGAGGAGCCT
6146	2644	GTTATTCCTGTTATT
6145	2970	TGATCAAGCTTGTCAAA
W13	3064	CACTAGTTTAACTCT
474	2317	GTGGGTACGATACAATAACGTAGATG
12615	3569	TTTGATGACCCAGCATG
AO42	3960	CTTTATGCGTACACGAA

(2.18) Polyacrylamide urea denaturing gels

Polyacrylamide denaturing urea gels were poured using 6% (w/v) acrylamide/bis-acrylamide (19:1), 8 M urea and 0.5 volume of TBE. To set the gels, 0.2% Temed (N,N,N,N-Tetramethylene-diamine) and 0.04% (w/v) ammonium persulphate were added. DNA sequencing products were analysed by electrophoresis on 20 cm x 40 cm polyacrylamide-urea denaturing gels. Gels were pre-run in 0.5 x TBE for at least 15 min to reach operating temperatures and then the wells were washed to remove excess urea. Immediately before electrophoresis the sequencing reactions were heated to 75°C for 2 mins to ensure that the DNA products were denatured. The heated sequencing samples were loaded onto the gel which was subjected to 40mA constant current for 1.5 to 5 hours. After electrophoresis, the glass plates were separated and the gels soaked in 10% acetic acid for 10 mins. The gel was then transferred to a sheet of Whatman 3MM paper and dried for 30 mins at 80°C under vacuum.

RADIOLABELLING OF DNA

(2.19) Random primer extension using ^{32}P- dCTP

This technique was used for labelling restriction fragments of double stranded DNA for use as probes. Using a method largely based on that of Feinberg and Vogelstein (1983,84) the DNA was restricted with the appropriate enzyme and run on low melting point agarose. The desired band was cut out and boiled in 500 μl of sterile water in a microfuge tube. The following mixture (See Appendix) were then mixed and incubated overnight: 2 μl of solution A, 5 μl of solution B, 3 μl of solution C, 30 ng DNA, 50 μCi ^{32}P- d-CTP and 1 μl Klenow. In order to stop the reaction and recover separate the labelled DNA, 50μl of water and 5 μl tRNA were then added followed by phenol/chloroform extraction and ethanol precipitation. The pellet was dissolved in 150 μl of sterile water and boiled in a microfuge tube (make a hole in the lid) prior to adding to the hybridisation solution.

(2.20) Labelling oligonucleotides with ^{32}P-dATP

Radiolabeled oligonucleotides were used for confirmation and identification of cDNA fragments (Chapter 6), *in vitro* transcribed RNA (Chapter 4) and bacterial colonies carrying the plasmid of interest (Section 4.3).

Labelling of oligonucleotides was carried out at 37°C for 1.5 hours in a microfuge tube containing the following: 2 μl of 10 X kinase buffer (supplied with the enzyme), 25 pmol oligonucleotide, 10 units of polynucleotide kinase, γ^{32} ATP and 11 μl water. 5 μl (5 μg)

of carrier *E.coli* tRNA was then added prior to ethanol precipitation. The pellet was resuspended in 100 µl of water prior to adding to the hybridisation solution.

METHODS USED IN *IN VITRO* ANALYSIS

(2.21) *In vitro* transcription

Synthesis and analysis of *in vitro* capped RNA transcripts were carried out according to Sambrook et al., (1989). *In vitro* transcription was carried out for 2 hours at 37°C in a sterile microfuge tube containing the following: 10 µl transcription 5X buffer (supplied with enzyme), 5 µl 100 mM DTT, 50U RNasin ribonuclease inhibitor, 10 µl nucleotides (2.5 µl each of 10 mM ATP, CTP, and UTP plus 2.5 µl of 1 mM GTP), 5 µl 5 mM m7G(5')G, 5 µg of linearized template DNA in TE buffer and 40 units of T3 polymerase. The reaction was then phenol/ chloroform extracted and subjected to formaldehyde gel electrophoresis.

(2.22) Formaldehyde gel electrophoresis

(1) Preparation of RNA samples

RNA was stored under ethanol at -20°C and aliquots centrifuged when required. The dried RNA pellet was dissolved in 10µl RNA buffer (1x MOPS, 50% formamide, 6.5% formaldehyde, 50 µg/ml ethidium bromide). Denaturation was carried out by warming to 65°C for 15 minutes and subsequently placed on ice. Afterwards, 1/10 volume of 10x sample buffer (50% glycerol, 1 mM EDTA, pH8.0, 0.25% bromophenol blue, 0.25% xylene

cyanol) was added to the sample, and the mixture was applied to the previously prepared formaldehyde gel.

(2) Preparation and running of a 1% formaldehyde gel.

The gel cassette and chamber were rinsed in DEPC-treated water. Based on a 50 ml gel, 500 mg agarose were melted in 5 ml 10X RNA running buffer and 36.25 ml DEPC-treated water. This solution was cooled to 65°C, 8.75 ml 37% formaldehyde were added while stirring, and then the gel was cast. MOPS buffer (1X) was used as running buffer and electrophoresis was carried out at 80 mA and continued until the bromophenol blue front had migrated 75% the length of the gel.

(3) Northern transfer to nylon membrane

Upward capillary transfer was carried out overnight. The gel was first washed twice in DEPC-treated water, 15 minutes per wash, to removed as much formaldehyde as possible from the gel. Subsequently the RNA was transferred by the capillary action of the 10x SSC transfer buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) being drawn upwards from a reservoir through the gel and then a nylon membrane (Hybond N) covering it, and into a stack of paper towels. This caused the RNA to be transferred from the gel to the membrane. Membranes were then fixed by UV irradiation to 0.4 J/cm^{-2}. Prehybrisation and hybridisation were carried out according to Section 2.14.

(2.23) Isolation of *in vitro* transcribed RNA from formaldehyde gels

The easiRNA Kit (Biorad) was used to isolate and purify RNA from formaldehyde gels. RNA bands were excised from the gels after electrophoresis and weighed. Three volumes of RNA binding salt/acetic acid mixture were added to the gel slice and left for 10 minutes at 37°C. For every μg of RNA isolated, 2 μl of easiRNA Silica were added, mixed, and again left for 10 minutes at 37°C. The silica with bound RNA was pelleted by centrifugation in a microfuge (13000 rpm) for 1 minute. The supernatant was discarded and the pellet washed in 500 μl ethanol. Finally the RNA was eluted in 20 μl sterile, DEPC treated water. Aliquots of the purified RNA were analysed on formaldehyde gels to check the amount (by eye), hybridisation pattern and integrity of the RNA.

(2.24) *In vitro* translation

Translation of *in vitro* transcribed RNA was performed with the Flexi Rabbit Reticulocyte System (Promega). *In vitro* translation was carried out for 90 minutes at 30°C in a sterile microfuge tube containing the following: 33 μl Flexi Rabbit Recticulocyte lysate (Promega), 1 μl amino acid (1 mM) mixture minus Methionine, 5 μl ^{35}S-Methionine (1,200 Ci/mmol at 10 mCi/ml), 1 μl 25 mM magnesium acetate, RNA substrate and sterile water making up the total volume to 50 μl. Prior to the addition RNA substrate was heated to 65°C for 10 min and cooled immediately on ice, to the minimize the formation of secondary structures. A 10 μl aliquot was transferred into 20 μl SDS sample buffer (100mM Tris-HCl,

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pH 6.8, 2% Beta-mercaptoethanol, 4 % SDS, 0.2% bromophenol blue and 20% glycerol)

and heated for 2 minutes at 100°C before loading onto an SDS-polyacrylamide gel.

(2.25) Preparation and running of SDS PAGE

CAT produced from *in vitro* translation was examined by SDS polyacrylamide gel

electrophoresis (SDS-page) (Laemmli, 1970) using a Bio-Rad "Protean" mini-gel system.

The separating gel mix was poured into the assembled gel plates, followed by an overlay

of 0.1% SDS, leaving space at the top for the stacking gel. The gel mix was left for 10 min

to polymerize and then the surface of the separating gel was rinsed with water.

The remaining space was filled with stacking gel mix and immediately afterwards the

comb was inserted. The wells were rinsed with 10 X SDS PAGE running buffer after

polymerization. Typically, electrophoresis was carried out at a constant current of 15 mA

during the stacking, and 30 mA therafter. Electrophoresis was usually performed until the

bromophenol blue dye front had run off the bottom of the gel. Gels were dried on a 3 MM

Whatman paper on a vacuum slab gel drier (Hoffmann Scientific) for 2 hours and exposed

to BIOMAX X-ray film (Kodak) at room temperature for 16 hours.

(2.26) Slot Hybridization of RNA

A piece of nitrocellulose was placed briefly in water and then soaked in 20 X SSC for 1 hour. Two sheets of 3 MM paper, prewetted with 20 X SSC, were placed on top of the vacuum unit. The wet nitrocellulose was then placed on the bottom of the sample wells. Air bubbles between the upper section of the manifold and the nitrocellulose were smoothed away. All the slots were filled with 10 X SSC and gentle suction was applied until all the fluid had passed through the nitrocellulose filter. This procedure was then repeated.

A sample (5 μl) of the *in vitro* transcribed RNA was mixed with the following and heated to 68°C for 15 minutes: 20 μl 100% formamide, 7μl formaldehyde and 2μl 20 X SSC. Two volumes of 20 X SSC were added to each sample before the samples were loaded into the slots. After all the samples had passed through the filter, each of the slots were rinsed twice with 1 ml of 10 X SSC. The nitrocellulose was then allowed to dry completely at room temperature before baking for 2 hours at 80°C in a vacuum oven.

Prehybridisation and hybrization were then carried out as in Section 2.14.

METHODS USED IN *IN VIVO* ANALYSIS

(2.27) Maintenance of cells

Mouse neuroblastoma cells (N2a) were obtained from ICN-Flow. Dulbecco's Modification of Eagle's medium (DMEM) was used as a growth medium and was supplemented with

10 % foetal calf serum and 1X non-essential amino acids. Cells were grown to confluence and were subcultured 1/6. Cells were released from the surface of the flasks by the action of a solution of trypsin TVP (Phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free, Chicken serum 2.5% Trypsin and 0.5 M EDTA). TVP was added at 1ml per 25 cm^2 flask and incubated at room temperature for one minute. At this stage the cells rounded up and detached from the bottom of the flask. The TVP was diluted by the addition of at least an equal volume of medium and clumps of cells broken up by repeated pipetting up and down. Fresh medium was added and the cells incubated at 37°C and in 5% CO_2 in a Heraeus 6000 incubator.

(2.28) Freezing cells

Frozen stocks of cells were prepared by detaching the cells from the flasks as described above and collecting them by centrifugation at 3,000 rpm for 3 mins. The cells were then resuspended in 10 ml (per 75 cm^2 flask) of freezing mix (Freezing mix MEEM, 6ml DMSO, 2 ml FCS) 2 ml and the centrifugation step repeated. The cells were then resuspended in 5 ml of the freezing mix and aliquoted into 0.5 ml lots in Nunc screw top freezing vials, which were then transferred into a polystyrene container and kept overnight at -70°C before transfer to liquid nitrogen where they could be stored indefinitely.

Before use, vials were removed from liquid nitrogen and quickly thawed at 37°C. The cells were diluted in 5 ml of medium and immediately collected by centifugation, before being

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resuspended in 10 ml of media and plated out in a 25 cm^2 flask. This step had the result of removing the DMSO in the freezing mix, which is toxic to the cells when unfrozen.

(2.29) Transfection of N2a cells

Two techniques were commonly used for the transfection of N2a cells:

(1) Electroporation

(2) Calcium Phosphate precipitation

Electroporation was used in Chapter 5 "*In vivo* transfection A" as the method to deliver constructs into N2a cells while calcium phosphate precipitation was used in "*In vivo* transfection B". Calcium phosphate precipitation was subsequently chosen because it was easier to use with a lot of samples.

(1) Electroporation

As described in Sambrook et al., (1989), N2a cells were washed twice with phosphate-buffered saline (PBS), scraped with a scraper in 2 ml of cold PBS and pelleted in a microcentrifuge (13000 rpm) for 30 seconds. At least 20 µg of plasmid DNA dissolved in 100 µl of TE buffer were then added to the cells. The plasmid DNA/ cell mixture was transferred into an cuvette and placed into the electroporator chamber. A charge of 0.15-0.20 Coulombs was most efficient at producing a high number of transfected cells. The

plasmid DNA/ cell mixture was finally transferred into a 100 mm petri dish containing 6 ml of medium and incubated for 48 hours at 37°C with 5% CO_2.

(2) Calcium Phosphate precipitation

The N2a cells were plated the day before transfection onto 100 mm petri dishes and were 30-60% confluent on the day of the transfection. A minimum of 10 ug of plasmid DNA was dissolved in 500 µl of 2X HeBs and 50 µl of 2.5M CaCl were then slowly added. The mixture was left at room temperature for 20 minutes before gently pipetting onto a 10 cm dish of cells. After 48 hours the cells were harvested for CAT assays or RNA analysis by RT-PCR

(2.30) <u>Preparation of cell extracts for Chloramphenicol acetyltransferase assays and β-Galactosidase Assays</u>

The methods were adapted from Sambrook et al., (1989). Transfected cells were washed twice in 4.5 ml PBS and scraped into 2 ml of cold PBS. The cells were then pelleted in a microcentrifuge for 20 seconds and resuspended in 100 µl of 0.25M Tris-HCl, pH 7.4. Lysis of the cells was achieved by three freeze-thaw cycles. Cellular debris was pelleted in a microcentifuge for 30 seconds and the supernatant removed for assay.

(2.31) CAT assay reaction

Endogenous acetylase in the lysate was heat inactivated at 65°C for 10 minutes. The assay was performed at 37°C for 30 minutes by the addition of 50 µl of cell extract to a microcentrifuge tube containing 65 µl of 0.25 M Tris-HCl, pH8, 5 µl of 5mg/ml n-butyryl coenzyme A and 2 µl of ^{14}C chloramphenicol (at 0.050mCi/ml). Termination of the reaction was achieved by adding 500 µl of ethyl acetate and votexing. The reaction mixture was then centrifuged in a microcentrifuge (13000 rpm) for 5 minutes, the upper organic phase was removed to a fresh tube and evaporated to dryness in a Speedvac. The residue was dissolved in 20 µl of ethyl acetate and spotted onto a TLC plate. Separation of acetylated ^{14}C chloramphenicol took place in a pre-equilibrated tank containing chloroform :methanol::97:3. Acetylated and non-acetylated products were scraped from TLC plates using the end of a pipette tip and placed into vials containing 1 ml of scintillation fluid, and the radioactivity was measured in a scintillation counter.

(2.32) β-Galactosidase Assay

The pSV-β-galactosidase positive control plasmid (Promega) contains the open reading frame of the bacterial *lacZ* gene which encodes the β-galactosidase enzyme. The SV40 early promoter and enhancer are used to drive transcription of the *lacZ* gene. The pSV-β-galactosidase positive control plasmid was used as an internal control for transient tranfection assays.

The following reaction mixtures were prepared in microfuge tubes: 25 μl of cell extract, 3 μl 100 X Mg buffer, 50 μl ONPG at 4 g ml^{-1} and 222 μl 0.1 M sodium phosphate buffer at pH 7.5. The samples were vortexed and incubated at 37°C for 30 mins. The reaction was stopped by the addition of 500 μl of 1 M sodium carbonate and the OD was measured at 420nm.

(2.33) Preparation of cytoplasmic RNA from tissue culture cells

N2a cells were washed twice with phosphate-buffered saline (PBS), scraped with a scraper in 2 ml of cold PBS and pelleted in a microcentrifuge (13000 rpm) for 30 seconds. From this point on, all manipulations, were carried out at 4°C. The cells were lysed by resuspending the pellet in 200 μl solution A (0.1 M NaCl, 10 M Tris-HCl, pH 7.9, 0.65% Nonidet P-40). Cellular debris and nuclei were pelleted in a microcentrifuge for 30 seconds. The supernatant was transferred to another tube containing an equal volume of solution B (7.0 M urea, 0.35M NaCl, 10 mM Tris-HCL, pH 7.4, 10 mM EDTA, 1% SDS) and phenol:chloroform (1:1). The resulting mixture was vortexed and centrifuged for 5 mins. The upper aqueous layer containing cytoplasmic RNA was removed and stored under ethanol until required.

(2.34) RT- PCR

For Reverse Transcription (RT), 1 µg of cytoplasmic RNA isolated from transiently transfected cells or 3 µl of a stock solution containing *in vitro* transcribed RNA were incubated with a RT primer (Section 2.34a). for 10 min at 70°C in a total volume of 11 µl. The reaction was immediately placed on ice. The following was added and incubated for 1 hour at 42°C: 200 units Superscript II, 1 mM dNTP and 40 units RNAsin (Pharmacia). The reaction was stopped and the cDNA purified by heating to 70°C for 10 minutes and ethanol precipitated twice. For PCR amplification, the cDNA pellet was dissolved in 50 µl sterile water and 25 µl were then used for amplification in a final volume of 100 µl containing 1X PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM MgCl and 0.01% (w/v) gelatin), 200 µM deoxynucleotide triphosphates, 2.5 U Taq DNA polymerase.

(2.34a) Primers used for Reversed Trasncription, Polymerase Chain

Reaction and making radioactive oligonucleotides probes

Primer	Sequence
anchored OligoT	CGTTTTTTTTTTTTTTTTTTT
Reverse primer	CATCGATGACAAGCTTAGGTATCGATACCA TTCATCCGCTTATTATC
Tailed Primer	CATCGATGACAAGCTTAGGTATCGATA
CAT start	CTAAAATGGAGAAAAAAATCACTGG
CAT ORF	TAAGCACATGCGTATTTC
PrP 3'UTR	GTAGCGAGACCTCACTTATG
BO11	ACACAGACGTCTCTTGATATTTCC

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(2.35) Southern Blotting

Separation of DNA fragments was carried out according to section 2.8. Upward capillary transfer was carried out overnight. The gel was first denatured in 500 ml denaturation solution (1.5 M NaCl and 0.5 M NaOH) and then neutralised in a solution containing 3 M NaCl and 0.5M Tris-HCl for 15 mins. Subsequently the DNA was transferred by the capillary action of the 10x SSC transfer buffer (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) being drawn upwards from a reservoir through the gel and nylon membrane (Hybond N) covering it, and into a stack of paper towels. This caused the DNA to be transferred from the gel to the membrane. Membranes were then fixed by UV irradiation to 0.4 J/cm^{-2}. Prehybridisation and hybridisation were carried out according to Section 2.14.

(2.36) Unidirectional deletions

The generation of ordered sets of deletions by this method relies on the uniform digestion rate of exonuclease III (Exo III) from appropriate DNA ends (Heniikoff et al., 1984). In order to make 5' protruding ends of DNA exonuclease resistant a method using alpha-phosphothiorate nucleotides was used. A *Bam*H I site between the CAT and the start of the PrP 3' UTR fragment was chosen on the plasmid pFr (Figure 15.) as the site resistant to Exo III. This restriction site was cleaved and filled in with α-phosphorothioate deoxyribonucleoside triphosphates as follows.

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The following reagents were added in the order listed to a microfuge tube, and incubated for 10 minutes at 37°C: all four α-phosphorothioate deoxyribonucleoside triphosphates to 40 µM each, dithiothreitol at 1 mM and Klenow fragment at 50 units/ml. The reaction mixture was ethanol precipitated, washed in 70% ethanol, air dried and the pellet dissolved in sterile water. *Hinc* II was then used to digest a second site on the plasmid pFR, which produced an exonuclease III sensitive 3' end. The DNA was then reprecipitated and redissolved.
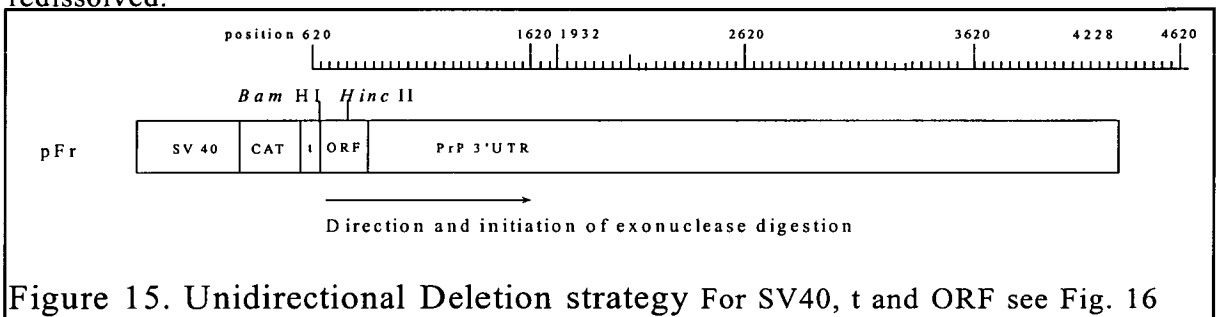
Figure 15. Unidirectional Deletion strategy For SV40, t and ORF see Fig. 16

In order to create the deletion mutants, 10 µg of doubly cut plasmid DNA was dissolved in 60 µl Exo III 1X buffer (66 mM Tris-HCl and 0.66 mM $MgCl_2$). Exo III (500 units) was added and incubated at 37°C. Samples (2.5 µl) were removed every minute and each placed into a microcentrifuge tube containing 7.5 µl of S1 nuclease mix (for 25 time points: 172 µl sterile water, 27 µl S1 buffer and 60 units S1 nuclease). S1 nuclease digestion removes single stranded DNA and was carried out for 15 minutes at room temperature. The reaction was stopped by adding 1µl of S1 stop buffer (0.3M Tris base, 0.05M EDTA) and ethanol precipitated before being added to a ligation reaction as in Section 2.11. An aliquot of the ligated products was used to transform *E.coli* according to Section 2.13 and the appropriate clones (a series of deletion mutants) selected by restriction analysis (Section 2.6) and DNA sequencing (Section 2.18).

Chapter 3   Sequencing the sheep PrP 3'UTR

(3.1) Introduction

In a study of PrP genotype, an association of natural and experimental scrapie was found with an *Eco*RI Restriction Fragment Length Polymorphism (RFLP). The polymorphic *Eco*RI site lies in the 3' UTR and gives rise to two alleles e1 and e3.

PrP-e1 was shown to be associated with disease in more than 30 different breeds (Hunter et al., 1992). Around 90% of the 167 diseased animals tested carried e1 with 60% homozygotes.

The studies undertaken in this thesis used a Suffolk sheep PrP clone containing the disease associated e1 fragment and was supplied by Wilfred Goldmann (Goldmann et al., 1990). The 3'UTR of this allele (e1) had not been sequenced and sequencing was therefore the first step of analysis. The e1 allele was shown to encode glutamine at position 171 in the PrP open reading frame while the e3 allele produces arginine at this position (Goldmann et al., 1990). The e1 association with disease could well be due to the linked missense mutation affecting position 171. The sequence of the e3 allele 3' UTR is known and published (Goldmann et al., 1990).

(3.2a) Sequencing of the 3' UTR of the e1 allele of the Suffolk sheep PrP gene

A *Pvu* II (ca 3.8Kbp) DNA fragment containing the entire PrP 3'UTR of the e1 allele

was isolated from plasmid p71 which originated from a larger PrP λ phage subclone

p32/21 (Goldmann et al., 1990). The fragment was ligated into chimaeric CAT

constructs, downstream of the CAT ORF and SV40 promoter. The PrP 3'UTR from this

construct pEYR (Section 5.2b) was sequenced on one strand (Methods Section 2.17) and

an alignment was made with the PrP 3' UTR of the e3 allele (Goldmann et al., 1990)

(Figure 15).

Figure 15. Sequence of the PrP gene 3' UTR (e1 upper, e3 lower)
The PrP 3'UTR sequence of the e1 allele (upper) is shown aligned with the published sequence of the e3 allele (lower) and differences are double underlined (Goldmann et al., 1990). The numbering of nucleotides is according to Goldmann et al. (1990) where position 1 corresponds to the 5' end of the mature mRNA and 840 is the start of the 3' UTR. Large lettering denote potential polyadenylation signals with any downstream G/T rich regions often found near strong signals denoted by underlining on the e1 sequence. ATTTA "instability" motifs are denoted in lower case. Restriction sites is noted in italics. Bold lettering denotes sequences that differ between e1 and the published sqeuence.

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       PrP  ORF                                        3'UTR
801 ATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAGGGGCAACC  850
    ||||||||||||||||||||||||||||||||||||||||||||||||||
801 ATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAGGGGCAACC  850

851 TTCCTGTTTTCATTATCTTCTTAATCTTTGCCAGGTTGGGGGAGGGAGTG  900
    ||||||||||||||||||||||||||||||||||||||||||||||||||
851 TTCCTGTTTTCATTATCTTCTTAATCTTTGCCAGGTTGGGGGAGGGAGTG  900

901 TCTACCTGCAGCCCTGTAGTGGTGGTGTCTCATTTCTTGCTTCTCTCTTG  950
    ||||||||||||||||||||||||||||||||||||||||||||||||||
901 TCTACCTGCAGCCCTGTAGTGGTGGTGTCTCATTTCTTGCTTCTCTCTTG  950
    AccI
951 TTACCTGTATAATAATACCCTTGGCGCTTACAGCACTGGGAAATGACAAG  1000
    ||||||||||||||||||||||||||||||||||||||||||||||||||
951 TTACCTGTATAATAATACCCTTGGCGCTTACAGCACTGGGAAATGACAAG  1000
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1001 CAGACATGAGATGCTGTTTATTCAAGTCCCATTAGCTCAGTATTCTAATG 1050
     ||||||||||||||| |  | ||||||||||||||||||||||||||||||
1001 CAGACATGAGATGCTatttaTTCAAGTCCCATTAGCTCAGTATTCTAATG 1050
                    ==

1051 TCCCATCTTAGCAGTGATTTTGTAGCAATTTTCTCATTTGTTTCAAGAAC 1100
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1051 TCCCATCTTAGCAGTGATTTTGTAGCAATTTTCTCATTTGTTTCAAGAAC 1100

1101 ACCTGACTACATTTCCCTTTGGGAATAGCATTTCTGCCAAGTCTGGAAGG 1150
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1101 ACCTGACTACATTTCCCTTTGGGAATAGCATTTCTGCCAAGTCTGGAAGG 1150

1151 AGGCCACATAATATTCATTCAAAAAAACAAAACTGGAAATCCTTAGTTCA 1200
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1151 AGGCCACATAATATTCATTCAAAAAAACAAAACTGGAAATCCTTAGTTCA 1200

1201 TAGACCCAGGGTCCACCCTGTTGAGAGCATGTGTCCTGTGTCTGCAGAGA 1250
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1201 TAGACCCAGGGTCCACCCTGTTGAGAGCATGTGTCCTGTGTCTGCAGAGA 1250

1251 ACTATAAAGGATATTCTGCATTTTGCAGGTTACATTTGCAGGTAACACAG 1300
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1251 ACTATAAAGGATATTCTGCATTTTGCAGGTTACATTTGCAGGTAACACAG 1300

1301 CCATCTATTGCATCAAGAATGGATATTCATGCAACCTTTGACTTATGGGC 1350
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1301 CCATCTATTGCATCAAGAATGGATATTCATGCAACCTTTGACTTATGGGC 1350

1351 AGAGGACATCTTCACAAGGAATGAACATAATACΔAAAGGCTTCTGAGACT 1399
     ||||||||||||||||||||||||||||||||| ||||||||||||||||
1351 AGAGGACATCTTCACAAGGAATGAACATAATACAAAAGGCTTCTGAGACT 1400

1400 AAAAAATTCCAACATATGGAAGAGGTGCCCTTGGTGGCAGCCTTCCATTT 1449
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1401 AAAAAATTCCAACATATGGAAGAGGTGCCCTTGGTGGCAGCCTTCCATTT 1450

1450 TGTATGTTTΔAAGCACCTTCAAGTGATATTCCTTTCTTTAGTAACATAAA 1498
     ||||||||| ||||||||||||||||||||||||||||||||||||||||
1451 TGTATGTTTAAAGCACCTTCAAGTGATATTCCTTTCTTTAGTAACATAAA 1500
              ==

1499 GTATAGATAATTAAGGTACCTTA ATTAAA CTACCTTCTAGACACTGAGAG 1548
     |||||||||||||| |||| ||| |||||| ||||||||||||||||||||
1501 GTATAGATAATTAAGGTACCTTA ATTAAA CTACCTTCTAGACACTGAGAG 1550
                    Kpn I         Pac I
1549 CAAATCTGTTGTTTATCTGGAAΔCCCAGGATGATTTTGACATTGCTTAGG 1597
     |||||||||||||||||||||| ||||||||||||||||||||||||||||
1551 CAAATCTGTTGTTTATCTGGAACCCCAGGATGATTTTGACATTGCTTAGG 1600

1598 GATGTGAGAGTTGGACTGTAAAGAAAGCTGAGTGCTGAAGAGTTCATGCT 1647
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1601 GATGTGAGAGTTGGACTGTAAAGAAAGCTGAGTGCTGAAGAGTTCATGCT 1650
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1648 TTTGAACTATAGTGTTGGAGAAAACTCTTGAGAGTCCCTTGGACTGAAAG 1697
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1651 TTTGAACTATAGTGTTGGAGAAAACTCTTGAGAGTCCCTTGGACTGAAAG 1700

1698 GAGATCAGTCCTGAATATTCATTGGAAGGACTGATGCTGAAGCTGAAACT 1747
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1701 GAGATCAGTCCTGAATATTCATTGGAAGGACTGATGCTGAAGCTGAAACT 1750

1748 CCAATACTTTGGTCACCTGATGGGAAGAACTGAAGGCAGGAGGGATGCTA 1797
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1751 CCAGTACTTTGGTCACCTGATGGGAAGAACTGAAGGCAGGAGGGATGCTA 1800
        =          Bst EII
1798 GGAAAGACTGAAGGCAGGAGGAGAAGGGGACGACAGAGGATGAGATGGCT 1847
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1801 GGAAAGACTGAAGGCAGGAGGAGAAGGGGACGACAGAGGATGAGATGGCT 1850

1848 AGATGGCATCATGGACTCAATGGACATGAGCTTAAGTAAACTCCAGGAGT 1897
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1851 AGATGGCATCATGGACTCAATGGACATGAGCTTAAGTAAACTCCAGGAGT 1900

1898 TGGCAATGGACAGGGAGACCTGGCGTCCTGCAGTCCATGGTGTCGCAGAG 1947
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1901 TGGCAATGGACAGGGAGACCTGGCGTCCTGCAGTCCATGGTGTCGCAGAG 1950
                             Pst I
1948 TCGGACACGATTGAGTGACTAAATTGAGGTGACCCAGatttaACATAGAG 1997
     ||||||||||||||||||||||||||||||||||||||||||||||||||
1951 TCGGACACGATTGAGTGACTAAATTGAGGTGACCCAGatttaACATAGAG 2000

1998 AATGCAGATACAAAACTCATATTCATTTGATTGAATCTTTTCCTGAACCA 2047
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2001 AATGCAGATACAAAACTCATATTCATTTGATTGAATCTTTTCCTGAACCA 2050

2048 GTGCTAGTGTTGGACTGGTAAGGGTATAACAGCATATATAGGTTATGTGA 2097
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2051 GTGCTAGTGTTGGACTGGTAAGGGTATAACAGCATATATAGGTTATGTGA 2100

2098 TGAAGAGATAGTGTACATGAAATATGTGCATTTCTTTATTGCTGTCTTAT 2147
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2101 TGAAGAGATAGTGTACATGAAATATGTGCATTTCTTTATTGCTGTCTTAT 2150

2148 AATTGTCAAAAAGAAAATTAGGTCCTTGGTTTCTGTAAAATTGACTTGA 2197
     |||||||||||||||||||||||||||||||||||||||||||||||||
2151 AATTGTCAAAAAGAAAATTAGGTCCTTGGTTTCTGTAAAATTGACTTGA 2200

2198 ATCAAAAGGGAGGCatttaAAGA AATAAA TTAGAGATGATAGAAATCTGA 2247
     |||||||||||||||||||||||||||||||    ||||||||||||||||||||
2201 ATCAAAAGGGAGGCatttaAAGA AATAAA TTAGAGATGATAGAAATCTGA 2250

2248 TCCATTCAGAGTAGAAAAAGAAATTCCATTACTGTTATTAAAGAAGGTAA 2297
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2251 TCCATTCAGAGTAGAAAAAGAA TTGCAT ACTGT ATTAA  GAAG TCA 2299
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2298 AAT**T**ATTCC**C**TGAATTGTTCAATATTGTCACCTAGCAGATAGACACTATT 2347
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2300 AAT ATTCC TGAATTGTTCAATATTGTCACCTAGCAGATAGACACTATT 2343

2348 CTGTACTGTTTTTACTAGCTTGCACCTTGTGGTATCCTATGTAAAAACAT 2397
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2344 CTGTACTGTTTTTACTAGCTTGCACCTTGTGGTATCCTATGTAAAAACAT 2393

2398 ATTTGCATATGACAAACTTTTTCTGTTAGAGCAATTAACATCTGAACCAC 2447
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2394 ATTTGCATATGACAAACTTTTTCTGTTAGAGCAATTAACATCTGAACCAC 2443

2448 CTAATGCATTACCTGTTTTTGTAAGGTACTTTTTGTAAGGTACTAAGGAG 2497
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2444 CTAATGCATTACCTGTTTTTGTAAGGTACTTTTTGTAAGGTACTAAGGAG 2493

2498 ATGTGGGTTTAATCCCTAGGTCAGGTAAATCCCCTAGAGGAAGAAATGGC 2547
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2494 ATGTGGGTTTAATCCCTAGGTCAGGTAAATCCCCTAGAGGAAGAAATGGC 2543

2548 AACCCACTCCAGTATTCTTGCCAGGAAAATCCAGTGGGCAGAGGAGCCTG 2597
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2544 AACCCACTCCAGTATTCTTGCCAGGAAAATCCAGTGGGCAGAGGAGCCTG 2593

2598 GCAGGGTACAGTCT**AA**GAGCATGGGGTTGCAAAGAGTGAGACAAGACTTG 2647
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2594 GCAGGGTACAGTCT__GAGCATGGGGTTGCAAAGAGTGAGACAAGACTTG 2641

2648 AGCTACTGAACAATAAGGAC**AATAAA**TGCTGGGTCGGCTAAAAGGTTCAT 2697
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2642 AGCTACTGAACAATAAGGAC**AATAAA**TGCTGGGTCGGCTAAAAGGTTCAT 2691

2698 TAGGTTTTTTTTCTGTAAGATGGCTCTAGTAGTACTTGTCTTTATCTTCA 2747
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2692 TAGGTTTTTTTTCTGTAAGATGGCTCTAGTAGTACTTGTCTTTATCTTCA 2741

2748 TTCGAAACAATTTTGTTAGATTGTATGTGACAGCTCTTGTATCAGCATGC 2797
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2742 TTCGAAACAATTTTGTTAGATTGTATGTGACAGCTCTTGTATCAGCATGC 2791

2798 ATTTGAAAAAAACATCACAATTGGTAAATTTTTGTATAGCCATCTTACTA 2847
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2792 ATTTGAAAAAAACATCACAATTGGTAAATTTTTGTATAGCCATCTTACTA 2841

2848 TTGAAGATGGAAGAAAAGAAGCAAAATTTTCAGCATATCATGCTGTACTT 2897
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2842 TTGAAGATGGAAGAAAAGAAGCAAAATTTTCAGCATATCATGCTGTACTT 2891

2898 ATTTCAAGAAAGATAACCAAAATGCAAAAATGTATTTGTGAAGTGTATGG 2947
     | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
2892 ATTTCAAGAAAGATAACCAAAATGCAAAAATGTATTTGTGAAGTGTATGG 2941
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2948 AGAAGGGGCTGCAACTGATCAAGCTTGTCAAAGTAGTTTGTGAAGTTTCG 2997
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2942 AGAAGGGGCTGCAACTGATCAAGCTTGTCAAAGTAGTTTGTGAAGTTTCG 2991

3998 TGCTGGAGATTTCTTATTGGACGATGCTCCACAGTTGGATATACCAGTTG 3047
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2992 TGCTGGAGATTTCTTATTGGACGATGCTCCACAGTTGGATATACCAGTTG 3041

3048 AAGTTGATAGTGATCAAATTGAGATATTGAGAATAATCGATGTTATACCA 3097
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3042 AAGTTGATAGTGATCAAATTGAGATATTGAGAATAATCGATGTTATACCA 3091

3098 CGCGGGAGATAGCTGACATACTCAAAATATCCAAATAGAACCTTGAAAAC 3147
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3092 CGCGGGAGATAGCTGACATACTCAAAATATCCAAATAGAACCTTGAAAAC 3141

3148 CATTTGCACCATCTCAGTTATGTTAATCACTTTGATGTTTGAGTTCCACA 3197
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3142 CATTTGCACCATCTCAGTTATGTTAATCACTTTGATGTTTGAGTTCCACA 3191

3198 TAAGCAAAAAAACAACAACAAAAAAAAATACAACCTTGACCATATTTGCG 3247
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3192 TAAGCAAAAAAACAACAACAAAAAAAAATACAACCTTGACCATATTTGCG 3241

3248 CATGCAGTTCTCTACTGAAATGATTGAAAACACTTTGTTTTTAAAAACAG 3297
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3242 CATGCAGTTCTCTACTGAAATGATTGAAAACACTTTGTTTTTAAAAACAG 3291

3298 ATTTTGATTAACAGTGGGTACGATACAATAACGTAGATGGAAGAAATTGT 3347
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3292 ATTTTGATTAACAGTGGGTACGATACAATAACGTAGATGGAAGAAATTGT 3341

3348 AGGGTGAGCAAAATGAACCACACCACCAAAGGCCAGTCTTCCTCTAAAGA 3397
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3342 AGGGTGAGCAAAATGAACCACACCACCAAAGGCCAGTCTTCCTCTAAAGA 3391

3398 AGATGTGTGTATGGTGGGATTGGAAAGTAATCCTCTATTATGGATTCTTC 3447
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3392 AGATGTGTGTATGGTGGGATTGGAAAGTAATCCTCTATTATGAATTCTTC 3441

3448 TGGAAAACACTGCTCCTAATTAGACCAACTGAAAACAGCACTCAACGAAA 3497
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3442 TGGAAAACACTGCTCCTAATTAGACCAACTGAAAACAGCACTCAACGAAA 3491

3498 AGCATCCAGAATTAGTCAATAGAAAACATAATCTTCCATCAGGATAACGC 3547
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3492 AGCATCCAGAATTAGTCAATAGAAAACATAATCTTCCATCAGGATAACGC 3541

3548 AAGACTACATATTTCTTTGATGACCCAGCATGGCTGGAGTTTCTGATTCA 3597
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3542 AAGACTACATATTTCTTTGATGACCCAGCATGGCTGGAGTTTCTGATTCA 3591
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3598 TCTGTTGTATTCAGACGTTGCATCTTTGGATTTTTTCCatttaTTTCAGT 3647
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3592 TCTGTTGTA TCAGACGTTGCATCTT GGATTTTT C atttaTT CAGT3635

3648 CTACAAAATTATCATAATGGAAAAAATTTCCATTCCCTΔGGAAGATGTAAA3697
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3636 CTACAAA TTAT ATA TG AAAA   TT   CAT  CCTTGTAAGATGTAA 3675
     Acc I
3698 GTGCATCTGGAAAATTTCTTTGCTCAAAAAGATAAAAAGTTTTGTGAACA  3747
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3676 GTGCATCTGGAAAATTTCTTTGCTCAAAAAGATAAAAAGTTTTGTGAACA  3725

3748 CAGAATTATGACGTTGCCTGAAAAATGGCAGAAGGTAGTGGAACAAAAGA  3797
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3726 CAGAATTATGACGTTGCCTGAAAAATGGCAGAAGGTAGTGGAACAAAAGA  3775

3798 GTGACTATGTTGTTTGGTAAAGTTCTTAGTGAAAATGAAAAATGTGTCTT  3847
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3776 GTGACTATGTTGTTTGGTAAAGTTCTTAGTGAAAATGAAAAATGTGTCTT  3825

3848 TTATTTTTatttaAACACCAAAGGCACATTTTAGCAACCCAATACTGAAT  3897
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3826 TTATTTTTatttaAACACCAAAGGCACATTT GCACACCAA  CTGTAA   3867

3898 ΔCTAAAGGAAACTCTTCTGTGTGTTGTCCTTACAGTGTGCACTGATAGTTT 3947
     |||||||||||||||||||||||||||||||||||||||||||||||||||
3868 TCTAA GAA   CCTCGGTGTCCTAGCCTTACAGTGTGCACTGATAGTTT  3917

3948 GTATAAGAATCCAGAGTGATATTTGAAATACGCATGTGCTTATATTTTTT  3997
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3918 GTATAAGAATCCAGAGTGATATTTGAAATACGCATGTGCTTATATTTTTT  3967

3998 ATATTTGTAACTTTGCATGTACTTGTTTTGTGTTAAAAGTTTATAAATat  4047
     ||||||||||||||||||||||||||||||||||||||||||||||||||
3968 ATATTTGTAACTTTGCATGTACTTGTTTTGTGTTAAAAGTTTATAAATat  4017

4048 ttaATATCTGACTAAA ATTAAA CAGGAGCTAAAAGGAGTATCTTCCACGG 4097
     |||||||||||||||| |||||| |||||||||||||||||||||||||||
4018 ttaATATCTGACTAAA ATTAAA CAGGAGCTAAAAGGAGTATCTTCCACGG 4067

4098 AGTTGTCTGGCTGTGTTCACCAGATGTGCACACΔATGTTGGCAGTCT     4144
     |||||||||||||||||||||||||||||||||||||||||||||||
4068 AG TGTCTGGCTGTGTTCACCAG TGTGCACACCATGTTGGCAG.CT     4111

4145 TCATTTGGGGGGTTAATATGAGAAAAGTGACACATTCAGTCCTCACACTG  4194
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4112 TCATTTGGGGGGTTAATATGAGAAAAGTGACACATTCAGTCCTCACACTG  4161

4195 CCAATTGCAGGAGGAGGGCTACTCCTGATCCTGCTTCAGCCTTATTCCCA  4244
     ||||||||||||||||||||||||||||||||||||||||||||||||||
4152 CCAATTGCAGGAGGAGGGCTACTCCTGATCCTGCTTCAGCCTTATTCCCA  4201
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4245 GTCACATGCCAGCCC  PvuII removed by blunt ending
     |||||||||||||
4202 GTCACATGCCAGCTG
              PvuII
```

(3.3) Results and Discussion

The PrP 3' UTR of the e1 allele was sequenced from the construct pEYR (Section 5.2b). The

PrP 3'UTR sequence of the e1 allele was aligned with the published sequece of the e3 allele

(Goldmann et al., 1990) Many seeming differences were originally found. These differences

were further investigated by resequencing the e3 allele from constructs bs10 and p23.4. and

most of them were found to reflect apparent sequencing errors in the published sequence

(noted in Figure 15). The genuine sequence differences identified between the e3 and e1 PrP

3' UTR are shown in Table 4. In total there were only six confirmed differences.

Table 4. Confirmed differences between the e3 allele and e1 allele sequenced in this study.

Position	Polymorphism
1015	A → G
1458	A → Δdel
1751	G → A
2613	Δdel → A
2614	Δdel → A
3440 *	A → G

Δdel = single base deletion * EcoR1 polymorphic site

In both alleles four alternative polyadenylation signals (ATTAAA or AATAAA) are present, upstream of the consensus sequence ATTAAA (position 4064) which is known to generate a 4.6kb mRNA (Table 5). These alternatives are candidates for generating the short PrP transcript (2.1kb) found in the peripheral tissues of sheep (Hunter et al., 1994; Iketa et al., 1995). The alternative polyadenylation signal at position 1522, unlike the other 3 alternative polyadenylation signals but like that at 4064,, contains G/T-rich stretches downstream. Of the poly A signals recognised, only that at 1522 would be likely to produce a polyadenylated mRNA of circa 2.1 kb.

Table 5. Position and sequence of potential alternative polyadenylation signals in the PrP 3' UTR.

Position	Potential alternative polyadenylation signals
1522	ATTAAA
2221	AATAAA
2284	ATTAAA
2668	AATAAA

Five "instability motifs" ATTTA were found in the e1 and six in the e3 3' UTR sequence (Table 6). The functional consequence of these differences between the PrP 3' UTRs is not known.

Table 6. Positions of the "instability motifs" in the e3 PrP 3' UTR (Goldmann et al., 1990) and the e1 allele.

Instability motifs ATTTA	e3 3'UTR	e1 3' UTR
1016	✓	X
1985	✓	✓
2213	✓	✓
3636	✓	✓
3856	✓	✓
4046	✓	✓

X - site not present

In summary, the two PrP 3' UTRs have been shown to contain polymorphisms, potential alternative polyadenylation signals and ATTTA instability motifs. The following chapters will attempt to address the importance of the PrP 3' UTR.

Chapter 4
Effects of the 3' UTR of PrP upon the *in vitro* translation of a linked CAT reporter message

(4.1) Introduction

The PrP 3' UTR sequence (chapter 3) was found to contain potentially interesting sequence elements eg. instability motifs 'ATTTA' (Table 6) and polymorphisms (Table 4). The potential for the 3' UTR to affect gene expression was investigated (this chapter) using a cell free system and (Chapter 5) using intact cells. Chimaeric CAT constructs including different parts of the 3' UTR of PrP were *in vitro* transcribed and translated in order to assess whether the 3' UTR can alter expression at the translational level.

In vitro translation (IVT) systems were initially used to identify and characterise mRNA, but now IVT is a tool for addressing basic questions about the mechanism and regulation of protein synthesis. Researchers wishing to express cloned genes in an *in vitro* system frequently use rabbit reticulocyte lysate (RRL) (Pelham and Jackson 1976). Unlike prokaryotic systems where transcription and translation are coupled, RRL has traditionally been a translation-only system requiring the addition of RNA templates and, prior to IVT analysis, *in vitro* transcription reactions employing T3, T7 or SP6 RNA polymerases are widely used to synthesize large amounts of specific RNA from recombinant vectors containing appropriate promoters.

The RRL translation system has played an important role in the investigation into the effects of untranslated regions from mRNA transcripts upon subsequent translation. RRL is pretreated with micrococcal nuclease to destroy endogenous mRNA and contains all the necessary components for translation of exogenously added transcripts. Incorporation of a radiolabelled amino-acid enables one to assess protein levels, for example, by scanning autoradiograms of SDS polyacrylamide gels using a densitometer or measuring

radioactivity incorporation by liquid scintillation counter.

Translation of human interferon mRNA was shown to be affected by its 3' UTR (Kruys et al., 1987) using the RRL system. The sequence responsible for the observed translational repression was rich in AUs, and was called an AU-rich element (Kruys et al., 1988). AU-rich elements repressed translation when placed at any region within the 3'UTR but not when placed in the 5'UTR. *In vitro* transcribed ferritin RNA was also translationally regulated in RRL and this was controlled by its 3' UTR (Dickey et al., 1987). Further analysis showed that a 28 nucleotide structure termed the "iron regulatory element" in the 5'UTR of ferritin mRNA interacted with a 90 KDa protein under low iron concentration and blocked initiation of translation in cells. (Rouault et al., 1988, 1989; Walden et al., 1989). It was thus hypothesized that ferritin can undergo a number of stages of translational control involving both the 3' and the 5' UTR (Harfford and Klausner 1990). In contrast to the previous two examples, *in vitro* transcribed Lox mRNA was translationally repressed through sequences in its 3' UTR only when the repressor protein Lox BP was added to the RRL (Ostareck-Lederer et al., 1994).

Models that have been put forward to explain translational regulation involve a protein-mRNA interaction and cytoplasmic polyadenylation which are elements absent from the RRL system (Zamore and Lehmann 1996). Moreover, Recent data suggest that translational regulation can be obscured in RRL due to the lack of competition for the translation machinery (Tanguay and Gallie 1996). Nevertheless, the simple *in vitro* translation system may be useful in terms of gaining results that may be further investigated in intact cells.

(4.2) Experimental approach

To determine if the 3'UTR of sheep PrP can have an effect upon *in vitro* translation of chloramphenicol acetyl transferase (CAT) mRNA, reporter gene constructs were made containing various sections of the PrP 3' UTR downstream of the CAT gene. Two constructs contained the entire PrP 3' UTR, one in reverse orientation, and a third construct contained the 3'UTR with a 2746 bp internal deletion. Chimaeric CAT transcripts were produced from the corresponding plasmid by *in vitro* run-off transcription, and the sizes of the transcript were predicted from the length between the T3 polymerase promoter and the recognition site for the restriction enzyme used to linearize the plasmid. The corresponding transcript were then isolated from formaldehyde gels. An aliquot of each gel-purified RNA was subjected to Northern transfer analysis to check the concentration, hybridisation pattern and integrity of the transcript. A positive control construct containing only the CAT gene fragment was used to establish a dose-response curve for this assay. The effects of increasing *in vitro* transcribed RNA upon the yield of CAT defined the limits of the experimental assay. Equimolar amounts of *in vitro* transcribed RNAs containing CAT fused to the 3'UTR of sheep PrP differing in length and orientation were *in vitro* translated using the RRL (Promega). The concentration of RNA was calculated and checked by spectrophotometry, formaldehyde gel electrophoresis and slot blotting. ^{35}S methionine labelled CAT was measured by scanning autoradiograms of SDS gels with a densitometer.

(4.3) Construction of plasmids

Construction of plasmids was carried out using standard procedures (Chapter 2) and involved the following steps: preparation, isolation and ligation of the vector and insert DNA, transformation of *E. coli* with the ligated products, screening of transformants by restriction analysis and partial sequencing. The numbering of nucleotides was as described in Chapter 3.

(a) pTCAT

The construct pTCAT contains no PrP 3' UTR and was made (Figure 16) by digesting pCAT Basic (Promega) with *Mae*I (restriction sites at position: 2267 and 3291) and isolating a 1024 bp fragment containing the entire open reading frame of CAT and "t" which is mainly the intron plus flanking sequences, whose consequent splicing is important for expression of the CAT fusion mRNA *in vivo*. The insert was blunt ended using Klenow fragment polymerase and subsequently ligated into the *Hind*II site of pT3T7α-19 (Gibco), such that the T3 promoter was located upstream from the start site of the CAT gene. Partial sequencing from the intron and restriction analysis confirmed the identity of this clone.

Mae I digestion

t: 316bp of UTR of SV 40 small t antigen gene containing the intron
T3, T7 : promoters

Figure 16. Schematic representation of the construction of plasmid pTCAT. The CAT ORF fragment was removed from the plasmid pCAT basic by *Mae* I restriction and subsequently ligated into the *Hind* II site of pT3T7α -19.

91
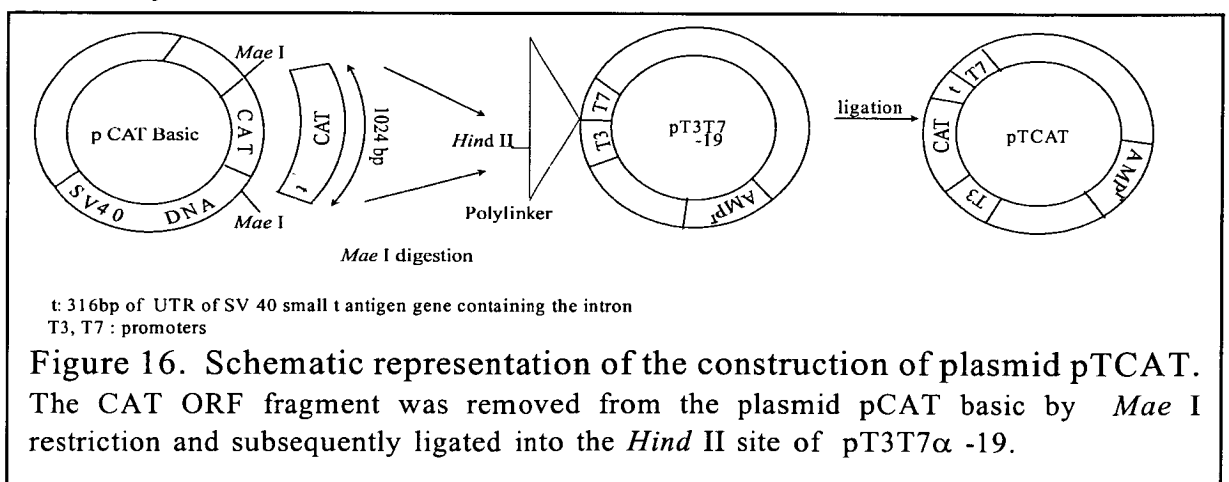
(b) pRAR and pRAW

The construct pRAR contains the full length e1 PrP 3' UTR. pRAR (Figure 17) was constructed by digesting p71 (Goldmann et al., 1989) with *Pvu*II and isolating a 3825 bp (position 432-4256) fragment containing the entire 3' UTR and 400 bp of the PrP open reading frame. This fragment was ligated into the *Sma*I site of pTCAT. Colonies were screened for inserted fragments in both orientations by colony hybridization. pRAW contains the same fragment as pRAR, but in the opposite direction as determined by restriction analysis.

Figure 17. Schematic representation depicting the cloning strategy for pRAR The PrP 3'UTR fragment was removed from p71 by *Pvu* II restriction and was subsequently ligated into the *Sma* I restriction site of the plasmid pTCAT.
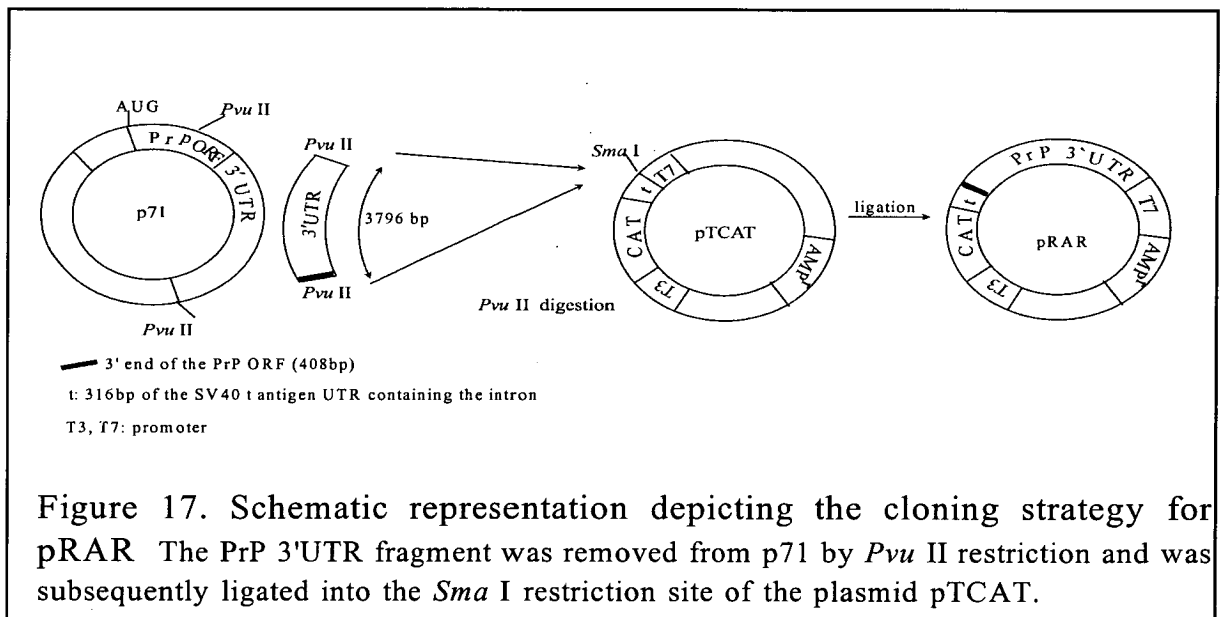
(c) pRAD

The construct pRAD contains the PrP 3' UTR with a 2734 bp deletion. pRAD was constructed (Figure 18) by digesting pRAR with *Acc*I overnight and religating, resulting in the deletion of 2746 bp (positions 901-3646) from the PrP 3'UTR. Restriction analysis confirmed the identity of this construct.
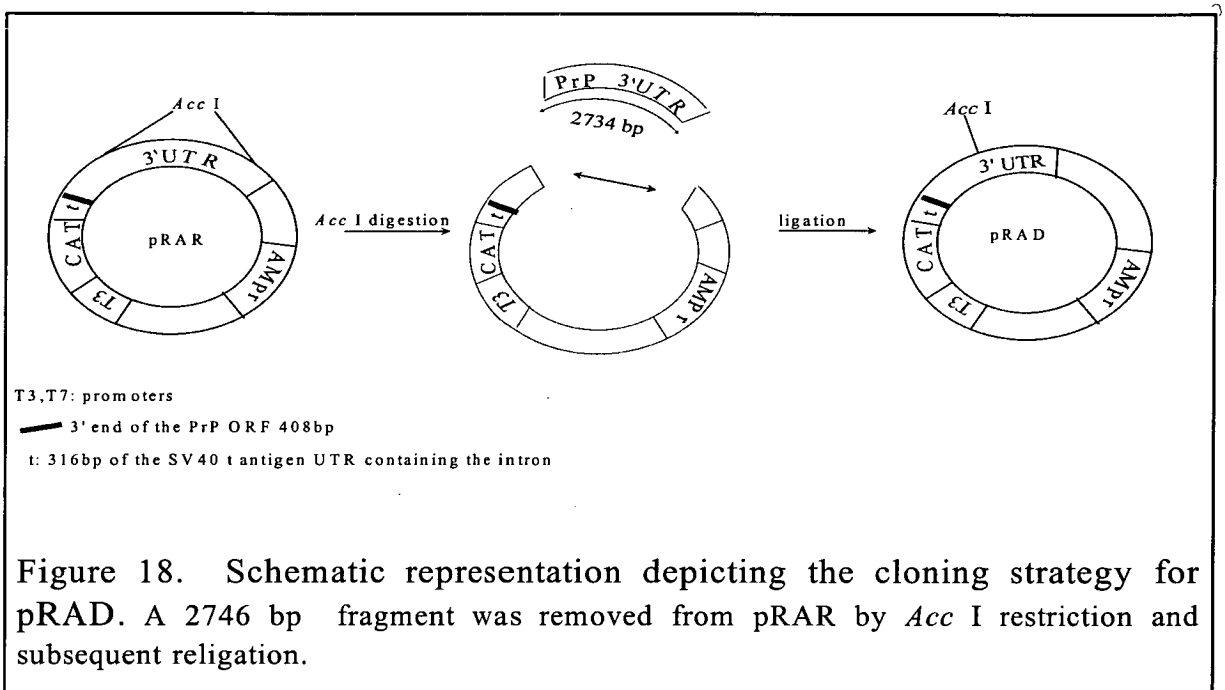
T3,T7: promoters

━━ 3' end of the PrP ORF 408bp

t: 316bp of the SV40 t antigen UTR containing the intron

Figure 18. Schematic representation depicting the cloning strategy for pRAD. A 2746 bp fragment was removed from pRAR by *Acc* I restriction and subsequent religation.

(d) Summary of constructs

Reporter gene constructs were made containing various sections of the el PrP 3' UTR downstream of the CAT gene (Figure 19). pRAR and pRAW contain the full length 3' UTR in the forward and reverse orientation respectively (Section 4.3b). pRAD has a 2746 bp deletion (position 901-3646) within the 3' UTR (Section 4.3c). pTCAT contains no sheep PrP 3' UTR (Section 4.3a).

Figure 19. Summary of constructs used to investigate the PrP 3'UTR

94
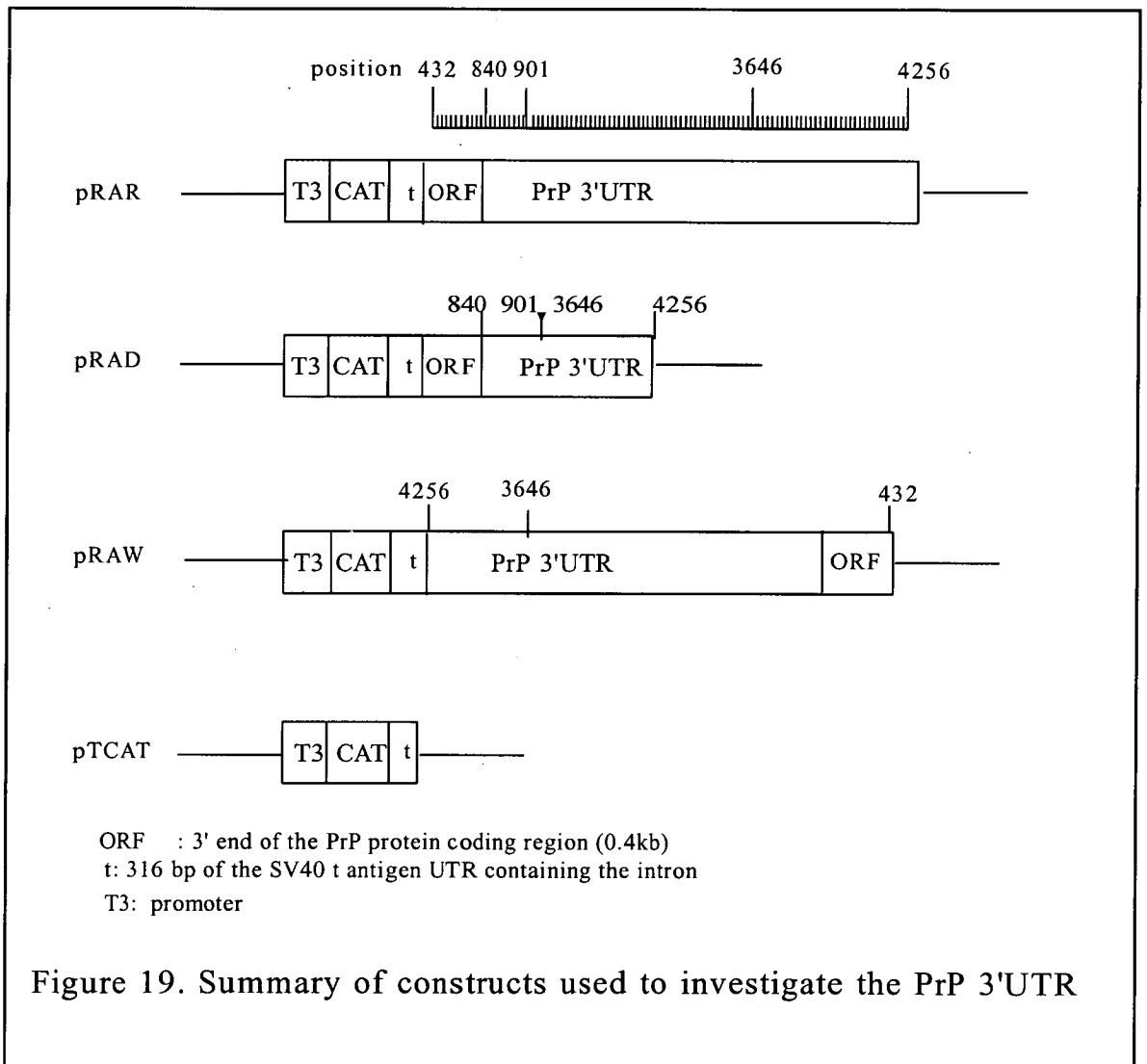
(4.4) Production of RNA

(a) *In vitro* transcription

The Chimaeric CAT constructs shown in Figure 19 were used in the following *in vitro* transcription and translation reactions. *In vitro* transcription from constructs pRAD and pTCAT produced single major species of transcript of approximately the expected sizes (2086 and 1024 nucleotides: Figure 20, lanes a and d respectively) in relation to RNA size markers. In contrast, pRAW and pRAR yielded not only transcripts of expected size (4820 and 4760 nucleotides: Figure 20, lanes b and c respectively), but also smaller transcripts.
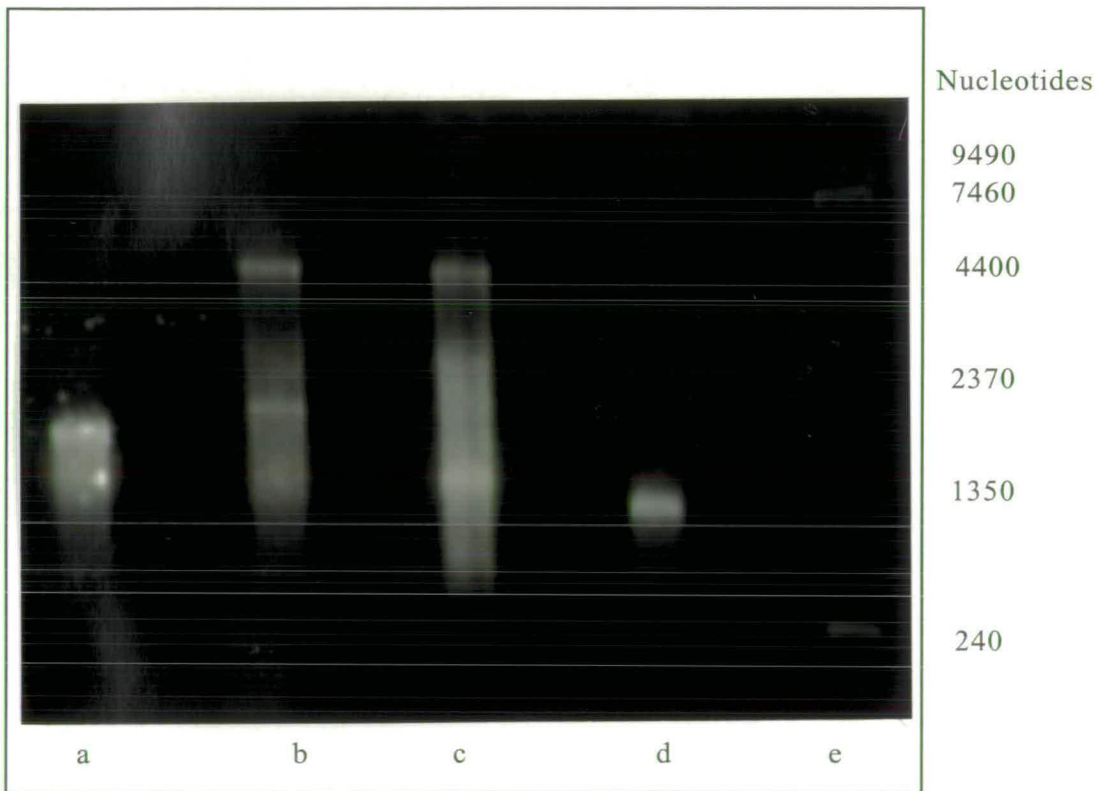
Figure 20. Formaldehyde gel analysis of *in vitro* transcribed RNA from chimaeric CAT constructs. The plasmid constructs used for RNA transcription were as follows: lane a ,pRAD; lane b, pRAW; lane c, pRAR and lane d, pTCAT; lane e, RNA markers. Transcripts visualised with ethidium bromide.

(b) Isolation of transcripts

All full length transcripts were purified from 1% formaldehyde gels. An aliquot was

subjected to 1% formaldehyde gel electrophoresis (Figure 21) and Northern analysis.

Hybridisation with an oligonucleotide anti-sense probe directed against the CAT

ORF gave a positive signal with RNA species from all four constructs (Figure 22A)

while hybridisation with an oligonucleotide anti-sense probe directed against

sequences just upstream of the main poly A signal took place only with transcripts

produced from constructs pRAD and pRAR (Figure 22B lane c and d respectively)

because pRAW has the PrP 3' UTR inverted and pTCAT contains no PrP 3' UTR

sequences. These results show that the transcripts recovered from the formaldehyde

gels exhibited homogeneity, the expected size, and correct hybridisation pattern

according to the constructs from which they were produced.
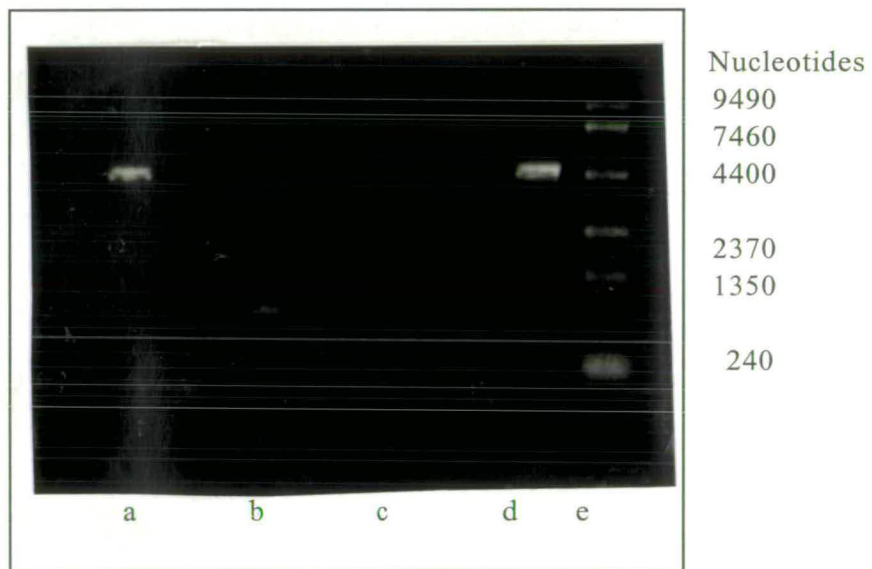
Figure 21. Formaldehyde gel analysis of gel purified *in vitro* transcribed RNA from chimaeric CAT constructs. The plasmid constructs used for RNA transcription were as follows: lane a: pRAW; lane b: pTCAT, lane c: pRAD and lane d: pRAR, lane e: RNA markers

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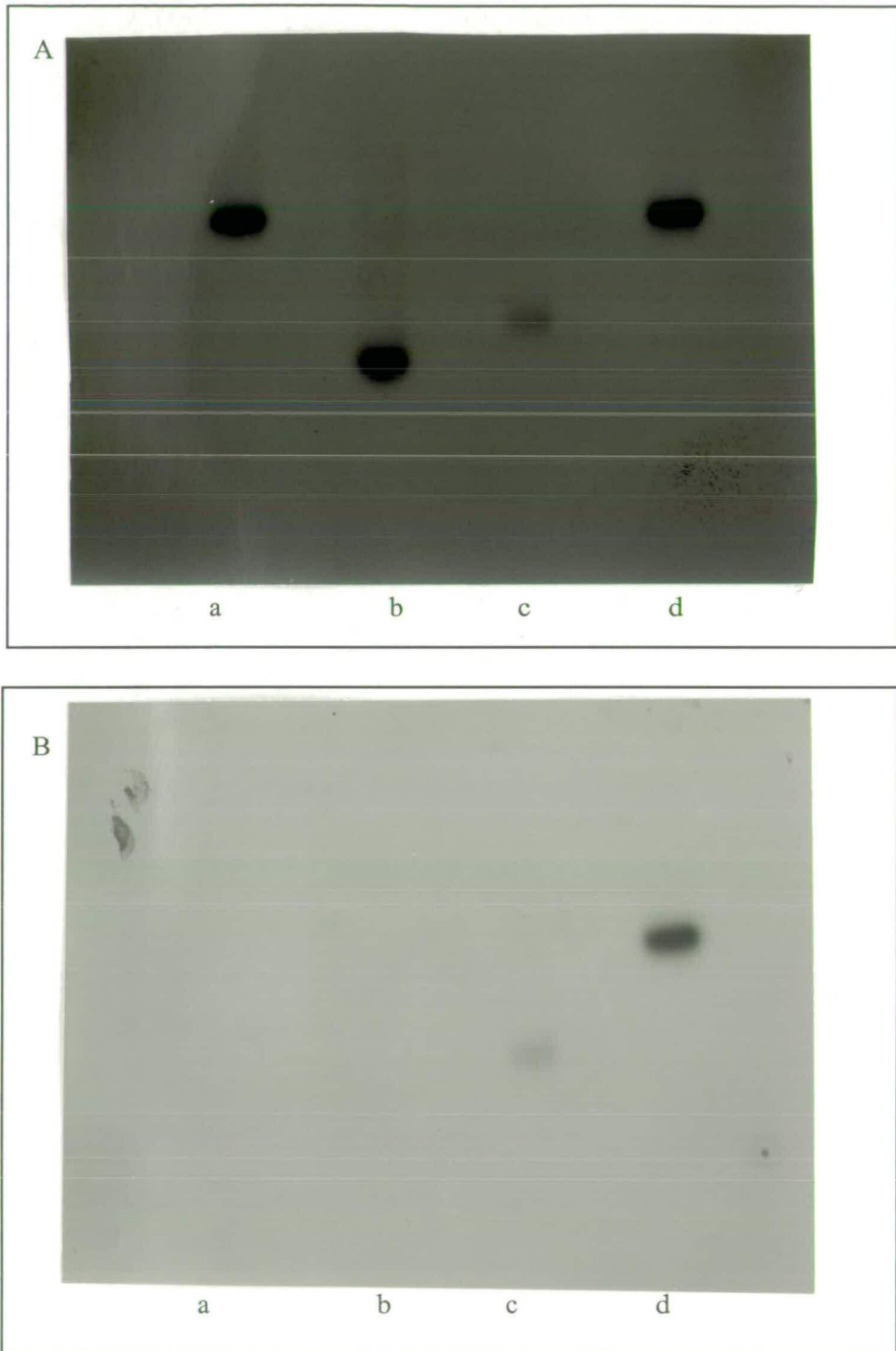
Figure 22. Northern analysis of gel-purified *in vitro* transcribed RNA. Aliquots of gel-purified *in vitro* transcribed RNA were separated on formaldehyde gels (Figure 21), before blotting onto Hybond N membranes. These were then hybridized sequentially with the following oligonucleotide probes: (A) directed against the CAT ORF; (B) directed against sequences just upstream of the main poly A signal. Constructs used for RNA transcription were as follows: lane a: pRAW, lane b: pTCAT, lane c: pRAD and lane d: pRAR.

(4.5) Effect of increasing RNA concentration upon the translational efficiency of CAT.

Increasing amounts of *in vitro* transcribed RNA from the plasmid pTCAT were *in vitro* translated (Figure 23). A dose response curve was obtained using TCAT RNA to assess the effect of an increase in RNA concentration upon the translation of CAT protein. *In vitro* translation of RNA containing the CAT or luciferase protein coding regions the latter a positive control supplied with the *in vitro* translation kit produced the appropriate sized protein bands, 27 and 64 Kda respectively (Figure 23 lanes a-d and lane e respectively) as judged by molecular weight markers. Increasing pTCAT RNA concentration resulted in increased amounts of CAT protein translated and this relationship was linear between 0.2-1.0 µg of RNA (Figure 24 and Appendix Table 7). Higher concentrations of RNA caused a decrease in translational efficiency and so an aliquot of 0.2µg of TCAT RNA and as nearly as possible equal molar concentrations of the other 3 species of RNA (Section 4.6) were used in the following experiments.
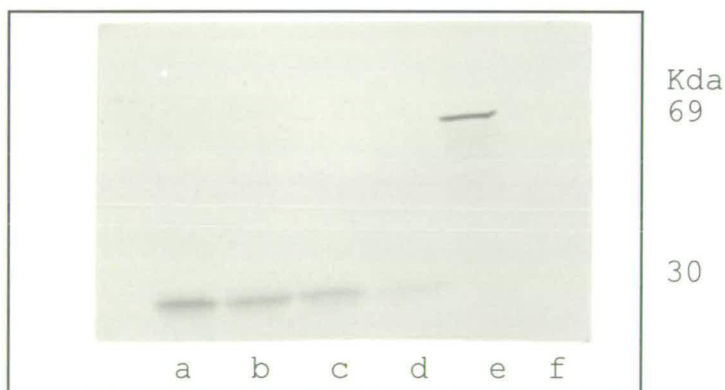
Figure 23. Representative autoradiogram showing the effect of increasing the concentration of *in vitro* transcribed RNA upon *in vitro* translation. After 90 mins at 30°C, the newly systhesized ^{35}S methionine labelled CAT was measured by scanning autoradiograms of SDS gels. Lane a: 2µg of pCAT RNA, b:1µg, c: 0.5µg, d: 0.2µg, e: Positive control with 1µg luciferase RNA and lane f: Negative control no RNA.
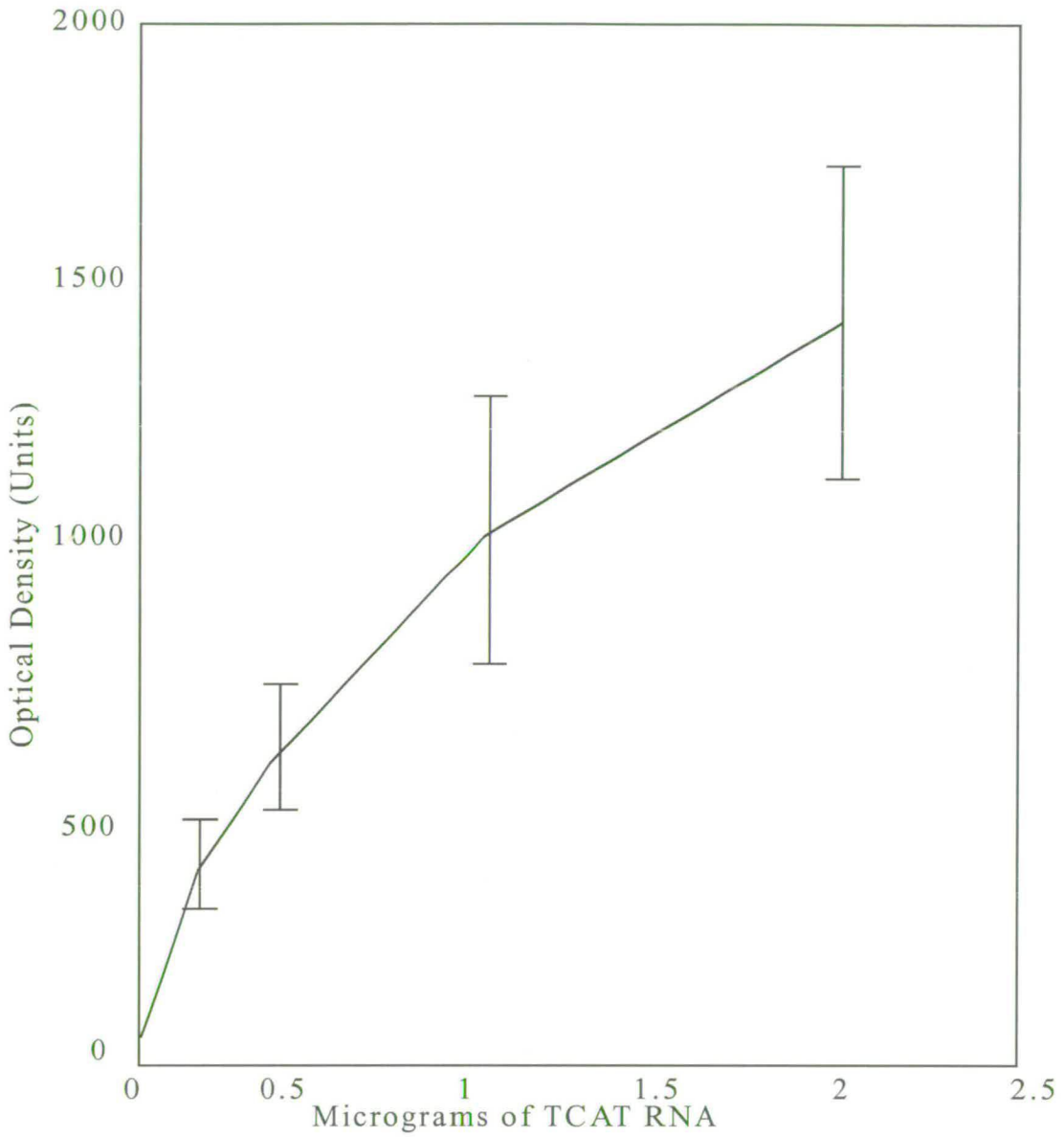
Figure 24. Dose response curve: effect of increasing the concentration of *in vitro* transcribed RNA produced from the construct pTCAT upon *in vitro* translation yield. Error bars represent standard errors from the means of three independent experiments. (See also Appendix Table 7).

(4.6) Relative Molar concentrations of mRNA by slot blot hybridisation

Accurate estimates of the relative molar concentrations of the mRNAs were required in order to make precise comparisons between RNAs of different lengths. Densitometry of autoradiograms from Northern- or slot blotted RNA has been used by others to follow relative (Mallet et al., 1983; Young and Anderson 1985 and Thompson et al., 1986) and absolute (Denman et al.,1991) amounts of mRNA. Hybridization was carried out with an oligonucleotide probe directed against the CAT open reading frame (GTAGCGAGACCTCACTTATG). Relative molar concentrations of full length *in vitro* transcribed RNAs were obtained by scanning an autoradiogram of a hybridised slot blot and dividing by the OD for TCAT:

$$\text{Relative concentration of RNA} = \frac{\text{OD of experimental RNA hybrid}}{\text{OD of TCAT RNA hybrid}}$$

Values were determined by obtaining the means from three experiments. An example of a slot blot autoradiograph is shown in Figure 25 and exact results are given in the Appendix (Table 8)
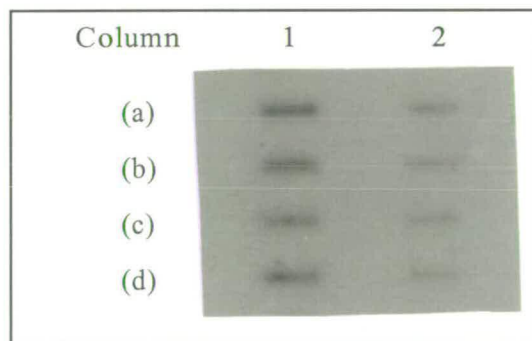
Figure 25. Sslot blot hybridization analysis.
Autoradiogram of a typical slot blot used to assess ralative molar concentrations of CAT mRNA. Lane a: pRAR, b: pRAD, c: pRAW, d: pTCAT. Column 1: Undiluted sample, Column 2: Same as column 1 but samples were diluted by 50% with 1X SSC.

(4.7) The presence of the sheep PrP 3' UTR downstream of the CAT gene does not significantly affect RNA translation in RRL

In vitro produced CAT mRNAs containing different lengths and orientations of the PrP 3'UTR downstream were assessed for their ability to be *in vitro* translated. The translatability of transcripts was measured in arbitrary units by the following equation:

Translatability = $\dfrac{\text{CAT Protein}}{\text{CAT RNA}}$

= $\dfrac{\text{O.D of autoradiographs of }^{35}\text{S labelled CAT protein bands after PAGE (Figure 26)}}{\text{Relative molar concentration of RNA (Section 4.6)}}$

The presence or absence of various lengths and orientations of the sheep PrP 3'UTR, downstream of the CAT ORF, had little or no effect upon the levels of methionine ^{35}S labelled CAT produced *in vitro*, as measured by scanning autoradiograms eg(Figure 26) of SDS gels with a densitometer. The experiment was repeated three times and values for translatability were averaged (see Figure 27 and Appendix, Table 9). pTCAT, with none of the PrP 3' UTR produced a mean value for translatability of 201 whereas the construct with the full PrP 3'UTR in the correct orientation (pRAR) had a translatability value of only 138. The other two constructs, with either a large deletion in the PrP 3' UTR (pRAD) or the whole 3'UTR in reverse orientation (pRAW) had higher translatability values (182 and 166, respectively). Although there was a suggestion of a negative effect on translation of CAT by the intact PrP 3'UTR in pRAR, the effect is not statistically significant (see error bars in Figure 27), as was confirmed by t-test analysis.

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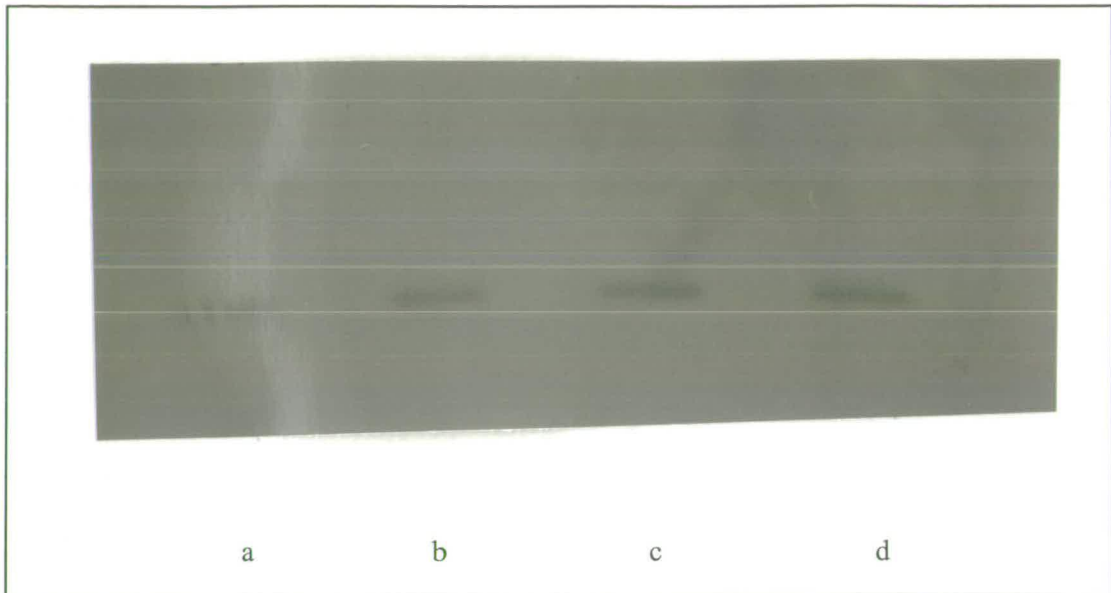
Figure 26. Effect of the PrP 3' UTR upon the *in vitro* translation of CAT. *In vitro* transcribed RNA differing in the 3' UTR length and orientation were compared in their ability to produce CAT protein. After 90 mins at 30°C, the newly synthesized ^{35}S methionine-labelled CAT was measured by scanning autoradiograms of SDS gels. (Constructs used for RNA syn thesis; lane a: pRAW, lane b: pRAD, lane c: pRAR and lane d: pTCAT.)
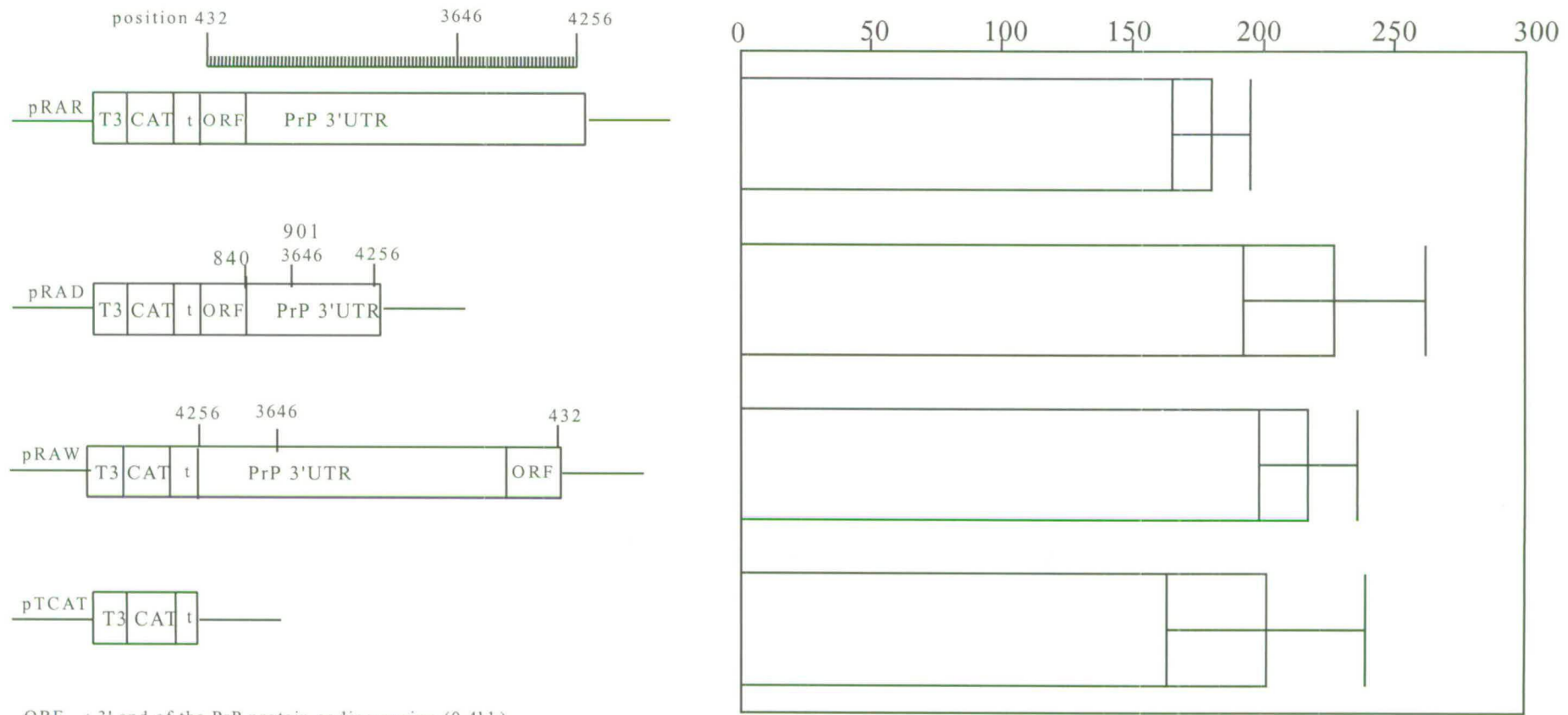
ORF : 3' end of the PrP protein coding region (0.4kb)
t: 316 bp of the SV40 t antigen UTR containing the intron
T3, T7: promoter

Figure 27. Effect of the 3'UTR of sheep PrP upon *in vitro* translation of CAT. *In vitro* transcribed RNA containing the full length of the 3'UTR of sheep PrP in the sense orientation (pRAR) or inverted orientation (pRAW), a 2.7kb deletion derivative of the 3'UTR(pRAD), or no 3'UTR of sheep PrP (pTCAT) were compared in their ability to produce CAT. After 90 mins at 30 C, the newly synthesized [35]S methionine-labelled CAT was measured by scanning autoradiograms of SDS gels. Values from 3 independent experiments have been pooled. Error bars represent standard errors from the mean. (See also Appendix Table 9)

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(4.8) Conclusion

To identify sequences within the 3' UTR of PrP which might affect translation, reporter gene constructs were made containing various sections of the 3' UTR downstream of the CAT ORF (Section 4.3d). Two constructs contained the entire PrP 3' UTR, one in reverse orientation. A third construct contained the 3' UTR with a large deletion. A control construct contained only the CAT gene with no PrP 3' UTR.

In vitro transcription from constructs containing the full length PrP 3' UTR produced full length RNA products (pRAW: 4820; pRAR 4760 nucleotides) in addition to smaller RNA species (approximately 2100 and 800 nucleotides) which are most likely the result of premature termination of RNA synthesis (Figure 20). *In vitro* transcription reactions were fractionated on formaldehyde gels (Section 4.4a) and the expected intact transcript were isolated and purified (Section 4.4b). Northern hybridisation analysis of formaldehyde gel suggested that the constructs all expressed mRNA containing both the CAT sequence and the appropriate 3' UTR (Figure 22A,B).

A dose response curve was performed with TCAT RNA to assess whether an increase in RNA concentration had an effect upon translational efficiency (Section 4.5). The effect of increasing TCAT RNA concentration upon *in vitro* translation was linear between 0.2-1.0 ug of RNA (Figure 24). Evidently the higher concentrations caused a decrease in translational efficiency. These results are compatible with the findings of Polayes (1994) and Krowczynska (1993), who showed that at high concentrations of mRNA the translational efficiency decreased, and attributed this in part to the presence

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of inhibitory contaminants in the mRNA preparations. Purity of a transcript is especially important when it is used in *in vitro* translation systems. Trace amounts of ethanol, phenol, salt or excess cap analogue (used during the synthesis of capped mRNA) can cause a dramatic decrease in translation efficiency.

Molar concentrations of RNA were quantified by scanning autoradiographs of a slot blot (Section 4.6) and checked by spectrophotometry and formaldehyde gel electrophoresis analysis. $0.2\mu g$ samples of TCAT RNA and effectively equal molar concentrations of the other 3 species of RNA were used to assess the effect of the 3'UTR of PrP upon *in vitro* translation.

The results show that the 3'UTR of sheep PrP has no major effect upon the *in vitro* translation of CAT in RRL (Section 4.7). The presence or absence of various lengths and orientations of the sheep PrP 3'UTR, downstream of the CAT ORF, had no significant effect upon the levels of methionine ^{35}S labelled CAT as measured by scanning autoradiogram of SDS gels with a densitometer. Initially it was planned that CAT protein would be quantified by measuring CAT activities, i.e the amount of functional protein. This would give a more accurate assay. However, in practice the results obtained by CAT activity measurements (not shown) were low and unreliable. The reasons for this were not fully understood. The stability of the RNA in the translation reaction was not quantified, but the RNAse inhibitor added to the reactions should efficiently counteract any selective degradation of RNA (Ostareck-Lederer et al., 1994).

At least two plausible explanations can be put forward to explain these results. Either the PrP 3' UTR does not contain sequences that can alter expression at the post-

transcriptional level or additional factors are required which are not present in the RRL system. It had been hoped that the *in vitro* translation studies might provide, in a relatively easy way, results which could be followed up in the more difficult systems utilising intact cells to study gene expression. The next Chapter describes experiments of the latter sort.

Chapter 5 Effect of the sheep PrP 3'UTR upon the expression of CAT in neuroblastoma cells

(5.1) Introduction

Results described in Chapter 4 showed that there were no significant differences in CAT translational efficiency caused by addition of PrP 3' UTR sequences in a cell free translation system. These results were followed up in a system that utilizes intact cells to direct synthesis of CAT mRNA and protein. Thus the principal question addressed was whether the sheep PrP gene 3' UTR can alter expression of a reporter gene in intact neuronal cells of mouse origin.

Delivery of foreign DNA into intact cells allows the study of control of gene expression. The types of analysis can be quite varied, including analysis of cis-acting mediators (promoters and enhancers), trans-acting mediators (eg. transcription factors), mRNA processing and translation, and the development of gene delivery methods. To provide accurate quantitative results, sequences of interest are normally fused to reporter genes eg. CAT, Beta-galactosidase, Luciferase or human growth hormone. Most commercially available reporter constructs are specifically designed to analyse promoter and enhancer regions. As yet there is not an equivalent construct for analysing 3' UTRs and so these have to be made for each project. At least two studies have investigated the potential of non-coding sequences to alter expression of PrP at the translation level in cell lines (Caughey et al., 1988; Scott et al., 1988). Only one of these studies provided satisfactory evidence that the non-coding sequences (from mouse PrP mRNA) could alter expression

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(Caughey et al., 1988). It is not known whether the 5' or the 3' UTR was responsible for this change in translation, nor whether these sequences were sufficient alone to alter expression.

By fusing the PrP 3'UTR to the CAT ORF one can address the question of whether the 3'UTR of sheep PrP alone can alter gene expression by itself. Results may have relevance for understanding variation in translational control in at least three different areas: during disease (Denman et al., 1992), between the two different PrP transcripts (Hunter et al., 1994 ; Horiuchi et al., 1995) and between different tissues (Horiuchi et al., 1995).

(5.2) Experimental Approach

Two sets of experiments were carried out to determine if the 3'UTR of sheep PrP can alter expression of CAT in neuroblastoma (N2a) cells of mouse origin. Reporter gene constructs were made containing various sections of the PrP 3'UTR downstream of the CAT gene and the SV 40 promoter. A positive control construct contained the SV 40 3' UTR.

The first set of experiments ('*In vivo* transfections: A'; Sections 5.3-5.9) tested the idea that deletion of 3'-UTR sequences may result in differences in the translatability of CAT reporter transcripts in N2a cells. The translatability of CAT mRNA was measured by dividing the amount of protein produced by the amount of RNA present. CAT activity and CAT transcripts were quantified from cell lysates that had previously been transfected with chimaeric CAT constructs differing in the PrP 3'UTR. The measurement of CAT is a standard procedure that involves incubating cell lysate with ^{14}C chloramphenicol and acetyl Coenzyme A. The amount of acetylated ^{14}C chloramphenicol is proportional to the amount of CAT protein. The amount of CAT mRNA is measured

by semi-quantatitive RT PCR.

The second set of experiments ("*In vivo* transfections: B," Sections 5.10-5.13) was designed to assess if the PrP 3'UTR can alter expression of CAT either at the transcriptional or post-transcriptional level. Constructs were introduced into N2a cells by calcium phosphate precipitation and transfection efficiency was normalized by co-transfection with the pSVlacZ plasmid.

Routine methods used for "*In vivo* transfections: A and B" are described in Chapter 2. Procedures that are not standard will be discussed in the relevant section.

(5.3) "*In Vivo* Transfections: A." Constructs used in N2a cells to investigate post-transcriptional effects of the PrP 3'UTR.

(a) pEYD

To produce pEYD, a 2806bp deletion (positions 840-3646) was made within the PrP 3'UTR. pEYD was constructed (Figure 28) by digesting the pCAT Control plasmid (Promega) with *Sca* I and isolating a 1781bp fragment containing 309 bp of the ampicillin gene, the entire SV40 promoter and 659bp of the CAT ORF. pRAD (Section 4.3d) was restricted with *Sca* I and a 3228bp fragment isolated and ligated to the 1781bp *Sca* I fragment so that both the CAT and ampicillin genes were repaired, with the introduction of the SV40 promoter immediately upstream of CAT ORF in the proper orientation (plasmid pFA). Removal of the *Bam* HI - *Acc* I fragment from pFA led to the construction of pEYD by removal of all of the PrP ORF and part of the 3' UTR leaving the sequence from position 3646-4256 intact (Figure 28 and 31). Plasmid

construction was confirmed by restriction analysis and partial sequencing.
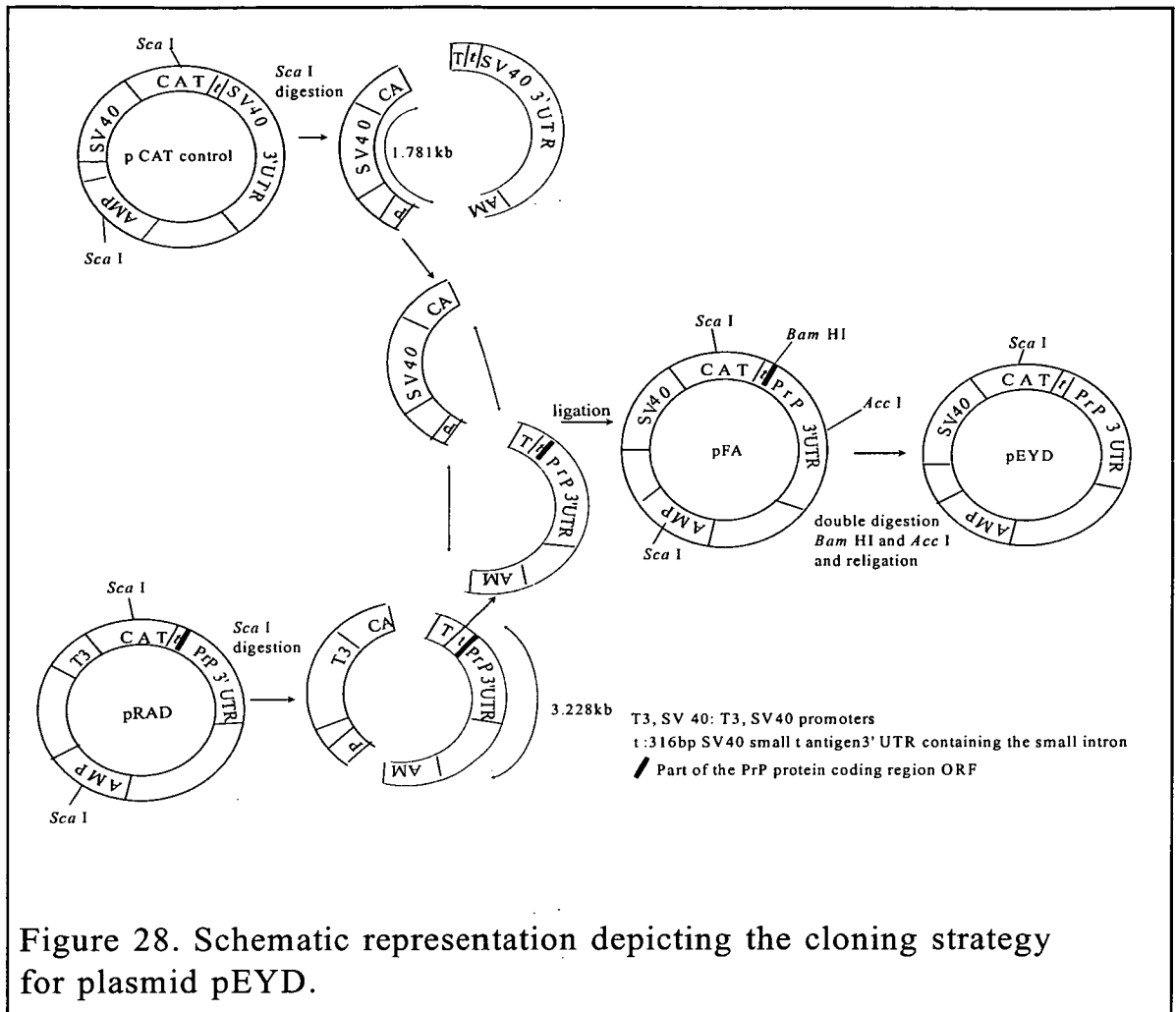
Figure 28. Schematic representation depicting the cloning strategy for plasmid pEYD.

(b) pEYR

pEYR contains the full length 3'UTR of the PrP gene and part of the PrP protein coding region ORF (positions 640-4256). pRAR (4.3b) was digested with *Acc*I and a 2746bp fragment isolated and inserted into the *Acc*I site of pFA. The resultant construct pFR was restricted with *Bam*HI and *Hin*dII the BamHI end filled in, and the product, religated leading to the removal of a 300bp fragment and producing construct pEYR (Figure 29 and Figure 31). Plasmid construction was confirmed by restriction analysis and partial sequencing.

T3, SV40: T3, SV40 Promoters

\ Part of the PrP protein coding region ORF

t: 316bp SV40 small t antigen UTR containing the intron
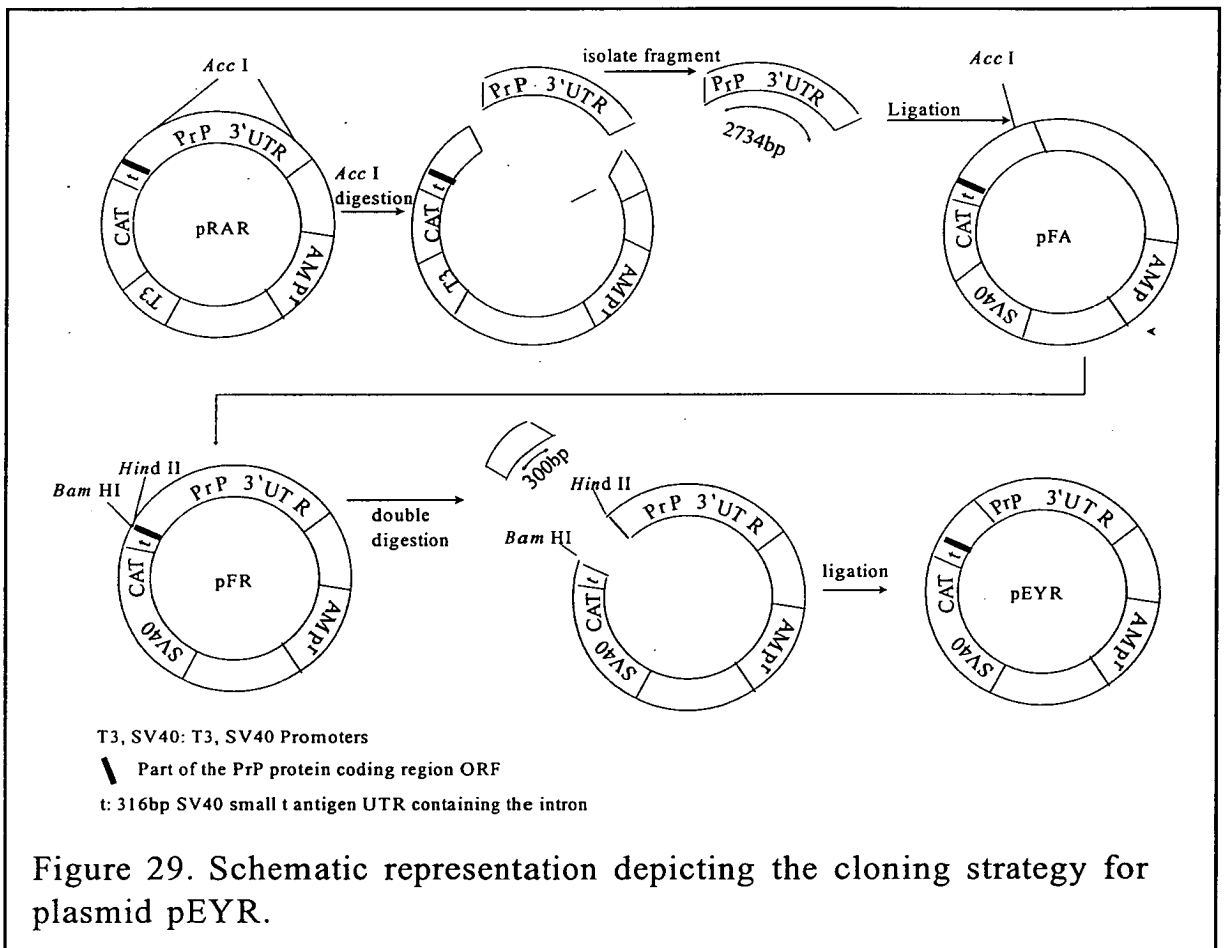
Figure 29. Schematic representation depicting the cloning strategy for plasmid pEYR.

(c) pEYP

To make pEYP a 1090 bp (position 840-1929) deletion was made in the PrP 3'UTR.

pEYP was constructed (Figure 30) by digesting pFR (Figure 29) with *Pst*I and *Bam* HI,

blunting the ends, and religating, leaving position 1930-4256 of the 3' UTR intact

(Summarised in Figure 31). Plasmid construction was confirmed by restriction analysis

and partial sequencing.

Figure 30. Schematic representation depicting the cloning strategy for plasmid pEYP
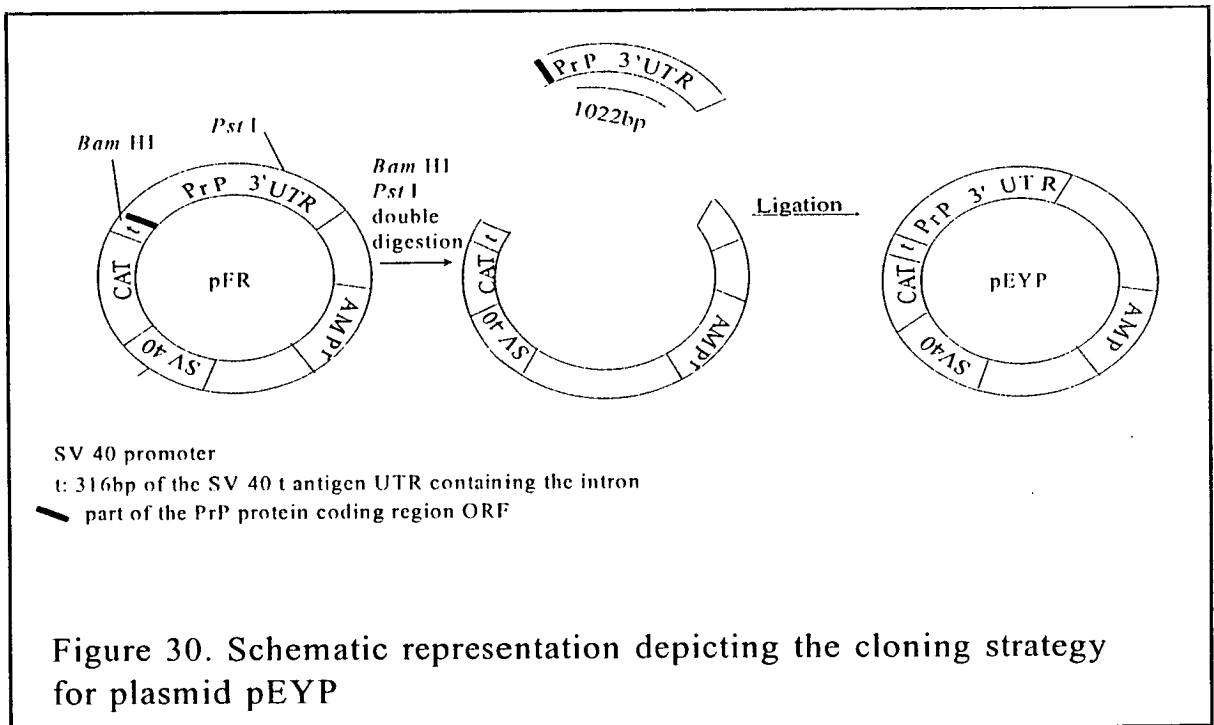
(5.3d)   Summary of constructs

Reporter gene constructs containing various sections of the e1 PrP 3' UTR downstream of the CAT gene are shown in Figure 31. pEYR contains the full length PrP 3'UTR (Section 5.3b) while pEYP (Section 5.3c) and pEYD (Section 5.3a) have 1090bp and 2806bp deletions respectively. pCAT control contains the SV 40 3' UTR.

Figure 31. Summary of constructs used to investigate the PrP 3'UTR.
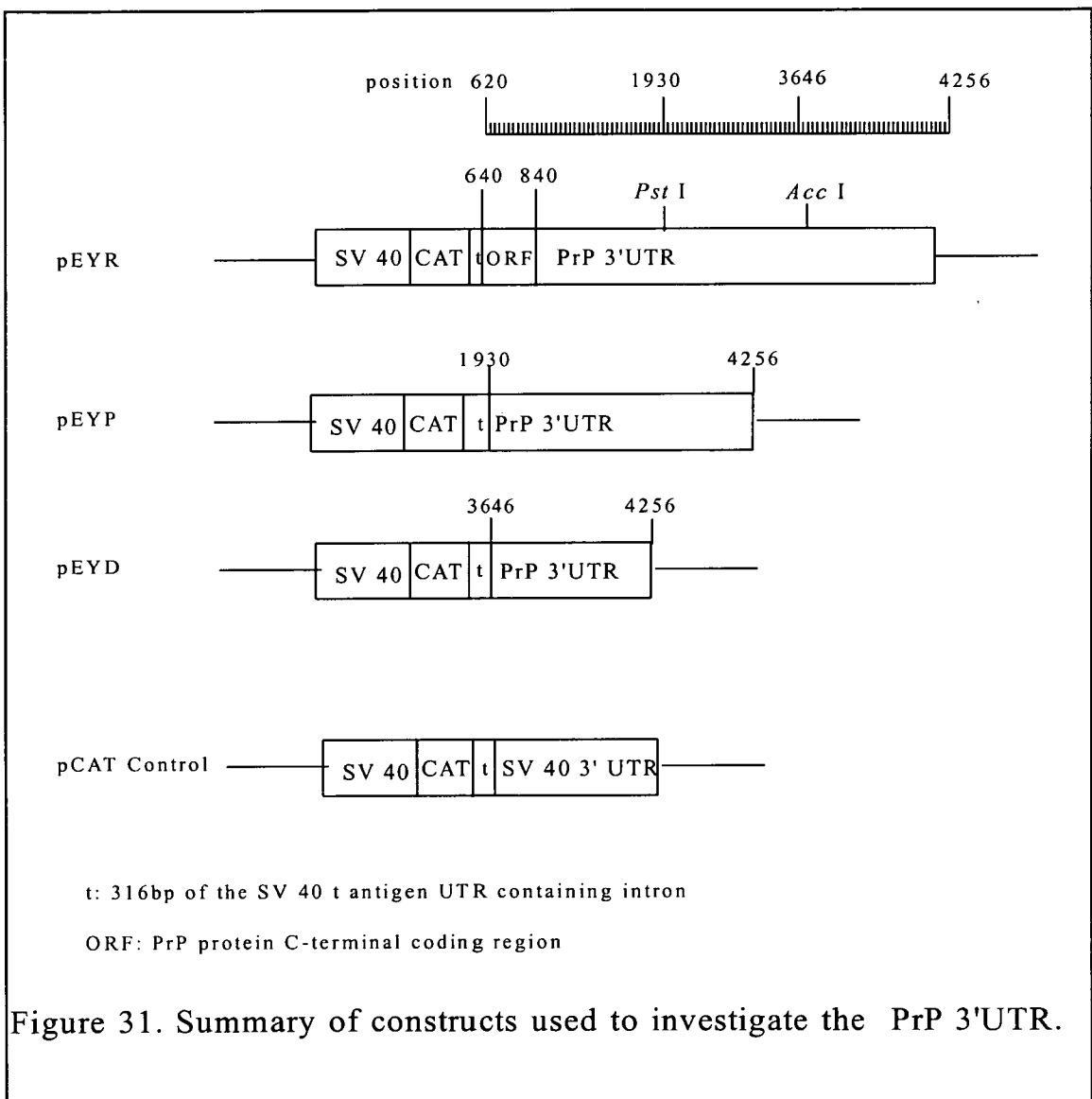
(5.4) Northern Hybridisation of CAT transcripts

Attempts were made by Northern blot hybridization to detect CAT RNA transcripts from cells that had been transfected with chimaeric CAT constructs. Cytoplasmic RNA from one petri dish (100mm) was isolated from cells harvested after 48 hours using the NP-40/urea method (Section 2.33) and subjected to formaldehyde gel electrophoresis to verify the quality and yield of RNA (Figure 31A). However a radiolabelled probe targetted against the ORF of the CAT gene produced no signal on autoradiographs after 1 week (not shown). In addition to absorbance ratios, RNA quality was evaluated by the formaldehyde agarose gel electrophoresis which showed the characteristic 2:1 ethidium bromide staining ratio of 28S to 18S RNA, indicating no significant degradation (Figure 31A).
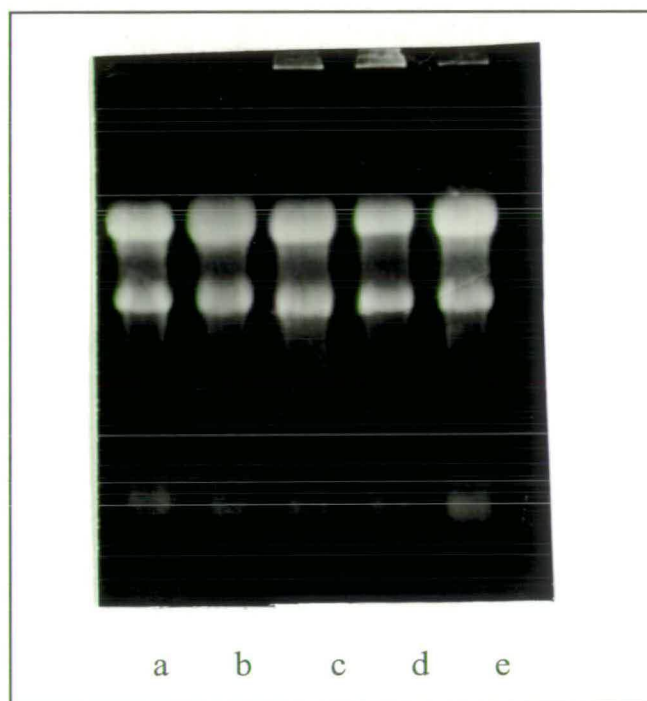
Figure 31A. Formaldehyde gel electrophoresis of total cytoplasmic RNA isolated from N2a cells transfected with chimaeric CAT constructs. N2a cells were transfected by electroporation with chimaeric CAT constructs.
(Lane a: pEYR, Lane b: pEYP, Lane c: pEYD, Lane d: pCAT control, Lane e: mock transfected.)

(5.5.a) Semi Quantatitive RT-PCR

Since the attempts to analyse CAT mRNA by Northern hybridisation were unsuccessful, semi-quantative RT-PCR was used to measure the relative concentrations of CAT mRNA from transfected cells in a method modified from that of Knuchel et al., 1994 (Figure 32, B). The relative abundance of CAT mRNA (target) among the samples can be determined by comparing the ratio of the amount of amplified target to an amplified competitor in each sample. The internal competitor consists of RNA transcribed *in vitro* from the plasmid pDRA I which has a 339bp deletion within the ORF of CAT (Figure 32, A). pDRA I was made by partial digestion of pTCAT (Section 4.3a) with *Dra* I and subsequently religating. Clones with the 339bp deletion (133 bp downstream of the start site of CAT ORF) were found by restriction analysis and double stranded sequencing. During RT-PCR, target and competitor sequences are amplified to a level consistent with their relative abundance at the start of the amplification reaction. To counteract intrinsic differences in the efficiency of reverse transcription and polymerase chain reaction between different samples, the CAT mRNA sequence (target) to be quantified competes for the same reverse transcription primer (tailed anti-sense primer) and PCR primers (Section 2.34a) with the *in vitro* transcribed competitor RNA fragment (Figure 32, C) during RT-PCR.

Before this method could be used, it was necessary to develop and optimise it for use with the RNA of interest. The following Sections describe these necessary steps.
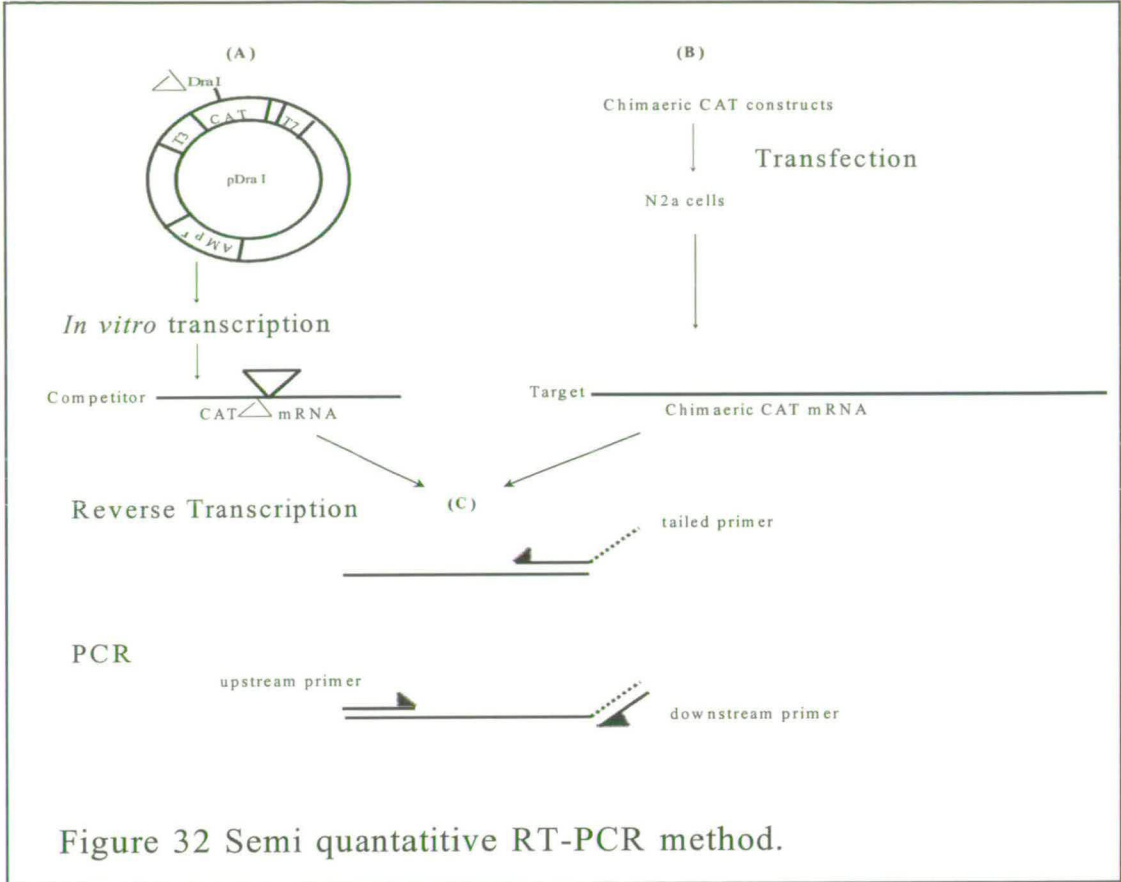
115

Figure 32 Semi quantatitive RT-PCR method.

(5.5.b) Dose reponse: effect of increasing the amount of target RNA upon the semi-quantatitive RT-PCR assay

It was first necessary to optimise the RT-PCR assay. In a dilution series CAT RNA (the target RNA) transcribed from a chimaeric CAT construct in N2a cells was added to a constant amount of competitor RNA transcribed from pDRA I in RT-PCR reactions. The amplification products were analysed by agarose gel electrophoresis and Southern blotting. The relative amounts of target and competitor products in each sample were compared by measuring the optical density.

The molar ratio of target and competitor products were judged from agarose gel electrophoresis and Southern blots to be equal, when between 0.5 and 1μg of target RNA was used in the dilution series (Figure 33, lanes 3 and 4). However the presence of one template in large excess, when the amount of cytoplasmic RNA added was below 0.1μg (Figure 33, lane 2) or above 5μg (Figure 33, lanes 5 and 6), inhibited product formation from the relatively underrepresented template. The positive controls consisted of RT-PCR reactions that were carried out using only RNA from transfected cells (Figure 33, lane 7) or only *in vitro* transcribed competitor RNA (Figure 33, lane 1). These produced one fragment in each case, sized at around 800 bp and 500 bp respectively. The negative control consisted of PCR without the addition of reverse transcriptase (RT) which produced no signal, as expected (Figure 33, lane 8). Therefore in order to quantify CAT mRNA from transfected cells, 1μg of target RNA and 3μl of *in vitro* transcribed competitor standard RNA were used in subsequent RT-PCR assays.
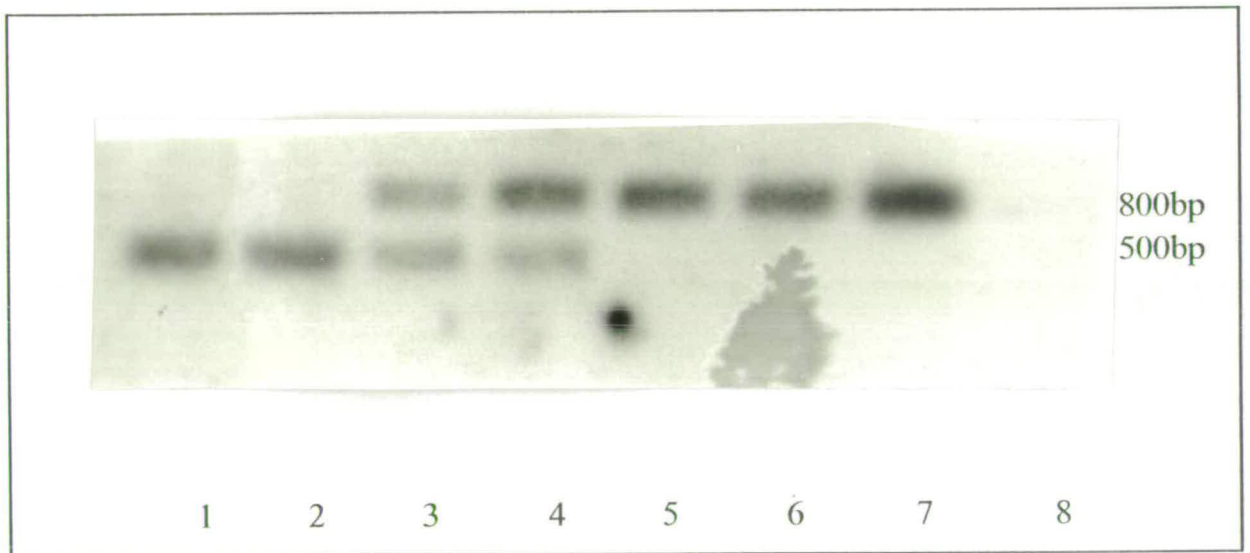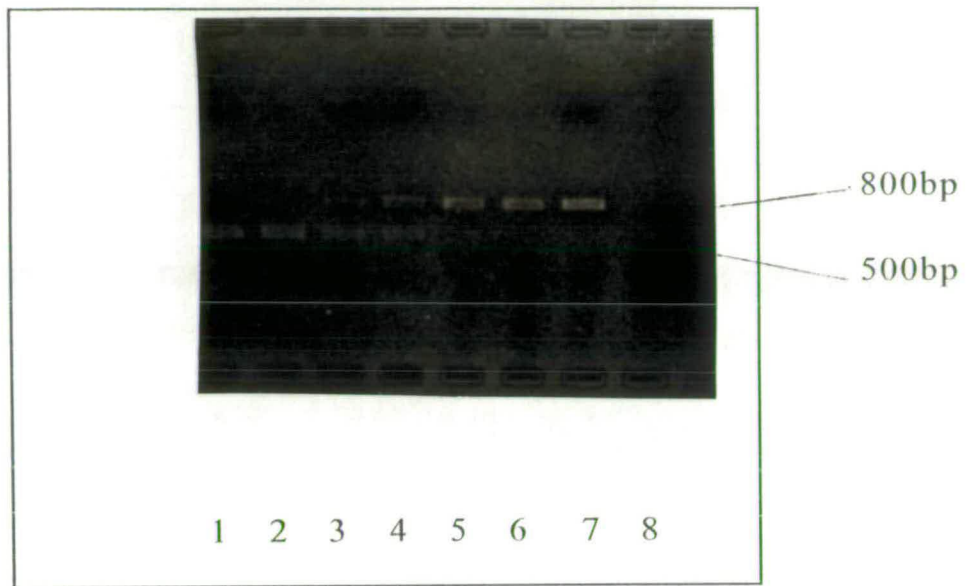
Figure 33. Dose Response Relationship- Effect of increasing target RNA
upon the RT-PCR using a constant amount of competitor.

Increasing amounts of target RNA isolated from transiently transfected cells after 48hrs
and were mixed with a constant amount of competitor (*in vitro* transcribed RNA
containing the ORF of CAT with a internal 339 nucleotide deletion.). RT-PCR was
carried out with Lane 1: 3μl competitor, 0μg target RNA, Lane 2: 3μl competitor, 0.1μg
target RNA, Lane 3: 3ul competitor, 0.5μg target RNA, Lane 4: 3μl competitor, 1.0μg
target RNA, Lane 5: 3μl competitor, 5μg target RNA, Lane 6: 3μl competitor, 7.0μg
target RNA, Lane7: 0μl competitor, 7μg target RNA, Lane 8: 0μl competitor, 7μg target
RNA, no RT.

(5.5.c) Southern blot analysis of PCR DNA fragments

In order to confirm the identity of RT-PCR fragments, samples (1μg) of cytoplasmic

RNA were isolated from the N2a neuroblastoma cells that had been transfected with

chimaeric CAT constructs and were used in semi-quantatitive RT-PCR assays. PCR

products were electrophoresed on agarose gels (Figure 34) and blotted onto a

nitrocellulose membrane. Hybridisation was carried out with two probes. The first probe

was antisense to the mRNA strand, 206 nucleotides downstream of the CAT ORF start

site, and was specific for the target fragment produced from cytoplasmic RNA as the

corresponding region had been deleted from the plasmid pDRA I (competitor). This

probe hybridised only to the circa. 800bp target fragment (Figure 35A). The upstream

PCR primer was used as the second probe and hybridised to both target and competitor

500bp DNA fragments, as expected (Figure 35B). The results demonstrate that target

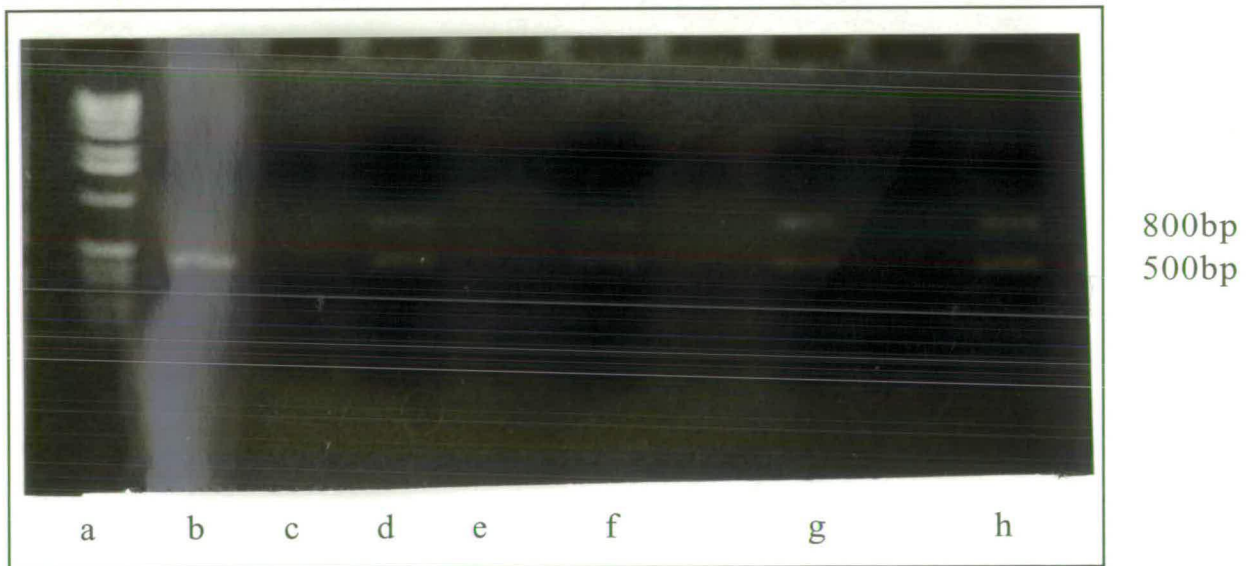and competitor DNA fragments had the correct size and hybridization patterns.

Figure 34. Agarose Gel analysis of PCR fragments. Target RNA prepared from N2a cells transiently transfected with the chimaeric CAT constructs and *in vitro* transcribed competitor RNA were subjected to RT-PCR. Lane (a) 1 kb DNA markers, (b) +ve control: competitor RNA (c) -ve control: mock transfection (d) pEYR (e) as in d, but no reverse transcriptase (f) pEYD (g) pEYP (h) pCAT control. Competitor RNA was also present in reaction (d) through (h).
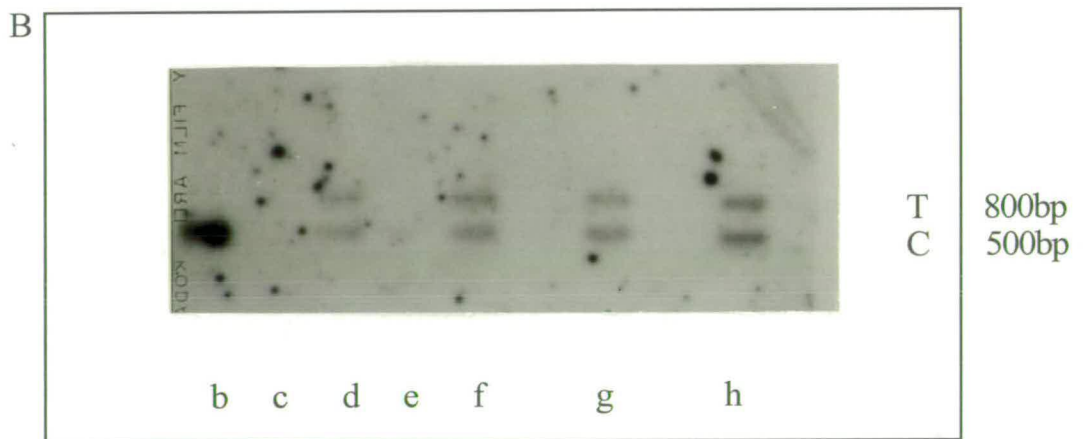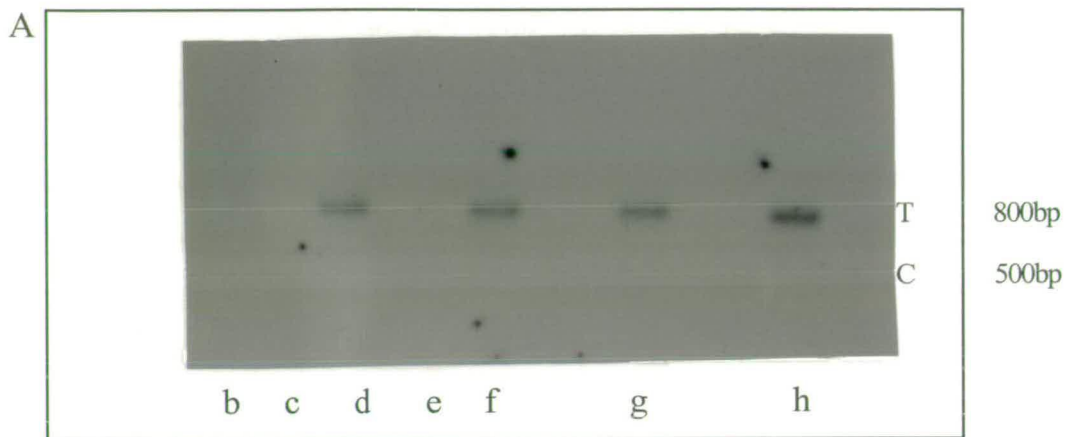
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Figure 35 Southern blot analysis of RT-PCR fragments
Target RNA prepared from N2a cells transiently transfected with the chimeric CAT constructs and *in vitro* transcribed competitor RNA were subjected to RT-PCR. After (Figure 34) gel electrophoresis, and Southern blotting, the DNA fragments were characterised by differential hybridisation: (A) Probe 1: Anti-sense CAT ORF Primer (B) Probe 2: Upstream PCR primer. Lane (b) +ve control: competitor RNA (c) -ve control: mock transfection (d) pEYR (e) as in d but no reverse transcriptase (f) pEYD (g) pEYP (h) pCAT control T: target fragment, C: competitor fragment

(5.5.d) Relative concentration of target mRNA

Quantification of the RT-PCR products was carried out by scanning an autoradiograph from a Southern blot (Figure 36). The ratio obtained between the OD value of the target and competitor gave the relative concentration of CAT mRNA from transfected cells. The upstream PCR primer was used as a probe which hybridised to both target and competitor DNA fragments. From each 100mm petri dish of N2a cell,s 2/3rds, were used in semi-quantatitive RT-PCR analysis and the rest were used in CAT activity assays (Figure 40).

Figure 36. Quantification of CAT mRNA by RT-PCR.
Target RNA prepared from N2a cells transiently transfected with the chimaeric CAT constructs and *in vitro* transcribed competitor RNA were subjected to RT-PCR. After gel electrophoresis, and Southern blotting, the DNA fragments were quantified by scanning autoradiographs. Lane: (a) pCAT control, (b) pEYP, (c) pEYD, (d) As in e but no reverse transcriptase, (e) pEYR, (f) -ve control: mock transfection and (g) +ve control competitor RNA.T: target fragment, C: competitor fragment

(5.6) CAT Assay: Dose Response Curve

A dose response curve was performed in order to assess the linearity of the CAT assay and to find the point at which there is a direct correlation between with the amount of lysate in the assay and the percentage acetylation of ^{14}C chloramphenicol. Lysates from N2a cells transiently transfected with pCAT control were incubated with ^{14}C chloramphenicol and acetyl Co-enzyme A for 20mins. The products were extracted with ethyl acetate and separated by TLC on silica plates using a chloroform: methanol :: 97:3 solvent system.

There are usually 3 radioactive spots visible when the TLC plate is exposed to X ray film and developed. The spot that has migrated the least distance from the origin consists of nonacetylated chloramphenicol partitioned into ethyl acetate (Figure 37, row 1). The two faster- migrating spots are modified forms of chloramphenicol that have been acetylated at either one or the other of the two potential sites (Figure 37, row 2 and 3). To quantitate the % ^{14}C chloroamphenicol acetylation, the acetylated and nonacetylated chloramphenicol products were scraped off TLC plates, the radioactivity measured by liquid scintillation counting and the results calculated.

Using increasing amounts of lysate from transfected cells the linearity of the CAT assay was found to be between 5 and 60 percent acetylation of ^{14}C chloramphenicol (Figure 37 and 38). A representative CAT assay is shown in Figure 37 and detailed results in Appendix Table 10. The dose response curve is shown in Figure 38.
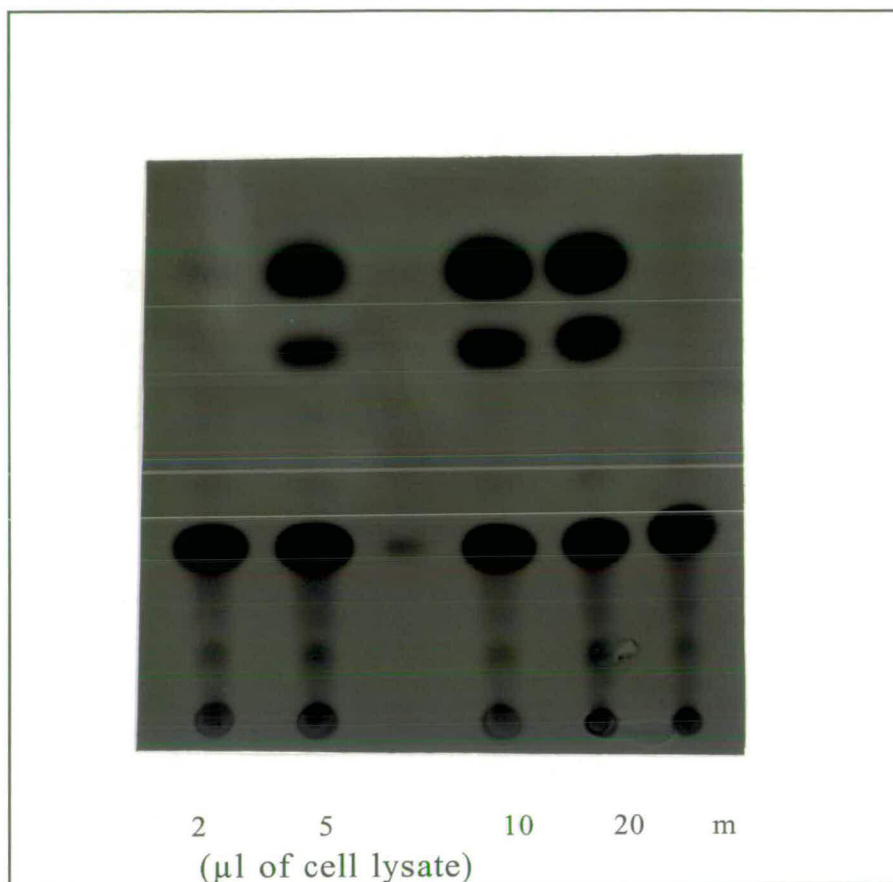
Figure 37. Representative autoradiograph of TLC showing the effect of increasing lysate upon the acetylation of ^{14}C chloramphenicol.

Increasing amounts (2, 5, 10 and 20µl) of cell lysate from cells transiently transfected with the construct pCAT were used to assess the linearity of the CAT assay. Lysate from mock infected cells was used as a negative control. Row 1: Non acetylated, Rows 2 and 3 acetylated, ^{14}C chloroamphenicol.
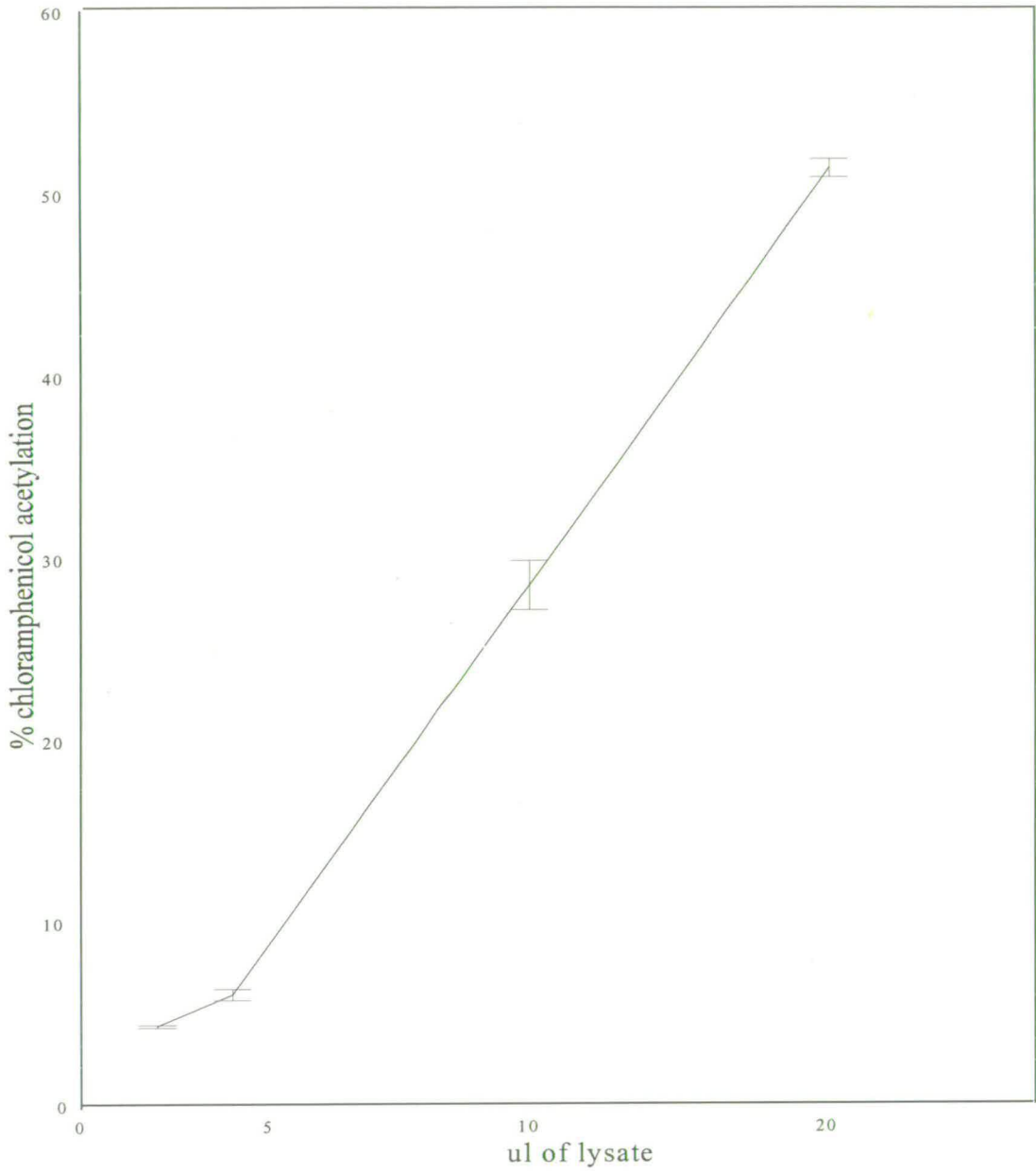
Figure 38. CAT assay: Dose Response Curve. Effect of increasing lysate upon the acetylation of [14]C chloramphenicol. Values were determined from 3 experiments. Error bars represent standard errors from the mean. (See also Appendix Table 10)

(5.7) The Effect of deletions` in the PrP 3' UTR on translational efficiency of CAT transcripts

Cells were transfected with various chimaeric CAT constructs (Figure 31) and harvested 48 hours post transfection. One third of the cells were used in CAT assays and the rest to isolate cytoplasmic RNA for the RT-PCR reaction. The translational efficiencies of the CAT transcripts in N2a cells were assessed by examining the ratio of CAT activity to cytoplasmic CAT mRNA expressed.

$$\text{Translational efficiency} = \frac{\text{CAT protein}}{\text{CAT RNA}}$$

$$\simeq \frac{\% \ ^{14}\text{C chloramphenicol acetylation}}{(\text{OD Target / OD Standard})}$$

Comparisons were made with constructs containing the full length PrP 3'UTR (pEYR, Section 5.3b) or derivatives with a 1090bp deletion (pEYP, 5.3c) or a 2806bp deletion (pEYD, Section 5.3a). CAT activity from lysates that had been transfected with pEYD or pCAT control (Figure 39, b and d respectively) consistently produced higher percentages of ^{14}C Chloramphenicol acetylation than lysates from pEYR and pEYP (lanes c and a respectively). When values for translational efficiency (translatability) were calculated from three experiments, the pCAT control gave a figure of 61 and the full length PrP 3' UTR (pEYR) reduced the translational efficiency to 33.6. Deleting 1090bp from the 5' region of the 3' UTR (pEYP) did not alter this significantly (31.3) but deleting 2806bp (pEYD), leaving only the most distal 600bp of UTR did have an

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effect, as the translational efficiency returned to a levels (67.6) equivalent to the pCAT control (Figures 39, 40 and Appendix Table 11). It may be that the 2734bp deletion from the PrP 3'UTR may have affected the stability of CAT mRNA or translational efficiency and it is not known if these effects are sequence specific or due simply to the shortening of the 3' UTR.
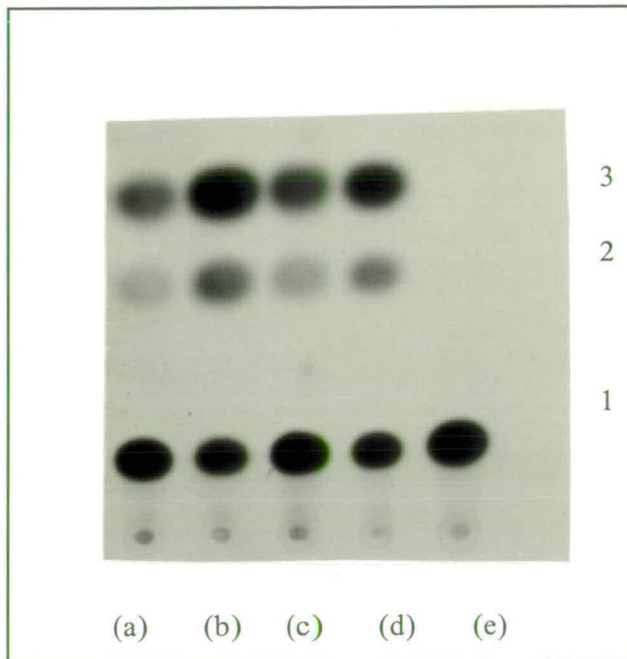

Figure 39. Quantitfication of CAT activity by TLC
Representative results depicting an autoradiogram of a CAT assay performed by TLC. lane a: pEYP, Lane b: pEYD, Lane c: pEYR, Lane d: pCAT control (e) mock transfection. 1: Non-acetylated 2 and 3: acetylated, ^{14}C chloramphenicol
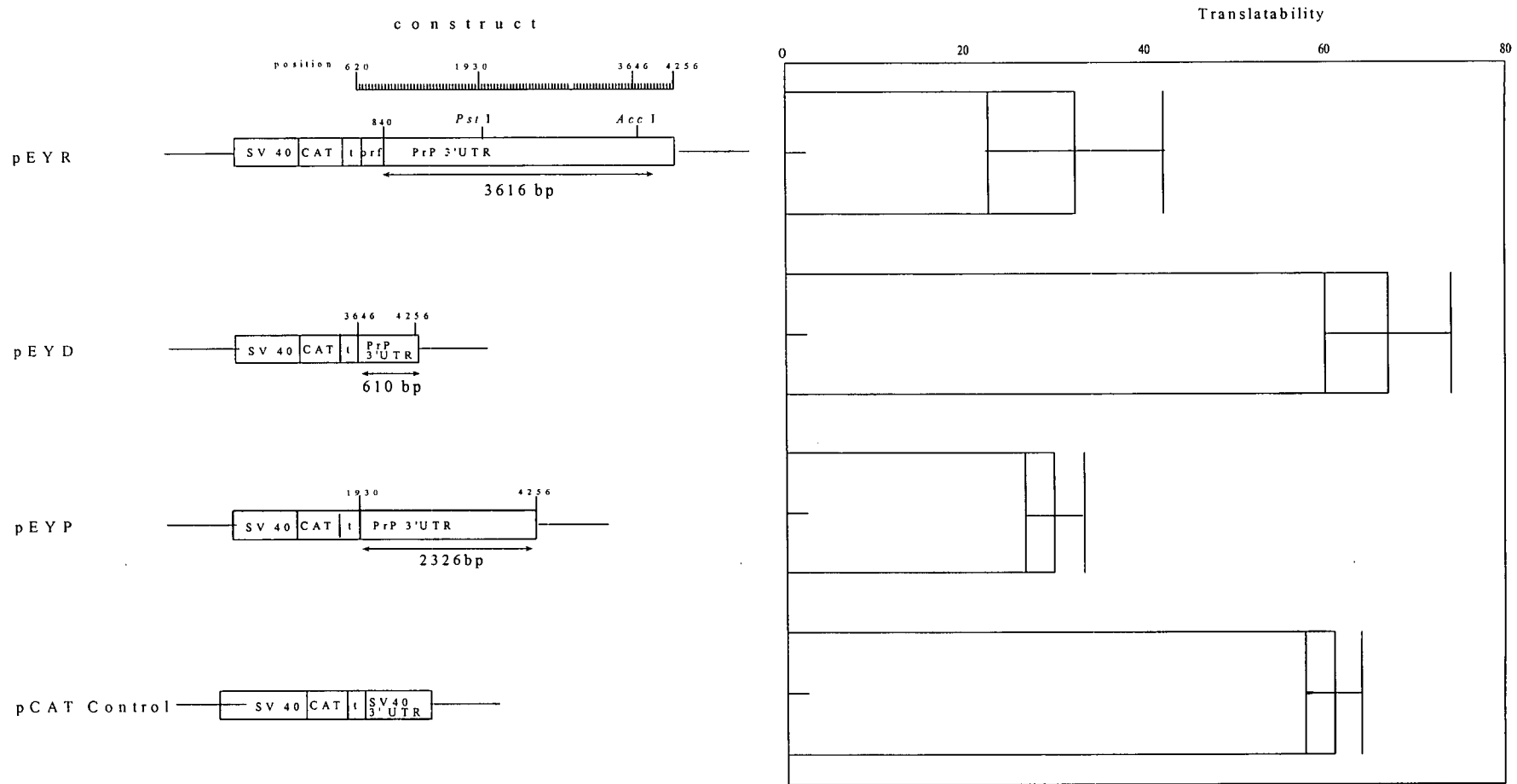
Figure 40. Effect of the 3'UTR of PrP upon the expression of CAT as measured by CAT assay . Equal molar amounts of plasmid DNA were used to transiently transfect N2a cells by electroporation. After 48 hours, the cells were harvested for CAT assays and RNA analysis by semi-quantitative RT-PCR. Values were determined from 3 independent experimental data. Error bars represent standard errors from the mean. (See also Appendix Table 11).

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(5.8) Levels of CAT mRNA in transiently transfected N2a cells

Semi-quantitative RT-PCR analysis did not show any significant differences in CAT mRNA produced by any of the chimaeric CAT constructs during the period between transfection and harvesting of the cells (Figure 41). Target (CAT mRNA) (-dervived) DNA fragments were first detected after 36 hours (Lanes d, h, m, q) and their intensities were unchanged at 48 hours (Lane e, i, n and r). Target fragments could not be detected before or 24 hours after transfection (Lanes b and c, f and g, k and l, o and p). Differences in CAT activity may not be attributed to differences in RNA levels.
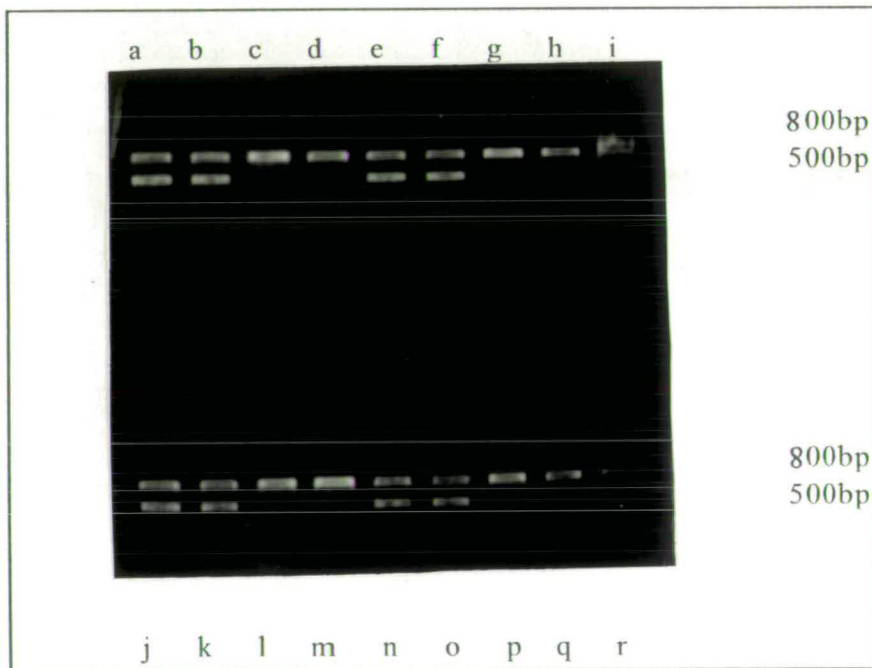

Figure 41: Levels of CAT mRNA
Lanes a, j: 1 kb DNA markers. Semi-quantitative RT-PCR was carried out with cytoplasmic RNA from cells that had been transfected with constructs at 0, 24, 36 or 48 hours earlier. The constructs used were pCAT control (lane b-e), pEYD (lane f-i), pEYR (lane k-n) and pEYP(lane o-r).

(5.9.) Conclusion and Discussion

Results from Chapter 4 using a cell free system suggested that there were no marked differences in translatability of mRNA from a CAT reporter gene attached to various sections of the e1 PrP gene 3'UTR. In this chapter the results were followed up using intact N2a cells to direct synthesis of CAT mRNA and protein from similar reporter gene constructs. To determine if the 3'UTR of sheep PrP could have an effect upon the expression of CAT in this system, three reporter gene constructs were made containing various sections of the PrP 3'UTR downstream of the CAT gene and the SV 40 promoter (Section 5.3). A positive control construct contained the SV 40 3' UTR. By measuring the enzymatic activity of CAT, and CAT mRNA levels, it is possible to determine if sequences within the PrP 3' UTR gene can alter expression. CAT activity was quantified by TLC (Section 5.7) and RNA levels measured by semi quantitative RT-PCR (Section 5.5d).

A dose response curve was made to assess the effects of increasing lysate from transfected cells upon the acetylation of ^{14}C chloramphenicol (Section 5.6). The linearity of the CAT assay was shown to lie between 5 and 60% acetylation of ^{14}C chloramphenicol. Attempts to probe for CAT mRNA by Northern hybridisation were unsuccessful. This confirms personal communications from Promega, in whose laboratories CAT mRNA had previously been shown to be difficult to detect in similar experiments.

A dose response experiment was then carried out to assess the effects of increasing cytoplasmic RNA inputs upon the semi-quantitative RT-PCR analysis of CAT transcripts

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(Section 5.5). When low (0.1 μg) or high (> 5μg) concentrations of cytoplasmic RNA were used, inhibition of product formation from the underrepresented template was seen. When the concentration of RNA was between 0.5μg and 1μg a clear signal was present for both the target and competitor DNA band. This parallels the results from Goswami et al., (1994) where it was shown that when a competitor RNA was used to quantitate target RNA, the presence of one template at a 10-fold to 100 fold higher level almost completely inhibited product formation from the underrepresented template. The translatability of CAT transcripts was assessed by dividing the % ^{14}C chloramphenicol acetylation, determined by TLC, by the optical density value from scanning autoradiographs of Southern blots of RT-PCR products. The data suggest that the sheep PrP gene 3'UTR reduces expression of CAT mRNA at the translational level in N2a cells (Section 5.7). This conclusion is based upon the following observations. CAT mRNA from constructs pEYP and pEYR showed about two-fold lower translatability when compared with pEYD or the pCAT control (Figure 40 and Appendix Table 11). pEYR encodes the full length (3616bp) PrP 3'UTR (840-4256) while pEYP and pEYD encode only the most distal 2326bp (1930-4256) or 610bp (3646-4256) (Figure 31). pCAT control contains the SV 40 3'UTR and was used as a positive control.

Analysis of CAT mRNA over the transfection period (48 hours) showed no significant differences in RNA produced from the different constructs, judged by RT-PCR analysis (Section 5.8). This further confirms that the reduced CAT activity produced by pEYR and pEYP can not be attributed to differences in the level of CAT mRNA.

Two hypotheses can be put forward to explain the restoration of translational efficiency by the deletion in pEYD. Firstly, the increase in translational efficiency may be a

function of specific sequence difference. The nucleotides between positions 1930 and 3646 may therefore be of particular interest, as these sequences are the only differences between the construct pEYP and pEYD. This region of DNA contains three e1/e3 polymorphisms at positions 2613, 2614 and 3440, three possible polyadenylation signals at 2221, 2284 and 2668 and three ATTTA instability motifs at position 1985, 2213, 3636 and is not conserved among other PrP genes. Alternatively, the observed increase in translatability in the shorter transcript may simply be a function of mRNA length: the shorter the mRNA, the more protein is produced.

(5.10) Experimental Approach

In an attempt to address whether the increase in translatability of CAT transcripts was dependent upon mRNA sequence or simply a function of mRNA length, a further series of deletion constructs was made. The new constructs, in addition to the three made previously (Summarised in Figure 30) were introduced into N2a cells by calcium phosphate precipitation. Transfection efficiency was normalized by co-transfection with the pSVlacZ plasmid, and the effects of the PrP 3' UTR upon the expression of CAT were again assessed. In this way it was hoped to validate and to extend the previous findings.

(5.11) Constructs used in the second series of *in vivo* transfections.

The Erase-a-base system was used to generate a nested set of deletions within the 3'UTR of the e1 PrP gene. The system is based on the procedure developed by Henikoff (1984), in which exonuclease III (ExoIII) is used to digest DNA unidirectionally from a 5' protruding or blunt end restriction site. The other DNA end is protected from digestion by using a restriction site which generates a four-base 3' overhang, or by filling a regressed 3' end using alpha phosporothioate nucleotides. The uniform rate of digestion of ExoIII allows deletions of predetermined lengths to be made by simply removing timed aliquots from the reaction to tubes containing S1 nuclease, which removes the remaining single-stranded tails. The low pH and the

presence of zinc cations in the exonuclease and S1 nuclease buffer effectively inhibit

further digestion by Exo III. After neutralisation and heat inactivation of the S1

nuclease, Klenow DNA polymerase is added to blunt the ends, which are then ligated

to circularize the deletion-containing vectors. The ligation mixtures are used directly

to transform competent cells (Method detailed in Section 2.36). Clones containing

unidirectional deletions from position 620 to 3940 were selected by DNA sequencing
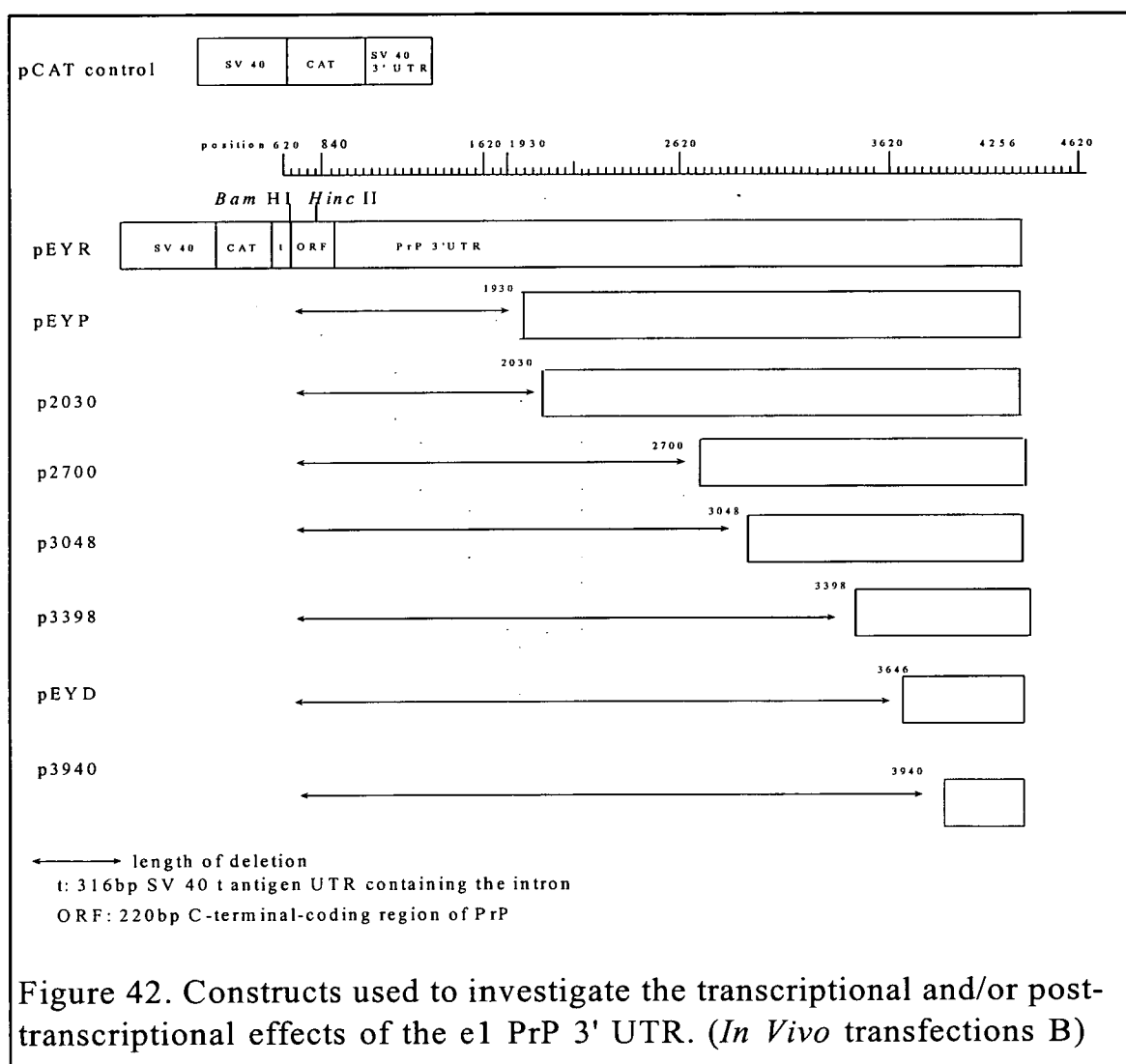
(Figure 42).

Figure 42. Constructs used to investigate the transcriptional and/or post-transcriptional effects of the e1 PrP 3' UTR. (*In Vivo* transfections B)

(5.12) β Galactosidase Assay: Dose Response Curve.

The pSV-β-galactosidase positive control plasmid (pSVlacZ) was used as an internal control for the *In vivo* B transient transfection assays and it was therefore necessary to carry out a dose response study. Lysate from cells transfected with pSVlacZ was incubated with ONPG at 37°C for 20 mins and then the absorbance was measured at 420nm. The linearity of the β Galactosidase Assay was found to be between OD (420nm) 0.14 and 0.73. (Figure 43 and Appendix Table 12)
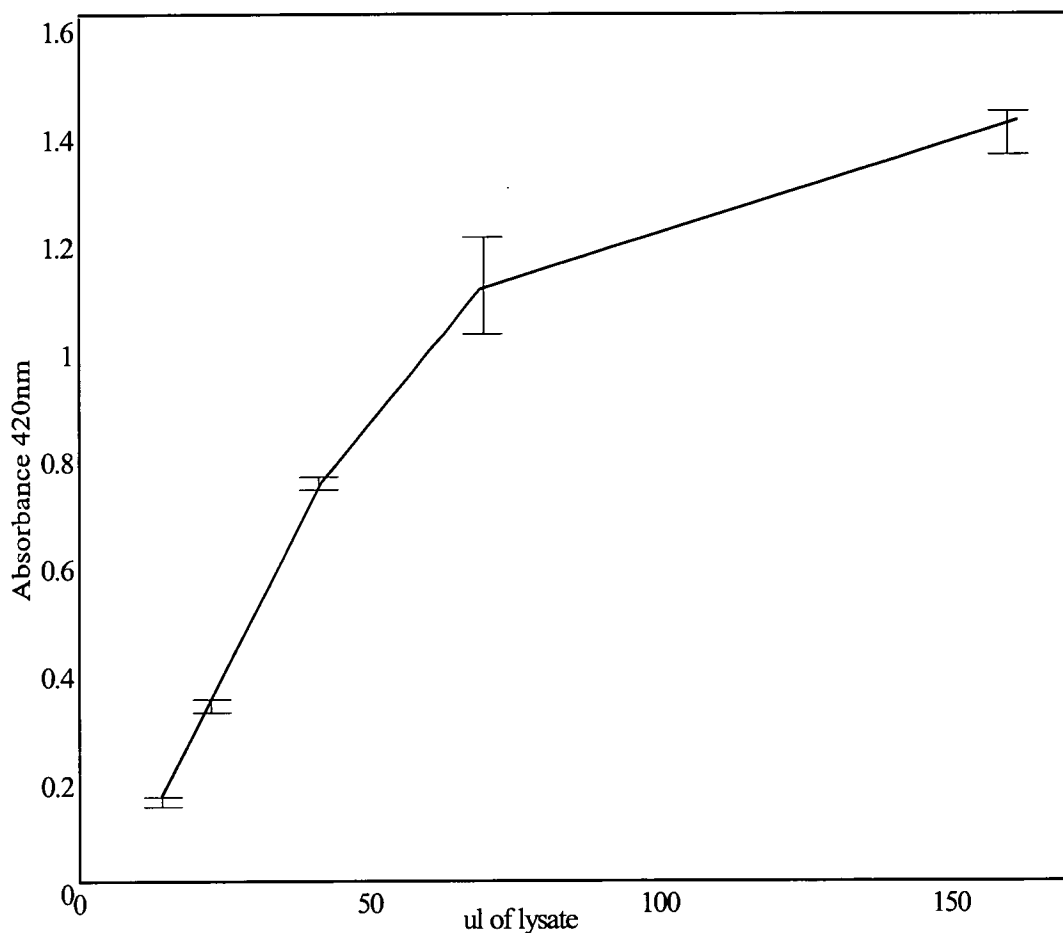

Figure 43. Effect of increasing pSVlacZ lysate upon the hydrolysis of ONPG. Values were determined from 3 independent experiments. Errors bars represent standard errors from the mean. (See Appendix Table 12)

(5.13) Effects of the PrP gene 3' UTR upon the expression of CAT

Constructs with the intact PrP 3' UTR or deletion derivatives lacking bp from 620 up to 2030 (Figure 44 lane f-h) showed low normalized CAT activity, while deletions that extended to position 2700 or beyond showed higher activity (Lanes a-e). The increase in the expression of CAT shown in Figure 45 (and Appendix Table 13) appears to be due to removal of specific sequences between 2030 and 3048, although the less likely possibility that it is simply correlated with shortening of the UTR cannot be excluded. However, extrapolating from the results reported for the pEY plasmids in section 5.8, a post-transcriptional effect(s) seem(s) very likely.
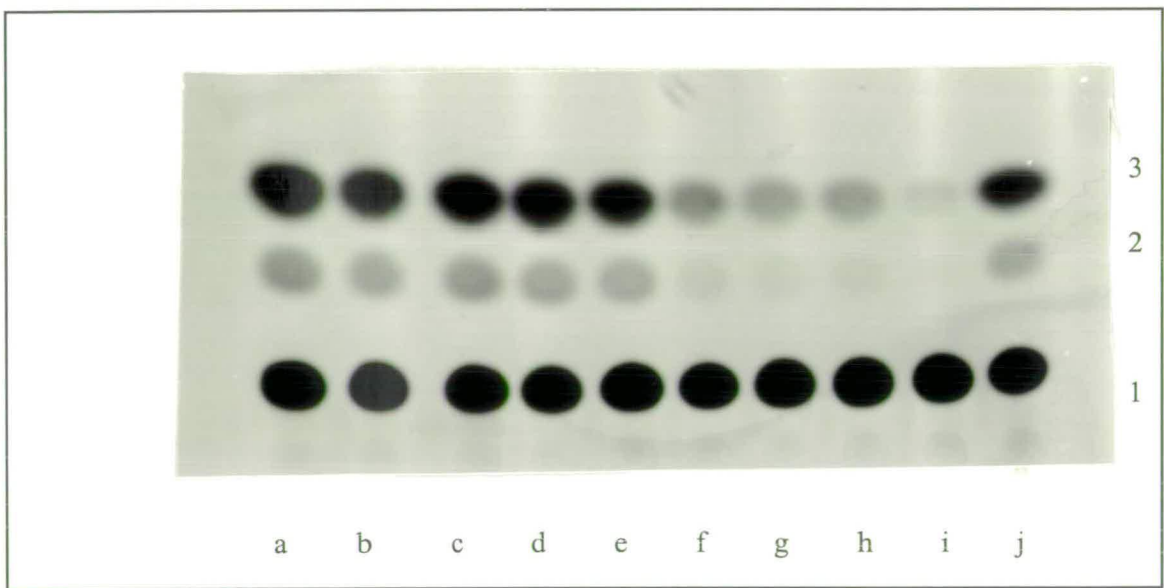
Figure 44. Effects of the PrP gene 3'UTR upon the expression of CAT at the transcriptional and/ or post-transcriptional level. Lysates from N2a cells that had been transfected with chimeric CAT constructs with progressive deletions in the PrP 3' UTR were assessed for their ability to acetylate ^{14}C chloramphenicol. lane a: p3940, lane b: pEYD, lane c: p3398, lane d: p3048, lane e: p2700, lane f: p2030, lane g: pEYP, lane h: pEYR, lane i: mock, lane j: pCAT control.

Figure 46. Effect of the PrP 3' UTR upon the translation of CAT in N2a cells. Lysates of N2a cells that had been transfected with chimaeric CAT constructs containing nested deletions in the PrP 3' UTR were assessed for their ability to acetylate ^{14}C chloramphenical. Experiments were repeated 3 times using different DNA preparations. Normalised by co-transfection with pSVlac Z anad assays for beta galactosidase.
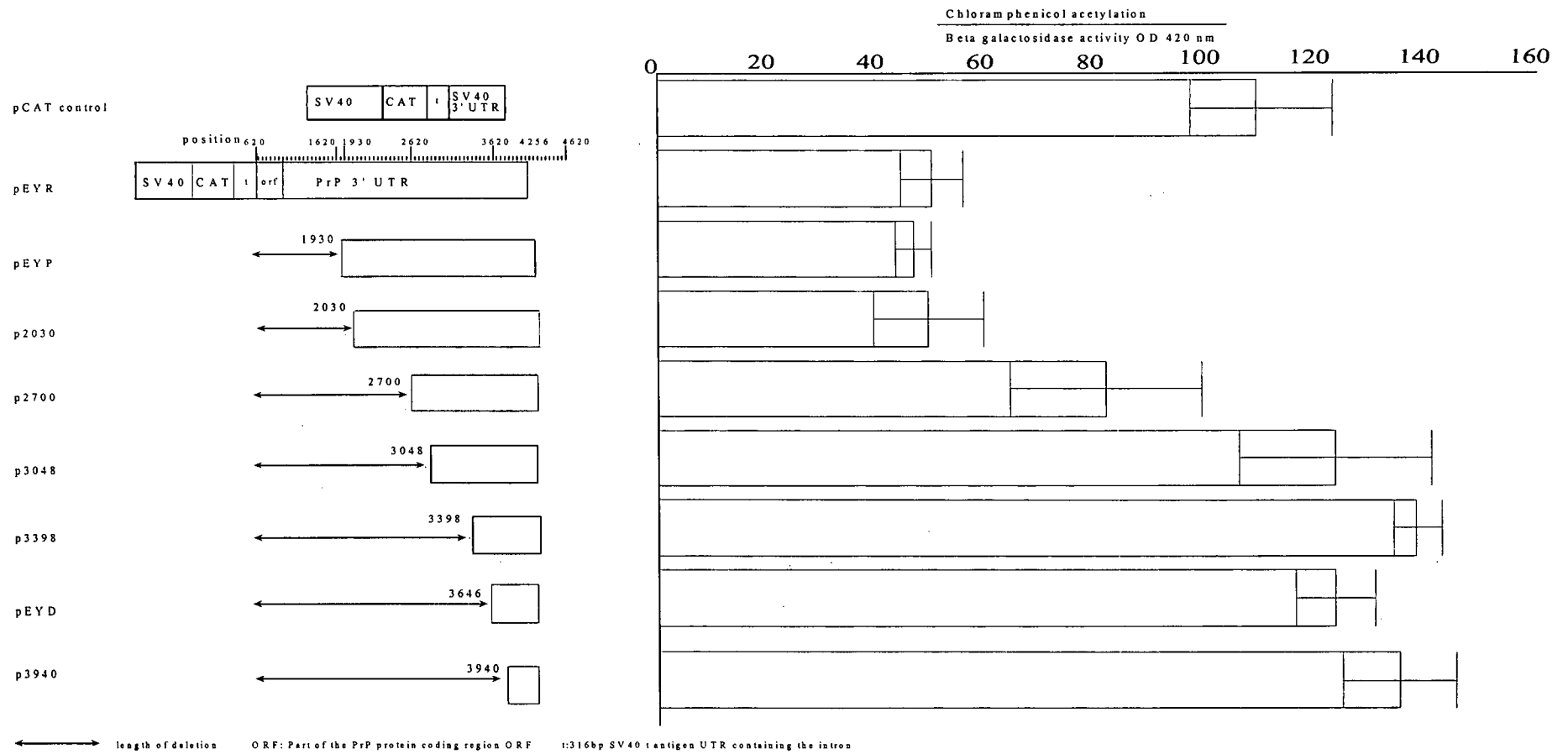
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(5.14) Conclusion and Discussion

Previous results showed that deletion of 2746 bp from the PrP 3' UTR could increase the expression of an upstraem CAT reporter gene (as measured by CAT activity) at the post-transcriptional level compared with that observed in the presence of the full length 3'UTR. Confirmation and extension of this result came from a further deletion series of constructs (Section 5.11). A different method of transfection and normalisation was used in order to rule out any artefacts resulting from the techniques involved. Calcium phosphate precipitation was used to introduce the DNA into N2a cells and co-transfection with the plasmid pSVlacZ was used to normalise transfection efficiency. A dose response curve was performed for β Galactosidase to assess the effects of increasing the amount of lysate from transfected cells upon the hydrolysis of ONPG. The linearity of the assay was shown to be between OD 0.14 and 0.73. (Section 5.12).

It had been found already that CAT mRNA from constructs pEYD and pCAT showed a significant increase in translatability when comparisons were made with pEYP and pEYR (Section 5.9). These results clearly are consistent with the new set (Figure 45). More generally the constructs with an intact PrP 3'UTR or with proximal deletions extending from position 620 to as much as 2030 (Figure 44 lanes f-h) showed low normalized CAT activity while deletions that extended to position 3940 showed higher activity (Lanes a-e).

 increase in the expression of CAT shown in Figure 45 (and Appendix Table 13) appears to be due to removal of specific sequences between 2030 and 3048, although the less likely possibility that it is simply correlated with shortening of the UTR cannot be excluded.

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However, extrapolating from the results reported for the pEY plasmids in section 5.8, a post-transcriptional effect(s) seem(s) very likely.

These results raise the interesting possibility that different lengths of the PrP gene 3' UTR may result in different levels of expression. In sheep brain, PrP protein is encoded primarily by a 4.6 kb mRNA transcript, while in peripheral tissues an additional 2.1 kb mRNA transcript is present. (Hunter et al., 1994; Horiuchi et al., 1995). At the time this work was started, it was thought that the two PrP transcripts differed in the length of the PrP 3' UTR and that an upstream polyadenylation signal might be used to generate the smaller transcript.

If so, allowing for the fact that the 2.1kb mRNA would include a significant length of poly (A), it is clear that it would not incorporate the inhibitory sequences (or unspecific length effect) discovered in Chapter 5. The following chapter will explore the polyadenylation signal used in generating an alternative polyadenylated PrP transcript.

Chapter 6 Investigations into the potential alternative polyadenylation signal at position 1522.

(6.1) Introduction

In sheep brain, PrP protein is encoded by a 4.6 kb mRNA transcript, while in peripheral tissues an additional mRNA transcript of apparent size 2.1 kb is present (Hunter et al., 1994; Hoiruchi et al., 1995). The origin of the smaller transcript was not certain known at the start of these studies but oligonucleotide hybridisation experiments suggested that it originated from alternative polyadenylation (Hunter et al., 1994). In Chapter 3 four potential polyadenylation signals were shown to be in both the e1 and e3 alleles present within the sequence of the long 3'UTR, Chapter 3, at positions 1522, 2221, 2284 and 2668 (Table 5). In this chapter the ability of the potential polyadenylation (poly A) signal at position 1522 to function in 3' end processing was investigated. Of the potential poly A signals recognised, only that at 1522 would be likely to produce a polyadenylated mRNA of circa 2.1 kb.

In eukaryotic cells, most mRNAs are post-transcriptionally modified, by, for example, 5'capping, intron splicing and 3'-end processing. The 3'-end processing involves cleavage of an mRNA precursor (pre-mRNA) and the addition of 200 -300 adenosine nucleotides at the cleavage site (polyadenylation) (review Wickens, 1990). All eukaryotic mRNAs, with the exception of histone mRNAs, have a poly (A) tail (review Wickens, 1990).

In eukaryotes mRNA processing occurs in the nucleus immediately after transcription and polyadenylation, or 3' end processing of mRNAs is absolutely essential for gene expression. mRNAs that are not correctly polyadenylated are unable to be correctly

expressed. This leads, for instance, to a number of diseases including leukaemias, anaemias and thalassaemias (Review: Cooper, 1993). In addition, polyadenylation can function as a mechanism for the control of gene expression; for example, the switch in expression of immunoglobulins from membrane-bound proteins to secreted proteins in B-cells of the immune system is controlled by a change in the site of polyadenylation (Peterson, 1994).

In mammalian genes, two well defined cis-acting sequences present in the 3' UTR are important for 3'-end processing (figure 47). The highly conserved polyadenylation (poly A) signals AAUAAA or AUUAAA are present in 85% and 14% (respectively), of all vertebrate mRNAs approximately 10 to 30 nt upstream of the polyadenylation site (Wilusz et al., 1989; Sheets et al., 1990). The second cis-acting sequence is less well conserved and consists of either G + U- or U- rich sequences approximately 1 to 30 nt downstream of the poly (A) site (McLauchlan et al., 1985; Gil and Proudfoot, 1987). Such sequences are present downstream of the potential polyA signal at 1522 in the sheep PrP 3'UTR, as well as the major signal at 4064 (Figure 15). A third cis- acting element, an upstream enhancer, has recently been found 3' to the poly (A) signal and is thought to increase poly (A) site efficiency (Moreira et al., 1995). The two key components responsible for the recognition of the pre-mRNA substrate are the cleavage and polyadenylation specificity factor (CPSF) (Wahle and Keller 1992) and the cleavage stimulation factor (CstF) (Gilmartin and Nevins 1989; Takagaki et al., 1990). The 3'-processing complexes that form around the poly (A) sites of pre-mRNAs also contain the cleavage factors (CFI and CFII) that carry out the endonucleolytic cleavage step (Figure 47). Surprisingly, poly (A) polymerase is also required for the cleavage of

most pre-mRNAs. Once cleavage has occurred, polyadenylation ensues in a closely coupled fashion. CPSF and poly (A) polymerase remain bound to the upstream cleavage product and carry out the next step, the addition of a short tract of poly (A) of approximately ten adenosine residues (Bienroth et al., 1993). The polyadenylation complex is then joined by an additional component, the poly (A) binding protein II (PABII) which binds to the oligo (A) tail (Wahle and Keller 1992). This causes a rapid burst of processive synthesis of a poly (A) tail of approximately 250nt.
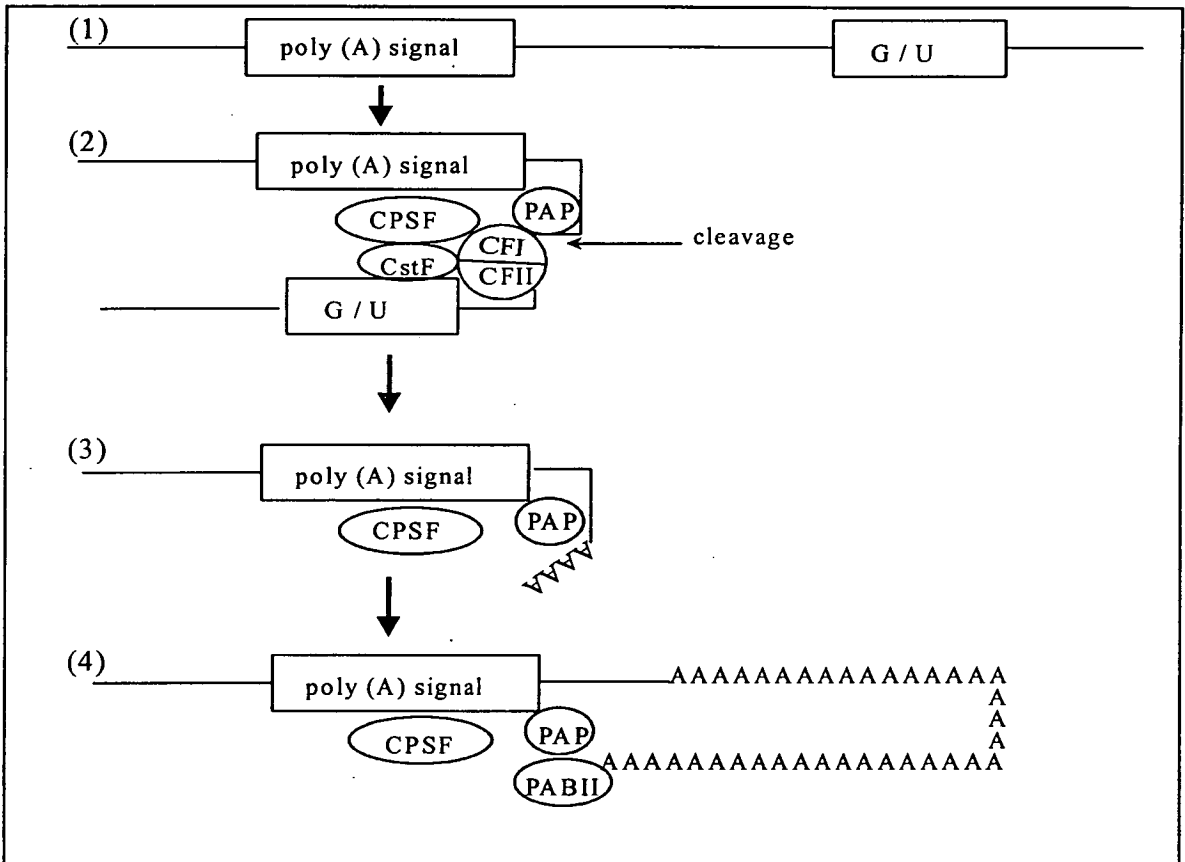
Figure 47. Schematic representation of the steps involved in the mammalian pre-mRNA 3' end processing reaction.

(1)Representation of the polyadenylation signal and G+U rich region at the 3' end of a primary transcript. (2)Complex of proteins that associate with polyadenylation signals to mediate cleavage and polyadenylation. (3) poly (A) polymerase (PAP) adding an oligo (A) tail to the cleaved mRNA. (4)Rapid burst of processive systhesis of a poly (A) tail caused by the binding of PABII to the oligo (A) tail (Terms defined in text).

(6.2) Experimental approach

Previous studies (oligonucleotide hybridisation) had suggested that the 2.1kb mRNA contained the protein coding region in full and was shorter than the 4.6kb mRNA simply because the 3' UTR was shorter. Probes directed against the extreme 3' end of the 4.6kb 3' UTR did not hybridise to the 2.1kb mRNA (Hunter, unpublished) and sequencing data suggested that an upstream poly A signal was used to ge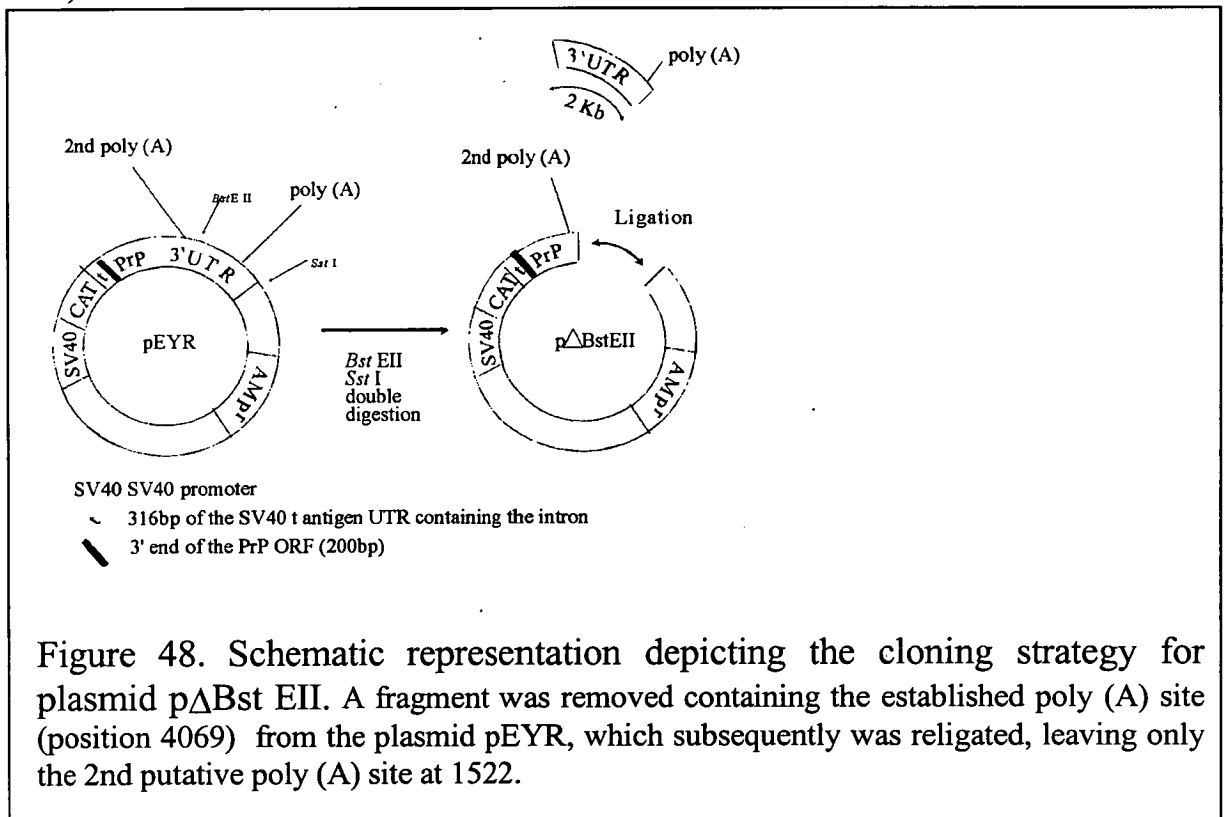nerate the 2.1kb mRNA. An attempt had been made to identify which upstream signal was used and one candidate at position 1253 (TATAAA whose sequence is indeed not classic for a poly A signal) had been discounted by the use of S1 mapping experiments (Goldmann et al., unpublished). However, the sequence at position 1523 (ATTAAA) is the second most common signal used in polyadenylation, and was investigated in this chapter in parallel with other studies using RT-PCR and sequencing (Goldmann et al., unpublished).

The ability of the position 1522 signal to direct polyadenylation in N2a cells was tested. The experimental approach involved introducing into N2a cells chimaeric CAT constructs containing the potential poly A signal for 3' end processing, with additional contructs which carried general or specific deletions affecting the putative poly (A) signal (Section 6.3). It was reasoned that if the poly A signal is functional in 3' processing, then transcripts of the correct size should be produced and site directed mutations should abolish 3' end processing of transcripts originating from this region (Section 6.4).

(6.3) Construction of plasmids

(a) pΔBstEII

pΔBstEII contains the 5' end of the PrP gene 3' UTR and terminates at position 1761. pΔBst EII was made by restriction and removal of a 2000 nucleotide *Sst* I *-Bst* EII fragment from the plasmid pEYR (Section 5.3b). The DNA ends were made blunt ended with Klenow fragment polymerase and religated. This construct does not contain the polyadenylation signal used to generate the full length 4.6kb PrP transcript (position 4064), but does contain the potential alternative signal at position 1522 (Figures 48 and 53).

SV40 SV40 promoter
  316bp of the SV40 t antigen UTR containing the intron
  3' end of the PrP ORF (200bp)

Figure 48. Schematic representation depicting the cloning strategy for plasmid pΔBst EII. A fragment was removed containing the established poly (A) site (position 4069) from the plasmid pEYR, which subsequently was religated, leaving only the 2nd putative poly (A) site at 1522.

(b) pΔKpnI

pΔKpnI contains the 5' end of the PrP gene 3'UTR and terminates at position 1519. This construct was made by restriction and removal of a 2400 nucleotide *Kpn* I fragment from the plasmid pEYR (Section 5.3b). This construct does not contain the polyadenylation signal used in the 4.6 kb PrP transcript (position 4071),n or the potential alternative signal at position 1522 (Figures 49 and 53).

SV40 SV40 promoter
ᴗ 316bp of the SV40 t antigen UTR containing the intron
◥ 3' end of the PrP ORF (200bp)

Figure 49. Schematic representation depicting the cloning strategy for plasmid pΔKpn I. A fragment was removed containing the established poly (A) site (4064) and a 2nd potential site (1522) from the plasmid pEYR.
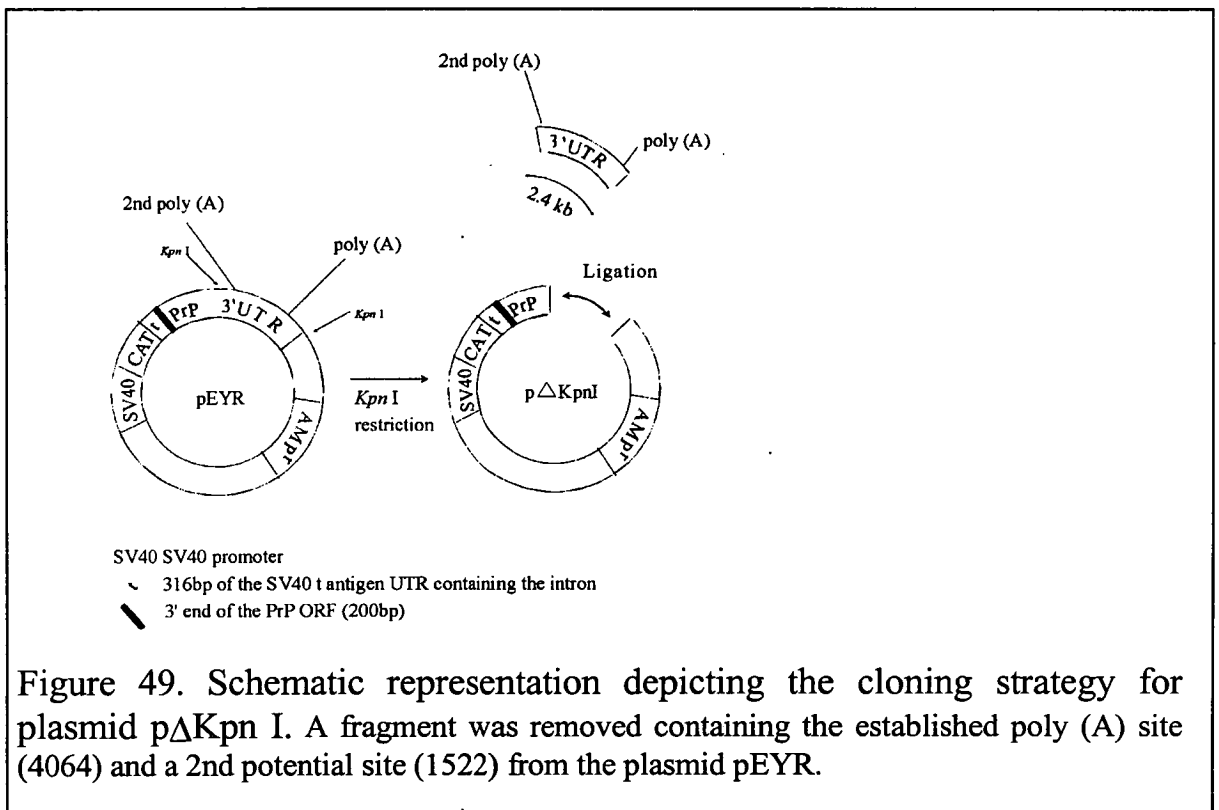
(c) pSVTC

pSVTC contains no 3' UTR. pSVTC was constructed (Figures 50 and 53) by digesting

pCAT Control plasmid (Promega) with *Sca* I and isolating a 1781bp fragment insert

containing 309 bp of the ampicillin gene, the entire SV40 promoter and 659bp of the CAT

ORF. pTCAT (Section 4.3) was restricted with *Sca* I and a 2228bp fragment was isolated.

The 1781bp and 2228bp fragments were ligated so that both the CAT and ampicillin genes

were repaired with the introduction of the SV40 promoter upstream of CAT in the proper

orientation. The construct was confirmed by restriction analysing and sequencing.

SV40, T3 : SV40, T3 promoter
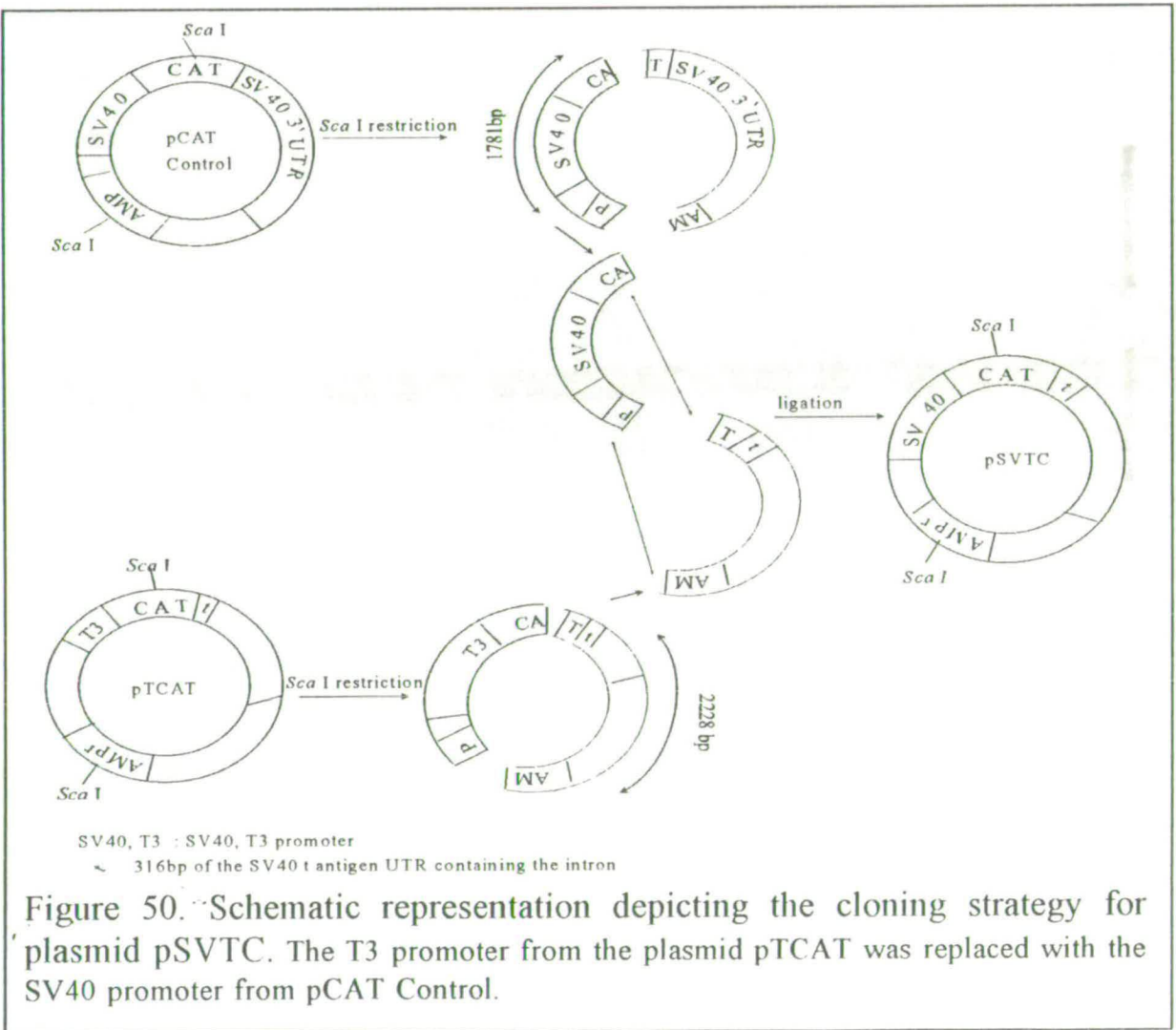316bp of the SV40 t antigen UTR containing the intron

Figure 50. Schematic representation depicting the cloning strategy for plasmid pSVTC. The T3 promoter from the plasmid pTCAT was replaced with the SV40 promoter from pCAT Control.

(d) pΔ2, pΔ3 and pΔ5

pΔ2, pΔ3 and pΔ5 contains 2, 3 and 5 bp deletions respectively, within the potential polyadenylation signal at position 1522 (Figures 51 and 53). The signal at position 1522 from plasmid pΔBstEII was mutated by *Pac*I restriction, treatment with T4 DNA polymerase and Klenow and subsequently religated. Clones containing mutations within the hexanucleotide signal were identified by double stranded DNA sequencing (Figure 52).
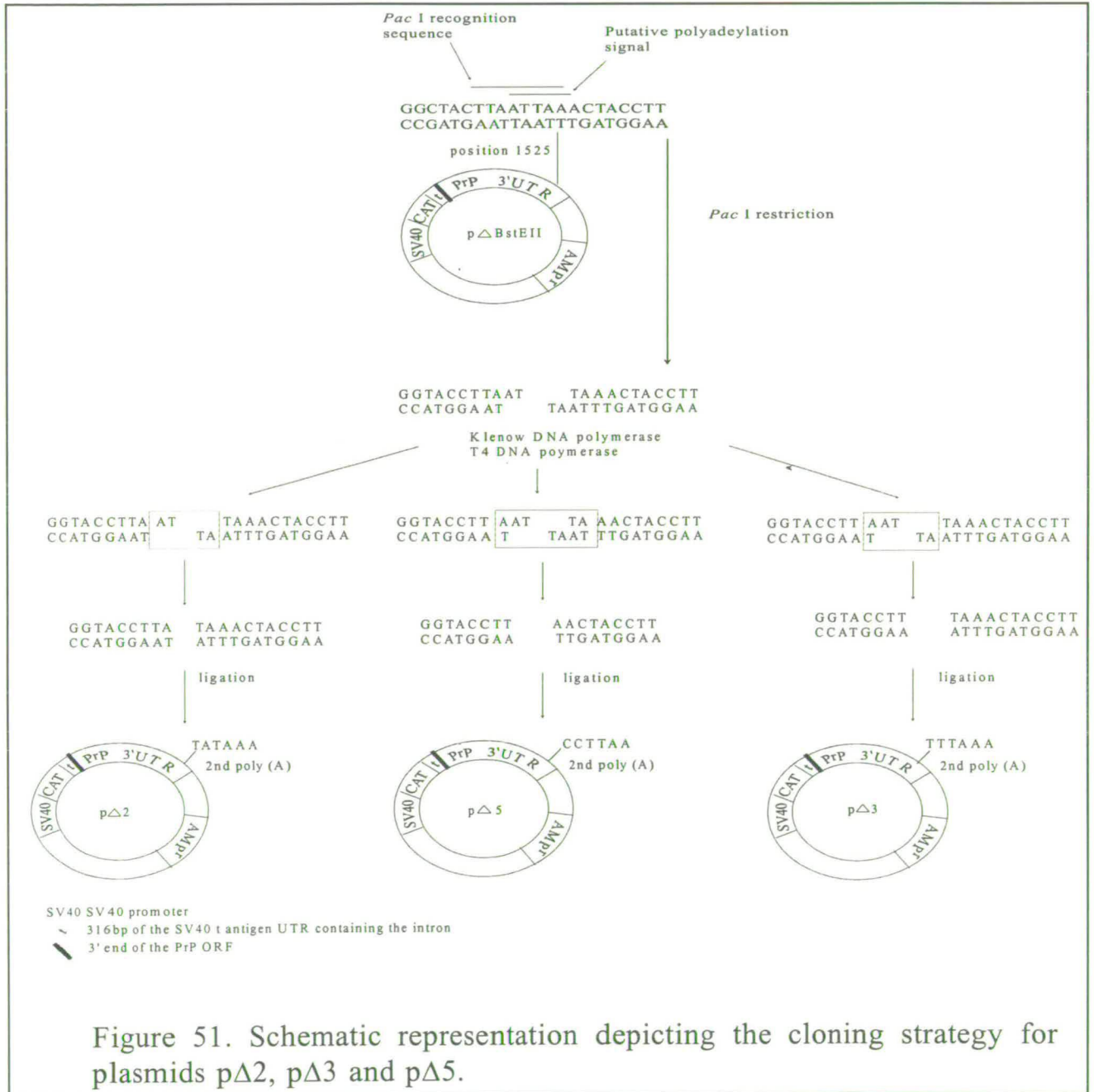
Figure 51. Schematic representation depicting the cloning strategy for plasmids pΔ2, pΔ3 and pΔ5.

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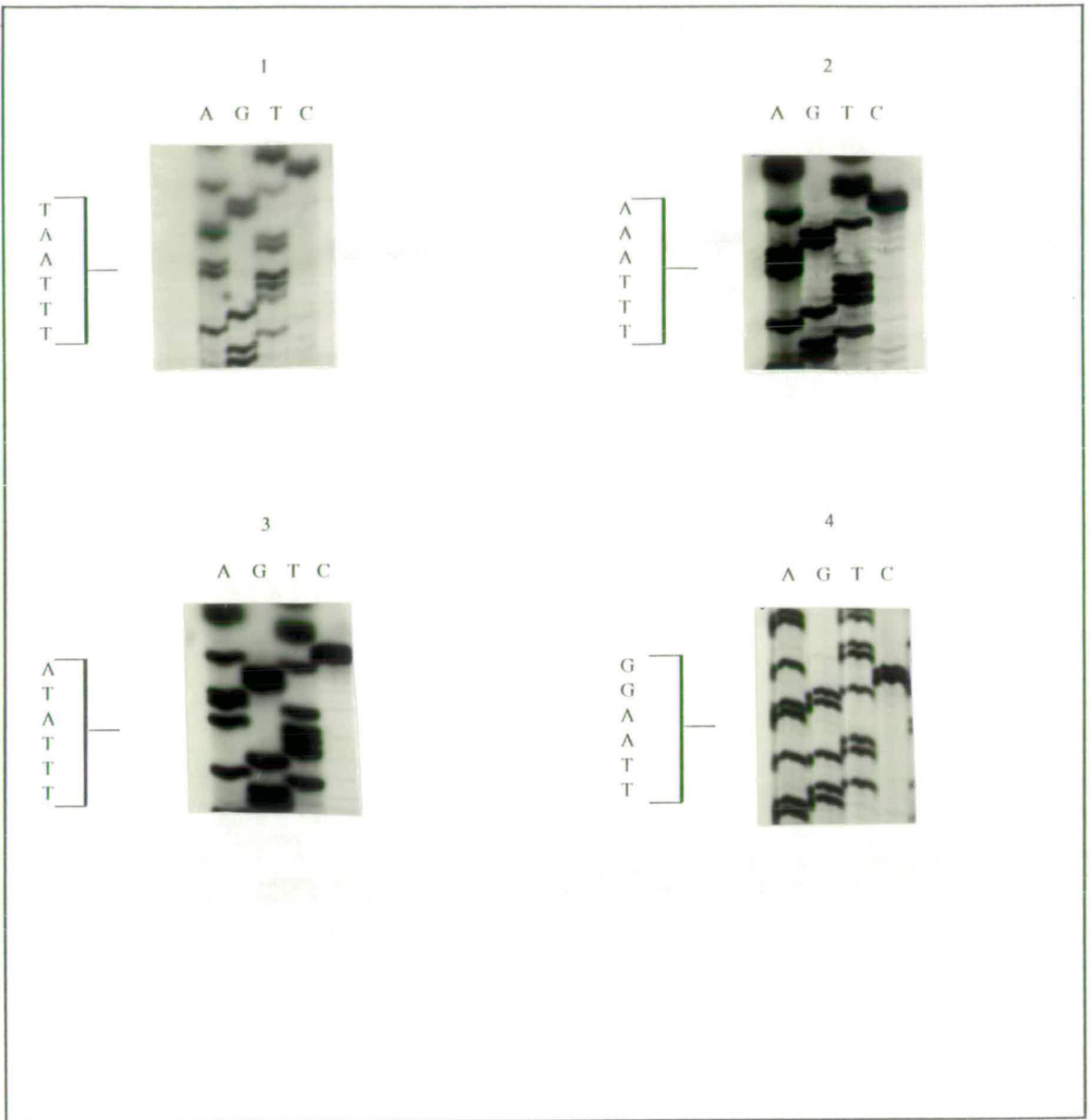
Figure 52. Sequencing autoradiographs of the potential poly A signal and the deletions from mutant chimeric CAT plasmids produced by restriction of pBst EII, blunting the ends with T4 DNA polymerase and religation. The sequences of 4 mutant clones are presented alongside the wildtype sequence. Clone 1 (pΔBstEII) contains the wild type hexanucleotide sequence while clone 2 (pΔ3) 3 (pΔ2) and 4 (pΔ5) contains 3, 2 and 5 bp deletion respectively.
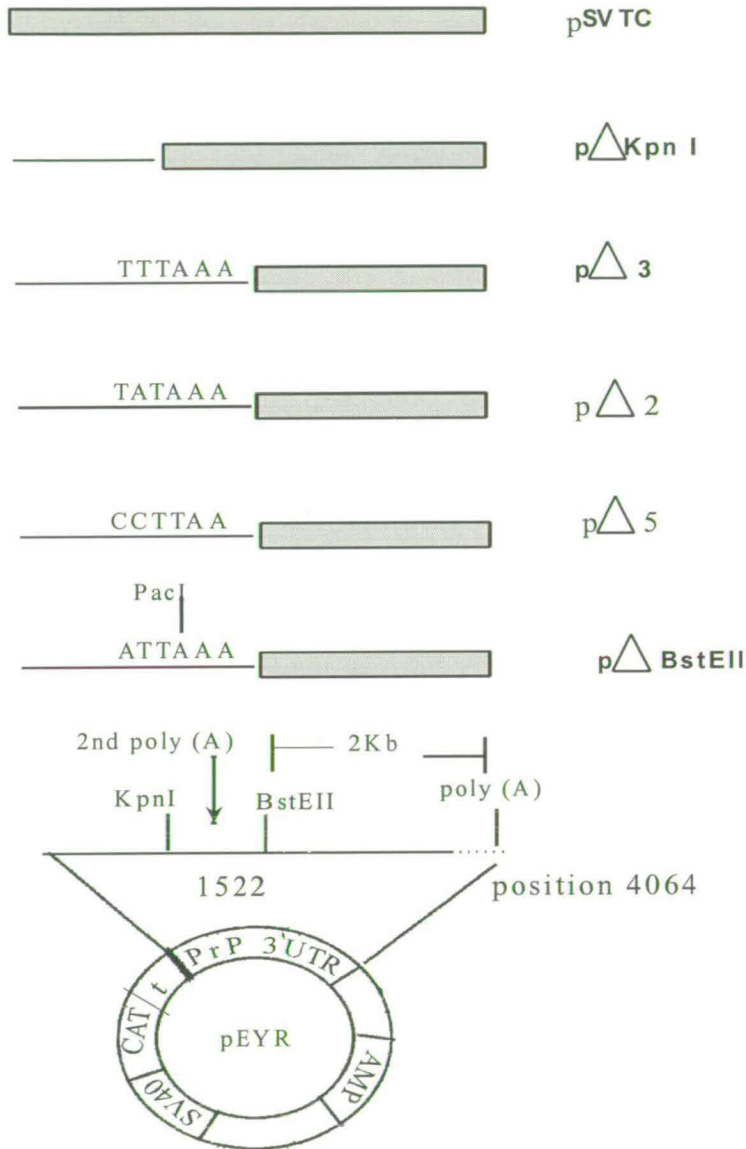
Figure 53. Summary of constructs used to investigate alternative polyadenylation sites

Constructs pΔBstEII has the complete poly A site and, pΔ5 , pΔ2, pΔ3 have no, 5, 2 and 3 base pair deletions respectively within the hexanucleotide ATTAAA region. While pΔKpn I and pSVTC have 400bp and 1Kb deletion with respect to pΔBstEII and to pol A signal.

(6.4) Investigation into the alternative polyadenylation signal at position 1522

To address whether the potential alternative polyadenylation signal at position 1522 was functional in mRNA 3'end processing, a series of fragments with deletions in this region were generated as described above. The deletion (Section 6.3e) fragments were inserted downstream of the CAT reporter gene, itself under control of the SV40 promoter. These chimeric constructs were introduced into N2a cells by calcium phosphate precipitation (Section Section 2.29). After 48 hours incubation, cytoplasmic RNA was isolated from the transfected cells and the CAT trancripts analysed by RT-PCR (Section 2.34). In each assay, cytoplasmic RNA from mock transfected cells was used as a control. The CAT mRNA transiently expressed from the pΔBstEII construct had evidently been subjected to 3' end processing and gave rise to 1 major band of the expected size (approx. 700bp) when reversed transcribed using an anchored oligo dT primer and PCR amplified using a specific downstream 3' UTR primer (A030) (Figure 53 lane b). This construct contains the 5' end of the PrP 3'UTR terminating at position 1716 (Section 6.3a). The constructs that terminate within the PrP 3' UTR at position 1519 (pΔKpn I Section 4.3b) or do not contain any 3' UTR (pSVTC, Section 4.3c) seem to abolished 3' end processing, since no DNA band was present in the RT-PCR assay (Figure 53 lanes c and d respectively). Constructs that contain specific deletions in the potential polyadenylation signal at position 1522, pΔ5 and pΔ3 (Section 4.3d) also produced no DNA product probed (Figure 54 Lane h and i) as judged from agarose gels and Southern blots with a ^{32}P labelled oligonucleotide BO11. However when the polyadenylation signal was changed from ATTAAA to TATAAA, in pΔ2 (Section 4.3d) 3' end polyadenylation in this region was retained, as judged from the RT-PCR assay (Figure 54, lane j).

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A
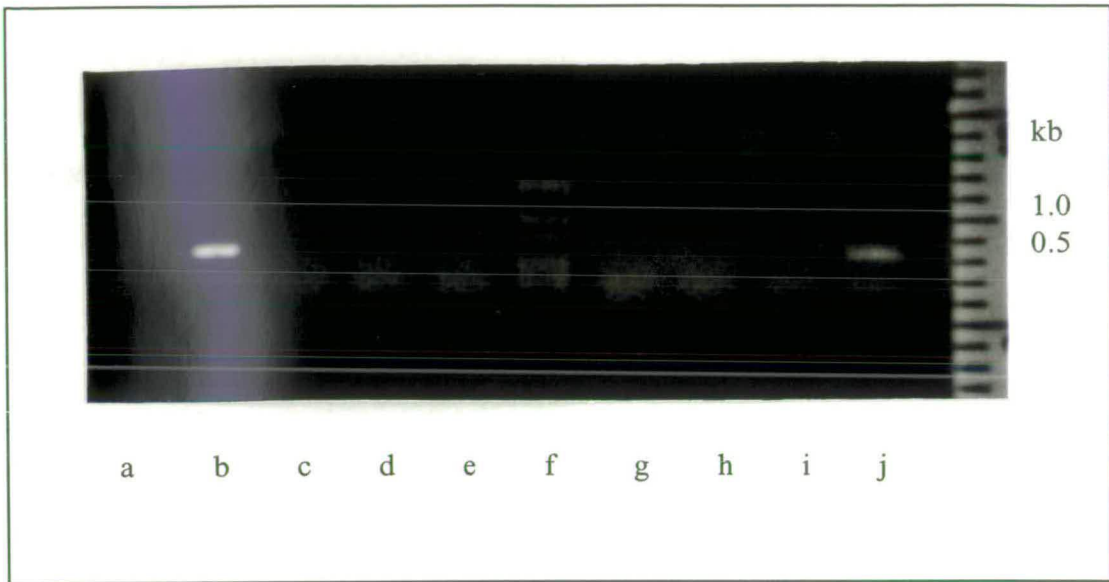
B
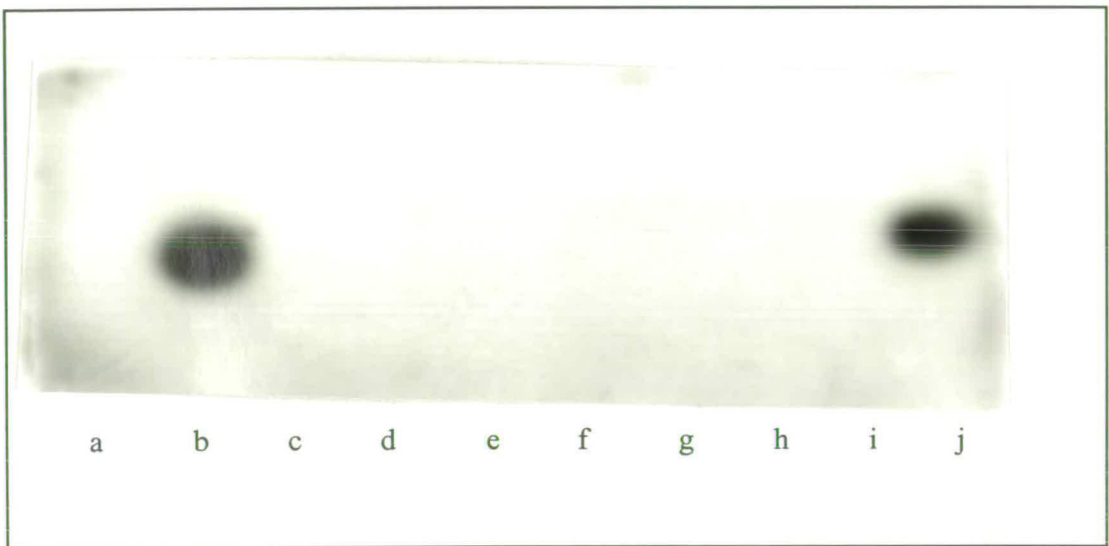
Figure 54. Polyadenylation of transcripts

(A) RNA transcribed from the chimaeric CAT constructs were reverse transcribed using an anchored oligo T primer then, PCR amplified and separated on 1.6% agarose (B) The gel was blotted onto a membrane using, probed with a ^{32}P labelled oligonucleotide BO11 (ACACACAGACGTCTCTTGATATTTCC) and exposed to X-ray film over night. (Lane a: No template, Lane b: pΔBstEII, lane c: pΔKpnI, Lane d: pSVTC, Lane e: As (i) but no Reverse transcriptase, Lane f: 1Kb markers, lane g: As (j), but no Reverse transcriptase, Lane h: pΔ5, Lane i: pΔ3 and Lane j: pΔ2.)

(6.5) Effect of mRNA 3' end processing upon gene expression

Abolition of 3' end processing has been shown in the previous section by mutation in the poly A signal at position 1522. This section will extend these results and measure the consequences for CAT protein production. Calcium Phosphate was used to introduce DNA into mouse neuroblastoma cells. After 48 hours lysates from neuro2a cells transiently transfected with chimaric CAT constructs (Section 6.3) were harvested and incubated with ^{14}C chloramphenical and acetyl Co-enzyme A for 20mins. The reaction was extracted with ethyl acetate and separated by TLC on silica plates using a chloroform: methanol :: 97:3 solvent system (Section 2.31).

CAT activty was abundant in cells transfected with the constructspΔBstEII and pΔ2 which contains the recognisable polyadenylation signal at position 1522 (Figure 55 lanes h and e respectively; Figure 56). CAT transiently expressed from the plasmid pΔBstEII produced the highest CAT activity . Other constructs, terminating in the PrP 3' UTR before position 1522 (pΔKpnI and pTCAT) or having specific short deletions inactivating that polyadenylation signal 1525, (pΔ3 and pΔ5) produced low CAT activity (Figure 55 lanes f, b, d and c respectively; Figure 56). However, when the polyadenylation signal was changed to TATAAA (pΔ3), CAT activity remained strong (Figure 55 lane e; Figure 56, Appendix Table 14). The experiment was done 3 times and co-transfection with the pSVlacZ plasmid accounted for differences in transfection efficiency by allowing parallel assays for beta galactosidase as described previously). The results are summarised in Figure 56 and Table Appendix Table 14.
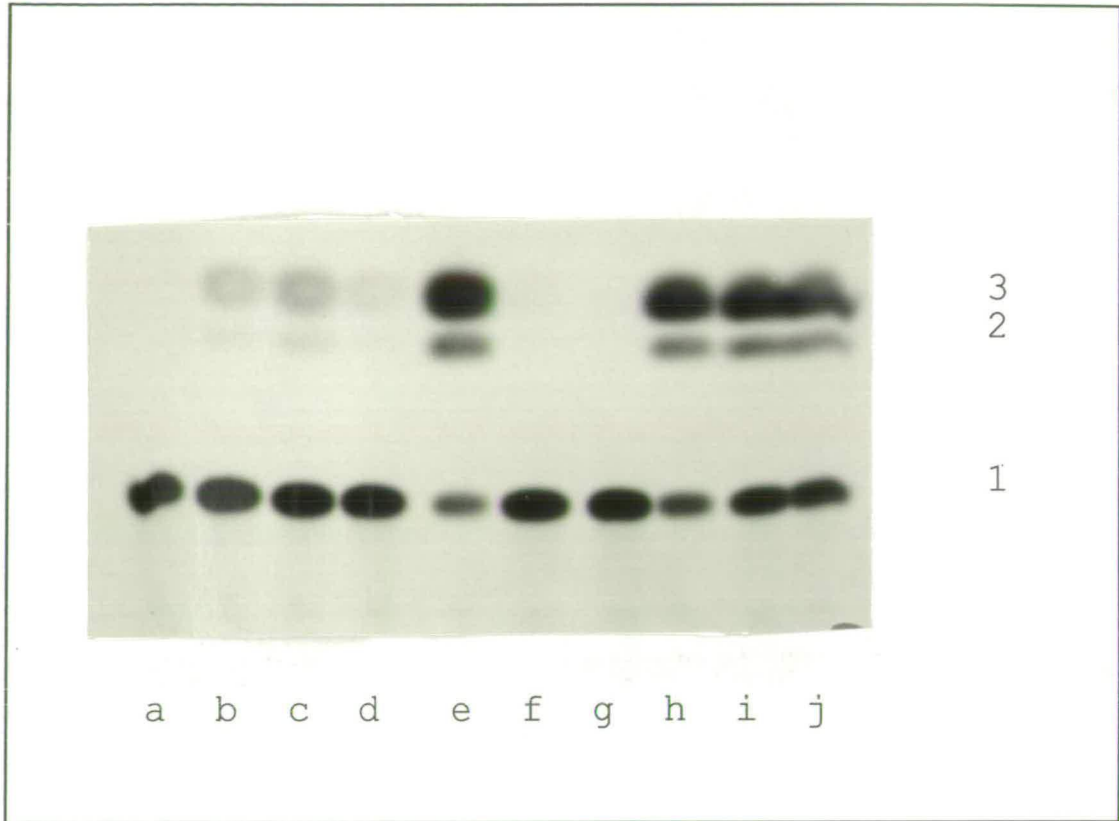
Figure 55. Effect of mRNA 3' end processing upon gene expression
Representative results depicting an autoradiogram of a CAT assay performed by TLC.
In all cases except for lane i and j transfected neuroblastoma cells were harvested after
48 hours. The constructs used were Lanes a: Mock transfection, Lane b: pSVTC, Lane
c: pΔ5, Lane d: pΔ3, Lane e: pΔ2, Lane f: pΔKpnI, Lane g: pSVlac Z, Lane h:
pΔBstEII, Lane i: pΔBstEII (24 hours), Lane j: pΔBstEII (36 hours). Row 1: Non-
acetylated; Row 2 and 3: acetylated [14] C chloroamphenicol.
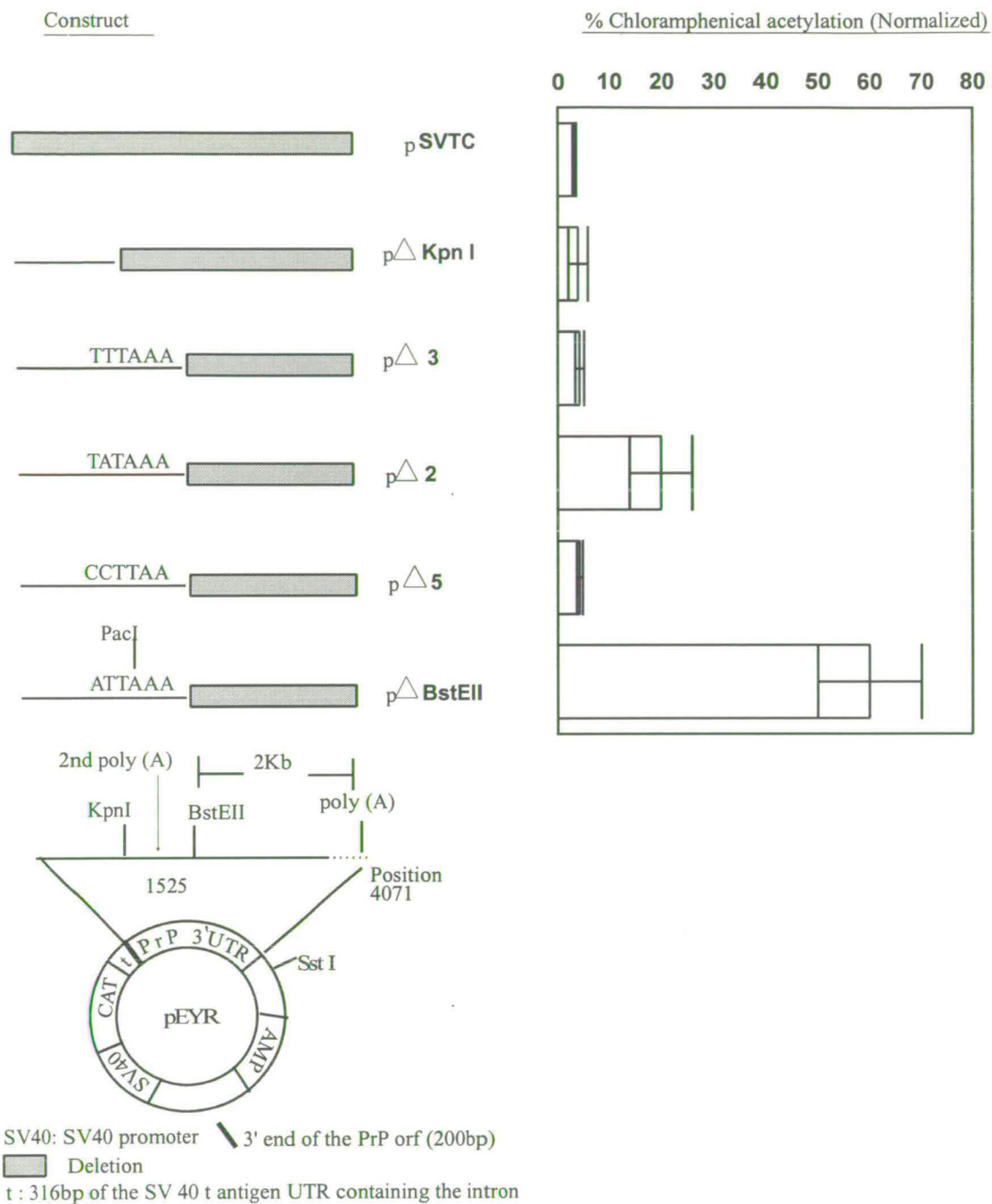
Figure 56. Effect of the putative alternative polyadenylation signal of PrP upon the translation of CAT. Transfection efficiency was normalized by co-transfection with a plasmid containing the beta-galactosidase ORF. Values were determined from 3 independent experiments. Error bars represent standard errors from the mean. Constructs pΔBst EII, pΔ5, pΔ2, pΔ3 have no, 5, 2 and 3 base pair deletions respectively within the hexanucleotide ATTAAA region. While pΔKpn I and pSVTC have 400bp and 1Kb deletions with respect to pΔBstEII.
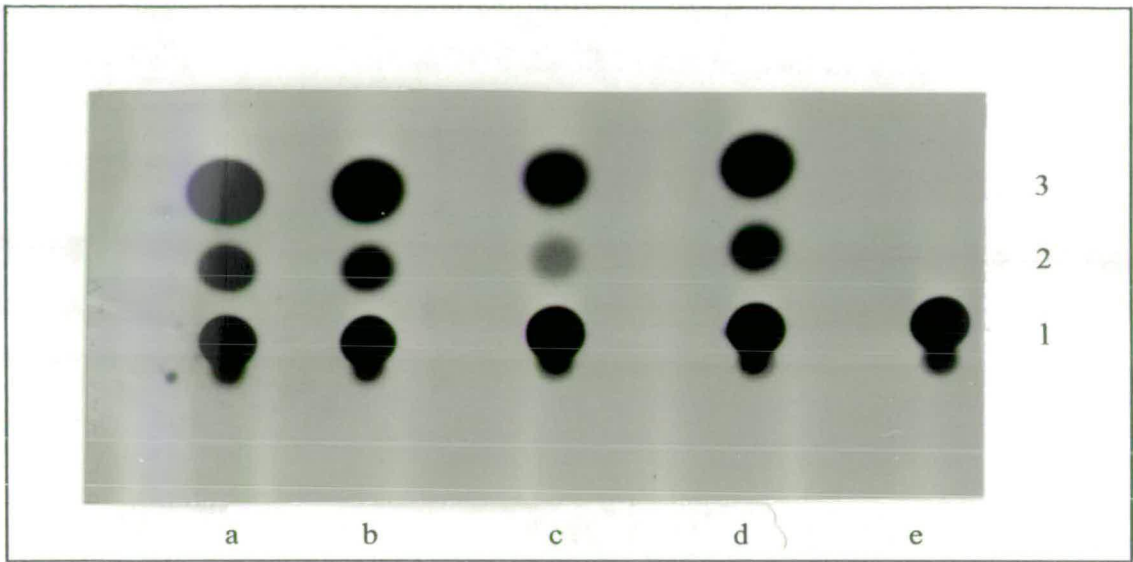
Figure 57. An autoradiograph of a TLC plate depicting the CAT activity from cells transfected with the constructs lane a: pEYD, lane b: pΔBstEII, lane c: pEYR, lane d: pCAT control. lane e: mock transfected. Row 1: non-acetylated 2: and 3 acetylated ¹⁴C chloroamphenicol.
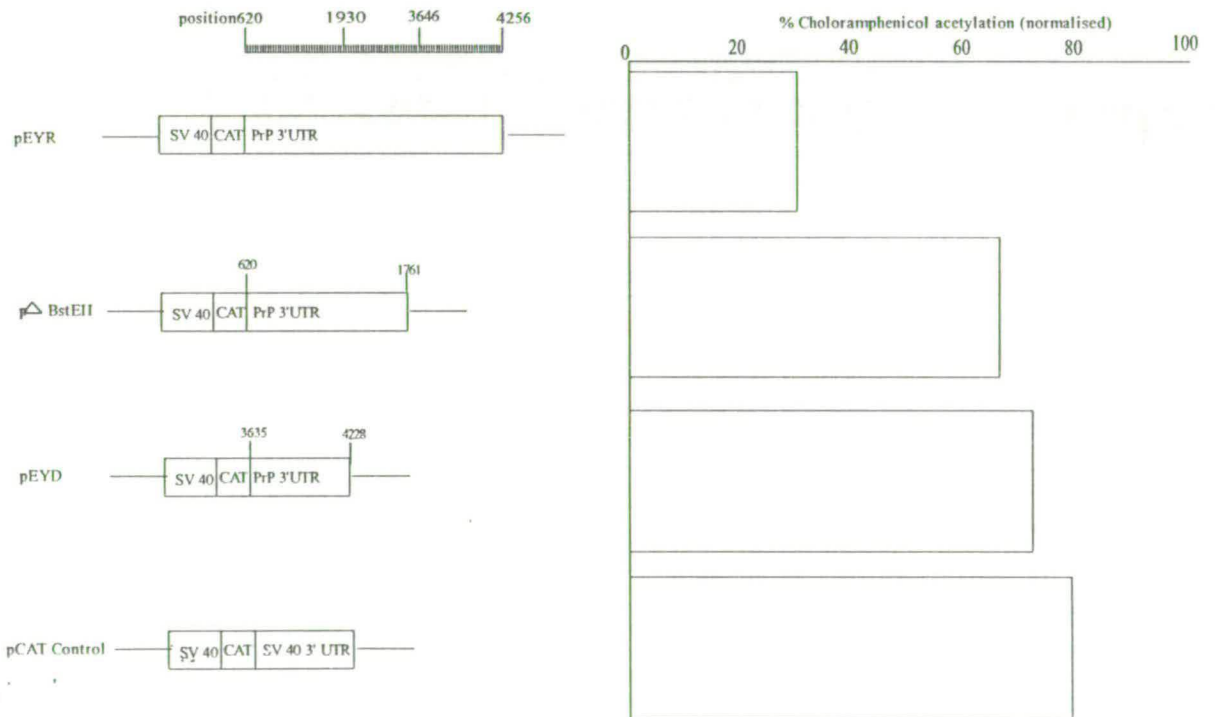
Figure 58.Comparison of the constructs pEYR and pBstEII in their ability to produce CAT activity. (See also Appendix Table 15)

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(6.6) Comparison of the constructs pEYR and pΔBstEII in their ability to produce CAT activity

A comparison was made between the constructs pΔBstEII (Section 6.3a) and pEYR (Section 5.3b) in their ability to produce CAT activity. The construct pEYR encodescontains the full length PrP 3' UTR which is present in the 4.6kb PrP transcript of 4.6 kb in length found in sheep brain. The construct pΔBstEII terminates 200bp downstream of the polyadenylation signal at position 1522 and encodes only the 3' UTR contained within the short PrP (2.1 kb) transcript found in peripheral tissues of sheep. The constructs pCAT control, containing the SV40 3' UTR, and pEYD which has a 2806 bp deletion in the PrP 3' UTR (Section 5.3a) were used as positive controls. Results shown in Figure 57 and 58 shows that the constructs pCAT control, pEYD and pΔBstEII (Figures 58 and 57 lanes d, a, b respectively) produce similar levels of CAT activity and all higher than the construct pEYR which encodes the whole PrP 3' UTR (Figure 57 lane c).

Based on the evidence from section 5.9, the difference between pEYR and pEYD (and, by extrapolation, probably pΔBstEII) is due to a posttranscriptional effect. Due to constraints of time this experiment was only carried out once but will be repeated by others before publication.

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(6.7) Conclusion

Numerous mutagenesis experiments as well as the analysis of naturally occurring mutations have confirmed that the sequences (AATAAA or ATTAAA), typically located 10-30 nucleotides upstream of the poly (A) site, are essential for 3'-end formation of polyadenylated RNA (Review: Wahle and Keller, 1992). The variant TATAAA has also been shown to have at least partial ability to act as a polyadenylation signal (Russnak and Ganem 1990).

If 3' end processing employing the polyadenylation signal ATTAAA at position 1523 of the PrP gene 3' UTR were taking place then by RT using an anchored oligo dT primer, followed by PCR amplification using a specific downstream primer AO30, a 700bp cDNA fragment should be produced. This was confirmed (Section 6.4). Deletion and site directed mutagenesis analysis further confirmed that the polyadenylation signal ATTAAA at position 1522 can be used in 3' end processing in N2a cells (Section 6.4). Constructs that terminated in the PrP 3' UTR upstream of position 1522 (Section 6.3b and c) produced no cDNA product and therefore seemed not to have been polyadenylated. Constructs that contained specific deletions in the polyadenylation signal at 1522, pΔ5 and pΔ3 (Section 6.3.d) also produced no cDNA product as judged from agarose gels and Southern blots. However, when the polyadenylation signal was changed from ATTAAA to (pΔ2) TATAAA (known to be partially functional in other systems: see above) 3' end processing in this region was retained. Southern blotting with a [32]P labelled oligonucleotide BO11 confirmed the identity of the DNA fragments. The overall results strongly suggest that the polyadenylation signal at position 1522 can be

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functional in 3' end processing in N2a cells.

CAT activity was abundant in cells transfected with the constructs pΔBstEII and pΔ2 which contain functional polyadenylation signals at position 1522. In contrast the other mutated chimaeric CAT constructs which do not have poly A signals, produced only low CAT activity (Figure 56) possibly due to the use of cryptic sites along the plasmid backbone .

These results strengthen the hypothesis that the short PrP transcripts seen in peripheral tissues of sheep originate from alternative polyadenylation of transcripts from a single PrP gene. The use of the polyadenylation signal at the position 1522 would produce a shorter PrP transcript similar to the size seen *in vivo*. Recently, in a follow up to this work the shorter 2.1 kb sheep transcript has been RT-PCR amplified and sequenced and the poly (A) tail shown to start approximately 16bp downstream of the poly(A) signal at position 1522 (Goldmann et al., unpublished). Thus in agreement with the literature, poly(A) addition occurs between 10 and 30 nucleotides downstream of the polyadenylation signal (Review: Whale and Keller 1992). The constructs pΔBstEII and pEYR in N2A cells produce CAT mRNAs carrying the PrP 3' UTR of the 2.1kb and 4.6kb transcripts respectively. A comparison was made between the construct pΔBstEII and pEYR in their ability to produce CAT activity. The results showed that pCAT control, pEYD and pΔBstEII produce similarly high levels of CAT activity whereas pEYR, with the full length PrP 3'UTR, produces lower levels of CAT (Figure 57). These results raise the interesting possibility that the two PrP transcripts in sheep, differing in the lengths of their 3' UTR may also differ in their expression of PrP protein.

Summary and Discussion

(7.1)   Summary

This project was designed to investigate the potential of the sheep PrP gene 3' UTR to control gene expression. Interest in this region of the PrP gene resulted from the following studies of sheep PrP genetics (Hunter et al., 1991), PrP mRNA (Hunter et al., 1994b) and protein (Horiuchi et al., 1995). There is an *Eco*RI RFLP which has good association with the incidence of both natural and experimental scrapie (Hunter et al., 1989, 1991) and is located in the 3' UTR region of the PrP gene. However, the association between e1 and disease may be due to the linked missense mutation affecting codon 171. Since the amino acid changes in the PrP protein does not always account for PrP predisposition to scrapie (Hunter, 1996), however, it should be noted that variation in the 3' UTR can affect mRNA localisation inside cells, and might therefore affect the pattern of PrP glycosylation with possible consequences for disease susceptibility (Jansen, 1995).

Furthermore, in sheep brain there is a single PrP mRNA of 4.6 kb, whereas in peripheral tissues (eg, spleen, kidney), the 4.6 kb mRNA is accompanied by a smaller (2.1 kb) PrP mRNA (Hunter et al., 1994b; Horiuchi et al., 1995). This differ in the length of the 3' UTR (Figure 14) therefore lacking several features which are present in the 4.6 kb mRNA including repetitive sequences and a highly conserved region at the 3' end of the UTR. The existence of both transcriptional and post transcriptional control of PrP expression in sheep was suggested by the finding that the brain expresses PrP mRNA at a level about fivefold higher than the kidney, and moreover produce at least fortyfold more PrP^C protein.
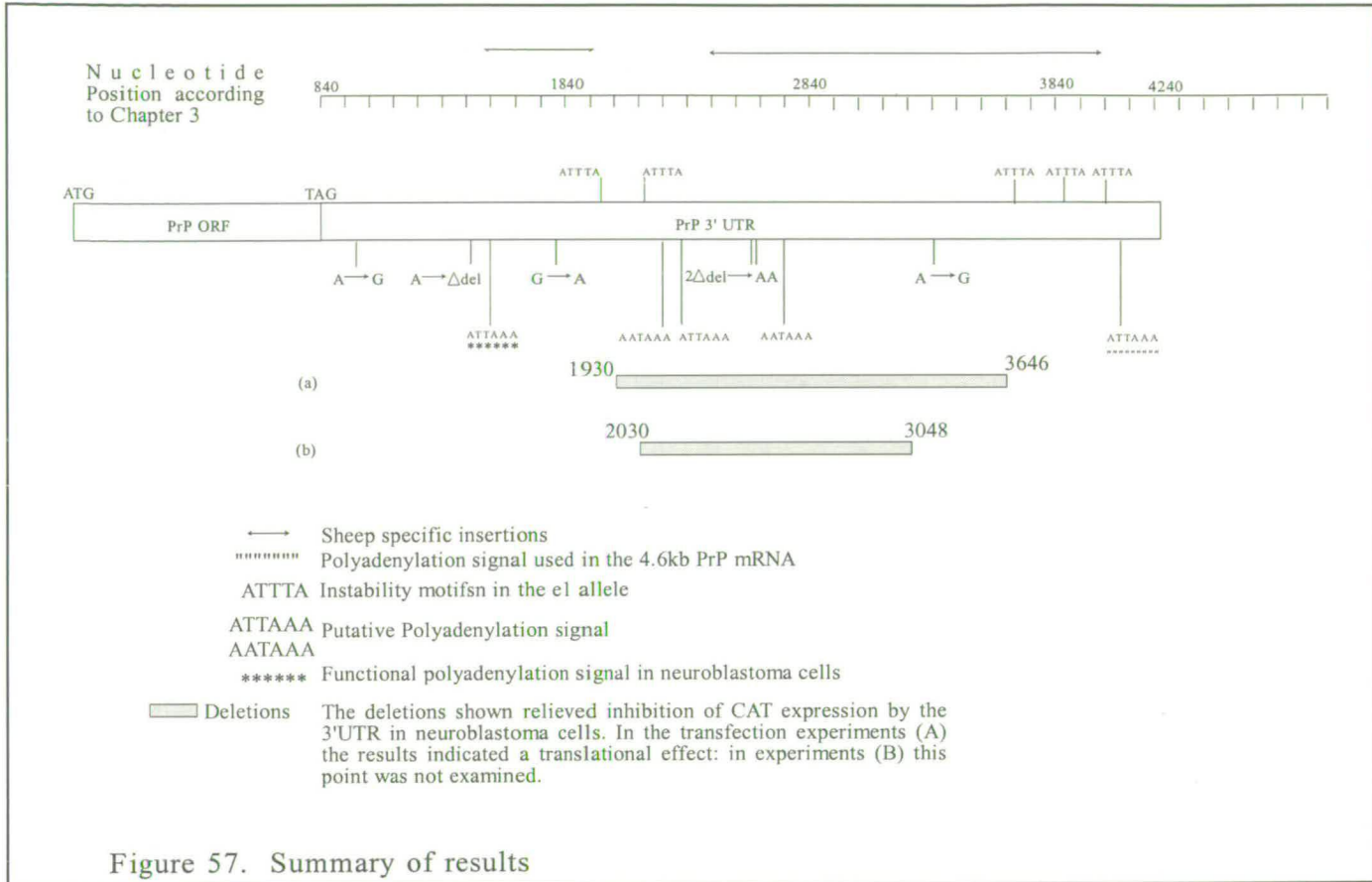
Figure 57. Summary of results

Expression might be important in at least three respects: the development of disease, differential expression from the two PrP transcripts and tissue specific expression. Results from this thesis have shown that the PrP gene 3' UTR is more polymorphic than was thought previously, can alter expression at the translational level of a reporter gene in neuroblastoma cell lines but not in cell free systems and contains a polyadenylation signal at position 1522 which is functional in 3' processing in the same cell line, when the polyadenylation signal at 4064 is deleted (Figure 57).

(7.2)   Cell Free System

The effect of the PrP 3' UTR upon translation was investigated initially using a cell-free translation system. Two constructs contained CAT fused to the entire PrP 3'UTR, one in reverse orientation. Another construct contained the 3'UTR with a 2.8kb section deleted. A control construct contained only the CAT gene with no PrP 3'UTR. In the rabbit reticulocyte cell-free system, expression of CAT protein was not significantly affected by any of the PrP 3'UTR sequences tested. It was also confirmed that all the constructs yielded expected 3'UTR sequences, when transcribed in vitro for the translation tests. It is thought possible that *in vitro* transcribed RNAs that differ by several knt in the 3' UTR would show differences in translational efficiency simply due to differences in the length of the 3' UTR. It has been shown that increasing the length of an artificial 3' UTR from 4 to 104 nucleotides increases both the translational efficiency and stability of an *in vitro* transcribed transcript (Tanguay and Gallie, 1996) and this effect is due simply to lengthening of the 3' UTR, independently of the sequence. This effects is thought to involve a passive mechanism. Ribosomes (or 40S Subunits) that transit a long 3' UTR following termination might be prevented from diffusing away from the mRNA and therefore might be more likely to reinitiate on the same mRNA than a ribosome that has already dissociated from a short 3' UTR. If the effect of a long 3' UTR serves to raise the local concentration of ribosomes, this model would predict that the effect of a long 3'UTR would be lost if the availability of ribosomes were no longer limiting. In other words, the extent to which the 3' UTR could raise the local concentration of ribosomes would be inconsequential in an environment

in which the concentration of available ribosomes was already high. These are the conditions that prevail in an *in vitro* lysate in which endogenous mRNA levels are low and translation is relatively non-competitive. Thus an unspecific PrP 3'UTR length effect might exist *in vivo*, and yet be undectable *in vitro*. Moreover, factors required for 3'UTR-dependent regulation might be missing or inactive in the *in vitro* system (not least a heterologous reticulocyte system): for example polyadenylation complexes, or specific 3'UTR-binding proteins. However, the use of *in vitro* lysate had been sucessful in showing translation regulation for transferrin (Dickey et al., 1987), interferon (Kruys et al., 1987) and Lox (Ostareck-Lederer et al., 1994) mRNA.

(7.3)   Intact Cells

The second experimental approach involved a study of chimaeric CAT constructs in intact N2a cells (Chapter 5). In the first (A) series of such experiments both CAT mRNA and enzyme activity were measured, to determine if the PrP 3' UTR sequences are controlling expression at the post-transcriptional level.

One construct (pEYR) contained the full length PrP 3'UTR which terminates at position 4256, beyond the major polyadenylation signal. Other constructs had progressive deletions from the start of the PrP 3'UTR (at position 840) to position 1930 (pEYP) and 3646 (pEYD). The control construct contained part of the SV40 3' UTR. CAT protein was produced at approximately two fold higher levels by the construct pEYD (and the control) than by pEYP and pEYR (judging by the activity of the CAT protein). Analysis

of mRNA levels showed that these were similar with all constructs and therefore the data suggest that the 3' UTR of sheep PrP can reduce expression of CAT mRNA at the translational level, at least in these N2a cells. It is not known if the increase in translatability produced by the deletion in pEYD results from removal of specific sequences or is simply due to the shortening of the PrP 3' UTR, but the former seems likelier. The nucleotides between the position 1930 and 3646 may be of particular interest as these sequences are the only differences between the construct pEYP and pEYD. This region of DNA is not conserved amongst PrP genes of other species, but in sheep it includes 3 polymorphisms (e1 versus e3) at positions 2613, 2614 and 3440 and three ATTTA instability motifs at positions 1985, 2213, and 3636.

Further constructs were made by unidirectional deletions in the PrP 3'UTR (Figure 43) for the second series (B) of transfection experiments. The results confirmed and extended the above data, in that constructs with deletions from the start (position 840) of the PrP 3' UTR to position 2030 gave lower expression of protein (CAT activity) when compared with those carrying deletions extending to position 3048 or beyond. Thus these results narrows down potentialy interesting region to between 2030 and 3048. This region may carry negative regulation signal(s), conceivably involved in binding proteins. Very little is known about the mechanism by which proteins bound to mRNA 3' UTR sequences alter translation. There are currently at least three hypotheses for such mechanisms of translational control. The first suggests that binding of specific proteins to the cis-acting 3' UTR signals, such as the CPE/ACE motifs in vertebrate oocyte mRNAs, is central to the mechanism of control of polyadenylation and translation initiation. For many developmentally important mRNAs, this type of translationally

silent ribonucleoprotein particle permits stockpiling of mRNAs during gametogenesis in a number of organisms (Standart and Jackson 1994). The second model suggests that the binding of a specific protein to a 3 'UTR acts as a nucleation signal for other proteins to bind to the mRNA, wrapping it up in a particle (mRNP) in which the RNA is inacessible to the translational machinery. Possible candidates for these structural proteins are the so-called Y-box proteins (Sommerville et al., 1996). A third hypothesis is that the 3' UTR serves as a transient reservoir of ribosomes (or 40S subunits) for recruitment. The high degree of functional codependence between the cap and poly (A) tail previously observed (Gallie 1991) suggested an interaction between these termini during translation. Recent evidence suggests that two elongation factors eIFs known to bind to the cap structure, i.e., eIF-4F and eIF-4B, also bind to poly(A) and may form part of the basis for the interaction for the interaction between the cap and the poly(A) tail (Gallie and Tanguay 1994). A consequence of this model would be that the interaction maintains the physical proximity of the termini during translation and thereby increases even further the local concentration of ribosomes for reinitiation. The poly(A) tail-mediated form of regulation might be an active mechanism in which a biochemically based interaction between the termini is used to promote reinitiation (Tanguay and Gallie 1996). It is not known whether any of these mechanisms are involved in regulation of sheep PrP gene expression. Future studies could include gel-shift assays to find out if the region from 2700-3048 in the 3' UTR specifically binds proteins.

(7.4)   Upstream polyadenylation signal

By deletion and site directed mutagensis analysis it has been shown in this thesis that

the polyadenylation signal ATTAAA at position 1525 can be used in 3' end processing

in neuroblastoma cells. Constructs that do not contain this sequence do not produce any

polyadenylated product. Conversion of the hexanucleotide sequence to TATAAA

maintains 3' processing but not when changed to TTTAAA or CCTTAA.

The use of the polyadenylation signal at the position 1522 in sheep would produce a

shorter PrP transcript similar to the size seen *in vivo*. The shorter transcript has been

subsequently, RT-PCR amplified and sequenced, the poly (A) tail was shown to occur

approx. 16 bp downstream of the poly (A) signal at position 1525 (Goldmann et al.,

unpublished). This is in an agreement with the literature, poly (A) addition naturally

occurs between 10 and 30 nucleotides downstream of the polyadenylation signal

(Review: Whale and Keller 1992). These findings are consistent with the work of Singh

et al., 1996 in which the protein BICP27 was shown to stimulate Bovine herpesvirus

(BHV) 3' end processing. Two chimeric CAT constructs differing only in the poly (A)

signal, one containing HGH (human growth hormone) and the other BHV-1 were

transiently introduced into insect cells. It was shown that the poly (A) signal of BHV-1

was inefficient in 3' end processing and consequently exhibited low CAT ativity.

However, upon co-transfection with the BICP27 open reading frame, CAT activity was

stimulated 7 fold. It was concluded that since these two plasmids differed only with

respect to the poly (A) signal present, that the increased CAT activity from the chimeric

CAT construct containing the poly (A) signal of BHV reflected enhanced 3' end

processing.

There are suggestions that polyadenylation stabilises transcripts and enables transport from the nucleus to the cytoplasm and high translational efficiencies (Tanguay and Gallie 1996). Many transcription units contain multiple polyadenylation sites, and the choice between them can play an important role in gene regulation (eg. Skadsen and Knauer 1995) and tissue specific expression (eg. Thekkumkara et al., 1992). Poly (A) sites vary in strength: when transcription units are engineered so that they differ only in their poly (A) sites, different steady state levels of mRNA result (eg., Carswell and Alwine 1989; Gimmi et al., 1988). When two sites are placed in tandem into the same transcription unit, one is used more often than the other. Although with two identical sites the upstream site has been preferred (Luo and Carmichael 1991), a strong poly(A) signal downstream can outcompete a weaker signal upstream (eg., Levitt et al., 1989). Why is one site more efficient than another? The *in vitro* system is still only partially defined, and its rate-limiting step is unknown. However sequences flanking the polyadenylation signal may play a part in influencing the site used (Moreira et al., 1995). Moreover, the *in vitro* system is limited to the investigation of 3'-processing as an isolated event, whereas *in vivo* the process is coupled to transcription and splicing. Thus, the determinants of poly (A) site strength might well be different under the two circumstances. Transcriptional pause sites enhance the use of upstream poly (A) sites *in vivo* and the strength of a site is correlated with the stability of the processing complexes it forms *in vitro*, either in nuclear extract or with partially purified fractions (Prescott and Falck-Pedersen 1992 and 1994; Gilmartin et al, 1992; Weiss et al., 1991). Upstream elements are as variable and can have similar effects (Gilmartin et al, 1992;

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Moreira et al, 1995). Even sequences surounding the AAUAAA element can influence poly(A) site strength (Prescott and Falck-Pedersen 1994; Gilmartin et al, 1992). The exact sequences that increase the use of one polyadenylation site over another are not fully understood. The potential implications of these results are discussed later (7.8).

(7.5)   Sequence Analysis

Five polyadenylation signals were identified within the 3' UTR of the PrP gene (Table 5), only two of which are now known to be used (Goldmann et al., personal communication and this thesis).

The findings of unused polyadenylation signals is not unique and has also been shown for the alpha-subunit of the eukaryotic initiation factor-2 (eIF-2 alpha). This is a single-copy gene expressing two mRNAs by alternative polyadenylation, 1.6 and 4.2 kb in size. Cloning and sequencing of the cDNA for the 4.2 kb mRNA revealed four polyadenylation sites within the 3' UTR of eIF-2 alpha, of which only two were found to be used in human and mouse tissues (Miyamoto et al., 1996).

Five copies of the pentamer AUUUA are present in the full length PrP 3' UTR of the e1 allele and only one of these is present in the 2.1 kb PrP transcript (Figure 57). Attempts made to investigate the ability of the PrP 3' UTR to alter the expression of a reporter gene in cell free systems and intact cells from Chapter 4 and 5 are discussed in Section 7.5 and 7.6 respectively. The finding of AUUUA motifs is interesting because they have been shown to be central to mRNA stability (Zubiaga al., 1995), localisation (Veyrune et al., 1996) and translational regulation (Wymore et al., 1996).

For example, in transfected fibroblasts the c-myc 3' untranslated region is able to localise beta-globin reporter sequences to the perinuclear cytoplasm (Veyrune et al., 1996). Constructs containing deletions within the 3' untranslated region identify the region between bases 194 and 280, which contains a ATTTA sequences, as critical for localisation. Transfection with constructs containing a mutated ATTTA sequence within the 194-280 base region showed that this conserved sequence is required for targeting of both c-myc mRNA and a chimaeric transcript of beta-globin linked to the c-myc 3' untranslated region.

In addition, labile mRNAs from cytokine and immediate-early genes often contain AU-rich (AT-rich on the DNA molecule) sequences within their 3' untranslated region (UTR) (Gehrke et al., 1994). These AU-rich sequences appear to be key determinants of the short half-lives of these mRNAs. Evidence suggests that the pentamer AUUUA, which previously was suggested to be the minimal determinant of instability present in mammalian AREs (AU rich elements), cannot direct rapid mRNA deadenylation and decay. Instead, the nonamer UUAUUUAUU is the essential AU-rich sequence motif that destabilizes mRNA (Zubiaga al., 1995).

(7.6) Sequence Differences between the e1 and e3 alleles of the sheep PrP gene 3' UTR

The *Eco* RI RFLP in the 3' UTR of the sheep PrP gene was shown to be loosely associated with experimental and natural scrapie (Hunter et al., 1991) although the linkage could well be due to the linked missense mutation affecting codon 171. The effects, if any, of addition polymorphisms found in the 3'UTR (Chapter 2) upon translation are currently not known. The allele tested in this thesis was the e1 allele, the one associated with incidence of scrapie. It would now be simple to insert the 3' UTR of the other allele, e3 (associated with disease resistance) into the chimeric constructs and compare its effects upon gene expression.

There are 6 copies of the ATTTA instability motif in the e3 allele, but only 5 in the e1 allele due to the polymorphism at position 1016 that results in a A toG substitution (Table 4). By site directed mutagensis it would be possible to repair the ATTTA pentamer at position 1016 from the construct pEYR which contains the full length PrP 3' UTR (Section 5.3b) and thus examine the functional importance of this polymorphism. Differences in protein levels may be linked to disease susceptibility. The level of PrP proteins have been shown to be directly linked to disease susceptibility (Manson et al., 1995). Studies on transgenic mice carrying different copy numbers of PrP genes and expressing PrP at different levels have shown that susceptibility to clinical disease, as evidenced by incubation time and disease progression, is correlated to PrP expression levels (Prusiner et al., 1990). It has been found that PrP null mice (Bueler et al., 1992),

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which are devoid of PrP protein, are completely resistant to scrapie and that mice carrying one copy of the PrP gene, contain about half the amount of PrP, show a very long incubation times as compared with wild-type mice (Bueler et al., 1993).

Transcripts that only differ in their 3' UTR can and have been shown in some genes to produce different protein levels. For example the casein gene has two alleles, which differ in that one has a 150b insertion in the 3' UTR, also differ in gene expression. The allele with the insertion has been associated with increased protein levels (Perez et al., 1994).

Differences in the 3' UTR can often result in more complex phenotypes. Breast cancer cell lines were found to be inhibited from traversing the cell cycle following the introduction of wild-type prohibitin transcripts. Functional mapping experiments showed that the 3' UTR is sufficient alone to inhibit cell cycle progression, indicating that the anti-proliferative activity of the prohibitin transcript is localised to this region (Jupe et al., 1996).

The presence of a second PrP transcript found in the peripheral tissues of sheep raises the possibility of functional differences between the two different PrP mRNAs due to the extended sequences. The functional significance of the PrP 3' UTR was investigated in chapter 4, 5 and 6 and its potential relevence to PrP expression is discussed below.

(7.7) Sequence Differences between the 4.6 and 2.1kb PrP transcripts

Sheep PrP mRNA is expressed, from both the e1 and e3 alleles, in peripheral tissues as two transcripts of 4.6 and 2.1 kb. Results presented in this thesis suggest that the shorter PrP transcript terminates using the polyadenylation signal at position 1525 while the 4.6 kb transcript ultilizes a signal much further downstream at position 4071 (Goldmann et al., 1991). It is not known if both transcripts are translated or if the two mRNAs are functionally different. Results presented in Chapter 6 suggest that they may produce different amounts of protein depending on the length of the 3' UTR.

DNA sequence analysis has shown the 4.6 kb PrP mRNA will contain 6 'AUUUA' instability motifs present in the extended 3' UTR but the 2.1 kb PrP transcript will contain none of these. Such 'AUUUA' motifs have been shown to be central to post transcriptional control of gene expression (Section 7.6). Additional sequences present in only the 4.6kb mRNA are repetitive sequences and a highly conserved region right at the 3' end of the UTR (Goldmann et al., 1991).Similar findings have been reported for the mouse voltage-gated K+ channel gene Kv1.4, which is expressed in brain and heart as a 4.5- and 3.5 kb transcripts. Both mRNAs begin at a common site 1338 bp upstream of the initiation codon and contain 3477 and 4411 nucleotides, respectively. The 3.5-kb transcript terminates at a polyadenylation signal 177 bp 3' of the stop codon, while the 4.5-kb mRNA utilizes a signal 94 bp further downstream. The extended region in the 3' UTR of the longer transcript contains five AUUUA repeats which were shown to inhibit translation (Wymore et al., 1996). The implications of these findings are discussed later (7.8).

(7.8.) Possible consequences of alternative polyadenylation and translation regulation

PrP gene coding region variation does not account for all observed differences in scrapie incidence in sheep, CJD in humans and doesn't seem to be linked at all in BSE infected cattle. As mentioned in Section   disease incidence in Suffolk sheep is linked to mutations at codon 171 while in Cheviots linkage is found with codon 136. Thus differences outside the PrP open reading frame may be of interest.

(7.8a) Expression of PrP in sheep

Iketa et al., 1995 have shown there to be both an increase in transcriptional and translational expression of sheep PrP in the brain compared with other tissues. There are appears to a 5 fold difference in the mRNA level between sheep brain and peripheral tissues and consequently, potentially a five fold difference in transcription. Additionally, the same comparisons made at the PrP protein level suggested that PrP mRNA was potentially translated aprroximately 8 fold more efficiently. The regions for this control are not apparent but this thesis has shown that PrP 3' UTR deletions can alter expression at least 2 fold of a reporter gene in neuroblatoma cells. The results from Chapter 6 using reporter gene constructs and neuroblatoma cells of mouse origin would predict that peripheral tissues would produce PrP protein more efficiently. The apparent discrepancy between these findings and with sheep tissues is not fully understood but may arise because of the use of cells of mouse origin and not sheep.

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(7.8b)   Alternative polyadenylation and differential expression of PrP

The presence of two PrP transcripts in peripheral tissues and one in brain of sheep raises some interesting possibilities. This different expression patterns in sheep peripheral and brain cells may have implications for natural scrapie which must result from a peripheral, rather than an intracerebral, infection. Understanding how the differential expression is controlled is basic information vital to the understanding of the natural disease as, whatever the nature of the infectious agent, its interaction with the endogenous PrP protein is required for successful infection.

The expression pattern of the PrP gene is different in mice from that seen in sheep. Unlike sheep scrapie, mouse scrapie is not tranmitted between animals even when in the same cage, nor is it maternally transmitted. Maternal transmission of scrapie is supposed to occur in sheep and the sheep placenta is highly infectious. The importance of the 2nd peripheral mRNA potentially translated more efficiently in sheep may be linked to these facts. There are may be at least two controlling mechanisms operating in the sheep. One has the results in the higher levels of the PrP mRNA and protein in the brain than in peripheral tissues and the other, possibly working through the differentail expression from the two mRNA, would have the effect of the slightly increasing the peripheral levels of protein expression although not so high as that seen in brain. In this respect, it is interesting that although the bovine PrP gene has similar structure to that of the sheep, cattle only produce the full length 4.6kb mRNA and not the "extra" peripheral 2.1kb mRNA (Hunter, unpublished). There is much less abnormal PrP produced in cattle spleen in BSE

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affected animals and much lower levels of infectivity compared with sheep spleen (Review, Hunter 1996). It may there fore be the 2.1kb mRNA in sheep spleen which produces enough PrP protein to allow the infectious agent to replicate in this tissue. Other forms of translational control by the 3' UTR of other genes are poorly understood. A form of lysosomal storage disease aspartylglucosaminuria has been described resulting from a homozygous deletion of most of the 3' UTR part of aspartylglucosaminidase mRNA. This deletion caused the synthesis of a stable, truncated but polyadenylated mRNA with a completely intact coding region, which was nethertheless not translated *in vivo*, whereas no defect in translational efficiency could be demonstrated *in vitro* (Ikonen et al., 1992). Another example is provided by the mRNA for the Alzheimers disease amyloid protein precursor (APP), which has two forms produced by alternative polyadenylation. Fusion of each 3' UTR of the APP mRNA to CAT had shown that the long 3'UTR was translated significantly more efficiently (Sauvage et al 1992). The extended sequences appeared to be responsible for this increase in translational efficiency independent of mRNA length. The results presented in Chapter 5 may play a part in differences seen in translation efficiency between peripheral and neuronal tissues. This will be tested (in a new PhD project) by the use of chimeric constructs already produced for their ability to express protein in neuronal and non-neuronal sheep cell lines.

The potential of regions other than the 3' UTR to affect expression (7.9-7.11)

(7.9)   5' UTR

The 5' UTR of the sheep PrP gene (131bp) consists of exon I and exon II and 10 bases of exon III (Westaway et al.,1994). This is another region which might regulate translation efficiency. Leader sequences of mRNA molecules encoding proteins with a strictly regulated expression, however are often larger than the average of 140 nucleotides (Kozak 1987; Pesole et al., 1994) contain upstream AUG codons and/ or are expected to form stable secondary structures by virtues of a high G + C content (Kozak 1989; Gray and Hentze 1994). A large number of mRNA molecules for proteins involved in growth and development fall in this category. Many of these long leaders have been shown to be detrimental to translation in cells (Kozak 1991). How can the translational efficiency of these "diffircult" leaders containing lots of secondary structure be improved ? One way is to increase the limiting factor for translation initiation eIF-4E, so that these highly structured leaders get a chance of entering translation (Kormilas et al., 1992). Ornithine aminotransferase and growth regulating proteins like cyclin D1 and transcription factors have been shown to be induced by eIF-4E overexpression (Rosenwald et al., 1993). Other methods include the addition of proteins that can bind specifically to the leader and thereby alters its conformation. Alternatively, a tightly bound RNA

binding protein can mimick secondary structure by obscuring the cap or stalling the scanning complex in the early stages, thereby hampering translation. The prototype for this idea is the translational regulation of ferritin expression by iron (Melefors and Hentze 1993).

Thermodynamically stable elements of secondary structure have been found in the 5' region including the ORF for PrP genes (Luck et al., 1996), however their functional significance is not known. It would be interesting to insert the PrP 5'UTR into the chimeric CAT constructs already produced in this thesis and assess whether it has potential to alter translation either alone or in conjunction with the 3'UTR.

(7.10)   Coding Region

Regulation of translation by sequences in the protein coding region is not a widespread phenomenon. Mechanisms that have been put forward have involved the presence of "ribosomal pause" sites, probably caused by secondary structures in the coding region. The TAR (trans-activator region) sequence of the human HIV (Okamoto et al., 1986) contain target sequences for trans-activator protein, Tat, which interacts with the TAR at the uridine rich bulge in the TAR RNA stem region (Dingwall et al., 1990). Wills and Hughes (1990) reported that the PrP mRNA of a range of species contained stem-loop structures. In the protein coding sequence of PrP mRNA, TAR-like stem-loop structure is present which have the pentanucleotide CUGGG in the loop and a so called uridine- and adenine bulge in the stem. In fact three CUGG sequences are located on the coding region of the mRNA at the nt

positions +170, +194 and +218. The nuclear beta galactoside-specific lectin CBP 35

(carbohydrate binding protein with a molecular mass of 35 kDa), which has been

identified in nuclear ribonuclearprotein (RNP) complexes from a variety of

mammalian tissues and cells, was among those proteins which bound to PrP RNA

and PrP^C. This association has led to the hypothesis of a possible role for prion

replication (for example, involved in PrP conversion) and/ or for translation

regulation (Schroder et al., 1995).
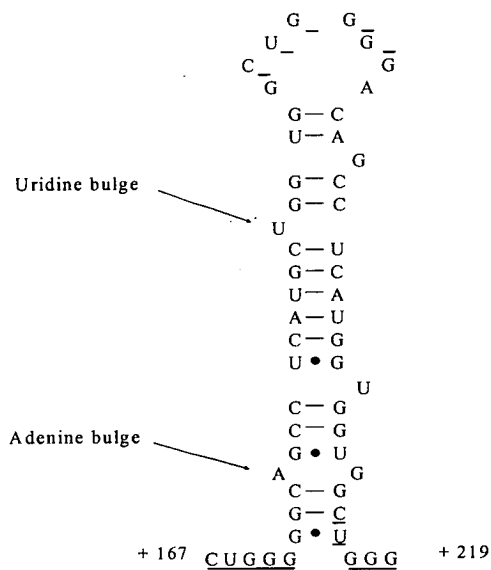
Figure 60. Proposed stem-loop structure of human PrP mRNA from the coding region ase +167 to +219. The characteristic CUGGG motif in the loop and two on the flanks are underlined and the uridine- and adenine bulge is labelled.

(7.11)   Introns

Almost all protein-coding genes in vertebrates have intervening sequences or introns

and artificial constructs lacking introns are often poorly expressed. Introns can

increase the yield of mature mRNA up to 50 fold by facilitating 3'-processing (Huang

and Gorman 1990; Chiou et al., 1991; Nesic et al., 1993; Nesic and Maquat 1994)

and a strict coupling of poly (A) site choice and recognition of the final intron *in*

vivo has been reported (Liu and Mertz 1993). *In vitro*, 3' processing is stimulated by

an upstream intron (Niwa et al., 1990), and splicing of a 3'-terminal intron is partially

dependent on the downstream poly (A) site (Niwa and Berget 1991). The 3'-splice

site is responsible for coupling to 3'-processing both *in vitro* (Niwa et al., 1990) and

in vivo (Nesic et al., 1993; Nesic and Maquat 1994; Luo and Carmichael 1991). In

contrast insertion of a 5'-splice site upstream of a polyadenylation (Niwa et al.,

1992). Histone genes are among the few genes that lack introns and they are also the

only genes which undergo a different form of 3' processing. As a particularly striking

example of the coupling between splicing and 3'-processing, the insertion of introns

into histones genes interferes with the histone-specific pathway of 3'end formation

and induces the use of a cryptic polyadenylation site further downstream (Pandey et

al., 1990).

Transgenic mice expressing lacking both intron I and II precluded expression of PrP.

These results, in agreement with earlier data (Scott et al., 1989), show that the

presence of at least one intron is essential for the efficient (Brinster et al., 1988) and

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or tissue-specific (Lozano and Levine, 1991) expression of some trangenes. However, recent results has shown that a PrP mini-gene functioning perfectly well without an intron (Race et al., 1995). These contradictory results cannot be fully understood.

(7.12)   Gene Therapy

Despite all the scientific research into TSEs, there is no cure for these neurodegenerative diseases. Since gene targeted mice with ablated PrP genes are resistant to scrapie prions (Bueler et al., 1993; Prusiner et al., 1993), one therapy would use the anti-sense approach. Despite the progress that has been made in attempting to develop efficient and safe gene vectors, none of the presently available vectors fulfills the stringent requirements that are necessary for human trials (Karparti et al., 1996). Other difficulties involve delivering antisense polynucleotides to all neurons of the CNS (Zon, 1995)

Other approaches include the design of "molecular glue" that would cross the blood brain barrier and bind to the hydrophobic core of PrP^C. Such a pharmacotherapeutic would stabilize the surrounding alpha helix and thus , might be effective in preventing PrP^{Sc} formation. An alternative approach is to design molecules that could bind to the interaction surface of the infectious PrP^{Sc} and thus terminate its ability to interact with and convert PrP^C. The approaches that have been put forward may be rather optimistic. More important studies must involve analysing the molecular and biochemical mechanism for infection since only then can efficient and effective targets be identified. Because PrP null mice are resistant to scrapie and yet otherwise unaffected, it may be possible to breed sheep or cattle that are resistant to this

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disease, either by PrP gene disruption or, because this methodology has not been implemented for animals other than mice, by the introduction of transgenes expressing PrP antisense RNA. Moreover, the fact that PrP hemizygous (PrP $^{+/-}$) mice show prolonged scrapie incubation times argues that a moderate reduction of PrP^C systhesis, as might be achieved by therapy with antisense oligonucleotides, would extend the preclinical phase. Since people at risk for inherited prion diseases can now be identified decades before neurologic dysfunction is evident, the development of an effective therapy is imperative. Other areas of interest for treatment include the use of Amphotericin B, which has been shown to prolong the incubation period of the disease and to delay the accumulation of the protease-resistant protein (PrP^{Sc}) in the central nervous system of scrapie-infected hamsters (Adjou et al., 1995)

(7.13)   Final conclusion and future work

The work in this thesis has suggested that the ideas which has prompted the study in the first place may be worth pursuing. PrP protein coding region variation does not alone account for all observed differences in scrapie incidence and the fact that the non-coding 3' untranslated region has the potential to control gene expression may have some importance in disease regulation. Present and future studies will analyse this potential using sheep cell lines, will look at the cellular localisation of protein produced from both the known sheep PrP transcripts, and will compare the translational effects of the e1 and e3 3'UTRs.

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Appendix

Table 7

µl of TCAT RNA	OD	Mean	Standard error of the mean
0.2	579 425 367	457	63
0.5	520 529 760	603	78
1	1299 532 955	928	222
2	1765 1174 845	1261	269

Effect of increasing TCAT RNA concentration upon the translation efficiency of CAT (Figure 24).

Table 8.

Construct	RNA levels OD, (arbitrary units)	Mean
pRAR	195, 175, 131	167
pRAD	150, 163, 192	168
pRAW	163, 170, 178	170
pTCAT	127, 141, 161	143

Relative molar Concentrations of RNA assessed by Slot blot hybridisation. The molar concentrations of *in vitro* transcribed RNA were compared by scanning the OD from autoradiographs of slot blots according to Section 4.6. Mean values were determined from 3 experiments an example of which is shown in Figure 25.

Appendix

Table 9

Construct	Protein levels (optical density)	Protein levels divided by mean RNA levels	mean (arbitrary units)	Standard errors of the mean
pRAR	184, 158, 138, 176, 176	153, 132, 115, 147, 147	138	7
pRAD	222, 118, 311, 220, 169	188, 100, 264, 186, 174	182	26
pRAW	191, 143, 248, 215, 206	159, 119, 264, 162, 187	166	15
pTCAT	262, 74, 285, 159,225	262, 74, 285, 159, 225	201	38

Effects of the PrP 3' UTR upon the translation of CAT (Figure 27).
[35]S-CAT protein levels measured by scanning autoradiographs of SDS polyacrylamide gels and the values were divided by the RNA levels from Table 8, according to Section 4.7. The values were determined from 3 independent experiments.

Appendix

Table 10

ul of lysate	% chloroamphenicol acetylation	Mean	standard error of the mean
2	4.2, 4.1, 3.9	4.1	.09
4	5.8, 5.8, 5.7	5.8	.04
12	25.6, 29.1, 30.0	28.2	1.4
20	50.6, 52.1, 50.6	51.1	.5

Effect of increasing lysate upon the acetylation of ^{14}C chloramphenicol-dose response curve (Figure 38)

Appendix

Table 11

Construct	% CAT conversion	RNA Levels	CAT/RNA	Mean	Standard Error of the mean
pEYP	22 17 25	920/1162 672/1455 1378/1706	28 37 31	31.3	2.6
pEYD	43 36 46	780/1189 702/1607 1344/1624	65 82 56	67.6	7.5
pEYR	17 22 25	672/1455 920/1162 1378/1706	48 21 31	33.6	7.9
pCAT control	46 42 50	767/1203 877/1333 1248/1652	61 58 66	61	2.9

Effect of the PrP 3' UTR upon the translation of CAT in N2a cells. (Figure 40)
target/competitor hybridisation ratio: see section 5.5(d)

Appendix

Table 12

ul of lysate	Absorbance 420 nm	mean	standard error of the mean
10	0.15, 0.13, 0.16	0.14	0.01
20	0.30, 0.33, 0.34	0.32	0.012
40	0.76, 0.72, 0.73	0.73	0.012
80	1.32, 1.04, 1.01	1.12	0.09
160	1.41, 1.45, 1.30	1.38	0.04

Effect of increasing lysate volume upon the hydrolysis of ONPG-Dose response curve for beta galactosidase (Figure 43).

Appendix Table 13.

Construct	% choramphenicol acetylation (normalised)	Mean	Standard error of the mean
pCAT control	97, 87, 143	109.0	15.9
pEYR	66,40,44	50.0	5.7
pEYP	52,50,39	46.6	3.3
p2030	28, 60, 60	49.3	10.6
p2700	79, 50, 116	81.6	17.4
p3048	152, 130, 88	123.3	17.6
p3398	144, 140,131	138.3	4.4
pEYD	125, 135, 110	123.3	7.2
p3940	151, 114, 140	135.0	10.4

Effect of the PrP 3' UTR upon the expression of CAT (Figure 45).

Appendix Table 14

Construct	%Chloramphenicol acetylation (normalised)	mean	standard error of the mean
pΔBstEII	77, 59, 49	61	8
pΔ2	31, 16, 15	20	5
pΔKpnI	5, 9, 2	5	2
pSVTC	2, 5, 6	4	1
pΔ3	2, 5, 7	5	1.5
pΔ5	4, 7, 7	6	1

Effect of the putative alternative polyadenylation signal of PrP upon the translation of CAT (Figure 56.)

Table 15

Construct	% chloramphenicol acetylation (Normalised)
pΔBstEII	66
pEYR	30
pEYD	72
pCAT control	79

Comparison of the constructs pEYR and pΔBstEI in their ability to produce CAT activity (Figure 58)

Appendix

Solutions (Apart from those supplied by manufacturers of kits or enzymes)

Agarose Gel loading dye

0.25% bromophenol blue

0.25% Xylene cyanol FF

30% glycerol in water

Alkaline lysis buffers

Solution 1

50mM glucose

25mM Tris.Cl pH8

10mM EDTA pH 8

Solution 2

0.2N NaOH

1% SDS

Solution 3

60 ml 5M potassium acetate

11.5 ml glacial acetic acid

28.5 ml H_2O

Bind silane

Bind-Silane

Ethanol

5ml 10% Acetic acid

170µl Bind-Silane

Denaturation solution

1.5 M NaCl

0.5 M NaOH

50 X Denharts solution

0.5g Ficoll

0.5g Polyvinylpyrrolidine

0.5g Bovine serum albumin (BSA)

water to 50 ml.

DEPC treatment

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

Dialysis tubing

Cut tubing into suitable lengths and boil for 10 mins in 1 litre in 10 % sodium carbonate. Discard the buffer and boil X 3 in 1 litre water. Store in TE.

0.5M EDTA (pH 8)

186.1g EDTA.H_2O pH 8 dissolve in 1 litre water and 20g NaOH Pellets

H_2O to 1 litre

2X HeBs

50mM HEPES

280 mM NaCl

1.5 mM Na_2HPO4

Neutralising solution

3 M NaCl

0.5M Tris-HCl

Polyacrylamide gels for sequencing

For an 8% gel:

7ml Acrylamide solution

9M Urea

170μl 10% Ammonium persulphate

30μl Temed

33ml Water

Prehybridisation solution

15 ml 6X SSC

0.1% SDS

1X Denhardts

5 mg boiled sheared herring sperm DNA

33 ml sterile water

10 X RNA running buffer

20.92 MOPS (3-(N-morpholino) propanesulphonic acid)

8.33 M sodium acetate

10ml 0.5 M EDTA in 500ml at pH 7.

RNA Loading dye

100 μl 10 X RNA running buffer

200 μl formamide

120 μl formaldehyde [37/40% w/v]

SDS-page separating buffer

Tris 8.2g

4ml 10% SDS

pH to 8.8 with HCl

H$_2$O to 100 ml

SDS-page stacking buffer

Tris 6.06g

4ml 10% SDS

pH to 6.8 with HCl

H$_2$O to 100 ml

5X SDS-page running buffer

Tris 15g

Glycine 72g

SDS 5g

SDS sample buffer

100 mM Tris-HCl, pH 6.8

2 % Beta-mercaptoethanol

4 % SDS

0.2 % bromophenol blue

20 % glycerol

Solutions for Random primer extension using d-CTP

Solution A:

470 μl of solution O,

9 μl mercaptoethanol,

12.5 μl 2mM dATP, dTTP, dGTP.

Solution O:

1.5 g Tris base,

0.25g MgCl$_2$.6H$_2$O

added to 10 ml H$_2$O at pH8.

Solution B:

6.5 g HEPES (N-2-Hydroxyethyl piperazine-N'-2-ethanesulphonic)

12.5ml dH$_2$O pH 6.6

Solution C:

Random hexamer primers pdN$_6$ (Pharmacia) at 50µg ml^{-1}

20 X SSC

175.3g NaCl

88.2g sodium citrate in 1 litre water

10 X TAE

900mM Tris-acetate

10 mM EDTA pH 8

10X TBE Tris-borate

400mM Tris-borate

10 mM EDTA pH 8

TE pH 7.4

10ml 1M Tris.HCl pH 8.0

2ml 0.5M EDTA

H$_2$O to 1 litre

TfbI

30 mM rubidium chloride

100 mM calcium chloride

10 mM manganese chloride

50 mM glycerol, altered to pH 5.8 with dilute acetic acid

TfbII

MOPS 0.21g

10 mM calcium chloride

75 mM rubidium chloride

10 mM glcerol, altered to pH 6.5 with dilute NaOH

Trypsin TVP

2ml Phosphate buffered saline (PBS) Ca^{2+} and Mg^{2+} free

2ml Chicken serum

2.5% Trypsin

0.5 M EDTA